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The Essential Role of Histone H3 Lys9 Di-Methylation and MeCP2 Binding in MGMT Silencing with Poor DNA Methylation of the Promoter CpG Island

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Silencing of the *O*^{6}-methylguanine-DNA methyltransferase (*MGMT*) gene, a key to DNA repair, is involved in carcinogenesis. Recent studies have focused on DNA hypermethylation of the promoter CpG island. However, cases showing silencing with DNA hypomethylation certainly exist, and the mechanism involved is not elucidated. To clarify this mechanism, we examined the dynamics of DNA methylation, histone acetylation, histone methylation, and binding of methyl-CpG binding proteins at the *MGMT* promoter region using four *MGMT* negative cell lines with various extents of DNA methylation. Histone H3K9 di-methylation (H3me2K9), not tri-methylation, and MeCP2 binding were commonly seen in all *MGMT* negative cell lines regardless of DNA methylation status. 5Aza-dC, but not TSA, restored gene expression, accompanied by a decrease in H3me2K9 and MeCP2 binding. In SaOS2 cells with the most hypomethylated CpG island, 5Aza-dC decreased H3me2K9 and MeCP2 binding with no effect on DNA methylation or histone acetylation. H3me2K9 and DNA methylation were restricted to in and around the island, indicating that epigenetic modification at the promoter CpG island is critical. We conclude that H3me2K9 and MeCP2 binding are common and more essential for *MGMT* silencing than DNA hypermethylation or histone deacetylation. The epigenetic mechanism leading to silent heterochromatin at the promoter CpG island may be the same in different types of cancer irrespective of the extent of DNA methylation.

Key words: DNA methylation, gene silencing, histone H3K9 di-methylation, MeCP2, *MGMT*.

It is well-known that genetic aberrations such as gene mutations, deletions or genomic rearrangements cause cancer. However, epigenetic gene silencing of tumor suppressors and/or DNA repair genes has also been shown to cause cancer (*1–3*). The *O*^{6}-methylguanine-DNA methyltransferase (*MGMT*) gene, a key member of the DNA repair network, is silenced in various cancers, associated with hypermethylation of the promoter CpG island. Since MGMT prevents G:C to A:T transition mutations by repairing the alkylated base, *O*^{6}-methylguanine, loss of MGMT function results in an accumulate of mutations leading to tumor induction. In fact, a correlation has been found between *MGMT* methylation and transition mutations of *K-ras* and *p53* in various cancers (*4*). So far, much attention has focused on hypermethylation of the CpG island in the promoter region to address the mechanism of the repression of DNA repair genes; however, some genes seem to be repressed without hypermethylation. For example, *p19*, *cyclin D2*, and *WT1* have been reported to be silenced with CpG island hypomethylation or unmethylation in cancer (*5–8*). In fact, the methylation status of the promoter CpG island among *MGMT* non-expressed cancer cells varies from hypermethylation to hypomethylation (*9*). The silencing mechanism associated with hypomethylation is unclear.

Histone modifications also play critical roles in epigenetic silencing (*10*). It is generally thought that acetylated histone H3 and H4 (H3Ac, H4Ac) and methylated H3 lysine 4 (H3meK4) are enriched in an open chromatin structure, and associated with active gene expression, whereas methylated H3 lysine 9 (H3meK9) is a marker of transcriptionally silent heterochromatin (*11–13*). For tumor suppressor and DNA repair genes in cancer cell lines, deacetylation of H3 and H4, demethylation of H3K4, and methylation of H3K9 correlate with promoter DNA methylation and gene silencing (*14*). However, it is not known how histone modifications work in silencing with a hypomethylated CpG island.

Methyl-CpG binding (MBD) proteins can mediate transcriptional repression by recruiting protein complexes, including histone deacetylases (HDACs). MBDs usually act as a mechanistic bridge between DNA methylation and histone deacetylation (*15*). Furthermore, MeCP2 and MBD1 mediate H3K9 methylation (*16*, *17*). It was recently reported that multiple MBDs bind to the methylated promoter region of many genes; however, a single

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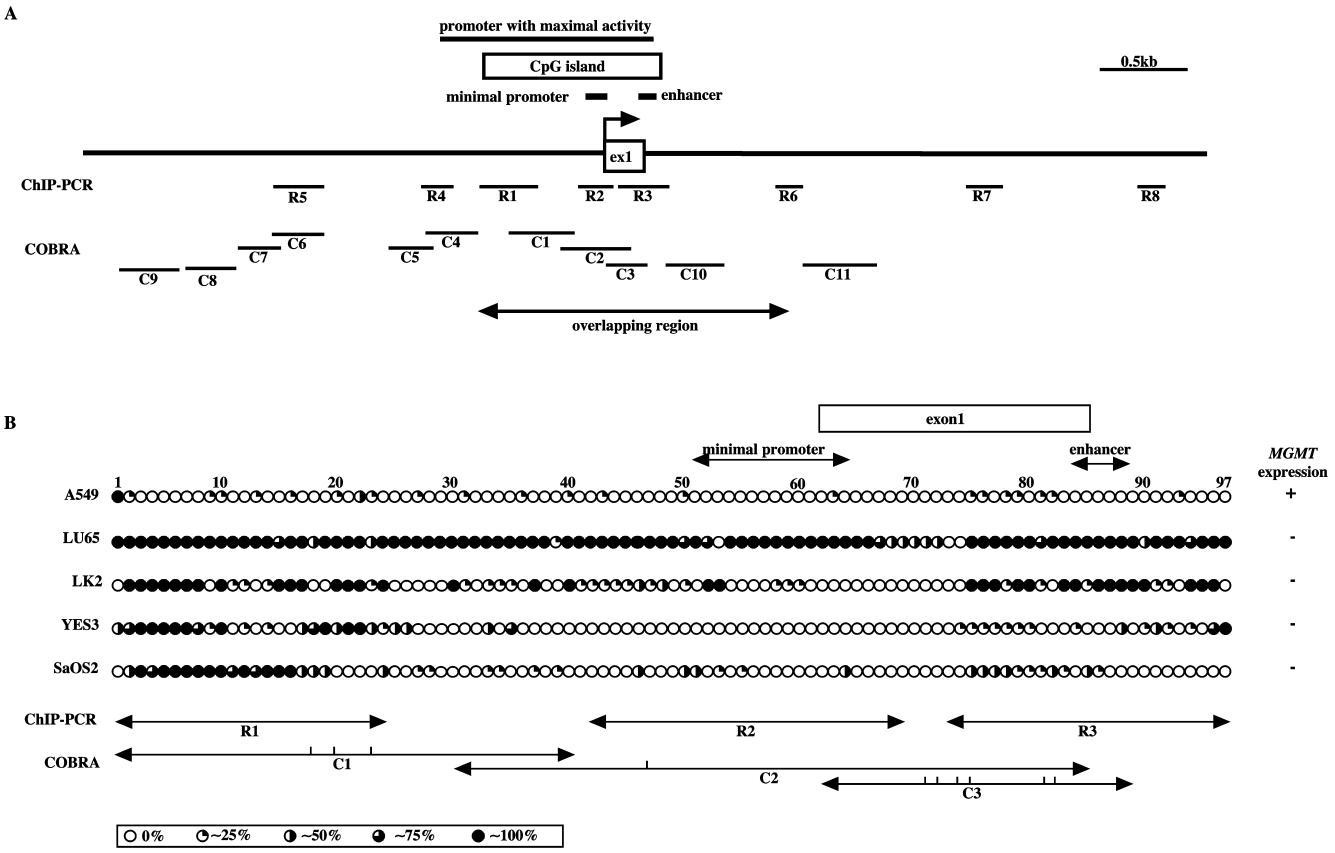

Fig. 1. **Map and methylation status of the *MGMT* CpG island.** (A) Map of the *MGMT* promoter region. The broken arrow indicates the transcription initiation site. The promoter with maximal activity, minimal promoter, and enhancer are indicated by thick lines. The upper and lower rows below the map indicate the locations of ChIP-PCR (R1–R8) and COBRA (C1–C11), respectively. Restriction enzymes for COBRA are as follows. *Acc*II for C3 and C10, *Dpn*I for C8, *Hin*fI for C2, C7, C9, and C11, *Nsp*V for C4, C5, and C6, *Rsa*I for C1. The arrow indicates the overlapping region of H3me2K9 and DNA methylation (see Fig. 5). (B) Detailed methylation status of the CpG island in cell lines used in this study. The *MGMT*(−) cell lines LU65 and LK2 showed promoter hypermethylation and moderate methylation, respectively, whereas YES3 and SaOS2 were hypomethylated. *MGMT*(+) A549 showed a mostly unmethylated CpG island. Regions analyzed by ChIP and COBRA are shown. Vertical bars indicate restriction enzyme cut sites.

MBD is associated with other genes in breast cancer (*18*). It is, however, still unknown how many MBDs binds to the *MGMT* promoter and whether a specific MBD(s) is associated with *MGMT* silencing, even in plural cancer cells with DNA hypomethylation.

In the present study, we examined the dynamics of histone acetylation, methylation, and binding of MBD proteins in several cancer cell lines with different CpG island methylation and *MGMT* expression statuses. We also monitored the effects of treatment with the DNA demethylating agent 5-Aza-2′-deoxycytidine (5Aza-dC) or histone deacetylase inhibitor trichostatin A (TSA) or both in combination. We found that di-methylation, but not tri-methylation, on H3K9 and MeCP2 binding at the promoter CpG island is common and plays an essential role in *MGMT* silencing in different types of cancer, regardless of DNA methylation status and histone deacetylation.

MATERIALS AND METHODS

Cell Lines—Five cell lines—A549, LU65, and LK2 derived from lung cancer; YES3 from esophageal cancer; SaOS2 from osteosarcoma—were cultured in RPMI-1640 medium (Sigma) with 10% FCS (Gibco). Cells were harvested at 70% confluence.

5Aza-dC and TSA Treatments—Cells were plated at 1 × 10^6 cells/10cm dish 12–24 h before treatment, and treated variously as follows: (i) 1 μM 5Aza-dC (Sigma) for 120 h with medium change every 24 h, (ii) 300 nM TSA (Wako) for 24 h, (iii) combination of the two drugs, 1 μM 5Aza-dC for 120 h with 300 nM TSA at 96 h. Two independent experiments were performed.

DNA Methylation Analysis—We used bisulfite-sequencing and combined bisulfite restriction analysis (COBRA) to analyze DNA methylation status. Genomic DNA extracted from the cells was modified by the urea/bisulfite method and amplified by PCR. For bisulfite-sequencing, the PCR product was cloned and sequenced as previously described (*19*). For COBRA, the PCR product of each region was digested with the appropriate restriction enzyme (Fig. 1A). The products were electrophoresed in 6% polyacrylamide gels and stained with ethidium bromide. Band intensity was quantified with NIH Image 1.63. The methylation status was calculated by (methylated band)/(methylated band + unmethylated band).

RNA Extraction and RT-PCR—Total RNAs were extracted with ISOGEN (NIPPON GENE). Five hundred

Table 1. **Primers and PCR conditions used in this study.**

Region	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)	Annealing temperature (PCR cycle No.)
ChIP assay			
R1	TGGACGGCATCGCCCACCAC	GCGCCGCCGCACAGGGCATG	66°C (31)
R2	GCGCCGCCGCACAGGGCATG	GGGGCCGAGGGGCACGGGGA	69.4°C (31)
R3	GCCCCGGATATGCTGGGACA	GGGCAACACCTGGGAGGCAC	66°C (31)
R4	TGTCTCACACTTCCGGGGGC	TGGGGAGGAGGATTGGAGGG	59°C (31)
R5	AGTAACAGGCTGTAAGTGGC	ATGTGGAAGGACAGATGAAA	57°C (31)
R6	TGTTAAGCACAGAGAGCTGA	AGTGTTTTAGCCATTGAGAG	59°C (31)
R7	GAAGGGCCTGAAAAAGAACT	ATGGAAGGTCTGAATACATG	59°C (31)
R8	AAATCAGAGAAGACACATAAACCATC	CTGCACAGCTCAAACCTCCT	57°C (31)
COBRA			
C1	TTTGGGGGTTTTTGATTAGG	TAAAACAATCTACICATCCTCICTA	56°C (45)
C2	TATTIGGGTTAGGIGTATAGGGTAG	ACCCAAACACTCACCAAATC	62°C (45)
C3	GTTTTCGGTTTCGTTTCGTTTTAGATT	AACTACCACCGTCCCGAAAAAAAAC	57°C (45)
C4	GGGTTAGTTTGAAATTTAGGTA	AATCCTAACTCCCAATACCTCACAA	60°C (40)
C5	TATGTGGGTTTTTGGATGGGA	CCTAACACACCTAAATTTCAAACTT	54°C (40)
C6	GTAGTAATAGGTTGTAAGTGGTTAG	AAACTAATATAAAAAAACAAATAAA	54°C (40)
C7	AAGGGTTTTTGGATGATTATATTGG	TAAAACCGAAATCTTAATAAAACCA	54°C (40)
C8	ATTTTGGAGTTTTTGATATAGTTTT	AACTAACCAATCTTAACATCTCTAA	54°C (40)
C9	TAGTTTTGTATTGTTATTTTATGAA	AAACTATCTAATAAACAAAACTCAA	54°C (40)
C10	GTGTTTTTTAGGTGTTGTTTAGTTT	ATACCAAACACCAACCATAATAAC	54°C (40)
C11	TTTTGGGAGATGAATAAGATTTTAA	CTTCAAATAATCCACCCACC	54°C (40)

All PCR reactions are performed with an initial denaturation at 96°C for 3 min, followed by appropriate amplification cycles of 96°C for 15 s, appropriate annealing temperature for 20 s, 72°C for 30 s, and a final extension at 72°C for 3 min.

nanograms of total RNA was treated with RNase-free DNaseI (Roche) and reverse-transcribed with ReverTra Ace reverse transcriptase (TOYOBO). PCR was performed with the primers as previously described (*9*).

Chromatin Immunoprecipitation (ChIP)—We performed ChIP assays as previously described (*9*, *20*, *21*). The regions amplified by PCR are shown in figure 1A. Antibodies used were as follows: anti-H3Ac, anti-H4Ac, anti–di-methylated histone H3K4 (Upstate Biotechnology), anti–di-methylated H3K9 (*9*), anti–tri-methylated H3K9 (Upstate Biotechnology), anti-MeCP2 (*22*, Abcam), two kinds of anti-MDB1 (Abcam), and anti-MBD2/3 (Upstate Biotechnology; Abcam). The 5′ CpG island of *G6PD* was amplified as a positive control for the H3Ac, H4Ac, and H3meK4 and as a negative control for the H3meK9 and MBD proteins. The human chromosome 16 centromere region (*16CEN*) was an inverse control for *G6PD* (*20*, *23*). Three independent ChIP assays were performed for each cell line, and PCR for each ChIP-DNA was performed in duplicate. Fold enrichment in each immunoprecipitation was determined as previously described (*9*, *21*)

Western Blotting—To detect MBD proteins, all cell lines were lysed in RIPA buffer as previously described (*9*). Equal amounts of protein were size fractionated by 10% SDS-PAGE and transferred onto PVDF membranes. The membranes were hybridized with antibodies against MeCP2, MBD1, and MBD2/3. The proteins were detected with the ECL plus Western blotting detection system (Amersham Biosciences).

Statistical Analysis—Differences among cell lines [*MGMT*(+) A549 *versus* each *MGMT*(–) cell line] were calculated by Student's *t*-test. Probability levels of <0.05 were considered statistically significant.

Primers and PCR Conditions—Primer sequences and PCR conditions are shown in a Table 1.

RESULTS

Profiles of Histone Modifications and DNA Methylation at the MGMT Promoter CpG Island—We previously examined the DNA methylation profile of the CpG island in the *MGMT* promoter region in 25 *MGMT* non-expressed [*MGMT*(–)] cell lines, showing variations in methylation (*9*). To assess the relationship between histone modifications and DNA methylation status at the *MGMT* promoter, we chose four *MGMT*(–) cell lines—LU65, LK2, YES3, and SaOS2—displaying different promoter methylation patterns (Fig. 1B). First, we rechecked the DNA methylation status of all cell lines by bisulfite-sequencing. LU65 has an entirely hypermethylated CpG island. LK2 shows a moderately methylated CpG island. YES3 and SaOS2 are the most hypomethylated cells among the 25 *MGMT*(–) cell lines, although several CpGs are hypermethylated. We chose A549, which has an unmethylated CpG island with a high level of *MGMT* expression [*MGMT*(+)], as a control. To confirm whether the *MGMT*(–) cell lines preserve the transcriptional machinery for *MGMT* transcription, the luciferase reporter construct, which carries an *MGMT* promoter region, was transfected into all cell lines as previously described (*9*). The luciferase activity in the *MGMT*(–) cell lines was the same or higher than *MGMT*(+) A549 (data not shown). The results indicate that the *MGMT*(–) cell lines preserve the constitutive transcriptional machinery for *MGMT* transcription, and that the silencing of *MGMT* in these cells is due to an epigenetic mechanism.

Three regions within the CpG island were amplified by ChIP-PCR to investigate histone modifications (Fig. 2, A and B). Since the standard deviations for several samples were striking, especially in region R3 (Fig. 2B), we also performed quantitative real-time PCR as ChIP-PCR for

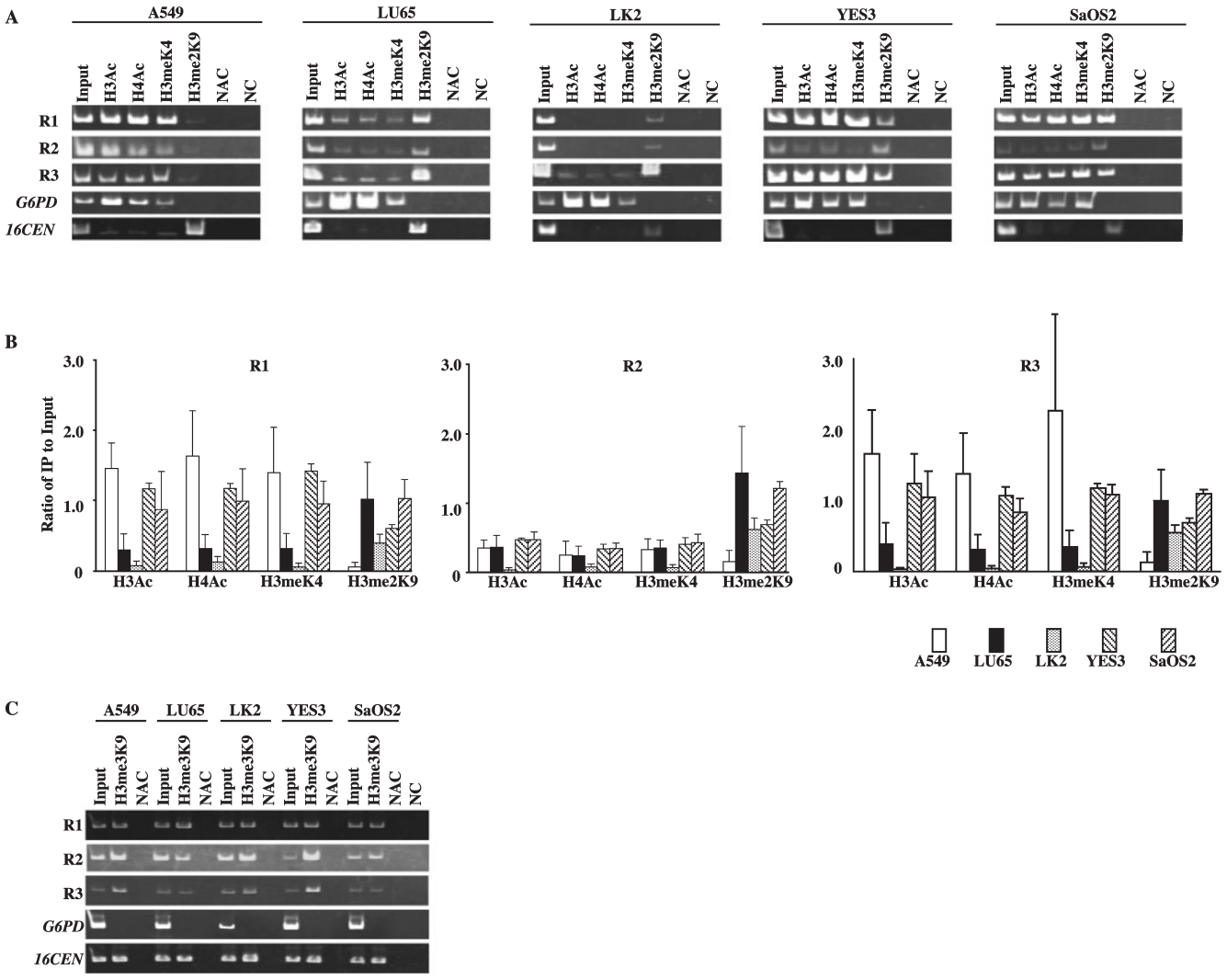

Fig. 2. **Histone modification status before drug treatments.** (A) Representatives of ChIP-PCR. NAC: no antibody control, NC: negative control. *G6PD* is a positive control for H3Ac, H4Ac, and H3meK4, and a negative control for H3meK9. *16CEN* is an inverse control for *G6PD*. (B) Quantification of ChIP-PCR results. The ratios of PCR from immunoprecipitated DNA versus input DNA (total chromatin) are shown on the y-axis. At R1 and R3, H3Ac, H4Ac, and H3meK4 in LU65 and LK2 are lower than in A549 ($p < 0.05$), whereas those of YES3 and SaOS2 are the same as in A549. H3me2K9 in all *MGMT*(–) cells is significantly higher than in A549 ($p < 0.05$). The ChIP procedure was performed independently three times, and PCR for each ChIP-DNA was performed in duplicate. (C) Representatives of ChIP-PCR for tri-methylation of H3K9. H3me3K9 is seen in all cell lines regardless of *MGMT* expression. The results of ChIP-PCR were confirmed by quantification (data not shown).

R3. We obtained the same results, indicating the accuracy and reliability of our results (data not shown). At region R1, A549 showed a high level of H3Ac, H4Ac, and H3meK4, but virtually no H3me2K9. In contrast, LU65 and LK2 showed exceedingly low H3Ac, H4Ac, and H3meK4, but significantly high H3me2K9, with statistical significance compared with A549. The two most hypomethylated cell lines, YES3 and SaOS2, also showed a significantly high level of H3me2K9. Surprisingly, regardless of *MGMT* silencing, the acetylations and H3meK4 were as high as in A549, with no statistical significance. The same pattern of modifications was seen at region R3. At R2, in all *MGMT*(–) cell lines, H3me2K9 was high, but acetylations and H3meK4 were low. Since a high level of H3me2K9 signified nucleosomal chromatin at the region, the low level of the acetylations and H3meK4 meant deacetylation and demethylation. On the other hand, A549 also showed a low level of acetylations and H3meK4 at R2 regardless of high expression. It is plausible that less-nucleosome chromatin due to the occupation of transcription factors would cause less immunoprecipitation during ChIP preparation, resulting in poor PCR amplification (*9*, *24*, *25*). We also investigated tri-methylation of H3K9 (H3me3K9) at the CpG island. H3me3K9 was surprisingly seen in all cell lines regardless of *MGMT* expression, suggesting that H3me3K9 at promoter CpG island is not correlated with silencing of *MGMT* (Fig. 2C). These results suggest that the high level of H3me2K9 through the entire CpG island and the low acetylations and H3meK4 at R2 are more important and common epigenetic factors for *MGMT* gene silencing in cancer than DNA hypermethylation.

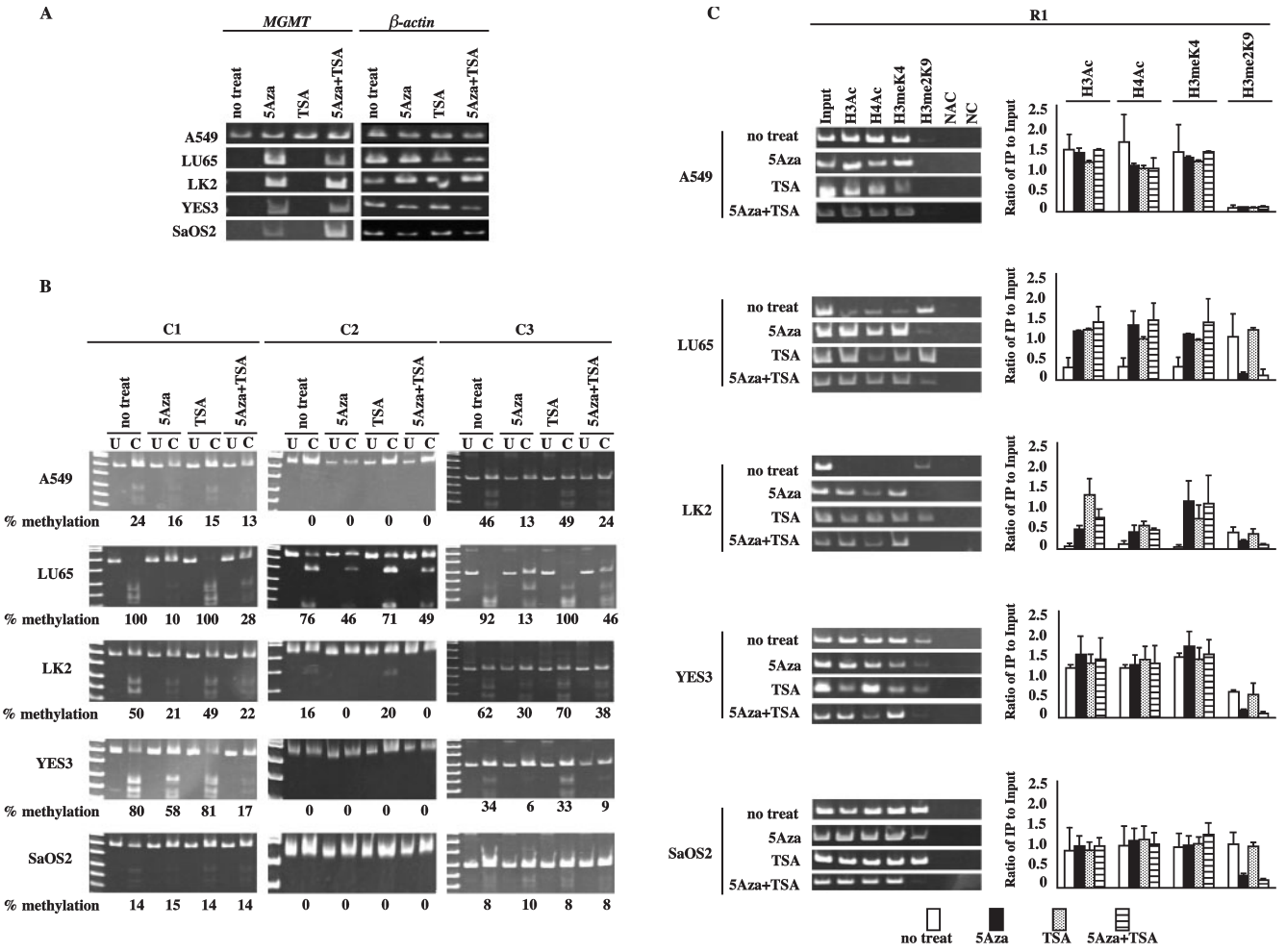

Fig. 3. ***MGMT* expression, DNA methylation, and histone modifications after drug treatment.** (A) Results of RT-PCR. β-actin is an internal control. (B) Results of COBRA in each region (see Fig. 1A). U: the PCR product was not digested with the restriction enzyme. C: the PCR product was digested. The numerals below each panel indicate the level of methylation. In SaOS2, note that the methylation of the promoter region was not influenced by drug treatment. In A549, YES3, and SaOS2, the higher methylation frequencies than observed by bisulfite sequencing at R1 and R3 may be due to multiple cut sites in each PCR product resulting in many sizes of DNA fragments; all digested fragments were considered methylated (see Fig. 1B). (C) Results of ChIP-PCR at R1 after drug treatment. Representative results of ChIP-PCRs are shown on the left and quantification of ChIP-PCR on the right. Only a decrease in H3me2K9 was commonly correlated with *MGMT* restoration in all *MGMT*(–) cells. Similar results were obtained from R2 or R3. Two independent drug treatment experiments were done. NAC: no antibody control, NC: negative control.

Alterations of MGMT Expression, DNA Methylation, and Histone Modifications by Drug Treatments—We then investigated the influence of the DNA demethylating agent, 5Aza-dC, and HDAC inhibitor, TSA, on *MGMT* expression and epigenetic modifications of the CpG island (Figs. 3 and 4). 5Aza-dC, but not TSA, could restore *MGMT* expression. Combining 5Aza-dC and TSA also restored the expression (Fig. 3A).

Subsequent COBRA indicated that DNA methylation was remarkably decreased in all but SaOS2 by treatment with 5Aza-dC or 5Aza-dC plus TSA, but not by TSA alone (Fig. 3B). The COBRA data suggest that 5Aza-dC but not TSA can decrease the overall methylation level, and that the demethylation of the promoter appears to be involved in the reactivation of *MGMT* expression in LU65, LK2, and YES3. However, in SaOS2, neither 5Aza-dC nor TSA influenced DNA methylation; nevertheless, 5Aza-dC restored *MGMT* expression. This suggests that a factor other than DNA methylation mediates *MGMT* silencing in SaOS2, and 5Aza-dC may have a function apart from the DNA demethylation effect.

We next examined the status of histone modification after drug treatment. Results for R1 are shown in Fig. 3C; similar results were obtained for R2 or R3 (Fig. 4). In A549, no histone modifications were changed by any drug treatment. In LU65 and LK2, all drug treatments significantly increased the levels of acetylations and H3meK4. H3me2K9 was remarkably decreased by 5Aza-dC and 5Aza-dC plus TSA. TSA alone had no effect on H3me2K9, although it enhanced the acetylations and H3meK4. On the other hand, in YES3 and SaOS2, no treatment had much effect on the acetylations and H3meK4. However, 5Aza-dC and 5Aza-dC plus TSA, but not TSA alone, markedly decreased H3me2K9. Therefore, a factor completely correlated with the restoration of *MGMT* expression is the decrease of H3me2K9 rather than the increase

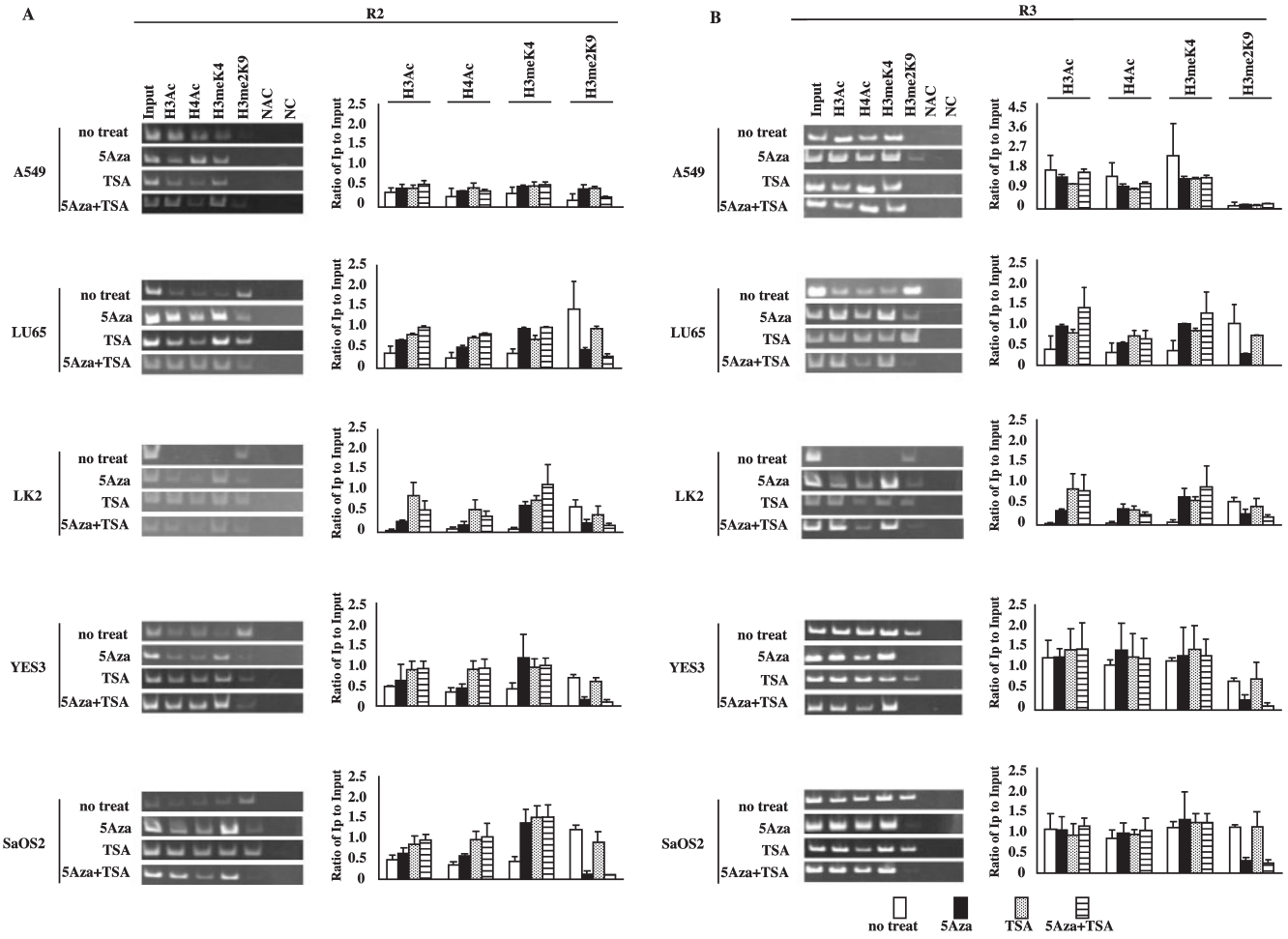

Fig. 4. **Results of ChIP-PCR at R2 (A) and R3 (B) after drug treatment.** Representative results of ChIP-PCRs are shown on the left and quantification of ChIP-PCR on the right. Only a decrease in H3me2K9 was commonly correlated with *MGMT* restoration in all *MGMT*(–) cells. Two independent drug treatment experiments were done. NAC: no antibody control, NC: negative control.

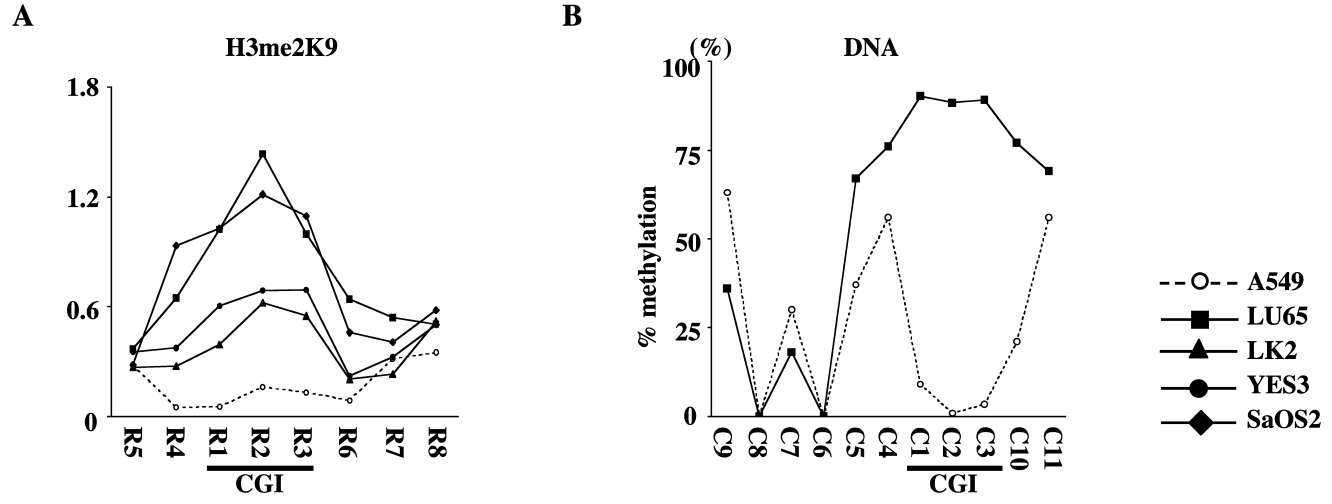

Fig. 5. **Epigenetic modifications around the CpG island.** (A) H3me2K9 is limited to in and around the CpG island. However, H3me2K9 at R5, R7, and R8 does not vary significantly among all cell lines. CGI: CpG island. (B) DNA methylation around the island. DNA methylation outside the island varies and is not correlated with *MGMT* expression. DNA methylated region in *MGMT*(–) LU65 cells mostly overlapped the region of H3me2K9. The arrow in Figure 1 indicates the overlapping region.

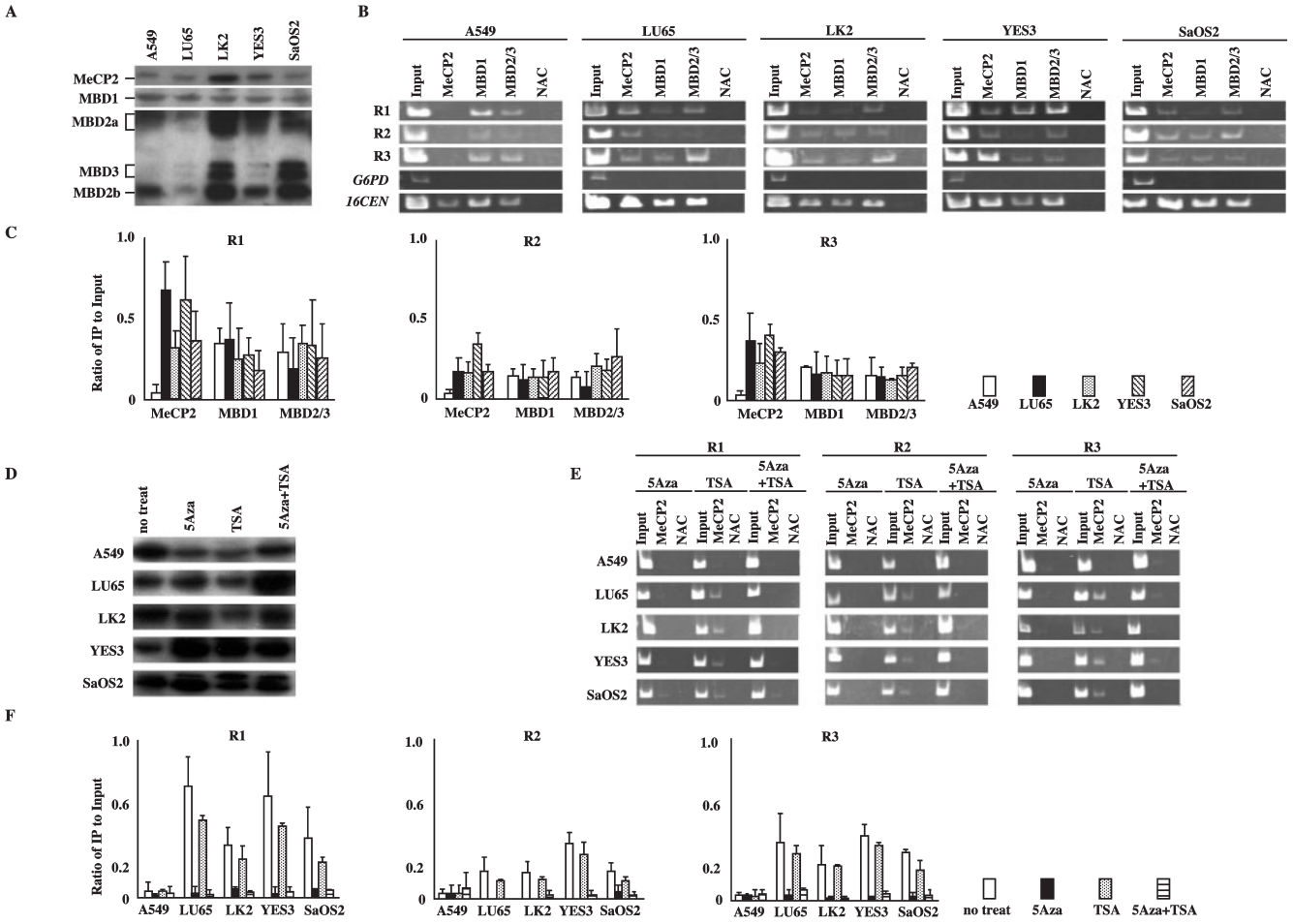

Fig. 6. **Association of MBD proteins with *MGMT* silencing.** (A) Western blotting of MBD proteins. MeCP2, MBD1, MBD2a, MBD2b, and MBD3 were expressed in all cell lines, although biased expression of MBD2/3 was seen. (B) Representative results of ChIP-PCR. NAC: no antibody control. *G6PD* and *16CEN* are a negative and a positive control for MBD proteins, respectively. Each ChIP was done twice, and duplicate PCR was performed for each ChIP-DNA. (C) Quantification of ChIP-PCR results. MeCP2 binds to the *MGMT* silent promoter with statistical significance. Other MBDs show no difference between *MGMT*(+) and *MGMT*(–) cells. (D) Western blotting confirmed the expression of MeCP2 after drug treatment in all cell lines. (E) Representative results of ChIP-PCR after drug treatments. See Figure 6B as no treatment control. NAC: no antibody control. Two independent experiments were done. (F) Quantification of ChIP-PCR after drug treatments. 5Aza-dC and 5Aza-dC plus TSA significantly decreased the binding of MeCP2 in all *MGMT*(–) cells with statistical significance.

of the acetylations and H3meK4, or the demethylation of DNA, indicating that H3me2K9 is a common and essential epigenetic factor in *MGMT* silencing. Further, 5Aza-dC reduced H3me2K9 at all silenced loci, regardless of DNA methylation status. TSA does not restore *MGMT* expression because it probably does not reduce H3me2K9, although it augments acetylations and H3meK4.

Epigenetic Modifications Limited to in and around the CpG Island—We also examined the H3me2K9 status upstream and downstream of the CpG island (Fig. 5A). The H3me2K9 patterns at R4 and R6, approximately 0.5 kb upstream and 1 kb downstream of the CpG island, respectively, were similar to the pattern at R1, R2, and R3 in the island, and higher than A549 in all *MGMT*(–) cells. On the other hand, at regions further outside, such as R5, R7, and R8, the H3me2K9 levels in each cell line were the same, showing no statistical significance. The acetylations and H3meK4 were also the same at R5, R7, and R8 in all cell lines (data not shown). This suggests that alterations in histone modifications could be restricted to in and around the CpG island. We carried out COBRA to evaluate the DNA methylation status outside the CpG island in unmethylated A549 and hypermethylated LU65 (Fig. 5B). DNA methylation in LU65 occurred in and around the CpG island. However, CpG sites outside the island were variously methylated from null to approximately 75%, and the levels of methylation were not correlated with *MGMT* expression. This DNA methylated region mostly overlapped the region where H3me2K9 altered. Thus, it is likely that a critical region for epigenetic modification is the promoter CpG island.

Association of Methyl-CpG Binding Proteins with MGMT Silencing: MeCP2 Links with Silencing—We assessed the binding of MBDs to the CpG island in all cell lines with various levels of DNA methylation. Western blotting showed that MeCP2, MBD1, MBD2a, MBD2b, and MBD3 were expressed in all cell lines, although biased

expression of MBD2 and MBD3 was seen (Fig. 6A). ChIP assays revealed that MeCP2 strongly binds to the entire CpG island in all *MGMT*(–) cell lines compared with A549 with statistical significance (Fig. 6, B and C). Notably, the level of MeCP2 occupancy was not associated with the density of DNA methylation in the *MGMT*(–) cell lines. A certain binding level of MBD1 and MBD2/3 was seen in all cell lines, but was not statistically different between *MGMT*(–) and *MGMT*(+). Therefore, MeCP2, rather than MBD1 and MBD2/3, is commonly involved in *MGMT* silencing, and the binding of MBD1 and MBD2/3 does not affect *MGMT* expression.

5Aza-dC, but Not TSA, Significantly Induces the Release of MeCP2 Binding from the MGMT Promoter—We next asked whether drug treatment affects the binding of MeCP2 to the *MGMT* CpG island. We first confirmed MeCP2 expression after drug treatment by Western blotting (Fig. 6D). ChIP assays revealed that 5Aza-dC and 5Aza-dC plus TSA significantly decrease the binding of MeCP2 at all regions of the CpG island in all *MGMT*(–) cell lines (Fig. 6, E and F). As expected, TSA did not release MeCP2 binding with statistical significance compared to untreated controls. In LU65, LK2, and at R1 and R3 in YES3, the release of MeCP2 is definitely associated with DNA demethylation and *MGMT* gene reactivation. It was intriguing that in SaOS2, although 5Aza-dC did not affect DNA methylation of the entire CpG island, it substantially induced the release of MeCP2, and expression was restored. So the release of MeCP2 is completely associated with *MGMT* restoration, and 5Aza-dC may induce the release of MeCP2 binding from silent loci regardless of DNA methylation status.

DISCUSSION

We investigated the dynamics of epigenetic modifications and binding of MBD proteins at the *MGMT* promoter region with and without treatment with 5Aza-dC and TSA. Our results revealed important points about *MGMT* silencing in cancer cells. First, the most critical and common epigenetic factor for silencing is H3me2K9. Second, MeCP2, rather than MDB1 and MBD2/3, is commonly and completely associated with silencing. Third, the epigenetic aberration is restricted to in and around the promoter CpG island. Last, 5Aza-dC may have functions in H3K9 demethylation and MeCP2 release independent of DNA demethylation.

Our results using five cancer cell lines indicated that the epigenetic factor correlated with *MGMT* silencing is H3K9 di-methylation, not DNA hypermethylation or histone deacetylation. Furthermore, among MBD proteins, MeCP2 is the most important and completely related to silencing. Generally, it is known that DNA hypermethylation at the promoter region is a key epigenetic factor for gene silencing. It seems, however, that DNA methylation does not lead to gene silencing by itself. The recruitment of proteins to methylated DNA is required for the formation of heterochromatin to silence genes. MBDs, which function as transcriptional repressors, bind to methylated CpG nucleotides and interact with a co-repressor complex containing HDACs, leading to deacetylation of H3 and H4. In addition, MBDs, such as MeCP2 and MBD1, interact with HMT(s), resulting in highly methylated H3K9 (*16*, *17*). Furthermore, hypo- or un-methylated H3K4 is associated with silencing. All interactions finally lead to transcriptionally inactive heterochromatin (*2*, *3*, *26*). The cases of LU65 and LK2 in our study are consistent with this mechanism, in which MeCP2 binds to a hypermethylated *MGMT* promoter. However, the results from YES3 and SaOS2 are inconsistent with the above mechanism in two respects—high levels of acetylations and H3meK4, and DNA hypomethylation. The high levels of acetylations and H3meK4 indicate that in these cells, deacetylation of H3 and H4 and demethylation of H3K4 are dispensable for the formation of heterochromatin. DNA hypomethylation suggests that MeCP2 binding and H3me2K9 may occur independent of DNA methylation. Especially, in SaOS2, 5Aza-dC did not affect DNA methylation of the entire CpG island, but decreased MeCP2 binding and H3me2K9 with a restoration of *MGMT* expression. Two previous reports support this idea. 1: MeCP2 can bind to unmethylated naked DNA and condense unmethylated nucleosomal array *in vitro*, repressing gene transcription in a histone deacetylation-independent manner (*27*, *28*). 2: H3me2K9 occurs at the *p16* promoter prior to DNA methylation and is associated with the silencing of *p16* in *DNMT1* and *DNMT3B* double knockout cancer cells (*29*). However, since YES3 and SaOS2 have several hypermethylated CpG sites, and MeCP2 can bind to one methylated CpG site, we can not rule out that a small number of methylated CpGs are still effective in MeCP2 binding and trigger heterochromatin formation without HDACs. Our findings suggest that the indispensable condition for *MGMT* silencing is a heterochromatin configuration marked by H3me2K9 and MeCP2, and that epigenetic mechanism(s) leading to such heterochromatin may be the same, irrespective of the extent of DNA methylation, in different types of cancer.

H3K9 and DNA methylation occurred in and around the CpG island, and the regions of each modification mostly overlapped. It is supposed that a boundary element outside the promoter CpG island prevents the promoter region from propagating histone deacetylation, H3K9 methylation, and DNA methylation to transcribe genes (*2*, *3*). An upstream and downstream boundary of the *MGMT* promoter must exist between R4 and R5 and between R6 and R7, respectively, because all cell lines investigated showed a certain level of histone modifications, and their levels were not different among cell lines in regions further upstream than R4 or further downstream than R6. In addition, DNA methylation in those regions varied and was not correlated with *MGMT* expression. Since a high level of H3me2K9 appears critical for *MGMT* silencing, DNA methylation in the CpG island or another unknown mechanism may compel H3me2K9 to overcome the boundaries in *MGMT*(–) cells. Identification of the boundary sequences and proteins bound to them will be helpful for understanding the epigenetic silencing mechanism further.

Numerous studies of drug treatments have shown that 5Aza-dC can induce a precipitous remodeling of chromatin structure of many tumor suppressor promoter regions, with increases in H3Ac, H4Ac, and H3meK4. However, decreases in H3me2K9 and MeCP2 binding by 5Aza-dC have been reported for only two genes—*p16* and *MDR1* (*30*, *31*). Our experiments show that 5Aza-dC

drastically decreases H3me2K9 and MeCP2 binding at the *MGMT* promoter CpG island regardless of H3Ac, H4Ac, H3meK4, or DNA methylation. The inhibition of H3K9 methylation by 5Aza-dC seems to be more powerful than its effect on DNA methylation, and 5Aza-dC induces chromatin remodeling in the absence of DNA methylation (*30*). 5Aza-dC can also induce histone hyperacetylation at pericentric heterochromatin in mice independent of its DNA demethylation activity (*32*). Overall, 5Aza-dC has functions in H3K9 demethylation and MeCP2 dissociation independent of DNA demethylation, though the precise mechanism is still unknown.

In conclusion, H3me2K9 and MeCP2 binding is common and essential for *MGMT* silencing regardless of DNA methylation status at the promoter CpG island in cancer cells. The epigenetic mechanism(s) leading to silent heterochromatin at the promoter CpG island may be the same, irrespective of the extent of DNA methylation. The roles of HMT(s) and MeCP2 in *MGMT* silencing should be investigated in different types of cancer cells to further elucidate the epigenetic mechanism of silencing.

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