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Valproate induces widespread epigenetic reprogramming which involves demethylation of specific genes

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Valproate (VPA)[1] has been used for decades in the treatment of epilepsy, and is also effective as a mood stabilizer and in migraine therapy. It has been shown that VPA is also a histone deacetylase (HDAC) inhibitor. We have previously shown that VPA could trigger active demethylation of ectopically methylated transiently transfected DNA in HEK 293 cells. We therefore tested whether VPA treatment could bring about stable changes in the epigenome by causing changes in the state of DNA methylation of genomic DNA. Using a microarray gene expression analysis we identified the genes whose expression is induced by VPA treatment in HEK 293 cells. We found that a subset of these genes could also be induced by the classical DNA methylation inhibitor 5-aza-2′-deoxycytidine (5-aza-CdR) suggesting that VPA can alter the state of expression of genes, which are stably suppressed by DNA methylation. We mapped the state of methylation of three of these genes, *MELANOMA ANTIGEN B2 GENE* (MAGEB2), *METALLOPROTEINASE 2* (MMP2) and WIF1, which are involved in tumor growth and metastasis. A chromatin immunoprecipitation (ChIP) assay revealed that VPA treatment caused as expected a change in the state of acetylation of these genes. Our data supports the concept that chromatin acetylation and DNA methylation are found in a dynamic interrelation and that the consequences of HDAC inhibitors are not limited to changes in histone acetylation but that they also bring about a change in the state of modification of DNA. The implications of our results on the future therapeutic utilities of VPA in cancer will be discussed.

Introduction

The epigenome is composed of chromatin structure, which packages DNA and defines its accessibility to transacting factors and DNA methylation, which is a covalent modification of DNA (1). A well-documented relationship exists between DNA methylation, chromatin structure and gene expression, such that methylated genes are generally transcriptionally silent (2). Although it is well established that DNA methylation precipitates closed configuration in chromatin through interaction with chromatin remodeling complexes containing methylated DNA binding proteins (MBD) (3,4), recent data suggests that this relationship is bidirectional. Several studies have now shown that closed chromatin configuration precedes DNA methylation (5,6) and that there is a physical interaction between DNA methylating enzymes (DNMT) and chromatin modifying enzymes, such as histone deacetylases (HDAC) and methyltransferases (7–10). Since changes in chromatin involve not only gene suppression but also gene activation, this relationship could also involve the possibility that open chromatin configuration would result in DNA demethylation. Typically the effects of HDAC inhibitors are rapid and transient (11), however if they could also cause changes in DNA methylation, HDAC inhibition might result in long-term consequences for the integrity of gene expression programming. It is therefore critical to determine whether HDAC inhibitors could also cause stable changes in genomic modification by DNA methylation.

HDAC inhibitors are being developed and are currently being tested in clinical trials as potential therapeutic agents in cancer (12,13) and psychiatric conditions (14). The general assumption is that these agents, which inhibit histone-modifying enzymes, would only target chromatin and not DNA methylation (15). Indeed, early studies have shown that HDAC inhibitors and DNA methylation inhibitors activated genes by different mechanisms and therefore targeted different sets of genes when applied as single agents although they could synergize when applied in combination (15). However, recent data suggests that DNMT inhibition and HDAC inhibition might target the same genomic targets (16). The possibility that HDAC inhibitor treatments would result in stable DNA methylation alterations has clear and important implications on the future therapeutic use of such agents. In addition, this concept is important for understanding the basic principles of maintenance of epigenetic states and how they might be influenced through life by environmental, behavioral, physiological and pathological signals.

An example of an HDAC inhibitor agent, which has been used for decades in therapy is Valproic acid also known as Valproate or 2-n-propylpentanoic acid (VPA). VPA has been used in the treatment of epilepsy, and is also effective as a mood stabilizer and in migraine therapy (17,18). Recent data suggests that, in addition to its known classical actions, this drug can modulate the epigenome by inhibiting HDACs (19,20). It must be noted however that VPA is far less potent inhibitor of HDAC than Trichostatin A (TSA). Similar to other HDAC inhibitors, such as TSA (21) and *n*-butyrate (22), VPA can induce gene expression. Recent studies suggested that VPA could activate genes, which were regulated by DNA methylation. It was demonstrated that both 5-aza-2′-deoxy-cytidine (5-aza-CdR) and VPA induced

Abbreviations: VPAvalproate; HDAC, histone deacetylase; 5-aza-CdR, 5-aza-2′-deoxy-cytidine; MAGEB2, melanoma antigen gene B2; MMP2, metalloproteinase 2, ChIP, chromatin immunoprecipitation; TSA, trichostatin A.

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the expression of *5-LIPOXYGENASE* in neuronal differentiated NT-2 cells [(23) 445, 149–150]. The REELIN gene, which encodes a neuronal protein that is downregulated in schizophrenia, could be induced by either 5-aza-CdR or VPA treatment in neuronal precursor cells NT-2 (23). Although, the promoter of *REELIN* is poorly methylated in these cells, and it is therefore unclear whether the minimal promoter is directly regulated by DNA methylation, some loss of the infrequently methylated CGs was observed following VPA treatment (24). VPA was also shown to reverse the down regulation of both *reelin* and *gad67* in mice treated by L-methionine, an agent shown to increase DNA methylation (25). The data presented above suggested the possibility that VPA may also be able to trigger DNA demethylation, thus causing a stable change in the covalent modification of the genome, however further evidence from more highly methylated genes was required to support this hypothesis.

We have previously developed a transient transfection assay system to measure active demethylation in living somatic cells without the confounding effects of DNA replication (26). An *in vitro* methylated CMV-GFP plasmid was transiently transfected into the human embryonal kidney cell line HEK 293 and its state of methylation was measured 48 h after transfection. Since we had shown that the plasmid did not replicate during the time frame of the experiment, nor was it *de novo* methylated by DNA methyltransferases, any loss of methylation measured by this assay was active demethylation (26). Using this assay we showed that increasing histone acetylation either pharmacologically using the HDAC inhibitor TSA, or by driving expression with a strong enhancer induced active demethylation of an *in vitro* methylated plasmid (26). We have recently used this assay to demonstrate the inhibition of active demethylation by inhibitors of histone acetylation (26). We have also shown that similar to TSA, VPA induced active demethylation of ectopically methylated DNA (27). We have recently confirmed that the results derived from our transient transfection system also apply to postmitotic tissues *in vivo* (28). We showed that TSA brought about demethylation of methylated CpG sites in the glucocorticoid receptor gene promoter in the adult rat hippocampus (28). However, although it is generally assumed that HDAC inhibitors only target chromatin, there is a rapidly expanding data that indicate in addition to histones there are many nonhistone protein targets among which could be proteins that are involved in methylation and demethylation of DNA.

It is clearly extremely important to determine whether VPA and similar agents which are now being tested in clinical trials as anticancer and anti-schizophrenic agents could reverse the DNA methylation marking of genomic targets and thus target the covalent structure of the genome itself, in addition to altering the state of modification of histones, which are associated with the genome. Since our previous study demonstrated that VPA triggered active demethylation of ectopically methylated DNA in HEK 293 cells, here we performed microarray gene expression analysis to determine whether VPA treatment results in stable epigenetic alterations of the cognate gene expression programs in the same cells. We then used bisulfite mapping to test whether this mechanism also involves changes in DNA methylation. We then determined whether classic DNMT inhibitors, such as 5-aza-CdR would also similarly reprogram genes identified to be reprogrammed by VPA. Our study demonstrates that VPA induces epigenetic reprogramming, which parallels in part the changes associated with DNMT inhibitors. This study suggests that the DNA methylation pattern is responsive to drugs, which target ostensibly unrelated mechanisms in somatic cells. Since VPA targets basic cellular mechanisms, this study further illustrates the dynamic nature of the DNA methylation pattern and its potential responsiveness to different cellular signaling pathways (29). The therapeutic implications of this data for anti cancer therapy will be discussed.

Materials and methods

In vitro methylation of plasmid DNA

CMV–GFP (pEGFP-C2 from Clontech; GenBank TM accession no. U55763) was methylated *in vitro* by incubating 10 μg of plasmid DNA with 12 U of SssI CpG methyltransferase (New England Biolabs) in the recommended buffer containing 800 μM of AdoMet for 3 h at 37°C. Twelve units of SssI and 0.16 μM of AdoMet were then added and the reaction was further incubated for additional 3 h at 37°C. The methylated plasmid was recovered by phenol/chloroform extraction and ethanol precipitation, and complete methylation was confirmed by observing full protection by HpaII digestion.

Cell culture and transient transfections

HEK 293 cells were plated at a density of 0.5×10^6 in a 10 cm plate and transiently transfected with 500 ng of methylated CMV–GFP using the calcium phosphate precipitation method as described previously (26). VPA (3 mM) was added 24 h post-transfection. Cells were harvested after different times of VPA treatment.

Northern blot analysis and RT-PCR

Approximately 10 μg of RNA was eletrophoresed on a 1.2% denaturing agarose gel and then transferred to Hidrobond-N+ membrane (Amersham Pharmacia Biotech). Blots were probed with a ^{32}P-labeled cDNA probe for GFP [a 529 bp Cfr10I–AvaII fragment from pEGFP-C1 (CLONTECH)] synthesized using a random priming labeling kit (Roche Molecular Biochemicals). The membranes were hybridized at 68°C for 4 h in a buffer containing 0.5 M sodium phosphate (pH 6.8), 1 mM EDTA, 7% SDS and 0.2 mg/ml herring sperm DNA. Following hybridization the membranes were washed twice for 10 min in a 5% SDS, 0.04 M sodium phosphate (pH 6.8), 1 mM EDTA solution and then four times for 10 min in the same solution containing 1% SDS. Each experiment was normalized for the amount of total RNA by hybridization with a ^{32}P-labelled 18 s ribosomal RNA oligonucleotide probe RT–PCR—total RNA was extracted using an RNeasy kit (Qiagen). cDNA was synthesized in a 20 μl reaction volume containing 2 μg of total RNA, 40 U of Moloney murine leukemia virus reverse transcriptase (MBI), 5 μM random primer (Roche Applied Science), 1 mM of each of the deoxynucleotide triphosphates and 40 U of RNase inhibitor (Roche Applied Science). mRNA was denatured for 5 min at 70°C, the random primers were annealed for 10 min at 25°C and the mRNA was reverse-transcribed for 1 h at 37°C. The reverse transcriptase was heat-inactivated for 10 min at 70°C, and the products were stored at −20°C until use. PCRs were performed in a 40 μl reaction mixture containing 2 μl of synthesized cDNA product, 4 μl of 10× PCR buffer with $(NH4)_2SO_4$, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1.5 U of *Taq* polymerase (all from MBI) and 0.4 μM of each primer. Amplifications were performed in a Biometra T3 thermocycler (Biomedizinische Analytik GmbH). The primer sequences and the amplification programs were as follows: MAGEB2 (melanoma antigen B2): (sense, 5′-AGCGAGTGTA-GGGGGTGCG-3′; antisense, 5′-TGAGGCCCTCAGAGGCTTTC-3′) 95°C 5 min, one cycle (95, 62 and 72°C for 30 s each), one cycle (95, 60 and 72°C for 30 s each), 30 cycles (95, 58 and 72°C for 30 s each), 72°C for 5 min; matrix metalloproteinase 2 (MMP2) (sense, 5′-GATGATGCCTTTGCT-CGTGCC-3′; antisense, 5′-CCCTGGAAGCGGAATGGAAAC-3′) 95°C for 5 min, one cycle (95, 64 and 72°C for 30 s each), one cycle (95, 62 and 72°C for 30 s each), 29 cycles (95, 60 and 72°C 30 s each), 72°C for 5 min; CD24 (CD24 antigen) (sense, 5′-TCCTTGAATGTGGCTTGAGA-3′; antisense, 5′-TGTCAAATCCAAAGCCTCAG-3′) 95°C 5 min, one cycle (95, 58 and 72°C for 30 s each), one cycle (95, 56 and 72°C for 30 s each), 26 cycles (95, 54 and 72°C for 30 s each), 72°C for 5 min; KIF5C (kinesin family member 5c) (sense, 5′-CGGATTCTTCAGGACTCTTT-3′; antisense, 5′-TTGTCTATGATGGGGGTGTT-3′) 95°C for 5 min, one cycle (95, 62 and 72°C for 30 s each), one cycle (95, 60 and 72°C for 30 s each), 32 cycles (95, 58 and 72°C for 30 s each), 72°C for 5 min; LUM (lumican) (sense,

5′-ATAAACCACAACAACCTGAC-3′; antisense, 5′-TTTCCAGGTATTC-CACTATC-3′) 95°C for 5 min, one cycle (95, 53 and 72°C for 30 s each), one cycle (95, 51 and 72°C for 30 s each), 40 cycles (95, 49 and 72°C for 30 s each), 72°C 5 min; WIF1 (WNT inhibitory factor 1) (sense, 5′-CCAACCGTCAATGTCCCTC-3′; antisense, 5′-GCAGGTGGTTGAGC-AGTTT-3′) 95°C 5 min, one cycle (95, 62 and 72°C for 30 s each), one cycle (95, 60 and 72°C for 30 s each), 30 cycles (95, 58 and 72°C for 30 s each), 72°C 5 min; β-actin (sense, 5′-GTTGCTAGCCAGGCTGTGCT-3′; antisense, 5′-CGGATGTCCACGTCACACTT-3′) 95°C 5 min, one cycle (95, 66 and 72°C for 30 s each), one cycle (95, 64 and 72°C for 30 s each), one cycle (95, 62 and 72°C for 30 s each) and 17 cycles (95, 60 and 72°C 30 s each), 72°C for 5 min. The number of cycles used for quantification per primer set was selected by a preliminary test determining the range of cycles where PCR amplification product was linear with increasing cDNA. A total of 20 µl of the PCR products were run on a 1.2% gel and visualized by ethidium bromide staining. Densitometric analysis was performed using MCID software (Imaging Research Inc.).

Chromatin immunoprecipitation (ChIP) assay

HEK293 cells were either treated with 3mM VPA or were left untreated as a control for 1 day or 5 days. Formaldehyde was added to a final concentration of 1%, and the cells were incubated at 37°C for 10 min. The cells were washed and harvested in cold phosphate-buffered saline (PBS) containing protease inhibitors (Complete mini, Roche Applied Science), were pelleted, resuspended in 300 µl of SDS lysis buffer [1% SDS, 10 mM EDTA and 50 mM Tris–HCl (pH 8.1)], and incubated on ice for 10 min. Lysates were sonicated with six 10 s bursts and debris were removed by centrifugation for 10 min at 13 000 r.p.m. at 4°C. Supernatants were diluted 10-fold in ChIP dilution buffer [0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl (pH 8.1) and 167 mM NaCl] and pre-cleared by incubating with 80 µl of agarose G mix [50% agarose G, 0.2 mg/ml sonicated herring sperm, 0.5 mg/ml BSA, 10 mM Tris–HCl and 1 mM EDTA (pH 8.0)] for 30 min at 4°C. Beads were pelleted for 5 min at 3000 r.p.m. at 4°C. A total of 100 µl of the supernatant was saved as input, and the rest was divided into equal aliquots and incubated by rocking with either no antibody (control), or with 10 µl of specific antibodies O/N at 4°C. The following antibodies were used: anti acetylated histone H3 (Upstate Biotechnology #06-599), anti acetylated histone H4 (Upstate Biotechnology #06-866). A total of 60 µl of agarose G mix was added, and the samples were rocked for 2 h at 4°C. The complexes on the beads were washed for 5 min at 4°C with the following buffers: low salt [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl (pH 8.1) and 150 mM NaCl], high salt [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl (pH 8.1), 500 mM NaCl], LiCl wash [0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA and 10 mM Tris–HCl (pH 8.1)], and six washes of TE [10 mM Tris–HCl and 1 mM EDTA (pH 8.0)]. Immune complexes were eluted twice with 250 µl of elution buffer [1% SDS and 0.1 M NaHCO3) for 15 min at room temperature. The input (volume adjusted to 500 µl with elution buffer) and combined eluates were de-cross-linked by adding 20 µl of 5 M NaCl and incubating for 4 h at 65°C. EDTA, Tris–HCl (pH 6.5) and proteinase K were then added to the final concentrations of 10 mM, 40 mM and 0.1 µg/µl, respectively, and the samples were incubated at 45°C for 1 h. Immunoprecipitated DNA was recovered by phenol/chloroform extraction and ethanol precipitation and was analyzed by PCR. For amplifying 287 bp region of MAGEB2 promoter and 308 bp region of MMP2 promoter the FailSafe PCR system (Epicenter) was used with pre-mix D, 0.5 µM of each primer and 0.5 µl of FailSafe polymerase. The primer sequences and the amplification programs were as follows for MAGEB2: (sense, 5′-AGTACCTCCTGTCA-CCATAG-3′; antisense, 5′-GGTAAACCAAGAAATGTGGGC-3′), 95°C 5 min, one cycle (95, 61 and 72°C for 30 s each), one cycle (95, 59 and 72°C for 30 s each), 36 cycles (95, 57 and 72°C 30 s each), 72°C for 5 min. The primers and amplification program for MMP2 were as follows: (sense, 5′-AGAGCGACAGATGTTTCCC-3′; antisense, 5′-GGAAGTCTGGATGC-AGCG-3′), 95°C for 5 min, one cycle (95, 60 and 72°C for 30 s each), one cycle (95, 58 and 72°C for 30 s each), 30 cycles (95, 56 and 72°C for 30 s each), 72°C for 5 min. A total of 20 µl of the PCR products was run on a 1.4% gel and visualized by ethidium bromide staining. Densitometric analysis was performed using MCID software (Imaging Research Inc.).

Bisulfite mapping of the CMV, MAGEB2, MMP2 and Wif1 promoters

HEK293 cells were treated with 1 mM VPA or 3 mM VPA for 5 days or were left untreated as a control. Bisulfite mapping was performed as described previously with minor modifications (30). A total of 50 ng of sodium bisulfite-treated DNA samples were subjected to PCR amplification using the first set of primers, followed by a nested PCR with the second set of primers. For MMP2 the primers were as follows: first set (sense, 5′-ATTTTAGGG-AGTGTAGGGTG-3′; antisense, 5′-CCCTTTATATATTTAAAACCCC-3′) and second set (sense, 5′-AGATGTTTTTTAGTAGGGG-3′; antisense, 5′-CCTAAAAAAATCTAAATACAA-3′). For MAGEB2 the primers were as follows: first set (sense, 5′-TTTAGATTTTTATAGTGGGG-3′; antisense, 5′-ACCAATCCTAAAAATTCACC-3′) and second set (sense, 5′-GGGTAT-TGTTTGGAGGTTGG-3′; antisense, 5′-TTCACCCCTAACTAACCAAA-3′). For GFP the primers were as follows: first set (sense, 5′-GTTATTATG-GTGAGTAAGGG-3′; antisense 5′-TATAACTATTATAATTATACTCCA-3′) and second set (sense, 5′-GGGGTGGTGTTTATTTTGG-3′; antisense 5′-CTTATACCCCAAAATATTACC-3′). For *WIF1* the primers were as follows: first set (sense, 5′-GTTTTGTTTGTTTTTTTTTTTTGT-3′; antisense, 5′-CTATAAATACTCTTACCTAATAA- 3′) and second set (sense, 5′-GTAGGTTTTTTGGTATTTAGG-3′; antisense, 5′-TAAATACAAACTC-TCCTCCTA-3′). The PCR products of the second reaction were subcloned into a TA cloning vector (Invitrogen), and the clones were sequenced using the T7 Sequencing kit (Amersham Biosciences).

Microarray analysis

HEK293 cells were treated with 1 mM VPA or left untreated as a control for 5 days. Total RNA was extracted with RNAeasy kit (Qiagen). Micoarray analysis was performed as described previously (31). Briefly, 20 µg of RNA was used for cDNA synthesis, followed by *in vitro* transcription with a T7 promoter primer having a poly(T) tail. The resulting product was hybridized and processed with the GeneChip system (Affymetrix) to a HuGeneFL DNA microarray containing oligonucleotides specific for ~25 000 human transcripts. Data analysis, average difference and expression for each feature on the chip were computed using Affymetrix GeneChip Analysis Suite version 3.3 with default parameters. The gene expression analysis was performed by the Montreal Genome Center.

Results

VPA treatment induces reprogramming of genome expression

We have previously shown that VPA triggers histone acetylation and active DNA demethylation of ectopically methylated DNA transfected into HEK293 cells (27). Whereas this study demonstrated the presence of demethylase activity in HEK293 cells, it was unclear whether this response was unique to ectopic DNA or whether it reflected the whole genome response to VPA. To determine whether VPA treatment could alter cognate gene expression programs, we utilized the HuGeneGL DNA microarray assay to determine the expression profile of ~25 000 human transcripts in both control HEK293 cells, and cells treated with 1mM VPA for 5 days. We chose a 5 day time point to focus on the long-term changes in gene expression, which would suggest the mechanism involving epigenomic reprogramming. Two independent VPA treatments and two independent control groups were compared and only differences in expression which were at least 2-fold different between treatment and control, and consistent in both experiments, were scored. 0.64% of the genes were induced (>2-fold) 5 days after initiation of treatment and a small component of this group was induced more than 10-fold from negligible basal expression, which is consistent with a change in methylation state (Figure 1 and Table I) since methylated genes are believed to be robustly silenced. Supplementary Table I lists all the genes that were induced 2-fold or more in both experiments, while Table I lists most highly induced representatives belonging to different functional groups. Among these are the genes that play important roles in the nervous system, such as GAP43, which is involved in neurite and axonal outgrowth (32), KIF5C, which is involved in neuronal trafficking (33), and NPTX2 which mediates synaptic uptake (34). A number of genes induced highly by VPA have been previously shown to be regulated by methylation, such as WNT inhibitory factor 1 (WIF1) (35), *UROPLAKIN 1B* (*UPK1B)* (36) and NPTX2 (37). In addition, proteins known to be associated with metastasis

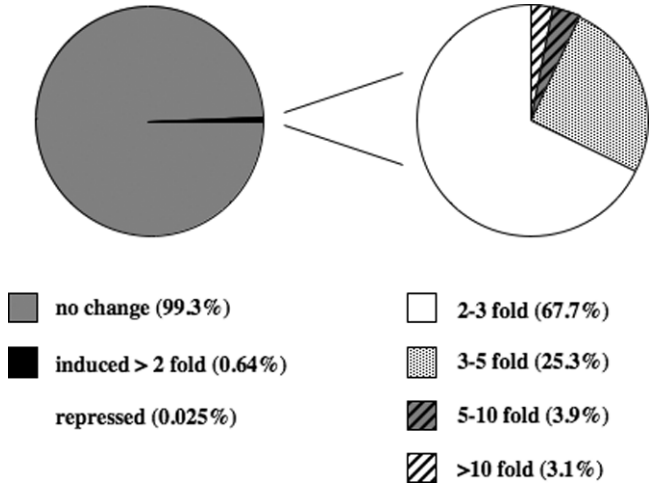

Fig. 1. Gene expression changes following VPA treatment. Pie chart summary of the changes in gene induction brought about by 1 mM VPA treatment as determined by a microarray gene expression analysis as described under 'Materials and methods'. Overexpression of MAGEB2 by VPA was found by a candidate gene approach using semi-quantitative RT–PCR.

and cell migration, such as the gelatinase *MMP2* (38,39) and bone matrix protein *LUMICAN* (40) were also induced. VPA also induced cancer-testis specific gene melanoma antigen MAGEB2, which was previously shown to be regulated by methylation (41) and was shown to be hypomethylated and induced in invasive cancers (38). It is important to note that VPA induces genes, which are closely associated with cancer and metastasis, since these findings might have implications on future use of VPA and other HDAC inhibitors in therapy.

A subset of the genes induced by VPA was also induced by the DNA demethylating agent 5-aza-CdR

We predicted that VPA affected two classes of genes: unmethylated genes which were induced by increased histone acetylation, and methylated genes which were demethylated and activated as a consequence of increased histone acetylation. Inspection of the table of genes whose expression was elevated by VPA revealed that many of the genes whose expression was elevated by VPA were expressed at the detectable levels in untreated cells (Table I and Supplementary Table). These genes were most probably unmethylated in untreated cells since genes regulated by methylation were believed to be stably silenced. Genes regulated by methylation should be silenced in untreated cells and induced manifold by VPA if VPA had triggered their demethylation. The inspection of the microarray data identified such candidate genes, which are listed in Table I. We first validated the microarray results by confirming the induction of several of these genes by using semi-quantitative RT–PCR. We treated HEK293 cells with either 1 or 3 mM VPA for 1, 3 and 5 days. RT–PCR was performed on the extracted RNA from duplicate experiments and the levels of expression of genes shown in Figure 2A were quantified, normalized to actin, and represented as fold induction relative to untreated control (Figure 2B). The indicated genes were induced from very low or undetectable basal levels up to 25-fold one day after addition of VPA. Genes regulated by methylation should be silenced in untreated cells and induced manifold by VPA if VPA had triggered their demethylation. To test whether these genes were suppressed in untreated HEK293 cells by DNA methylation, we determined whether these genes could also be induced by the classic DNA methylation inhibitor 5-aza-CdR. As shown in Figure 2, five out of six genes that were induced by VPA were also induced by 5-aza-CdR, supporting the hypothesis that VPA activated a set of genes, which were also regulated by DNA methylation.

Table I. Representative genes induced by VPA treatment in HEK 293 cells

Gene	Encoded protein	Biological roles	Fold induction
LUM	Lumican	Cell proliferation and migration	75
RALDH1	Retinal dehydrogenase	Signal trasduction	56
CLU	Clusterin/ApoJ	Tissue remodeling, membrane recycling, cell–cell interactions	12.5
CD24	CD24	Cell surface antigen	10
KIF5C	Kinesin family member 5c	Neuronal trafficking	27.5
UPK1B	Uroplakin 1B	Structural protein, urothelium specific marker	5
MMP2	Matrix metalloproteinase2	ECM remodeling/cell migration	5
WIF1	WNT inhibitory factor 1	Embryonal development	5
BASP1	Brain abundant membrane attached signal protein 1	Signal transduction in neurite outgrowth/plasticity	5
EEF1A2	Eukaryotic translation elongaion factor 1 alpha 2	Protein synthesis putative oncogene	5
FEZ1	Fasciculation and elongation Protein zeta 1 (zygin I)	Axonal outgrowth Putative tumor suppressor	5
SEPP1	Selenoprotein P plasma1	Free radical scavenger	4
GAP-43	Growth-associated protein3	Neurite growth/plasticity	4
NPTX2	Neuronal pentraxin 2	Uptake of synaptic material	4.5

HEK293 cells were either treated with 1 mM VPA for 5 days in duplicate or were left untreated as a control. Total RNA was subjected to a differential expression microarray analysis using HuGeneFL DNA microarrays containing oligonucleotides specific for approximately 25 000 human transcripts as described under 'Materials and methods'. The table lists representative genes, which showed consistent changes in both experiments and their known biological roles. The entire list of genes, which were induced more than 2-fold by VPA in two independent experiments are listed in a Supplementary Data sheet.

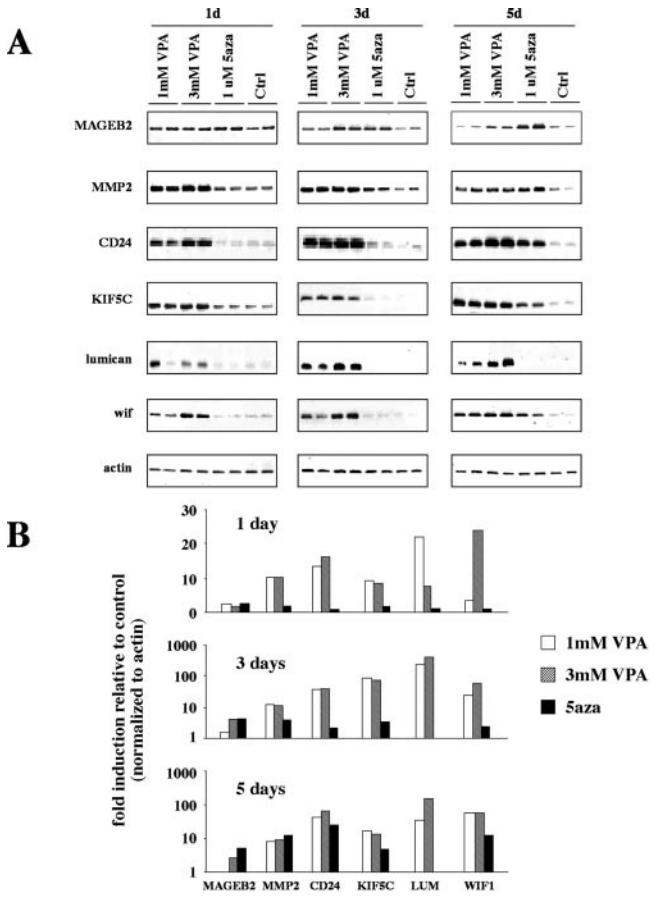

Fig. 2. A subset of genes induced by VPA is regulated by DNA methylation as indicated by their induction with DNA demethylating agent 5-aza-CdR. Total RNA was isolated from untreated control HEK 293 cells or cells treated with either 1 mM VPA, 3 mM VPA or 1 μM 5aza for 1, 3, and 5 days. (**A**) Semi-quantitative RT–PCR was performed for the indicated genes as described under 'Materials and methods'. A total of 10 μl of PCR products were run on 1.2% agarose gel. (**B**) PCR products were quantified by densitometry, normalized to β-actin, and presented as a fold induction relative to controls. The bars represent averages of duplicate determinations.

VPA treatment brought about demethylation of MAGEB2 gene promoter and selective demethylation of specific sites in MMP2 and WIF1 promoter 24 h after initiation of VPA treatment. MAGE genes were originally identified as tumor-specific antigens, which were recognized by autologous cytolytic T cells on melanoma cells but were normally expressed in the testes. MAGE antigens and specifically MAGEB2 were shown to be expressed in different tumor types but never in normal tissue except the testes (41–44). Interestingly, it was shown that MAGE antigens, which bear CG rich promoters could be induced by the DNA demethylating agent 5-aza-CdR. It was proposed that the expression of MAGE antigens was silenced by methylation in somatic tissues and was activated by the global hypomethylation, which was shown to be a property of most cancers (42,43). Expression of MAGE antigens is one of the most common consequences of pharmacological demethylation (45). Similarly, in our study 5-aza-CdR induced the expression of MAGEB2 (Figure 2). The appearance of MAGEB2 following VPA treatment is therefore a striking indication that VPA acts upon the DNA demethylation machinery.

We therefore performed a bisulfite mapping analysis of the *MAGEB2* promoter in untreated and 3 mM VPA treated HEK293 cells at 24 h and 5 days following initiation of treatment with VPA or HEK293 cells treated with the classic demethylating agent 5-aza-CdR. In parallel we confirmed that VPA induced active demethylation in our system by measuring the state of methylation of a nonreplicating methylated CMV–GFP plasmid transiently transfected into the same cells. The results in Figure 3 show extensive replication-independent demethylation of the GFP reporter plasmid 24 h after administration of VPA, thus demonstrating that an active demethylation activity was triggered in the cells by VPA at this time point. We then determined whether VPA induced demethylation of MAGEB2 in a similar time frame. Our analysis showed that VPA treatment resulted in extensive demethylation of the *MAGEB2* gene promoter as early as 24 h after initiation of treatment (Figure 4) in parallel to the active demethylation of the ectopically methylated DNA shown in Figure 3. There was a slight increase of demethylation of the promoter between 1 and 5 days. 5-aza-CdR the classic demethylation agent which is known to act by inhibiting DNA methyltransferase activity during DNA synthesis also induced demethylation of MAGEB2 promoter but to a lesser extent than VPA.

The *MMP2* gene is expressed in metastasizing cancer cells and contributes to extracellular matrix degradation, which facilitates tumor cell invasion and metastasis (46). *MMP2* expression is correlated with higher tumor grade and larger tumor size in breast cancer (39). The possibility that VPA induced genes that are involved in metastasis is particularly interesting. 5-aza-CdR was previously shown to induce the expression of *MMP2* in pancreatic cancer cell lines, which resulted in increased invasiveness (37). Both 5-aza-CdR and VPA induce *MMP2* in HEK 293 cells, suggesting that this gene is silenced by DNA methylation and that its induction by VPA involves DNA demethylation. We therefore performed bisulfite mapping to determine the state of methylation of CG sites residing upstream of the transcription initiation site of *MMP2* in response to 24 h and 5 days treatment of VPA as well as 24 h treatment with 5-aza-CdR. We analyzed the state of methylation of 17 CG sites upstream of the transcription initiation site as shown in Figure 5. Whereas the proximal 9 CG sites are unmethylated in both control and treated cells, the upstream 8 CG sites are methylated in a heterogeneous manner in control DNA (between 20 and 73% for different CG sites). A reduction in methylation of 6 out of 8 sites was observed 24 h following VPA treatment. There was no additional demethylation of these sites between 24 h and 5 days, which is consistent with the time frame required for active demethylation of GFP (Figure 3). The classic demethylating agent 5-aza-CdR demethylated the same sites to a similar extent. Whereas it is difficult to demonstrate that these specific sites are critical for the expression of *MMP2*, this experiment is consistent with the hypothesis that VPA can alter DNA methylation pattern of a gene, which is regulated by DNA methylation, as indicated by its induction with 5-aza-CdR (Figure 2).

We then examined the state of methylation of the proximal promoter and first exon of the *WIF1* gene, which is induced in response to both VPA and 5-aza-CdR treatment and is therefore a candidate for activation by DNA demethylation. The data presented in Figure 6 shows that this region is generally unmethylated in control cells. However, two sites,

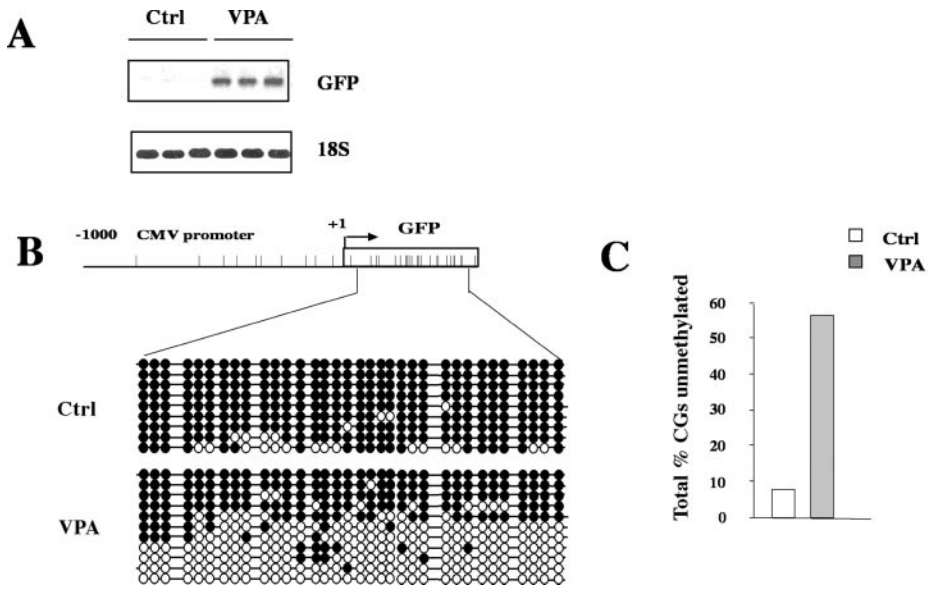

Fig. 3. Bisulfite mapping of CG sites in GFP exon, (**A**) HEK293 cells were transfected with *in vitro* methylated pCMV-GFP plasmid and 24 h post-transfection were either left untreated as a control or were treated with 3 mM VPA for 1 day. RNA was extracted and northern blot for GFP and 18S rRNA was performed. (**B**) Physical map of CMV promoter-GFP exon showing the density of CG sites (indicated as small vertical lines). The transcription start site is indicated with upper arrow. The region tested for its methylation status is shown. HEK 293 cells were treated as in A and DNA was isolated and treated with sodium bisulfite, which converts all unmethylated cytosines into thymidines while methylated cytosines remain intact. The specific methylation pattern is revealed by PCR amplification, subcloning, and sequencing of the region of interest. The methylation pattern of 34 CG sites in the GFP exon is shown (9-11 clones for each treatment). Open and closed circles indicate unmethylated and methylated sites, respectively. (**C**) Total percentage of unmethylated CGs across all sites is shown for untreated control cells, and cells treated with 3 mM VPA for 1 day.

which are methylated about 40% in control cells are demethylated in response to 1 day treatment of cells with VPA (sites 18 and 23) while only site 23 is demethylated in response to 5-aza-CdR. It is unclear whether this demethylation is important for activation of this gene. It is possible that other differentially methylated regions, which are yet to be characterized are involved in suppression of this gene in HEK293 cells and its activation in response to 5-aza-CdR and VPA. It is also possible that this gene is regulated exclusively by the chromatin modification state. Nevertheless this experiment further demonstrates that VPA induces demethylation of CG sites in endogenous genes at the same time frame when active demethylation activity is triggered by VPA (Figure 3) and further illustrates that similar to the classic inhibitor of DNA methylation 5-aza-CdR activation of genes by VPA involves DNA demethylation.

VPA caused hyperacetylation of induced genes

What is the mechanism by which VPA induced DNA demethylation? We have previously demonstrated that *in vitro* methylated plasmid transfected into HEK293 cells undergoes demethylation following treatment with classic HDAC inhibitor TSA. This demethylation was shown to be dependent on histone acetylation, since inhibition of histone acetylation by INHATs prevented active demethylation (47). In accordance with its activity as an HDAC inhibitor, VPA was also shown to induce both acetylation and demethylation in the same assay system (27). Here, we determined whether similar events that occur at the ectopically transfected plasmids also occur at the chromatin of the endogenous genes following VPA treatment. Therefore, we tested whether histones associated with 5′ regulatory regions of *MAGEB2* and *MMP2* genes were acetylated following VPA treatment. By using ChIP assay, we showed that VPA treatment increased the state of acetylation of H3 and H4 histones associated with the regulatory regions of both *MAGEB2* and *MMP2* genes, and that this state of hyperacetylation was maintained for 5 days (Figure 7). These data are consistent with the hypothesis that VPA triggered demethylation of these genes through histone acetylation.

Discussion

HDAC inhibitors were generally believed to act exclusively on the chromatin modification machinery by altering the state of histone acetylation. They were therefore postulated to change the state of expression of genes, which were silenced by histone deacetylation, but not genes, which were stably silenced by DNA methylation (15). VPA has been used for decades in therapy as an antiepileptic agent and a mood stabilizer, but was later discovered to be an HDAC inhibitor (19,20). We had previously shown that VPA could bring about active demethylation of ectopically methylated DNA (27), but it was not known whether it could similarly act on endogenous genes. We therefore used the same cellular system, HEK293 cells, to address the question of whether VPA treatment results in stable epigenetic reprogramming by altering DNA methylation patterns. First, we show using a microarray gene expression analysis that VPA causes widespread changes in gene expression in HEK293 cells and that these changes include many different categories of genes, including genes involved in metastasis, such as MMP2, and genes specifically expressed in tumors, such as MAGEB2 (Table I). Second, we showed that DNA demethylating agent, 5-aza-CdR, could also induce a subset of the genes induced by VPA, suggesting a mechanism involving DNA demethylation (Figure 2). This shows

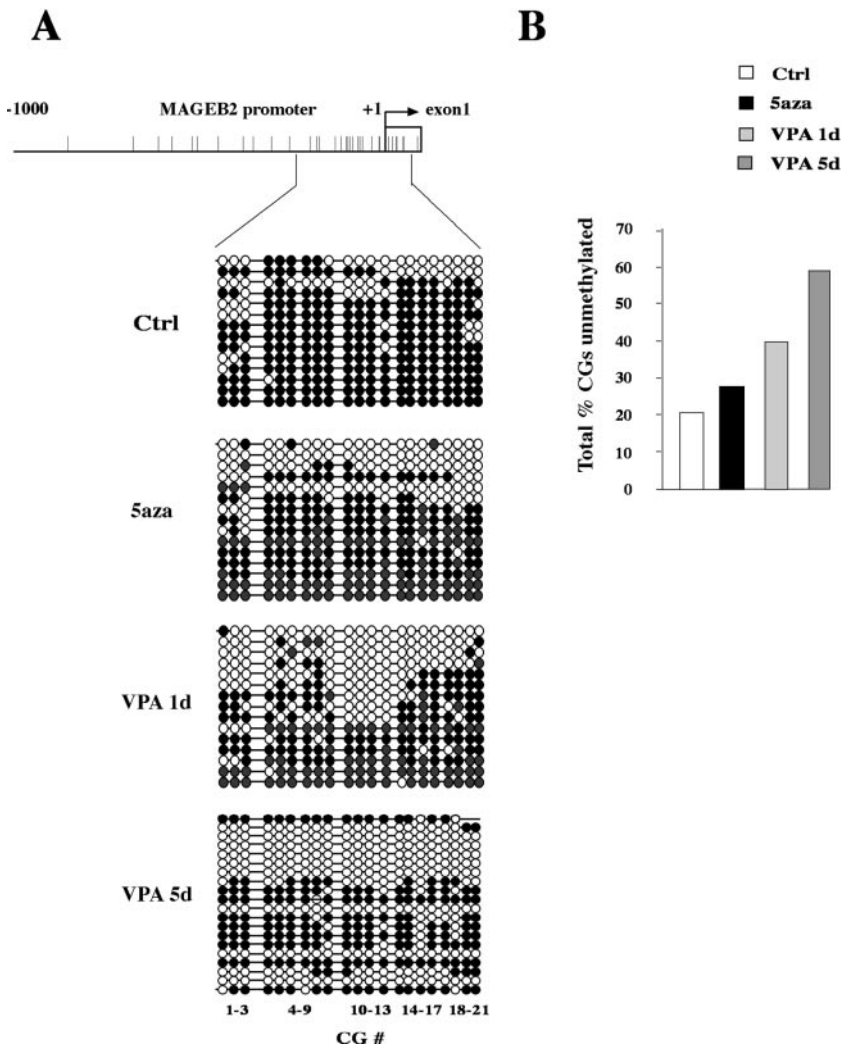

Fig. 4. Bisulfite mapping of CG sites in the MAGEB2 promoter. (**A**) Physical map of the MAGEB2 promoter (1000 bp) and the first exon, showing the density of CG sites (indicated as small vertical lines). The transcription start site is indicated with upper arrow. The region tested for its methylation status is shown. HEK293 cells were treated with 1 μM 5azaCdR for 24 h (1 day) or 3 mM VPA for 1 or 5 days or were left untreated as a control. DNA was isolated and treated with sodium bisulfite, which converts all unmethylated cytosines into thymidines while methylated cytosines remain intact. The specific methylation pattern is revealed by PCR amplification, subcloning and sequencing of the region of interest. The methylation pattern of 21 CG sites in the MAGEB2 promoter is shown (15–20 clones for each treatment). Open and closed circles indicate unmethylated and methylated sites, respectively. (**B**) Total percentage of unmethylated CGs across all sites is shown for control untreated cells, 1 day 5azaCdR, and 1 day and 5 days VPA treated cells.

cross-reactivity between HDAC inhibitors and DNA demethylation inhibitors, which must be considered for assessing both the mechanism of action of these drugs as well as the potential clinical outcomes of their use in therapy. Third, we demonstrated that both VPA and 5-aza-CdR treatment results in demethylation of 5′ regulatory regions of *MAGEB2* (Figure 4) and *MMP2* (Figure 5) genes and few sites in *WIF1* gene (Figure 6). These three genes show a different pattern of demethylation in response to VPA treatment, which probably reflects differences in the mode of interaction of these genes with DNA demethylation machinery. Fourth, we showed that demethylation of endogenous genes occurs rapidly within 24 h after initiation of treatment at the same time frame that an active demethylation activity is triggered toward a nonreplicating ectopically methylated CMV–GFP plasmid by VPA in the same cells (Figure 3). Although, it is hard to determine whether an endogenous gene undergoes active or passive demethylation in a replicating cell, the parallel use of an ectopic nonreplicating DNA as a probe of active demethylation (26) shows that the demethylation of endogenous genes occurs concurrently with the active demethylation of our probe. This is consistent with an active demethylation of these genes although our data does not preclude the possibility that VPA causes both passive and active demethylation.

A general response to VPA was observed with *MAGEB2*. All CG sites showed reduced demethylation following VPA and 5-aza-CdR treatment. In the case of *MMP2* and *WIF1,* VPA caused demethylation of certain sites but not all sites. Further experiments are required to understand why demethylation is processive in some 5′ regions but more site-selective in others. In addition, certain sites show increased methylation following VPA treatment in both *MAGEB2* and *MMP2* genes (Figures 4 and 5). It is possible that the changes in chromatin structure caused by VPA might result in

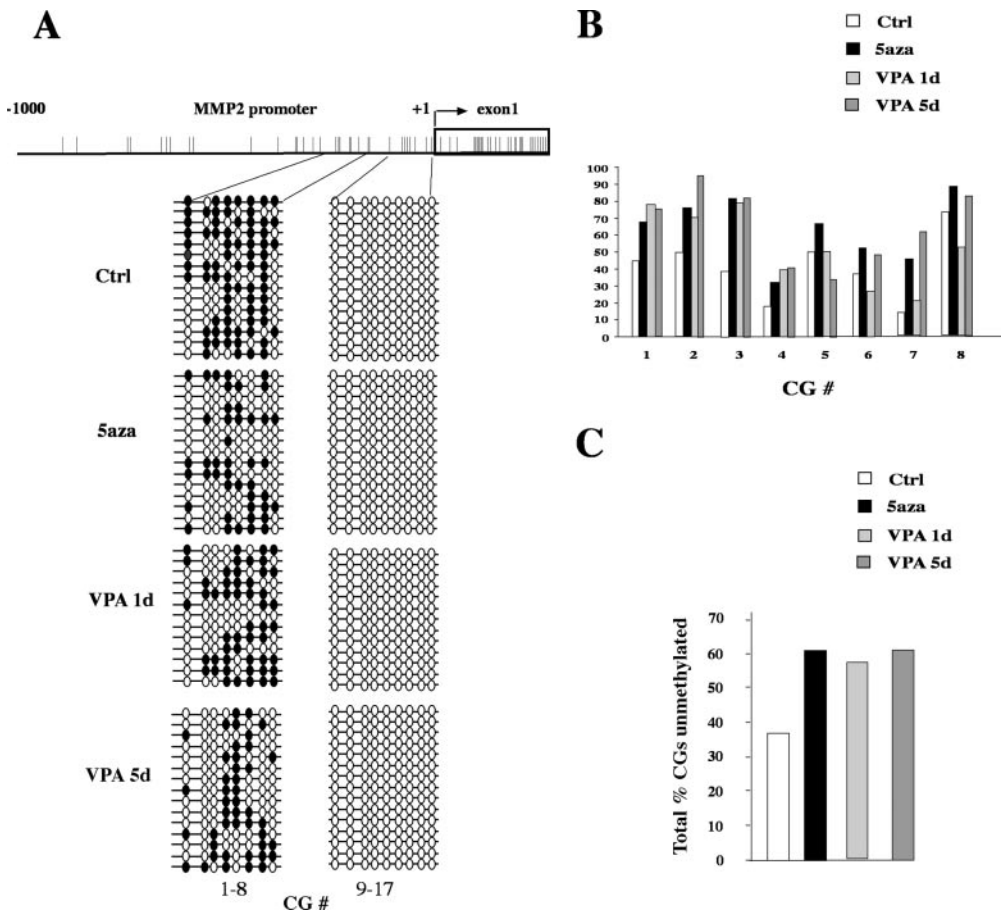

Fig. 5. Bisulfite mapping of CG sites in the MMP2 promoter. (**A**) Physical map of the 1 kb of the promoter and the first exon of MMP2 gene, showing the density of CG sites (small vertical lines). The transcription start site is indicated with the upper arrow. The two regions tested for their methylation status are shown. HEK 293 cells were treated for 1 day with 1 μM 5azaCdR, 1 day or 5 days with 3 mM VPA or were left untreated as a control. DNA was isolated and treated with sodium bisulfite, which converts all unmethylated cytosines into thymidines while methylated cytosines remain intact. The specific methylation pattern is revealed by PCR amplification, subcloning, and sequencing of the region of interest. The methylation pattern of 17 CG sites in the MMP2 promoter is shown (15–13 clones for each treatment). Open and closed circles indicate unmethylated and methylated sites, respectively. (**B**) Percentage of unmethylated CGs is shown for each CG site in control, 5 azaCdR 1 day, VPA 1 and 5 days for the first region (CGs 1–8). The second region (CGs 9–17) was completely unmethylated in all conditions. (**C**) Total percentage of unmethylated CGs across sites 1–8 is shown for control, 5 azaCdR 1 day and VPA 1 or 5 days treated cells.

increased accessibility to demethylase around some sites, together with increased accessibility to DNMTs around other sites. Lastly, we showed that VPA treatment altered the state of chromatin modification by increasing the acetylation of histones associated with these genes. These data are consistent with the hypothesis that the induction of these genes is mediated by changes in histone acetylation.

What is the functional significance of the demethylation observed in *MAGEB2, MMP2* and WIF1 genes. Although we observed either site-specific or regional changes in DNA methylation in response to VPA (Figures 4–6), this on its own does not necessarily mean that DNA demethylation plays a causal role in the activation of these genes. Since both histone acetylation and DNA demethylation are known to activate gene expression, it is difficult to determine which of these changes is directly responsible for activation of *MAGEB2* and *MMP2* genes. However, the fact that a DNA demethylation inhibitor, 5-aza-CdR, also induces these genes supports the hypothesis that they are regulated by DNA methylation. In summary, our data shows that VPA can induce genes by causing changes in both chromatin, through altering its state of histone acetylation, and DNA itself, through DNA methylation. The fact that VPA similar to 5-aza-CdR can change the DNA methylation pattern of the genome raises the possibility that it could compromise the integrity of epigenomic programs in the cell.

What are the implications on therapy and the clinical potential of VPA and other HDAC inhibitors? Traditionally, drugs such as VPA were believed to act on their target proteins by changing their activity only transiently, while leaving the genome intact. New drugs are routinely subjected to mutagenicity assays, which eliminates those ones that cause stable genomic alterations. However, it is becoming clear that in many instances DNA methylation changes could have similar effects as genetic changes. Spurious DNA methylation can silence a gene similarly to a silencing genetic mutation, while demethylation can function as an activating mutation, or could cause gene rearrangements. Therefore, it is clear that in addition to genotoxic effects one must also consider DNA methylation when assessing the safety and therapeutic utility of future drugs. Our data illustrates that drugs, which do not directly interact with the DNA methylation machinery, could alter the DNA methylation steady-state. Our data emphasizes that there is a cross

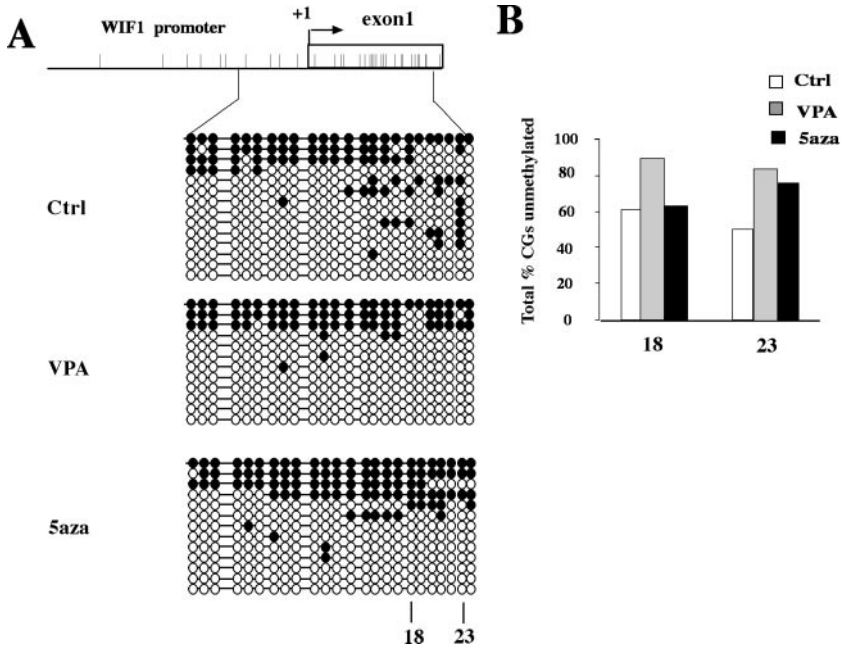

Fig. 6. Bisulfite mapping of CG sites in the WIF1 promoter and first exon. (**A**) Physical map of the WIF promoter and first exon, showing the density of CG sites (indicated as small vertical lines). The transcription start site is indicated with the upper arrow. The region tested for its methylation status is shown. The methylation pattern of 24 CG sites in the WIF1 promoter and first exon is shown (13–15 clones for each treatment). HEK293 cells were treated with 1 μM 5-aza-CdR or 3 mM VPA for 1 day or were left untreated as a control. (**B**) Quantification of unmethylated CGs in sites 18 and 23 in control, 5 azaCdR and VPA treated cells for 24 h (% of total).

talk between DNA methylation and chromatin modification, and that drugs that act seemingly transiently and reversibly on chromatin, could also alter DNA methylation, which is a far more stable modification of the DNA itself. It is known that the state of chromatin modification might be altered by other signaling pathways raising the specter that drugs which are tightly specific to their targets might indirectly cause wide spread and stable changes in the epigenome. Further experiments are required to address this possibility. Thus, the epigenome might serve as a possible route whereby a highly specific and targeted agent could indirectly bring about extensive off-target effects. In this paper we showed that VPA, which is broadly used in psychiatric therapy, could cause epigenetic changes and induce the expression of a wide range of genes. Since some of these changes might involve DNA demethylation, this raises a concern that VPA might have long-term consequences.

An interesting observation is that VPA induces MMP2, a gene known to be required for tumor invasion and metastasis. This is especially important since HDAC inhibitors are currently used as anticancer agents in clinical trials. DNA demethylation was previously shown to induce pro-metastatic genes, and 5-aza-CdR was shown to induce the protease *uPA*, which plays a critical role in breast cancer metastasis (48–50). We have proposed that demethylation might be a critical mechanism in metastasis and that DNA demethylating agents should be avoided when there is a concern for the development of metastasis (29,51). Therefore, the use of VPA and other HDAC inhibitors might be associated with similar problems.

Whereas DNA demethylation effects of VPA should raise concerns, they could nevertheless be of value in certain circumstances. First, since VPA causes active demethylation and is not dependent on replication, it could potentially be used in non-dividing cells. This is different from the classical DNA demethylating agents, such as 5-aza-CdR, which is a nucleoside analog and needs to be incorporated into DNA during replication in order to inhibit DNA methylation. In accordance with this hypothesis we have shown that a methylated glucorticoid receptor could be demethylated in the adult hippocampus by administration of the HDAC inhibitor TSA into the rat brain (28). A second application for VPA might be in cancer therapy. Methylation of tumor suppressor genes is suggested to play a causal role in cancer (52) and demethylation of such genes might be possible by VPA treatment either as monotherapy, or in synergy with other demethylating agents. However, it is vital to consider the possibility that VPA, as well as other HDAC inhibitors, might induce pro-metastatic genes, such as *MMP2* and convert a nonmetastatic tumor into a metastatic tumor.

Our microarray analysis raises an important issue regarding the scope of the effects of VPA on gene expression. Since VPA inhibits HDAC1 and potentially other HDACs, it should have general effects on the basic processes of gene regulation through histone acetylation. However, only a small subset of genes is induced by VPA (Figure 1). It is too early to speculate on the mechanisms that render genes resistant to HDAC inhibition. It is important to bear in mind that HDAC inhibitors are not acetylating the histones; rather they are allowing histone acetyltransferases to do so by inhibiting the opposing deacetylation reaction. Therefore, if regulatory regions of a certain gene were not engaged with histone acetyltransferases, HDAC inhibition would have no effect on its acetylation. Another possibility is that genes may be protected from the effects of HDAC inhibition by proteins, which inhibit histone acetylation. Such proteins were identified and termed InHATs (inhibitors of histone acetylation) and were shown to protect genes from the effects of

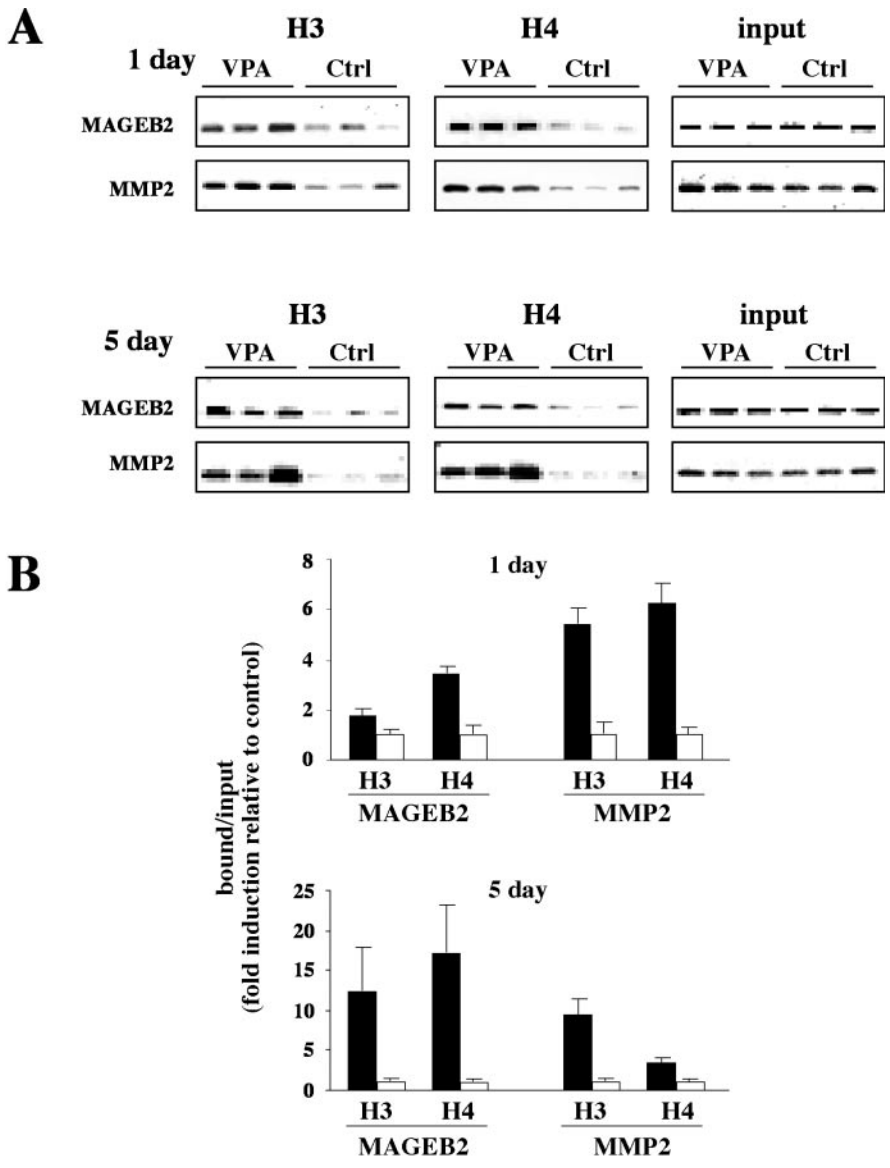

Fig. 7. Histone acetylation of promoters of MAGEB2 and MMP2 is induced by VPA. HEK293 cells were treated with 3 mM VPA for 1 or 5 days or were left untreated as a control. Chromatin was isolated and immunoprecipitated with antibodies specific for acetylated histones H4 and H3. Associated DNA was analyzed by PCR using primers that amplify 287 and 308 bp regions of MAGEB2 and MMP2 promoters, respectively. Triplicates are shown for each experiment. (**B**) The relative occupancy of the promoters with acetylated H3 and H4 histones is determined by densitometric quantification of PCR products and normalization to the input DNA. The fold induction in acetylated H3 and H4 following VPA treatment is shown relative to the control. The error bars are SEMs of the triplicates. There was no PCR amplification in no antibody control immunoprecipitations (data not shown).

TSA on histone acetylation (53) and DNA demethylation (47). It is possible that other such factors exist and protect genes from the effects of global HDAC inhibitors.

Although HDAC inhibitors lead to wide spread increase of H3 and H4 histone acetylation in chromatin only a select number of sites are demethylated. It is clear that our understanding of the processes leading to demethylation following global inhibition of HDAC activity is very limited. Nevertheless, recent data suggests that DNA methyltransferases and possibly demethylases need to be targeted to genes. Only sites associated with demethylases or DNA methyltransferases would be affected. Further experiments are required to identify the factors, which confer selectivity to the response to HDAC inhibitors.

Although a full understanding of the gene-selectivity of VPA epigenomic effects is required to explain the effects of VPA, the data presented here provides an illustration of how a classic drug such as VPA might have long-term effects on gene expression, which might be similar to the consequences of genetic alterations. The crosstalk between drugs affecting chromatin modification and DNA demethylation raises the possibility that other signaling pathways, which directly or indirectly affect chromatin structure, might also in turn cause changes in DNA methylation patterns. As DNA methylation is a stable mark on the genome this might be a mechanism through which transient interaction of a drug and its target protein is translated into a long-term reprogramming of gene expression.

Supplementary data

Supplementary data are available at Carcinogenesis Online.

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