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DNA methyltransferase inhibitor assay system based on the HBx-induced DNA methylation of E-cadherin

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ABSTRACT

We here report a simple assay system for DNA methyltransferase (DNMT) inhibitors based on the HBx-induced DNA methylation of E-cadherin. A stable cell line named G1 was generated by co-transfecting E-cadherin luciferase reporter and HBx-expression plasmid into HepG2 cells. Treatment of G1 cells with DNMT inhibitors, 5-azacytidine, 5-aza-2′-deoxycytidine, and procainamaid, dose-dependently inhibited DNA methylation of E-cadherin promoter in the reporter, resulting in up-regulation of luciferase levels and its enzyme activity. Treatment with all-*trans* retinoic acid that is known to inhibit DNMT expression, also induced similar effects. Our system can be useful for development of epi-drugs targeting DNA methylation in malignancies.

1. 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 (DNMTs) that use S-adenosylmethionine as the methyl donor [1]. DNMT1 is responsible for copying and maintaining methylation patterns after DNA replication whereas both DNMT3a and DNMT3b function as de novo methyltransferases [1,2]. Hypermethylation of 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].

DNA methylation is recognized as an important epigenetic control over different genome functions including differential gene expression [3], allele-specific expression in parental imprinting [4] and X inactivation (6). In addition, recent studies have revealed how methylation anomalies play a direct causal role in tumorigenesis and genetic diseases [5,6]. Aberrant hypermethylation of tumor suppressor genes involved in the cell cycle, DNA repair, metabolism of carcinogens, cell-to-cell interaction, apoptosis and angiogenesis is frequently detected in human tumors [2,5,6], resulting in the loss of the corresponding gene function in a tumor-type-specific pattern. Therefore, alterations in DNA methylation are widely recognized as an important factor involved in the initiation and progression of cancer in addition to abnormal genetic events.

The prevalence of DNA methylation in human cancers and their general reversibility has urged the development of drugs that target the enzymes that mediate epigenetic modifications. Two prominent examples are the cytosine analogs 5-azacytidine (5-aza-C) and 5-aza-2′-deoxycytidine (5-aza-2′dC), which are potent inhibitors of DNMTs and have been approved for the treatment of myeloid malignancies [7–9]. However, several drawbacks of these nucleoside analogs limit their usages in cancer treatment. Both 5-aza-C and 5-aza-2′dC can be incorporated into RNA and DNA, increasing their side effects by acting in resting and dividing cells [10]. In addition, these nucleoside analogs are quite unstable because they are readily hydrolyzed in aqueous solution and subject to deamination by cytidine deaminase. To improve the stability and efficacy of 5-azanucleosides, some non-nucleoside DNMT inhibitors have been developed and are under preclinical or clinical stages on the purposes of cancer treatment [7]. It is quite difficult to obtain new DNMT inhibitors because the current assay systems to measure the potential to inhibit DNA methylation both *in vivo* and *in vitro* are too complicate to be employed in the high throughput screening of chemical libraries. Therefore, it is necessary to develop a simple and rapid assay system for DNMT inhibitors that facilitates development of new drugs targeting DNA methylation in tumors.

According to previous reports, hepatitis B virus X protein (HBx) up-regulates levels of DNMT1 and 3b to induce promoter hypermethylation of tumor suppressor genes including E-cadherin [11–13]. The elevated DNMT activity in the presence of HBx thus can provide an excellent target for the development of potential DNMT inhibitors. Based on the HBx-induced DNA methylation of E-cadherin, we attempted in the present study to establish a

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simple and rapid assay system for DNMT inhibitors. First, we verified that the E-cadherin promoter not only in its original context but also in front of luciferase gene in the reporter plasmid is epigenetically regulated by HBx. Second, we generated a stable cell line by cotransfecting a luciferase reporter containing E-cadherin promoter and HBx-expression plasmid into a human hepatoma cell line, HepG2. Third, we tested whether both levels of luciferase and its activity in the stable cell line are strictly dependent to the DNA methylation status of E-cadherin promoter after treatment with several different kinds of DNMT inhibitors.

2. Materials and methods

2.1. Plasmids

pCMV-3 × HA1-HBx (HBx) encodes HBx downstream of three copies of the influenza virus heamagglutinin (HA) epitope [14]. The E-cad-luc, which contains the sequence from −420 to +32 of E-cadherin promoter, was described before [11].

2.2. Cell lines and transfection

HepG2 (KCLB No. 58065), a human hepatoblastoma-derived cell line, was obtained from the Korean Cell Line Bank. Stable cell lines were established by transfection of pCMV-3 × HA1-HBx alone or in a combination with E-cad-luc followed by selection with 500 μg/ml G418 (Gibco). For transient expression, 2×10^5 cells per 60-mm dish were transfected with an increasing amount of HBx-expression plasmid with the use of the WelFect-EX™PLUS (WelGENE) following the manufacturer's instructions. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum.

2.3. Western blot analysis

Cells were lysed in buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP-40) supplemented with protease inhibitors. Cell extracts were separated by SDS–PAGE and transferred onto a nitrocellulose membrane (Hybond PVDF, Amersham). Membranes were then incubated with DNMT1, DNMT3b, E-cadherin, luciferase antibodies (Santa Cruz Biotechnology), HA antibody for HBx (Roche) and γ-tubulin antibodies (Sigma) and subsequently with the appropriate horseradish peroxidase-conjugated secondary antibodies: anti-mouse IgG (H + L)-HRP (Bio-Rad) and anti-rabbit IgG (H + L)-HRP (Bio-Rad). The ECL kit (Amersham) was used to visualize protein bands via the ChemiDoc XRS imaging system (Bio-Rad).

2.4. Polymerase chain reaction (PCR)

For semi-quantitative RT-PCR, total RNA (3 μ) extracted using RNeasy mini kit (Qiagen) was reverse transcribed with the corresponding antisense primer. One-quarter of the reverse-transcribed RNA was amplified with *Taq* polymerase to detect levels of E-cadherin, HBx, and GAPDH as described before [11]. To detect DNA levels of HBx, E-cadherin, and luciferase in G1 cells, genomic DNA (100 ng) isolated from the cells using QIAamp DNA mini kit (Qiagen) was amplified with *Taq* polymerase (95 °C for 5 min; 30 cycles at 95 °C for 30 s, 56 °C for 30 s; 72 °C for 30 s; 72 °C for 5 min) using the following primer pairs: HBx, 5′-ACC GAA TTC CCA TGG CTG CT-3′ and 5′-AAC TCT AGA TGA TTA GGC AGA GGT-3′; E-cadherin, 5′-TGC AGG TAC CAT AAC CCA CC-3′ and 5′- CCC TCA GCC AGG CAG CGG T-3′; luciferase, 5′-TGG TCT GCC TAA AGG TGT CG-3′ and 5′-ATG TAG TCT CAG TGA GCC C-3′.

2.5. Methylation-specific PCR (MSP) and bisulfite DNA sequencing

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 °C for 16 h. For MSP, the modified DNA (100 ng) was amplified with *Taq* polymerase using both methylated and unmethylated primer pairs of DAPK, E-cad, RAR-β, Rassf1a, p14, p16, and p73 as described before [15]. For bisulfite DNA sequencing, modified genomic DNA was amplified by PCR using the sense primer 5′-ATT TTA GGT TAG AGG GTT AT-3′ and either 5′-CAA ACT AAA TAC TTT AC-3′ [15] or 5′-TTA CAT CTT CCA TAA TAA CT-3′ to detect the GpC-rich region of E-cadherin promoter (−179 to +30) from either genomic DNA or E-cad-luc, respectively. The PCR products were subcloned into the pGEM-T Easy vector (Promega) and their nucleotide sequences were determined.

2.6. Luciferase and DNMT activity assays

2×10^5 cells per 60-mm diameter plate were either mock-treated or treated with the indicated concentrations of 5-aza-2′dC (Sigma), 5-aza-C (Sigma), procainamaid (Sigma) or all-*trans* retinoic acid (ATRA; Sigma) for 48 h. Luciferase assay was performed using a luciferase assay system (Promega). DNMT activity in the cells prepared as above was measured using EpiQuick DNMT activity/inhibition Assay Ultra Kit (Epigentek) following the manufacturer's instructions.

2.7. Statistical ananlysis

The values indicate means ± S.D. from at least three independent experiments prepared in duplicate. The difference between the means of the treatment group and the control was assessed with the paired two tailed *t* test; difference was considered to be significant if $P < 0.05$.

3. Results

3.1. HBx down-regulates E-cadherin expression via DNA methylation

Initially, we examined whether HBx induces promoter hypermethylation of tumor suppressor genes that are known to be hypermethylated in the HCC associated with HBV infection [16,17]. Both transient and stable expression of HBx in HepG2 cells invariably induced promoter hypermethylation of E-cadherin, Rassf1a, RAR-β, p16, p14, DAPK in a dose-dependent manner (Fig. 1A). These effects were almost completely abolished when cells were treated with a universal DNMT inhibitor, 5-aza-2′dC. In contrast, the DNA methylation of p73 was little affected by HBx, indicating that HBx specifically induces DNA methylation of tumor suppressor genes. E-cadherin was selected for further studies because its DNA methylation by HBx is relatively well understood [11–13].

To confirm that HBx actually induces DNA methylation of E-cadherin, we performed bisulfite DNA sequencing analysis of genomic DNA isolated from HepG2 cells with or without HBx expression (Fig. 1B). DNA methylation was detected in a few CpG sites (5.1%) of the E-cadherin promoter in the control cells while it was much higher in the HBx-expressing cells (37.5%). In addition, both transient and stable expression of HBx resulted in down-regulation of RNA and protein levels of E-cadherin (Fig. 1C). These effects were almost completely abolished by treatment with 5-aza-2′dC, confirming that HBx down-regulates E-cadherin expression via DNA methylation.

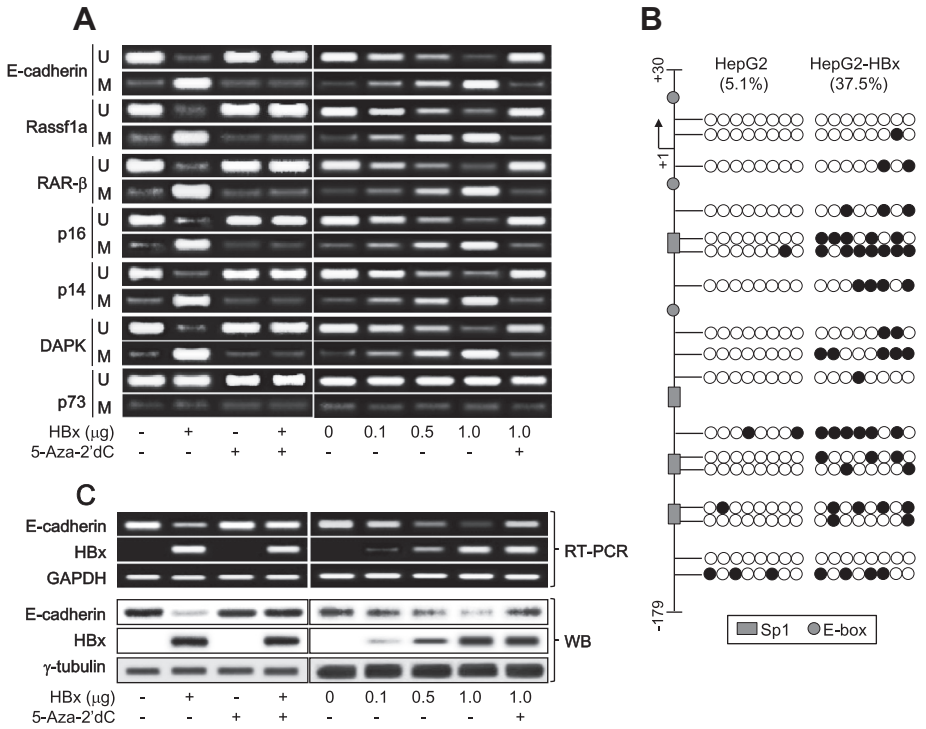

Fig. 1. HBx induces DNA methylation of E-cadherin to down-regulate its expression. (A) HepG2 cells either stably (lanes 2 and 4) or transiently (lanes 6 to 9) transfected with HBx-expression plasmid were either mock-treated (lanes 2, 6, 7, and 8) or treated with 5 μM 5-aza-2′dC (lanes 4 and 9) for 48 h. Genomic DNA isolated from these and their control cells was subjected to MSP to determine the methylation status of E-cadherin, Rassf1a, RAR-β, p16, p14, DAPK, and p73. (B) Bisulfite sequencing of the E-cadherin promoter region in HepG2 cells with or without HBx expression. The CpG sites in a 209 bp region (−179 to +30) of the E-cadherin promoter from eight different clones from each sample are shown as unmethylated (open circles) or methylated (filled circles). The positions of the Sp1 binding sites, E-boxes, and transcription initiation site are indicated. (C) HepG2 cells prepared as in A were subjected to RT-PCR (upper panel) and Western blot analysis (lower panel) to measure RNA and protein levels of E-cadherin and HBx, respectively. GAPDH and γ-tubulin were used as internal controls.

3.2. Generation of a stable cell line reporting the status of E-cadherin promoter methylation in the presence of HBx

As the HBx-induced DNA methylation of E-cadherin can be abolished by 5-aza-2′dC in a dose-dependent manner, we attempted to establish a simple assay system to evaluate the potentials of DNMT inhibitors based on this phenomenon. For this purpose, we first generated a stable cell line by co-transfecting pCMV-3 × HA1-HBx [14] and E-cad-luc [11] into HepG2 cells. After several steps of selection procedures, we finally obtained a stable cell line named G1. The G1 cells exhibited altered morphologies probably due to impaired E-cadherin expression by HBx, which makes them loosely interconnected each other (Fig. 2A and F). The stable integration of both HBx-expression plasmid and E-cadherin reporter plasmid was confirmed by detecting them in the chromosomal DNA of G1 cells (Fig. 2B). Both the enzyme activity and protein levels of DNMT1 and 3b were up-regulated in the G1 cells compared to the control cells (Fig. 2C and D). As a consequence, E-cadherin promoter from the endogenous gene (Fig. 2E) and the transfected reporter plasmid (Fig. 3) was hypermethylated in the G1 cells. In addition, both luciferase protein and its enzyme activity were detected in the G1 cells but not in the control cells (Fig. 2F and G). These results suggest that G1 cells harbor a stably integrated form of E-cadherin reporter plasmid in which luciferase expression is down-regulated by HBx via DNA methylation.

3.3. The expression of luciferase gene in the G1 cells is regulated by the DNA methylation status of the E-cadherin promoter

Next, we examined whether the G1 cell line is suitable for the measurement of the potential of DNMT inhibitors. Treatment with 5-aza-2′dC lowered DNMT activity in the G1 cells in a dose-dependent manner (Fig. 3A). Accordingly, the DNA methylation of the E-cadherin promoter from the reporter plasmid in the G1 cells was dramatically decreased by treatment with 5-aza-2′dC, as demonstrated by both bisulfite DNA sequencing (Fig. 3B) and MSP analyses (Fig. 3C). As a consequence, both the luciferase protein level and its enzyme activity in the G1 cells were dose-dependently up-regulated by 5-aza-2′dC (Fig. 3D and E). These results indicate that 5-aza-2′dC up-regulates luciferase expression by abolishing the HBx-mediated promoter hypermethylation of the reporter plasmid as demonstrated in the endogenous E-cadherin (Fig. 2E and 3D).

Finally, we attempted to show that our system can be applied to general DNMT inhibitors but not restricted to 5-aza-2′dC. For this purposes, we employed two other DNMT inhibitors to test their potentials to up-regulate the luciferase expression in the G1 cells by inhibiting DNA methylation. Treatment of G1 cells with either another nucleoside analog 5-aza-C or a non-nucleoside analog procainamaid [7] induced promoter hypomethylation of E-cadherin promoter from the reporter plasmid (Fig. 4A and B), resulting in up-regulation of luciferase activity (Fig. 4D and E) and its protein levels (Fig. 4G and H) in the G1 cells. In addition, treatment with ATRA, which is recently known to induce promoter hypomethylation of E-cadherin promoter by down-regulating levels of DNMTs [18] also up-regulated luciferase expression in the G1 cells (Fig. 4C, F and I). These results indicate that our system can be applied not only to compounds that directly inhibits DNMTs but also to those that indirectly inhibits DNMT activity via modulation of DNMT expression.

4. Discussion

Epigenetic alterations are involved in the initiation and progression of cancer in addition to abnormal genetic events. Many tumor

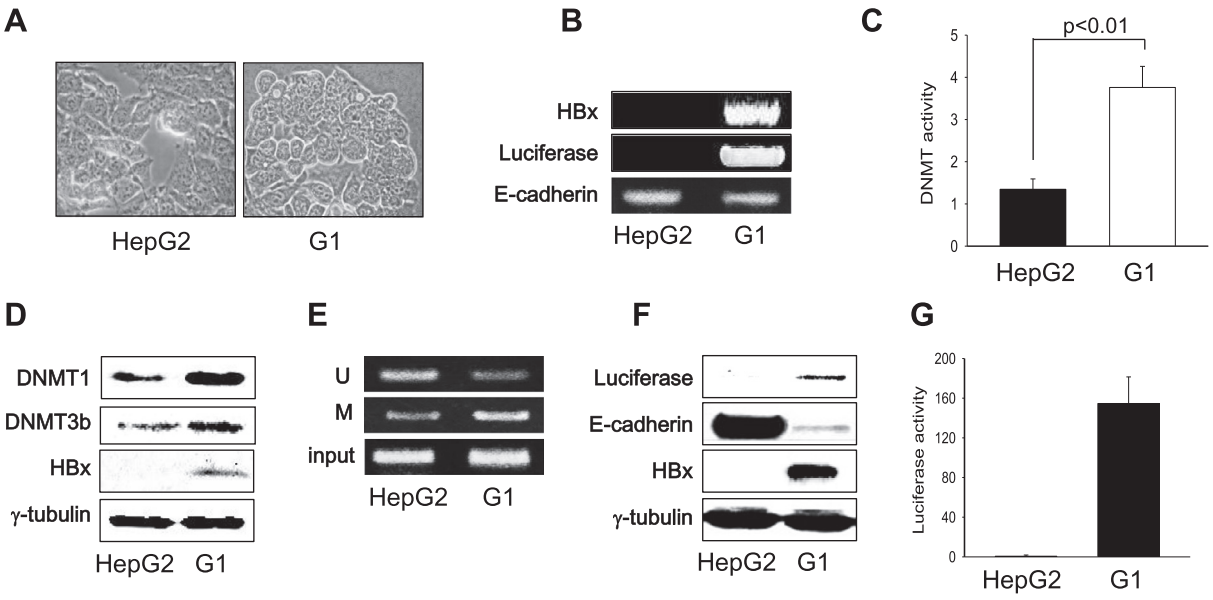

Fig. 2. Construction of a stable cell line reporting the extent of DNA methylation in the E-cadherin promoter in the presence of HBx. HepG2 and G1 cells were compared in the following aspects: (A) Cell morphology; (B) DNA levels of luciferase and E-cadherin; (C) DNMT activity; (D) protein levels of DNMT1 and DNMT3b; (E) DNA methylation of chromosomal E-cadherin promoter; (F) protein levels of luciferase and E-cadherin; (G) luciferase activity.

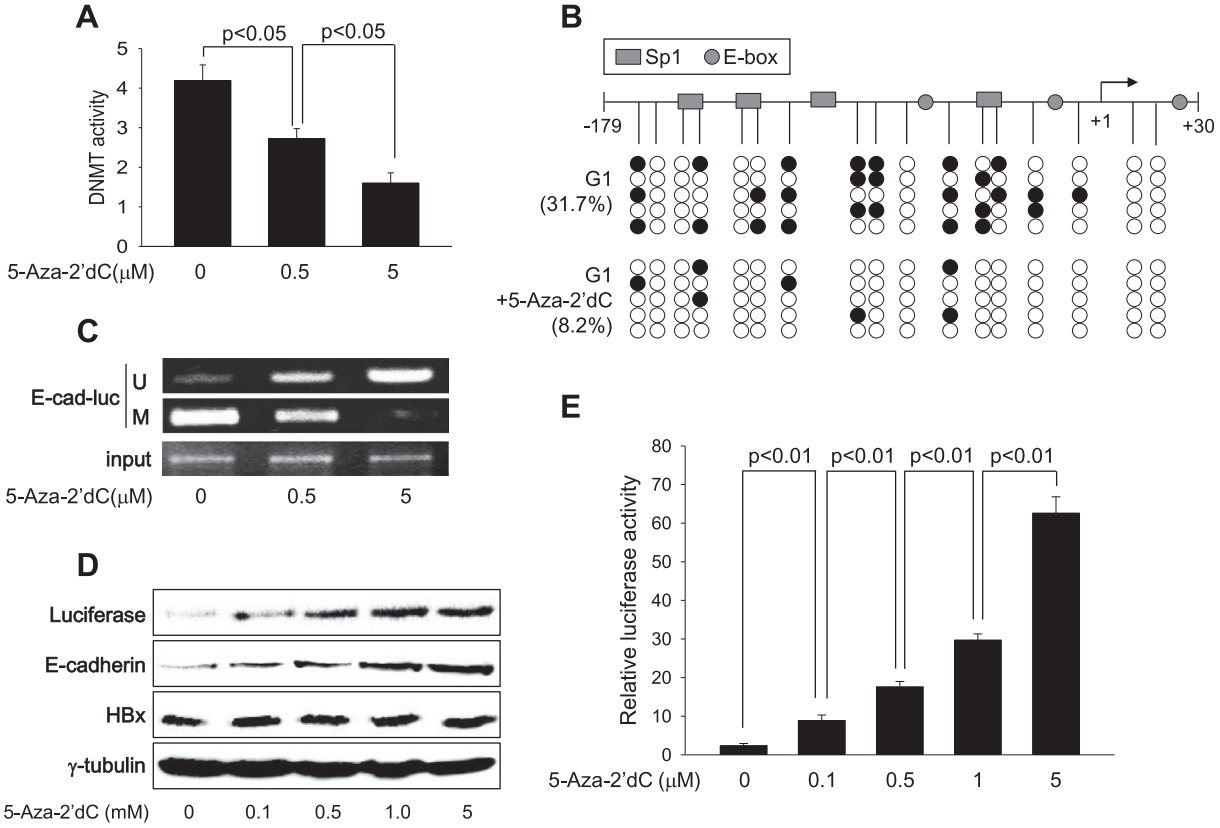

Fig. 3. DNA methylation of E-cadherin promoter in front of luciferase reporter gene negatively regulates luciferase expression. Treatment of G1 cells with increasing amounts of 5-aza-2′dC for 48 h resulted in step-wise decrease in DNMT activity (A), promoter hypomethylation of the reporter gene (B and C), enhanced luciferase and E-cadherin expression (D), and elevated luciferase activity (E). Cells were either mock-treated or treated with 5 μM 5-aza-2′dC for B.

suppressor genes, cellular functional genes, and miRNAs are known to be silenced by promoter hypermethylation in human malignant tumors [5,6,17]. In particular, epigenetic alterations, in contrast to genetic changes, can be pharmacologically reversed, restoring the function of silenced genes in the cancer cells [19]. Therefore, DNA methylation serves an excellent target for anticancer therapies. Several approaches have been tested to inhibit DNMT activity in the cancer cells [20]. These include small interfering RNA–mediated depletion of DNMTs [21–23], the use of nucleoside analogs such as 5-aza-C and 5-aza-2′dC for covalent enzyme trapping [8,24], and the rational development of small-molecule inhibitors such as RG108 [24,25], procainamaid [26] and hydralazine

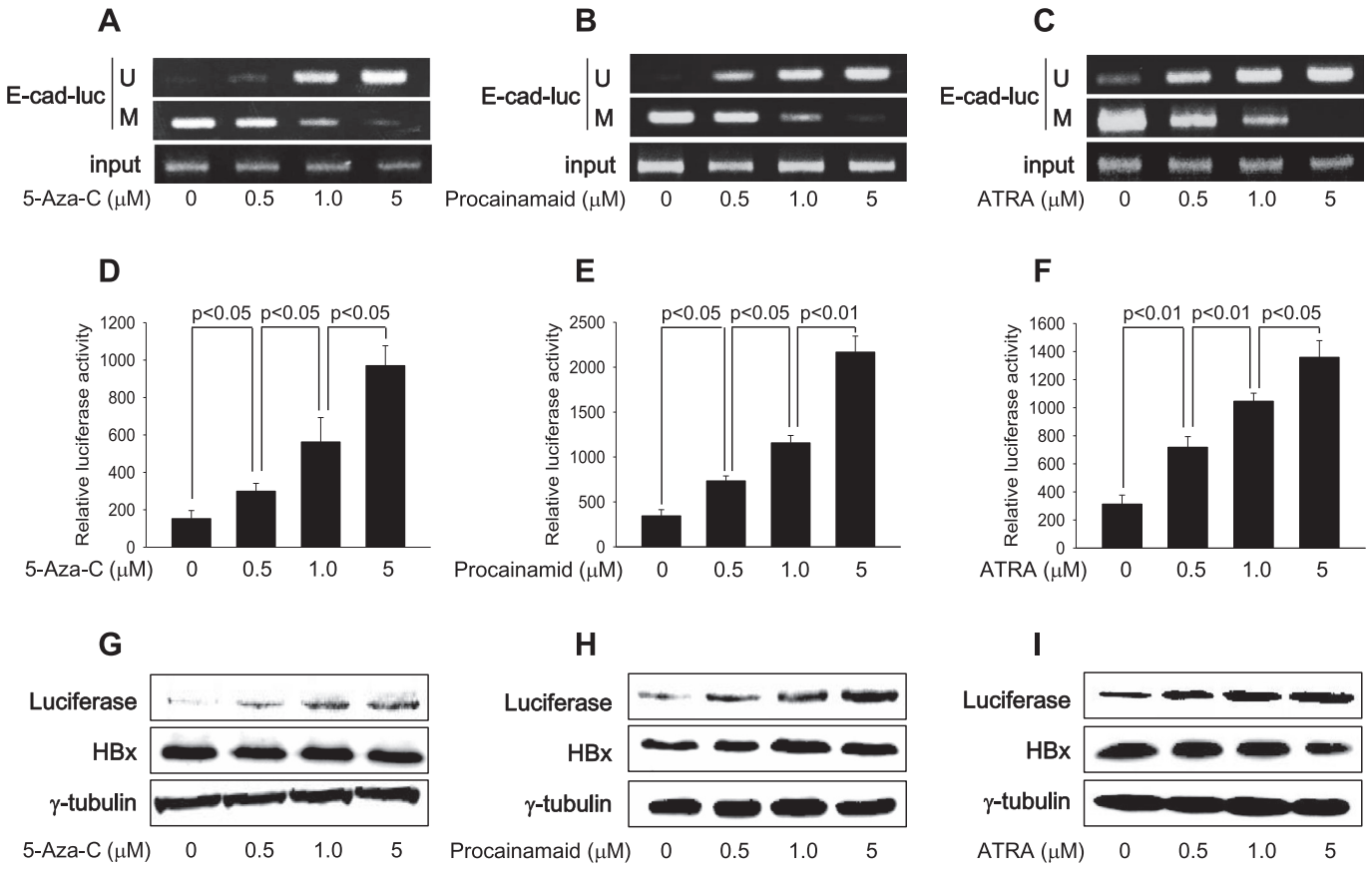

Fig. 4. The G1 cells serve a general DNMT inhibitor assay system. G1 cells were treated with increasing amounts of 5-aza-C (A, D, and G), procainamaid (B, E, and H), or ATRA (C, F, and I) for 48 h and were subjected to MSP (A, B, and C), luciferase assay (D, E, and F), and Western blot analysis (G, H, and I).

[27,28]. Some of these drugs were approved by the US Food and Drug Administration for the treatment of human malignancies like myelodysplastic syndrome or under clinical trials for other purposes [7,20]. In particular, the family of nonnucleoside DNMT inhibitors comprising a large variety of different chemical scaffolds is steadily growing [7].

A number of different approaches can be employed to test the potentials of chemical compounds to inhibit DNA methylation. In general, a relatively high enzyme activity is required to establish a cell-based assay system for DNMT inhibitors. In this respect, human malignant cells should be suitable as they are known to express relatively high levels of DNMTs [5,29]. However, most of the currently available human cancer cell lines seem to lose their potentials to overexpress DNMTs during cell culture adaptation as demonstrated in the HepG2 cells (Fig. 2). In addition, although MSP, bisulfite DNA sequencing analysis and DNMT activity assay are widely employed to measure DNA methylation in the cells, these procedures are too complicate to be employed in the high throughput screening system. In addition, DNMT inhibitors generally inhibit cell growth via up-regulation of tumor suppressors such as p14, p16 and p21 [20,30,31]. However, it is difficult distinguish DNMT inhibitors from the cytotoxic compounds if simply considering their effects on cell growth.

Several previous reports have demonstrated that HBx, the principal viral oncoprotein of HBV, induces promoter hypermethylation of tumor suppressor genes including E-cadherin [11–13]. For this effect, HBx is likely to elevate DNMT activity via activation of DNMT1 and DNMT3a [12,32]. HBx appears to activate DNMT1 expression by up-regulating its promoter activity via the E2F site [12] while the mechanism by which HBx activates DNMT3b expression remains to be clarified. Knock-down of either DNMT1 or DNMT3a almost completely abolishes the potential of HBx to induce DNA methylation of tumor suppressor genes [33], indicating that upregulation of both enzymes is required for the promoter hypermethylation of tumor suppressor genes by HBx. The elevated DNMT activity in the presence of HBx thus may provide an excellent target for the development of potential DNMT inhibitors.

The present study provides an effective DNMT assay system based on the potential of HBx to induce promoter hypermethylation by up-regulating levels of DNMTs. This system enables us to identify DNMT inhibitors simply by measuring their potentials to increase reporter activity. Cytotoxic compounds without the potential to inhibit DNMTs can be excluded simply because they cannot elevate the reporter activity. Using this system, we could isolate two novel DNMT inhibitors from a chemical library (data not shown). In addition, our screening system makes it possible to screen compounds like ATRA (Fig. 4C) that interrupt the HBx-mediated signaling pathway(s) leading to up-regulation of DNMTs. This kind of compounds may serve drug candidates for the treatment of HBV-associated HCC because the HBx-induced DNA methylation is considered to play essential roles during HCC development. Our system thus can be applied to compounds either directly inhibiting DNMTs or indirectly inhibiting the HBx-induced DNA methylation, serving an ideal assay system especially at the initial stage of DNMT inhibitor development.

Conflict of interest statement

None declared.

Acknowledgments

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