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Differential DNA methylation patterns in the CD86 gene controls its constitutive expression in keratinocytes

M.A. Romero-Tlalolini, P. Chávez Olmos, Efraín Garrido *

Department of Genetics and Molecular Biology, CINVESTAV-IPN, Mexico City, Mexico

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ABSTRACT

The interaction of B7 family members with appropriate receptors is essential for an effective T cell response. CD80 and CD86 are the principal co-stimulatory molecules of this family and they are mainly expressed on professional antigen presenting cells (APCs), but also on several non-lymphoid cells. CD86 is constitutively expressed in keratinocytes from the spinous layer of normal cervical epithelium. However, the mechanisms that control the expression of this gene in epithelial cells remain unknown. We analyzed the DNA methylation status of the CD86 promoter and a CpG island located in the upstream intergenic region in keratinocyte-derived cell lines. In those cell lines where CD86 is expressed, a high degree of methylation in the CpG island was observed. However, a CpG dinucleotide within the cAMP response element (CRE) in the promoter region was consistently unmethylated and associated to the transcription factor CREB, as demonstrated by ChIP assays. The opposite methylation pattern was observed in cell lines where CD86 is not expressed, affecting also the binding of CREB. The analysis of the DNA methylation pattern of this gene in cells from the spinous and basal layers of normal cervical epithelium showed a similar profile to that observed in cell lines with and without expression of CD86 respectively. Our results indicate that the methylation pattern in the CD86 promoter and CpG island is closely related to the expression of this co-stimulatory molecule in keratinocytes.

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1. Introduction

The interaction of B7 family members with appropriate receptors is essential for the initiation, modulation and regulation of an effective T cell response. CD80 and CD86 are the principal co-stimulatory molecules of this family. Despite their homology, CD80 and CD86 have non-redundant roles in immune modulation and their interactions results in distinct T cell functional outcomes [1]. Most APCs constitutively express low levels of CD86, but following activation they are rapidly up-regulated whereas *de novo* expression of CD80 is just observed [2]. CD86 up-regulation after activation is mainly induced by cytokines [3–6] and this "maturation" process has been largely studied. However, despite the impact of this co-stimulatory molecule on immune response, the mechanisms for loss or down-regulation of its constitutive expression are poorly understood.

Besides their presence in APCs, CD80 and CD86 expression has also been detected in non-lymphoid cells [7–10], but its role in these cells remains unknown. In normal cervical epithelium, keratinocytes from the spinous layer express CD86, but not CD80 [11]. CD86 expression in such specific epithelial layer suggests a relationship with the keratinocyte differentiation process. Considering the critical role that epigenetic modifications play in cellular differentiation [12], DNA methylation might be a mechanism that regulates the constitutive expression of CD86. Furthermore, a marked reduction of this co-stimulatory molecule has been observed in HPV-16-positive low squamous intraepithelial lesions (LSIL) [11], suggesting that viral oncoproteins could be involved in CD86 down-regulation [13,14].

In the present study, and trying to contribute to the knowledge of the mechanisms that regulate CD86 expression in cervical epithelium, we have assessed the methylation pattern of the CD86 promoter and upstream CpG island in keratinocytes from normal cervical tissue and keratinocyte-derived cell lines.

2. Materials and methods

2.1. Cells and culture conditions

HeLa, SiHa, C-33A, and Raji cell lines were obtained from ATCC and HaCaT cell line was a donation from Dr. Norbert Fusenig. HeLa and SiHa cells were cultured in Dulbecco's modified Eagle's

* Corresponding author. Address: Laboratory of Research in Cancer Molecular and Cell Biology, Departamento de Genética y Biología Molecular, CINVESTAV-IPN, Av. Instituto Politécnico Nacional 2508, Col. San Pedro Zacatenco, C.P. 07360 México D.F., Mexico. Fax: +52 55 5747 3891.

E-mail addresses: aromero@cinvestav.mx (M.A. Romero-Tlalolini), pchavez@cinvestav.mx (P. Chávez Olmos), egarrido@cinvestav.mx (E. Garrido).

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medium (DMEM), C-33A cell line in Dulbecco's modified Eagle's medium: Nutrient Mixture F12 (DMEM-F12) and Raji in RPMI-1640 medium. Media were supplemented with 10% fetal bovine serum, 100 units/L penicillin, 100 μg/ml streptomycin and 0.3 mg/ml glutamine. All cell lines were maintained in a humidified atmosphere at 37 °C and 5% CO_2. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation with Hystopaque-1077 (Sigma Aldrich).

2.2. RNA isolation, reverse transcription and PCR (RT-PCR)

Total RNA from cell lines and PBMC was obtained by using TRIzol reagent (Invitrogen) according to the manufacturer's conditions. DNA contamination was removed with Turbo DNA-free kit (Ambion Inc.). First strand complementary DNA (cDNA) was synthesized using 2 μg of total RNA, oligo dT_{15} primer and M MLV-RT (Promega). PCR reactions were performed using Taq DNA polymerase (Promega) and primers designed to amplify a 367 bp fragment of CD86: CD86Ex7S 5′-TGG AGA GGG AAG AGA GTG AAC A-3′ and CD86Ex8AS 5′-GCC CAT AAG TGT GCT CTG AA-3′. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression was evaluated as an internal control using the previously reported primers and conditions [15].

2.3. DNA methylation analysis

Genomic DNA was obtained from cell lines or keratinocytes separated by laser capture microdissection (LCM) from cervical epithelium, by alkaline lysis and phenol–chloroform–isoamyl alcohol extraction. Two micrograms of DNA were subjected to bisulfite conversion using the EZ DNA Methylation-Direct™ kit (Zymo Research). Bisulfite-converted DNA was amplified by PCR using the primer set designed for the CD86 CpG island: 5′-TTT AGG AAA GTT AGA ATT TTG GTT T-3′ and 5′-ATA CTA ACA CAT TCC TCC CAA CTA-3′; or the primer set for the CD86 promoter region: 5′-GAA TGT TTT TTT ATT TTA TTT TGT TG-3′ and 5′-CTC TTT TCT CTA CAA ATT TTA ACC A-3′. After 40 cycles of amplification, PCR products were cloned into bacteria using the pGEMT-Easy kit (Promega). Clones were sequenced using commercial services from INMEGEN (Mexico City, Mexico). The sequence of each clone was compared with the *in silico* bisulfite converted DNA sequence.

2.4. Chromatin immunoprecipitation (ChIP) assay

Approximately 1×10^6 cells were used for each immunoprecipitation assay. Nuclear proteins were crosslinked to DNA by incubating the cells with 1% paraformaldehyde for 10 min at room temperature. Cells were rinsed twice with ice-cold PBS, resuspended in lysis buffer and then sonicated to produce 200–500 bp DNA fragments. ChIP was performed using 2 μg of anti-CREB or anti-CREM antibodies (CREB-1 [X-12] and CREM [C-12], Santa Cruz Biotechnology), as well as normal mouse and normal goat IgGs as negative controls. The primers used, sense 5′-TGA GGG AAT TTC AAG AGG GGA GAT-3′ and antisense 5′-AGA TTT TGA CCA CAC TTG AGG AAG A-3′, spanned both CRE sequences (−190 and −141) in the promoter region.

2.5. 5-Aza-2′-deoxycytidine treatment (5-aza)

HaCaT, C-33A, SiHa and HeLa cells were seeded on 6-well plates for 24 h before incubation with 5 μM or 100 μM 5-aza. Medium containing 5-aza was changed every 24 h. Cell cultures treated with vehicle (DMSO) or without treatment were used as controls. Following 72 h after treatment, cells were either collected, washed twice and harvested using TRIzol reagent to start RNA extraction, or used for methylation analysis as described previously.

2.6. Immunohistochemistry and laser capture microdissection (LCM)

Five micrometer-thick sections from a formalin-fixed paraffin-embedded biopsy of normal cervical epithelium were collected on silane pre-coated slides, or on slides covered with a polyethylene napthalate (PEN) membrane (Applied Biosystem). Sections were dewaxed and re-hydrated prior to performing antigen retrieval in citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 20 min. Non-specific binding of the primary antibody was blocked with 3% normal pig serum for 1 h. Primary antibody anti-CD86 (B7-2, [C19] Santa Cruz, CA) was added for 1 h at room temperature. Bound primary antibody was detected using LSAB + System-HRP (DAKO) according to the manufacturer's instructions. Sections were counterstained with hematoxylin and either mounted or subjected to LCM. Analysis of sections was performed using a NIKON eclipse 80i microscope, and images were digitally captured with the accompanying camera and software NIS-Elements 3.2. LCM was performed using the Arcturus Veritas 704 microdissection system (Arcturus Bioscience) with CapSure Macro LCM Caps (Applied Biosystems). CD86-negative or CD86-positive cell layers were separated by laser UV with 80 mW pulse power, 8000 μs pulse duration and 2 μm laser spot. Tissues were digested directly on LCM caps and bisulfite converted as mentioned above.

3. Results

3.1. Lack of CD86 expression in cervical cancer derived cell lines

In order to know the ability of keratinocyte-derived cell lines to express CD86, we determined its mRNA levels in HaCaT, C-33A, SiHa, and HeLa cell lines by RT-PCR. CD86 mRNA was detected in the positive controls PBMC and Raji cells, but only at low levels in the immortalized HaCaT cell line (Fig. 1A).

3.2. Loss of methylation in a CpG island upstream the CD86 promoter region in cervical cancer derived cell lines

The human CD86 genomic region (GenBank ID: 942) including the coding sequence, the promoter and the upstream intergenic region was analyzed *in silico* using three different software programs: Methyl Primer Express Software v1.0 (Applied Biosystems), Softberry CpG Finder (Softberry Inc., Mount Kisco, NY, USA) and the CpG island searcher http://cpgislands.usc.edu. A consensus CpG island was located upstream the promoter region, between positions −2795 and −2598 relative to the transcription start site +1 (TSS) (Fig. 2A).

Following genomic DNA bisulfite modification, PCR amplification and cloning, the sequence of five clones from each cell line and PBMCs was obtained. In HaCaT cells, where a low but evident expression of CD86 was observed, almost all of the CpG

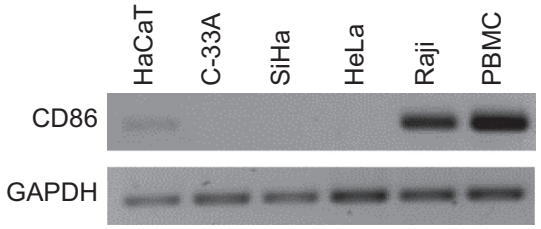

Fig. 1. CD86 mRNA expression in epithelial cell lines. CD86 gene expression detected by RT-PCR in HaCaT, C-33A, SiHa and HeLa cell lines. Raji cells and PBMCs were used as controls for CD86 expression and GAPDH expression was included as a loading control.

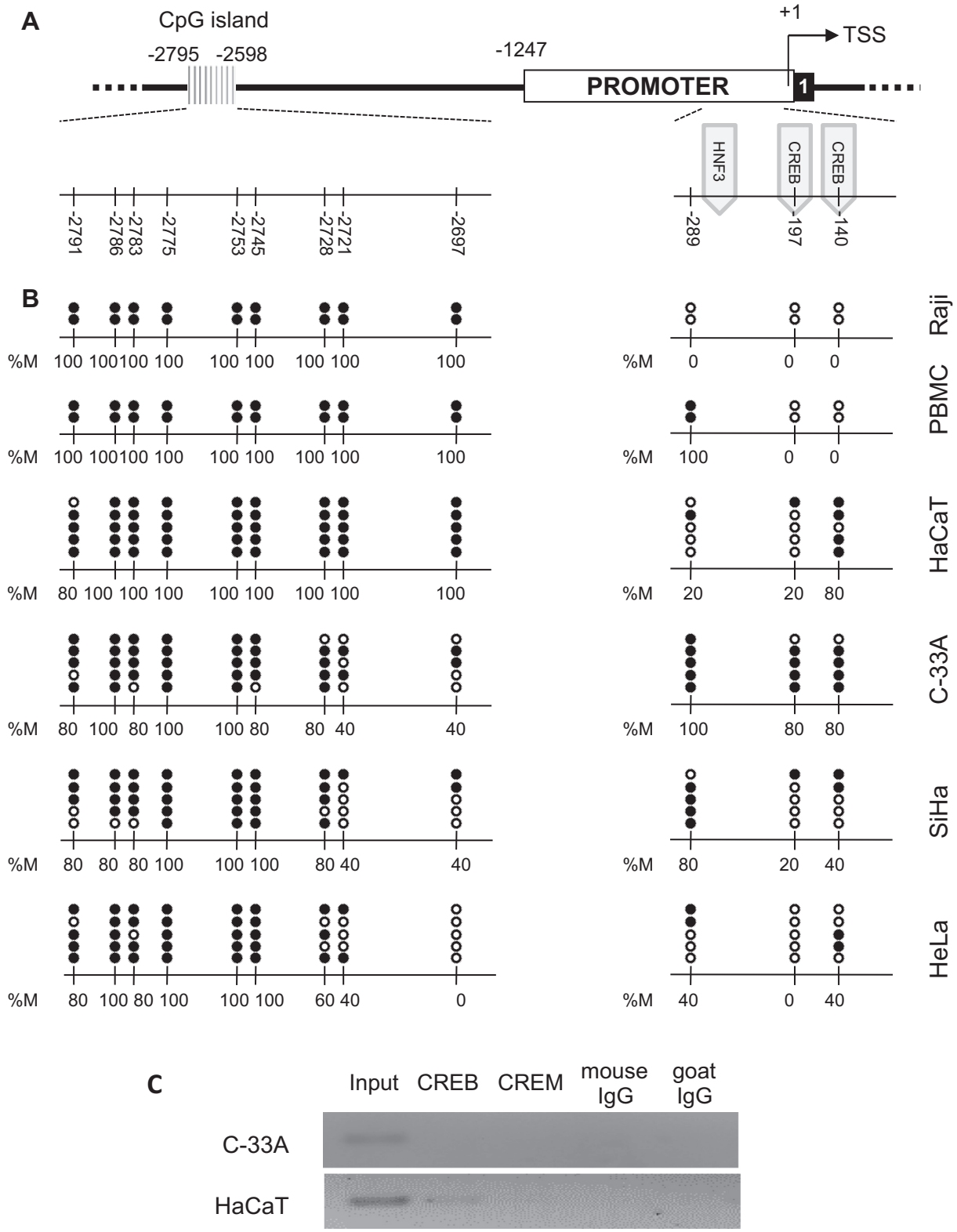

Fig. 2. Methylation status of the human CD86 genomic region in epithelial cell lines. (A) Scheme of the human CD86 genomic region, indicating the CpG island located upstream the promoter. Numbers −2795 and −2598 indicate the boundaries of the CpG island, and −1247 and +1 indicate the promoter.+1, transcriptional start site (TSS); exon 1, black box. HNF and CRE sites are indicated. Position of CpG dinucleotides are indicated as vertical lines across the horizontal. (B) Schematic representation of the methylation status in the CpG island and CpG dinucleotides in the promoter of CD86 gene. Empty circles, cytosines; black circles, methylcytosines. Five individual clones of each cell line were analyzed and numbers below the lines indicate methylation percentage at individual sites (%M). (C) Chromatin immunoprecipitation from C-33A and HaCaT cell lines with CREB and CREM antibodies.

dinucleotides included in this island were found methylated. This pattern was highly similar to that obtained in PBMCs and Raji cells used as positive controls, where CD86 was highly expressed. In contrast, C-33A, SiHa and HeLa cell lines, where CD86 expression was not detected, showed reduced levels of methylation mainly in the last three CpG dinucleotides in the island (Fig. 2B).

3.3. Specific methylation status is required for CD86 expression

Although the CD86 gene does not contain a CpG island in the promoter region, several CpG dinucleotides are located within the consensus sequences for the binding of important transcription factors. For this reason, we evaluated the methylation status of three CpG sites located close to the transcriptional start site (TSS) within the CD86 promoter. Nucleotides in positions −197 and −140 (numbers correspond to the C in the CpG dinucleotide) are part of two different consensus sequences for the cAMP Response Element (CRE), and at least one of them (−197) has been shown to be functional [21]. In addition, the CpG dinucleotide in position −289 is located near to the putative binding site for the transcriptional factor HNF3 (Fig. 2A).

As expected, the −197 and −140 CpG dinucleotides were found unmethylated in PBMC and Raji cells. However, the −289 site was found unmethylated in Raji cells, but methylated in PBMCs. In the HaCaT cell line, where the gene is moderately expressed, the −289 and −197 sites were almost completely unmethylated and methylation of both sites was found in only one clone (20%). Interestingly, the −140 CpG site was found methylated in 80% of the sequenced clones of this cell line. In contrast, the C-33A cell line was almost completely methylated in all the CpG dinucleotides analyzed, finding only one unmethylated clone (20%) in the last two positions. Finally, SiHa and HeLa cells showed a methylation profile slightly similar to that observed in the HaCaT cell line where −197 site was methylated in only one clone (Fig. 2B).

To determine whether the methylation of the CRE sites in CD86 promoter can affect the DNA binding of CREB family members, ChIP assays were performed using HaCaT and C-33A cell lines. Anti-CREB and anti-CREM antibodies were used, followed by oligonucleotides to amplify, from the immunoprecipitated material, a fragment of the CD86 promoter region that spans the two CRE sites. In HaCaT cell line where at least one CRE site is poorly methylated (−197), CREB was bound, but not in C-33A, where these sites are highly methylated (Fig. 2C).

3.4. 5-Aza treatment induced changes in CD86 expression

In order to confirm the involvement of DNA methylation on CD86 expression, we treated HaCaT, C-33A, SiHa and HeLa cells with different concentrations (5 μM and 100 μM) of the general demethylating agent 5-aza-2′deoxycitidine (5-aza) and evaluated CD86 mRNA level.

In C-33A cells, 5-aza treatment was induced CD86 expression even at the lowest concentration tested (5 μM). However, the expression of CD86 in SiHa cells was only observed after treatment with the highest concentration tested (100 μM), whereas none of the conditions used was able to induce CD86 mRNA expression in HeLa cells (Fig. 3A). Interestingly, treatment of HaCaT cells with 5-aza induced the downregulation of CD86 mRNA levels at 5 μM and almost abolished its detectable expression at 100 μM. The effect of 5-aza on the methylation status of CD86 was confirmed by treating C-33A or HaCaT cells with this demethylating agent (5 μM and 100 μM) and sequencing the CpG island and promoter of CD86 from five individual clones (Fig. 3B).

3.5. Methylation status in the CpG island and CD86 promoter in normal cervical epithelium correlates with that observed in cell lines

To determine if such apparent epigenetic regulation mechanism also occurs in tissue, we evaluated the methylation status of the CpG island and the selected CpG dinucleotides located in the promoter region of CD86 in normal cervical epithelium. The presence of CD86 protein in a normal cervical biopsy was initially confirmed by immunohistochemistry (Fig. 4A) and then basal (negative for CD86 protein) and spinous (positive for CD86 protein) layers of the epithelium were separated by LCM (Fig. 4B). The analysis of the CpG island showed that in the basal layer the methylation percentage in all the CpG dinucleotides was considerably lower than the one observed in the spinous layer. This pattern, as well as the particular CpG sites with low and high methylation in each specific epithelial layer was consistent with the patterns observed in cell lines regardless their expression of CD86.

The analysis of the CpG dinucleotides located in the promoter showed that those dinucleotides that are part of the CRE sites are methylated similarly in cells of the basal layer and in C-33A cells. However, the methylation pattern in cells from the spinous layer was highly consistent with that observed in the HaCaT cell line, but also similar to SiHa and HeLa cells where the −197 site was unmethylated too (Fig. 4C).

4. Discussion

Here we have shown the existence of different DNA methylation patterns in the genomic region of the CD86 gene in cells from normal cervical epithelium and keratinocyte-derived cell lines, suggesting an epigenetic mechanism regulating the constitutive expression of this co-stimulatory molecule in epithelial cells.

None of the cervical carcinoma derived cell lines included in our study express CD86 mRNA in normal conditions. In agreement with this observation, a very low or undetectable expression of the molecule has been reported in malignant epithelial cells, suggesting that this mechanism as a means to evade the immune response [7,8,10,11].

It is well known that CD86 can be up-regulated by different stimuli not only in APC, but also in some other cell types [3,4,8]. However, a decrease of CD86 expression in cells constitutively expressing this molecule is less common, and this has only been reported in diseases where the immune response is severely compromised. The mechanisms controlling this down-regulation or total shut off of CD86 expression remain unclear [18,19].

The expression of CD86 of a specific layer in the normal cervical stratified epithelium suggests that its presence could be associated to the differentiation process of such epithelium. Dynamic DNA methylation is closely related to changes in gene expression during differentiation [12] and the involvement of this epigenetic mechanism has been observed in the regulation of CD86 in dendritic cells [20]. Our analysis of the CpG island located in the upstream intergenic region of CD86 showed a hypomethylated pattern in those cell lines where CD86 is not expressed, whereas a methylated pattern was found in those cells that express this gene. Although it is commonly accepted that most of the CpG islands located in the proximity of the promoter in expressed genes are unmethylated, it is also true that each cell type has its specific heritable pattern of methylation, which determines a specific configuration of expressed genes [21]. Moreover, global hypomethylation and promoter specific hypermethylation has been reported in cancer cells as frequent epigenetic aberrations [22].

Although a formal CpG island is not present in the CD86 promoter, we found that the CpG dinucleotides overlapping with CRE sequences were consistently methylated in those cell lines where CD86 is not expressed. ChIP assays demonstrated that the transcription factor CREB binds to these sequences on the CD86 regulatory region, where at least one CRE site (−197) is unmethylated in HaCaT cells, but not in C-33A cells where both sites were methylated. In fact, it has been demonstrated that methylation of the CRE sites in different promoters could inhibit DNA binding of CREB, decreasing the transcriptional activity [23]. Since it has been reported that CREB positively regulates the expression of CD86

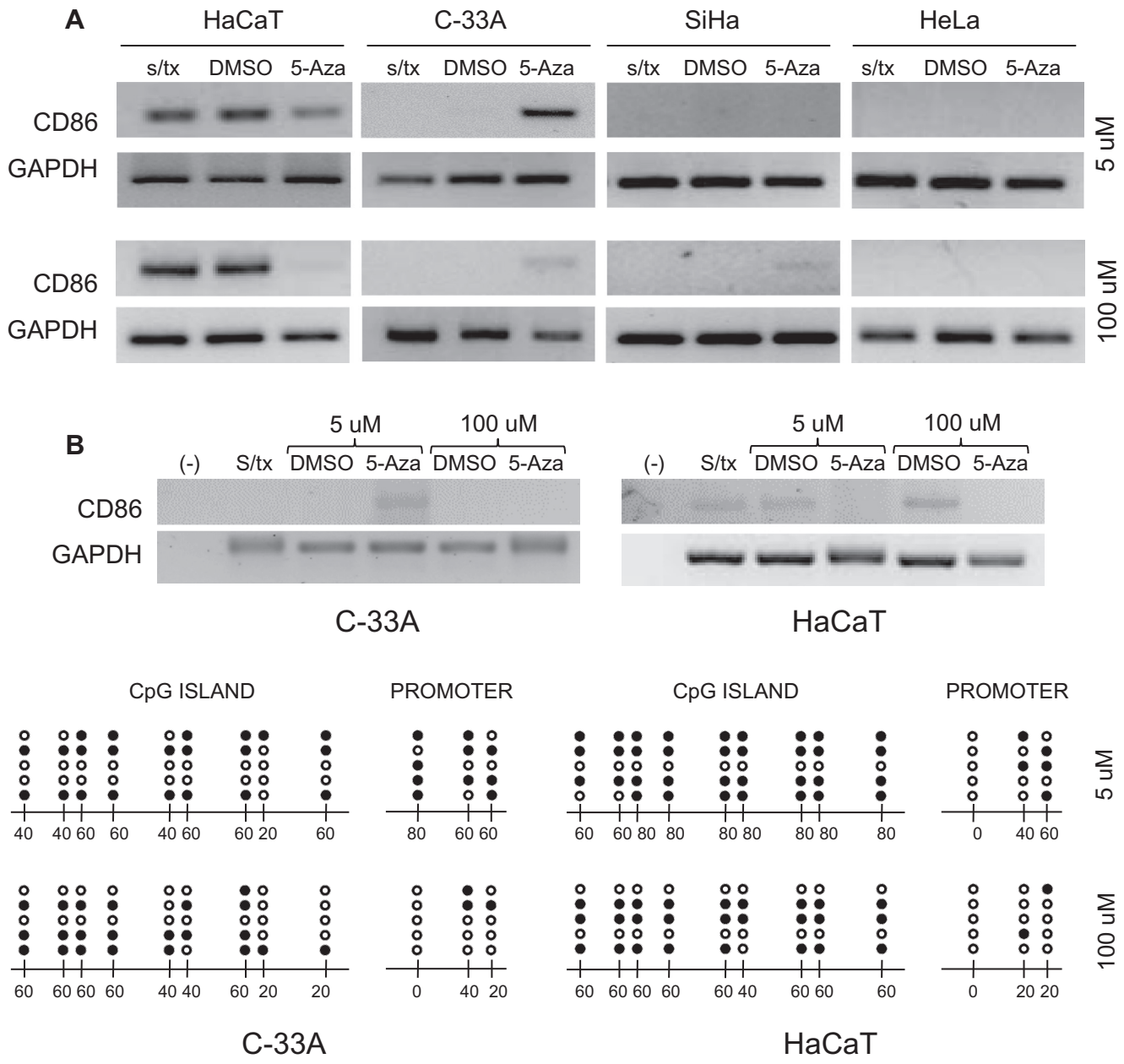

Fig. 3. CD86 mRNA expression in epithelial cell lines after treatment with 5-aza. (A) CD86 mRNA level evaluated by RT-PCR in HaCaT, C-33A, SiHa and HeLa cell lines after 72 h 5-aza or DMSO treatment. GAPDH expression was included as a loading control. (B) CD86 mRNA expression and schematic representation of the methylation status in the genomic CD86 regulatory region after 5-aza treatment of C-33A and HaCaT cell lines. Empty circles, cytosines; black circles, methylcytosines. Five individual clones were analyzed and numbers indicate the methylation percentage at individual sites (%M).

[5,16], the methylation of CRE, lack of CREB binding and a poor expression of CD86 were the expected results.

On the other hand, it has been demonstrated that the methylation of CRE enhances the binding of different transcription factors to the DNA, such as C/EBPα, [24] repressing transcription. Even though the binding of CREM to the CRE sites on the CD86 promoter induces a suppression of its transcriptional activity [16], we could not detect CREM bound to the CD86 promoter in the analyzed epithelial cell lines and this is independent from the expression level of this gene. Thus, our results indicate that the lack of expression of CD86 in those cells is mainly due to the inability of CREB to bind to the methylated consensus sequences rather than a transcriptional repression mediated by CREM.

The lack of CD86 expression in SiHa and HeLa cell lines, where a CRE site in the promoter region of the gene is unmethylated, could be explained by the activity of viral proteins encoded by human papillomavirus (HPV) genomes in these cell lines. In consistence with this idea, we have previously reported a low or absent expression of CD86 in HPV-positive cervical tissues [11]. In fact, it has been demonstrated that E7 interacts and inhibits P/CAF, while E6 does the same with CBP/p300, whose functions are required to activate NF-kB and CREB [25,26]. Both of these transcription factors have been reported to positively regulate CD86 expression [5,6,16].

As expect in genes where methylation is determinant to keep its expression limited, low concentrations of 5-aza were able to induce the expression of CD86 in C-33A cells. However, SiHa and HeLa cell lines showed a low (SiHa) or even lack of response (HeLa) to the 5-aza treatment. The unmethylated status found in the CRE sites of the promoter region before the treatment of both cell lines

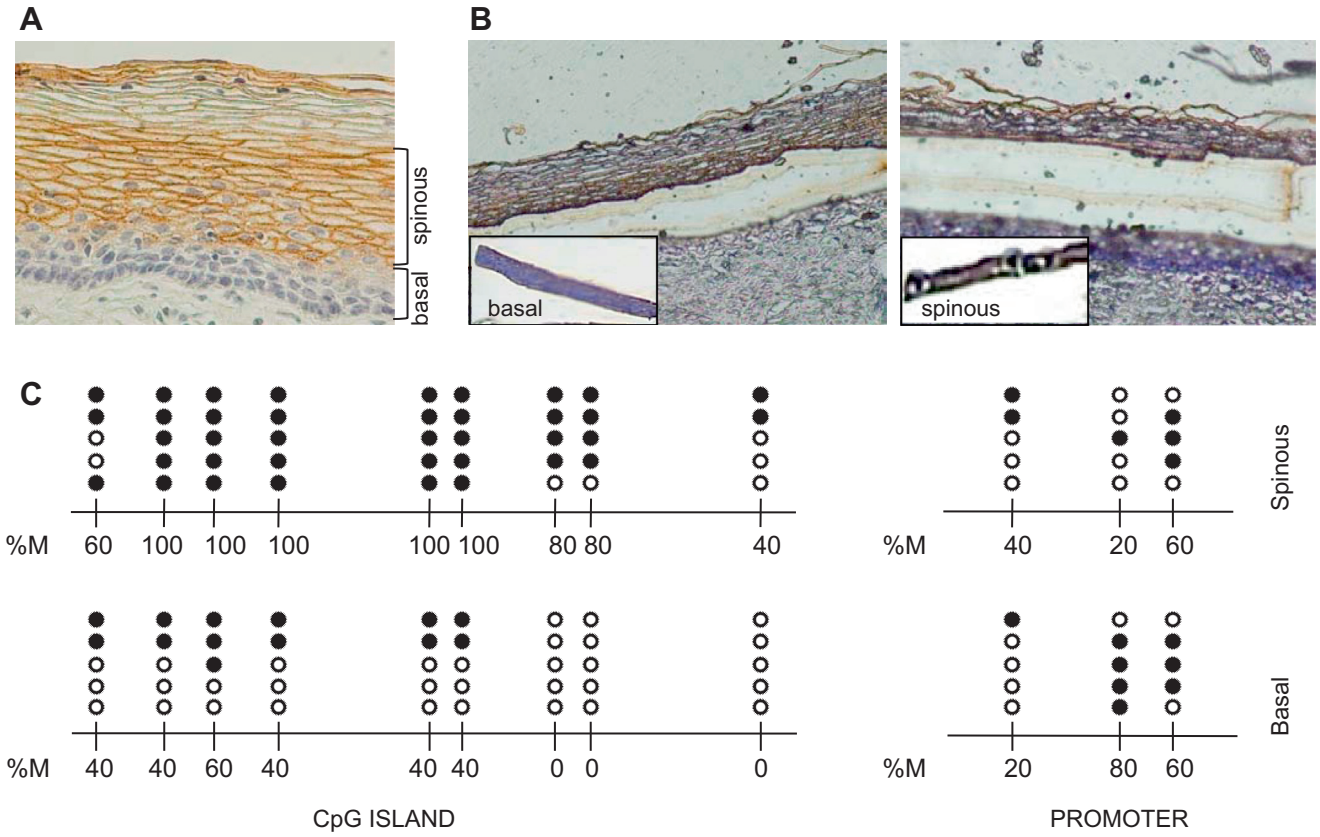

Fig. 4. Methylation status of the CpG island and CpG dinucleotides in the CD86 gene promoter in normal cervical tissue. (A) CD86 immunohistochemistry showing the protein expressed in the spinosum stratum of normal cervical tissue. (B) Image showing the LCM of the spinosum (CD86+) and basal (CD86−) stratus. (C) Schematic representation of the methylation status of the CpG island and CpG dinucleotides in the promoter for each epithelial stratum. Vertical lines across the horizontal indicate CpG sites. Empty circles, cytosines; black circles, methylcytosines. Five individual clones of each region, in both layers, were analyzed and numbers indicate methylation percentage at individual sites (%M).

indicates that demethylation in the rest of the CpG dinucleotides located in the promoter region has little or no influence on the onset of CD86 in these cells.

Interestingly, 5-aza treatment in HaCaT cells or high concentration of treatment in C-33A cells induced an important decrease (greater than 50%) in CD86 mRNA levels. These results correlate with the demethylation observed in the CpG Island after treatment, result that is more evident in the HaCaT cell line. This suggests that DNA methylation in intergenic regions is necessary to maintain CD86 expression. Although methylation in CpG island located in the proximity of promoters has been often associated with no expression, methylation in intergenic regions could also interfere with the binding of factors that isolate these genes from external enhancers, activating its expression [27,28].

Finally, the CD86 methylation pattern observed in cells from basal and spinous layers in normal cervical epithelium was consistent with that observed in C-33A and HaCaT cell lines respectively; suggesting that this epigenetic mechanism could also regulate CD86 expression in normal tissues.

The early differentiation status in the HaCaT cell line [17] makes its gene expression profile similar to that observed in the spinous layer in normal cervical epithelium. On the contrary, C-33A cells, despite its tumoral origin, preserve characteristics from the basal epithelial layer where they were originated from and show a similar gene expression profile to the one observed in cells from this epithelial layer.

In conclusion, our data suggest that methylation status in the genomic region of CD86 is closely related to its expression in keratinocytes. In those cells where CD86 needs to be expressed, the DNA is methylated in the intragenic region and unmethylated in the promoter, thus allowing the regulation of CD86 expression by other mechanisms. This methylation pattern differs from that observed in cells where CD86 is not expressed. Further studies are needed to ascertain the mechanisms underlying this hypothesis.

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