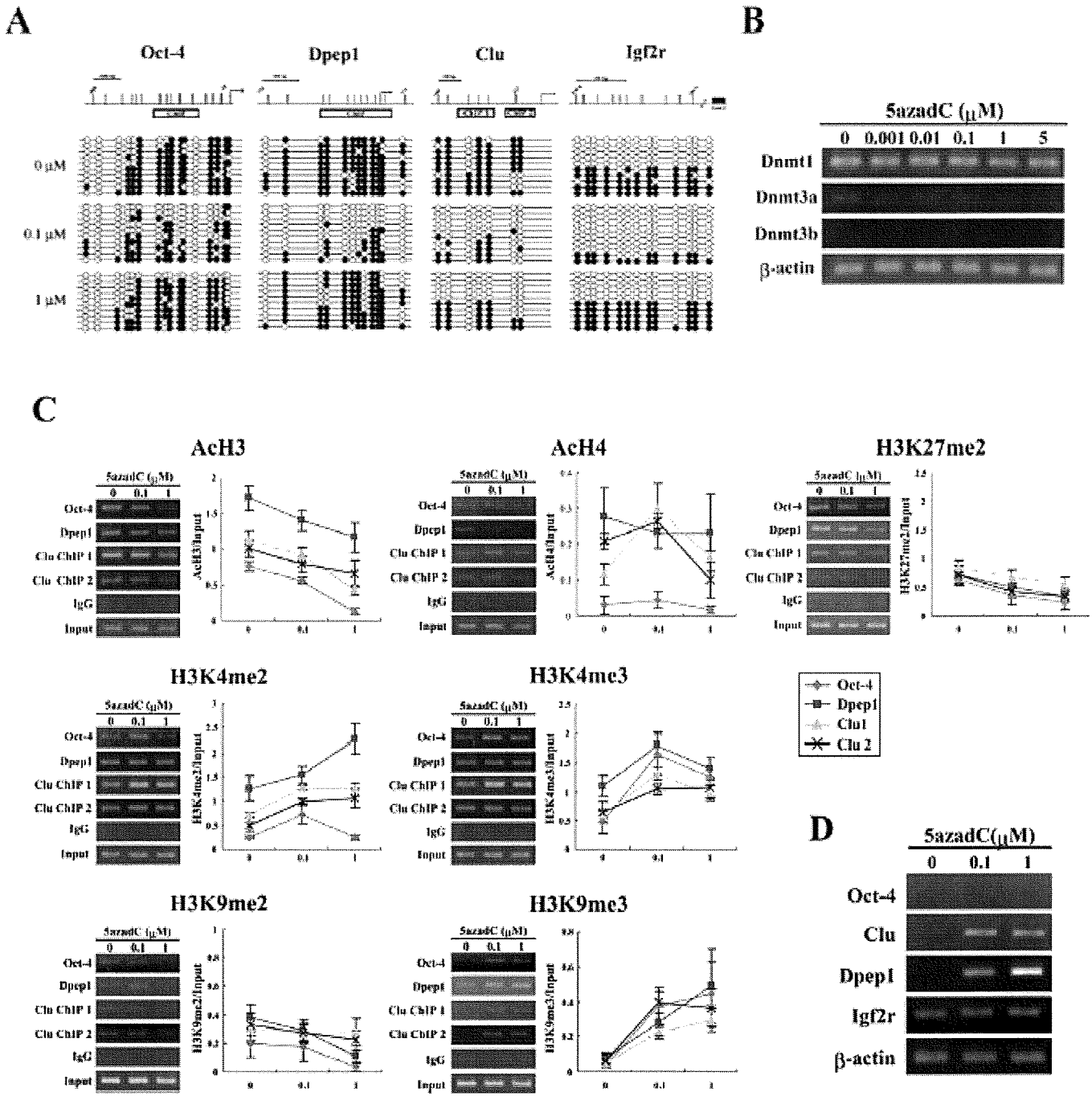

Fig. 2. Effect of 5azadC on DNA methylation, histone modification and gene expression. A: DNA methylation analysis of 4 gene loci, *Oct-4*, *Clu*, *Dpep1* and *Igf2r*, using bisulfite sequencing. The top panel shows the location of individual CpG sites (vertical lines) in genic regions amplified by PCR. The 0, 0.1 and 1 μM 5azadC-treated DNA was bisulfite converted and sequenced. The methylation status of each CpG site is represented by open (unmethylated) or closed (methylated) circles. B: Expression of *Dnmt1*, *Dnmt3a* and *Dnmt3b* at 0–5 μM 5azadC analyzed by RT-PCR. The expression level of *β-actin* was used as the internal control. C: Histone modification analysis of 0, 0.1 and 1 μM 5azadC-treated samples by ChIP. Analyzed regions are indicated by boxed "ChIP" in the schematic diagram in A. DNA immunoprecipitated with respective antibodies was amplified by PCR and electrophoresed (left panels). The relative intensities of PCR bands to those of the input DNA, presented as the means ± S.E. of 3 independent PCRs of 3 cultures, are shown in the right panels. D: RT-PCR results for the expression of each gene following 5azadC treatment.

of *Dnmt*1, *Dnmt*3a and *Dnmt*3b were not affected by 5azadC at concentrations from 0.001 to 5 μM (Fig. 2B), indicating that the partial retention of methylation observed at high concentrations of 5azadC was not due to increased Dnmt expression.

5azadC induced a unique combination of histone tail modifications

Since 0.1 and 1 μM 5azadC showed rather unexpected, different demethylation effects on genic regions, we investigated changes in histone modifications by performing a ChIP assay with antibodies against euchromatic and heterochromatic marks. The AcH3 level in genic regions continuously declined as the concentration of 5azadC increased (Fig. 2C), which is in contrast to the condition in decondensed chromatin [26]. Such changes were not observed in AcH4, which was less enriched in genic regions.

Both the H3K4me2 and H3K4me3 levels increased with 5azadC treatment, in almost all investigated regions, and high levels were correlated with increased gene expression (Fig. 2D). Elevation of H3K4me2 in *Dpep1* at 1 μM 5azadC corresponded to a remarkable transcriptional increase, and the relatively low abundance of H3K4me2 in the *Oct-4* promoter region at any level of 5azadC did not induce *Oct-4* expression. H3K4me3 was more enriched by 0.1 μM than by 1 μM 5azadC treatment.

Heterochromatin-associated H3K9me2 and H3K27me2 marks continuously decreased with 5azadC treatment. In contrast, elevation of H3K9me3 was observed, with higher enrichment at 1 μM compared with 0.1 μM 5azadC in most regions. Altogether, 5azadC treatment at different concentrations was accompanied by a distinct combination of changes in euchromatic and heterochromatic histone marks in genic regions.

Increase of H3K9me3 correlates with partially methylated regions

Previous studies show that H3K9 methylation directs DNA methylation [34, 35]. To date, our data have shown that 5azadC induces partial demethylation, not complete demethylation, in genic regions. To determine whether increased H3K9me3 is associated with DNA methylation, we investigated enrichment of H3K9 methylation in the *Dpep1* and *Clu* promoter regions in Dnmt-deficient cells. The promoter regions of *Dpep1* and *Clu* were heavily methylated in wild type ES cells and were demethylated in *Dnmt1*$^{-/-}$ and *Dnmt 3a*$^{-/-}$*3b*$^{-/-}$ (Fig. 3A). The increase of H3K9me3 in the genic regions of Dnmt knockout cells was not as obvious as those in the 5azadC-treated NIH/3T3 cells (Fig. 3B). Therefore, increased H3K9me3 correlated with partially methylated regions, such as in 5azadC-treated cells. Similar to 5azadC-treated cells, H3K9me2 decreased in *Dnmt1*$^{-/-}$ cells, but was increased in *Dnmt3a*$^{-/-}$*3b*$^{-/-}$ cells.

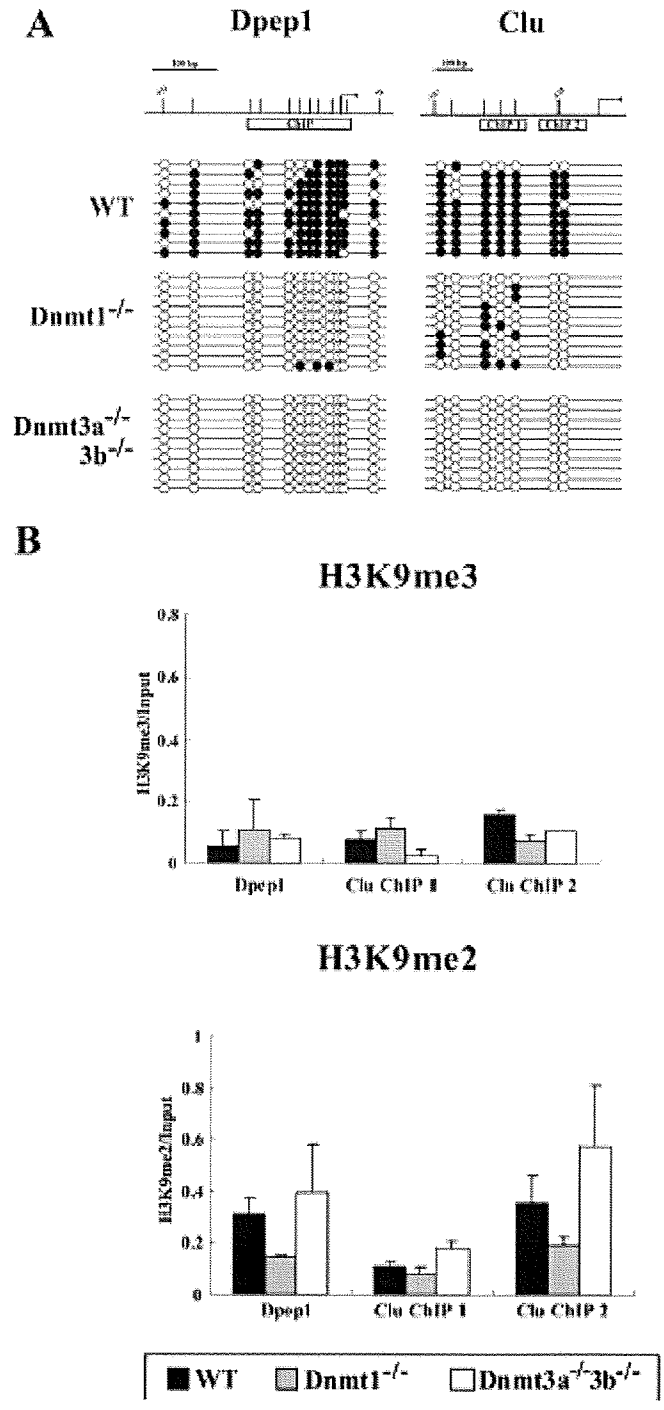

Fig. 3. DNA methylation and H3K9 methylation status of *Dpep1* and *Clu* in Dnmt knockout ES cells. A: DNA methylation analysis of *Dpep1* and *Clu* by bisulfite sequencing. B: H3K9 di- and trimethylation levels in the regions shown in panel A assessed by ChIP. The bar charts show the relative intensities of PCR bands to those of the input; the values are presented as means ± S.E. of 3 independent PCRs of 2 cultures.

Discussion

The current results demonstrate that 5azadC has differential effects on non-genic repetitive sequence and genic regions (Fig. 4). Importantly, non-genic repetitive sequences are susceptible to 5azadC, whereas genic regions are only demethylated by effective low doses.

Demethylation of repetitive sequences was concentration-dependent, whereas genic regions were only demethylated at effective concentrations, but not at higher concentrations. Thus,

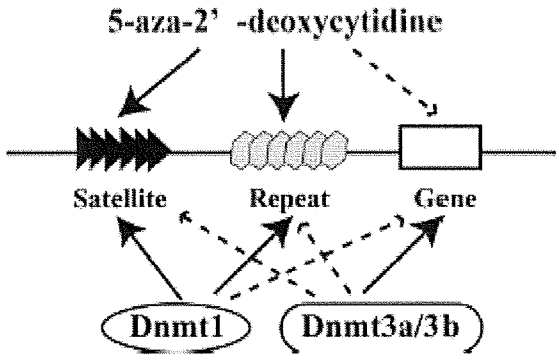

Fig. 4. Summary of the effect of 5azadC on repetitive sequences and genic regions. Repetitive sequences (black arrows) are strongly demethylated by 5azadC, but genic regions are only demethylated at low doses (dotted arrow). The present study suggests the preference of 5azadC for Dnmt1, as Dnmt1 has a functional preference for repeats, whereas Dnmt3a and Dnmt3b functions on genic regions [24].

increasing the treatment dosage might not effectively induce demethylation in targeted genic regions, but will lead to genome-wide hypomethylation in non-cancer cells. Loss of methylation promotes additional genomic changes, including increased mutation rate and pericentromeric rearrangement [17, 36]. Thus, before using 5azadC, precise doses should be determined to achieve localized hypomethylation, but avoid concurrent global hypomethylation.

Some reports have shown that 5azadC treatment is followed by decreased H3K9 methylation [37–39]. Our results also showed decreased H3K9me2, but increased H3K9me3 in all genic regions after 5azadC treatment. H3K9me3, but not H3K9me2, is a mark for cytosine methylation in chromatin regions in *Neurospora crassa* [35]. Thus, increased H3K9me3 might be associated with partial methylation in 5azadC-treated cells, indicating a functional difference between H3K9me2 and H3K9me3 in mammalian cells.

5azadC treatment, in contrast to their authentic roles in DNA methylation and gene silencing [25, 26]. Such changes might reflect mechanisms to prevent full demethylation in genic regions. As DNA methyltransferases are associated with H3K9 methyltransferases [40, 41], accumulation of histone methyltransferases, which confers increase in H3K9me3, may also attracts Dnmts. Recruitment of MeCPs to the methylated DNA region is associated with a corepressor complex containing mSin3 and histone deacetylases (HDACs), causing reduction of AcH3 [42, 43]. Given that Dnmt1 has preference for repetitive sequence, whereas Dnmt3a and 3b favor gene regions [24], the current study may reflect the preference of 5azadC for Dnmt1 (Fig. 4). Thus, Dnmt3 might be associated with H3K9 methyltransferases for maintaining the methylation level in genic regions due to the functional cooperation of Dnmt1 and Dnmt3 [22–24, 44, 45].

Gene activation by 5azadC seems to involve a complex mechanism of action, in addition to DNA demethylation [37, 39]. We showed that genes were upregulated at 0.1 μM and that expressions were maintained or increased at 1 μM, despite higher methylation levels observed at 1 μM. Although transcriptional activation could be explained by an increase of H3K4 methylation marks [46], AcH3 concurrently decreased drastically. Interestingly, H3K4me3 abundance was lower in the 1 μM treatment than in the 0.1 μM treatment and was accompanied by DNA hypermethylation. A similar study on cancer genes suggested that histone modifications of demethylated genes do not fully recover to euchromatic states [47]. Interestingly, recent findings have shown that H3K9me3 is associated with active genes [48–49] and is dynamically present in active transcribed regions together with heterochromatin protein, HP1γ [50], suggesting the role of H3K9me3 in 5azadC-induced gene activation. Thus, gene activation by 5azadC seems to be more complex than the authentic model of epigenetic gene activation and silencing.

Although studies have shown that the toxicity of the 5azadC treatment is not due to a demethylating effect [51], our results raise questions concerning the 5azadC toxicity mechanism. Repetitive sequences were partially demethylated at 0.1 μM and were extensively demethylated at 1 and 5 μM. Concurrent with demethylation, cell viability was minimally affected at 0.1 μM, but was retarded at 1 and 5 μM, reflecting that the induced cytotoxicity might be an effect of global demethylation in the repetitive sequences. In contrast, genic regions were hypomethylated at 0.1 μM, and were methylated at 1 and 5 μM 5azadC, probably as a resistance mechanism against cell death.

This study provides an explanation of the mechanisms behind the dual modes of action of the demethylating drug 5azadC. At low concentrations, the demethylating effect is potent in both repetitive and genic regions. At high concentrations, repetitive sequences are highly demethylated, which potentially leads to toxicity. DNA methylation in genic regions is less affected, which might be due to changes in histone modification attempting to maintain the DNA methylation level. Thus, an appropriate dose of 5azadC should be wisely chosen for therapeutic use.

Acknowledgments

We appreciate Dr En Li for providing us with the ES cells and probes. This work was supported in part by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN), Japan (to KS); Health Science Research Grant from Ministry of Health, Labor and Welfare, Japan (to KS); Program for Promotion of Fundamental in Studies in Health Sciences of the National Institute of Biomedical Innovation, Japan (to KS); and a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (20062003 to ST).

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