BIOCHEMICAL PHARMACOLOGY 73 (2007) 1297–1307

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Histone deacetylase inhibitor Trichostatin A induces global and gene-specific DNA demethylation in human cancer cell lines

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ARTICLE INFO

Article history:
Received 27 October 2006
Accepted 27 December 2006

Keywords:
Histone deacetylase inhibitor
Tumor suppressor gene
DNA methylation
Histone acetylation
Hypomethylation
Hydroxyurea

ABSTRACT

DNA methylation and chromatin structure are two modes of epigenetic control of genome function. Although it is now well established that chromatin silencing could lead to DNA methylation, the relation between chromatin activation and DNA demethylation is unclear. It was generally believed that expression of methylated genes could only be restored by demethylating agents, such as 5-aza-deoxycytidine (5-azaCdR), and that inhibition of histone deacetylation by Trichostatin A (TSA) only activates transcription of unmethylated genes. In this report, we show that increase of histone acetylation by TSA was associated with a significant decrease in global methylation. This global demethylation occurs even when DNA replication is blocked by hydroxyurea, supporting a replication-independent-mechanism of demethylation. TSA also induces histone acetylation, demethylation and expression of the methylated *E-CADHERIN* and *RARβ2* genes. However, the genome-wide demethylation induced by TSA does not affect all methylated tumor suppressor genes equally suggesting that induction of acetylation and demethylation by TSA shows some gene selectivity. Taken together, our data provide evidence for a reversible crosstalk between histone acetylation and DNA demethylation, which has significant implications on the use of HDAC inhibitors as therapeutic agents.

1. Introduction

The epigenome is comprised of modifiable chromatin and DNA methylation, which are two major regulatory elements that interact to direct gene expression and other biological processes in somatic cells [1]. The link between DNA methylation and chromatin structure was initially believed to be unidirectional, whereby DNA methylation leads to chromatin inactivation, which in turn causes gene repression [2]. In accordance with this hypothesis, cytosine methylation was shown to target methyl-CpG binding proteins (MBD) to methylated genes. These MBD proteins then recruit a complex of transcription repressors including histone deacetylase (HDAC) to suppress genes transcription by inactivating

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Abbreviations: TSA, Trichostatin A; 5-azaCdR, 5-aza-deoxycytidine; HDACi, histone deacetylase inhibitor; HAT, histone acetyltransferase; DNMT, DNA methyltransferase

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doi:10.1016/j.bcp.2006.12.032

chromatin structure [3,4]. However, several studies have raised the possibility that the relation between chromatin and DNA methylation could be bi-directional and, a number of chromatin modifying enzymes were shown to recruit DNA methyltransferases (DNMTs) to specific genes and thus target DNA methylation [5,6].

It was previously described that inhibition of histone deacetylation is not sufficient to reactivate genes silenced by promoter hypermethylation, and that only DNA-demethylating agents, or a combination of HDAC inhibitors and DNA demethylating agents were able to restore the expression of silent genes [2]. For instance, it is commonly accepted that histone deacetylase inhibitors (HDACi), which are used extensively in both research and clinical trials target histone acetylation. Therefore, the possibility that histone acetylation could cause gene demethylation was generally not considered. Nevertheless, earlier data provided some evidence to the contrary. Sodium butyrate, a nonspecific inhibitor of HDAC was shown to induce genomic hypomethylation of Epstein Barr virus DNA in the absence of DNA replication [7]. More recently it was shown that the HDAC inhibitor Trichostatin A (TSA) could cause selective loss of DNA methylation in *Neurospora* [8].

We had previously shown that increasing histone acetylation by treating cells with TSA, brought about demethylation of non-replicating methylated DNA in HEK 293 cells, which suggested that histone acetylation could cause active DNA demethylation [9]. Furthermore, we also demonstrated that demethylation induced by TSA through histone acetylation could be blocked by ectopic expression of a histone acetyltransferase inhibitor protein INHAT [10]. However, the question remained whether these effects were limited to ectopically introduced plasmid, and whether TSA would confer a similar effect on fully chromatinized genomic DNA. The nature of the relationship between chromatin and DNA demethylation is important for understanding the programming of gene expression within the epigenome, as well as for revealing the potential impact of HDACi used in clinic on genomic methylation patterns.

We therefore addressed in this paper the issue of whether HDACi could alter global genomic methylation, as well as methylation of specific tumor suppressor genes. Furthermore, we also tested whether this effect could occur in the absence of DNA replication. Our results support the hypothesis that histone modifications could reverse the state of methylation, which suggest a true bilateral relationship between histone acetylation and DNA demethylation [8–14].

2. Materials and methods

2.1. Cell lines

Human bladder carcinoma, T24 cells were grown in McCoy 5A medium (Gibco), and human breast carcinoma, MDA-MB-231 cells were cultured in RPMI-1640 medium (Gibco), both supplemented with 10% fetal calf serum, L-glutamine, penicillin and streptomycin (Life Technologies). The cells were grown in regular culture medium in the presence of 1 μM of 5-azaCdR (Sigma), 2 mM of hydroxyurea (Sigma), or 50–300 nM of TSA (Sigma).

2.2. Reverse transcription-PCR

Total RNA was extracted using RNAeasy kit (Qiagen). The primers used for amplifying *E-CADHERIN*, sense: 5′-TACACCATCCTCAGCCAAGATCCT-3′; antisense: 5′-GTTCACTGGATTTGTGGTGACGAC-3′. For *P16*, sense: 5′-AGCCTTCGGCTGACTGGCTGG-3′; antisense: 5′-CTGCCCATCATCATGACCTGGA-3′. For *RARβ2*, sense: 5′-AGAGTTTGATG GAGTTGGGTGGAC-3′; antisense: 5′-GACGAGTTCCTCAGAGCTGGTG-3′. For *β-ACTIN*, sense: 5′-GTTGCTAGCCAGGCTGTGCT-3′; antisense: 5′-CGGATGTCCACGTCACACTT-3′, at an annealing temperature of 60 °C. A triplicate PCR was performed for each sample; the intensity of signal obtained for each message was determined by densitometry (NIH Image Software) and normalized to the intensity obtained from *β-ACTIN*.

2.3. 5-Methylcytosine quantification by nearest neighbor analysis

5-Methylcytosine level was quantified by nearest neighbor analysis as described previously [15]. The intensity of 5-methylcytosine and cytosine mononucleotide spots was measured using a phosphoimager and Image Quant image analysis program. Cytosine levels are determined as percentage of [cytosine]/[cytosine + methylcytosine].

2.4. Western blot analysis

Total protein extract was obtained by five freeze and thaw cycles in Tris-lysis buffer: (Tris–HCl 10 mM pH 7.6, $MgCl_2$ 5 mM, NaCl 300 mM, Tween-20 0.05%, glycerol 10% and complete protease inhibitors® (Roche)). The total protein yield was determined using bradford reagent (Biorad). Fifty micrograms of proteins were loaded on a 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were immunoblotted with an anti-*E-CADHERIN* antibody (BD Transduction Laboratories), followed by a secondary anti-mouse IgG antibody (Jackson ImmunoResearch). The membranes were blotted with an anti-β-ACTIN antibody as loading control (Sigma–Aldrich).

2.5. Sodium bisulfite mapping of DNA methylation and combined bisulfite restriction analysis (COBRA) of satellite 2

Sodium bisulfite mapping was performed as described previously with minor modification [16]. Two rounds of PCR were carried out to amplify the 5′ upstream sequence of *E-CADHERIN* and *RARβ2* from sodium bisulfite treated DNA. The outside primers used for *E-CADHERIN* were, sense: 5′-GAATTAGAATTGTGTAGGTTTT-3′ (nucleotide position 799–820); antisense: 5′-CTACAACAACAACAACAAC-3′ (nucleotide position 1175–1193). For *RARβ2*, sense: 5′-GAGAAGTTGGTGTTTAATGTGAGTT-3′ (nucleotide position 544–568); antisense: 5′-CATAAATTATAACAAACAAACCAAC-3′ (nucleotide position 1148–1172). The PCR product was used as a template for subsequent PCR using nested primers, for *E-CADHERIN*, sense: 5′-TTTAGTAATTTTAGGTTAGAGG-3′ (nucleotide position 836–857); antisense: 5′-ACTCCAAAAACCCATAACTAA-3′ (nucleotide position 1140–1160). For *RARβ2*, sense: 5′-GTTATTTGAAGGTTAGTAG-3′ (nucleotide position 763–790); antisense:

5′-TTACCATTTTCCAAACTTAC-3′ (nucleotide position 1098–1117). The PCR cycling conditions were 95 °C for 1 min, 52 °C for 2 min 30 s, and 72 °C for 1 min. The PCR product was then cloned using Original TA cloning kit (Invitrogen), and the clones were sequenced using the T7 sequencing Kit (Amersham Biosciences). Bisulfite PCR were performed using previously described primers targeting repetitive sequence of Alu [17,18], and satellite 2 [19,20]. The primers used to amplify Alu consensus sequence are listed in Table 1. For *COBRA*, the PCR products were purified by QIAquick gel extraction kit (Qiagen), and digested with methylation-sensitive enzyme *Hin*fI (MBI). The digested PCR fragments were separated by 0.8% polyacrylamide gel.

2.6. Chromatin immunoprecipitation assay (ChIP)

The assay was performed following the ChIP assay protocol recommended by the manufacturer (Upstate). Chromatin was immunoprecipitated using a rabbit anti-acetylated lysine 9 (K9) of histone H3 antibody (Upstate), or non-immune rabbit IgG antibody (Santa Cruz). The recovered DNA was subjected to PCR amplification using primers targeting the promoter region of the *E-CADHERIN* and *P16* genes. Primers for *E-CADHERIN* promoter, sense: 5′-ACTCCAGCTTGGGTGAA-3′; antisense: 5′-CACAGGTGCTTTGCAGTT-3′, at an annealing temperature of 57 °C. For *P16* promoter, the primers were sense: 5′-GAGATACCGCGGTCCCTC-3′; antisense 5′-ACCGT-AACTATTCGGTGCGTTGG-3′, at an annealing temperature of 63 °C. We performed real-time-qPCR in light cycler (Roche), using real-time SYBR green PCR master mix (SuperArray). The primers used for *E-CADHERIN*, sense: 5′-CTCAGCCAAT-CAGCGGTACG-3′; antisense: 5′-CTCCAAGGGCCCGGC-TGG-3′. For *P16*, sense: 5′-CACATTCGCTAAGTGCTCGGAGTT-3′; antisense: 5′-CGGTCCGCCCCACCCTCTGGTGAC-3′.

2.7. Acidic extraction for histone proteins

Cells were lysed in NP-40 lysis buffer (10 mM Tris pH 7.4, 3 mM $MgCl_2$, 10 mM NaCl, and 0.5% NP-40). The nuclei pellets were resuspended in nuclear wash buffer (10 mM Tris pH 7.4, 4 mM $MgCl_2$, 10 mM sodium butyrate, and 0.1 mM PMSF), and an equal volume of ice-cold 0.8 M HCl solution was added to extract histones. The proteins were blotted with anti-acetylated K9-H3 histone antibody (Upstate), followed by a secondary anti-rabbit IgG antibody (Sigma). The membranes were blotted with an anti-histone H3 antibody as loading control (Upstate).

Table 1 – Primers used to amplify Alu-repetitive sequence

Alu subfamily	No. of CpGs	Primers sequences
Sq	15	F: 5′-cctgtaatcccagcactttgg-3′ R: 5′-ccactgcactccagcctggg-3′
Sb	18	F: 5′-cctgtaatcccagcactttgg-3′ R: 5′-ccactgcactccagcctggg-3′
Sp	17	F: 5′-cctgtaatcccagcactttgg-3′ R: 5′-ccactgcactccagcctggg-3′

2.8. [^{3}H]-thymidine incorporation DNA synthesis assay

T24 cells were plated in six-well plates at a density of 5×10^4 per well, and were incubated for 4 h with 1 μCi/ml of [^{3}H]-thymidine (Perkin-Elmer). The cell lysate was collected and applied onto a liquid scintillation cocktail and [^{3}H]-thymidine incorporation was measured using a liquid scintillation counter (1211Rackbeta-LKB Wallac).

2.9. Luminometric methylation assay (LUMA)

The assay was performed as previously described [21]. Genomic DNA (500 ng) was cleaved with *Hpa*II and *Eco*RI, and then subjected to polymerase extension assay using the pyrosequencing platform, which can distinguish the *Hpa*II and *Eco*RI cutting sites. The level of cytosine methylation was determined by comparing the ratio of *Hpa*II over *Eco*RI in the different treated samples.

3. Results

3.1. TSA enhances global histone acetylation levels and reduces global DNA methylation

We first verified that inhibition of histone deacetylation by the HDACi, TSA altered global histone acetylation in the human bladder cancer cell line, T24. A Western blot analysis using anti-acetyl H3-K9 antibodies demonstrated that acetylation at lysine-9 (K9) of H3 histones was substantially increased by 150 nM of TSA (Fig. 1A). We then tested whether this change of acetylation has an effect on genomic DNA methylation by nearest neighbor analysis, which directly measures the extent of methylation at all CpG dinucleotides [15]. This assay showed that the level of unmethylated cytosine in CpG dinucleotides in the genome was increased from 30% in control cells to 50% in TSA-treated cells (Fig. 1B). Similarly, TSA treatment also results in genomic demethylation in the breast cancer cell line MDA-MB231 as determined by a nearest neighbor analysis (Fig. 1C), suggesting that this effect of TSA is not limited to T24 cells. The global methylation analyses demonstrate that the inhibition of HDAC by TSA triggers a reduction in fraction of methylated CG dinucleotides in the genome.

3.2. Inhibition of histone deacetylation affects the methylation level of repetitive elements

Since a major fraction of methylated CpGs is localized at repetitive sequences, we determined whether the global demethylation induced by TSA is predominantly localized to repetitive elements. However, in cancer cells repetitive sequences are hypomethylated [22], thus the effect of HDACi on these sequences in cancer cells is not expected to be dramatic. We first performed an analysis of pericentromeric satellite 2 using combined bisulfite restriction assay (COBRA), which indicates that TSA triggers a small change in methylation in the repetitive sequence (Fig. 2B and C). The difference between 5-azaCdR and control is significant as determined by a student *t*-test, statistically significant differences are

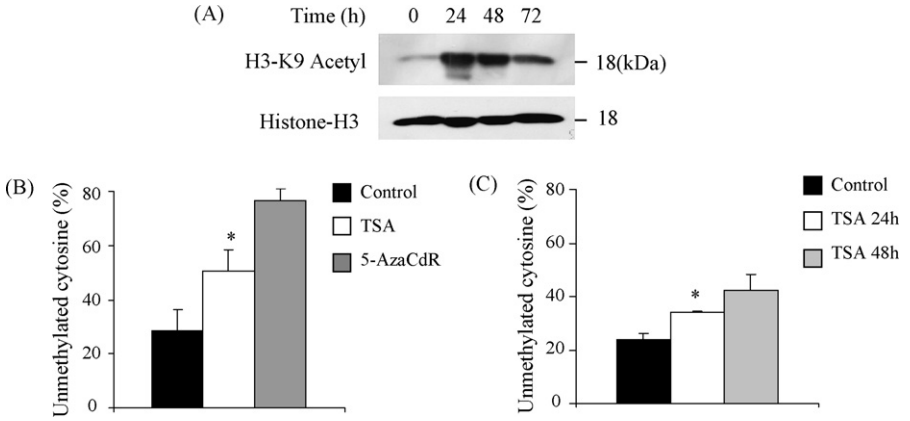

Fig. 1 – TSA enhances histone acetylation and reduces global DNA methylation. (A) T24 cells were treated with 150 nM of TSA for 24–72 h. The level of acetylation of K9 residue of histone H3 was then quantified by Western blot analysis. Pan histone H3 protein level is used as a loading control. (B and C) T24 (B) and MDA-MB231 (C) cells were treated with either TSA (150 nM), or 5-azaCdR (1 μM) for 24 h, and the level of cytosine methylation of each sample was determined by nearest neighbor analysis. The graph represents an average of three determinations ±S.E.M. (*$p < 0.01$).

indicated by an asterisk (*$p < 0.01$). However, there is no significant difference between TSA-treated sample and the control. A more sensitive and quantitative assay sodium bisulfite sequencing, revealed a 10% decrease in the average methylation of CpGs in satellite 2 following TSA induction (Fig. 2D and E). We also mapped the state of methylation of repetitive Alu sequences by bisulfite mapping using consensus primers, which amplified a range of Alu-repeat subfamilies. The sequencing results demonstrate that TSA only causes minor demethylation of certain Alu sequences (Fig. 2F and G), which cannot account for all the global changes in methylation observed in the global analyses. We therefore considered the possibility that this TSA-induced demethylation might have targeted unique genes across the genome.

3.3. *TSA treatment induces expression of E-CADHERIN in human bladder cancer T24 and breast cancer MDA-MB231 cells*

A number of studies have previously shown that tumor suppressor genes that are methylated in cancer cell lines could only be activated either by a DNA methylation inhibitor 5-azaCdR by itself, or by a combination of HDACi and 5-azaCdR [23,24]. Since we observed global demethylation induced by TSA in Fig. 1, we inquired whether certain tumor suppressor genes would also be demethylated by TSA. We focused on genes previously shown to be methylated and silenced in different cancer cell lines, such as *E-CADHERIN*, *RARβ2* or *P16*, and their transcription was generally restored by demethylating agent 5-azaCdR [23,25,26]. All three genes were thought to play a role in suppressing tumorigenesis by different mechanisms [27–30]. We therefore tested whether inhibition of deacetylation by TSA could reactivate these genes. We first confirmed that these genes were silenced by DNA methylation by verifying that their expression was inducible by 5-azaCdR, as previously shown (data not shown). T24 and MDA-MB231 cells were then treated with 150 nM of TSA for the indicated time points or with increasing concentrations of TSA (50 nM to 300 nM) for 24 h, and the mRNA levels of the three methylated tumor suppressor genes were determined by RT-PCR (Fig. 3A, B and D). The expression of these genes is barely detected in untreated cells, whereas TSA treatment induces the transcription and protein levels of *E-CADHERIN* gene in a dose and time-dependent manner, with a peak of induction at or before 24 h (Fig. 3A–C).

RARβ2 is similarly induced by TSA, as determined by RT-PCR (Fig. 3D). In contrast, the expression of *P16* is not induced even using 1 μM of TSA for up to 96 h of treatment (data not shown), which is consistent with previous reports [23,24]. These data suggest that the inhibition of histone deacetylation by TSA could restore the transcription of certain methylated genes, such as *E-CADHERIN* and *RARβ2*.

3.4. *TSA triggers demethylation of E-CADHERIN gene in certain sites at the 5′ region flanking the transcription initiation position*

To test whether activation of *E-CADHERIN* by TSA involved demethylation of its transcriptional regulatory regions, we performed sodium bisulfite mapping of the proximal promoter and the region flanking the transcription start site in DNA extracted from T24 and MDA-MB231 cells, treated with TSA for 24 h (Fig. 4A). Our analyses revealed that methylation of the promoter in cancer cell lines T24 and MDA-MB231 is concentrated at specific positions (863, 873, 879, 887) upstream to the transcription initiation site, and specific positions downstream to the trancription initiation sites (1101, 1105, 1111, 1114). TSA induced demethylation at these positions, as well as a general hypomethylation of the 5′ region of *E-CADHERIN* in both T24 and MDA-MB231 cell lines (Fig. 4B and C). For example, CpGs at positions 863 and 873 are nearly 70% methylated in untreated cells, and their methylation level decreases to 15–17% in response to TSA treatment (Fig. 4B and D). Interestingly a standard *t*-test reveals significant differences between untreated T24 and TSA treated cells in methylation of specific CpG located around core promoter

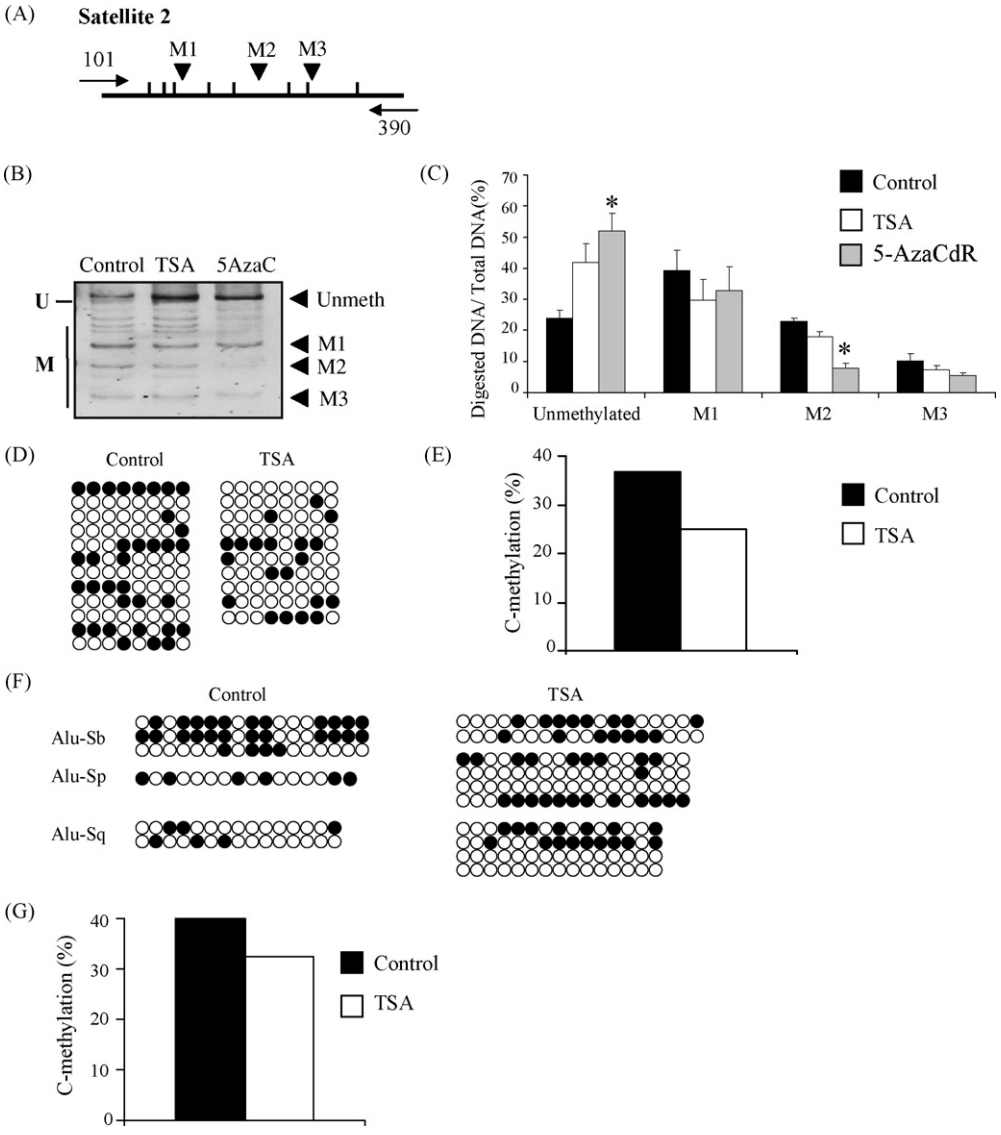

Fig. 2 – Inhibition of histone deactylation reduces cytosine methylation of repetitive sequences. Sodium bisulfite treated DNA were amplified using primers targeting pericentromeric satellite 2. (A) A physical map of satellite 2 sequence analyzed by COBRA, where *arrows heads* indicate the restriction sites of methylation-sensitive enzyme *Hin*fI containing CpG dinucleotide. Only methylated CGs which are not converted by sodium-bisulfite treatment are cleaved with *Hin*fI. (B) The upper band (280 bp) represents unmethylated fragment, whereas the lower bands (130 bp, 110 bp and 80 bp) correspond to methylated fragments digested by *Hin*fI (M1, M2, M3). (C) The results from COBRA are quantified by dividing the *Hin*fI digested fragments (M1, M2, and M3) over undigested DNA. The graph represents quantified values derived from three independent experiments. (D) The diagrams represent methylation profiles of satellite 2 determined by sodium bisulfite mapping, where each *line* represents an independent clone, *filled circles* represent methylated CpG dinucleotides, and *empty circles* represent unmethylated CpG dinucleotides. (E) The average methylation is represented by the bar graph. (F) Bisulfite mappings of Alu consensus sequences. The consensus sequences are subdivided into three subgroups, based on their sequence homology. (G) The methylation of all consensus sequences is averaged based on the number of clones sequenced.

binding protein consensus sequence (CPBP) and Sp1 binding sites (Fig. 4A and D). The demethylation of *E-CADHERIN* promoter region in MDA-MB231 cells (Fig. 4C and E), suggesting that TSA effects on methylation of selective genes are not limited to one cell type. The methylation status of *RARβ2* in MDA-MB231 following TSA treatment was determined by sodium bisulfite sequencing as well. Two sites were found to be highly methylated (60%) in control cells at positions 1010 and 1016. The results revealed a decrease in CpG methylation at these two positions, which is less extensive than the demethylation observed in *E-CADHERIN* (Fig. 4F and G).

In accordance with previous reports and our RT-PCR assay (Fig. 3A), TSA alone is unable to induce the *P16* gene. Previous studies have indicated that the promoter is not demethylated by TSA [23,24], and we confirmed this by a methylation-specific-PCR (data not shown). In summary, the results

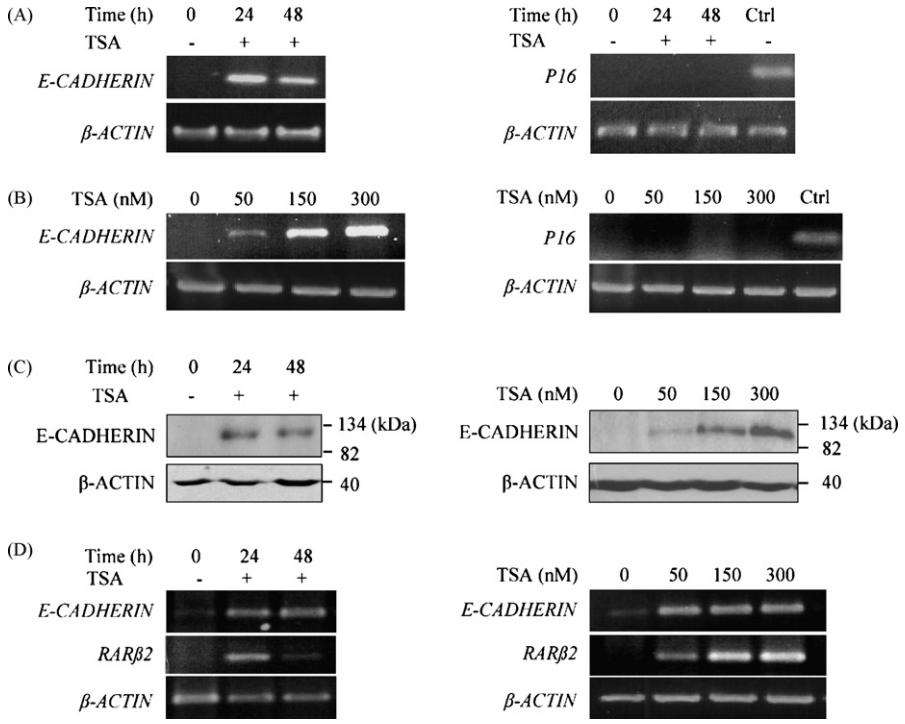

Fig. 3 – TSA selectively induces expression of *E-CADHERIN* and *RARβ2*. (A) T24 cells were treated with TSA (150 nM) for the indicated time intervals. (B) T24 cells were treated with the indicated concentrations of TSA for 24 h. Representative RT-PCR amplifications of *E-CADHERIN* and *P16* mRNA are shown. (C) Western blot analysis of E-CADHERIN protein in T24 cells following TSA treatment, β-ACTIN protein is used as a loading control. The positions of molecular weight markers are indicated. (D) MDA-MB231 cells were similarly treated with TSA for the indicated time intervals and concentrations. mRNA levels of *E-CADHERIN* and *RARβ2* was determined by RT-PCR. The size of the PCR product corresponding to *E-CADHERIN* is 420 bp, *RARβ2* is 330 bp and *P16* is 138 bp.

presented here provide evidence that HDAC inhibitor could trigger selective demethylation of certain but not all methylated CGs in cancer cells.

3.5. *TSA stimulates acetylation of histones associated with E-CADHERIN*

The observation that a selective HDAC inhibitor would affect a different enzymatic process such as DNA methylation was unanticipated. One possible explanation is that TSA facilitates loss of DNA methylation indirectly by altering chromatin structure through histone acetylation. To determine the state of H3 histone K9 acetylation at *E-CADHERIN* and *P16* promoters in response to TSA treatment, we performed a chromatin immunoprecipitation (ChIP) analysis using an anti-acetyl H3-K9 antibody. The ChIP analysis reveals that TSA increases K9 acetylation of H3-histones associated with the promoter of *E-CADHERIN* by 4.5-fold in T24 cells, as quantified by semi-quantitative and quantitative real-time PCR (Fig. 5B and C). This enriched state of acetylation was maintained through the entire treatment period, although there was a reduction in the extent of acetylation with time. We have consistently seen a slight decrease in *E-CADHERIN* expression at longer time points after TSA treatment (Fig. 3B). This might reflect the half-life of TSA, since a single treatment was used in our experiments. As histone acetylation is a balance of HDAC and HAT activity, a decrease in TSA concentration would result in increased HDAC activity and reduced acetylation and expression after time (Fig. 5D).

In contrast, TSA has a noticeably smaller effect on H3-K9 acetylation of *P16* gene, whereas 5-azaCdR, which demethylates and activates this gene increases acetylation of *P16* promoter (Fig. 5D), as previously reported [31]. The selectivity of TSA to a subset of genes has been previously described, and only a small fraction of the genome is activated in response to TSA [32]. Our data is consistent with the hypothesis that selective acetylation of tumor suppressor genes following TSA treatment, is associated with selective demethylation and gene activation.

3.6. *TSA induces global hypomethylation in the absence of DNA replication*

We tested whether the genomic demethylation induced by HDAC inhibitor occurred by a replication independent mechanism similar to what was previously shown in a transient system with plasmid DNA [9]. In order to determine whether the effects of TSA would remain in the absence of DNA replication, T24 cells were grown in the presence of the DNA synthesis inhibitor hydroxyurea, which completely blocked DNA replication as early as 2 h after its addition to the culture medium, as measured by [^{3}H]-thymidine incorporation assay (Fig. 6A). TSA

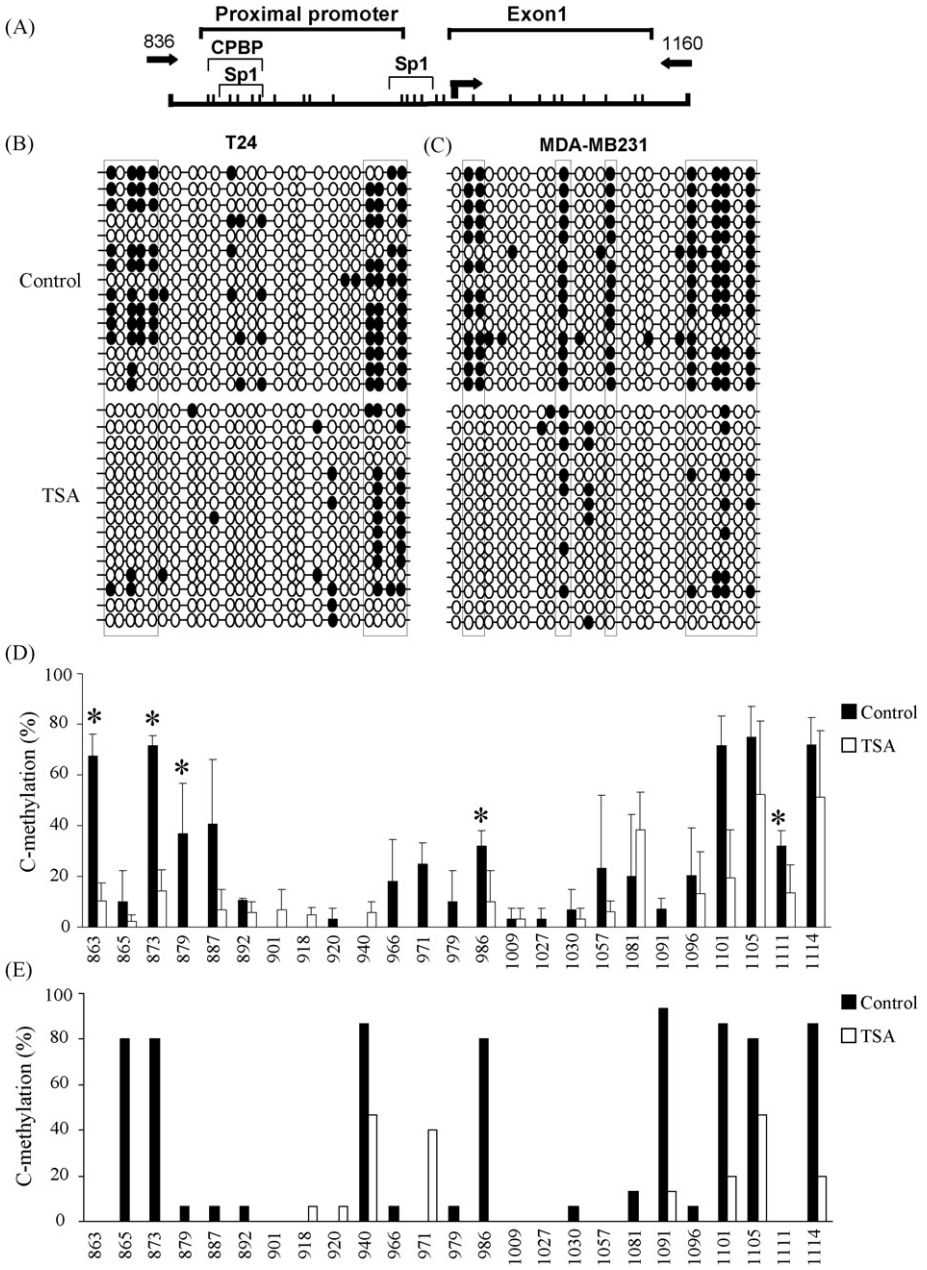

Fig. 4 – Inhibition of histone deacetylation by TSA induces demethylation at the proximal promoter and the region flanking the transcription start site of *E-CADHERIN* in T24 and MDA-MB231 cell lines. (A) Physical map of the *E-CADHERIN* promoter region analyzed by bisulfite mapping. (B) T24 and MDA-MB231 cells were treated with 150 nM of TSA, and harvested 24 h after initiation of treatment. DNA was isolated and treated with sodium bisulfite. *E-CADHERIN* gene sequences were amplified, cloned and sequenced. Specific regions around the transcription start site are indicated in the physical map. The positions of the CpG sites are numbered according to GenBank accession number L34545 (*E-CADHERIN*), or X56849 (*RARβ2*). (B) Beads on strings scheme of the state of promoter methylation of *E-CADHERIN* in control T24 and TSA treated cells, where each *line* represents an independent clone, *filled circles* represent methylated CpG dinucleotides, and *empty circles* represent unmethylated CpG dinucleotides. (C) A schematic representation of methylation state of *E-CADHERIN* in MDA-MB231 cells. Boxes indicate the cluster of CpG sites, which are methylated in the silenced gene and become demethylated upon TSA treatment. (D) The chart represents an average of three independent experiments performed in T24 cell line (±S.E.M), the significance of the difference in methylation between the control and TSA-treated sample, is determined by standard *t*-test. Statistically significant differences are indicated by an asterisk (*$p < 0.01$). (E) The graph represents the percentage of methylation at each CpG site in the *E-CADHERIN* gene in control and TSA treated MDA-MB231 cell line. (F) A physical map of the *RARβ2* promoter region analyzed by sodium bisulfite mapping. (G) A beads on strings scheme of the state promoter methylation of RARβ2 in control MDA-MB231 and TSA treated cells (empty circles represent an unmethylated CG site and a filled circle represents a filled CG site, each line represents one sequenced clone). The percentage of methylation at each CpG site is presented by the bar graph. Arrows indicate sites that are demethylated by the treatment.

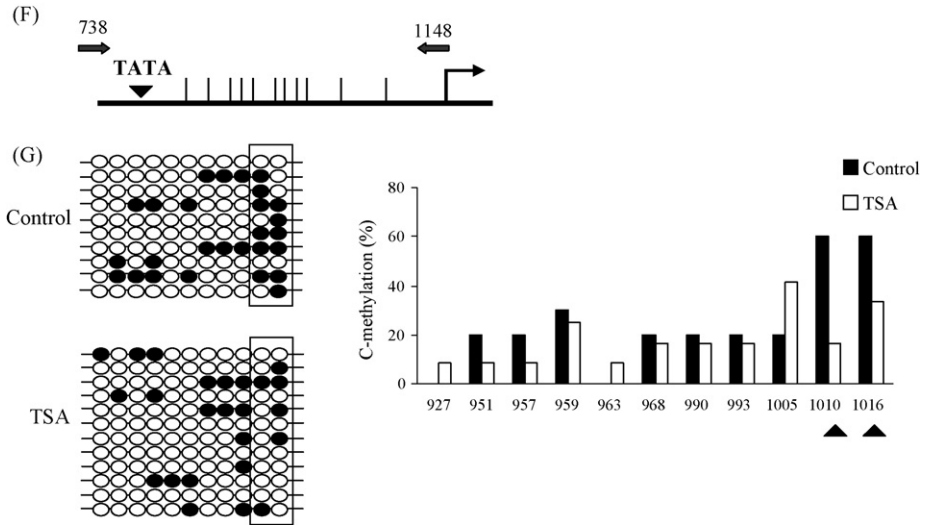

Fig. 4. (Continued).

was added 2 h following addition of hydroxyurea when a complete arrest of DNA replication was observed. This DNA replication arrest was maintained throughout the TSA treatment period (Fig. 6A). The state of cytosine methylation of treated cells was measured by two different methods, nearest neighbor analysis and a lately developed Luminometric Methylation Assay (LUMA) which measured the level of CG demethylation by the extent of cleavage of CCGG sites by *Hpa*II [21]. The fraction of unmethylated cytosines in the genome increased from 30 to 50%, as determined by nearest neighbor analysis, while hydroxyurea did not cause global hypomethylation by itself (Fig. 6B). Both assays indicated that TSA could induce global demethylation in the absence of DNA synthesis (Fig. 6B and C). Since hydroxyurea had a TSA independent effect on expression and state of DNA methylation of *E-CADHERIN* (data not shown), we were unable to study the effect of TSA on *E-CADHERIN* methylation in the presence of hydroxyurea. These results provide evidence for the presence of a mechanism in the cell for removal of methylation upon change in the state of histone acetylation, which is independent of DNA replication.

4. Discussion

DNA methylation is associated with different states of chromatin, and the nature of this relationship remains to be defined. The accepted model is that DNA methylation is a dominant mechanism in silencing of tumor suppressor genes in cancers [2,23]. This model has two important implications. First, the activation of methylated tumor suppressor genes could only be accomplished, if DNA methylation is first inhibited during DNA replication by DNMTs inhibitors, and then it could synergize with an HDACi. Second, drugs that affect chromatin modification would not alter DNA methylation patterns unless used in combination with DNA methylation inhibitors. HDACi by themselves were shown to induce only genes, which were not silenced by DNA methylation [2]. HDACi would be therefore predicted to target a different subset of genes than DNA methylation inhibitors. However, recent studies using microarray-based gene expression analysis demonstrated that similar expression profiles were obtained from HCT-116 cells following their exposure to 5-azaCdR or TSA [32]. Furthermore, previous data also suggested that altering chromatin modifications resulted in extensive changes in DNA methylation patterns in both mammalian cells[12], and in *Neurospora* [8]. Recent studies also illustrated that TSA could induce active demethylation of ectopically methylated genes *in vitro* [9,10], and demethylation of the glucocorticoid receptor gene in adult rat hippocampi *in vivo* [14]. The question of whether drugs targeting chromatin modification might affect the DNA methylation patterns as well, has obvious clinical implications, since HDACi are now being tested in different clinical trials, under the assumptions that when these drugs are used on their own, they only affect chromatin structure, but not DNA methylation.

In this report, we test whether a chromatin-modifying drug can also induce a change in DNA methylation, and whether the expression of methylated tumor suppressor genes can be restored by increasing histone acetylation. Our results show that the inhibition of histone deacetylation by TSA could induce hyperacetylation of histones, restore the expression of methylated *E-CADHERIN*, and bring about global hypomethylation, as well as demethylation of specific genes (Figs. 1–4). These data suggest that the state of histone modifications defines the state of gene expression, and that a change in the state of histone acetylation could reverse DNA methylation patterns of target sites. It was previously suggested that histone acetylation is secondary to DNA methylation since methylated tumor suppressor genes such as *P16* were resistant to TSA [2]. The fact that the state of acetylation of *P16* is not affected by TSA is not unprecedented, a number of microarray analyses of genes induced by TSA revealed that only a certain fraction of the transcriptome is influenced by TSA [32]. We propose that *P16* gene escapes the demethylation effects of TSA (data not shown) since it is specifically resistant to the effects of TSA on histone acetylation (Fig. 5). The sensitivity to TSA might depend on the composition of the transcription repressor complexes and HDACs associated with

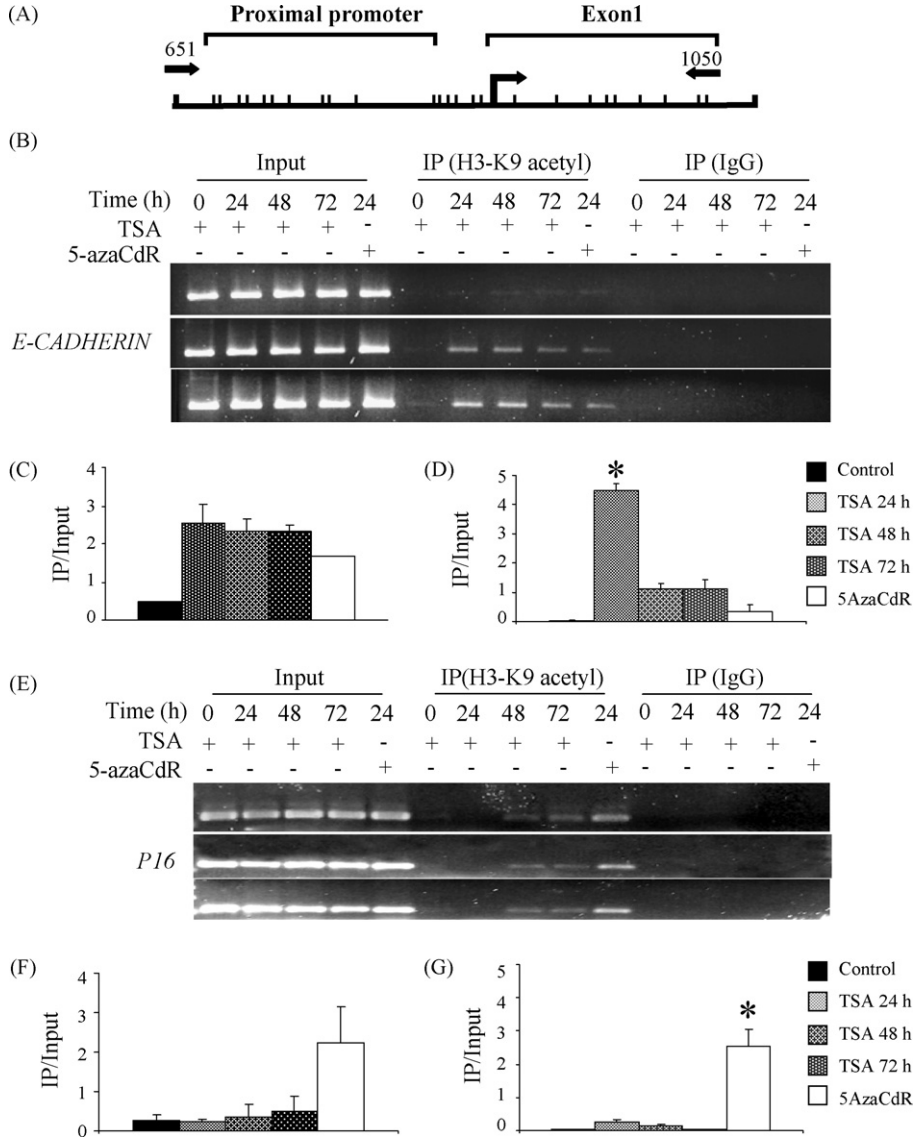

Fig. 5 – Histone acetylation within *E-CADHERIN* promoter is induced by TSA. T24 cells were grown in the presence of either 5-azaCdR (1 μM), or TSA (150 nM), and subjected to a ChIP assay using an antibody against acetylated K9 of histone 3. (A) The amplified region for *E-CADHERIN* is indicated by arrows. The promoter region of the *E-CADHERIN* and *P16* sequence were amplified from the DNA samples by PCR. (B and E) Semi-quantitative PCR was performed using different number of cycles (28, 30 and 32) to ascertain linear dose response. A representative PCR is shown: Input, 10% of total DNA prior to immunoprecipitation; IgG, reactions carried out with a non-specific antibody, and IP, DNA immunoprecipitated by anti-acetylated K9 H3 histone antibody. The length of the PCR product corresponding to *E-CADHERIN* is 400 bp, and to *P16* promoter is 312 bp. (C and F) The results from PCR are quantified as the ratio of *E-CADHERIN* (C) or *P16* (F) DNA amplified from the acetylated histone immunoprecipitated relative to the corresponding input. (D and G) Quantitative real-time PCR determination of *E-CADHERIN* (D) and *P16* (G) promoter sequences immunoprecipitated by anti-acetylated histone antibody normalized to input in control and TSA treated cells. The ChIP experiments were repeated three times, and the results are an average of quantified values derived from three determinations per experiment ±S.E.M (*$p < 0.01$).

different genes, and the availability of histone acetyltransferase (HATs) to acetylate a gene following inhibition of HDAC, which might explain why previous studies failed to show demethylation of tumor suppressor genes by TSA [2,23,24].

Despite the fact that TSA has a global effect on both histone acetylation and DNA methylation, it is evident that the action of the drug also exhibits gene specificity and site selectivity. At the global level, we observe that pericentromeric sequences satellite 2 are more sensitive to the effect of TSA as compared to euchromatic Alu repetitive elements. This is surprising considering the overall effect on global DNA demethylation. Previous studies have similarly shown that repetitive sequences located in euchromatic regions exhibit weak demethylation in cells treated by 5-azaCdR [19]. We do not

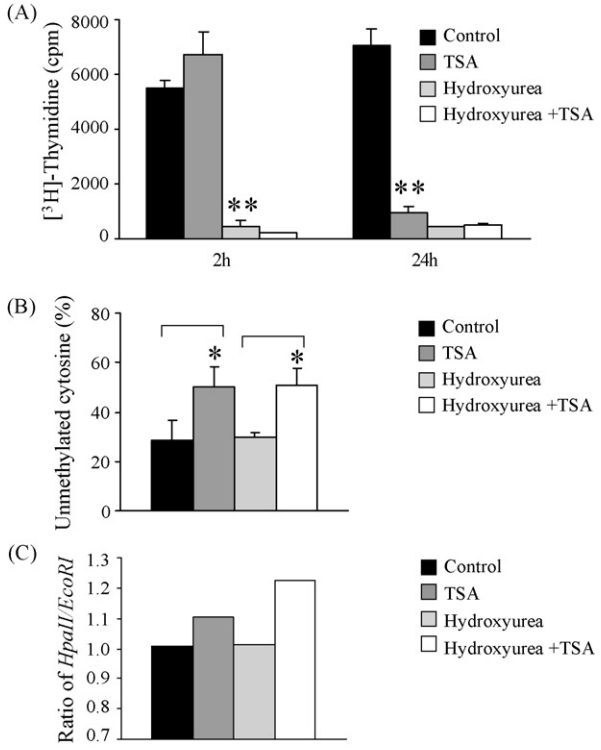

Fig. 6 – TSA induces global hypomethylation in the absence of DNA replication. T24 cells were treated with 2 mM of hydroxyurea for 2 h, followed by 150 nM of TSA for 24 h. (A) DNA replication was measured by [^{3}H]-thymidine incorporation assay. The results presented are mean ± S.E.M of triplicate determinations ($p < 0.005$) from one of three independent experiments with similar results. (B) Cytosine methylation of DNA following hydroxyurea and TSA treatment was determined by nearest neighbor assay. The graph represents the average methylation from three independent experiments ±S.E.M (*$p < 0.01$). (C) The global methylation is tested by LUMA, where the ratio of genomic DNA digested by methylation-sensitive enzyme *Hpa*II over *Eco*RI indicates the level of cytosine demethylation.**

have any evidence-based explanation for this selectivity of TSA and 5-azaCdR induced demethylation for certain repetitive sequences and not others. The *E-CADHERIN* gene bears a CpG island within its promoter region, which also contains multiple Sp1 sites [33]. Although methylation of *E-CADHERIN* is rather heterogeneous in T24 cells, certain specific sites tend to be hypermethylated (indicated by boxes in Fig. 4B). These CpGs are preferentially demethylated upon TSA treatment. Interestingly, these sites are located upstream of Sp1 recognition elements, previous reports have demonstrated that methylation occurring outside of consensus Sp1 sites might interfere with the binding of transcription factor Sp1 [34].

TSA is an HDAC inhibitor, and there is no evidence that it directly interferes with DNA methyltransferases. Although it was theoretically possible to explain TSA induced demethylation in cancer cells by the classic passive loss of methylation model, it was critical to test the hypothesis that TSA could evoke replication-independent demethylation. In this report, we used hydroxyurea to block DNA synthesis, and the results show that demethylation triggered by TSA did not require DNA replication (Fig. 6). This has important pharmacological implications. First, it implies that HDAC inhibitors might alter the state of DNA modification in non-dividing cells and cause stable changes in DNA methylation. Second, if TSA could alter methylation pattern in absence of DNA synthesis it might be possible to activate methylated genes therapeutically in non-mitotic tissues. It has been generally believed that DNA methylation could only be modified in replicating cells using DNMT inhibitors, the current data forces us to revisit this concept.

TSA is not a demethylating agent; it only facilitates demethylation by other mechanisms. This drug could only trigger demethylation if DNA demethylation machinery is constitutively present in cells. We propose that a change in chromatin modification induced by an HDAC inhibitor would render a gene preferentially susceptible for demethylation by the demethylation machinery. Similarly, a change in histone acetylation brought about by recruitment of a histone acetyltransferase to a gene will result in site-specific demethylation in a gene. Indeed, it has been reported that recruitment of transactivating factor to specific sites triggered demethylation in IgKappa genes [35]. The nature of the cellular demethylase involved is still unclear, and is still a contentious topic.

One interesting possibility that should be considered in future experiments is that TSA might affect the state of acetylation of enzymes and proteins involved in the DNA methylation-demethylation machinery. Several proteins interacting with DNA and gene expression machineries were previously shown to be regulated by histone acetylation. For example, acetylation sequence-specific DNA binding of the p53 protein is activated by acetylation of its C-terminal domain [36].

In summary, our data illustrates the presence of default demethylation machinery in cancer cells, and demonstrates how chromatin modification could affect DNA demethylation, thus establishing a bilateral relation between DNA demethylation and chromatin modification. Since chromatin states are responsive to different cellular signaling pathways, this establishes a mechanism through which DNA methylation might be altered by different cellular signals.

Acknowledgements

This project was supported by a grant from the National Cancer Institute of Canada to M.S., from ''Fondation pour la Recherche Medicale'' to J.T., and from the Swedish Cancer Foundation to T.J.E.

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