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CpG site-specific alteration of hydroxymethylcytosine to methylcytosine beyond DNA replication

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ABSTRACT

Hydroxymethylcytosines (hmC), one of several reported cytosine modifications, was recently found to be enriched in embryonic stem cells and neuronal cells, and thought to play an important role in regulating gene expression and cell specification. However, unlike methylcytosines (mC), the fate of hmC beyond DNA replication is not well understood. Here, to monitor the status of hmC during DNA replication, we prepared a stable episomal vector-based monitoring system called MoCEV in 293T cells. The MoCEV system containing fully hydroxymethylated-cytosine fragments revealed a significant modification towards mC after several rounds of DNA replication. Strikingly this modification was specifically observed at the CpG sites (71.9% of cytosines), whereas only 1.1% of modified cytosines were detected at the non-CpG sites. Since the unmodified MoCEV did not undergo any DNA methylation during cell division, the results strongly suggest that somatic cells undergo hmC to mC specifically at the CpG sites during cell division.

1. Introduction

DNA modifications are known to have critical roles in controlling and maintaining the pattern of chromatin structure, gene expression and cell specification. Methylation of DNA in mammals is a well-characterized modification, which predominantly occurs at the 5th-position of cytosine in CpG dinucleotides (mCpG). This epigenetic modification has been thought to play an important role in gene regulation and it is highly correlated to a variety of human diseases such as cancers and imprinting disorders [1–6]. In addition to the commonly known methylcytosine (mC), the existence of 5-hydroxymethylcytosine (hmC), which is produced through the oxidation of mC by the ten–eleven translocation family of dioxygenases, was recently reported in mammalian cells [7,8]. Although hmC is widely detected in many tissues and cell types, differential hmC contents were observed across multiple tissues, which is contrary to the mC class [9]. The integration of genomic hmC signals with a histone enrichment map suggested that hmC might play diverse roles in regulating specific promoters, gene bodies and enhancers in embryonic stem cells (ESCs) [10]. Also, since it has been reported that mCpG binding proteins do not interact with hmC containing DNA substrates [11,12], modifications of methylation and hydroxymethylation are likely to play distinct roles in biological systems [13]. During the cell proliferation, the mC type on the daughter strand of DNA is methylated by DNA methyltransferase 1 (DNMT1), while the fate of hmC during cell propagation remains unclear. One report showed the persistence of hmC in ESCs beyond DNA replication [14], suggesting that ESCs may require the existence of hmC after DNA replication. On the other hand, hmC in mouse preimplantation embryos, which have relatively low expression levels of DNMTs than the other cells, reported loss of hmC during DNA replication [15]. Here, to address the fate of hmC in somatic cells, we improved a previously reported episomal plasmid-based method [16] to monitor hmC status of any desired DNA fragments beyond DNA replication using 293T cells.

2. Materials and methods

2.1. Cell culture

Human embryonic kidney cell line, 293T, was provided from Cell Bank of RIKEN BioResource Center. 293T cells were grown in

Abbreviations: hmC, hydroxymethylcytosine; mC, methylcytosine; mCpG, methylcytosine in CpG dinucleotides; DNMTs, DNA methyltransferases; ESCs, embryonic stem cells; T4-BGT, T4 β-glucosyltransferase; GFP, green fluorescent protein; MoCEV, modified cytosine in episomal vector.

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DMEM (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Life Technologies, Grand Island, NY) and were incubated at 37 °C in a humidified 5% CO_2 incubator.

2.2. PCR products with cytosine modification

The 1.8 kbp of CAG promoter sequence, a strong promoter consisting of chicken β-actin promoter and cytomegalovirus early enhancer element, with vector shared homologous 15-bp fragments on both ends was obtained by PCR amplifying the pEBMulti-Hyg/Venus+ vector as a template and specific primers (5′-TTGATTATTGACTAGTTATTAATAGTAATCAATTAC-3′, 5′-CCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGG-3′) using PrimeSTAR GXL DNA polymerase (Takara Bio, Otsu, Japan). To make a CpG methylated CAG promoter, 1 μg of PCR product was treated with 4 units M.SssI CpG methyltransferase (New England BioLabs, Ipswich, MA) together with 0.64 mM S-adenosylmethionine at 37 °C for 4 h, followed by purification using QIAquick PCR purification kit (Qiagen, Valencia, CA). Fully hmC modified linear CAG promoter product was generated using hydroxymethyl dCTP (Nippon gene, Tokyo, Japan) in PCR amplifications and purified using QIAquick PCR purification kit.

2.3. Transfection of In-fusion-based constructed vector with cytosine modified DNA fragments

For a new construct of vector, pEBMulti-Hyg/Venus+/Promoterless, the EB virus based pEBMulti-Hyg expression vector [17] (Wako Pure Chemical Industries) was modified. To perform In-Fusion assembly, 9 μg of pEBMulti-Hyg/Venus+/Promoterless vector was digested overnight by SpeI and purified by phenol/chloroform extraction, followed by resuspension in a total volume of 45 μl of autoclaved water. Four-microliters of purified PCR product of CAG promoter as described above, 800 ng of the purified vector and 8 μl of 5x In-Fusion HD enzyme (Takara Bio) were mixed and incubated at 50 °C for 15 min [18]. To transfect assembled vector into 293T cells, the samples were purified by phenol/chloroform extraction and ethanol precipitation, and then resuspended in 50 μl Opti-MEM (Life Technologies). Two-microliter of Lipofectamine 2000 (Life Technologies) was diluted into 50 μl Opti-MEM and kept for 5 min at room temperature. The preincubated Lipofectamine 2000 solution was mixed with 50 μl of the In-Fusion assemble samples in Opti-MEM and incubated for 30 min. For the transfection, the cells were plated in 24 well plate at a density of 5×10^4 293T cells per well and the medium was exchanged for 400 μl Opti-MEM just before transfection, followed by addition of the incubated mixture of the assembled vector and Lipofectamine. The transfected 293T cells were cultured in Opti-MEM for 24 h before replacing it by DMEM medium with 250 μg/ml Hygromycin B (Life Technologies).

2.4. Bisulfite sequencing

Genomic DNAs from all episomal vector transfected 293T cells were isolated with the NucleoSpin Tissue (Macherey-nagel, Düren, Germany). Bisulfite treatment for 400 ng of each genomic DNA was performed using an EZ DNA Methylation-Gold Kit in accordance with the manufacturer's instructions (Zymo Research, Irvine, CA). The converted samples were amplified by Blend Taq Plus (Toyobo, Tokyo, Japan) using specific primers as follows: Region1 (5′-TTGTATTAATTAATTGAAGGGATTATATGT-3′ and 5′-AAAATCATATACTAAACATAATACCAAAC-3′), Region2 (5′-AAAGAATTAATTTTTATTAAAGGGGTAT-3′ and 5′-AAATCAACTTCAAAATCAACTTACC-3′). The PCR amplicons were subcloned into TArget vector (Toyobo) and isolated clones were sequenced using ABI3730xl DNA Analyzer (Life technologies). Visualization of bisulfite sequence data for CpG methylation was performed using the QUMA web-based tool [19].

2.5. Determination of the existence of hmC

The presence of hmC at specific MspI sites were analyzed using EpiMark 5-hmC and 5-mC Analysis Kit (New England BioLabs) following the manufacturer's recommended protocol, followed by quantitative real-time PCR. Genomic DNA from 293T-CAG-hmC and control DNA, which was provided in the kit, was treated with T4 β-glucosyltransferase (T4-BGT) in the presence of UDP-glucose in parallel with a mock reaction containing only UDP-glucose. Then, one fourth of test and control reactions were digested with MspI. Whereas MspI cleave a recognition sequence (CCGG) containing either mC or hmC, glucosylated hmC containing MspI site is resistant to the digestion. The real-time PCR analyzed the amount of DNA template cut by MspI with/without T4-BGT treatment using specific primers for 293T-CAG-hmC genomic DNA (5′-CGGGCCCCCCCTCGAGGTCG-3′ and 5′-GCCGCTCTAGAACTAGTGG-3′) or the manufacturer provided primers for control DNA (5′-CAGTGAAGTTGGCAGACTGAGC-3′ and 5′-CTGACTTGCCACCTATAGACAGC-3′). Relative quantification of hmC was calculated as the ratio of the real-time PCR results (Ct [non-glucosylated samples]/Ct [glucosylated samples]).

2.6. MBD real-time PCR

Genomic DNA from 293T-CAG-C, 293T-CAG-hmC and 293T-CAG-mCpG were prepared using NucleoSpin Tissue Kits. One-microgram of isolated genomic DNA was sonicated for 5 min with a Branson 450 Sonicator (Branson Ultrasonics, Danbury, CT). PCR products (CAG-C, CAG-hmC or CAG-mC) were mixed human genomic DNA and fragmented by Covaris S2 sample preparation instrument system (Covaris, Woburn, MA) as control fragments. Sheared genomic DNA or control fragments was subjected to methyl-CpG binding domain DNA capture (MBD) using the MethylMiner Methylated DNA Enrichment Kit (Life Technologies) following the manufacturer's recommended protocol. The bound fraction was eluted by proteinase K and purified on a Qiaquick MinElute column (Qiagen, Valencia, CA). Real-time PCR reaction were carried out using SYBR Premix ExTaq (Takara Bio) and specific primers for control fragments (5′-GCTAACCATGTTCATGCCTTC-3′ and 5′-TGCCAAAATGATGAGACAGC-3′) or for genomic DNA from 293T-CAG-C, 293T-CAG-hmC or 293T-CAG-mCpG (5′-CGATAAGCTTGATATCGAATTCCT-3′ and 5′-CTCACCATGGTTGTGGGGTA-3′) on the ABI PRISM 7500 Fast Real-Time PCR System (Life technologies) by denaturation at 95 °C for 10 s, followed by running for 40 cycles at 95 °C for 5 s and 64 °C for 30 s. The amount of target DNA recovered in the captured fraction was quantified by calculating the percent input recovery.

3. Results

3.1. Preparation of an In-fusion-based vector construction method for cytosine modified DNA fragments

Hsieh and colleagues had previously reported that methylated DNA patches on the episomal plasmid are stably maintained for a long period after transfection into human somatic cells [16]. However, the restriction enzyme digestion-based system is not suitable for PCR products containing fully modified cytosines because the cleavage site is blocked when a cytosine in the enzyme recognition site is modified. To develop a more simple and effective method to directionally incorporate cytosine-modified PCR

fragments, we utilized the In-fusion cloning system [18], which enables fast directional cloning without the additional restriction enzyme digestion step (Fig. 1). To check the assembly reaction rates between the inserts with and without mCpG, we performed the In-fusion reaction with the *in vitro* methylated and untreated CAG promoters, respectively. The number of transformed colonies on LB plates resulted in no difference between the two vectors (data not shown). Then, to examine whether the In-fusion assembled vectors maintain the status of DNA methylation *in vivo*, 5×10^4 293T cells were transfected with the mCpG or unmodified promoter carrying vectors, and were grown to more than 1×10^7 cells, replicating more than 7 times in average during the cultivation. Subsequently, we performed down-stream gene expression analysis, bisulfite sequencing and 5-aza-2′-deoxycytidine treatments, and revealed that CpG DNA methylation status, both DNA methylated and unmethylated, were stably maintained throughout replication (Supplemental Figs. S1–S4). We thus established an In-fusion-based vector construction methods for cytosine modified DNA fragments designated as MoCEV (Modified Cytosine in Episomal Vector).

3.2. Observation of hmC status beyond DNA replication

Next, to observe the status of hmC throughout multiple cell divisions in 293T cells, we first synthesized CAG promoter containing fully hydroxymethylated-cytosine by replacing deoxycytidine triphosphates with 5-hydroxymethyl-deoxycytidine triphosphates using PCR. For checking the incorporation of hmC, PCR products were digested with the cytosine-modification sensitive restriction enzymes, HhaI and HpaII. Reasonably, the unmodified CAG promoter was completely digested by both enzymes, while hmC containing CAG promoter was resistant to these enzymes (Supplemental Fig. S5A). In addition, the incorporation of hmC was validated by the dot blots analysis using the antibody against the hmC epitope (Supplemental Fig. S5B). The assembly reaction rates were also analyzed, resulting in no difference in the number of transformed colonies (data not shown). Thereafter, the MoCEV containing hmC CAG promoter was transfected into 293T cells (293T-CAG-hmC). In order to observe the maintenance of hmC *in vivo*, genomic DNA from 293T-CAG-hmC was extracted after 2 weeks of cultivation and subjected to bisulfite treatment. The bisulfite-treated samples were amplified in two regions (Region1 and Region2), including the vector junction and the CAG promoter sequences, followed by the nucleotide sequencing analysis (Supplemental Fig. S3). The Region1 amplicon covered 79 cytosines on the CAG promoter (17 CpGs and 62 non-CpGs across the 360-bp region), and the Region2 amplicon contained 28 cytosines on the CAG promoter (7 CpGs and 21 non-CpGs across the 220-bp region). The hmC in the non-CpG contexts showed a higher frequency of loss-of-resistance against bisulfite treatment (1232 out of 1246 cytosines; 98.9%), indicating that hmC was changed to cytosine predominantly at non-CpG residues (Fig. 2A and B). However, hmC in the CpG context showed a significantly dense bisulfite resistant cytosine. We found that 71.9% of CpG in the 293T-CAG-hmC vectors were resistant to bisulfite treatment, while all of cytosines were converted in 293T-CAG-C (Fig. 2B and C). This outcome demonstrates that hmC in the CpG sites is selectively maintained to resist bisulfite treatment throughout DNA replication.

3.3. Determination of modified form of cytosines

Because bisulfite sequence analysis cannot distinguish hmC from mC [20], we performed MspI restriction enzyme digestion followed by real-time PCR to determine the existence of hmC. T4 β-glucosyltransferase (T4-BGT) with UDP-glucose is able to add a glucose group to the hydroxyl group of hmC. Unlike the MspI cleavage sites in hmC, mC and unmodified cytosines, glucosylated hmC cannot be cleaved by MspI (Fig. 3A). By applying this sensitivity change, real-time PCR was used to determine the amount of DNA template digested by MspI before and after the treatment with T4-BGT, allowing the quantification of hmC levels at the MspI sites [19]. As shown in Fig. 3B, we found clear changes in the enzyme digestion rates of hmC control fragments after T4-BGT treatments. However, although 63.6% of cytosines at the MspI sites were resistant to bisulfite treatment in 293T-CAG-hmC (Fig. 2C, blue box), no difference was observed between unglucosylated and glucosylated DNA from 293T-CAG-hmC, suggesting that the bisulfate resistant cytosine at the MspI site is not hmC. Because the above analysis showed modification of only one cytosine residue at the MspI site, we further examined the modification of entire hmC. We prepared a DNA precipitation method using the methyl binding Domain 2 (MBD2) protein, followed by the amplification of the Region2 using real-time PCR (MBD real-time PCR). Since MBD2 has no affinity for hmC and unmodified cytosine [11], *in vitro* synthesized control DNA fragments of CAG-mCpG were enriched higher than those of CAG-C and CAG-hmC by

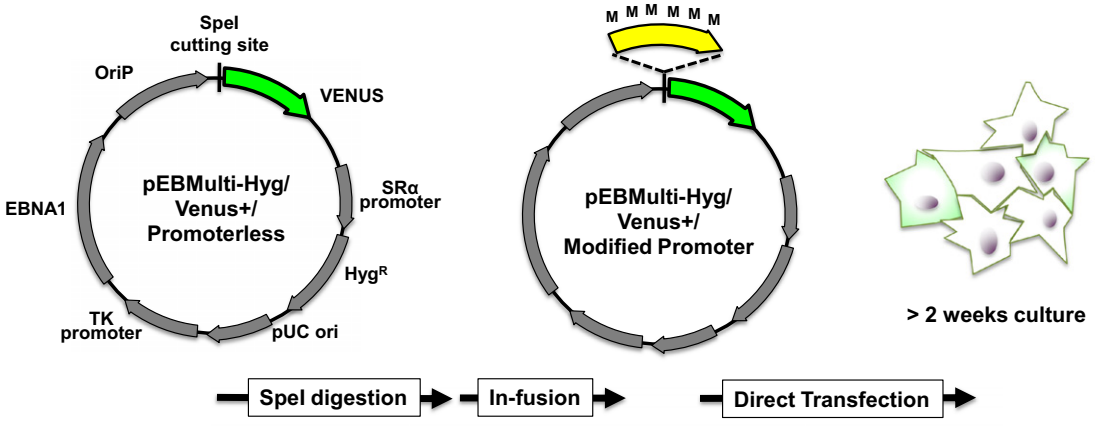

Fig. 1. Schematic illustrations of the MoCEV system construction process. The pEBMulti-Hyg/Venus+/Promoterless vector contains a SpeI recognition sequence (A|CTAGT). The SpeI digested vector and the desired PCR products with or without cytosine modifications were assembled by the In-fusion treatment to produce the pEBMulti-Hyg/Venus+/Modified Promoter vector. After purification, the assembled vectors are directly transfected into 293T cells. Hyg^R: Hygromycin B resistant gene, OriP: replication origin, VENUS: enhanced yellow fluorescent protein, pUC ori: *Escherichia coli* origin of replication, EBNA1: Epstein–Barr virus associated nuclear antigen 1, M: a modified cytosine. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

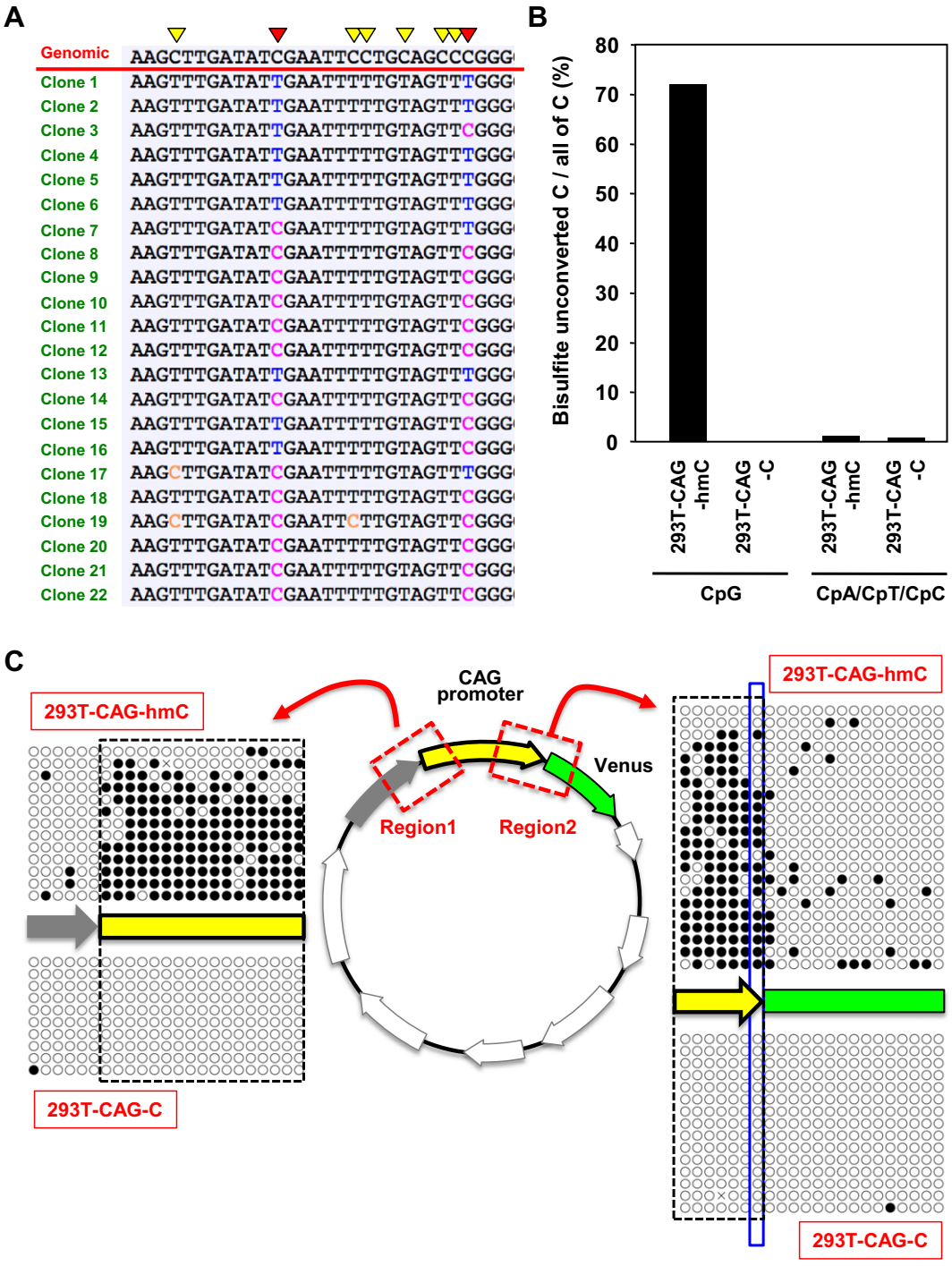

Fig. 2. Observation of hmC status beyond DNA replication. (A) Multiple alignment of bisulfite sequencing results using the QUMA software. Twenty-two Region2 individual clones from 293T-CAG-hmC were demonstrated. Yellow arrowhead, non-CpG; red arrowhead, CpG; Orange C, unconverted cytosine in non-CpG; pink C, unconverted cytosine in CpG; blue T, converted cytosine in CpG. (B) The distribution of unconverted cytosines in CpG and non-CpG after bisulfite treatment. (C) Bisulfite sequencing analysis on the isolated episomal vectors from the 293T-CAG-hmC (top panels) and 293T-CAG-C (bottom panels) cells 2 weeks after transfection. Both amplicons, Region1 and Region2, are indicated by red dashed boxes. Black broken line boxes demonstrate CAG promoter regions on both sides. Black and white circles indicate methylated and unmethylated CpGs, respectively. A yellow bar and arrow exhibit CAG promoter. Venus and OriP are depicted as a green bar and a gray arrow, respectively. A blue box shows the cytosine at MspI site for relative quantification analysis of hmC. ○, unmethylated CpG site; ●, methylated CpG site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

MBD2 precipitation (Fig. 4A). Subsequently, MBD real-time PCR analysis revealed that using sheared DNA samples from 293T-CAG-C, 293T-CAG-mCpG and 293T-CAG-hmC, and the precipitated DNA from 293T-CAG-hmC showed almost the same enrichment to that of 293T-CAG-mCpG, but not from 293T-CAG-C (Fig. 4B). Collectively, these data revealed that most of the bisulfite resistant cytosines at the CpG sites of CAG promoter were methylcytosines.

4. Discussion

The hmC was initially discovered as a new constituent of mammalian DNA [7,8]. Current studies suggested that hmC might play a role in regulating the status of DNA methylation [21]. Guo and colleagues previously demonstrated a replication-independent hmC-to-cytosine transformation using hmC-modified green

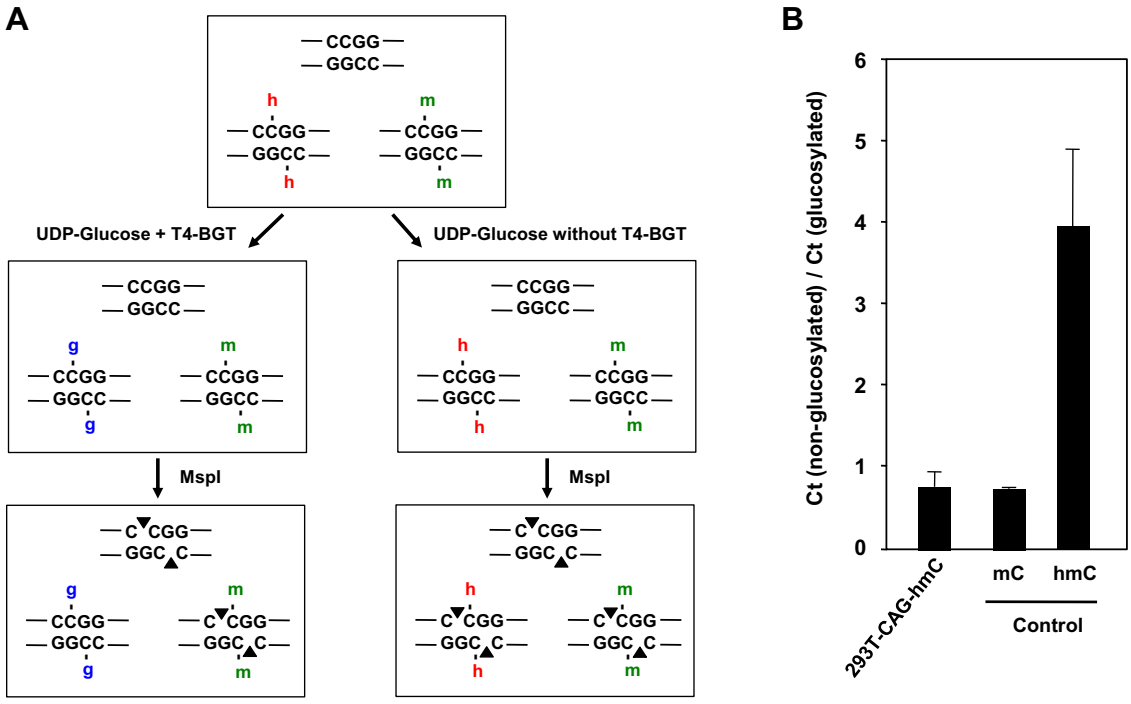

Fig. 3. Relative quantification analysis of hmC and mC at specific MspI sites. (A) Overview of hmC-qPCR analysis. All of hmC sites are converted to glucosylated hmC by treatment with T4-BGT and UDP-glucose. Unlike hmC, mC and unmodified cytosine, glucosylated hmC is refractory to digestion by MspI. h-C, 5-hydroxymethylcytosine (red); m-C, 5-methylcytosine (green); g-C, glucosylated 5-hydroxymethylcytosine (blue); T4-BGT, T4 β-glucosyltransferase. (B) Relative quantification of hmC and mC at specific MspI sites were analyzed using glucosylation reaction followed by quantitative PCR. The level of hmC was calculated as the ratio of the real-time PCR results (Ct [non-glucosylated samples]/Ct [glucosylated samples]). Mean ± SD, $n = 3$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

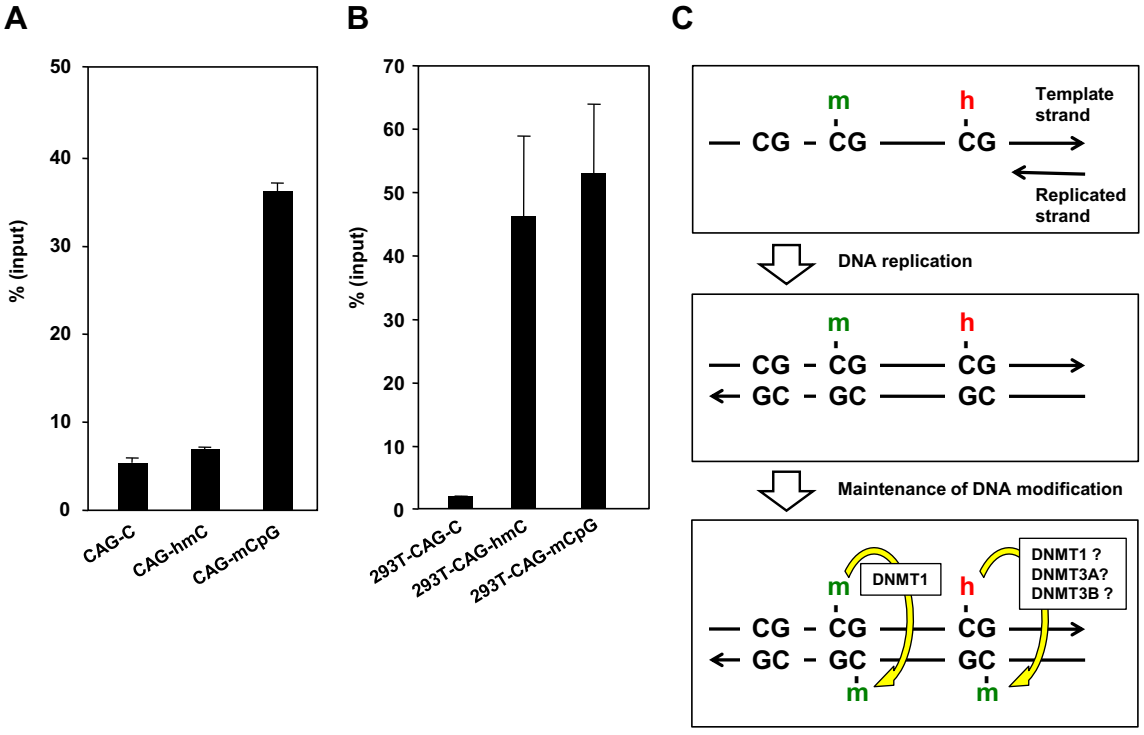

Fig. 4. Alteration of hmC to mC at CpG sites beyond DNA replication. (A) Precipitation of control fragments of CAG-C, CAG-hmC and CAG-mCpG by MBD2 protein. Enriched DNAs were analyzed using real-time PCR with appropriate primers. Results are presented as percentage of input material (mean ± SD, $n = 3$). (B) Enrichment of MBD2 protein captured episomal vectors from 293T-CAG-C, 293T-CAG-hmC and 293T-CAG-mCpG. Sheared genomic DNAs precipitated by MBD2 were analyzed using real-time PCR with appropriate primers. Results are presented as percentage of input material (mean ± SD, $n = 3$). (C) A proposed model of the alteration of hmC to mC at CpG sites beyond DNA replication. h-C, 5-hydroxymethylcytosine (red); m-C, 5-methylcytosine (green); DNMT, DNA methyltransferase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

fluorescent protein (GFP) DNA fragments in 293T cells [22]. Following this report, we hypothesized that a few bisulfite-resistant cytosines would be detected using the MoCEV system during several cycles of DNA replication in 293T cells. But to our surprise, bisulfite-resistant cytosines in the CpG sites, but not in non-CpG, were clearly detected. Moreover, our experimental results notably indicated that bisulfite-resistant cytosines in the CpG sites were mainly mCpGs after the several rounds of DNA replication. Since we showed that both methylated and unmethylated cytosines on the MoCEV were maintained beyond DNA replication (Supplemental Fig. S3), hydroxymethylated CpGs in the parental strand may dictate methylation sites on the daughter strand in somatic cells, which is consistent with the enrichment of hmC in non-proliferative neuronal cells (Fig. 4C). These findings suggest the existence of molecular machinery for hemi-hydroxymethylated CpG recognition. Although further experiments are needed to elucidate the mechanisms of this finding, we speculate that the maintenance DNMTs may participate in the alteration of hmC to mC at the CpG regions. In mammals, three catalytically active DNMTs have been identified and characterized. DNMT1 is responsible for the maintenance of DNA methylation pattern beyond DNA replication, whereas, DNMT3A and DNMT3B are *de novo* methyltransferases that have activities for establishing cytosine methylation patterns at unmethylated CpG sites. Valinluck and Sowers reported that hmC prevents DNMT1 recognition *in vitro* [23], however, UHRF1, an essential factor in DNA methylation maintenance, was reported to recognize hemi-hydroxymethylated CpG sites as well as hemi-methylated CpG sites [24]. This report suggested that UHRF1 may bind to the hemi-hydroxymethylated CpG sites and recruit DNMT1 to newly methylated CpGs in the daughter strand after DNA replication. On the other hand, Hashimoto and colleagues reported that DNMT3A and DNMT3B were capable of acting on the hemi-hydroxymethylated CpG site *in vitro* [25]. However, since 293T cells have low expression of DNMT3A, DNMT3B and DNMT3L according to the public expression database [26], DNMT3 proteins may not contribute to the alteration of hmC to mC in 293T cells.

In this study, we established the In-fusion based MoCEV system, which is simple and stable method to track modified cytosines. We believe that the MoCEV system may help to further elucidate the functional mechanism and regulatory mechanisms of cytosine modification during cell proliferation as well as differentiations. ESCs, which have relatively high levels of hmC and maintain them after DNA replication [27], are one of good cell model for these experiments. For instance, since the study of genome-wide distribution of hmC as well as mC was published [28], we are able to select the desired regions that are enriched for cytosine modifications in ESCs. In addition, since 5-formylcytosine and 5-carboxylcytosine were identified as new constituents of mammalian DNA [29,30], the MoCEV system also has great potentials to facilitate numerous research applications for cytosine modification and DNA demethylation.

In conclusion, to investigate the fate of hmC after DNA replication, we first prepared a simple and stable episomal vector-based system to monitor the status of cytosine modification, and we successfully demonstrated the maintenance of DNA methylation status at the CpG sites throughout multiple cell divisions. Using the system, we revealed that mCs were observed at the hydroxymethylated CpG sites, whereas the modification was not detected in the non-CpG sites after several rounds of DNA replication in 293T cells. This observation indicated somatic cells undergo hmC at the CpG sites to mC during cell division and is important to understand the regulation of DNA modification.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.08.053.

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