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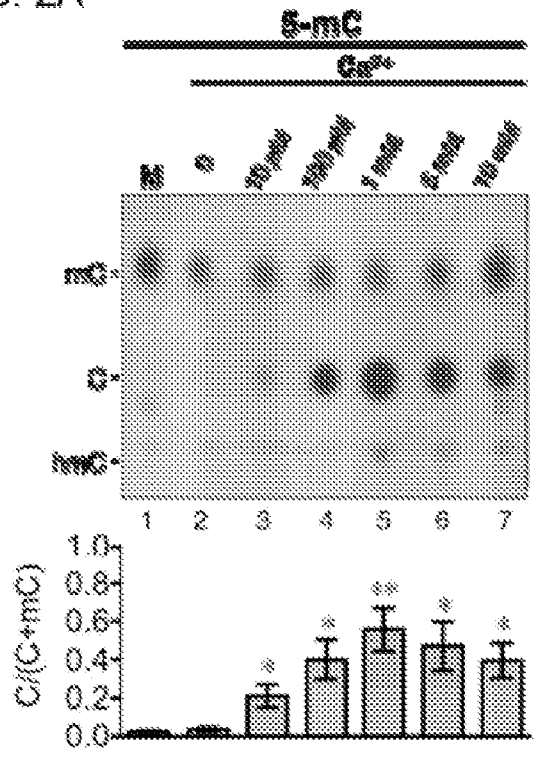
[Continued on next page]

(54) Title: DNA 5-METHYL CYTOSINE DEMETHYLATION ACTIVITY OF VERTEBRATE DNA METHYLTRANSFERASES

FIG. 2A



(57) Abstract: Methods for identifying a test agent as a modulator of the active DNA demethylation activity of a DNA methyltransferase are disclosed. The method comprises: a) providing a methylated DNA; b) providing the DNA methyltransferase; c) allowing the methylated DNA to react with the DNA methyltransferase for a sufficient time to perform a demethylation reaction and generate a demethylated DNA product in the presence or absence of a test agent; d) analyzing the extent of demethylation; and d) comparing the extents of the demethylation in the presence and absence of the test agent, and thereby identify the test agent as a modulator of the DNA demethylation activity of the DNA methyltransferase.

WO 2014/093351 A1

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DNA 5-METHYL CYTOSINE DEMETHYLATION ACTIVITY OF VERTEBRATE DNA METHYLTRANSFERASES

FIELD OF THE INVENTION

The present invention relates generally to DNA demethylation activity of DNA methyltransferases, therapeutic and diagnostic uses thereof.

BACKGROUND OF THE INVENTION

In vertebrates, DNA methylation occurs primarily at the 5-position of cytosine (C) in CpG dyads and their genomic methylation patterns are established/maintained by the DNA (C-5)-methyltransferases, or DNMTs. Of the known vertebrate DNMTs, DNMT1 shows a substrates preference for hemi-methylated DNA and maintains the methylation patterns during DNA replication. DNMT3A and DNMT3B show equal C-5 methylation activities toward unmethylated and hemi-methylated DNA *in vitro*, and they are essential for de novo genomic DNA methylation as well development of the early embryos. The vertebrate DNA methylation system comprising the above three essential DNMTs is indispensible for the establishment of the genomic DNA methylation patterns, globally and locally, and consequently the processes of gene expression, neuroplasticity, differentiation, carcinogenesis, imprinting, X-inactivation and development.

It has remained elusive in literature before 2012 whether there exists enzyme(s) in vertebrates that could actively and directly convert 5-mC on DNA into C.

SUMMARY OF THE INVENTION

In one aspect, the invention relates to a method for identifying a test agent (or a compound) as a modulator of the active DNA demethylation activity of a DNA methyltransferase. The method comprises:

a) providing a methylated DNA;

b) providing a DNA methyltransferase;

c) allowing the methylated DNA to react with the DNA methyltransferase for a sufficient time to perform a demethylation reaction and generate a demethylated DNA product in the presence or absence of a test agent;

d) analyzing the extent of demethylation; and

d) comparing the extents of the demethylation in the presence and absence of the test agent, and thereby identify the test agent as a modulator of the DNA demethylation activity of the DNA methyltransferase;

wherein the test agent is identified as an inhibitor of the active DNA demethylation activity of the DNA methyltransferase when the extent of the demethylation is less in the presence of the test agent; or the test agent is identified as a stimulator of the active DNA demethylation activity of the DNA methyltransferase when the extent of demethylation is more in the presence of the test agent.

In one embodiment of the invention, the analyzing step is performed by a technique selected from the group consisting of a restriction digestion-polymer chain reaction (PCR) assay, a hydrolysis-thin layer chromatography assay, an antibody-based analysis, scintillation counting, autoradiography, dot blotting, a liquid chromatography-based analysis, and a Na bisulfite-based analysis.

In another embodiment of the invention, the demethylation reaction occurs in the presence of a calcium ion concentration of around 10 μM to 10 mM.

In another embodiment of the invention, the DNA methyltransferase is an isolated vertebrate DNA methyltransferase, or a recombinant DNA methyltransferase, or is present in a nuclear extract or is present in a cellular extract. The vertebrate DNA methyltransferase, the nuclear extract, or the cellular extract may be prepared from cells selected from the group consisting of vertebrate cell cultures, vertebrate tissues, insect cells, worm cells, insect tissues, worm tissues, plant cells, plant tissues, yeast cells, and bacterial cells. The nuclear extract may be a sperm extract.

In another embodiment of the invention, the methylated DNA comprises a labeled methyl group. The methyl group in the methylated DNA may be radioactive-labeled.

In another embodiment of the invention, the aforementioned method steps (a) to (d) possess the following technical features: wherein:

(a) the methylated DNA provided in step (a) is a methylated reporter gene operably linked to a constitutive promoter and is present in a cell;

(b) the DNA methyltransferase provided in step (b) is operably linked to a constitutive promoter and is also present in the cell, or is endogenously present in the cell as an endogenous DNA methyltransferase;

(c) the demethylated DNA product generated in step (c) is a demethylated reporter gene, which expresses a reporter protein in the cell.

(d) the analyzing step is performed by a technique selected from the group consisting of:

(i) analyzing the signal of the reporter protein encoded by the demethylated reporter gene in the cell;

(ii) analyzing the reporter protein expression by Western blot; and

(iii) isolating the methylated and demethylated reporter gene and analyzing the extent of demethylation by a restriction digestion-polymer chain reaction (PCR) assay, a hydrolysis-

thin layer chromatography assay, an antibody-based analysis, scintillation counting, autoradiography, dot blotting, a liquid chromatography-based analysis, or a Na bisulfite-based analysis.

In one embodiment of the invention, the methylated reporter gene and the DNA methyltransferase are present in the cell via transfection. Alternatively, the DNA methyltransferase is an endogenous enzyme present in the cell.

The cell is transfected with the methylated reporter gene, and the DNA methyltransferase may be an exogenouse enzyme transfected into the same cell, or may be an endogenous enzyme already present in the same cell. In one embodiment, the methylated reporter gene and the DNA methyltransferase are co-transfected into the cell.

In one embodiment of the invention, the test agent is introduced into the cell or added to a culture medium bathing the cell.

In another embodiment of the invention, the methylated reporter gene comprises a DNA sequence of a gene selected from the group consisting of a fluorescent protein-encoding gene, a luciferase gene, a drug-resistant gene, and genes of cell survivals.

In another embodiment of the invention, the methylated DNA comprises 5-methylcytosine (5mC)-containing DNA.

In another embodiment of the invention, step (c) is performed under a condition that is free of a reducing agent or under a non-reducing condition.

The DNA methyltransferase may be a modified form of a wild-type DNA methyltransferase, said modified form retaining the active DNA demethylation activity of the wild-type DNA methyltransferase.

The DNA methyltransferase may be selected from the group consisting of DNA methyltransferase 1, DNA methyltransferase 3A, DNA methyltransferase 3B, and any combination thereof.

The constitutive promoter may be, but not limited to, a cytomegalovirus promoter.

In another aspect, the invention relates to a method for identifying a test agent as a modulator of the active DNA demethylation activity of a DNA methyltransferase, in which the method comprises:

(I)

a) admixing a first composition comprising a methylated DNA with a second composition comprising the DNA methyltransferase in the presence or absence of a test agent;

b) allowing a demethylation reaction to occur by reacting the methylated DNA with the DNA methyltransferase for a sufficient time to generate a demethylated DNA product;

c) analyzing the extent of demethylation; and

d) comparing the extents of the demethylation in the presence and absence of the test agent, and thereby identify the test agent for modulating active DNA demethylation activity of the DNA methyltransferase;

wherein the test agent is identified as an inhibitor of the active DNA demethylation activity of the DNA methyltransferase when the extent of the demethylation is less in the presence of the test agent; or the test agent is identified as a stimulator of the active DNA demethylation activity of the DNA methyltransferase when the extent of demethylation is more in the presence of the test agent; or **(II)**

1) providing a cell culture medium containing cells transfected with a reporter gene that is methylated and operably linked to a constitutive promoter, said cells containing an endogenous DNA methyltransferase or exogenously expressing a wild-type, a modified or a genetically engineered DNA methyltransferase;

2) exposing the cells to a test agent;

3) allowing a demethylation reaction to occur to generate a demethylated reporter gene, which expresses a reporter protein in the cells; and

4) analyzing the extent of demethylation of the reporter gene by examining the signal intensity of the reporter protein expressed by the demethylated reporter gene in the cells;

5) comparing the extents of the demethylation of the reporter gene in the presence and absence of the test agent, and thereby identify the test agent as a modulator of the DNA demethylation activity of the DNA methyltransferase;

wherein the test agent is identified as an inhibitor of the active DNA demethylation activity of the DNA methyltransferase when the extent of the demethylation is less in the presence of the test agent; or the test agent is identified as a stimulator of the active DNA demethylation activity of the DNA methyltransferase when the extent of demethylation is more in the presence of the test agent.

The wild-type DNA methyltransferase may be selected from the group consisting of DNA methyltransferase 1, DNA methyltransferase 3A, and DNA methyltransferase 3B.

The accompanying drawings illustrate one or more embodiments of the invention and, together with the written description, serve to explain the principles of the invention. Wherever possible, the same reference numbers are used throughout the drawings to refer to the same or like elements of an embodiment.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows DNA 5-mC demethylation activities of the mammalian DNMTs. The 5-mC-containing DNA substrates were subjected to incubation in 293T nuclear extracts in buffer B with 10 mM $CaCl_2$ that contained the exogenously expressed EGFP (lanes 1 and 2), mouse DNMT1 (lanes 3

and 4), DNMT3A (lanes 5 and 6), DNMT3B (lanes 7 and 8), and their site-directed mutants (lanes 9-14), respectively. The extents of conversion of 5-mC to C were analyzed by hydrolysis-TLC assay and quantitatively shown in the histogram. The amounts of the exogenous wild type enzyme in lanes 4, 6, and 8 were similar to those of the mutant enzymes in lanes 10, 12, and 14, respectively (Western blotting data not shown). M, mock control without incubation; R, with incubation. Error bars indicate S.D..*,$p<0.05$ by t-test comparing bars 4, 6, and 8 to bar 2.

FIG. 2A shows calcium-dependence of the DNA 5-mC demethylation activities of recombinant DNMTs. Hydrolysis-TLC assay of conversion of 5-mC to C by recombinant DNMT3B. The DNA demethylation activity of the recombinant mouse DNMT3B was assayed by incubation of 40 nM 5-mC containing DNA substrate with 40 nM of the enzyme in buffer B containing 100 μg/ml BSA and increasing concentrations (0, 10 μM, 100 μM, 1 mM, 5 mM and 10 mM) of $CaCl_2$. The incubations were all at 37°C for 4h. The quantitative results are presented in the histogram. M, mock control without incubation. Error bars indicate S.D. *, $p<0.05$; **, $p<0.01$ by t-test comparing bars 3-7 to bar 2.

FIG. 2B shows a comparison of the DNA demethylation activities of recombinant hDNMT1, hDNMT3A and DNMT3B by hydrolysis-TLC assay. The 5-mC containing substrate was incubated at 37°C for 4h with 40 nM of each of the recombinant hDNMT1 (lane 2), hDNMT3A (lane 3) and DNMT3B (lane 4) in buffer B containing 100 μg/ml BSA and 1 mM of $CaCl_2$, and then analyzed by hydrolysis-TLC assay. The quantitative analysis is presented in the histogram. M, mock control without incubation; R, with incubation. Error bars indicate S.D. *, $p<0.05$; ***, $p<0.005$ by t-test comparing bars 2-4 to bar 1.

FIGs. 3A-B show the effects of a reducing condition and SAM on the DNA demethylation and methylation activities of DNMT3B. FIG. 3A: The 5-mC containing DNA substrate was subjected to the demethylation reactions with 40 nM recombinant DNMT3B in buffer B containing 100 μg/ml BSA with or without the inclusion of 1 mM $CaCl_2$, 5 mM DTT, or 160 μM SAM. After incubation at 37°C for 4h, the DNA products were analyzed by the hydrolysis-TLC assay. The results are quantitatively presented in the histogram. Error bars indicate S.D.. *, $p<0.05$ by t-test comparing bars 2-4 to bar 1. FIG. 3B: Unmethylated pMR1-8 plasmid DNA was incubated with 40 nM recombinant DNMT3B in buffer B containing 100 μg/ml BSA with or without 1 mM $CaCl_2$, 5 mM DTT, or 160 μM SAM. After incubation at 37°C for 4h, the extents of C methylation of the DNAs from the different reactions were determined by hydrolysis-TLC assay and quantitatively compared in the histogram. Error bars indicate S.D.

FIGs. 4A-B show Reversibility of the DNA demethylation and methylation reactions *in vitro*. FIG. 4A: Strategy of a series of reactions testing the reversibility of DNA demethylation and

methylation (see text for more details). Briefly, 5-mC containing DNA substrate was incubated at 37°C for 2h with 40 nM recombinant DNMT3B in buffer B containing 100 μg/ml BSA and 1 mM $CaCl_2$ (reaction 1). Then, 160 μM SAM were added and the incubation was continued for another 1h (reaction 2). Finally, 1 mM H_2O_2 was added to the reaction mixture and the incubation continued for another 2h (reaction 3). Rx, reactions. FIG. 4B: The DNA products from the 3 reactions outlined in 4A were purified and analyzed by hydrolysis-TLC assay. The data are quantitatively compared in the histogram. M, mock control without incubation. Error bars indicate S.D.. *, $p<0.05$; ***, $p<0.005$.

FIGs. 5A-B show Experimental strategies for assay of the inter-conversions of 5-mC and C. FIG. 5A: Schematic diagram illustrating the stepwise processes of restriction digestion-PCR assay. This assay was used to estimate the extents of demethylation of methylated plasmid upon incubation with the porcine sperm nuclear extract under different conditions (FIG. 6). 20 ng of the 5-mC containing plasmid pMR1-8, with or without DNA demethylation reactions in the sperm nuclear extract, was digested by HpaII overnight at 37°C, and purified with Qiaquick Nucleotide Removal Kit (Qiagen). The proportion of HpaII-insensitive DNA substrate was analyzed by 12-15 cycles of PCR using a primer set bracketing the HpaII restriction cutting sites followed by the gel electrophoresis. The band intensities were further quantitatively estimated and compared. FIG. 5B: Schematic diagram illustrating the stepwise processes of hydrolysis-TLC assay. The double-stranded DNA substrates were digested by MspI at 37°C overnight, and then dephosphorylated with calf intestine phosphatase (New England Biolabs) followed by purification by Qiaquick Nucleotide Removal Kit (Qiagen). The purified DNAs were end-labeled by using γ^{32}P-ATP and T4 polynucleotide kinase (New England Biolabs), and then digested by snake venom phosphodiesterase (Worthington) and DNaseI (Roche) overnight. The hydrolyzates were loaded onto PEI cellulose plates (Merck) and separated in the buffer isobutyric acid: water: ammonia (66:18:3) for 16-18h. Autoradiography of the plates was carried out and the signals on the plates were quantitated using the Alpha Imaging 2200 (Alpha Innotech Crop.).

FIGs. 6A-C show the DNA demethylation activity of porcine sperm extracts. FIG. 6A: Fully methylated plasmid DNA pMR1-8 was incubated at 37°C for 2h in the porcine sperm nuclear extract with increasing concentrations (0, 10 μM, 100μM, 1mM and 10mM) of $CaCl_2$ (lanes 1-5), $MgCl_2$ (lanes 6-10) or $Fe(NH_4SO_4)_2$ (lanes 11-15). After the reactions, the extent of DNA demethylation of the plasmid DNA was analyzed by the restriction digestion-PCR assay outlined in FIG. 5A. The histograms show the relative proportions of the plasmid DNA resistant to HpaII cleavage. For comparison of bars 2-5 to bar 1, *, $p<0.05$; **, $p<0.01$ by the t-test. FIG. 6B: Effects of BER inhibitors on the demethylation activities of the porcine sperm nuclear extract. The DNA demethylation reactions were carried out by incubating fully-methylated plasmid DNA pMR1-8 and

sperm nuclear extract containing 10 mM $CaCl_2$ and increasing concentrations of APE-i (0, 10 μM, 100 μM, and 1 mM, lanes 2-6) or 3-AB (0, 5 μM, 500 μM, 5 mM, and 50mM, lanes 8-12). After the reactions, the extents of demethylation of the plasmid DNA were analyzed by the restriction digestion-PCR assay. FIG. 6C: Effect of the CDA inhibitor THU on the demethylation reaction in the porcine sperm nuclear extract. The fully-methylated pMR1-8 DNA was subjected to incubation in the extract at 37°C for 2h containing increasing concentrations of THU (0, 30 μM, 100 μM and 1mM, lanes 1-4). The extents of demethylation of the plasmid DNA were analyzed by the restriction digestion-PCR assay. The histogram shows the relative proportions of plasmid DNA resistant to HpaII cleavage. M, mock control without incubation. Error bars indicate S.D..

FIG. 7 shows the results of SDS-PAGE analyses. The recombinant human hDNMT1 (500 ng), human hDNMT3A (250 ng), and mouse DNMT3B (250 ng) were subjected to SDS-PAGE followed by the coomassie blue staining. The arrows indicate the expected bands of the three DNMTs, respectively. Protein markers were loaded in the M lanes.

FIG. 8 shows DNA demethylation by recombinant DNMT3B in the presence of ^{3}H-SAM. The reaction conditions were the same as those described in FIG.3A, except that 1μCi ^{3}H-SAM was also included in each of the reaction mixtures. After the reactions, the mixtures were further incubated with 100 μl of 0.5N NaOH at 55°C for 10 mins and neutralized with 100 μl of 1M Tris-Cl pH7.0. The DNA substrates were then precipitated with 10% trichloroacetic acid (TCA) in 5 mM Na pyrophosphate on ice for 15 mins, and loaded onto DE-81 ion-exchange papers followed by air drying. The DE-81 papers were washed 5 times with 5% TCA in 5 mM Na pyrophosphate, twice with 100% EtOH, air dried, and the ^{3}H counts on DNA were determined in a liquid scintillation counter.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is more particularly described in the following examples that are intended as illustrative only since numerous modifications and variations therein will be apparent to those skilled in the art. Various embodiments of the invention are now described in detail. Referring to the drawings, like numbers indicate like components throughout the views. As used in the description herein and throughout the claims that follow, the meaning of "a", "an", and "the" includes plural reference unless the context clearly dictates otherwise. Also, as used in the description herein and throughout the claims that follow, the meaning of "in" includes "in" and "on" unless the context clearly dictates otherwise. Moreover, titles or subtitles may be used in the specification for the convenience of a reader, which shall have no influence on the scope of the present invention. Additionally, some terms used in this specification are more specifically defined below.

DEFINITIONS

The terms used in this specification generally have their ordinary meanings in the art, within the context of the invention, and in the specific context where each term is used. Certain terms that are used to describe the invention are discussed below, or elsewhere in the specification, to provide additional guidance to the practitioner regarding the description of the invention. For convenience, certain terms may be highlighted, for example using italics and/or quotation marks. The use of highlighting has no influence on the scope and meaning of a term; the scope and meaning of a term is the same, in the same context, whether or not it is highlighted. It will be appreciated that same thing can be said in more than one way. Consequently, alternative language and synonyms may be used for any one or more of the terms discussed herein, nor is any special significance to be placed upon whether or not a term is elaborated or discussed herein. Synonyms for certain terms are provided. A recital of one or more synonyms does not exclude the use of other synonyms. The use of examples anywhere in this specification including examples of any terms discussed herein is illustrative only, and in no way limits the scope and meaning of the invention or of any exemplified term. Likewise, the invention is not limited to various embodiments given in this specification.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. In the case of conflict, the present document, including definitions will control.

As used herein, "around", "about" or "approximately" shall generally mean within 20 percent, preferably within 10 percent, and more preferably within 5 percent of a given value or range. Numerical quantities given herein are approximate, meaning that the term "around", "about" or "approximately" can be inferred if not expressly stated.

The term "5mC" refers to the 5 position of cytosine.

The term "agent" refers to any matter, substance or thing producing or used for obtaining specific results, which includes, but not limited to, compounds, co-factors, etc.

The invention relates to the discovery that mammalian DNMTs, including DNMT1, DNMT3A, and DNMT3B can actively and directly convert 5-mC on DNA into C biochemical reaction. See Chen *et al.* (2013) THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 13, pp. 9084–9091, which is incorporated herein by reference in its entirety.

Specifically, the invention relates to the discovery that in *in vitro* reactions, the mouse and/or human DNMT1, DNMT3A, and DNMT3B all could act as an active DNA demethylase, removing the methyl group from 5-mC on DNA in an Ca^{2+} ion and redox state-dependent manner.

The invention also relates to the discovery of the DNA 5-mC demethylation activities of DNMTs that could be used to screen, identity, and design reagents, including chemical compounds and

therapeutic agents, which would modulate the DNA 5-mC demethylation activities of DNMTs and their modified forms/ derivatives, for the purpose of regulating the different cellular processes including gene expression, chromosome structure, carcinogenesis (metastasis), cell division, cell motility, neuronal plasticity, etc.

The protein sequences of DNMTs are as follows: DNMT1 [*Homo spaines*] SEQ ID NO:4; DNMT1 [*Mus musculus*] SEQ ID NO: 5; DNMT3A [*Homo spaiens*] SEQ ID NO: 6; DNMT3A [*Mus musculus*] SEQ ID NO: 7; DNMT3B [*Homo spaiens*] SEQ ID NO: 8; DNMT3B [*Mus musculus*] SEQ ID NO: 9.

To generate modified DNMTs, we serially deleted 10 amino acids from N-terminal of the full-length DNMTs. For example, the modified forms of human DNMT1 is DNMT1(11-1620), DNMT1(21-1620), DNMT1(31-1620) . . . , etc. All the deletion-modified DNMT1s are tested for DNA methylation and demethylation activities *in vitro* and *in vivo*. In addition, DNMT1 may be modified by deletion of the internal 10 amino acid of the full length DNMT1, and the modified forms are DNMT1(Δ11-20), DNMT1(Δ21-30), DNMT1(Δ31-40) . . . , etc. The same deletion modifications may be used to generate deletion modified forms of DNMT3A and DNMT3B.

To screen, identify, and/or design reagents that modulate the DNA 5-mC demethylation activities of DNMTs, several assays can be used, which include *in vitro* and *in vivo* assays.

(1) *In vitro*

The DNMTs and 5-mC containing DNA substrates are incubated with the test agents under proper reaction conditions. The DNMTs used may be in the forms of either recombinant wild-type/modified/ genetically engineered enzymes or as ectopically expressed wild type/ modified/ genetically engineered enzymes in cellular or nuclear extracts prepared from vertebrate cell cultures/ tissues, yeast cells, or bacterial cells. The methyl group on the 5-mC-containing DNA substrates can be radioactive, or labeled by other methods.

After the reaction, the DNA substrates are isolated manually or automatically, and the content of methylation/demethylation is determined by several methods, such as restriction digestion-based analysis, antibody-based analysis, Scintillation counting, autoradiography, dot blotting, liquid chromatography-based analysis, Na bisulfite-based analysis and hydrolysis-TLC (thin layer chromatography). The effect of each test agent on the DNA 5-mC demethylation activities of the DNMT enzymes is determined by comparison of the content of 5-mC on the DNA substrate with the control (mock), before and after the *in vitro* reaction.

(2) *In vivo*

The reporter DNAs methylated at 5-C are introduced into cells containing either the endogenous DNMTs alone or with exogenously expressed wild type/modified/ genetically engineered DNMTs.

The reagents to be tested are added into the cell culturing medium, or sent into the cells, before triggering the DNA demethylation activities of the DNMTs.

The methylated reporter DNAs carry genes, such as different fluorescence genes, luciferase gene, drug-resistant gene, or genes of cell survivals etc., the expression levels of which can indicate the methylation/demethylation status of the DNA substrates. Alternatively, the methylated report DNAs can be isolated manually or automatically, and analyzed with respect to their 5-mC content by methods described above.

EXAMPLES

MATERIALS AND METHODS

Recombinant Plasmids and Recombinant Proteins. Constructions of expression plasmids used in this study were described in Chen et al. (*J Biol Chem.* VOL. 287, pp. 33116-33121, 2012. It is incorporated herein by reference in its entirety), which included plasmids for exogenously expressing DNMT1, DNMT3A, and DNMT3B. The DNA methylation-inactive mutants of the DNMTs, i.e. DNMT1-PSC, DNMT3A-PS and DNMT3B-PS, were generated by insertion of a serine residue before the Cys-1229 at the catalytic site of DNMT1 or by replacing the cysteine residue Cys-706 and Cys-657 in the catalytic domains of DNMT3A and DNMT3B, respectively, with a serine residue.

All of the recombinant enzymes DNMTs, including hDNMT1 (purity ~78%), hDNMT3A (purity~90%), and mouse DNMT3B (purity~50%) (FIG. 7), were purchased from BPS Bioscience.

Cell Culture and DNA Transfection. The human embryonic kidney 293T cells were cultured under 5% CO_2 at 37°C in DMEM medium supplemented with 10% FBS and 1% Penicillin-Streptomycin. For DNA transfections, different expression plasmids were delivered into cells using either LIPOFECTAMINE® 2000 or MAXIFECT™. The transfected cells were collected 2 days afterwards for further experimentation.

Preparation of Nuclear Extracts. The nuclear extracts were prepared from the porcine sperms and 293T cells, respectively, by a modified method. Briefly, the porcine semen was washed three times by PBS buffer and the sperm pellet isolated with FICOLL®. The pellet was resuspended in a hypotonic buffer (10mM Tris-HCl, pH7.4, 10mM NaCl, 10 mM EDTA and EDTA-free protease inhibitors) on ice for 15 minutes. The resuspended sperms were passed through a G21 needle 10 times and then centrifuged at 13,200 rpm at 4°C for 10 min. The supernatant was removed and the pellet of the nuclei was resuspended in a resuspension buffer (10 mM Tris-HCl, pH7.4, 10 mM NaCl, 1.5 mM $MgCl_2$ and EDTA-free protease inhibitors), and an equal volume of 1M NaCl was added for 30 min incubation on ice. The solution was centrifuged at 13,200 rpm at 4°C for 30 min and the supernatant (nuclear extract) was dialyzed at 4°C in buffer B (10mM Tris-HCl, pH7.4, 50

mM NaCl, 1.5 mM $MgCl_2$ and EDTA-free protease inhibitors) overnight with 2 changes of the dialysis buffer.

The preparation of nuclear extract from 293T cells followed the procedures described above. The transfected cells were washed 3 times with PBS and resuspended in the hypotonic buffer on ice for 10 mins. The solution was centrifuged at 4,000 rpm for 10 mins and the supernatant were removed. The nuclear pellet was resuspended in the resuspension buffer and then an equal volume of 1M NaCl was added for 30 min incubation on ice. The lysate was centrifuged at 13,200 rpm at 4°C for 30 min and the supernatant was collected as a nuclear extract, which was then dialyzed at 4°C in buffer B overnight.

DNA Substrates for in vitro DNA demethylation assay. The 5-mC-containing substrate for DNA demethylation assay of the porcine sperm nuclear extract was prepared from the 2,819 bp pMR1-8 plasmid containing 185 CpG dyads and 11 MspI restriction sites. The unmodified pMR1-8 plasmid was amplified in the SCS110 bacteria and then methylated by the bacterial methyltransferase M.SSS I in NEB buffer 2 supplemented with 160 μM SAM (S-adenosylmethionine). The extent of methylation of the plasmid was checked by HpaII digestion.

C-5 methylated double-stranded DNA substrate was used in the demethylation reactions with 293T nuclear extract or the recombinant DNMTs (see below), and then analyzed by the hydrolysis-TLC assay.

In Vitro Reactions of 5-mC to C Conversion on DNA. For DNA demethylation reactions, 40 ng of the methylated pMR1-8 plasmid DNA or 5-mC containing double-stranded DNA substrate was incubated with 100 μg of the nuclear extracts or 40 nM recombinant DNMTs proteins in 50 μl of buffer B containing 100 μg/ml BSA at 37°C up to 4h. When needed, 10 μM-10 mM of three divalent cations (Ca^{2+}, Mg^{2+}, or Fe^{2+}), 10 μM-1 mM CRT0044876 (APE-i), 0.5 μM-50 mM 3-aminobenzamide (3-AB), 30 μM 1 mM tetrahydrouridine (THU), 5 mM DTT, or 160 μM SAM was included in the reaction mixtures. To assay the effect of redox-state of the enzymes, 40 nM recombinant DNMTs was pre-treated with 10 μM-10 mM H_2O_2 in 50 μl of buffer B containing 100 μg/ml BSA at 15°C for 30 min. Forty ng of the 5-mC containing double stranded DNA substrate was then added and the reaction mixtures were incubated at 37°C for 4h.

All reactions were stopped by 1.3% SDS and treated with proteinase K at 50°C for 20 min. The DNA substrates and demethylated products at the end of the reaction were isolated using the Qiaquick Nucleotide Removal Kit, and subjected to restrictions digestion-PCR assay or hydrolysis-TLC assay (see below).

C-5 methylated double-stranded DNA substrate preparation. The 5-mC-containing DNA substrate was prepared by PCR amplification of a 561 bp fragment from the pMR1-8 plasmid

containing MspI/HpaII sites (SEQ ID NO: 1). During the PCR amplification, a 5-mC-containing dNTP mix (Zymo Research) was used. Primers for PCR were: pMR1-8 F, 5'- aaagataccaggcgtttcccc -3' (SEQ ID NO: 2); and pMR1-8 R, 5'- gagttttcgttccactgagcgtc -3' (SEQ ID NO: 3).

In Vitro Reactions of C to 5-mC Conversion on DNA. Methylation *in vitro* of unmodified pMR1-8 plasmid DNA by the DNMTs were carried out and analyzed by the hydrolysis-TLC assay. When needed, 1 mM $CaCl_2$ or 5 mM DTT was also included in the reaction mixture.

Restriction Digestion-PCR Assay of C-5 methylation on Double-Strand DNA Substrate(s). The procedures are as described previously (Chen et al., 2012, ibid). See FIG. 5A legend for more details.

Hydrolysis-TLC (Thin Layer Chromatography) assay of 5-mC, 5-hmC, and C on DNA. The procedures were similar to those described in Chen *et al.* (2012). ibid. See FIG. 5B legend for details.

RESULTS

In vitro DNA demethylation by porcine sperm extract. We performed *in vitro* DNA C-5 demethylation reactions using the nuclear extract prepared from porcine sperms. The effect of Ca^{2+} ion were also tested. Remarkably, inclusion of 1-10 mM of Ca^{2+}, but not Mg^{2+} (compare lanes 7-10 to lane 6) or Fe^{2+} (compare lanes 12-15 to lane 11, FIG. 6A), significantly reduced the extent of DNA methylation by 20-50% (compare lanes 4 and 5 to lane 1, FIG. 6A). Furthermore, inclusion of inhibitors of either the BER pathway, CRT0044876 (APE-i) and 3-aminobenzanide (3-AB), or the cytidine-deaminase (CDA), tetrahydrouridine (THU), in the reactions had little effect on the *in vitro* DNA demethylation activity of the sperm nuclear extract (FIGs. 6A and 6B). The data of FIGs. 6A-B suggested that Ca^{2+} ion stimulated a BER-independent and CDA-independent DNA demethylation activity in the nuclear extract of the porcine sperms.

It was not trivial to purify the factor(s)/enzyme(s) in the nuclear extract of the porcine sperms that was responsible for the *in vitro* conversion of 5-mC to C on DNA. Since the porcine sperm nuclear extract contained DNMT1/DNMT3A/DNMT3B (data not shown) and the murine/human orthologs of the latter two DNMTs acted *in vitro* as DNA 5-hydroxymethylcytosine (5-hmC) dehydroxymethylases under oxidative conditions in the absence of SAM, we suspected that under appropriate conditions, these DNMTs might also be capable to convert other modified forms of cytosine, e.g., 5-mC, to C.

In view of the data of FIG. 6, we performed *in vitro* DNA demethylation reactions in the presence of 10 mM of Ca^{2+}. 5-mC-containing double-stranded DNA substrate was incubated with nuclear extracts prepared from 293T cells transfected with plasmids overexpressing EGFP (a negative control), mouse DNMT1, mouse DNMT3A, mouse DNMT3B, as well as their mutants, respectively. After the reactions, the DNA products were hydrolyzed as depicted in FIG. 5B and the nucleotides were analyzed by TLC (FIG. 1). As shown, under the reaction conditions tested, the

nuclear extracts containing the exogenously expressed DNMT1 (lane 4, FIG. 1), DNMT3A (lane 6, FIG. 1), and DNMT3B (lanes 8, FIG. 1) all could remove the 5-methyl group from approximately 30% of the 5-mC residues on the DNA substrates.

Remarkably, the DNA demethylase activities of the 3 mouse DNMTs were greatly diminished, by approximately 73% to 88%, when amino acid substitutions or insertion were introduced into the known catalytic sites of C-5 methylation of these enzymes (compare lanes 10, 12, 14 to 4, 6, 8, respectively, FIG.1). The data of FIG. 1 suggested that the two de novo DNMTs as well as the maintenance DNMT1 could act as active DNA 5-mC demethylases under appropriate conditions. The de novo DNMTs, DNMT3A and DNMT3B can transfer the methyl-group to DNA which do not contain any DNA methylation on both strands. After de novo methylation, the DNA is methylated on both DNA strands. The maintenance DNMT1 can methylate the hemi-methylated (one of double strands is methylated) DNA during the DNA replications.

Ca^{2+}-and redox state-dependent DNA 5-mC demethylase activities of partially purified recombinant DNMTs. To further confirm the result of FIG.1, recombinant mouse DNMT3B, human DNMT1 (hDNMT1), and human DNMT3A (hDNMT3A) partially purified from recombinant baculovirus-infected Sf9 insect cells were examined for their DNA demethylation activities. First, the recombinant mouse DNMT3B (~50% purity, FIG. 7) was subjected to incubation with 5-mC-containing DNA substrate in buffer B containing increasing concentrations (0, 10 μM, 100 μM, 1 mM, 5 mM and 10 mM) of $CaCl_2$ (FIG.2A). As seen, the recombinant DNMT3B exhibited significant DNA demethylation activity only in the presence of Ca^{2+} (compare lanes 3-7 to lane 2, FIG.2A), with the activity highest in the presence of 1 mM Ca^{2+} (lane 5, FIG.2A). We tested and compared the DNA demethylation activities of DNMT3B, hDNMT1 (~70% purity, FIG. 7) and hDNMT3A (~90% purity, FIG. 7) in buffer B containing 1 mM $CaCl_2$ (FIG.2B). Both hDNMT1 and DNMT3B exhibited high DNA demethylation activity, converting at least 50% of 5-mC on DNA to C (lanes 2 and 4, FIG. 2B), while the recombinant hDNMT3A showed relatively lower activity (approximately 20% conversion, lane 3 of FIG. 2B). Demethylation reaction by the recombinant mouse DNMT3B also removed the HpaII resistance of the methylated DNA substrate (data not shown). These data together demonstrated that mammalian DNMTs could function as active DNA demethylases *in vitro*.

The DNA demethylation activities of the DNMTs appeared to be affected by the redox state of the enzymes. As exemplified by DNMT3B, pre-incubation of the enzyme with the reducing dithiothreitol (DTT) greatly decreased the extent of conversion of 5-mC to C (compare lane 5 to lane 3, FIG. 3A), although addition of H_2O_2 as high as 10 mM to the reaction mixture did not affect the DNA demethylation activity of the enzyme (data not shown). In contrast to 5-mC demethylation, the

5-C methylation reaction of DNMTs did not require Ca^{2+}, nor was it affected by DTT (compare lane 6 to lane 4, FIG. 3B).

Reversibility of the DNA 5-mC demethylation and 5-C methylation reactions catalyzed by DNMTs. As exemplified for DNMT3B in FIG. 3A, the inclusion of SAM, the methyl donors needed for DNA 5-C methylation by the DNMTs, in the reaction mixture greatly reduced the extent of conversion of 5-mC to C (compare lanes 4 and 6 to lane 3, FIG. 3A). This could be due to the inhibition of the demethylation activity of the DNMTs by SAM. Alternatively, the presence of SAM in the demethylation reaction might favor the methylation function of DNMTs, thus pushing the demethylation backwards. The latter scenario was confirmed with inclusion of radioactive ^{3}H-SAM in the reaction mixtures and quantitation of ^{3}H-labled-CH_3 on DNA after the reactions (lanes 4 and 6, FIG. 8). This result suggested that the DNA 5-mC demethylation reaction was reversible, with the presence SAM pushing the DNMT3B to re-methylate the demethylated cytosine on the DNA substrate.

The reversibility of the DNA methylation-demethylation reactions as catalyzed by the mammalian DNMTs was further studied by an analysis of the dynamic changes of the DNA methylation *in vitro*. In FIG. 4A, the double-stranded DNA substrate containing 5-mC was first incubated with the recombinant DNMT3B in a demethylation buffer for 2h. SAM was then added and the incubation continued for 1h. Finally, H_2O_2, which was known to inhibit the methylation reaction, was added and the reaction continued for another 2h. As exemplified in the TLC plate and statistically presented in the histogram of FIG. 4B, approximately 60% of 5-mC on the DNA substrate were demethylated by DNMT3B at the end of the first reaction (compare lane 1/bar 1 to lane M/bar M, FIG. 4B). The continued 1h incubation in the presence of SAM converted more than 60% of the C back to 5-mC (compare lane 2/bar 2 to lane 1/bar 1, FIG. 4B). Finally, the addition of H_2O_2 led to the switch of the enzyme activity of DNMT3B from methylation to demethylation again (compare lane 3/bar 3 to lane 2/bar 2, FIG. 4B), presumably due to loss the DNA methylation function of the oxidized enzyme. The data of FIGs. 3 and 4 altogether suggested that the switch of the catalytic functions of the DNMTs in between DNA methylation and demethylation was flexible, subjecting to the regulation by a range of factors including the local concentration of Ca^{2+}, the presence of SAM, and the redox-state of the DNMTs.

Generation of Radioactive-labeled methylated DNA substrate. Two different methods were used to generate radioactive-labeled methylated DNA as follows: The unmethylated DNA is incubated with M.SSSI and radioactive SAM, such as ^{3}H-SAM or ^{14}C-SAM. After the incubation and DNA isolation, the radioactive signal of the DNA was determined by autoradiography or scintillation counting to quantitate methylated DNA. Optionally, the extent of the methylation of the DNA may

be observed by HpaII digestion followed by gel electrophoresis. Alternatively, to make radioactive methylated DNA, PCR amplification is performed by using dNTP mix containing 5-[^{3}H]-methyl-dCTP or 5-[^{14}C]-methyl-dCTP. After purification of the PCR product, the extent of methylation of the DNA is analyzed by radioactive signal autoradiography or scintillation counting, and optionally observed the methylation level by HpaII digestion followed by gel electrophoresis.

Methylated reporter gene DNA substrate. A plasmid containing a reporter gene expressing a reporter protein such as EGFP or GFP (e.g., commercial available pEGFP-C1) was highly methylated by the enzyme M.SSSI in the presence of SAM using a method described above. Then the highly methylated and unmethylated reporter plasmids were transfected into 293T cell by Lipofectamine 2000, respectively. To detect expression of the reporter gene, the transfected cells were analyzed by a fluorescence microscopy and FACS flow cytometry. The results showed that the unmethylated EGFP reporter gene could strongly express the EGFP protein in the cells, but the highly methylated EGFP reporter gene expression of the EGFP protein was severely suppressed.

Based on the correlation between the DNA methylation level of the reporter gene and gene expression, we co-transfect the highly methylated reporter plasmid EGFP and the DNMTs-expression plasmids to 293 cells by Lipofectamine 2000. After 24h, the transfected cells are treated with different test agents (chemical compounds) or different genetically modified viruses transduced with exogenous cDNA encoding a known protein of interest.

The cells co-transfected with methylated EGFP plasmids and DNMT3B-expression plasmid without any chemical or virus treatment showed 2~3 folds of EGFP-positive cell population comparing to the cells co-transfected with methylated EGFP plasmids and control plasmids (without DNMTs) without any chemical or virus treatment.

If the compound (test agent) or genetically modified virus could affect the DNA demethylation, the population of EGFP-positive cells will be changed. By comparing the EGFP-positive cell populations in the presence and absence of chemical (or virus) treatment, it is feasible to identify a compound (a test agent) which may enhance DNA demethylation (i.e., increasing GFP-positive cell population) or reduce the DNA demethylation (decreasing GFP-positive cell population).

In addition, the expression of reporter (EGFP) can be analyzed by western blotting.

The DNA methylation level of the reporter plasmids could also be determined by an *in vitro* method. After chemical or virus treatments, the methylated plasmids are isolated from the 293 cells. The DNA methylation level of isolated reporter plasmids can be determined by the several methods described above, such as a restriction digestion-polymer chain reaction (PCR) assay, a hydrolysis-thin layer chromatography assay, antibody-based analysis, scintillation counting, autoradiography, dot blotting, liquid chromatography-based analysis, Na bisulfite-based analysis.

Other candidate genes for reporters may be used, for example: (1) Neomycin-resistance gene, which can keep the cell survive under Neomycin (G418) treatment; (2) Puromycin resistance gene. The cell with expression of this gene can survive under Puromycin treatment; (3) Blasticidin resistance gene, which can make cell survive under Blastindin selection; (4) Luciferase gene- pGL3 reporter vector (Promega). The firefly (*Photinus pyralis*) luciferase can catalyze the two-step oxidation reaction to yield light (550-570nm); (5) Red Fluorescence gene-pDsRed-C1. The protein is red fluorescence protein (excitation-557nm, emission- 592nm); and (6) Green fluorescent protein (GFP)-encoding gene.

Antibody-based analysis. DNAs with different methylation levels are immunoprecipitated by the 5-mC antibody. The precipitated DNA may be analyzed by PCR or pyro-sequencing to determine the relative methylation level.

Dot blotting. DNAs with different methylation levels are loaded onto NC membranes in serial diluted amounts and cross-linked by UV. The membranes are hybridized with 5-mC antibody and exposed with X-Ray films. The strong dot signal indicates a strong methylation level on the DNA.

Scintillation counting, autoradiography. A Scintillation counter is a common-used instrument that can mesure ionizing radiation. A radioactive-labelled DNA, such as ^{3}H or ^{14}C methylated DNA, is load into a container containig a scintillation cocktail. The radioactive signal is determined with a Scintillation counter by detection of light emssion. In addition, radioactive-labelled DNA may be load into a gel or on a NC membrane, and exposed with an X-Ray film.

Liquid chromatography-based analysis. The affinity of each deoxynuclesides (dC, dmC, dT, dG, and dA) to bind a C18 column is different. In this assay, DNA is degradated by DNaseI and Nuclease P1 to generate deoxynucleasides. The degradated mixture is subjexted to an HPLC system with a C-18 column. After injection of the mixture, deoxynucleasides are eluted individually at different time points by a hydrophilic buffer.

Na bisulfite-based analysis. DNAs with different methylation levels are treated with Na-bisulfilte. The unmethylated cytosine is deaminated to uracil, but not the methylated DNA. The bisulfite treated DNAs are further amplified by PCR and sequenced by cloning. In the final sequence result, the original un-methylated cytosine will present "T", and the original methylated cytosine will present "C". Based on this bisulfite-generated polymorphisms, the DNA methylation level can be analyzed by sequencing, PCR amplification with site-specific primers, HRM (high resolution melt), restriction digestion (COBRA), non-denaturing gel(MS-SSCA), or MALDI-TOF.

The current study has revealed a totally unexpected characteristic of mammalian DNMTs, likely those of the vertebrates in general. That is, the vertebrate DNMTs, in addition to converting C to 5-mC on DNA, can also actively demethylate 5-mC on DNA under specific conditions (FIGs. 1 and 2),

in particular in the presence of Ca^{2+} ion and under non-reducing condition (FIG. 3). In other words, the covalent addition of the methyl group to the C-5 position of cytosine on DNA, as catalyzed by DNMTs, is reversible (FIG. 4). The loss of the DNA demethylation activities of the mutant forms in comparison to the wild type enzymes (FIG. 1) also suggests that each of the 3 DNMTs utilizes the same domain or overlapping domains to catalytically methylate and demethylate DNA.

Based on our data presented above, in particular the Ca^{2+} dependence of the DNA demethylation activities of the 3 DNMTs and in the porcine sperm nuclear extract, we suggest that in addition to other pathways, e.g. the conversion of 5-mC to 5-hmC by TET and 5-hmC to C by DNMT3A and DNMT3B, direct conversion of 5-mC to C by the active DNA demethylation activities of the 3 DNMTs also play a major role in the genome-wide demethylation during early embryonic development of the vertebrates.

In summary, we have discovered that the mammalian DNMT1, DNMT3A, and DNMT3B, contrary to the conventional thought of their being mainly DNA methyltransferases, also act *in vitro* as active DNA demethylases in a Ca^{2+} ion- and redox state-dependent manner.

CLAIMS

What is claimed is:

1. A method for identifying a test agent as a modulator of the active DNA demethylation activity of a DNA methyltransferase, comprising:
a) providing a methylated DNA;
b) providing the DNA methyltransferase;
c) allowing the methylated DNA to react with the DNA methyltransferase for a sufficient time to perform a demethylation reaction and generate a demethylated DNA product in the presence or absence of a test agent;
d) analyzing the extent of demethylation; and
d) comparing the extents of the demethylation in the presence and absence of the test agent, and thereby identify the test agent as a modulator of the DNA demethylation activity of the DNA methyltransferase;
wherein the test agent is identified as an inhibitor of the active DNA demethylation activity of the DNA methyltransferase when the extent of the demethylation is less in the presence of the test agent; or the test agent is identified as a stimulator of the active DNA demethylation activity of the DNA methyltransferase when the extent of demethylation is more in the presence of the test agent.

2. The method of claim 1, wherein the analyzing step is performed by a technique selected from the group consisting of a restriction digestion-polymer chain reaction (PCR) assay, a hydrolysis-thin layer chromatography assay, an antibody-based analysis, scintillation counting, autoradiography, dot blotting, a liquid chromatography-based analysis, and a Na bisulfite-based analysis.

3. The method of claim 1, wherein the demethylation reaction occurs in the presence of a calcium ion concentration of around 10 μM to 10 mM.

4. The method of claim 1, wherein the DNA methyltransferase is an isolated vertebrate DNA methyltransferase or a recombinant DNA methyltransferase, or is present in a nuclear extract or present in a cellular extract.

5. The method of claim 4, wherein the vertebrate DNA methyltransferase, the nuclear extract, or the cellular extract is prepared from cells selected from the group consisting of vertebrate cell

cultures, vertebrate tissues, insect cells, worm cells, insect tissues, worm tissues, plant cells, plant tissues, yeast cells, and bacterial cells.

6. The method of claim 4, wherein the nuclear extract is a sperm extract.

7. The method of claim 1, wherein the methylated DNA comprises a labeled methyl group.

8. The method of claim 7, wherein the methyl group in the methylated DNA is radioactive-labeled.

9. The method of claim 1, wherein:

(a) the methylated DNA provided in step (a) is a methylated reporter gene operably linked to a constitutive promoter and is present in a cell;

(b) the DNA methyltransferase provided in step (b) is operably linked to a constitutive promoter and is also present in the cell;

(c) the demethylated DNA product generated in step (c) is a demethylated reporter gene, which expresses a reporter protein in the cell; and

(d) the analyzing step is performed by a technique selected from the group consisting of:

(i) analyzing the signal of the reporter protein encoded by the demethylated reporter gene in the cell;

(ii) analyzing the reporter protein expression by Western blot; and

(iii) isolating the methylated and demethylated reporter gene and analyzing the extent of demethylation by a restriction digestion-polymer chain reaction (PCR) assay, a hydrolysis-thin layer chromatography assay, an antibody-based analysis, scintillation counting, autoradiography, dot blotting, a liquid chromatography-based analysis, or a Na bisulfite-based analysis.

10. The method of claim 9, wherein the methylated reporter gene and the DNA methyltransferase are present in the cell via transfection.

11. The method of claim 1, wherein:

(a) the methylated DNA provided in step (a) is a methylated reporter gene operably linked to a constitutive promoter and is present in a cell having an endogenous DNA methyltransferase;

(b) the DNA methyltransferase provided in step (b) is endogenously present in the cell as the endogenous DNA methyltransferase;

(c) the demethylated DNA product generated in step (c) is a demethylated reporter gene, which expresses a reporter protein in the cell.

(d) the analyzing step is performed by a technique selected from the group consisting of:

(i) analyzing the signal of the reporter protein encoded by the demethylated reporter gene in the cell;

(ii) analyzing the reporter protein expression by Western blot; and

(iii) isolating the methylated and demethylated reporter gene and analyzing the extent of demethylation by a restriction digestion-polymer chain reaction (PCR) assay, a hydrolysis-thin layer chromatography assay, an antibody-based analysis, scintillation counting, autoradiography, dot blotting, a liquid chromatography-based analysis, or a Na bisulfite-based analysis.

12. A method as claimed in any one of claims 9 to 11, wherein the test agent is introduced into the cell or added to a culture medium bathing the cell.

13. A method as claimed in any one of claims 9 to 11, wherein the methylated reporter gene comprises a DNA sequence of a gene selected from the group consisting of a fluorescent protein-encoding gene, a luciferase gene, a drug-resistant gene, and genes of cell survivals.

14. A method as claimed in any one of claims 1 to 11, wherein the methylated DNA comprises **5**-methylcytosine (5mC)-containing DNA.

15. A method as claimed in any one of claims 1 to 10, wherein the DNA methyltransferase is a modified form of a wild-type DNA methyltransferase, said modified form retaining the active DNA demethylation activity of the wild-type DNA methyltransferase.

16. A method as claimed in any one of claims 1 to 11, wherein the DNA methyltransferase is selected from the group consisting of DNA methyltransferase 1, DNA methyltransferase 3A, DNA methyltransferase 3B, and any combination thereof.

17. A method as claimed in any one of claims 9 to 11, wherein the constitutive promoter is a cytomegalovirus promoter.

18. A method for identifying a test agent as a modulator of the active DNA demethylation activity of a DNA methyltransferase, comprising:

(I)

a) admixing a first composition comprising a methylated DNA with a second composition comprising the DNA methyltransferase in the presence or absence of a test agent;

b) allowing a demethylation reaction to occur by reacting the methylated DNA with the DNA methyltransferase for a sufficient time to generate a demethylated DNA product;

c) analyzing the extent of demethylation; and

d) comparing the extents of the demethylation in the presence and absence of the test agent, and thereby identify the test agent for modulating active DNA demethylation activity of the DNA methyltransferase;

wherein the test agent is identified as an inhibitor of the active DNA demethylation activity of the DNA methyltransferase when the extent of the demethylation is less in the presence of the test agent; or the test agent is identified as a stimulator of the active DNA demethylation activity of the DNA methyltransferase when the extent of demethylation is more in the presence of the test agent;

or

(II)

1) providing a cell culture medium containing cells transfected with a reporter gene that is methylated and operably linked to a constitutive promoter, said cells containing an endogenous DNA methyltransferase or exogenously expressing a wild-type, a modified or a genetically engineered DNA methyltransferase;

2) exposing the cells to a test agent;

3) allowing a demethylation reaction to occur to generate a demethylated reporter gene, which expresses a reporter protein in the cells; and

4) analyzing the extent of demethylation of the reporter gene by examining the signal intensity of the reporter protein expressed by the demethylated reporter gene in the cells;

5) comparing the extents of the demethylation of the reporter gene in the presence and absence of the test agent, and thereby identify the test agent as a modulator of the DNA demethylation activity of the DNA methyltransferase;

wherein the test agent is identified as an inhibitor of the active DNA demethylation activity of the DNA methyltransferase when the extent of the demethylation is less in the presence of the test agent; or the test agent is identified as a stimulator of the active DNA demethylation activity of the DNA methyltransferase when the extent of demethylation is more in the presence of the test agent.

19. The method of claim 18, wherein the wild-type DNA methyltransferase is selected from the group consisting of DNA methyltransferase 1, DNA methyltransferase 3A, and DNA methyltransferase 3B.

20. A method as claimed in any one of claims 1 to 10, wherein step (c) is performed under a condition that is free of a reducing agent or under a non-reducing condition.

FIG. 1

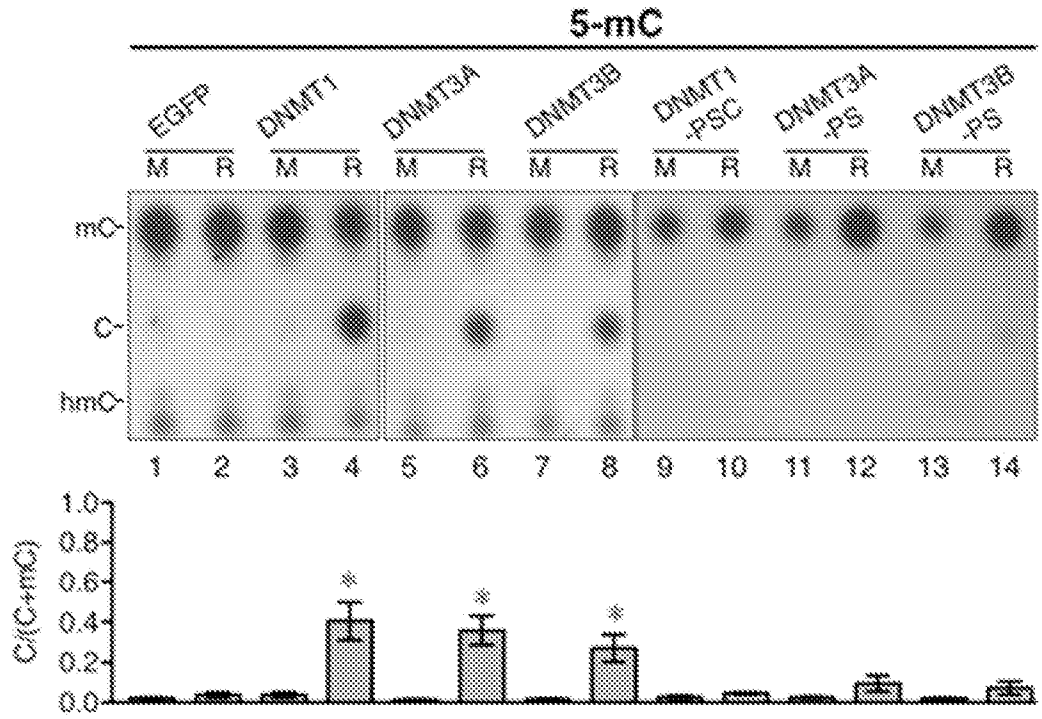


FIG. 2A

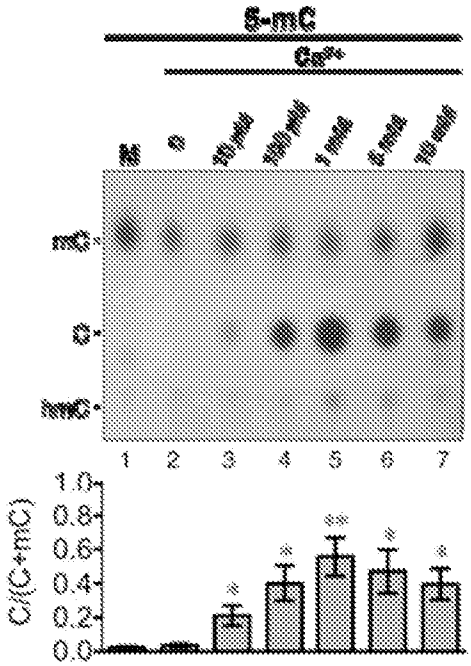


FIG. 2B

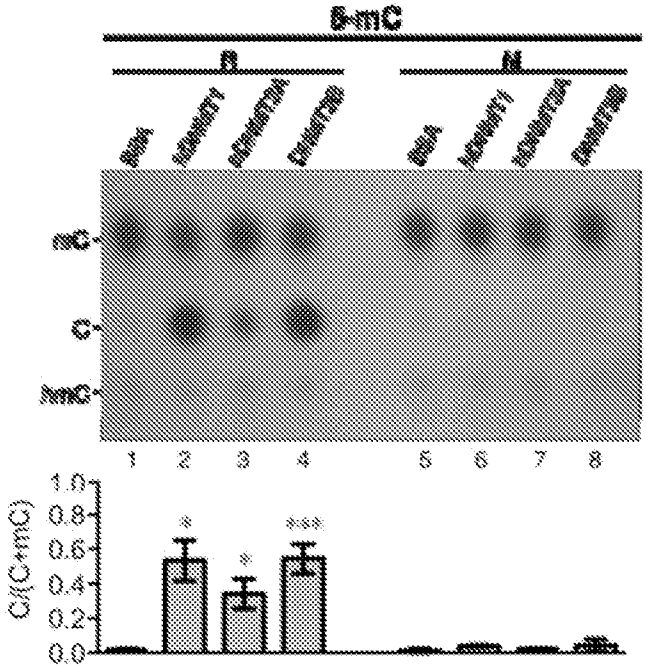


FIG. 3A

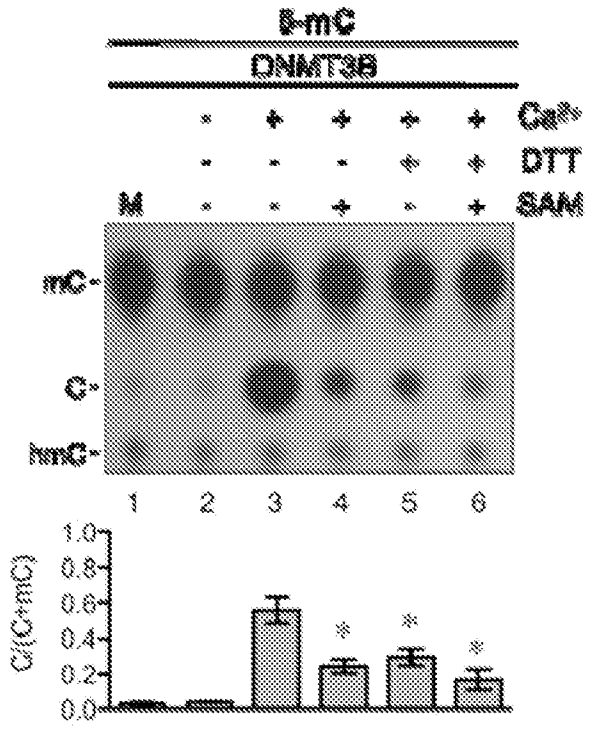


FIG. 3B

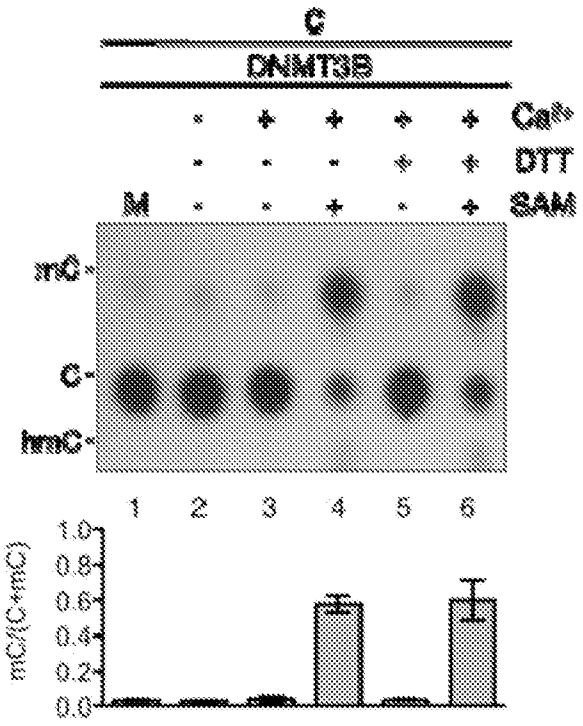


FIG. 4A

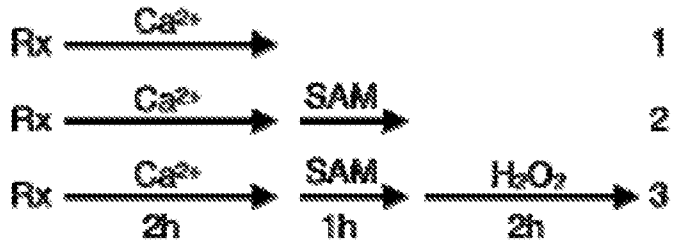


FIG. 4B

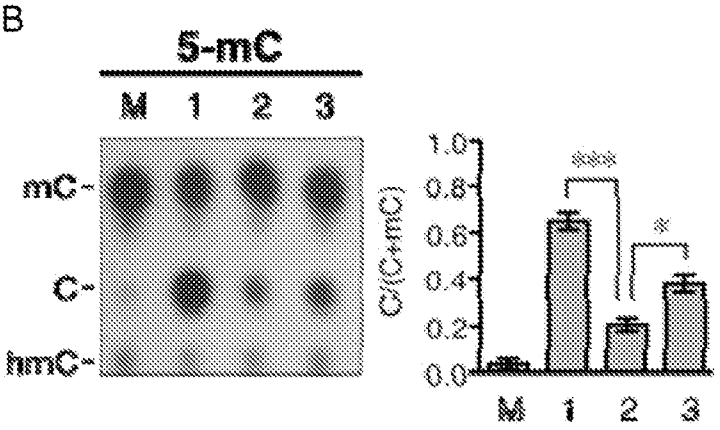


FIG. 5A

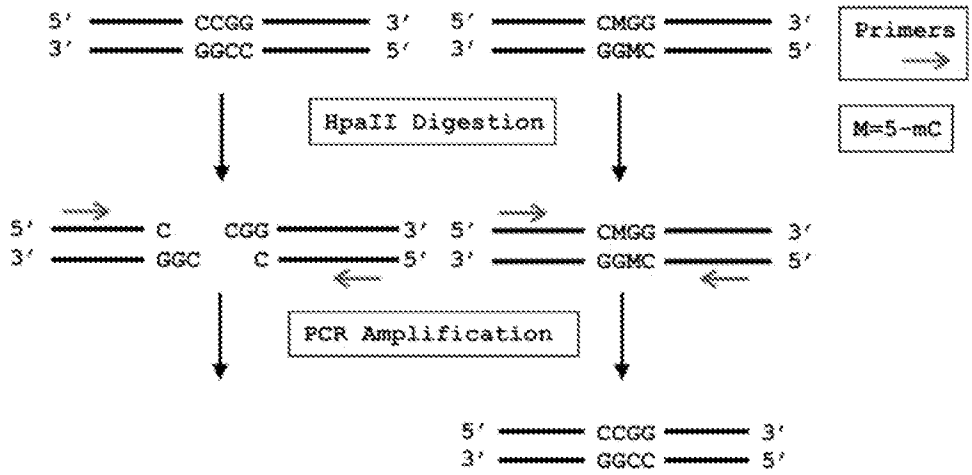


FIG. 5B

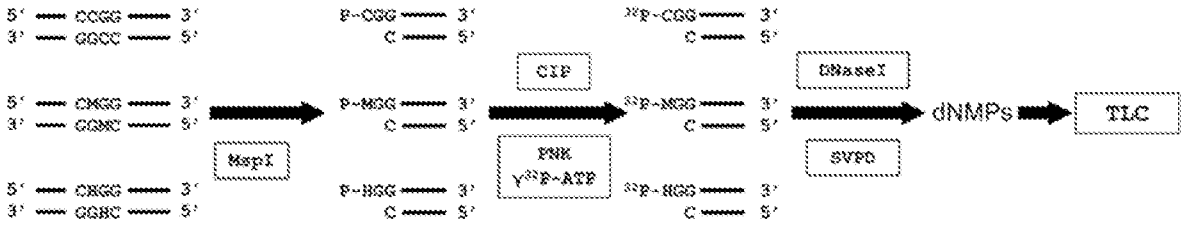


FIG. 6A

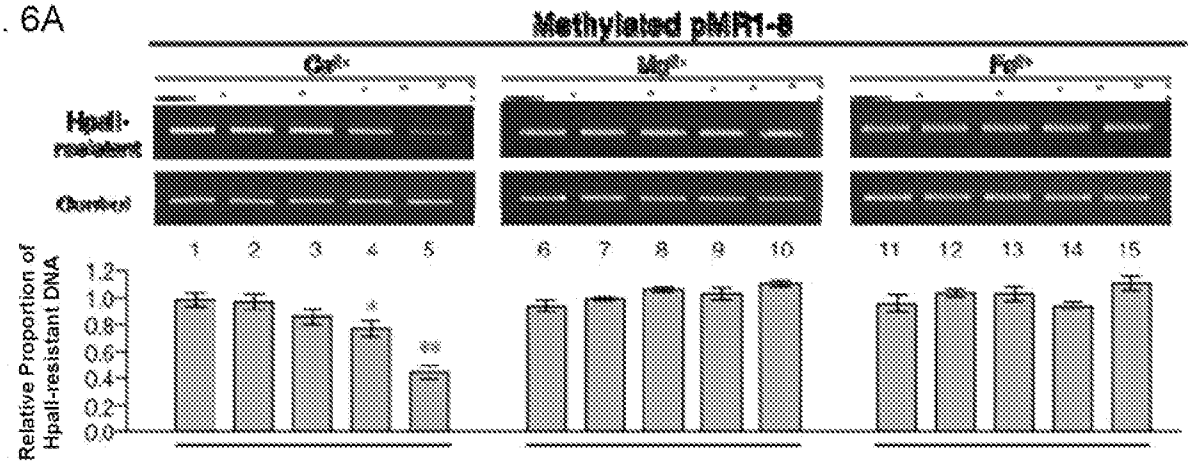


FIG. 6B

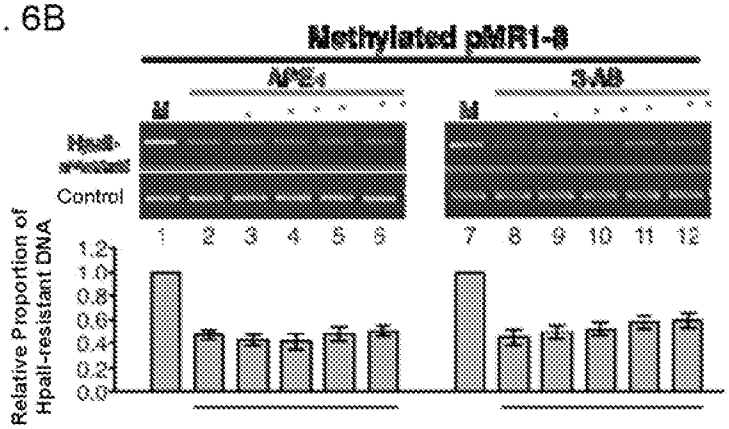


FIG. 6C

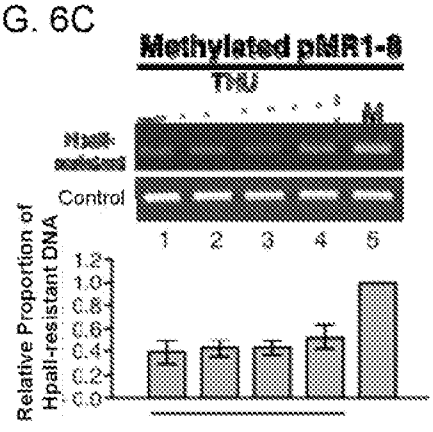


FIG. 7

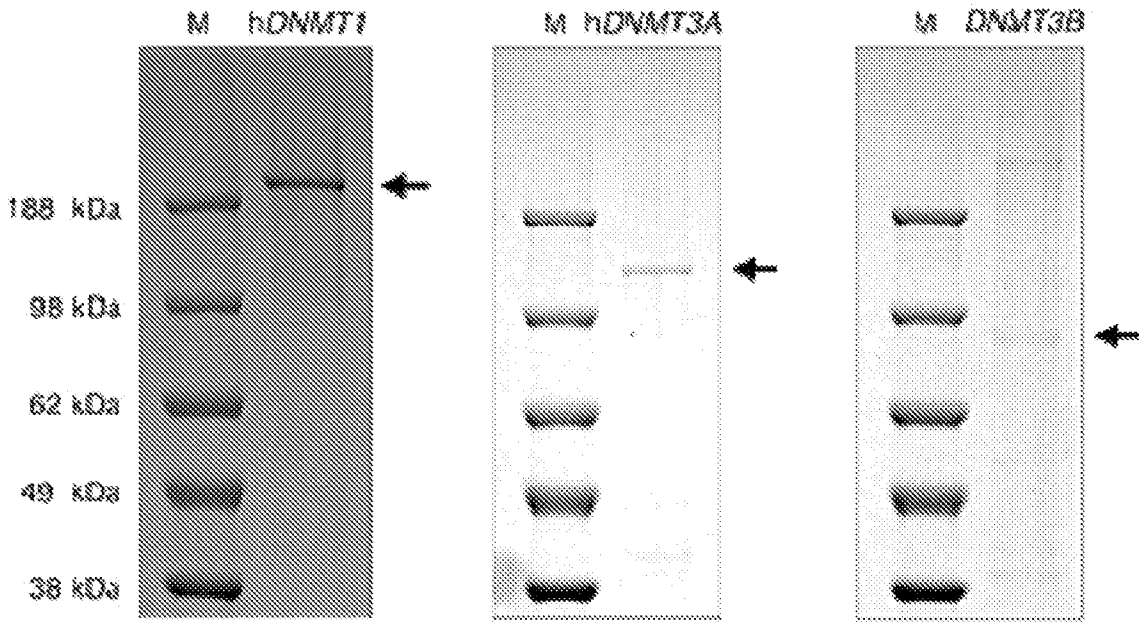
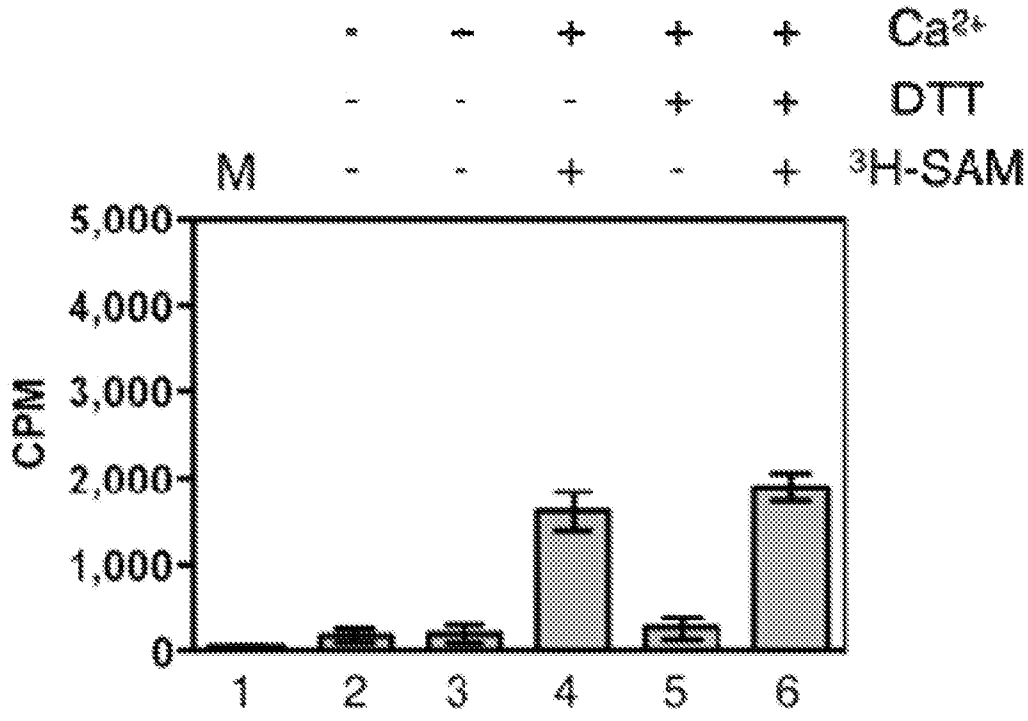

M hDNMT1
M hDNMT3A
M DNMT3B
188 kDa
98 kDa
62 kDa
49 kDa
38 kDa

FIG. 8


Ca2+
DTT
M
3H-SAM
CPM
5,000
4,000
3,000
2,000
1,000
0
1
2
3
4
5
6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US13/74141

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/48, 1/68 (2014.01)
USPC - 435/4, 6.18, 6.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): C12Q 1/48, 1/68 (2014.01)
USPC: 435/4, 6.18, 6.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MicroPatent (US-G, US-A, EP-A, EP-B, WO, JP-bib, DE-C,B, DE-A, DE-T, DE-U, GB-A, FR-A); Google Patents, PatFT, AppFT, Google Scholar, PubMed, ProQuest, Kegg (www.genome.jp), NCBI pdb; methylation, methyltransferase, DNA, 'constitutive promoter', calcium, 'sperm nuclear extract', demethylation, 'DNMT3B', 'DNMT3A', 'DNMT1'

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	CHEN, CC et al. The Mammalian de Novo DNA Methyltransferases DNMT3A and DNMT3B Are Also DNA 5-Hydroxymethylctosine Dehydroxymethylases. J Biol Chem. 16 August 2012, Vol. 287, No. 40, pp 33116-331121; abstract; page 33116, right column, second paragraph; page 33118, figure 1. DOI: 10.1074/jbc.C112.406975	1, 2, 3, 4, 5, 14/1-5, 15/1-5, 16/1-5, 18, 19, 20/1-5 --- 6-11, 14/6-11, 15/6-10, 16/6-11, 17/9-11, 20/6-10
Y	JOST, JP et al. 5-Methyldeoxycytidine monophosphate Deaminase and 5-methylcytidyl-DNA Deaminase Activities Are Present In Human Mature Sperm Cells. FEBS Lett. 22 May 2002, Vol 519, pp 128-134; abstract; page 128, right column, second to third paragraph. DOI: 10.1016/S0014-5793(02)02737-0	6-8, 14/6-8, 15/6-8, 16/6-8, 20/6-8
Y	US 2006/0166909 A1 (SZYF, M et al.) July 27, 2006; paragraph [0097]	9-11, 12/9-11, 13/9-11, 14/9-11, 15/9, 15/10, 16/9-11, 17/9-11, 20/9, 20/10
Y	Technical Bulletin: pCI and pSI Mammalian Expression Vectors, Instructions for Use Of Products E1721 And E1731. Datasheet [online]. Promgea Corportation. July 2009 [retrieved on 11-02-2014]. Retrieved from Internet: <URL: https://www.promega.com/resources/protocols/technical-bulletins/0/pci-and-psi-mammalian-expression-vectors-protocol/>	9-11, 12/9-11, 13/9-11, 14/9-11, 15/9, 15/10, 16/9-11, 17/9-11, 20/9, 20/10

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 February 2014 (11.02.2014)	24 FEB 2014
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774