Expression of DNA Methyltransferase 1 Is Activated by Hepatitis B Virus X Protein via a Regulatory Circuit Involving the p16^{INK4a}-Cyclin D1-CDK 4/6-pRb-E2F1 Pathway

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Abstract

DNA methyltransferase 1 (DNMT1) is responsible for copying DNA methylation patterns to the daughter strands during DNA replication. Its expression is frequently up-regulated in human tumors, including hepatocellular carcinoma, but the mechanism of overexpression and its biological significance remain unclear. Here, we show that hepatitis B virus X protein (HBx) activates DNMT1 expression via a regulatory circuit involving the p16^{INK4a}-cyclin D1-cyclin-dependent kinase (CDK) 4/6-retinoblastoma protein (pRb)-E2F1 pathway. HBx induced DNA hypermethylation of p16^{INK4a} promoter to repress its expression, which subsequently led to activation of G_1-CDKs, phosphorylation of pRb, activation of E2F1, and finally transcriptional activation of DNMT1. Inhibition of DNMT1 activity by either treatment with 5′-Aza-2′dC or introduction of DNMT1 small interfering RNA not only abolished the DNA methylation-mediated p16^{INK4a} repression but also impaired DNMT1 expression itself, suggesting a cross-talk between DNMT1 and p16^{INK4a}. The up-regulation of cyclin D1 by HBx is likely to serve as an initiative impulse for the circuit because it was absolutely required for the activation of DNMT1 expression. We also observed that accumulated DNMT1 via this pathway inactivates E-cadherin expression through promoter hypermethylation. Considering that the pRb-E2F1 pathway is commonly activated in human tumors, activation of this circuit might be widespread and a potential therapeutic target. [Cancer Res 2007;67(12):5771–8]

Introduction

DNA methylation involves the addition of a methyl group to the 5′ position of the cytosine ring in the CpG dinucleotide. It is catalyzed in mammalian cells by a family of highly related DNA methyltransferases (DNMT) that use S-adenosylmethionine as the methyl donor (1, 2). DNMT1 is responsible for copying and maintaining methylation patterns after DNA replication, whereas both DNMT3a and DNMT3b function as *de novo* methyltransferases (1). Hypermethylation of CpG-rich regions (CpG islands) residing within the promoter of a gene is generally associated with transcription repression and thus serves as an alternative mechanism for gene inactivation (3, 4). It is recognized as an important epigenetic control over different genome functions, including differential gene expression (4), allele-specific expression in parental imprinting (5), and X inactivation (6).

Recent studies have revealed how methylation anomalies play a direct causal role in tumorigenesis and genetic diseases (3, 7, 8). Especially, aberrant hypermethylation of tumor suppressor genes (TSGs) is frequently detected in human tumors, including hepatocellular carcinoma (HCC; refs. 8–11). DNMT1 is considered to play an essential role in aberrant DNA methylation in tumors. Its expression is significantly higher in HCC when compared with nontumor tissues (12). In addition, the increased DNMT1 expression is associated with increased cell proliferation (13), tumorigenesis (14), and tumor progression (15). Therefore, elucidation of the mechanism for DNMT1 up-regulation may provide an important clue for the understanding of epigenetic alterations in tumors. Interestingly, a strong correlation between hepatitis B virus (HBV) infection and epigenetic alterations of TSGs, including p16^{INK4a}, has been shown (16, 17). In addition, our previous report showed that HBV X protein (HBx) activates expression of DNMT1, which results in repression of E-cadherin through promoter hypermethylation (18). However, the mechanism by which HBx activates DNMT1 expression remains unknown.

Based on the nearly ubiquitous alterations both of DNA methylation and of the retinoblastoma protein (pRb)-E2F pathway in human cancer, McCabe et al. (19) showed that E2F regulates DNMT1 transcription through its binding site on the DNMT1 promoter. HBx, like other DNA tumor virus oncogenes, is capable of activating the pRb-E2F pathway (20). HBx activates several intracellular signal transduction pathways that can lead to up-regulation of cyclin D1 (21, 22). In addition, p16^{INK4a} is frequently inactivated in tumors, including HCC, via promoter hypermethylation (16). Thus, it is quite logical to hypothesize that HBx activates DNMT1 expression through the pRb-E2F pathway. In the present study, we show that HBx activates expression of DNMT1 via a regulatory circuit involving the p16^{INK4a}-cyclin D1-cyclin-dependent kinase (CDK) 4/6-pRb-E2F1 pathway. In addition, we observed that activation of DNMT1 through this circuit leads to the DNA methylation-mediated inactivation of E-cadherin.

Materials and Methods

Plasmids. pCMV-3×HA1-HBx encodes the full-length HBx sequence (nucleotides 1,374–1,838) downstream to three copies of the influenza virus hemagglutinin (HA) epitope (23). For DNMT1$_{-25/+232}$, the sequence from −25 to +232 of the DNMT1 promoter from genomic DNA of HepG2 cells was amplified with DNMT1$_{-25}$F (5′-AACGCGTGCAGCTTGGACGAGCC-3′) and DNMT1$_{+232}$R (5′-GAAGCTTCAGCAGACGCGGCG-3′) and subcloned into pGL2-basic vector (Promega). For DNMT1ΔE2F, the sequence from −25 to +173 of DNMT1$_{-252/+232}$ was amplified with DNMT1$_{-25}$F and DNMT1$_{+173}$R (5′-TCTCGAGGGGCGATGAGC-3′). DNMT1mE2F is derived

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from DNMT1$_{-25/+232}$ by substituting the sequence $_{+177}$TTTC*CGCGCG*$_{+186}$ to $_{+177}$TTTC*GAATTC*$_{+186}$. Both E2F1-luc and E2F1 expression plasmid were gifts from Dr. Chang-Woo Lee (Sungkyunkwan University, Seoul, Korea). For construction of pCMV-3×HA1-p16, a full-length p16^{INK4a} cDNA prepared from total RNA of HepG2 cells was amplified with the use of INK4aF (5′-GCCTCGAGCCGGCGGCGGGGA-3′) and INK4aR (5′-CTCTAGATCTTTCAATCGGGGATGTC-3′) and subcloned, in frame, into pCMV-3×HA1 (24). For p16-luc, the sequence from −880 to +2 of p16^{INK4a} promoter was amplified with p16$_{-880}$F (5′-GCCTACAGGCAGATTTCTCC-3′) and p16$_{+2}$R (5′-CAAGCTTCTCCCCGCCGCCC-3′).

Cell culture. HepG2 (KCLB 58065), a human hepatoblastoma-derived cell line, was obtained from the Korean Cell Line Bank. Stable cell lines were established by transfection with either pCMV-3×HA1 or pCMV-3×HA1-HBX3 followed by selection with 500 μg/mL G418 (Life Technologies; ref. 23). Cells were maintained in DMEM supplemented with 10% FCS.

Transient transfection and luciferase assay. For transient expression, 2×10^5 cells per 60-mm-diameter plate were transfected with 2 μg of appropriate plasmid(s) using WelFect-EX PLUS (WelGENE) following the manufacturer's instructions. pCH110 (0.1 μg; Pharmacia) containing the *Escherichia coli lacZ* gene under the control of the SV40 promoter was cotransfected as an internal control. At 48 h after transfection, luciferase assay was done and the value obtained was normalized to the β-galactosidase activity measured in the corresponding cell extract. Each experiment was repeated at least thrice prepared in triplicate.

Western blot analysis. Cells were lysed in buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.1% SDS, 1% NP40] supplemented with protease inhibitors. Cell extracts (20 μg) were separated by SDS-PAGE and transferred onto a nitrocellulose membrane (Hybond polyvinylidene difluoride, Amersham). Membranes were incubated with DNMT1, E2F1, cyclin D1, p16, p21, and p27 antibodies (Santa Cruz Biotechnology); HA antibody (Roche); γ-tubulin and phosphorylated pRb antibodies (Sigma); pRb and E-cadherin antibodies (Calbiochem); and subsequently with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies: rabbit IgG(H+L)-HRP (Bio-Rad), mouse IgG(H+L)-HRP (Bio-Rad), and goat IgG-HRP (Santa Cruz Biotechnology). The enhanced chemiluminescence kit (Amersham) was used to visualize protein bands.

Real-time PCR analysis. Total RNA (3 μg) was reverse transcribed with the corresponding reverse primer. The real-time PCRs were done in a volume of 20 μL containing forward and reverse primers (5 μmol/L each), one tenth of the cDNA (2.5 μL), and 2× SYBR Premix Ex Taq (TaKaRa). Forward primers 5′-GAGGAAGCTGCTAAGGACTAGTTC-3′ and 5′-ACCACAGTCCATGCCATCAC-3′ and reverse primers 5′-ACTCCACAATTTGATCACTAAATC-3′ and 5′-TACAGCAACAGGGTGGTGGA-3′ were used for DNMT1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), respectively. Amplification was carried out in two steps: preincubation at 95°C for 10 min and 40 cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 20 s. Each reverse transcription-PCR was repeated at least thrice to ascertain reproducibility, and data were analyzed using MJ Opticon Monitor analysis software (Bio-Rad). A normalized value was obtained by subtracting the threshold cycle (*C*t) of GAPDH from the *C*t of target genes, resulting in a Δ*C*t, and the $2^{-\Delta Ct} \times 1{,}000$ was used as an indication of the relative transcript level.

RNA interference. SilenCircle RNA interference (RNAi) system (Allele Biotech), a plasmid-based RNAi system that uses U6 RNA-based polymerase

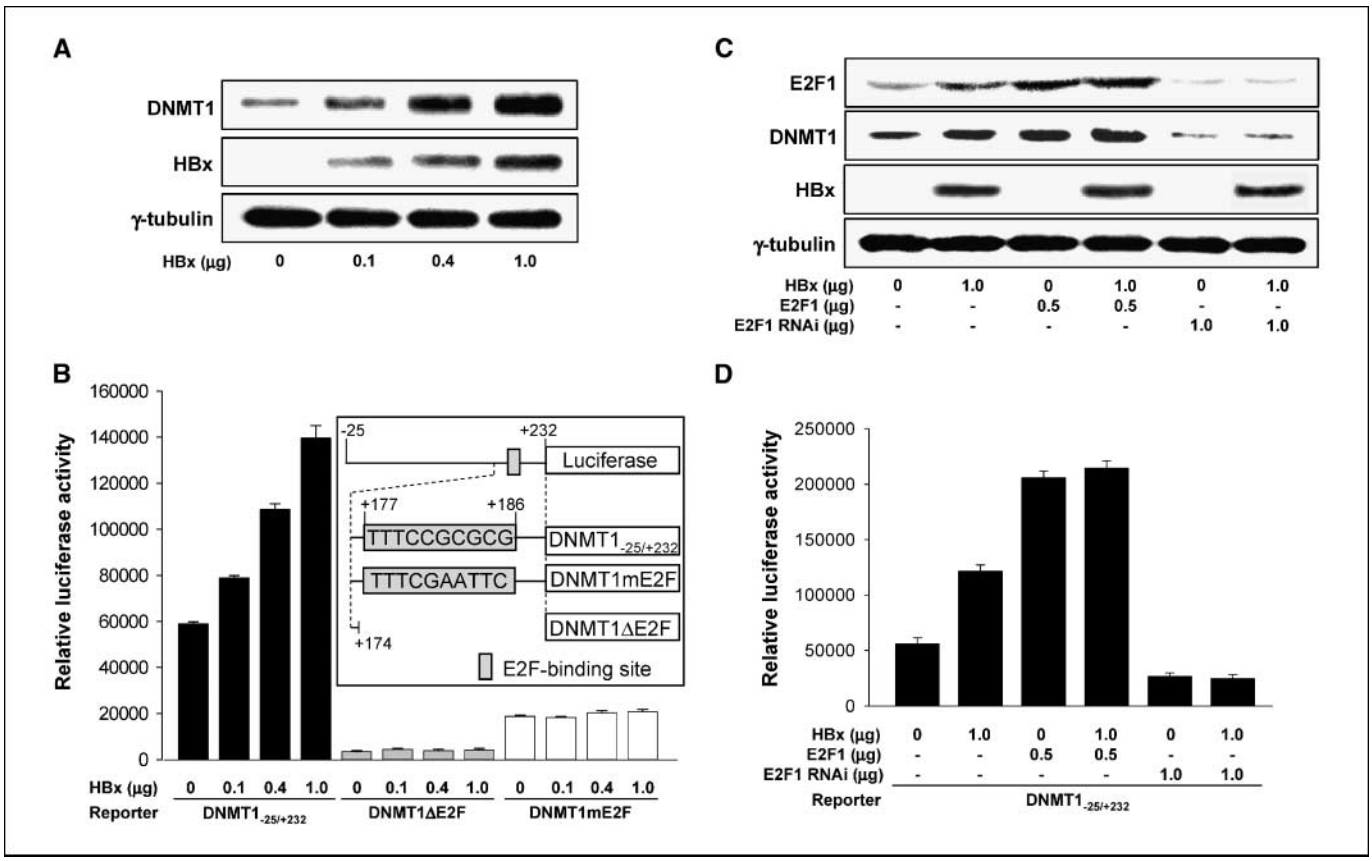

Figure 1. HBx activates DNMT1 expression through the E2F1 pathway. *A,* HepG2 cells were transiently transfected with increasing amounts of HBx-expressing plasmid (23) for 48 h and analyzed by Western blots for DNMT1, HBx, and γ-tubulin. *B,* increasing amounts of HBx were cotransfected with DNMT1$_{-25/+232}$ or its derivatives into HepG2 cells followed by luciferase assay. *C,* HepG2 cells were transfected with either E2F1 expression plasmid (*lanes 3* and *4*) or E2F1 RNAi plasmid (*lanes 5* and *6*) in the presence or absence of HBx. *D,* HepG2 cells were transiently transfected with DNMT1$_{-25/+232}$, HBx, E2F1, and E2F1 RNAi followed by luciferase assay.

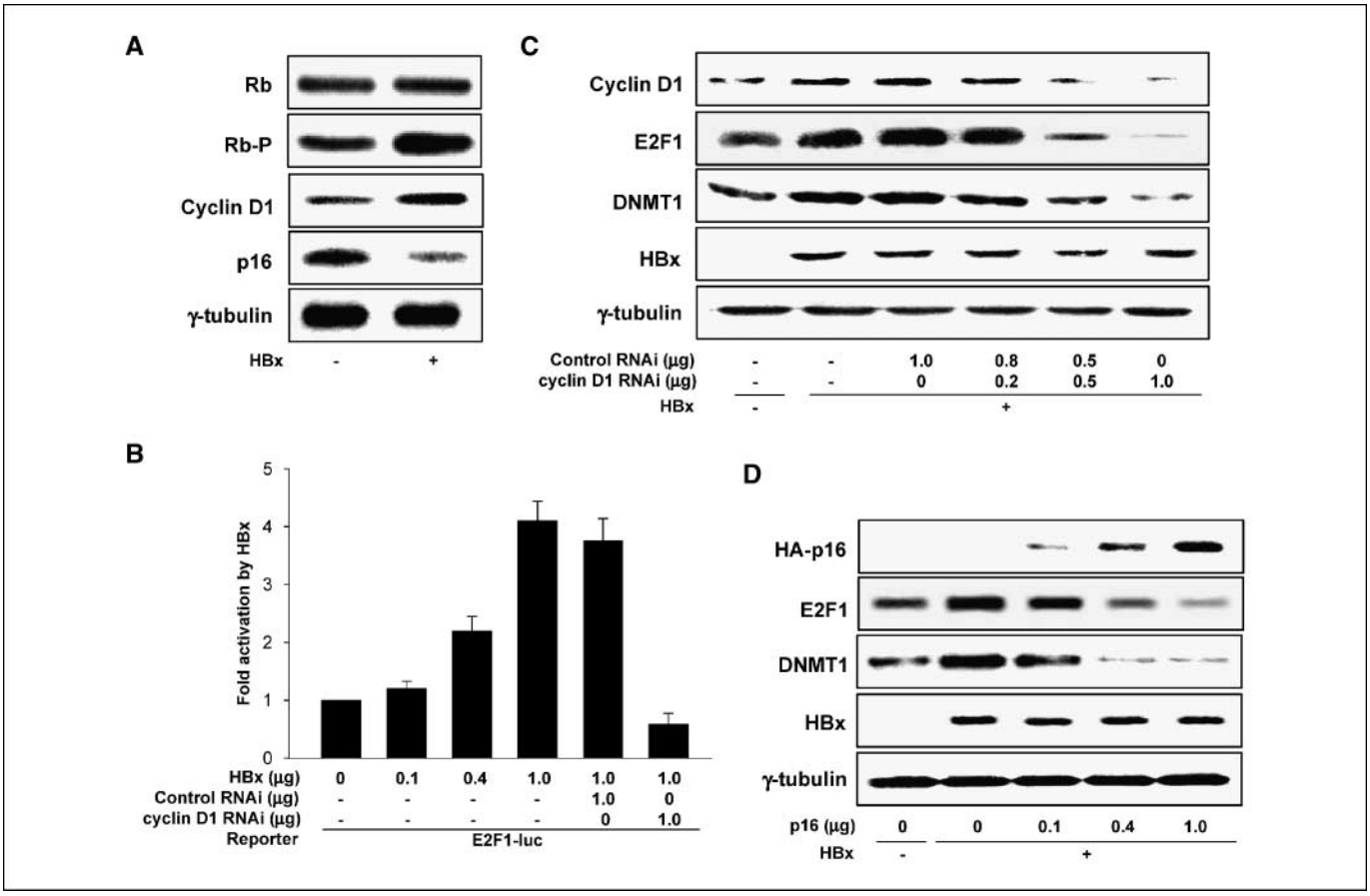

Figure 2. HBx elevates E2F1 level through either up-regulation of cyclin D1 or down-regulation of $p16^{INK4a}$. *A,* levels of pRb (total and phosphorylated form), cyclin D1, and $p16^{INK4a}$ in stable cell lines with or without HBx were measured by Western blots. *B,* HepG2 cells were cotransfected with E2F1-luc and increasing amounts of HBx. Either empty vector (*column 5*) or cyclin D1 RNAi plasmid (*column 6*) was included. Fold activation by HBx indicates the relative luciferase activity of HBx-expressing cells compared with the control. *C,* HepG2-HBx stable cells were transfected with increasing amounts of cyclin D1 RNAi plasmid (*lanes 3–6*). *D,* HepG2-HBx stable cells were transiently transfected with increasing amounts of HA-tagged p16-expressing plasmid (*lanes 3–5*). Levels of E2F1, DNMT1, and HBx were determined by Western blots. HA-p16 was probed with HA antibody.

III promoter and modified terminator for high-level small interfering RNA (siRNA) in a hairpin format inside target cells, was used to knock down specific gene expression. Based on the siRNA sequence of cyclin D1 (5′-CAAGCUCAAGUGGAACCUG-3′; ref. 25), DNMT1 (5′-CCAUGAGCACC-GUUCUUC-3′; ref. 26), and E2F1 (5′-GUCACGCUAUGAGACCUCA-3′; ref. 27), siRNA inserts composed of both sense and antisense sequences separated by a central loop sequence were designed.

CDK assay. CDK activity was measured with CDK Assay kit (Invitrogen) and pRb peptide (GNIYISPLKSPYKIS; Invitrogen) as a substrate following the manufacturer's instructions. In brief, 100 μg cell lysate, 5 μL ATP (1 μmol/L), and 10 μg pRb peptide were mixed and incubated for 1 h at 37°C, and then 25 μL 4× anti-phosphothreonine peptide-specific antibody was added. After incubation for 1 h at room temperature, the polarization values of samples were measured with VICTOR3 V Multilabel Counter (Wallac 1420, Perkin-Elmer).

Methylation-specific PCR. Genomic DNA (1 μg) denatured in 50 μL of 0.2 N NaOH was modified by treatment with 30 μL of 10 mmol/L hydroquinone (Sigma) and 520 μL of 3 mol/L sodium bisulfite (Sigma), pH 5.0, at 50°C for 16 h. The modified DNA (100 ng) was amplified with Taq polymerase using methylation-specific PCR (MSP) primer sets for $p16^{INK4a}$, p15, p73, and E-cadherin as described previously (28). For MSP analysis of the transfected E-cadherin promoter, total DNA was purified from HepG2 cells transfected with $Ecad_{-420/+32}$. To exclude the amplification of endogenous E-cadherin promoter, a fragment from E-cadherin promoter to luciferase open reading frame in $Ecad_{-420/+32}$ was amplified as described previously (18).

Results

HBx activates DNMT1 expression through the E2F1 pathway. First, we investigated the mechanism by which HBx activates DNMT1 expression. Transient expression of HBx in HepG2 cells resulted in up-regulation of DNMT1 level in a dose-dependent manner (Fig. 1*A*). As HBx activates DNMT1 expression at the transcription level (18), we attempted to map the HBx-responsive element on the DNMT1 promoter. The full-length (−252 to +232) DNMT1 promoter was activated ~3-fold by HBx (data not shown). Analysis of a truncated promoter construct ($DNMT1_{-25/+232}$) disclosed a similar level of activation (Fig. 1*B*). However, the effect was not observed with DNMT1ΔE2F, which is derived from $DNMT1_{-25/+232}$ by deleting the sequence from +174 to +232 (Fig. 1*B*). This is substantiated by the fact that the deleted fragment contains a putative E2F-binding site ($_{+177}$TTTCCGCGCG$_{+186}$), which is responsible for DNMT1 regulation by the pRb-E2F pathway (19). Destruction of the E2F site in DNMT1mE2F not only resulted in ~65% loss of basal activity but also completely abolished the effect of HBx (Fig. 1*B*). These results suggest that HBx activates DNMT1 expression by up-regulating its promoter activity via the E2F site.

According to McCabe et al. (19), stable overexpression of E2F1 up-regulates DNMT1 levels. Thus, we investigated whether HBx

up-regulates E2F1 to activate DNMT1 expression. Both transient (Fig. 1*C, lanes 1* and *2*) and stable expression (Fig. 2*C, lanes 1* and *2*) of HBx up-regulated E2F1 level. In addition, transfection with exogenous E2F1 increased both the protein level and promoter activity of DNMT1 (Fig. 1*C*, and *D* and *lanes 3* and *4*). On the other hand, silencing E2F1 expression through transient introduction of E2F1 siRNA down-regulated DNMT1 expression (Fig. 1*C* and *D* and *lanes 5* and *6*). Moreover, the DNMT1 activation by HBx was almost completely abolished under the condition that levels of E2F1 in the control and HBx-expressing cells were equalized (Fig. 1*C* and *D*). Like E2F1, other members of the activator E2F family, such as E2F2 and E2F3, which can bind and activate E2F target promoters (29), may also participate in HBx-induced DNMT1 activation. However, their protein levels were little affected by HBx (data not shown), suggesting that the effect is E2F1 specific. Based on these data, we conclude that HBx activates DNMT1 expression via up-regulation of E2F1.

HBx activates E2F1 by up-regulating G_1-CDK activity. Next, we examined the mechanism by which HBx activates E2F1. During G_1 phase of cell cycle, E2F1 is mainly activated by the coordinate action of G_1-CDKs CDK4 and CDK6 (29). In our *in vitro* kinase assay, HBx-expressing cells exhibited ~2.5-fold higher CDK activity than control cells (Fig. 3*B*). HBx also induced hyperphosphorylation of pRb without affecting its total protein level (Fig. 2*A*) and up-regulated E2F1 transcriptional activity as shown by the increased luciferase activity from E2F1-luc in the presence of HBx (Fig. 2*B*). These results suggest that HBx up-regulates both protein level and transcriptional activity of E2F1 via G_1-CDK–mediated phosphorylation/inactivation of pRb.

HBx activates the E2F1 pathway by modulating levels of cyclin D1 and $p16^{INK4a}$. G_1-CDK is normally activated by either increased synthesis of cyclin D or inactivation of CDK inhibitors (CKI; ref. 29). Both transient (data not shown) and stable expression of HBx up-regulated cyclin D1 level (Fig. 2*A*). To investigate whether this effect is responsible for the activation of E2F1 by HBx, we attempted to silence cyclin D1 expression in HBx-expressing cells by transient transfection of a specific siRNA-producing plasmid. Consequently, both transcriptional activity and protein level of E2F1 were markedly down-regulated (Fig. 2*B* and *C*). Accordingly, levels of DNMT1 were decreased in a similar manner (Fig. 2*C*).

To investigate the possible roles of CKIs in the activation of E2F1, levels of $p16^{INK4a}$, p21, and p27 were examined in the presence or absence of HBx. Among the CKIs tested in this study, only $p16^{INK4a}$ was down-regulated by HBx (Fig. 2*A*). Addition of

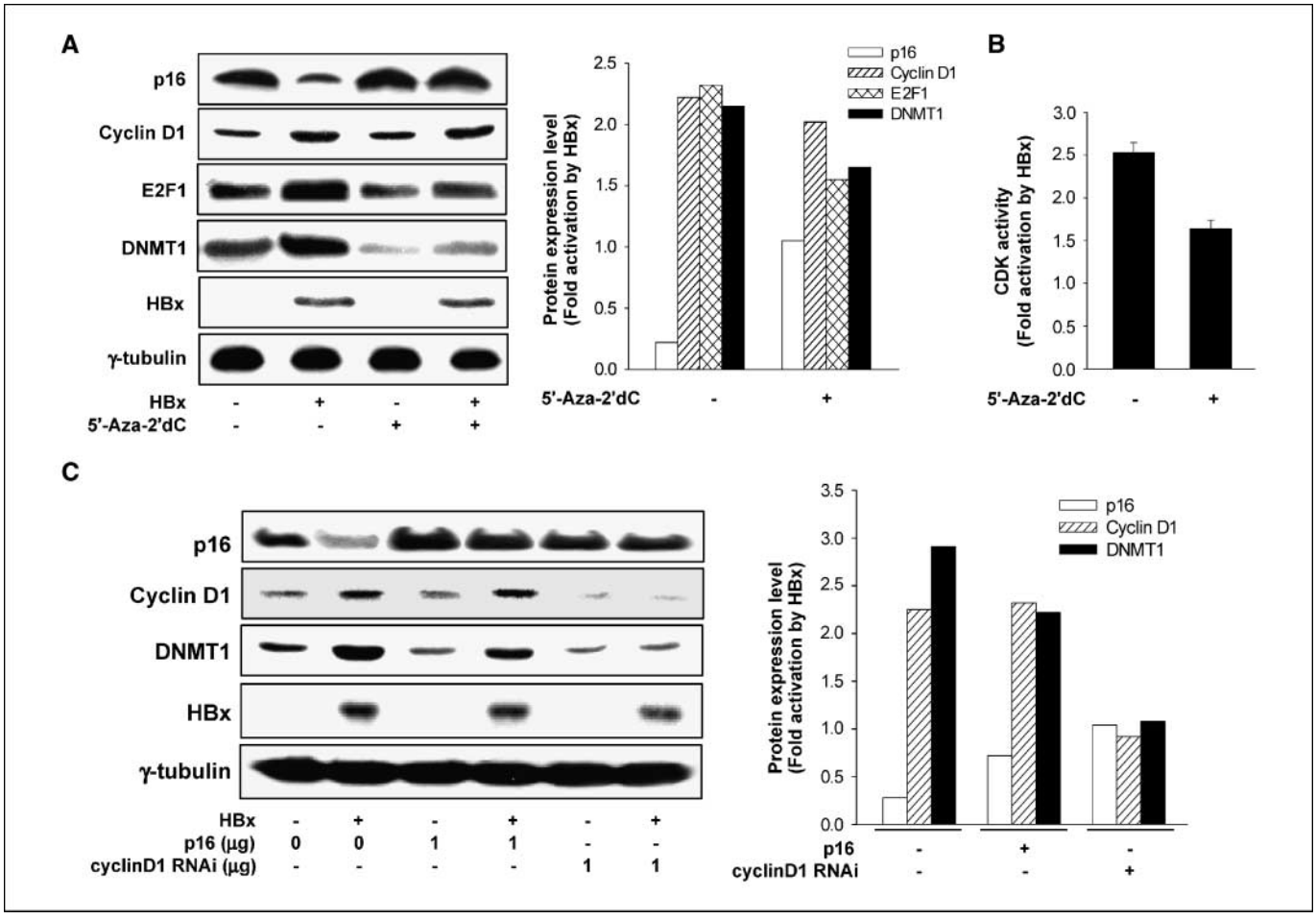

Figure 3. Up-regulation of cyclin D1, but not down-regulation of $p16^{INK4a}$, is required for DNMT1 activation by HBx. *A,* HepG2 stable cells were treated with 5′-Aza-2′dC as described in Fig. 4*A*. Cell lysates were probed with $p16^{INK4a}$, cyclin D1, E2F1, and DNMT1 antibodies. *B,* CDK activity was presented as fold activation by HBx. *C,* HepG2 cells with or without HBx were transfected with either $p16^{INK4a}$ (*lanes 3* and *4*) or cyclin D1 RNAi (*lanes 5* and *6*). *Right,* protein bands of $p16^{INK4a}$, cyclin D1, and DNMT1 in (*A*) and (*C*) were quantified with the use of BIOPRORIL BIO 1D image analysis software (Vilber Lourmat).

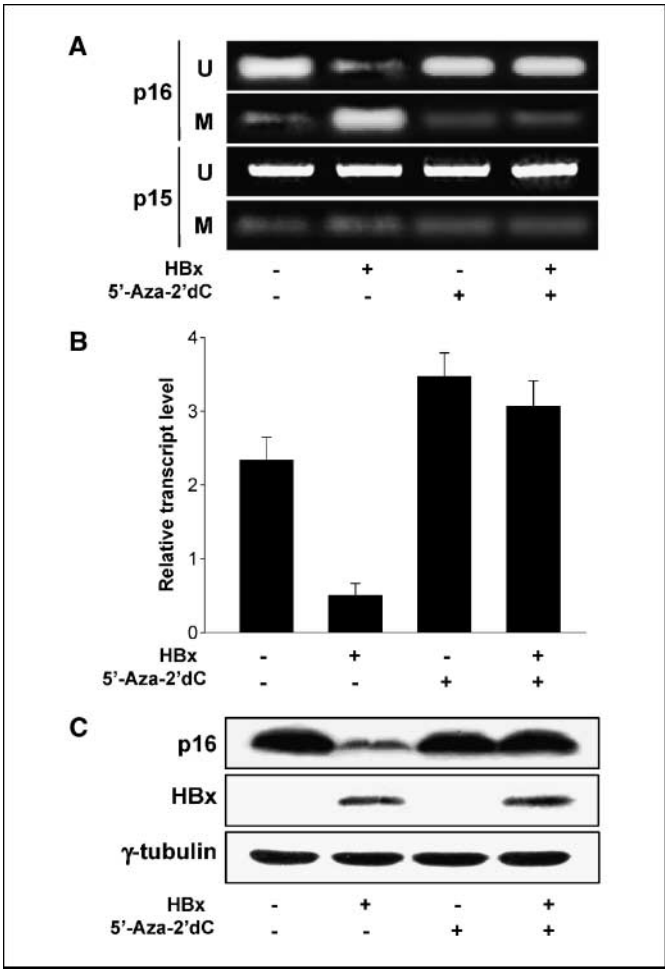

Figure 4. HBx represses $p16^{INK4a}$ expression via promoter hypermethylation. HepG2 stable cells with or without HBx were either mock treated (*lanes 1* and *2*) or treated with 5 μmol/L 5′-Aza-2′dC (*lanes 3* and *4*) for 24 h. *A,* MSP analysis was done to determine the methylation status of $p16^{INK4a}$. As a control, the methylation pattern of p15 promoter was analyzed. *B,* RNA levels of $p16^{INK4a}$ were determined by real-time PCR analysis. *C,* protein levels of $p16^{INK4a}$ were determined by Western blots.

exogenous $p16^{INK4a}$ to HBx-expressing cells also abolished the potential of HBx to up-regulate levels of E2F1 and DNMT1 (Fig. 2*D*). Thus, we conclude that HBx increases CDK activity required for DNMT1 activation through either up-regulation of cyclin D1 and/or down-regulation of $p16^{INK4a}$.

HBx represses $p16^{INK4a}$ expression via promoter hypermethylation. HBx activates several intracellular signal transduction pathways that can lead to up-regulation of cyclin D1 (21). Recently, Park et al. (22) have shown that up-regulation of NF-κB2(p52)/Bcl-3 in the nucleus by HBx results in cyclin D1 up-regulation. However, the mechanism by which HBx down-regulates $p16^{INK4a}$ is unknown. Several reports have shown that $p16^{INK4a}$ is inactivated in tumors, including HCC, via promoter hypermethylation (16). Thus, we explored the possibility that HBx inactivates $p16^{INK4a}$ via promoter hypermethylation. According to MSP analysis of $p16^{INK4a}$ promoter, significantly higher frequencies of 5-methylcytosine residues were detected on the $p16^{INK4a}$ promoter derived from HBx-expressing cells as represented by the level of 169-bp PCR products (Fig. 4*A, lanes 1* and *2*). This effect was completely abolished in the presence of a DNMT inhibitor 5′-Aza-2′dC (Fig. 4*A, lanes 3* and *4*). In contrast, HBx did not affect the methylation pattern of p15 promoter. Consistent with MSP results, both RNA (Fig. 4*B*) and protein levels (Fig. 4*C*) of $p16^{INK4a}$ were down-regulated by HBx but was completely restored after treatment with 5′-Aza-2′dC. Thus, we conclude that HBx represses $p16^{INK4a}$ expression via promoter hypermethylation.

DNMT1 is responsible for the promoter hypermethylation of $p16^{INK4a}$ by HBx. 5′-Aza-2′dC is a universal inhibitor that can affect all DNMTs, including DNMT1. Thus, we attempted to knock down DNMT1 expression in HBx-expressing cells to show whether the activated DNMT1 is responsible for the hypermethylation of $p16^{INK4a}$ promoter by HBx. Introduction of DNMT1 siRNA into HBx-expressing cells almost completely abolished the HBx-mediated induction of $p16^{INK4a}$ promoter methylation (Fig. 5*A*) and repression of its promoter activity (Fig. 5*B*). Silencing DNMT1 expression also up-regulated $p16^{INK4a}$ protein level in the presence or absence of HBx (Fig. 5*C*). Accordingly, both E2F1 level (Fig. 5*C*) and DNMT1 promoter activity (Fig. 5*D*) were down-regulated. These results suggest that DNMT1 not only down-regulates $p16^{INK4a}$ via DNA methylation but also activates its own expression via a positive feedback loop involving E2F1.

The increased cyclin D1, but not the decreased $p16^{INK4a}$, is essential for DNMT1 activation by HBx. According to Fig. 2, activation of DNMT1 by HBx results from either the increased cyclin D1 and/or the decreased $p16^{INK4a}$. To evaluate their relative importance in DNMT1 activation by HBx, we attempted to remove each effect and observed changes in the activation of DNMT1 by HBx. Either treatment with 5′-Aza-2′dC or expression of exogenous $p16^{INK4a}$ almost completely abolished the effect of HBx on $p16^{INK4a}$, whereas neither affected the up-regulation of cyclin D1 in HBx-expressing cells (Fig. 3*A* and *C*). Under these conditions, the effects of HBx on both E2F1 and DNMT1 were partially (~32% and ~25%, respectively) decreased. The effect of HBx on CDK activity was similarly affected by treatment with 5′-Aza-2′dC (Fig. 3*B*). These results suggest that the decreased $p16^{INK4a}$ plays a role in the activation of DNMT1 but is not essential for it. In addition, the increased cyclin D1 alone seems to be sufficient to activate DNMT1 expression as observed in the absence of $p16^{INK4}$ down-regulation (Fig. 3*A* and *C*).

In contrast, removal of the effect of HBx on cyclin D1 using a specific siRNA completely abolished activation of DNMT1 by HBx (Fig. 3*C, lanes 5* and *6*), suggesting that up-regulation of cyclin D1 by HBx is indispensable for the activation of DNMT1. Under this condition, both control and HBx-expressing cells expressed high levels of $p16^{INK4a}$ presumably due to the decreased DNMT1. Moreover, the $p16^{INK4a}$ down-regulation by HBx was not observed under this condition, suggesting that it relies on the activation of DNMT1 initiated by up-regulation of cyclin D1 by HBx. Based on these observations, we suggest that up-regulation of cyclin D1 is a prerequisite step for the activation of DNMT1 expression by HBx.

E2F1-mediated activation of DNMT1 is responsible for the inactivation of E-cadherin expression by HBx. Finally, we attempted to provide biological significance of DNMT1 activation by HBx. According to our previous report, HBx represses E-cadherin expression via DNA methylation (18). Therefore, we investigated whether this effect results from the E2F1-mediated activation of DNMT1 by HBx. According to MSP experiments, E-cadherin promoter was mostly unmethylated in the control cells but was converted to methylated state in the presence of HBx (Fig. 6*A, lanes 1* and *2*). This effect was completely abolished

when DNMT activity was inhibited by treatment with 5′-Aza-2′dC (Fig. 6*A, lanes 3* and *4*). When E2F1-expressing plasmid was transfected into the control cells, the methylated E-cadherin promoter became dominant over the unmethylated one (Fig. 6*A, lanes 5* and *6*). In addition, E-cadherin promoter in HBx-expressing cells reverted to the unmethylated state when levels of endogenous E2F1 in HBx-expressing cells decreased by a specific E2F1 siRNA (Fig. 6*A, lanes 7* and *8*). A similar result was obtained with the ectopic E-cadherin promoter (Fig. 6*B*). In contrast, the promoter of p73 was highly methylated irrespective of HBx and was unaffected by the level of E2F1 (Fig. 6*A*), suggesting that its methylation status is not regulated by DNMT1. Consistent with MSP results, both the promoter activity and protein level of E-cadherin in the control cells were down-regulated by the exogenous E2F1, whereas both were up-regulated when levels of the endogenous E2F1 in HBx-expressing cells were decreased by the action of E2F1 RNAi (Fig. 6*B* and *C*). Thus, we conclude that HBx inactivates E-cadherin expression via DNA methylation through the E2F1-mediated activation of DNMT1.

Discussion

As the principal oncoprotein of HBV, HBx has been implicated in a variety of functions, which seems to reflect its differential distribution within cells (30). In the nucleus, HBx can affect transcription of several genes involved in transformation, cell cycle regulation, apoptosis, and cell adhesion through its association with transcriptional activators (31). However, most of its roles in cancer progression seem to be executed in the cytoplasm where it interacts with and activates several signal signaling molecules that lead to the transcriptional up-regulation of several cellular genes, including those for growth factors and oncogenes (30, 32). The present study proposed a novel epigenetic regulatory mechanism of HBx, which leads to DNA methylation-mediated repression of TSGs.

Aberrant DNA methylation is the most common molecular lesion of tumor cells, and the potential causative factor investigated is overexpression of one or more DNMT enzymes (7, 8). Indeed, elevated expression of DNMT1 has been reported in carcinomas of the colon, lung, liver, and prostate (13, 15, 33). However, the molecular mechanism that leads to this phenomenon is unknown. Recently, McCabe et al. (19) showed that E2F up-regulates DNMT1 expression in murine and human cell lines of epithelial and fibroblast origin. Based on this finding, they argued that activation of the pRb-E2F pathway is closely linked to induction of DNA hypermethylation of TSGs in tumorigenesis. This concept is in correlation with our recent finding that HBx activates DNMT1 expression (18).

According to our present study, HBx activates DNMT1 expression via a positive regulatory circuit (Fig. 6*D*). Up-regulation

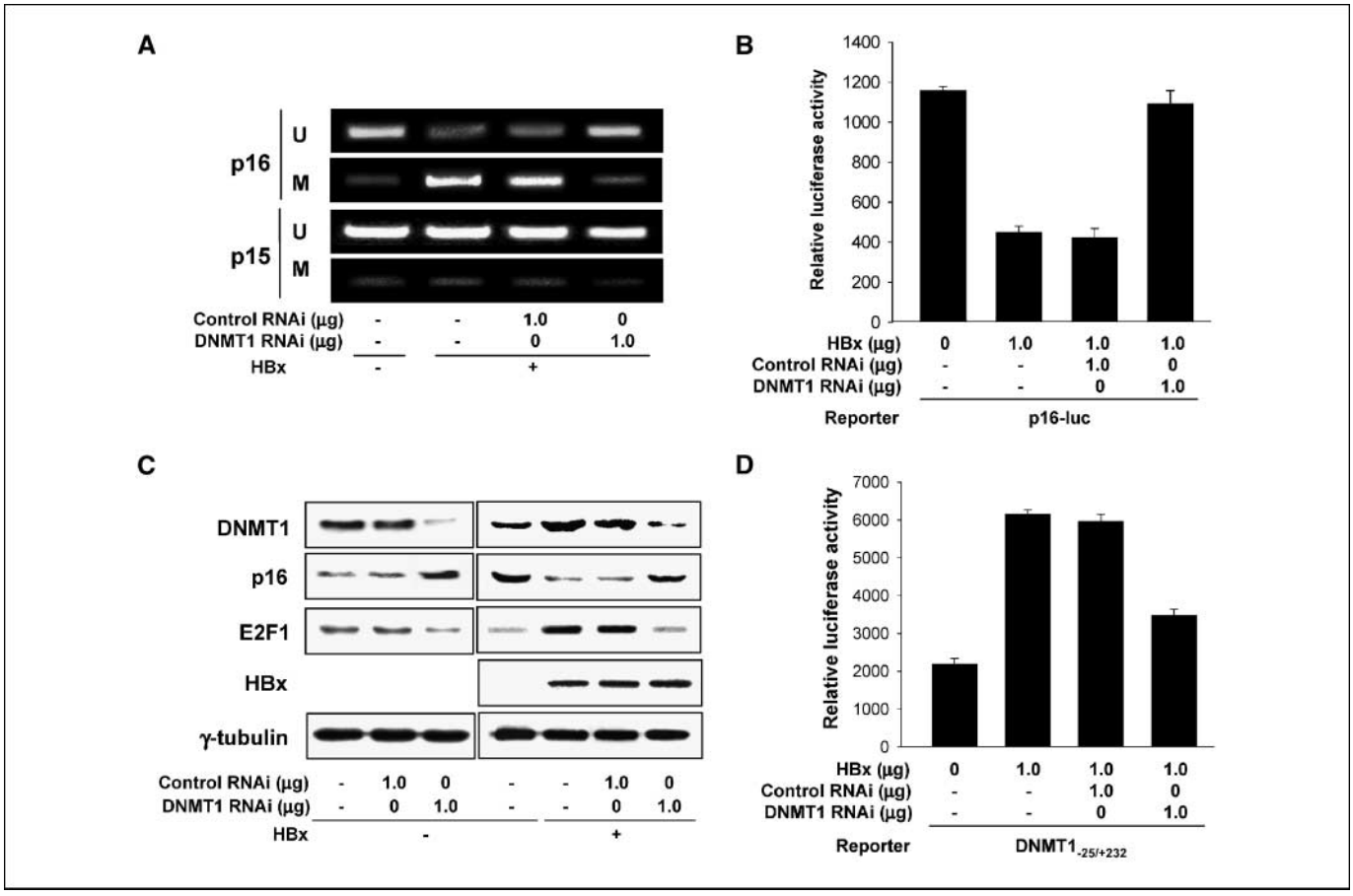

Figure 5. DNMT1 is responsible for the promoter hypermethylation by HBx. Either empty vector or DNMT1 RNAi plasmid was transfected into HepG2 stable cell lines. *A,* MSP analysis was done as described in Fig. 4*A*. *B,* HepG2 cells were transfected with p16-luc in the presence or absence of HBx followed by luciferase assay. *C,* levels of DNMT1, $p16^{INK4a}$, and E2F1 were determined by Western blots. *D,* HepG2 cells were transfected with $DNMT1_{-25/+232}$ in the presence or absence of HBx followed by luciferase assay.

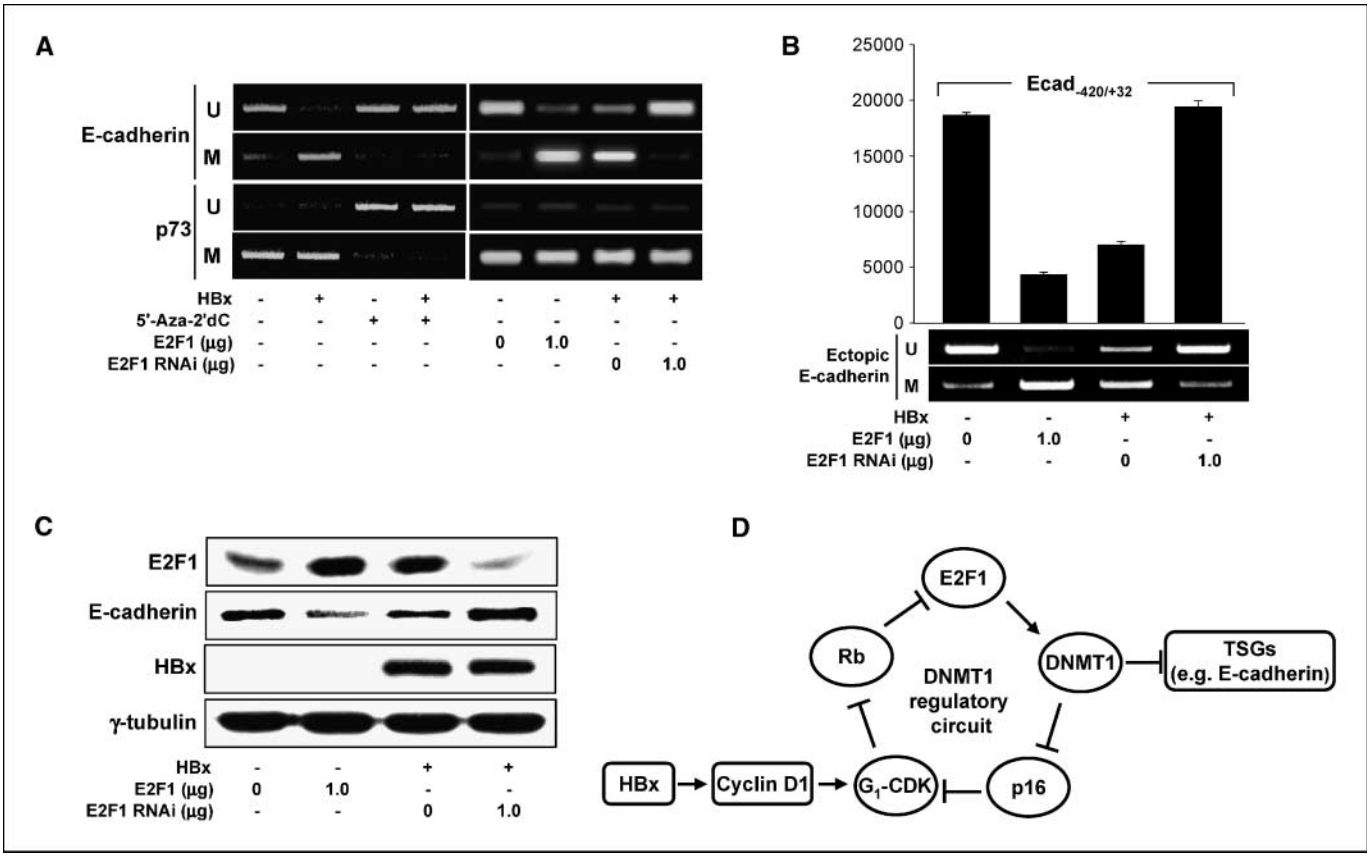

Figure 6. HBx inactivates E-cadherin expression via E2F1-mediated activation of DNMT1. *A,* HepG2 stable cells with or without HBx were treated with 5′-Aza-2′dC as described in Fig. 4*A* (*lanes 1–4*). For *lanes 6* and *8*, HepG2-vector and HepG2-HBx were transfected with E2F1 expression vector and E2F1 RNAi, respectively. MSP analysis was done to determine the methylation patterns of E-cadherin and p73. *B,* $Ecad_{-420/+32}$ (18) was cotransfected with either E2F1 or E2F1 RNAi as in (*A*) and luciferase assay was done. To determine the methylation pattern of ectopic E-cadherin promoter in $Ecad_{-420/+32}$, MSP was done as described in Materials and Methods. *C,* Western blots were done to measure levels of E2F1, E-cadherin, and HBx. *D,* a proposed model for the DNMT1 regulatory circuit and its activation by HBx. HBx initiates the circuit via up-regulation of cyclin D1, which leads to activation of G_1-CDK. Following phosphorylation/inactivation of pRb, the active E2F1 transcriptionally activates DNMT1 expression. The increased DNMT1 then inhibits expression of TSGs, such as $p16^{INK4a}$ and E-cadherin, via promoter hypermethylation. In the absence of $p16^{INK4a}$, G_1-CDK reinitiates another round of the circuit, which results in amplification of DNMT1 expression.

of cyclin D1 by HBx is likely to initiate the circuit and thus is obligatory for the activation of DNMT1 expression (Fig. 3*C*). The active CDK4/6 in a complex with cyclin D1 phosphorylates pRb, resulting in its dissociation from E2F1 (Fig. 2*A*). The free E2F1 then activates transcription of DNMT1 through binding at its cognate site on the DNMT1 promoter (Fig. 1). The increased DNMT1 expression then induces DNA hypermethylation of TSGs, including E-cadherin, to inhibit their expression (Fig. 6), which can result in several transformation phenotypes, as observed previously by the exogenous supplementation of DNMT1 (34, 35). Interestingly, $p16^{INK4a}$, an important TSG, also serves as a potential target for DNMT1 and is inactivated through hypermethylation on DNMT1 activation (Fig. 4). As a member of Ink4 family of CKIs, $p16^{INK4a}$ acts as a negative regulator of cell cycle progression through the inhibition of CDK4/6 and interactions with cyclin D1 (29). Thus, in the absence of $p16^{INK4a}$ expression, G_1-CDK can trigger another round of the circuit to amplify the activation of DNMT1. Without this amplification process, HBx can execute its potential to up-regulate DNMT1 only through the cyclin D1–dependent E2F1 activation and thus the effect is expected to be lower, as shown by either treatment with 5′-Aza-2′dC (Fig. 3*A*) or complementation of $p16^{INK4a}$ (Fig. 3*C*). In addition, a strong correlation between the protein level and promoter activity of DNMT1 was observed (Fig. 5*C* and *D*), which confirms that expression of DNMT1 is regulated via a positive feedback loop.

According to our model, expression of DNMT1 can be activated by a stimulus that either directly or indirectly modulates a member of the circuit. The increased DNMT1 in turn activates the pRb-E2F pathway, which can lead to cell cycle progression through the restriction point in late G_1 (29). Thus, the circuit may provide a reasonable explanation for the ubiquitous alterations of both DNA methylation and the pRb-E2F pathway in human tumors. In addition, our model may serve as a generic pathway by which a viral oncoprotein could activate DNMT1 expression and stimulates tumorigenesis. Indeed, large T antigen of human polyomavirus BKV activates DNMT1 through the pRb-E2F pathway (36). In addition, several types of cancer exhibit activation of the pRb-E2F pathway independently of viral infection (37). For example, in a variety of human malignant tumors and cell lines, the *$p16^{INK4a}$* gene is inactivated by various genetic mechanisms, including point mutations, homozygous deletions, and hypermethylation of CpG islands in the promoter (9, 26). In addition, overexpression of cyclin D1 is one of the most commonly observed alterations in human tumors (25, 38). Therefore, activation of DNMT1 through this circuit might be generic mechanism in human cancer and a potential therapeutic target.

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