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Uncoupling of promoter methylation and expression of *Period1* in cervical cancer cells

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Abstract

We investigated possible epigenetic regulation of *Period1* (*PER1*), a key circadian regulator gene, in six cervical cancer cell lines which showed up to 15.4-fold differences in *PER1* mRNA levels. Genomic methylation analysis showed that a discerned CpG island in the *PER1* promoter remained hypomethylated in five of the cell lines. In contrast, C33A cells that showed maximal *PER1* expression was hypermethylated; however, demethylation treatment of C33A cells resulted in small but significant elevated *PER1* mRNA levels suggesting a secondary role for promoter hypermethylation in *PER1* transcriptional regulation. A discerned hypomethylated zone that harbours crucial transcriptional elements including the critical proximal E-box progressively diminished in size in the cell lines until a methylation-resistant core was retained in C33A. Our data indicate that *PER1* transcription is mainly uncoupled from promoter methylation but probably involves availability and interactions of *trans*-acting factors with differentially methylated *cis* elements in the promoter hypomethylated zone.

Keywords: Cervical cancer; CpG islands; E-box; Methylation analysis; *Period1* gene; Promoter methylation; Transcription factors; Transcriptional regulation

In the genesis of cervical cancer, infections by the human papillomavirus (HPV) alone are insufficient; host factors are obligatory in the cellular transformation processes. Notably, the E6 and E7 oncoproteins of high-risk HPVs interact with the tumor suppressor proteins TP53 and RB1, respectively, in effecting a cascade of molecular events leading to transformation [1,2]. Cell cycle proteins and proteins pertaining to cellular differentiation are also tightly coupled to HPV gene expression and functionality [3,4]. Demonstration of loss of heterozygosity in cervical cancer lends further support that alterations in cellular genes may be the major causative factors in the HPV-free, or in HPV-infected, cancer cases [5]. More recently, the circadian clock genes are emerging in importance in the regulation of normal cellular process; dysregulated expression of circadian genes has been associated with human cancers [6–9].

In mammals, a wide range of physiological, hormonal, and metabolic processes and behavior patterns are governed by self-perpetuating circadian rhythms under the control of an intrinsic master clock [reviewed in 10–12]. The circadian genes collectively participate in an autoregulatory transcription–translation feedback loop that generates the circadian rhythm [10–12]. Central to the circadian clock mechanism are the three *Period* (*PER*) genes that encode unique PAS-domain proteins with distinct functions. Homozygous *PER* mutations have been shown to disrupt the circadian clock resulting in a shorter circadian period with reduced precision and consistency [13]. The *PER1* gene, the focus of the present study, is

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ubiquitously expressed in various tissues [14]. The *PER1* promoter is *trans*-activated by the heterodimeric complex constituting the circadian proteins CLOCK and BMAL1 *via* interactions with the E-boxes located in the *PER1* promoter [15–17]. In turn, accumulating PER1 and other PER proteins associate with the negative regulators, CRY1 or CRY2, to block CLOCK/BMAL1-activated *PER1* transcription completing the circadian auto regulatory feedback loop [16,18,19].

Our group has previously demonstrated reduced expression of the *PER* and other circadian genes in breast and endometrial cancers, and that the perturbed expression is associated with promoter hypermethylation [6–9,20]. In this work, we further investigated possible molecular mechanisms that modulate *PER1* expression in cervical cancer cells.

Materials and methods

Cervical cancer cell lines. All the cervical cancer cell lines used in this work, except CC7T, were originally obtained from the American Type Culture Collections. The CC7T cell line has previously been characterized [21,22].

Quantitative real-time RT-PCR (qRT-PCR). RNA preparation, qRT-PCR, and statistical analysis were performed as described [6–8]. For use as internal controls, primers specific for the glyceraldehydes-3-phosphate dehydrogenase gene were included. Triplicate samples were routinely performed; the data presented were derived from three to five independent experiments.

Mutational analysis. PCR amplification of a 1.1-kb sequence of the *PER1* promoter-exon 1 sequence between nucleotides −847 and +260 including the promoter and the exon 1 was carried out using *PER1*-specific primers and a high-fidelity DNA polymerase. PCR was performed in a reaction mixture containing 0.4 μM of each primer, 0.2 mM of each dNTP, 0.02 U/μl Phusion DNA polymerase, and 1× HF PCR buffer (Finnzymes, Keilaranta, Finland). The PCR products were gel purified and cloned into the pGEM®-TEasy vector (Promega, Madison, WI). Sequences of the PCR products were then determined.

CpG methylation analysis. A CpG island (CGI) was identified in the *PER1* promoter using the algorithm at www.urogene.org/methprimer. Methylation analysis was performed by bisulfite modification followed by PCR cloning and sequence analysis as described [6,7,20] with modifications. In brief, genomic DNA was sheared by passing through a 25 G needle ten times. Four micrograms of DNA was resuspended in 40 μl 10 mM Tris–Cl (pH 8.0) to which was added 5 μl 3 M NaOH, and the mixture was incubated at 37 °C for 10 min. Thirty microliter of freshly prepared 10 mM hydroquinone (Sigma–Aldrich, St. Louis, MO) and 520 μl freshly prepared 2.35 M sodium metasulfite (Merck, Darmstadt, Germany), pH 5.0, were added. The DNA solution was overlaid with 100 μl mineral oil and incubated at 50 °C for 12–16 h in the dark. The bisulfite-modified DNA was purified using a Wizard® minicolumn (Promega). The purified DNA was denatured by adding 10 μl 3 M NaOH and incubated at room temperature for 5 min. DNA was finally ethanol precipitated and the DNA pellet obtained was dissolved in 40 μl 10 mM Tris–Cl, pH 8.0. The modified DNA was used in PCR amplifications using the following primers (nucleotides T or A that are underscored and in heavy letters incorporated changes to allow primer annealing with bisulfite-modified genomic sequences: BSM-IF: 5′-TTTGTAGT**TT**TGG**T**TT**TT** TGGTTTT-3′; BSM-IR: 5′-CT**AAA**TTCCTTTCCCAACCTT**A**TTC-3′; BSM-IIF: 5′-GAA**T**AAGGTTGGGAAAGGAA**TTT**AG-3′; BSM-IIR: 5′-ACT**A**CCCCCTCATT**AA**CCTCTT**A**C-3′; BSM-IIIF: 5′-**T**AAGAGG **TT**AATGAGGGGG**T**AGTG-3′; BSM-IIIR: 5′-AA**A**T**AAA**TCA**AA** **A**TACA**A**ACTCCC-3′. Note that the Roman letters I, II, and III in the primer designations indicate segments I, II, and III of the *PER1* promoter as depicted in Fig. 2. The PCR products were cloned and up to eight random clones were picked for sequence analysis.

5-Aza-2′-deoxycytidine C (5AC) treatment of cultured cells. Cells were seeded on a 60 mm petri-dish and grown overnight to 60–80% confluency before the addition of 5AC (Sigma–Aldrich, St. Louis) dissolved in DMSO. Fresh doses of 5AC were added to the cells after 24 h, and the cells were harvested 48 h after the initial 5AC treatment. RNA was extracted for qRT-PCR analysis. Triplicate samples were routinely performed and the data presented were derived from four to five independent experiments.

Results

Varied PER1 expression levels in cervical cancer cell lines

To elucidate possible genetic and epigenetic changes that may have contributed to the regulated *PER1* transcription, six well-characterized cervical cancer cell lines were used. Each of the cell lines harbours either HPV16 (CC7T, SiHa, and CaSki), HPV18 (HeLa), HPV68 sequences (ME180), or is HPV-free (C33A). The relative *PER1* mRNA levels in the cell lines were first determined by real-time quantitative reverse transcription-PCR (qRT-PCR). In the experiments, the CC7T cells showed the lowest *PER1* mRNA level. On the other hand, C33A cells expressed 15.4-fold and the highest *PER1* mRNA level relative to that of CC7T; the relative *PER1* expression levels of the remaining cell lines fell in between these levels (Table 1). Our results demonstrated varied *PER1* expression levels in cervical cancer cell lines.

Absence of major genetic changes in the PER1 promoter in cervical cancer cell lines

To determine if the varied *PER1* expression is a result of genetic alterations in the promoter and transcriptional regulatory sequences, the −847 and +260 sequence derived from each of the cell lines was cloned and sequenced (Table 2). No major genetic aberrations were detected; only three single-nucleotide polymorphic nucleotide changes and a CT dinucleotide insertion in a pyrimidine tract were detected in SiHa, C33A, and CC7T. We conclude that, with the possible exception of a C to T polymorphic change in a GC-box in C33A, genetic changes in the *PER1* promoter do not contribute to regulated expression of *PER1* in cervical cancer cells [6–8].

The PER1 promoter is generally hypomethylated in cervical cancer cells

Tight clusters of CpG dinucleotides, called CpG islands (CGIs), are found in the promoter sequences of approximately half of the genes in the human genome; CGI hypermethylation has been linked to reduced gene expression [23,24]. Examination of the *PER1* promoter sequence has revealed a prominent CGI situated at −163 to +134 (Fig. 1A). The CGI is composed of 28 CpG sites and encompasses the basal *PER1* promoter and a cluster of cru-

Table 1
PER1 expression and methylation levels in cervical cancer cell lines

Cell line	HPV	*PER1* expression[a]		Methylation analysis[b]	
		Relative expression level	*p* Value	No. of CpG sites analyzed	Methylation level (%)
Normal cells	None	—	—	222	5.9
CC7T	16	1	—	347	5.5
SiHa	16	1.25	0.41	336	11.0
ME180	68	2.19	0.0015	87	n.d.
CaSki	16	3.75	4.97E−05	360	20.8
HeLa	18	7.00	6.17E−06	216	20.8
C33A	None	15.40	6.44E−08	352	80.7

[a] The *PER1* mRNA level observed in CC7T was arbitrarily set as 1; for other cell lines, the *p* values were derived in comparison with the CC7T level.
[b] Methylation level was determined as the fraction of methylated CpG sites amongst the total number of CpG sites analyzed in segments II and III (CpG sites #9–#53) of the *PER1* promoter as displayed Fig. 1B. For normal cells, data from the normal cervix and oral epithelial cells were included. The methylation level of ME180 was not computed (n.d.) due to the small number of CpG sites analyzed.

cial *cis*-acting transcriptional elements (see below). To establish if *PER1* expression is related to promoter methylation, the methylation status of the discerned CGI and 53 CpG dinucleotides in the *PER1* promoter and transcriptional regulatory sequence in the cervical cancer cell lines was determined using the bisulfite-modified genomic sequencing (BGS) approach. To optimize BGS analysis, three sets of overlapping primers were designed to generate three linking segments I–III covering 1092 bp (Fig. 1A). Genomic DNAs prepared from a normal cervix and from oral exfoliated epithelial cells taken from a healthy individual were included in the analysis as a reference. The results showed that the CpG site #1–#8 in segment I were mostly methylated. The methylation levels of the cancer cell lines were, therefore, computed for segments II and III which contained the CGI and the bulk of the CpG dinucleotides (CpG sites #9–#53) (Figs. 1A and B and Table 1, right panel). Only a low level of 5.9% CpG methylation was detected in the normal cells in this region. On the other hand, the methylation levels of the cervical cancer cell lines varied by about 15-fold from 5.5% in CC7T to 80.7% in C33A. The mean *PER1* promoter methylation level, if excluding the extreme case of C33A, was 20.6%; inclusion of the C33A data raised the methylation level to 27.8%. BGS analysis, thus, indicates that the *PER1* promoter generally remains hypomethylated with only a few exceptions. More significantly, the *PER1* expression levels did not appear to correlate with the extents of promoter methylation; on the contrary, there was a directly proportionality between hypermethylation of the *PER1* promoter and increased *PER1* expression, as reflected in the C33A cells.

Identification of a hypomethylated zone (HoZ) in the PER1 promoter

BGS analysis showed that in normal cells, the first 8–11 CpG sites were methylated (Fig. 1B). In the hypomethylated CC7T and SiHa cells, the boundary of the hypermethylated region was also mapped at CpG #11 (Fig. 1B). A downstream shift of the hypermethylation boundary to CpG #19 and beyond was discerned in HeLa, ME180, and CaSki. In contrast, and recalling that CpG #19 of C33A had been abolished due to a mutation (Table 2), 47 of the remaining 52 CpG sites in C33A were methylated; only CpG site #29 remained absolutely unmethylated in the 8 C33A clones and in 40 clones from other cell lines sequenced (Fig. 1B). The 3′-end of the hypermethylation boundary in the cervical cancer cell lines, except for C33A, appeared to lie beyond the region analyzed; however, low levels of CpG methylation were beginning to be detected in the cancer cells. Taken together, methylation analysis has revealed the existence of a hypomethylated zone (*HoZ*) in the *PER1* promoter at least 600 bp in size bound by CpG site #12 at about −320 at the 5′-end and a downstream boundary that lies beyond +300 in intron 1 (Figs. 1B and C). However, a methylation-resistant core, designated as *HoC*, bracketed by CpG #24–#29, was found embedded within the *HoZ* sequence. In particular, site #29 that lied within *MRC* remained absolutely unmethylated. More important is the observation of a progressive contraction of *HoZ* in the cervical cancer cell lines that were derived from different cancer stages and with varied pathologies until

Table 2
Nucleotide changes in the *PER1* promoter and 5′-regulatory sequence in cervical cancer cell lines[a]

Nt position	Nt change	Cell line	Consequence[b]
−21	T to C	SiHa	Creation of a new CpG site between sites #34 and #35 (unmethylated)
−234	C to T	C33A	Loss of CpG site #19; mutated GC-box-2
−283 to −284	CT insertion	CC7T, SiHa	Insertion in a pyrimidine (CT) tract
−295	G to T	CC7T	Loss of CpG site #16

[a] The region of the *PER1* promoter analyzed was −847 to +260; no polymorphic changes were detected in the promoter of CaSki, HeLa, and ME180.
[b] The CpG sites are designated as displayed in Fig. 1B.

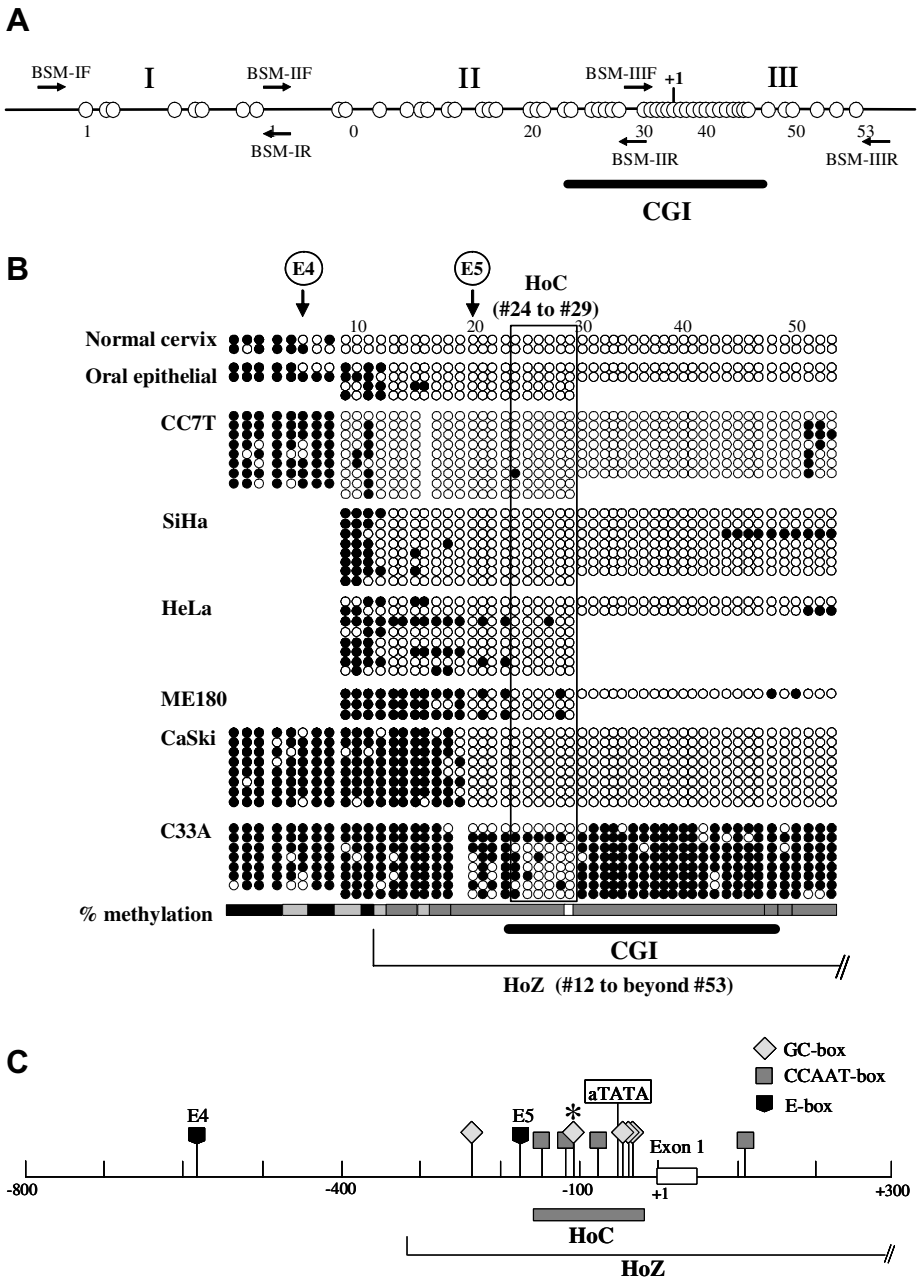

Fig. 1. Methylation analysis of the *PER1* promoter and 5′-regulatory sequence between −800 and +300 in cervical cancer cell lines. (A) Locations of 53 CpG dinucleotides (open circles) and the discerned CpG island (CGI). For PCR amplification and cloning purposes, the sequence was dissected into three segments (I–III); locations of the PCR primers are denoted by horizontal arrows with the primer designations. (B) Bisulfite-modified genomic sequence analysis. Filled circles denote methylated and open circles denote unmethylated CpG sites. CpG sites #16 and #19 in CC7T and C33A, respectively, are abolished through mutations (Table 2) and are not shown. Locations of the E-boxes-4 and -5 (E4 and E5), the proposed hypomethylated zone (*HoZ*) and hypomethylated core (*HoC*) (boxed) are indicated. The bar below with different shadings show the overall methylation level of each CpG site: Unfilled bar, 0% methylation; dotted, 0.125%; hatched, 25.1–50%; grey, 50.1–75%, filled, 75.1–100%. (C) Location of crucial transcriptional elements in the hypomethylation zone. The atypical TATA-box (aTATA), the E-box, GC-box and CCAAT-box motifs are shown at approximate locations. E4 and E5 denote E-boxes-4 and -5; the GC-box that has been mutated in C33A (Table 2) is marked by an asterisk.

the extreme hypermethylated state in C33A cells that showed maximal *PER1* expression but still retained the *HoC* sequence. Our results are reminiscent of previous reports that boundaries that safeguard unmethylated genomic sequences may progressively collapse with progressive cancer development and with ageing reviewed in [24,25].

5-Aza-2′-deoxycytidine C (5AC) treatment increased PER1 expression in C33A cells

To further clarify relationship between promoter methylation and *PER1* expression, three cell lines, CC7T, CaSki, and C33A, with distinctive *PER1* expression and methylation patterns were treated with the demethylation

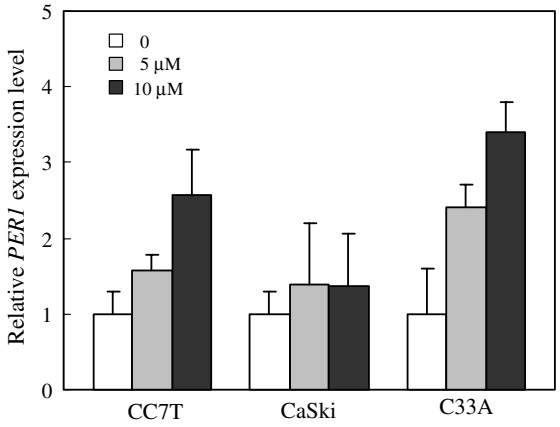

Fig. 2. Effects of 5-aza-2′-deoxycytidine C on *PER1* expression in selected cervical cancer cell lines. The cells were treated for 48 h with the solvent DMSO (unfilled bars), or with 5 or 10 μM 5AC dissolved in DMSO.

drug, 5AC, and the *PER1* mRNA levels in the treated cells were determined by qRT-PCR (Fig. 2). Relative to untreated cells, treatment with 5 or 10 μM 5AC resulted in no discernible *PER1* changes in the hypomethylated CaSki cells; the *PER1* mRNA level in the similarly hypomethylated CC7T cells was about 2.5-fold that of untreated cells. The *PER1* mRNA level in 5AC-treated hypermethylated C33A cells was about 3.5-fold of untreated cells suggesting that DNA demethylation could exert a small but significant effect on *PER1* transcription. The data may be taken to indicate that promoter methylation probably plays a secondary role in the regulation of *PER1* transcription in cervical cancer cells.

Discussion

In this work, we show that despite the presence of a CpG island in the *PER1* promoter, elevated promoter methylation is correlated with increased PER1 expression. Data from 5AZ demethylation experiments further suggest promoter methylation plays only a secondary role in *PER1* transcriptional regulation in this cancer. A hypomethylated zone (*HoZ*) was identified in the *PER1* promoter that harbours crucial *PER1* transcriptional regulatory elements including a putative atypical TATA-box (aTATA) in the configuration 5′-ATTAT-3′ which is the presumptive core promoter of the *PER1* gene (Fig. 1C). Clustering within about 200 bp around aTATA are five GC-box and four CCAAT-box (also known as Y-box) motifs. Many of the GC-boxes are putative Sp1-binding motifs essential for the operation of atypical TATA-box or TATA-less promoters [26]. It is further noted that CpG #26 that lies within the methylation-resistant hypomethylated core (*HoC*) coincides with a GC-box (GC-box-2 in Fig. 1C) raising the possibility that the methylation status of this GC-box and other GC-box motifs may have consequences in *PER1* transcription. CCAAT-boxes are targeted by members of the ubiquitous and highly selective CCAAT-box transcription factor family [27]. In *HoZ*, the four discernible CCAAT-boxes are flanked by CpG sites; methylation of these CpG sites may also have regulatory effects on *PER1* transcriptional regulation.

The most crucial *cis*-acting elements in *PER1* transcriptional regulation are the E-box motifs which are targeted by the CLOCK–BMAL1 complex [15–17]. Amongst the five E-boxes that have been identified, the 3'-most proximal E-box (E-box-5 in Figs. 1B and C) appears to be most important due to its close proximity to the core promoter: mutations in this motif resulted in the most severe suppression of *PER1* promoter activities [17,18]. It is noted that E-box-5 overlaps with CpG #20 and lies well within the hypomethylated zone (Fig. 1B). Analysis of the 41 BGS clones clearly indicated that CpG #20 was absolutely unmethylated in the five hypomethylated cell lines. It is likely that CpG #20 remains methylation-free so that E-box-5 may properly function as a positive or negative *cis* element. In C33A, however, three of the eight clones (37.5%) were methylated (Fig. 1B) and may have a direct consequence on *PER1* transcription.

It may be important to systematically evaluate the effects of E-box methylation on differential interactions of the E-boxes with positive- or negative-acting transcription regulatory complexes in normal and cancer cells. Furthermore, we cannot rule out contributions from exon 1 and intron 1 that are also encompassed in *HoZ* (Fig. 1C) to *PER1* transcriptional regulation [17,26,28]. A negative regulatory *cis*-element has, indeed, been identified in the *PER1* intron 1 [17]. Reduced *PER1* expression despite promoter hypomethylation may also have resulted from impaired DNA methyltransferases, or transcriptional suppression through a long-range epigenetic silencing mechanism that affects both methylated and unmethylated physically distant gene clusters [29].

In conclusion, *PER1* transcription in cervical cancer cells is mainly uncoupled from promoter methylation and may primarily be related to the availability and interactions of crucial positive or negative *trans*-activating transcription factors targeting at their respective binding sites in the hypomethylated zone located in the *PER*1 promoter.

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