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Original Article

Virion protein 16 induces demethylation of DNA integrated within chromatin in a novel mammalian cell model

Lu Yang[1†], Huijun Wang[2†], Xin Luo[1], Pengliang Mao[1], Weidong Tian[3], Yujiang Shi[4], Guoying Huang[2], Jin Zhang[1*], and Duan Ma[1,2,4*]

[1]Key Laboratory of Molecular Medicine, Ministry of Education, Department of Biochemistry and Molecular Biology, Shanghai Medical College, Fudan University, Shanghai 200032, China
[2]Children's Hospital, Fudan University, Shanghai 200032, China
[3]School of Life Sciences, Institute of Biostatistics, Fudan University, Shanghai 200433, China
[4]Institutes of Biomedical Science, Fudan University, Shanghai 200032, China
[†]These authors contributed equally to this work.
*Correspondence address. Tel: +86-21-54237631; Fax: +86-21-54237632; E-mail: duanma@shmu.edu.cn (D.M.)/Tel: +86-21-54237860; Fax: +86-21-54237632; E-mail: jeanzhj@gmail.com (J.Z.)

DNA methylation and demethylation play important roles in mediating epigenetic regulation. So far, the mechanism of DNA demethylation remains elusive and controversial. Here, we constructed a plasmid, named with pCBS-luc, that contained an artificial CpG island, eight Gal4 DNA-binding domain binding site, an SV40 promoter, and a firefly luciferase reporter gene. The linearized pCBS-luc plasmid was methylated *in vitro* by DNA methyltransferase, and transfected into the HEK293 cells. The stable HEK293 transfectants with methylated pCBS-luc (me-pCBS-luc) were selected and obtained. The methylation status of the selected stable cell lines were confirmed by bisulfite sequencing polymerase chain reaction amplification. The methylation status could be maintained even after 15 passages. The virion protein 16 (VP16) was reported to enhance DNA demethylation around its binding sites of the promoter region in *Xenopus* fertilized eggs. Using our me-pCBS-luc model, we found that VP16 also had the ability to activate the expression of methylated luciferase reporter gene and induce DNA demethylation in chromatin DNA in mammalian cells. Altogether, we constructed a cell model stably integrated with the me-pCBS-luc reporter plasmid, and in this model we found that VP16 could lead to DNA demethylation. We believe that this cell model will have many potential applications in the future research on DNA demethylation and dynamic process of chromatin modification.

Keywords epigenetic; cell model; DNA methylation; demethylation; VP16

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Introduction

Heritable alterations in gene expression caused by mechanisms other than changes in the DNA sequence are called epigenetic alterations. Epigenetic events are reversible and contribute to chromatin structure stability, genome integrity, the modulation of tissue-specific gene expression, embryonic development, replication timing, genomic imprinting, as well as X-chromosome inactivation in females [1–4]. DNA methylation is one of the most important molecular mechanisms mediating epigenetic regulation. In mammals, DNA methylation often occurs at the cytosine residue in CpG islands (CGIs), which are short fragments of CpG-dense DNA. Approximately 50,200 such CGIs exist in the human genome, and at least 29,000 are in repeat-masked sequences, many of which surround the 5′ end of genes, particularly the promoter region or the first exon of a gene [5]. Methylation and demethylation of the promoter CGIs are alternative mechanisms of gene inactivation and activation. For example, the silence of a significant proportion of tumor-suppressor genes in human embryonic and adult stem cells has been associated with promoter DNA hypermethylation [6,7]. However, most hypermethylated promoters are occupied by methyl-CpG-binding domain proteins which have a high affinity *in vivo* for binding hypermethylated promoter CGIs [8]. Several studies have revealed that both global DNA hypomethylation and regional hypermethylation occur in tumorigenesis [9,10], highlighting the important roles of DNA methylation and demethylation in the carcinogenesis of human tumors [11]. DNA demethylation is a process of removal of methyl group from the nucleotide in DNA, which could be passive or active. Passive process takes place in the absence of

methylation of newly synthesized DNA strands by DNA methyltransferase 1 (DNMT1) during several replication rounds, such as using 5-aza-2′-deoxycytidine [12,13], trichostatin A [14,15], RNA interference [16], and microRNA to down-regulate DNMT [17]. Active DNA demethylation occurs via active dismiss of methyl group, which is involved in many vital developmental and physiological processes of plants and animals [18]. After the protamine–histone exchange, the sperm-derived paternal pronucleus undergoes active genome-wide DNA demethylation, an event that occurs quite rapidly within 4–8 h post-fertilization [19]. Active cytosine demethylation can be also triggered by a nuclear receptor, such as retinoic acid receptor, oestrogen receptor, etc. [20].

Li *et al*. [21] first reported that chimeric DNMTs, fused with Gal4 DNA-binding domain (DBD), could target DNA methylation to specific DNA sequences and repress the expression of target gene, which indicated that epigenetic modifiers fused with DBD domain have the ability to induce epigenetic alteration around the DBD-binding site. To study the mechanisms of local targeted DNA demethylation and the relation between DNA demethylation and chromatin histone modification in living cells, we constructed the cell lines stably transfected with methylated luciferase reporter plasmid, with DBD-binding sites in its promoter regions. For the first time, we found that chimeric virion protein 16 (VP16) transcription activating domain, fused with Gal4 DBD, could induce the DNA demethylation in mammalian cells.

Materials and Methods

Plasmid construction

The pCBS-luc plasmid used in this study was derived from the vector pcDNA 3.1, and consisted of a CpG island from *Streptomyces coelicolor*, eight Gal4 DBD-binding sites, an SV40 promoter, and a luciferase reporter gene. pMVP16 was constructed by digesting the vector pM (Clontech, Palo Alto, USA) with *EcoR*I (TaKaRa, Dalian, China), and ligated with an *EcoR*I fragment from the polymerase chain reaction (PCR) product which using plasmid pVP16 as the template. pMIP was derived from the vector pM and contained IRES-puromycin (IP), which was derived from pCAG-IP. pMIPVP16 was constructed based on pMIP, which was digested using *EcoR*I and ligated with an *EcoR*I fragment from the plasmid pMVP16.

The primers used for PCR were: VP16-F, 5′-GGG GAATTCATGGGCCCTAAAAAGAAGCGTAAAGTC-3′ and VP16-T, 5′-TCAGGTTCAGGGGGAGGT-3′.

In vitro methylation and methylation-sensitive restriction enzyme assay

The CpG sites in pCBS-luc were methylated *in vitro* using CpG methylase (*M.Sss*I) according to the manufacturer's instructions (New England Biolabs Inc., Beverly, USA). The product was purified using the Axygen DNA gel extraction kit (Axygen Biosciences, Shanghai, China). The efficiency and amount of methylation were determined by restriction analysis using the methylation-sensitive restriction endonuclease *Hpa*II (New England Biolabs Inc.).

A total of 1 μg methylated pCBS-luc (me-pCBS-luc) and unmethylated pCBS-luc (unme-pCBS-luc) were digested at 37°C for 3 h using 20 U *Hpa*II. Restriction enzyme digestion results were analyzed using 1% agarose electrophoresis.

Cell culture and transfection

The HEK293 cell line was obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, USA) supplemented with 5% fetal bovine serum and 5% new bovine serum (Gibco). All cell lines were incubated at 37°C in 5% CO_2.

All plasmid DNA used for transfection were extracted using a DNA Midiprep kit (Qiagen, Hilden, Germany). For both transient and stable transfections, cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions.

Monoclonal cell and stable cell line selection

To generate me (or unme)-pCBS-luc stable cell lines, the pCBS-luc plasmids were linearized with restriction enzyme *Pst*I and methylated *in vitro* using *M.Sss*I. HEK293 cells were co-transfected with linearized me (or unme)-pCBS-luc plasmid and anti-hygromycin gene expression vector in the ratio of 9:1. Stable pCBS-luc cell lines were selected with 100 μg/ml hygromycin B (Sigma, St Louis, USA) and, 3 weeks after transfection, surviving cell clones were selected and expanded.

The selected stable HEK293 transfectants were screened using a luciferase assay and PCR. PCR was performed to detect the stable integration of pCBS-luc into the HEK293 cells. Genomic DNA extracted from cell colonies was used as the template. PCR conditions were as follows: 1 cycle of 94°C for 3 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 30 s, and a final extension at 72°C for 1 min. The primers used for detection were CBS-F2, 5′-TCTGCGATCT GCATCTCAAT-3′ and CBS-T, 5′-AACAGTACCGGAAT GCCAAG-3′.

To select the stable cell lines containing both pCBS-luc and pMVP16, we transfected pMIPVP16 into the me-pCBS-luc stable HEK293 transfectants. As a negative control, we transfected pMIP into the me-pCBS-luc stable HEK293 transfectants. These cell lines were selected with 1 μg/ml puromycin.

Luciferase assay

To confirm the luciferase activity of pCBS-luc plasmid (me-pCBS-luc/unme-pCBS-luc), HEK293 cells were seeded in a 96-well plate. The cells were co-transfected with 2 ng of the internal control vector pRL-*Renilla* (Promega, Madison, USA), 40 ng of pCBS-luc (me-pCBS-luc/unme-pCBS-luc), and 160 ng of different plasmids (pM or pMVP16). To screen the me (or unme)-pCBS-luc stable HEK293 transfectants with luciferase assay, the me (or unme)-pCBS-luc stable HEK293 transfectants were seeded in a 96-well plate, followed by co-transfection with 2 ng of the internal control vector pRL-*Renilla* (Promega), and 200 ng of different plasmids (pM or pMVP16). pM was used as a negative control, and pMVP16 was used for transcriptional activation. At 48−72 h after transfection, the firefly and *Renilla* luciferase activities were assayed according to the manufacturer's protocol (Promega). Firefly luciferase activity was normalized on the basis of *Renilla* luciferase activity values for each sample. Relative luciferase value = firefly luciferase value/*Renilla* luciferase value.

DNA extraction, bisulfite treatment, and nested bisulfite sequencing PCR amplification

Genomic DNA was extracted from cells using the TIANamp Genomic DNA kit (Tiangen Biotech Beijing Co., Ltd, Beijing, China) according to the manufacturer's instructions. All extracted DNA samples were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA).

A total of 500 ng of extracted genomic DNA was bisulfite-converted using the EZ DNA methylation-Gold Kit (Zymo Research, Orange, USA) according to the manufacturer's recommendations. To obtain products for sequencing, the nested PCR amplification was performed with 50 ng of bisulfite-treated DNA. The first round of PCR was performed in a reaction volume of 20 μl consisting of 1× Taq reaction buffer, 0.25 mM of each dNTP, 0.4 μM of each primer, and 1.5 U of Taq DNA polymerase (Tiangen Biotech Beijing Co., Ltd). PCR conditions were as follows: 1 cycle at 94°C for 3 min followed by 45 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. A final extension was performed at 72°C for 1 min. The second round of PCR was performed in a 50-μl mixture containing 2 μl of first-round PCR products under the previously described conditions. The amplified fragments were purified using the Axygen DNA gel extraction kit (Axygen Biosciences), subcloned into the pTA2 vector (Toyobo Inc., Osaka, Japan) and sequenced.

The primers used for PCR were: CBSB-wF, 5′-TTATG GATTTGYGATTTGTATT-3′, CBSB-wT, 5′-TAAAAAC AATTATTCCAAAAACCA-3′, CBSB-F, 5′- TTGTATTT TAATTAGTTAGTAATTATAGTT-3′, and CBSB-T, 5′- TT ACAAAAACCTAAACCTCCAAAAA-3′.

Results

Construction of a luciferase reporter gene plasmid with methylated promoter

To obtain a stable cell model for studying DNA demethylation, we constructed a luciferase reporter gene vector, with eight Gal4 DBD-binding sites in the upstream of its SV40 promoter region [**Fig. 1(A)**]. The SV40 promoter had moderate activity and could activate the transcription of the luciferase reporter gene. DBD-binding sites can recruit the epigenetically modifying enzymes fused with DBD. DNA methylation and demethylation events can be easily monitored by measuring the expression of the luciferase reporter gene. pCBS-luc plasmid was linearized and methylated by DNMT *M.Sss*I *in vitro*. The efficiency and the level of methylation of me-pCBS-luc plasmid were verified with methylation-sensitive restriction enzyme *Hpa*II. As expected, unme-pCBS-luc plasmid was cleaved completely, with the bands ∼500 bp, whereas the bands of digested me-pCBS-luc ranged mainly from 2000 to >4500 bp [**Fig. 1(B)**]. The result indicated that *M.Sss*I-mediated CG methylation effectively inhibited *Hpa*II digestion, and we also noticed that me-pCBS-luc was partially digested by *Hpa*II, which might be resulted from incomplete methylation by *M.Sss*I because of high contents of CpG in pCBS-luc (502 CpGs in total). But the incomplete methylation status would not affect our subsequent experiments, because we would select the stable HEK293 transfectants according to the luciferase activity, theoretically by which the selected transfectants can be divided into three different methylation states: unmethylation, complete methylation, and partial methylation, and the partial methylation cells would also be useful for other experiments as a transition state. It is the premise for our subsequent experiments that DBD can bind the methylated DBD-binding sites, so we detected whether the fusion protein DBD-VP16-expressing plasmid (pMVP16) can activate luciferase reporter gene of me-pCBS-luc by transient transfection. As shown in **Fig. 1(C)**, luciferase activity was observed for both plasmids (me-pCBS-luc and unme-pCBS-luc), with the unmethylated plasmid showing significantly higher activity than the methylated plasmid.

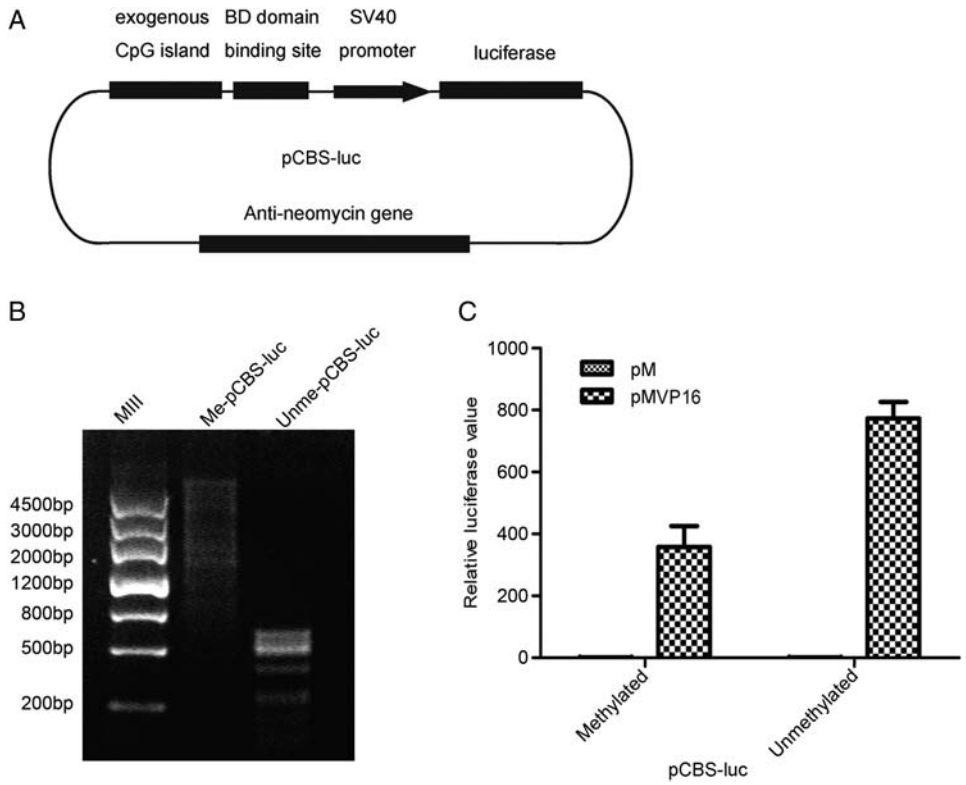

Figure 1 Methylate the luciferase reporter gene plasmid pCBS-luc (A) Schematic of the plasmid pCBS-luc used in this study. (B) Methylation-sensitive restriction enzyme assay of me-pCBS-luc and unme-pCBS-luc plasmids. Methylation of pCBS-luc inhibits the digestion by *Hpa*II, while unme-pCBS-luc was cleaved completely. (C) Luciferase expression of me-pCBS-luc plasmid can also be induced by pMVP16. me-pCBS-luc or unme-pCBS-luc was co-transfected with pMVP16 plasmid into HEK293 cells, respectively, and the firefly luciferase activity was assessed at 72 h after transfection. The pM plasmid was used as the negative control. The luciferase value of HEK293 transfected with pM plasmid was set as 1. *Renilla* luciferase was used to normalize the values of the experimental reporter gene. Normalized data were expressed as the mean ± SD.

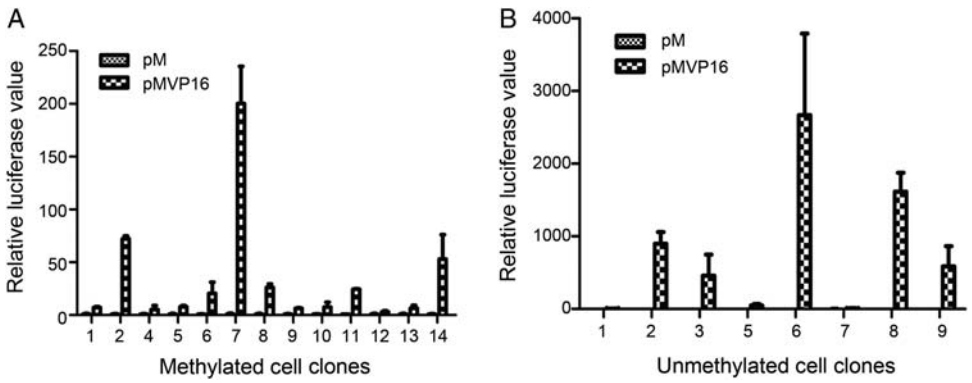

Figure 2 Selection of stable transfected cell lines (A) Select the stable me-pCBS-luc transfectants based on whether the luciferase expression can be induced by pMVP16. As described in the Materials and Methods, stable me-pCBS-luc HEK293 transfectants were generated by selection of hygromycin B. pMVP16 was transfected into the selected clones, and the luciferase activity was assayed at 72 h after transfection. The pM plasmid was used as the negative control, and the luciferase value of HEK293 transfected with pM plasmid was set as 1. *Renilla* luciferase was used to normalize the values of the experimental reporter gene. Normalized data were expressed as the mean ± SD. (B) Similar to the selection of me-pCBS-luc transfectants, stable unme-pCBS-luc transfectants were selected according to the luciferase activity induced by pMVP16.

Selection of cell lines with me-pCBS-luc and unme-pCBS-luc

As described in the Materials and Methods, stable methylated (or unmethylated) pCBS-luc HEK293 transfectants were generated by selection of hygromycin B. To simplify the first-round screening process, pMVP16 was introduced into the stable cell lines and cell clones were selected based on whether pMVP16 can induce the luciferase expression [**Fig. 2(A,B)**]. Two me-pCBS-luc stable cell lines were selected, with three unmethylated ones as

Figure 3 The characteristics and the stability of the cell model (A) The linearized methylated and unme-pCBS-luc stably existed in the selected transfectants. Six weeks after selection by hygromycin B, genomic DNA was extracted from the selected stable transfectants, and the promoter region of pCBS-luc was amplified by PCR (the upper). After 10 and 15 passages of growth, the genomic DNA of methylated cell line 7 was detected by PCR (the lower). pCBS-luc plasmid was used as the positive control. (B) BSP analysis of the promoter region of the pCBS-luc in methylated cell line 9, methylated cell line 7 and unmethylated cell line 8, respectively. (C) BSP analysis of the promoter region of the pCBS-luc in the 15th generation of methylated cell line 7 and unmethylated cell line 8. The black squares correspond to methylated Cs, the white squares correspond to unmethylated Cs, and the gray squares correspond to missing values.

Table 1 The methylation status and the relative luciferase value of three cell lines

	Methylated cell line 9	Methylated cell line 7	Unmethylated cell line 8
Methylation status (%)	100	64	0
Unmethylation status (%)	0	32	96
Relative luciferase value	6.41	200.36	1614.23

negative controls. Comparing the two methylated cell lines, we found that methylated cell line 9 showed less luciferase activity than line 7 [**Fig. 2(A)**].

Confirming the methylation status of the selected cell lines

The existence of pCBS-luc in the selected stable transfectants was confirmed by PCR. The linearized pCBS-luc still can be detected even after 15 passages of growth, which indicated that they have integrated into chromatin [**Fig. 3(A)**]. In addition, no mutation was found in pCBS-luc, which was confirmed by PCR product sequencing (data not shown). Nested bisulfite sequencing polymerase chain reaction amplification (BSP) was then used to detect the methylation level of the promoter region of pCBS-luc in the cell lines. As shown in **Fig. 3(B)**, methylated cell line 9 was 100% methylated, whereas methylated cell line 7 was partially methylated, and unmethylated cell line 8 was 100% unmethylated. As shown in **Table 1**, the basic and VP16-induced expression of luciferase in me-pCBS-luc cells was lower than in unme-pCBS-luc ones, which indicated that DNA methylation suppressed the expression of reporter genes. This result further confirmed that pMVP16 can bind the methylated promoter and activate luciferase expression in the context of chromatin. Methylated cell line 9 was 100% methylated and had almost no luciferase activity. However, the lack of reporter gene activity did not make it an ideal target for studying the dynamic changes in DNA demethylation. In contrast, methylated cell line 7 had moderate luciferase activity,

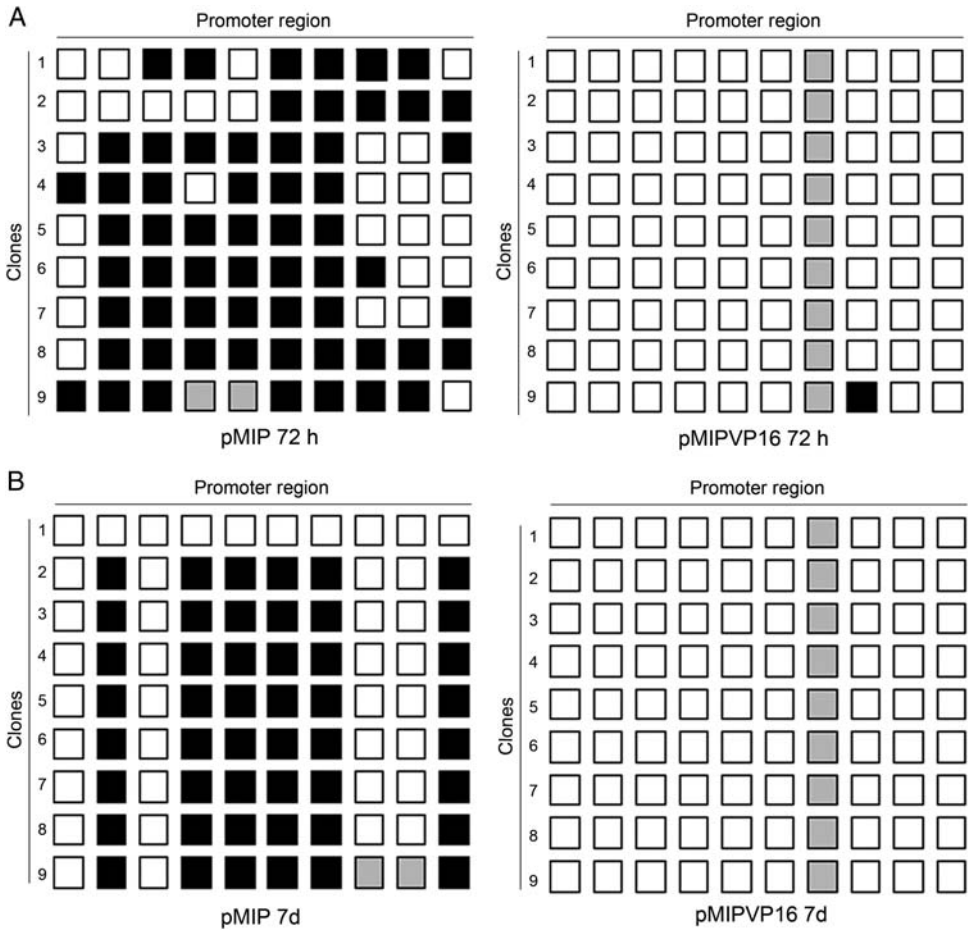

Figure 4 VP16 can induce demethylation of the promoter region in the me-pCBS-luc cell model (A) Methylation status of the pCBS-luc promoter region in methylated cell line 7 was analyzed by BSP at 72 h after transfection with pMIPVP16, using pMIP as negative control. (B) Seven days after transfection, methylation status was tested. The black squares correspond to methylated Cs, the white squares correspond to unmethylated Cs, and the gray squares correspond to missing values.

making it a more convenient tool to study both DNA demethylation and DNA methylation. Therefore, we selected methylated cell line 7 as the methylation model. For the unmethylated cell line, we selected cell line 8.

Before using the selected cell lines to study DNA methylation and demethylation, we sought to verify whether their methylation status was stable throughout generations of cell growth. For methylated cell line 7 and unmethylated cell line 8, we verified the methylation level of pCBS-luc using BSP sequencing [**Fig. 3(C)**]. The BSP sequencing results suggested that the methylation level of both two cell lines were stable even after 15 passages of cell growth.

Application of the constructed cell model to study DNA demethylation by VP16

VP16 is a viral protein that has a strong activation domain and can activate transcription by interacting with a DNA-binding domain [22,23]. In the experiments described above, VP16 was mainly used as a strong activator in the reporter luciferase assay to select cell clones. In addition to its role in transcriptional activation, VP16 has also been reported to enhance DNA demethylation in its binding promoter region in *Xenopus* fertilized eggs [24]. However, little is known about whether VP16 can also enhance DNA demethylation in mammalian cells. To demonstrate the applicability of our cell model for studying DNA demethylation, we used the cell model to investigate whether VP16 could also enhance DNA demethylation in mammalian cell lines. After transfecting pMIPVP16 into methylated cell line 7, we observed that there was only one methylation site at 72 h post-transfection and no methylation site at 7 days. In contrast, the transfection of pMIP did not lead to any change in the DNA methylation status [**Fig. 4(A,B)**]. Thus, we conclude that, using our constructed cell model, we observed for the first time that VP16 can induce DNA demethylation in mammalian cells under our experiment condition.

Discussion

In this study, we developed a cell model with stable DNA methylation status. The reporter gene vector, pCBS-luc,

was constructed, and then was linearized and methylated using *M.Sss*I *in vitro*, following by transfected into HEK293 cell lines, and the stable me (or unme)-pCBS-luc HEK293 cell lines were selected. The results of genomic PCR showed that the originally linearized pCBS-luc stably existed in the selected transfectants after 15 passages of growth, which indicated that the pCBS-luc had been integrated into chromatin [**Fig. 3(A)**]. Three types of cell lines (completely methylated, partially methylated, and completely unmethylated) were selected for BSP analysis to confirm their methylation status [**Fig. 3(B)**]. Mammalian DNMTs can be divided into: *de novo* DNMTs that could methylate hemimethylated and unmethylated CpG at the same rate (including DNMT3a and DNMT3b); and maintenance DNMTs that predominantly methylate hemimethylated CpG di-nucleotides in the mammalian genome and maintain the methylation pattern that had been established by the *de novo* DNMTs, including DNMT1, which is the most abundant DNMT in mammalian cells [25]. The existence of mammalian maintenance DNMTs is our theoretical basis for the establishment of stable me-pCBS-luc cell line. The BSP sequencing results suggested that the methylation status was maintained even after 15 passages [**Fig. 3(C)**]. As a control, the unmethylated cell line 8 was also stable.

The stable me-pCBS-luc cell model has two major advantages: (i) unlike the transiently transfected plasmid, the integrated pCBS-luc is packaged into chromatin, which more resembles the state of endogenous promoters; (ii) this is an easy-to-manipulate model. The luciferase reporter gene is easy to detect and quantify, by which the epigenetic modification's effects on gene expression can be easily analyzed and in turn epigenetic modification can be predicted according to the luciferase expression.

In our primary screening for the me-pCBS-luc stable transfectants, pMVP16 was introduced into the stable cell lines and shown to activate luciferase expression even at me-pCBS-luc stable transfectants. VP16 was reported to enhance DNA demethylation in the promoter region after interaction with a host DNA-binding domain in *Xenopus* fertilized eggs [24]. Our studies further revealed that pMVP16 can lead to a dramatic demethylation of the artificial CGIs of the promoter in our construct [**Fig. 4(A,B)**]. This result gives the protein experts a new sight to study the functional mechanism about VP16 in mammalian cells. Whether the DNA demethylation was activated by VP16-induced transcription activation as in *Xenopus* fertilized eggs needs further studies. The change of chromatin modification caused by the VP16-induced DNA demethylation is also an interesting project. Therefore, we have not only constructed a novel cell model for studying DNA demethylation but also demonstrated for the first time that VP16 can induce DNA demethylation in mammalian cells. Recently, two cell models were reported to study DNA demethylating agents [26,27]. We proposed that the me-pCBS-luc cell model can also be suitable for high-throughout screening of DNA methylation inhibitors and some putative DNA demethylase, such as the recently discovered ten-eleven translocation family proteins [28].

Furthermore, as described in the study by Li *et al*., [21] DBD-fused DNMTs can methylate the transiently transfected plasmids. However, transient transfected DNA plasmids are not able to reflect the real status of chromatin DNA, which can be solved in our unme-pCBS-luc cell lines. Me-pCBS-luc and unme-pCBS-luc also are suitable to study the relationship between DNA methylation and histone modification, such as histone methylation, phosphorylation, acetylation, and sumoylation, etc. By comparing the difference of the histone modification in these two cell models, we can tell how DNA methylation affects histone modification. Or in contrast to VP16-induced DNA demethylation, the DBD-fused DNMT can be introduced into unme-pCBS-luc cells to induce *de novo* promoter methylation, then the dynamic process of how DNA methylation regulates histone modification can be observed.

DNA methylation and demethylation play important roles in chromatin remodeling. We believe that this cell model will have many potential applications in the future research on DNA demethylation and dynamic process of chromatin remodeling.

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References

1 Chow JC and Brown CJ. Forming facultative heterochromatin: silencing of an X chromosome in mammalian females. Cell Mol Life Sci 2003, 60: 2586–2603.
2 Biliya S and Bulla LA, Jr. Genomic imprinting: the influence of differential methylation in the two sexes. Exp Biol Med (Maywood) 2010, 235: 139–147.
3 Asadollahi R, Hyde CA and Zhong XY. Epigenetics of ovarian cancer: from the lab to the clinic. Gynecol Oncol 2010, 118: 81–87.
4 Hirabayashi Y and Gotoh Y. Epigenetic control of neural precursor cell fate during development. Nat Rev Neurosci 2010, 11: 377–388.
5 Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J and Devon K, *et al*. Initial sequencing and analysis of the human genome. Nature 2001, 409: 860–921.
6 Calvanese V, Horrillo A, Hmadcha A, Suarez-Alvarez B, Fernandez AF, Lara E and Casado S, *et al*. Cancer genes hypermethylated in human embryonic stem cells. PLoS One 2008, 3: e3294.

7 Clark SJ and Melki J. DNA methylation and gene silencing in cancer: which is the guilty party? Oncogene 2002, 21: 5380–5387.
8 Lopez-Serra L, Ballestar E, Fraga MF, Alaminos M, Setien F and Esteller M. A profile of methyl-CpG binding domain protein occupancy of hypermethylated promoter CpG islands of tumor suppressor genes in human cancer. Cancer Res 2006, 66: 8342–8346.
9 Esteller M. CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. Oncogene 2002, 21: 5427–5440.
10 Rush LJ and Plass C. Alterations of DNA methylation in hematologic malignancies. Cancer Lett 2002, 185: 1–12.
11 Takada H, Wakabayashi N, Dohi O, Yasui K, Sakakura C, Mitsufuji S and Taniwaki M, *et al*. Tissue factor pathway inhibitor 2 (TFPI2) is frequently silenced by aberrant promoter hypermethylation in gastric cancer. Cancer Genet Cytogenet 2010, 197: 16–24.
12 Chuang JC, Yoo CB, Kwan JM, Li TW, Liang G, Yang AS and Jones PA. Comparison of biological effects of non-nucleoside DNA methylation inhibitors versus 5-aza-2′-deoxycytidine. Mol Cancer Ther 2005, 4: 1515–1520.
13 Mossman D, Kim KT and Scott RJ. Demethylation by 5-aza-2′-deoxycytidine in colorectal cancer cells targets genomic DNA whilst promoter CpG island methylation persists. Biomed Chromatogr Cancer 2010, 10: 366.
14 Jiang S, Ma X, Huang Y, Xu Y, Zheng R and Chiao JW. Reactivating aberrantly hypermethylated p15 gene in leukemic T cells by a phenylhexyl isothiocyanate mediated inter-active mechanism on DNA and chromatin. J Hematol Oncol 2010, 3: 48.
15 Wu Y, Alvarez M, Slamon DJ, Koeffler P and Vadgama JV. Caspase 8 and maspin are downregulated in breast cancer cells due to CpG site promoter methylation. Biomed Chromatogr Cancer 2010, 10: 32.
16 Saze H, Sasaki T and Kakutani T. Negative regulation of DNA methylation in plants. Epigenetics 2008, 3: 122–124.
17 Benetti R, Gonzalo S, Jaco I, Munoz P, Gonzalez S, Schoeftner S and Murchison E, *et al*. A mammalian microRNA cluster controls DNA methylation and telomere recombination via Rbl2-dependent regulation of DNA methyltransferases. Nat Struct Mol Biol 2008, 15: 998.
18 Zhu JK. Active DNA demethylation mediated by DNA glycosylases. Annu Rev Genet 2009, 43: 143–166.
19 Wu SC and Zhang Y. Active DNA demethylation: many roads lead to Rome. Nat Rev Mol Cell Biol 2010, 11: 607–620.
20 Kress C, Thomassin H and Grange T. Active cytosine demethylation triggered by a nuclear receptor involves DNA strand breaks. Proc Natl Acad Sci USA 2006, 103: 11112–11117.
21 Li F, Papworth M, Minczuk M, Rohde C, Zhang Y, Ragozin S and Jeltsch A. Chimeric DNA methyltransferases target DNA methylation to specific DNA sequences and repress expression of target genes. Nucleic Acids Res 2007, 35: 100–112.
22 Arendt ML, Nasir L and Morgan IM. A novel two-step transcriptional activation system for gene therapy directed toward epithelial cells. Mol Cancer Ther 2009, 8: 3244–3254.
23 Sadowski I, Ma J, Triezenberg S and Ptashne M. GAL4-VP16 is an unusually potent transcriptional activator. Nature 1988, 335: 563–564.
24 Matsuo K, Silke J, Georgiev O, Marti P, Giovannini N and Rungger D. An embryonic demethylation mechanism involving binding of transcription factors to replicating DNA. Embo J 1998, 17: 1446–1453.
25 Hermann A, Gowher H and Jeltsch A. Biochemistry and biology of mammalian DNA methyltransferases. Cell Mol Life Sci 2004, 61: 2571–2587.
26 Okochi-Takada E, Ichimura S, Kaneda A, Sugimura T and Ushijima T. Establishment of a detection system for demethylating agents using an endogenous promoter CpG island. Mutat Res 2004, 568: 187–194.
27 Si J, Boumber YA, Shu J, Qin T, Ahmed S, He R and Jelinek J, *et al*. Chromatin remodeling is required for gene reactivation after decitabine-mediated DNA hypomethylation. Cancer Res 2010, 70: 6968–6977.
28 Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y and Agarwal S, *et al*. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science 2009, 324: 930–935.