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Resistance to 5-aza-2'-deoxycytidine in Genic Regions Compared to Non-genic Repetitive Sequences

Hui Wen LIM¹⁾, Misa IWATANI¹⁾, Naoko HATTORI¹⁾, Satoshi TANAKA¹⁾, Shintaro YAGI¹⁾ and Kunio SHIOTA¹⁾

¹⁾Laboratory of Cellular Biochemistry, Animal Resource Sciences/Veterinary Medical Sciences, The University of Tokyo, Tokyo 113-8657, Japan

Abstract. The DNA methyltransferase (Dnmt) inhibitor and demethylating agent 5-aza-2'-deoxycytidine (5azadC) has been used to induce cellular differentiation and gene activation. It has been approved for treating several kinds of malignancies due to its ability to reactivate silenced tumor suppressor genes. Considering the potential effect of 5azadC on non-targeted genomic regions in normal cells, we investigated its effect on repetitive sequences and selected gene loci, *Oct-4*, *Sall3*, *Per1*, *Clu*, *Dpep1* and *Igf2r*, including tissue-dependent and differentially methylated regions, by treating mouse NIH/3T3 fibroblast cells with concentrations of 5azadC ranging from 0.001 to 5 μ M. Demethylation of minor satellite repeats and endogenous viruses was concentration dependent, and the demethylation was strong at 1 and 5 μ M. In genic regions, the methylation level decreased only at 0.1 μ M, but was minimally altered at concentrations lower or higher, regardless of the abundance of CpG sites. Thus, repeats are strongly demethylated, but genic regions are only demethylated at effective doses. Genes were activated by 5azadC treatment and were accompanied by a unique combination of histone modifications in genic regions, including an increased level of H3K9me3 and a decreased level of AcH3. Increase of H3K9me3 in genic regions was not observed in Dnmt knock out cells. We identified differential effects of 5azadC on repetitive sequences and genic regions and revealed the importance of choosing appropriate 5azadC doses to achieve targeted gene recovery.

Key words: 5-aza-2'-deoxycytidine, Decitabine, DNA methylation, Epigenetics, Histone modification

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DNA methylation is one of the epigenetic events associated with gene regulation and function. Hypermethylation of promoter regions of tumor suppressor genes causes silencing of the genes that lead to cancer [1–3]. Thus, reversing the methylation status of gene promoters to their prevalent methylation states has become a treatment option for certain cancer types. To date, there are many types of demethylating agents that have been shown to inhibit promoter methylation and reactivate silenced genes [4–6]. Some of these have been approved or are in clinical studies to be developed as cancer drugs [7].

The cytosine analog 5-aza-2'-deoxycytidine (5azadC), also known as decitabine, has been widely used as a DNA methyltransferase (Dnmt) inhibitor to reverse aberrant hypermethylation [8, 9]. It has been approved for hematological malignancies, showing favorable results with low dose treatment [10, 11]. Known to have dual modes of action, 5azadC at low doses induces gene hypomethylation, whereas high doses of 5azadC induce cytotoxicity and cause severe side effects in patients [12, 13].

Nearly 40% of the mouse genome is composed of repetitive sequences including different classes of interspersed repeats, such as LINEs, SINEs, LTR elements and satellites, that are mainly found in heterochromatin regions [14]. Most repeats are densely methylated, and methylation in repeats reflects the global methyla-

tion level [15, 16]. Loss of methylation in repeats causes genomic instability [17, 18]. Conversely, genes comprise only a small portion of the genome. Tissue-dependent and differentially methylated regions (T-DMRs) are unique sequences in genic regions that are methylated depending on tissue or cell types. T-DMRs have been widely observed, including in undifferentiated embryonic stem cells, normal tissues and even in cloned mice [19–21]. Both repetitive regions and T-DMRs serve as important markers for methylation analysis, as repeats could be used to estimate global methylation, whereas T-DMRs could serve as references for cell- or tissue-specific methylation.

Previous reports show that Dnmts exhibit functional cooperation on genomic regions [22, 23]. We reported previously that Dnmt1, Dnmt3a and Dnmt3b share targets in the same CpG islands with T-DMRs, and each Dnmt has target preferences depending on the genomic regions [24]. Dnmt3a and Dnmt3b prefer T-DMRs of genic regions, whereas Dnmt1 prefers repetitive sequences.

The demethylating effect of 5azadC is exerted by binding to Dnmts [4]. Since Dnmts have multiple targets, there is the potential of having a genome-wide demethylating effect when using 5azadC. Demethylation of non-targeted genomic regions might occur, not only in cancer cells but also in normal cells. In addition, there are diverse interactions between DNA methylation and histone modifications in euchromatic and heterochromatic regions [25, 26]. The epigenetic status of T-DMRs is regulated by the interplay between DNA methyltransferases, histone modification enzymes, nuclear proteins and other epigenetic factors that cooperate to form cell- and tissue-specific DNA methylation profiles [27,

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Correspondence: K Shiota (e-mail: ashiota@mail.ecc.u-tokyo.ac.jp)

28]. It may be possible to induce hypomethylation-independent activation of gene expression and downstream responses. To know whether 5azadC induces an invariable effect on different genomic regions, we investigated the effect of 5azadC on non-genic repetitive sequences and some genic regions including T-DMRs in fibroblast cells.

Materials and Methods

Reagents, cell culture and genome extraction

All reagents were purchased from Wako Pure Chemicals (Osaka, Japan) unless stated otherwise.

NIH/3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (JRH, Lenexa, KS, USA) and 50 unit/ml penicillin / 50 µg/ml streptomycin (Invitrogen) at 37 C in 5% CO₂ in air. Prior to treatment with 5-aza-2'-deoxycytidine (5azadC; Sigma-Aldrich, St. Louis, MO, USA; diluted with sterile water to the concentrations required), cells were plated at 1 × 10⁵ cells/150 mm dish and cultured for 24 h. Cells were treated with 5azadC to final concentrations ranging from 0.001 to 5 µM. Sterile water was substituted for 5azadC in the untreated control. The medium was changed every 24 h, and cells were collected after 3 days for DNA extraction.

Wild type ES cells (J1) and mutant ES cells deficient in *Dnmt1* (*Dnmt1*^{-/-}; c/c), *Dnmt3a* and *Dnmt3b* (*Dnmt3a*^{-/-}*3b*^{-/-}; 7aabb) were cultured on gelatin coated dishes with ES medium containing 1000 U/ml leukemia inhibitory factor (Chemicon, Temecula, CA, USA) as previously described [24]. J1, c/c and 7aabb cells were harvested at passage numbers 32, 17 and 17, respectively.

Cells were incubated in lysis buffer (150 mM EDTA, 10 mM Tris-HCl, pH 8.0, and 1% SDS) containing 10 mg/ml proteinase K (Merck, Darmstadt, Germany) at 55 C for 20 min. Following phenol/chloroform/isoamyl alcohol extraction twice, genomic DNA was precipitated with ethanol and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Cell proliferation assay

NIH/3T3 cells were seeded into 96-well plates at 1 × 10³ cells per well, 24 h before 5azadC was added. Cells were treated with 5azadC at final concentrations of 0 (as the control), 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1.0, 5.0 and 10.0 µM for 3 or 4 days at 37 C in 0.5% CO₂ in air with medium changes every 24 h. Four hours before plate reading, 10 µl of Cell Proliferation Reagent WST-1 (Roche, Penzberg, Germany) was added. The absorbance of each sample was measured against a background control using an ELISA reader at an absorption wavelength of 450 nm.

Analysis of the methylation status of repetitive sequences by Southern blotting

Genomic DNA (5 µg) was digested with the restriction enzyme *MspI* (Takara, Kyoto, Japan) or *HapII* (Takara) and electrophoresed on a 0.8% agarose gel. Following hydrolyzation with 0.25 N HCl and denaturation with 1.5 M NaCl/0.5 N NaOH, the DNA was transferred onto a nylon membrane. The membrane was probed with pMO for endogenous C-type retrovirus (MoMuLV; Genbank

accession NC_001501) and pMR150 for minor satellite repeats (X14469 and X07949). Probes were labeled with the Gene Images random prime labeling module (Amersham Pharmacia, Little Chalfont, Buckinghamshire, UK). Hybridization and detection were performed using the Gene Images CDP-star detection module (Amersham Pharmacia) according to the manufacturer's instructions.

Bisulfite restriction mapping and sequencing

Genomic DNA, digested with *EcoRI*, was denatured by incubating with 0.3 M NaOH at 37 C for 15 min. Sodium metabisulfite (pH 5.0) and hydroquinone were added to a final concentration of 2 M and 0.5 mM, respectively, and the mixture was incubated at 55 C for 18 h in the dark. Bisulfite modified DNA was purified with the Wizard DNA Clean-Up System (Promega, Madison, WI, US), and the bisulfite reaction was terminated with NaOH at a final concentration of 0.3 M at 37 C for 15 min. The sample was neutralized by adding NH₄OAc, pH 7.0 (3 M, final concentration), and was precipitated with ethanol. Purified DNA was dissolved in sterile water and amplified using Immolase (Bioline, Tokyo, Japan) with the primer sets as follows: 5'-TAAGGGTAGGTATATAGGTGTGGT-3', F, and 5'-TCTACCCCCTTTAAAAATCACTTTAA-3', R, for ODE; 5'-TGGGTTGAAATATTGGGTTTATTT-3', F, and 5'-CTAAAACCAAATATCCAACCATA-3', R, for OPR; 5'-GGGAAGGGGATTTTGTTATTGTAGT-3', F, and 5'-CATAAACCCAACAACAACCCATCT-3', R, for *Per1*; 5'-GTTAGGGTTTTTTAG-GGTATTAGT-3', F, and 5'-CCCTAATCTACCCAACATATACAAA-3', R, for *Sall3*. The PCR conditions were as follows: 95 C for 10 min, followed by 40 cycles of denaturation at 94 C for 30 sec, annealing at 55 C for 30 sec and extension at 72 C for 1 min, with a final extension at 72 C for 10 min.

Oct-4 distal enhancer, *Oct-4* proximal enhancer and promoter PCR products were digested with *TaqI* (Takara) at 65 C, and *Per1* and *Sall3* PCR products were digested with *HpyCH4IV* (NEB, Ipswich, MA, USA) at 37 C for 3 h. Restricted fragments were assessed by agarose gel electrophoresis. Images were recorded and semi-quantified using the ImageJ software provided by the National Institutes of Health (<http://rsbweb.nih.gov/ij/>). The relative DNA methylation level of each genic region was calculated by the formula: DNA methylation status (%) = 100 × I^C / (I^{UC} + I^C), where I^C and I^{UC} represent the intensities of the digested and undigested bands, respectively.

For bisulfite sequencing, PCR products were cloned into pGEM T-Easy vector (Promega, Madison, WI, USA), and 10 clones were sequenced for each sample. The primer sets used were 5'-TGGGCTGAAATACTGGGTTTACCC-3', F, and 5'-CTGAAGCCAGGTGTCCAGCCATG-3', R, for *Oct-4*; 5'-GGT-TGGGGAATTGGTTGTT-3', F, and 5'-CAACCTACTCCTAAATCCTCCA-3', R, for *Dpep1*; 5'-TAGTGAGTGGGGATGTAGTATTATGG-3', F, and 5'-AACCCCTAAACA-CTTCAAAATTTT-3', R, for *Clu*; and 5'-GTTTAGAATATTGTGAGTAGTGGG-3', F, and 5'-CCTTAAATAAAAAATAAACATCTTAAA-3', R, for *Igf2r*, with the following PCR conditions: 95 C for 10 min, followed by 40 cycles of denaturation at 94 C for 30 sec, annealing at 55 C for 30 sec and extension at 72 C for 1 min, with a final extension at 72 C for 10 min.

RNA extraction and RT-PCR

Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. First strand cDNA was synthesized with SuperScriptTM III First-Strand Synthesis System for RT-PCR (Invitrogen), and RT-PCR was performed using Taq DNA Polymerase (Promega) with primers as follows: 5'-CAGGAGTGTGTGAGGGAG-3', F, and 5'-GGTGTCACTGTCGACTTGC-3', R, for *Dnmt1*; 5'-ACCCATGCCAAGACTCACCTTC-3', F, and 5'-TCCACCTTCTGAGACTCTCAG-3', R, for *Dnmt3a*; 5'-TCAGACACGAAGGATGCTCC-3', F, and 5'-ACAGGGTACTCCTGCACATG-3', R, for *Dnmt3b*; 5'-TTCTACAATGAGCTGCGTGTGG-3', F, and 5'-ATGGCTGGGGTGTGAAGGT-3', R, for *β -actin*; 5'-GGCGTTCGCTTTGGAAAGGTGTTCC-3', F, and 5'-CTCGAACCACATCCTTCTCT-3', R, for *Oct-4*; 5'-CCAGTCGAAGATGCTCAACA-3', F, and 5'-TGTGATGGGGTCAGAGTCAA-3', R, for *Clu*; 5'-ATGCGGTATCTGACCCTCAC-3', F, and 5'-ATCTGCAAAGCGTCCTTCAT-3', R, for *Dpep1* and 5'-CAACGCTGTGGAAATGTGG-3', F, and 5'-CAGCCCATAGTGGTGTGAA-3', R, for *Igf2r*. The PCR conditions were as follows: 95 C for 1 min, followed by 30 cycles of denaturation at 94 C for 30 sec, annealing at 60 C for 30 sec and extension at 72 C for 1 min, with a final extension at 72 C for 5 min.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed as described previously [20] using a Chromatin Immunoprecipitation (ChIP) Assay Kit (Cat. No. 17-295; Upstate Biotechnology, Lake Placid, NY, USA) with anti-acetylated histone H3 and H4 antibodies (Cat. No. 06-599 and 06-598; Upstate Biotechnology), anti-trimethylated H3K4 and H3K9 (Cat. No. ab8580 and ab8898; Abcam, Cambridge, UK) and anti-dimethylated H3K4, H3K9 and H3K27 (Cat. No. 07-030, 07-212 and 07-452; Upstate Biotechnology). Normal rabbit IgG (Cat. No. 12-370; Upstate Biotechnology) was used as a negative control to verify immunoprecipitation specificity. PCR was performed using primers as follows: 5'-GTGAGGTGTCCGGTGACCCAAGGCAG-3', F, and 5'-CGGCTCACCTAGGGACGGTTTCACC-3', R, for *Oct-4*; 5'-TGCTCTGGAGACACAGGAAA-3', F, and 5'-CTGGGGAAGAAAGCCAAGAT-3', R, for *Clu* ChIP 1; 5'-ATTGCAGTGATGCCAGATGA-3', F, and 5'-ACGCACAGCAGGAGAATCTT-3', R, for *Clu* ChIP 2; 5'-CTCCTCTGTGGCTCCCTAA-3', F, and 5'-GGCTCCACAGAGTGCCAAG-3', R, for *Dpep1*. PCR was performed under the following conditions: 95 C for 10 min, followed by 30 cycles of denaturation at 94 C for 30 sec, annealing at 55 C for 30 sec and extension at 72 C for 1 min, with a final extension at 72 C for 10 min. The amount of each PCR product on an ethidium bromide-stained gel image was evaluated using the ImageJ software.

Results

Demethylating effect of 5azadC on repetitive sequences

We examined cell survival under different concentrations of 5azadC by WST-1 assay. After 72 h, the viable cell number was largely reduced at concentrations higher than 0.5 μ M and was severely affected at higher concentrations (Fig. 1A). A similar

effect was observed in the 96-h culture. Only minimal effects on viability were observed in cells treated with less than 0.1 μ M 5azadC.

To determine the effect of 5azadC on the methylation of repetitive sequences, cells were treated with 0.001 μ M to 5 μ M 5azadC, and the methylation status of repetitive sequences was analyzed using methylation-sensitive restriction enzymes. Southern hybridization was performed using two probes of differentially localized repetitive sequences, minor satellite repeats located in the centromeric regions and endogenous C-type viruses interspersed across the mouse genome [14, 24, 29]. Minor satellite repeats were demethylated extensively starting at the 0.1 μ M concentration (Fig. 1B), and 1 μ M was sufficient to induce the maximum demethylating effect. Similarly, endogenous viruses showed aggressive loss of methylation at the 0.1 μ M to 5 μ M treatment levels, but slight demethylation could be observed at a concentration as low as 0.001 μ M. Thus, the repetitive sequences were strongly demethylated by 5azadC. The results confirmed a previous report that 5azadC is effective in inducing demethylation dose-dependently from 0.1 μ M to 5 μ M [30].

Effect of 5azadC on genic regions

We next examined the effect of 5azadC on T-DMRs of genic regions. *Oct-4* (*Pou5f1*) has T-DMRs in the CpG-rich promoter/proximal enhancer region and distal enhancer region (Fig. 1C; 20). The DNA methylation status of the T-DMRs was analyzed using bisulfite restriction mapping focusing on *TaqI* restriction sites. At 0.001 and 0.01 μ M 5azadC, the methylation levels of the investigated regions changed little compared to the untreated ones. A significant loss of methylation in both regions was observed at 0.1 μ M, indicating that this concentration was able to induce demethylation in both genic regions and the repetitive sequences. In the 1 and 5 μ M treated samples, however, both T-DMRs had nearly the same methylation levels as in the untreated control, in contrast to the extensive demethylation observed in the repetitive sequences at these concentrations.

Sall3 has a T-DMR located at an edge of a CpG island, which is methylated only in the trophoblast cell lineage [19, 21]. The T-DMR is aberrantly methylated in the placental genome of cloned mice [21]. *Per1*, which is involved in generating circadian rhythm, has a few CpGs in the upstream promoter region. Similar to *Oct-4*, 0.001 and 0.01 μ M 5azadC had minimal effects on these loci. The methylation levels decreased significantly following 0.1 μ M 5azadC treatment, but were only slightly decreased at 1 μ M, and remained unchanged at 5 μ M.

Bisulfite sequencing was performed on several gene loci containing CpG-rich promoters, including *Oct-4*. Hypomethylation of the *Clusterin* (*Clu*) promoter is associated with high gene expression in the rat testis and epididymis [31]. *Dpep1*, a renal *Dipeptidase* gene, has been reported to be a tumor marker candidate in malignancies [32]. All investigated loci had hypermethylated promoter regions in NIH/3T3 cells (Fig. 2A). Bisulfite sequencing results for the *Oct-4* promoter region validated the restriction mapping results showing that 0.1 μ M was more effective in inducing demethylation in genic regions compared with 1 μ M. Similar dose-dependent demethylation patterns were also

Fig. 1. Different demethylating effects of 5azadC on repetitive sequences and genic regions. **A:** A WST-1 cell proliferation assay was performed on cells treated with 5azadC at the indicated concentrations for 72 and 96 h. Cell number was estimated by the absorbance, and then represented as the ratio relative to the untreated control, the absorbance of which was arbitrarily set to 100. The values represent means $\pm$ S.E. of 3 independent cultures. **B:** Analysis of the methylation status of repetitive sequences by Southern hybridization. Genomic DNA (5 μ g), digested with *HapII*, was hybridized to probes for minor satellite repeats and endogenous viruses. As a control for complete digestion, DNA from the untreated control was digested with *MspI*. **C:** Methylation analysis of T-DMRs by bisulfite restriction mapping. A schematic diagram of each investigated locus is shown in the top left panel. T-DMRs are represented by bars, and CG sites are represented by vertical lines. The locations of the amplified regions relative to each respective transcription start site are as follow: *Oct-4* distal enhancer (DE), -3086 to -2646; *Oct-4* proximal enhancer/promoter (PE/P), -420 to +31; *Sall3*, +1253 to +1665; and *Per1*, -1087 to -929. Each locus contains 2 *TaqI* or *HpyCH4IV* restriction sites, respectively (triangles). PCR products were digested with restriction enzymes and electrophoresed on agarose gel (top right panel). The relative DNA methylation level was calculated based on the relative intensities of cut to uncut bands, indicated below each cut lane. M, marker; U, uncut; C, cut with restriction enzyme. The bottom panel indicates the methylation level of each gene locus at each concentration; the values are presented as means $\pm$ S.E. of 3 independent PCRs of 2 cultures. * $P < 0.05$ (Student's *t*-test).

observed in the promoter regions of *Clu* and *Dpep1*.

We also analyzed the DMR2 region of the *Igf2r* imprinted gene, which is differentially methylated depending on its parental origin [33]. Due to allele-specific methylation, half of the clones were methylated in the untreated control. As observed in other genes,

the DMR2 region appeared demethylated at 0.1 μ M, but was not affected by 1 μ M 5azadC.

These results provide evidence that 5azadC has a strong demethylating effect on repetitive sequences, but its effect on genic regions is limited to a certain effective dose. The expression levels

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 μ M 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 μ M 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 μ M 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 $\pm$ 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 *Dnmt1*, *Dnmt3a* and *Dnmt3b* were not affected by 5azadC at concentrations from 0.001 to 5 μ M (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 μ M 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 μ M 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 μ M than by 1 μ M 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 μ M compared with 0.1 μ M 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 *Dnmt3a*^{-/-}*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.

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.

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 tri-methylation 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 $\pm$ S.E. of 3 independent PCRs of 2 cultures.

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.

Unusual histone tail modifications were observed following 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 μ M and that expressions

were maintained or increased at 1 μ M, despite higher methylation levels observed at 1 μ M. 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 μ M treatment than in the 0.1 μ M 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, HP1 γ [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 μ M and were extensively demethylated at 1 and 5 μ M. Concurrent with demethylation, cell viability was minimally affected at 0.1 μ M, but was retarded at 1 and 5 μ M, 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 μ M, and were methylated at 1 and 5 μ M 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.

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