Hepatitis B virus X protein overcomes the growth-inhibitory potential of retinoic acid by downregulating retinoic acid receptor-β2 expression via DNA methylation

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INTRODUCTION

Retinoids are vitamin A derivatives and analogues that are involved in many important biological processes, including vision, morphogenesis, differentiation, growth, metabolism and cellular homeostasis (De Luca, 1991). In addition, they act as inhibitors of carcinogenesis by blocking the promotion of initiated or transformed cells by three mechanisms: induction of apoptosis, arrest of further growth of abnormal cells and induction of abnormal cells to differentiate back to normal (Hansen et al., 2000). These effects are executed mainly by regulating gene expression primarily through two classes of nuclear receptors, including retinoic acid (RA) receptors (RARs) and retinoid X receptors (RXRs) (Chambon, 1996). As either homodimers or heterodimers, RARs and RXRs modulate the expression of target genes by acting on the RA response element (RARE) located on the promoter regions of target genes.

RAR-β contains four isoforms with different affinities to retinoids and different biological functions (Chambon, 1996). In particular, RAR-β2 has attracted attention as a major executor of the anti-tumour potential of retinoids in a wide variety of cancers (Houle et al., 1993; Xu, 2007). Loss of RAR-β2 is closely associated with retinoid resistance during tumorigenesis (Gebert et al., 1991; Hoffman et al., 1996; Hu et al., 1991; Xu et al., 1994). In addition, exogenous introduction of RAR-β2 into cervical, breast and lung cancer cells enhances cell responsiveness to growth inhibition and induction of apoptosis by retinoids (Seewaldt et al., 1995; Si et al., 1996). Therefore, its loss of function is now considered to play a critical role in tumorigenesis. aberrant promoter methylation of RAR-β2, which can lead to transcriptional downregulation of its expression, is frequently detected in human malignant tumours including hepatocellular carcinoma (HCC) (Lee et al., 2003; Yang et al., 2003). However, the mechanism of methylation and its biological significance are unknown.

Hepatitis B virus (HBV) is a major risk factor for the development of HCC (Tang et al., 2006). Interestingly, the incidence of promoter methylation in RAR-β2 is higher in HBV-positive HCC (>30 %) than in HBV-negative HCC (<10 %) (Yang et al., 2003). In addition, according to recent reports, HBx, the principal oncogene product of HBV, induces promoter hypermethylation of other tumour suppressor genes, resulting in downregulation of their expression (Jung et al., 2007; Lee et al., 2005; Park et al., 2007). These observations prompted us to investigate whether HBx induces promoter hypermethylation of the RAR-β2 gene and downregulates its expression. We also attempted to determine the biological significance of RAR-β2 downregulation by showing that the anti-growth potential of RA is inactivated in HBx-expressing cells.

Aberrant promoter methylation of retinoic acid receptor-β2 (RAR-β2) is frequently detected in hepatitis B virus (HBV)-positive hepatocellular carcinoma (HCC); however, the mechanism of methylation and its biological significance are unknown. This study showed that HBx, the principal oncogene product of HBV, induced promoter hypermethylation of RAR-β2 via upregulation of DNA methyltransferases 1 and 3a, resulting in downregulation of its expression in human HCC cells. In addition, HBx abolished the potential of retinoic acid (RA) to downregulate levels of G1-checkpoint regulators including p16, p21 and p27, resulting in activation of E2F1 in the presence of RA. As a consequence, HBx-expressing cells were less susceptible to RA-induced cell growth inhibition compared with control cells. These effects almost completely disappeared when levels of RAR-β2 in HBx-expressing cells were restored by treatment with a universal DNA methylation inhibitor, 5-aza-2′-deoxycytidine. As RAR-β2 is a major executor of the anti-tumour potential of RA, its epigenetic downregulation by HBx is likely to be an important step during HBV-mediated tumorigenesis.
RESULTS

HBx induces promoter hypermethylation of RAR-\(\beta_2\) via activation of DNA methyltransferase (DNMT) 1 and DNMT3a

To investigate whether HBx induces DNA methylation on the promoter of RAR-\(\beta_2\) in human hepatoma cells, we examined the methylation status of the RAR-\(\beta_2\) promoter in relation to expression of HBx. Genomic DNA extracted from HepG2 cells transiently transfected with HBx (Fig. 1a, lanes 1–5) or stable cell lines (Fig. 1a, lanes 6–9) was treated with sodium bisulfite, which converts deoxyctosine but not 5-methylcytosine residues into uracil by deamination. When methylation-specific PCR (MSP) for amplification of methylated regions on the RAR-\(\beta_2\) promoter was performed, we observed a significant increase in the methylation of the RAR-\(\beta_2\) promoter in the HBx-expressing cells compared to the control cells. This result suggests that HBx activates DNMT1 and DNMT3a, leading to hypermethylation of the RAR-\(\beta_2\) promoter.

![Methylation pattern of RAR-\(\beta_2\) promoter](image)

**Fig. 1.** HBx induces DNA methylation of RAR-\(\beta_2\) via activation of DNMT1 and DNMT3a. (a) Genomic DNA purified from HepG2 cells transiently transfected with empty vector (pCMV-3×HA1) (lane 1) or with increasing amounts of HBx-expressing plasmid (pCMV-3×HA1-HBx) (lanes 2–5) and from the stable cell lines HepG2-vector (lanes 6 and 8) and HepG2-HBx (lanes 7 and 9), was modified by treatment with sodium bisulfite as described in Methods. For lanes 5, 8 and 9, cells were treated with 5 \(\mu\)M 5-Aza-2\'-dC for 24 h before harvesting. MSP analysis was performed to determine whether CpG islands within the RAR-\(\beta_2\) promoter were methylated (M) or unmethylated (U). The methylation pattern of the p15 promoter was included as a control. (b) Bisulfite sequencing of the endogenous RAR-\(\beta_2\) promoter region in HepG2-vector and HepG2-HBx cells with or without 5-Aza-2\'-dC treatment. The CpG sites in a 595 bp region (−327 and +268) of the RAR-\(\beta_2\) promoter from five different clones are shown as unmethylated (open ovals) or methylated (filled ovals). The positions of the RARE, TATA box and transcription initiation site (arrow) are indicated. (c) HepG2-HBx cells were transfected with 1 \(\mu\)g each of empty vector (lane 3) or pSilence plasmid producing siRNA against DNMT1 (lane 4) or DNMT3a (lane 5). The methylation status of RAR-\(\beta_2\) in genomic DNA from HepG2-vector (lane 1) and HepG2-HBx (lanes 2–5) cells was determined by MSP analysis. Protein levels of RAR-\(\beta_2\), DNMT1, DNMT3a and \(\gamma\)-tubulin were determined by Western blotting (WB). Each band was quantified using BIOPORRIL BIO 1d image analysis software (Vilber Lourmat). The values shown beneath the protein bands indicate the level of each protein relative to the control (\(\gamma\)-tubulin). (d) HepG2-HBx cells (lanes 1, 3, 5 and 7) or HepG2-HBx (lanes 2, 4, 6 and 8) cells were mock-treated (lanes 2, 7 and 8) or treated with 10 \(\mu\)M ATRA for 48 h (lanes 3–6). For lanes 5 and 6, cells were treated with 5 \(\mu\)M 5-Aza-2\'-dC for 24 h before harvesting. ChIP 1 (lanes 1–6) and 2 (lanes 7 and 8) were performed to determine levels of DNMT1, MeCP2 and RAR-\(\beta_2\) bound to the positions of RAR-\(\beta_2\) promoter shown in Fig. 1(b).
performed, significantly higher frequencies of 5-methylcytosine residues were detected in the HBx-expressing cells, as represented by the level of 186 bp PCR products (Fig. 1a). In addition, the effect of HBx was almost completely abolished when a universal DNMT inhibitor, 5-aza-2'-deoxycytidine (5-Aza-2'dC), was administered (Fig. 1a, lanes 5 and 9). In contrast, the methylation pattern of p15 was minimally affected by the expression of HBx. According to bisulfite sequencing analysis, the CpG sequences of the RAR-β2 promoter were mostly unmethylated in the control cells (Fig. 1b). In contrast, over 50% of the CpG sites in the RAR-β2 promoter were found to be methylated in the HBx-expressing cells. Consistent with the MSP data, the effect of HBx on the methylation of CpG sites in the RAR-β2 promoter was almost completely abolished by treatment with 5-Aza-2'dC (Fig. 1b).

Next, we investigated the mechanism by which HBx induces DNA methylation of RAR-β2 in HepG2 cells. According to previous reports, HBx elevates DNMT activity via activation of DNMT1 and DNMT3a (Jung et al., 2007; Park et al., 2007). Consistent with this, the levels of these two enzymes were dramatically upregulated by the expression of HBx (Fig. 1c, lanes 1 and 2). To determine the enzyme responsible for the promoter hypermethylation of RAR-β2 in HBx-expressing cells, we conducted RNA interference experiments (RNAi) using a small interfering RNA (siRNA) specific for each enzyme. When DNMT1 or DNMT3a was knocked down by a specific siRNA, the effects of HBx on methylation of the RAR-β2 promoter and its expression almost completely disappeared (Fig. 1c, lanes 4 and 5). These results suggested that upregulation of both enzymes is required for the promoter hypermethylation of RAR-β2 by HBx.

RARE is an important transcriptional regulatory element for RAR-β2 promoter activity (Sun et al., 2000). Thus, it was expected that DNA methylation of the region including RARE would result in decreased RAR-β2 recruitment and subsequent inactivation of the RAR-β2 promoter. According to the chromatin immunoprecipitation (ChIP) assay, the levels of DNMT1 and methyl CpG-binding protein 2 (MeCP2) recruited to the RAR-β2 promoter were upregulated by HBx (Fig. 1d, lanes 1 and 2). Enhanced binding of DNMT1 to the CpG islands outside RARE (ChIP 2) was also observed in the presence of HBx (Fig. 1d, lanes 7 and 8), indicating that DNMT1 recruitment to the RAR-β2 promoter is CpG specific. In addition, the binding of RAR-β2 to its own promoter was dramatically reduced in the presence of HBx. All of these effects disappeared almost completely when DNA methylation was inhibited by treatment with 5-Aza-2'dC (Fig. 1d, lanes 5 and 6). Interestingly, treatment with all-trans RA (ATRA) reduced the binding of DNMT1 and MeCP2 to the promoter in the control cells but not in the HBx-expressing cells (Fig. 1d, lanes 3 and 4), suggesting an inhibitory effect of RA on DNA methylation in the former but not in the latter. Taken together, we concluded that HBx inactivates the RAR-β2 promoter by DNA methylation.

**HBx downregulates RAR-β2 expression via DNA methylation**

We next examined whether the HBx-mediated promoter methylation resulted in a decrease in RAR-β2 expression. Both RNA and protein levels of RAR-β2 were downregulated by HBx in a dose-dependent manner (Fig. 2a, lanes 1–4). The effects were almost completely abolished by treatment with 5-Aza-2’dC (Fig. 2a, lanes 4 and 5), suggesting that HBx downregulates RAR-β2 expression via DNA methylation.

To investigate whether the transcriptional activity of RAR was also downregulated by HBx, we performed a luciferase assay using a reporter construct, (RARE)₃–TK–luc, which

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**Fig. 2.** HBx downregulates RAR-β2 expression via DNA methylation. (a) RNA and protein levels of RAR-β2, HBx and GAPDH were determined by RT-PCR and Western blotting (WB), respectively, in cells prepared as described in Fig. 1(a). (b) Increasing amounts of HBx-expressing plasmid were transiently co-transfected with TK–luc or (RARE)₃–TK–luc into HepG2 cells. The data indicate the mean values of luciferase activity from two independent experiments carried out in triplicate.
contains a luciferase gene under the control of the herpes simplex virus thymidine kinase (TK) promoter fused to three copies of RARE from RAR-β2 (Sun et al., 2000). As shown in Fig. 2(b), HBx specifically downregulated the reporter activity from (RARE)<sub>3</sub>-TK–luc in a dose-dependent manner. In addition, this effect was almost completely abolished by treatment with 5-Aza-2′dC. As levels of RAR-α and RAR-γ were minimally affected by HBx (data not shown), it is likely that the lower transcriptional activity of RAR in the presence of HBx is primarily due to the downregulation of RAR-β2 via promoter hypermethylation.

**HBx suppresses the anti-growth potential of RA**

It is widely known that RA inhibits cell growth and induces cell-cycle G1 arrest (Soprano et al., 2006). In general, RA-induced G1 arrest is associated with multiple changes in G1-checkpoint regulators, i.e. either upregulation of cyclin-dependent kinase inhibitors such as p27 and p21, or downregulation of positive G1 regulators such as cyclin D1 and cyclin-dependent kinase 2 (Dietze et al., 2002; Wang et al., 2008; Zancai et al., 1998). Consistent with this, ATRA upregulated the levels of p16, p21 and p27 in HepG2 cells without HBx expression (Fig. 3a, lanes 1 and 3). In contrast, HBx downregulated the levels of p16 and p27 and upregulated the levels of p21 in the absence of RA (Fig. 3a, lanes 1 and 2), as demonstrated in our previous studies (Jung et al., 2007; Kwun & Jang, 2004). Interestingly, upregulation of the G1 regulators by ATRA was not observed in the HBx-expressing cells (Fig. 3a, lanes 2 and 4). In addition, when 5′Aza-2′dC was administered to inhibit DNA methylation, the levels of p16, p21 and p27 in the HBx-expressing cells were effectively upregulated by ATRA, up to the level in the control cells (Fig. 3a, lanes 5 and 6). These results suggested that HBx suppresses the potential of RA to alter levels of G1 regulators through DNA methylation-mediated downregulation of RAR-β2.

To provide more direct evidence that HBx-mediated RAR-β2 downregulation is closely associated with its ability to modulate levels of G1 regulators, we exogenously complemented RAR-β2 in the HBx-expressing cells. As a result, the effect of ATRA on p16 and p21 in the HBx-expressing cells was fully recovered by the expression of exogenous RAR-β2 in a dose-dependent manner (Fig. 3b). Taken together, we concluded that the potential of HBx to suppress the RA-mediated modulation of G1 regulators involves downregulation of RAR-β2 via DNA methylation.

We next examined whether the modulation of G1 regulators by HBx in the presence of RA resulted in activation of E2F, which is required for cell-cycle progression from G1 to S phase. For this purpose, we examined the phosphorylation status of retinoblastoma protein (Rb), which is affected by the levels of G1 regulators (Murphree & Benedict, 1984). As shown in Fig. 3(a), HBx-induced Rb phosphorylation without affecting its protein level in both the presence and absence of ATRA. As a consequence of Rb inactivation, levels of E2F1 increased significantly in the presence of HBx (Fig. 3a). In addition, exogenous RAR-β2 expression almost completely abolished the ability of HBx to modulate Rb and E2F1 (Fig. 3b).

**Fig. 3.** HBx suppresses the potential of RA to modulate G1 checkpoint regulators by downregulating RAR-β2 via DNA methylation. (a) HepG2-vector and HepG2-HBx cells were prepared as described in Fig. 1(d). The levels of the indicated proteins were determined by Western blotting. (b) HepG2-vector (lane 1) and HepG2-HBx (lanes 2–5) cells were treated with 10 μM ATRA for 48 h, and the levels of the indicated proteins were determined by Western blotting. For lanes 3–5, HepG2-HBx cells were transiently transfected with increasing amounts of RAR-β2-expressing plasmid (HA–RAR-β2).
Finally, we examined whether downregulation of RAR-β2 expression in HBx-expressing cells resulted in a lower susceptibility to the growth-inhibitory effect of RA, as demonstrated in other types of tumour (Hoffman et al., 1996; Houle et al., 1993; Liu et al., 1996a; Seewaldt et al., 1995; Si et al., 1996). For this purpose, we compared the proliferation of HepG2 cell lines with or without HBx expression after treatment with RA. According to data from direct counting of cell numbers (data not shown) and MTT analysis of cell proliferation (Fig. 4a), the growth of HBx-negative cells was markedly downregulated by treatment with 10 μM ATRA for 72 h (~75% reduction in MTT value), whilst the HBx-expressing cells showed a much lower susceptibility to ATRA-induced growth inhibition (~20% decrease). In addition, according to the results of the bromodeoxyuridine (BrdU) incorporation assay shown in Fig. 4(b), DNA synthesis in HBx-expressing cells was less severely affected by treatment with ATRA, with a 61.2 and 15.8% decrease in the control and HBx-expressing cells, respectively. In the presence of 5-Aza-2’dC, however, both cell growth and DNA synthesis in the two cell lines were similarly affected by ATRA. Taken together, we concluded that HBx overcomes the RA-induced G1 arrest through inhibition of RAR-β2 expression via promoter hypermethylation.

**DISCUSSION**

HBx is strongly associated with the development of HCC (Tang et al., 2006). HBx is encoded by the smallest open reading frame of the HBV genome, termed X, which is the most frequently integrated viral sequence found in HCCs (Paterlini et al., 1995). As a multifunctional regulatory protein, it can activate several transcription factors including AP-1, NF-κB, CREB and TBP (Benn et al., 1996; Maguire et al., 1991; Qadri et al., 1995). In addition, HBx has been implicated in the activation of several signal transduction pathways that lead to the transcriptional upregulation of a number of cellular genes, including those of growth factors and oncogenes (Benn et al., 1996; Lee & Yun, 1998; Shih et al., 2000). Moreover, HBx is able to induce HCC in transgenic mice (Kim et al., 1991). However, some lineages of HBx transgenic mice failed to develop liver tumours unless exposed to additional hepatocarcinogenic influences (Slagle et al., 1996; Terradillos et al., 1997). Despite extensive studies on the role of HBx during HBV-mediated hepatocellular carcinogenesis, the mechanisms of action remain controversial.

DNA methylation provides an alternative mechanism for inactivation of tumour suppressors during tumorigenesis (Palii & Robertson, 2007; Tischoff & Tannapf, 2008). The genes frequently methylated in HCC are APC (81.7%), GSTP1 (76.7%), RASSF1A (66.7%), p16 (48.3%), COX-2 (35%) and E-cadherin (33.3%) (Lee et al., 2003). However, the mechanism and its biological significance are largely unknown. The incidence of promoter methylation in RAR-β2 is higher in HBV-positive HCC (>30%) than in HBV-negative HCC (<10%) (Yang et al., 2003), suggesting that HBV is involved at least in part in this process. In addition, HBx has been implicated as a potential inducer of DNA methylation in some genes (Jung et al., 2007; Park et al., 2007). However, the underlying mechanisms remain unknown. It has been reported recently that HBx recruits DNMT3A via direct interaction with the regulatory promoters of target genes, resulting in repression of their transcription via de novo DNA methylation (Zheng et al., 2009). In addition, HBxs is known to elevate DNMT activity via upregulation of DNMT1 and DNMT3a (Jung et al., 2007; Park et al., 2007), and this has also been detected in HCC compared with non-neoplastic liver tissue (Park et al., 2006; Saito et al., 2003). Therefore, overexpression of these enzymes might be a potential mechanism of HBx-mediated epigenetic deregulation of cellular gene expression.

The present study showed that HBx upregulates levels of DNMT1 and DNMT3a to induce DNA methylation of the RAR-β2 promoter in human hepatoma cells, leading to downregulation of its expression. Both DNMT1 and DNMT3a are involved in DNA methylation of the RAR-β2 promoter (Fig. 1c). However, it is unclear why knockdown of the individual DNMTs almost completely abolished the potential of HBx to induce DNA methyla-

**Fig. 4.** HBx-expressing cells are resistant to RA-mediated growth inhibition. HepG2-vector and HepG2-HBx cells were incubated in six-well plates for 72 h in the presence or absence of 10 μM ATRA. Some cells were also cotreated with 5 μM 5-Aza-2’dC. Cell growth was measured by performing an MTT assay (a) or a BrdU incorporation assay (b) as described in Methods. Results are shown as means ± SD obtained from three different experiments.
tion. Previous reports have shown that both DNMT1 and DNMT3a siRNAs act specifically on the corresponding mRNA (Robert et al., 2003; Ting et al., 2004). Our study also showed that DNMT is specifically knocked down by the corresponding siRNA (Fig. 1c). Therefore, the possibility of cross-reaction of siRNA due to sequence redundancy between DNMT1 and DNMT3a could be excluded. In addition, the two DNMTs may not act on the different regions of the RAR-β2 promoter because knockdown of either enzyme almost completely abolishes the effects of HBx on the DNA methylation and expression of RAR-β2 (Fig. 1c). Instead, our data support the model that DNMT1 and DNMT3a form a complex to induce DNA methylation (Kim et al., 2002; Seo et al., 2008; Tsai et al., 2002). Further studies are required to understand the roles and action mechanisms of DNMT enzymes in the presence of HBx.

We also attempted to show the biological significance of DNA methylation-mediated downregulation of RAR-β2 by HBx. Because RAR-β2 is a major mediator for the growth-inhibitory effect of RA, the decrease in RAR-β2 expression usually leads to resistance to RA (Gebert et al., 1991; Hoffman et al., 1996; Hu et al., 1991; Xu et al., 1994). Indeed, HBx-expressing cells exhibited resistance to RA-induced growth inhibition (Fig. 4). According to previous reports, RA induces cellular growth arrest by modulating expression of G1 regulators (Dietze et al., 2002; Wang et al., 2008; Zancai et al., 1998). We also observed that RA upregulates levels of p16, p21 and p27 (Fig. 3a). However, RA could not effectively upregulate levels of those G1 CDK inhibitors in the presence of HBx due to downregulation of RAR-β2. The ability of HBx to overcome the growth-inhibitory effect of RA is likely to be directly associated with its potential to downregulate RAR-β2 expression via DNA methylation. First, as levels of RAR-β2 were restored by exogenous complementation, levels of G1 regulators including p16, p21, phosphorylated Rb and E2F1 were recovered to levels observed in the control cells (Fig. 3b). Furthermore, HBx-expressing cells became susceptible to RA-induced growth inhibition when levels of RAR-β2 were restored by treatment with a universal DNMT inhibitor, 5-Aza-2’dC (Fig. 4). Taken together, we concluded that HBx can overcome the growth-inhibitory effect of RA through downregulation of RAR-β2 via DNA methylation.

The mechanisms of how RAR-β2 activates the G1 regulators are unknown. It is unlikely that RAR-β2 binds directly to the promoters of every G1 regulator gene to activate their expression, as most of the RA-response genes do not contain a RARE sequence. Considering the numerous genes whose expression is regulated by RA, it is assumed that a regulator(s) mediating the roles of RAR/RXR complexes exists. More extensive studies are necessary to elucidate the action mechanisms of RAR and HBx in modulating levels of G1 regulators in the presence of RA.

Vitamin A/retinoids are important physiological regulators of embryonic development, vision, reproduction, bone formation, haematopoiesis, differentiation, proliferation and apoptosis. Therefore, they have served as prime chemoprevention. Retinoids suppress carcinogenesis in various organs including oral, skin, bladder, lung, prostate and breast cancers in experimental animals (Lotan, 1995). Clinically, they reverse pre-malignant human epithelial lesions and prevent lung, liver and breast cancer and second primary tumours in the head and neck (Hansen et al., 2000). Malfunction of nuclear receptors is closely associated with retinoid resistance during tumorigenesis (Gebert et al., 1991; Hoffman et al., 1996; Hu et al., 1991; Xu et al., 1994). In the case of HCC, it has been reported that post-translational modification of RXR by phosphorylation impairs its function, which leads to uncontrolled cell growth (Matsushima-Nishiwaki et al., 2001). We have shown here that downregulation of RAR-β2 via aberrant promoter methylation also resulted in retinoid resistance. As RAR-β2 is a major executor of the anti-tumour potential of retinoids, our results may provide an important target for the development of therapeutic drugs against HCC associated with infection by HBV.

METHODS

Plasmids. Plasmids pCMV-3 × HA1-HBx (Kwun & Jang, 2004) and pCMV-3 × HA1-RAR-β2 (Seo et al., 2008) encode HBx and RAR-β2, respectively, downstream of three copies of the influenza virus haemagglutinin (HA) epitope. Both TK–luc and (RARE)₃–TK–luc have been described previously (Sun et al., 2000).

Cell lines and transfection. HepG2, a human hepatoblastoma-derived cell line, was obtained from the Korean Cell Line Bank (KCLB no. 58065). Stable cell lines were established by transfection with either pCMV-3 × HA1 or pCMV-3 × HA1-HBx3, followed by selection with 500 μg G418 (Gibco) ml⁻¹ (Kwun & Jang, 2004). Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. For transient expression, 2 × 10⁵ cells per 60 mm dish were transfected with 1 μg of appropriate plasmid(s) using WelFect-EX PLUS (WelGENE) following the manufacturer’s instructions.

DNA methylation analysis. 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 MSP primer pairs for RAR-β2 and p15 as described previously (Herman et al., 1996). For bisulfate DNA sequencing, modified genomic DNA was amplified by nested PCR using the first primer set, RAR-β2 forward 1 (5’-GTATAGGAAGTTAAAGTTGTTGGG-3’) and RAR-β2 reverse 1 (5’-CTTTATAAATTCCAAATATCATTTACC-3’), followed by the primer set RAR-β2 forward 2 (5’-GTAGGG/TGGAATTTGTTTTTAAGTTAG-3’) and RAR-β2 reverse 2 (5’-AATTACCATTTACATTTCACAAAACTACTC-3’) (Ren et al., 2005). A 595 bp region (−327 and +268), encompassing 27 CpG sites in the RAR-β2 regulatory region, was subcloned into the pGEM-T Easy vector (Promega) and five individual clones were sequenced per sample.

Western blot analysis. Cells were lysed in 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 incubated with antibodies to RAR-β, DNMT1, DNMT3a, p16, p21, p27 and E2F1 (Santa Cruz Biotechnology), and to HA (Roche), Rb (Calbiochem), γ-tubulin (Sigma) and phosphorylated Rb (Sigma). Primary antibodies were detected with the appropriate horseraishid peroxidase (HRP)-conjugated secondary antibodies: anti-mouse IgG (H+L) and anti-rabbit IgG (H+L) (Bio-Rad). An ECL kit (Amersham) was used to visualize the protein bands.

RNAi. The SilenCircle RNAi system (Allele Biotech), a plasmid-based RNA interference system that uses U6 RNA-based polymerase III promoter, was employed to knock down specific gene expression. Based on the target sequences of DNMT1 (5'-CCATGAGCA-CCGGTTCCTCC-3') (Ting et al., 2004) and DNMT3a (5'-CAGGAGATGTGTCACCACCC-3') (Robert et al., 2003), siRNA inserts composed of sense and antisense sequences separated by a central loop sequence were designed. The siRNA inserts were ligated into the pre-cut pSilenCircle vector and positive clones were selected.

ChiP assay. ChiP assays were performed using a ChiP assay kit (Upstate Biotechnology) according to the manufacturer's specifications. The sheared chromatin was immunoprecipitated with anti-DNMT1 and anti-RAR-β antibodies (Santa Cruz Biotechnology), anti-MeCP2 antibody (Upstate Biotechnology) and a negative-control rabbit IgG (Santa Cruz Biotechnology). DNA released from the precipitated complexes was amplified by PCR using ChIP 1 primers (–165F, 5'-CTGGATTTGGTCCTCTGACT-3' or ChIP 2 primers (–833F, 5'-CAGCTGTTAAGTGCAGACCC-3') or ChIP 2 primers (–833F, 5'-CAGCTGTTAAGTGCAGACCC-3') or ChIP 2 primers (–833F, 5'-CAGCTGTTAAGTGCAGACCC-3').

Luciferase reporter assay. Cells (2 x 105 per 60 mm diameter plate) were transfected with 0.5 μg TK-luc or (RARE)-TK-luc along with the indicated amounts of HBx expression plasmid as described above. To control for transfection efficiency, 0.1 μg pCH110 (Pharmacia) containing the Escherichia coli lacZ gene under the control of the simian virus 40 promoter was co-transfected as an internal control. At 48 h after transfection, a luciferase assay was performed and the value obtained was normalized to the luciferase activity measured in the corresponding cell extract. Each experiment was prepared in triplicate and repeated at least three times.

RT-PCR. Total cellular RNA was extracted from cells using an RNaseasy Mini kit (Qiagen). DNase I-digested RNA (3 μg) was reverse-transcribed with the corresponding antisense primer. One-quarter of the reverse-transcribed RNA was amplified with Taq DNA polymerase (95 °C for 10 min; 30 cycles of 95 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min; 10 cycles of 95 °C for 30 s, 72 °C for 2 min) using sense primers 5'-CTGGAGGTCTCGTGCTGAT-3' and 5'-ATGGGGAAGGTAGGATGGCG-3' and antisense primers 5'-TCATGTGAGGCTTGCTGGTTC-3' and 5'-AACCTCTAGA-TGATTAGGCGAGG-3' and 5'-TGGAGGGATGCTGGTC-3' for RAR-β2, HBx and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), respectively.

Growth inhibition assay. For the determination of cell growth, cells were seeded at 1 x 104 cells per well in 96-well plates and incubated for 72 h under the indicated conditions. The MITT-derived formazan sued for 72 h and treated with 10 μM BrdU for an additional 24 h. The fixed cells were reacted with anti-BrdU–HRP for 2 h and the colour developed by adding trimethyl benzidine was measured at 490 and 405 nm.

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