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Effects of Tet-mediated Oxidation Products of 5-Methylcytosine on DNA Transcription *in vitro* and in Mammalian Cells

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5-methylcytosine (5-mC) is a well-characterized epigenetic regulator in mammals. Recent studies showed that Ten-eleven translocation (Tet) proteins can catalyze the stepwise oxidation of 5-mC to produce 5-hydroxymethylcytosine (5-HmC), 5-formylcytosine (5-FoC) and 5-carboxylcytosine (5-CaC). The exciting discovery of these novel cytosine modifications has stimulated substantial research interests about their roles in epigenetic regulation. Here we systematically examined the effects of the oxidized 5-mC derivatives on the efficiency and fidelity of DNA transcription using a recently developed competitive transcription and adduct bypass assay. Our results showed that, when located on the transcribed strand, 5-FoC and 5-CaC exhibited marginal mutagenic and modest inhibitory effects on DNA transcription mediated by single-subunit T7 RNA polymerase or multi-subunit human RNA polymerase II *in vitro* and in human cells. 5-HmC displayed relatively milder blocking effects on transcription, and no mutant transcript could be detectable for 5-HmC *in vitro* or in cells. The lack of considerable mutagenic effects of the oxidized 5-mC derivatives on transcription was in agreement with their functions in epigenetic regulation. The modest blocking effects on transcription suggested that 5-FoC and 5-CaC may function in transcriptional regulation. These findings provided new evidence for the potential functional interplay between cytosine methylation status and transcription.

Methylation at the C5 position of cytosine residues represents a major epigenetic mark that has important regulatory functions in gene expression and developmental processes^{1,2}. It was recently discovered that Ten-eleven translocation (Tet) family dioxygenases can catalyze the sequential oxidation of 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-HmC), 5-formylcytosine (5-FoC) and 5-carboxylcytosine (5-CaC) (Figure 1)^{3–9}. The resulting 5-FoC and 5-CaC in DNA can be recognized and excised by thymine DNA glycosylase (TDG) and ultimately converted back to unmodified cytosine^{8,10,11}. Thus, 5-HmC, 5-FoC and 5-CaC constitute important intermediates of a new pathway of active cytosine demethylation in mammals¹².

These Tet-mediated oxidation products of 5-mC can be readily detected in various mammalian tissues and cells. In this regard, it was reported that 5-HmC is present in mouse brain genomic DNA at a level of 5.6 modifications per 10⁴ nucleosides and occurs at substantial frequencies in other types of mammalian tissues and cells^{13–16}. Although much less abundant than 5-HmC, the levels of 5-FoC and 5-CaC in mammalian genomic DNA are comparable to those of the common types of DNA damage induced by endogenous and environmental sources^{3,5,7,8,13,17–20}. Emerging evidence indicates that 5-HmC, 5-FoC and 5-CaC may carry distinct regulatory functions in addition to being demethylation intermediates^{1,12,21–23}. For instance, 5-HmC was reported to have important roles in epigenetic regulation and reprogramming of somatic cells to pluripotent cells^{24–28}. In addition, a proteome-wide analysis revealed that 5-HmC, 5-FoC and 5-CaC can be recognized by some cellular proteins, which may confer specific biological functions²⁹. Moreover, a recent study showed that the presence of 5-FoC and 5-CaC on a template DNA strand are able to reduce the rate and substrate specificity of transcription mediated by yeast or rat RNA polymerase II *in vitro*¹⁹. It is therefore of interest to investigate how these oxidized 5-mC derivatives modulate the process of transcription in human cells.

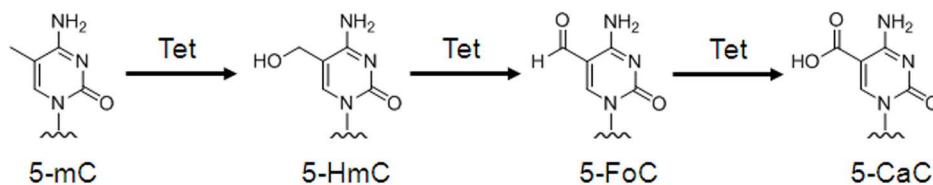


Figure 1 | Tet protein-mediated oxidation of 5-mC to produce 5-HmC, 5-FoC and 5-CaC.

Results

We employed a recently developed competitive transcription and adduct bypass (CTAB) assay³⁰ to investigate how the oxidation products of 5-mC affect the efficiency and fidelity of DNA transcription *in vitro* and in human cells. To this end, we constructed nonreplicative double-stranded plasmids containing a single site-specifically incorporated 5-HmC, 5-FoC, or 5-CaC (Figure 2a), as well as the corresponding unmodified control and competitor plasmids. These cytosine derivatives were located on the transcribed strand 58 and 39 nucleotides downstream of the cytomegalovirus (CMV) and T7 promoters, respectively (Figure 2b). We premixed the cytosine derivative-bearing or control plasmid with a competitor construct and used them as templates for the transcription assays. The run-off transcripts of interest were subjected to reverse transcription PCR (RT-PCR), followed by polyacrylamide gel electrophoresis (PAGE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses of the restriction digestion mixture of RT-PCR products (Figure 2b).

We firstly used the CTAB assay to quantitatively assess the effects of the oxidation products of 5-mC on transcription catalyzed by purified T7 RNA polymerase (T7 RNAP) or human RNA polymerase II (hRNAPII) in HeLa cell nuclear extract. In this regard, we chose to use T7 RNAP, a structural homologue of single-subunit eukaryotic mitochondrial RNA polymerases³¹, as a model to examine the impact of the 5-mC oxidation products on transcription of the mitochondrial genome.

PAGE analysis showed that, when located on the template DNA strand, a single 5-HmC did not substantially compromise the efficiency of transcription by T7 RNAP, whereas 5-CaC and 5-FoC impeded modestly the T7 RNAP-mediated transcription elongation, with relative bypass efficiencies (RBE) being ~50% and 66%, respectively (Figure 3a–c). Similarly, 5-FoC and 5-CaC, but not 5-HmC, inhibited considerably the transcription by hRNAPII (Figure 3a–c). We also employed LC-MS/MS analysis to detect the potential mutant products induced by these cytosine derivatives during transcription *in vitro*. We found that transcriptional bypass of 5-HmC with T7 RNAP or hRNAPII did not generate any detectable mutant transcripts; however, 5-FoC and 5-CaC were able to induce at least one type of mutant transcript (G→A), which contains an adenosine misincorporation opposite the 5-FoC and 5-CaC and occurs at frequencies of ~1–1.7% (Figure 4a,b, and Supplementary Figure S1–4).

We further asked how 5-HmC, 5-FoC, and 5-CaC affect DNA transcription in human cells. To this end, we premixed either cytosine derivative-bearing or control plasmids with the competitor vector and co-transfected the mixed DNA substrates into human cells. After a 24-h incubation, we extracted the RNA products from the cells and prepared the restriction digestion mixture of RT-PCR products for PAGE and LC-MS/MS analyses as described above.

Our results showed that the bypass efficiency for 5-HmC is comparable to that of unmodified control, whereas 5-FoC and 5-CaC constitute modest blocks to DNA transcription machinery in human 293T cells, with the RBE values being ~69% and 55%, respectively (Figure 3d and Supplementary Figure S5). Given that 5-FoC and 5-CaC are considered as substrates for TDG-mediated base excision repair (BER) pathway^{8,10,11}, we used siRNAs to efficiently knockdown the expression of TDG in 293T cells (Supplementary Figure S6) and tested its effect on the transcription bypass of 5-FoC and 5-CaC. We

found that, compared with the control siRNA treatment, depletion of TDG did not cause significant changes in the transcriptional bypass efficiencies of these modified cytosines (Figure 3d and Supplementary Figure S5). With the LC-MS/MS method, we also determined the mutagenic properties of these cytosine derivatives in TDG siRNA-treated human cells. Similar to what we observed from *in vitro* transcription reaction, we found that 5-HmC did not induce any detectable transcription errors, whereas transcriptional bypass of 5-FoC and 5-CaC could induce G→A mutations at frequencies of ~0.7–0.9% in the tested human cells (Figure 4c).

We next examined the potential role of nucleotide excision repair (NER) in transcriptional alternations induced by the oxidized 5-mC derivatives in human cells. Xeroderma pigmentosum group A (XPA) is a core and essential component of NER machinery³², we therefore compared the RBE values for 5-HmC, 5-FoC or 5-CaC in XPA-deficient (XP12RO) and XPA-complemented (GM15876A) cells. The results from PAGE analysis revealed no significant difference in transcriptional bypass efficiencies between XPA-deficient and XPA-complemented cells for 5-HmC, 5-FoC or 5-CaC (Figure 3e and Supplementary Figure S7). This result suggests that NER pathway may not be involved in the removal of these cytosine modifications from the template strand of an actively transcribed gene in human cells.

Discussion

The Tet-mediated oxidation products of 5-mC, including 5-HmC, 5-FoC and 5-CaC, have attracted substantial research interests regarding their potential biological functions^{1,12}. In this study, we systematically investigated the effects of these cytosine derivatives on the process of DNA transcription *in vitro* and in human cells.

It has been recently shown that 5-FoC and 5-CaC, but not 5-HmC, are able to reduce the rate of DNA transcription mediated by yeast or rat RNA polymerase II *in vitro*¹⁹. 5-FoC and 5-CaC were also found to decrease the rate of DNA replication in mammalian cells^{33,34}. In agreement with these studies, our results revealed that, when located on the transcribed strand, 5-FoC and 5-CaC, but not 5-HmC, constituted modest blocks to transcription mediated by single-subunit T7 RNA polymerase (T7 RNAP) or multi-subunit human RNA polymerase II *in vitro*. Moreover, we demonstrated the modest inhibitory effects of 5-FoC and 5-CaC on DNA transcription in human cells. Therefore, our findings provided new evidence to support the notion that, apart from their roles as intermediates of active cytosine demethylation, 5-FoC and 5-CaC may have important functions in other biological processes including regulation of gene expression in mammalian cells. On the other hand, it was reported that mitochondrial DNA is rich in 5-HmC and Tet proteins can be detected in mammalian mitochondrial extracts, suggesting the potential formation of Tet-mediated 5-mC oxidation products in mitochondria^{35,36}. Our findings that 5-FoC and 5-CaC had substantial impact on T7 RNAP-mediated transcription are of interest, due to the high degree of homology between T7 RNAP and eukaryotic mitochondrial RNA polymerases³¹. Although future studies are needed, it is reasonable to deduce that, aside from regulating nuclear gene expression, 5-FoC and 5-CaC might also be involved in transcriptional control in mitochondria.

We also found that 5-FoC and 5-CaC could weakly induce G→A mutations opposite the modified cytosine sites *in vitro* and in human

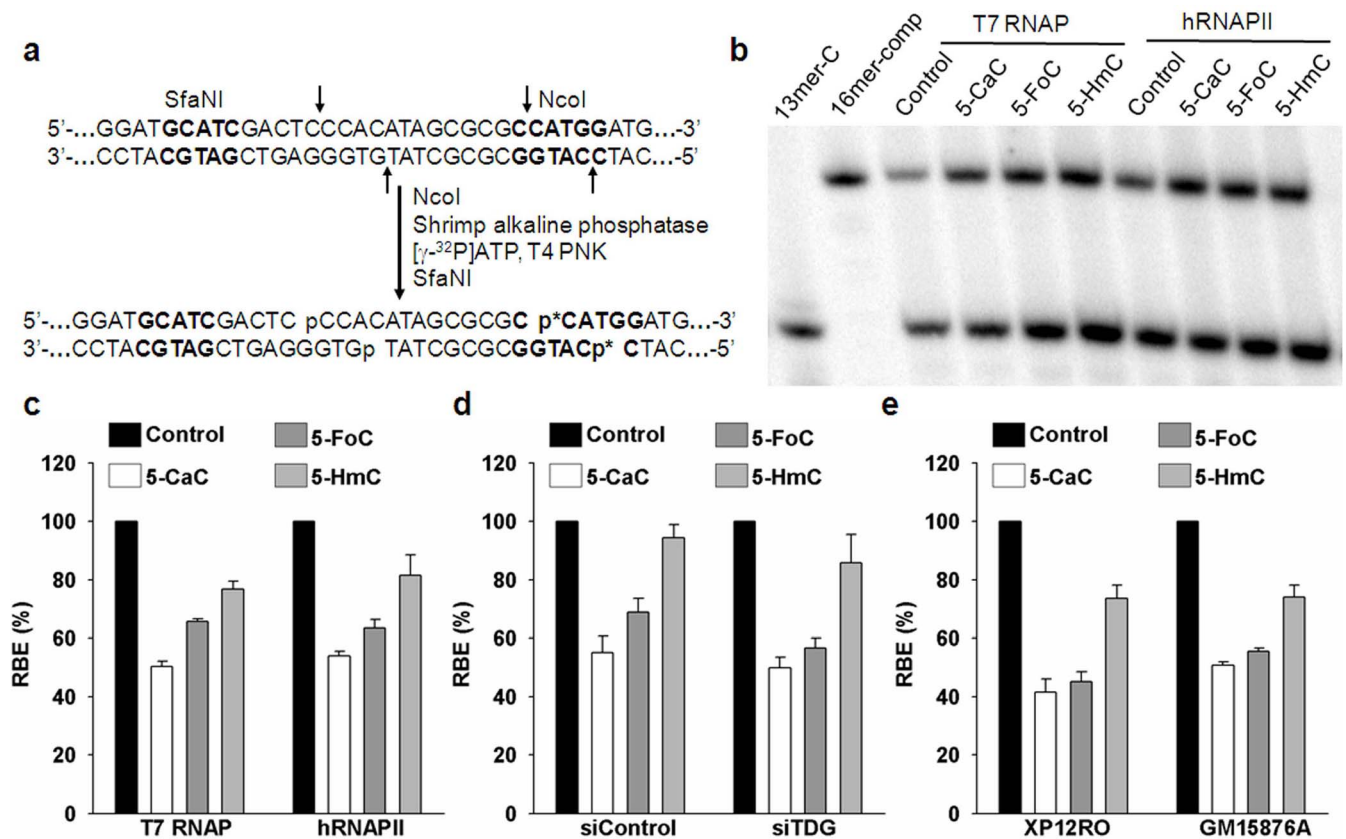


Figure 3 | The effects of cytosine derivatives on transcriptional efficiency. (a) Sample processing for PAGE analysis (p* indicates ^{32}P -labeled phosphate group). Only the wild-type sequence arising from the cytosine derivative-containing vector is shown. (b) Representative gel images showing the restriction fragments of interest. ‘13mer-C’ represents the standard ODN d(CATGGCGCGCTAT). ‘16mer-Comp’ represents the standard ODN d(CATGGCGATATGCTAT), which corresponds to the restriction fragment arising from the competitor vector. (c) The RBE values of cytosine derivatives in *in vitro* transcription systems using T7 RNAP and HeLa nuclear extract (hRNAPII). (d) The RBE values of cytosine derivatives in 293T cells treated with siRNA targeting TDG (siTDG) or nontargeting control siRNA (siControl). (e) The RBE values of cytosine derivatives in XPA-deficient (XP12RO) and XPA-complemented (GM15876A) cells. The data represent the mean and standard error of results from three independent experiments.

on transcription in human cells. This result may be attributed in part to the incomplete knockdown of TDG and the removal of 5-FoC and 5-CaC by residual TDG in human cells. Alternatively, the knockdown of TDG may be compensated by the presence of other cellular proteins that can remove these cytosine derivatives^{50–52}.

In conclusion, we showed here that 5-FoC and 5-CaC, two recently identified oxidative products of 5-mC, exhibited marginal mutagenic and modest inhibitory effects on transcription mediated by single-subunit T7 RNA polymerase or multi-subunit human RNA polymerase II *in vitro* and in human cells. This result indicates that these two oxidized 5-mC derivatives may play a role on transcriptional regulation. In addition, the presence of 5-HmC on template DNA does not perturb appreciably the efficiency or fidelity of transcription *in vitro* or in cells. Recent LC-MS/MS quantification studies revealed the presence of markedly higher levels of 5-HmC than 5-FoC and 5-CaC in genomic DNA from mammalian cells and tissues^{3,5,7,8,13,18,19}. The lack of adverse effects of 5-HmC on transcription and replication, as discussed above, may enable mammalian cells to maintain a relatively high level of 5-HmC in their genome during evolution. In addition, the weak mutagenic effects of 5-FoC and 5-CaC on transcription and replication may constitute another justification for the mammalian cells to evolve with a BER-based machinery for the efficient removal of these modified nucleobases from their genome. Together, our findings provide important insights into the potential functional interplay between active cytosine demethylation and transcription.

Methods

Materials. Unmodified oligodeoxyribonucleotides (ODNs) were purchased from Integrated DNA Technologies. [γ - ^{32}P]ATP was obtained from Perkin-Elmer. All enzymes and chemicals unless otherwise specified were obtained from New England Biolabs and Sigma-Aldrich, respectively. ON-TARGETplus SMARTpool siRNA against human TDG (L-003780) and Non-Targeting control siRNA (D-001210) were from Thermo Scientific Dharmacon. The 293T human embryonic kidney epithelial cells were purchased from ATCC. XPA-deficient (XP12RO) and XPA-complemented (GM15876A) human fibroblast cell lines were kindly provided by Prof. Karlene A Cimprich⁵³. Cells were cultured in Dulbecco’s Modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen), 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin (ATCC), and incubated at 37°C in 5% CO_2 atmosphere.

Transcription template preparation. To construct the unmodified control vector, a 50-mer ODN with the sequence of 5’-CTAGCGGATGCATCGACTCCCACATAGCGCGCCATGGATGACTCGCTGCG-3’ was annealed with its complementary strand and ligated to a restriction fragment from the NheI-EcoRI-treated pTGFPP-T7-Hha10 plasmids³⁰. Using the same method, a competitor vector was constructed to carry three more nucleotides than the control plasmid between the two restriction sites used for CTAB assay (Figure 2). We next nicked the unmodified control plasmid with Nt.BstNBI and produced a gapped vector by removing the Nt.BstNBI-generated 25-mer single-stranded ODN, followed by filling the gap with a 13-mer unmodified ODN (5’-GTGGGAGTCGATG-3’) and a 12-mer modified cytosine-containing ODN (5’-ATGGCGXGCTAT-3’, X=5-HmC, 5-FoC or 5-CaC)^{33,54}. We incubated the ligation mixture with ethidium bromide and purified the resulting supercoiled cytosine derivative-bearing plasmids by agarose gel electrophoresis, as described elsewhere^{55–57}. Purified control or cytosine derivative-bearing plasmids were mixed with the competitor genome at 3:1 ratio and used as DNA templates for all transcription assays in this study.

In Vitro Transcription assay. Multiple rounds of transcription reactions catalyzed by purified T7 RNA polymerase (T7 RNAP) or human RNA polymerase II (hRNAPII)

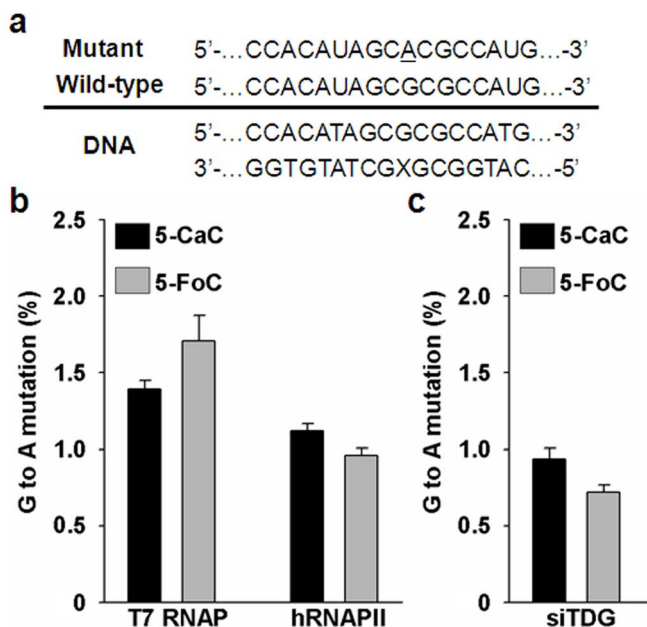


Figure 4 | The effects of 5-FoC and 5-CaC on transcriptional fidelity.

(a) The sequences of wild-type and mutant (G→A) transcripts are indicated above the double-stranded DNA construct. The underlined base (i.e., A) indicates an adenosine misincorporation opposite a 5-FoC or 5-CaC. (b) The mutation (G→A) frequencies of 5-FoC and 5-CaC in *in vitro* transcription systems using T7 RNAP and hRNAPII. (c) The mutation (G→A) frequencies of 5-FoC and 5-CaC in 293T cells treated with siRNA targeting the TDG gene (siTDG).

in HeLa nuclear extract (Promega) were performed as described previously^{30,58}. Briefly, T7 RNAP-mediated transcription reaction was carried out at 37°C for 1 h in a 20-μL mixture containing 50 ng of NotI-linearized DNA templates, 20 U of T7 RNAP, 10 U of RNase inhibitor, and 0.5 mM each of ATP, CTP, GTP and UTP. The hRNAPII-mediated transcription reaction was performed at 30°C for 1 h in a 25-μL mixture containing 50 ng of NotI-linearized DNA templates, 8 U of HeLa nuclear extract, 10 U of RNase inhibitor, and 0.4 mM each of the four ribonucleotides.

In Vivo transcription assay. XPA-complemented (GM15876A) and XPA-deficient (XP12RO) cell lines were grown to about 70% confluence in 24-well plates and co-transfected with 50 ng mixed DNA templates and 400 ng carrier plasmid (self-ligated pGEM-T, Promega) with Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. For siRNA experiments, 293T cells were grown to 40–60% confluence in 24-well plates and transfected with 25 pmol siRNAs directed against TDG or non-targeting control siRNAs. After a 48-h incubation, 50 ng DNA templates, 400 ng carrier DNA and another aliquot of siRNAs were co-transfected into the cells with Lipofectamine 2000 (Invitrogen). For all *in vivo* transcription assays, the cells were harvested for RNA extraction 24 h after transfection with the mixed DNA templates.

RNA extraction and RT-PCR. We used the Total RNA Kit I (Omega) to purify the RNA products arising from *in vitro* and *in vivo* transcription, and we subjected the RNA products to two rounds of DNase I treatment using the DNA-free kit (Ambion) to eliminate DNA contamination. We then produced cDNA by using a gene-specific primer (5'-TCGGTGTGCTGTGAT-3') and M-MLV reverse transcriptase (Promega). RT-PCR amplification was then performed by using Phusion high-fidelity DNA polymerase and a pair of primers spanning the lesion site as described in a recent study³⁰. Real-time quantitative RT-PCR for evaluating the efficiency of siRNA knockdown was conducted by using the iQ SYBR Green Supermix kit (Bio-Rad) and gene-specific primers for TDG or the control gene GAPDH as described elsewhere³³.

PAGE analysis. PAGE analysis of transcription products was performed as described elsewhere^{30,58}. Briefly, a portion of the above RT-PCR fragments was incubated in a 10-μL mixture containing 1× NEB buffer 3, 1 U shrimp alkaline phosphatase and 5 U NcoI at 37°C for 1 h, and then at 70°C for 20 min. The resulting dephosphorylated DNA was incubated in a 15-μL solution containing 1× NEB buffer 3, 10 U T4 polynucleotide kinase and ATP (50 pmol cold, premixed with 1.66 pmol [γ -³²P]ATP). The mixture was incubated at 37°C for 30 min and then at 70°C for 20 min, after which 2 U SfaNI was added and incubated at 37°C for 2 h. The resulting ³²P-labeled restriction fragments were resolved by using 30% polyacrylamide gel (acrylamide:bis-acrylamide=19:1) with 5 M urea and quantified by

phosphorimager analysis. The bypass efficiency was calculated using the following formula, bypass efficiency = (cytosine derivative signal/competitor signal)/(unmodified control signal/competitor signal)^{30,58,59}.

LC-MS/MS analysis. To identify the transcription products using LC-MS/MS, RT-PCR products were treated with 50 U NcoI, 50 U SfaNI and 20 U shrimp alkaline phosphatase in 250 μL NEB buffer 4 at 37°C for 4 h, followed by heating at 80°C for 20 min. The resulting solution was extracted with phenol/chloroform/isoamyl alcohol (25:24:1, v/v) and the aqueous portion was dried with Speed-vac, desalted with HPLC and dissolved in water. The resultant ODN mixture was subjected to LC-MS/MS analysis following previously described conditions^{30,55,58}. Briefly, a 0.50 × 150 mm Zorbax SB-C18 column (Agilent Technologies) was used. The flow rate was 8.0 μL/min, and a 5-min linear gradient of 5–20% methanol followed by a 25 min of 20–60% methanol in 400 mM 1,1,1,3,3,3-hexafluoro-2-propanol buffer (pH was adjusted to 7.0 by the addition of triethylamine) was employed for the separation. The LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific) was set up for monitoring the fragmentation of the [M-3H]³⁺ ions of 13-mer ODNs, i.e., d(CCACATAGCMCGC), where “M” designates A, T, C, or G.

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Author contributions

C.Y. and Y.W. designed the research, analyzed the data and wrote the paper; C.Y., D.J. and X.D. performed the research; Y.W. conceived and supervised the study.

Additional information

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