CDH1 and SNAI1 are regulated by E7 from human papillomavirus types 16 and 18

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Abstract. A common characteristic of cancer types associated with viruses is the dysregulated expression of the *CDH1* gene, which encodes E-cadherin, in general by activation of DNA methyltransferases (Dnmts). In cervical cancer, E7 protein from high risk human papillomaviruses (HPVs) has been demonstrated to interact with Dnmt1 and histone deacetylase type 1 (HDAC1). The present study proposed that E7 may regulate the expression of *CDH1* through two pathways: i) Epigenetic, including DNA methylation; and ii) Epigenetic-independent, including the induction of negative regulators of *CDH1* expression, such as Snail family transcriptional repressor Snai1 and Snai2. To test this hypothesis, HPV16- and HPV18-positive cell lines were used to determine the methylation pattern of the *CDH1* promoter and its expression in association with its negative regulators. Different methylation frequencies were identified in the *CDH1* promoter in HeLa (88.24%) compared with SiHa (17.65%) and Ca Ski (0%) cell lines. Significant differences in the expression of *SNAI1* were observed between these cell lines, and an inverse association was identified between the expression levels of *SNAI1* and *CDH1*. In addition, suppressing E7 not only increased the expression of *CDH1*, but notably decreased the expression of *SNAI1* and modified the methylation pattern of the *CDH1* promoter. These results suggested that the expression of *CDH1* was dependent on the expression of *SNAI1* and was inversely associated with the expression of E7. The present results indicated that E7 from HPV16/18 regulated the expression of *CDH1* by the two following pathways in which Snai1 is involved: i) Hypermethylation of the *CDH1* promoter region and increasing expression of *SNAI1*, as observed in HeLa; and ii) Hypomethylation of the *CDH1* promoter region and expression of *SNAI1*, as observed in SiHa. Therefore, the suppression of *CDH1* and expression of *SNAI1* may be considered to be biomarkers of metastasis in uterine cervical cancer.

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Introduction

According to current research, 15-20% of all cases of cancer can be attributed to infectious agents, including *Helicobacter pylori* and human papillomavirus (HPV), followed by hepatitis B virus (HBV), hepatitis C virus (HCV) and the Epstein-Barr virus (EBV) (1-3). In HPV, specifically types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 that are commonly referred to as high-risk (HR), are not only associated with cervical cancer (CC), the third most prevalent type of cancer worldwide in women in 2017, but also with other tumor types, including anal, penile, vulvar, vaginal and head and neck cancer (1,4).

Persistent infections with HR-HPVs are necessary, but not sufficient to cause cancer, indicating the existence of multistep actions in viral carcinogenesis that contribute to the characteristic hallmarks underlying the phenotype of tumors (3,5). Thus, it has been of great interest to study mechanisms by which persistent infections with these viruses contribute to cancer development. HPV has been demonstrated to induce a series of mechanisms that contribute to the evasion of the immune response and apoptosis-activated cell death, and finally the transformation, proliferation and cellular immortalization of the host cell (6,7).

HPV life cycle disruption due to integration of the viral genome into the cellular genome (8,9) and activation of the cell methylation machinery have been demonstrated to be involved in the carcinogenic process of CC among other factors (10-12). Specifically, the HR-HPV E7 oncoprotein has been reported to serve a crucial role in oncogenic transformation due to its ability to form complexes with members of the retinoblastoma protein (pRB) family and destabilize them (13,14), in addition to the ability to interact with other proteins, including histone deacetylase 1 (HDAC1) (15) and DNA methyltransferase 1 (Dnmt1) (16).

E7 binds to HDAC1 via its zinc finger-like motif through an intermediary protein Mi2β, which is a component of the nucleosome remodeling and histone deacetylation (NURD) complex that has the ability to modify chromatin structure trough both deacetylation of histones and ATP-dependent nucleosome repositioning (17,18). The formation of this complex is necessary for the maintenance of viral episomes, controlling cell proliferation and extending cell life (15,17). Consistent with this, a chromatin immunoprecipitation assay in Ca Ski cells demonstrated that E7 and HDACs are associated with the major histocompatibility complex (MHC) class I promoter and histone deacetylation (19), as well as chromatin repression and the downregulation of *MHC* class I genes and MHC class I heavy chain, and repression of the genes encoding the transporter associated with antigen processing subunit 1 (TAP1) and low molecular weight protein 2 (LMP2) (20). Furthermore, an association with Dnmt1 is directed and mediated by the conserved region 3 (CR3) zinc-finger region of E7, which is known to contribute to E7 transformation functions and stimulate the methyltransferase activity of Dnmt1, which may lead to aberrant genome methylation and cellular transformation as a consequence of the silencing of tumor-suppressor genes (16). Another study has demonstrated that in samples of normal cervix and cervical cancer, HPV types 16 and 18 activate the cell methylation machinery to methylate viral DNA, as well as the promoter regions of cellular genes, including cyclin A1, Rubicon-like autophagy enhancer, retinoic acid receptor β2, cadherin 1 (*CDH1*), death-associated protein kinase 1, human telomerase reverse transcriptase 1 (*hTERT1*), *hTERT2*, hypermethylated in cancer 1 and Twist Family BHLH Transcription Factor 1 (21). Therefore, previous evidence suggests that HR-HPV *E7* serves an important role in the activation of the cellular methylation machinery, which regulates the transcription of viral and cellular genes either during their productive infection during its life cycle or during the carcinogenic process.

The mechanisms by which E7 is involved in the regulation of gene expression at the chromatin level are not well understood. It has been observed that a common characteristic of several cancer types associated with viruses is the decreased expression of *CDH1*, which encodes E-cadherin, through epigenetic mechanisms (22-24). In the case of cancer types associated with HPV infections, it has been demonstrated that HPV16 *E7* suppresses the transcription of *CDH1*, which reduces protein expression of E-cadherin (25,26). In addition, HPV16 *E7* has been reported to increase the amount and activity of Dnmt1 in NIKS cells, which are derived from foreskin keratinocytes transfected with HPV16 *E7* or NIKS bearing episomal HPV16 DNA (26); however, NIKS cells not infected with HPV16 and NIKS bearing episomal HPV16 DNA did not exhibit any differences in the CpG methylation status of the *CDH1* promoter regions, as all CpG sites were unmethylated (26). It is evident that HPV activates the methylation machinery via E7/Dnmt1; however, it is not clear how HPV induces *CDH1* repression by epigenetic mechanisms.

Since HR-HPVs E7 has been demonstrated to interact with Dnmt1 and HDAC1, the aim of the current study was to determine the methylation pattern of the *CDH1* promoter region in HeLa, SiHa and Ca Ski cell lines positive for HPV16 and HPV18. Additionally, associations with transcription factors Snai1 and Snai2, which are negative regulators of *CDH1* expression and inducers of the epithelial-mesenchymal transition (EMT) process (27), were evaluated. HeLa, SiHa and Ca Ski cell lines were selected for the present study, as they are representative of the most frequent cancer types of the uterine cervix with positive HR-HPV infection with different viral loads and epithelial origins, including cervical adenocarcinoma, cervical squamous cell carcinoma and cervical epidermoid carcinoma (28-34).

Materials and methods

Cell lines. As reported by the American type culture collection, HeLa cells are derived from a female African-American patient with uterine cervical adenocarcinoma and are reported to contain 10-50 integrated copies of HPV18 per cell (30-36). SiHa cells are derived from a female Asian patient with grade II cervical squamous cell carcinoma and are reported to contain 1-2 integrated copies of HPV16 per cell (29,30,33,36). Ca Ski cells are derived from a female Caucasian patient with cervical epidermoid carcinoma and are reported to contain 500-600 integrated copies of HPV16 per cell (28,30,36). HaCaT is a non-tumorigenic immortalized human epidermal cell line derived from skin keratinocytes. All cell lines were authenticated through STR DNA profiling (ID no. DP0297) by the University of Colorado DNA Sequencing & Analysis Core. HeLa, SiHa and HaCaT cell lines (ATCC) were cultured in DMEM (cat. no. 12800-058; Gibco; Thermo Fisher Scientific, Inc.); Ca Ski cells were cultured in RPMI medium (cat. no. 31800-014; Gibco; Thermo Fisher Scientific, Inc.). All cell lines were supplemented with 10% fetal bovine serum (FBS; cat. no. 16000-044; Gibco; Thermo Fisher Scientific, Inc.) and 1X penicillin-streptomycin (cat. no. 15140122; Gibco; Thermo Fisher Scientific, Inc.) and were incubated in a humidified chamber at 37˚C with 5% CO_2.

Untransfected MCF-7 cells and MCF-7 cell stable clones transfected with a pcDNA 3.1 expression vector (Invitrogen; Thermo Fisher Scientific, Inc) with the bicistronic E6/E7 region from HPV18 (MCF-7 pE6/E7) were kindly provided by Dr Erick de la Cruz Hernández (Juarez Autonomous University of Tabasco, Villahermosa, Mexico) and were cultured with 800 μg/ml Geneticin in DMEM/F12 for 3 weeks as previously described (37-39). Untransfected C33-A cells and C33-A cell stable clones transfected with a pcDNAE7 plasmid (C33-A pE7/HPV16) were kindly provided by Dr Patricio Gariglio (CINVESTAV-IPN, Mexico City, Mexico) and were cultured with 800 μg/ml Geneticin in DMEM for 2 weeks as previously described (40).

Treatments with 5-aza-2'-deoxycytidine (5-AzadC) and trichostatin A (TSA). 5-AzadC causes DNA demethylation or hemi-demethylation that results in gene activation by inhibiting Dnmt activity (41). TSA has been used as a histone deacetylase inhibitor, which causes histone hyperacetylation that leads to chromatin relaxation and modulation of gene expression (42). 5-AzadC and TSA were obtained from Sigma-Aldrich; Merck KGaA (cat. nos. A3656 and T8552, respectively) and resuspended in DMSO (Sigma-Aldrich; Merck KGaA; cat. no. D8418) to obtain working stock solutions (10.0 mM 5-AzadC and 1.0 mM TSA). The 5-AzadC and TSA stock solutions were aliquoted, protected from light and stored at -80˚C for later use. A total of $4.5x10^5$ HeLa and $5x10^5$ SiHa cells were seeded in p60 boxes in triplicate and were treated with 5 and 10 μM 5-AzadC and 200 and 500 nM TSA. Untreated cells or cells treated with DMSO were used as controls. The total volume of culture medium was 3 ml, which was supplemented with 10% FBS and did not contain any antibiotics. Assays were performed protected from light and the cells were incubated for 48 h at 37˚C with 5% CO_2. The culture medium was replaced after 24 h due to the half-life of 5-AzadC and TSA.

Transfection with short interfering RNA (siRNA). siRNAs targeting HPV16 and HPV18 E7 were designed as previously described (43,44) and obtained from Ambion (Thermo Fisher Scientific, Inc.; cat. nos. s237642 and s237640). Silencer® Select *GAPDH* siRNA (Hs, Mm, Rn) (cat. no. 4390849; Ambion; Thermo Fisher Scientific, Inc.) was used as a positive transfection control to select the transfection agent (Lipofectamine® 2000 or siPORT™ NeoFX™) and to optimize gene silencing without affecting cell viability according to the manufacturer's protocol and as previously described (45-50). siRNAs were resuspended in UltraPure™ DNase/RNase-free distilled water (cat. no. 10977-015; Thermo Fisher Scientific, Inc.) to obtain a working stock of 10 μM. siRNAs were then aliquoted and stored at -80˚C for later use.

Transfection with siRNA was performed in triplicate using the siPORT™ NeoFX™ kit (cat. no. AM4511; Ambion; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. A total of $7.5x10^4$ HeLa and $8.0x10^4$ SiHa cells/well were seeded in a 12-well plate. HeLa cells were transfected with 30 nM siRNA against HPV18 E7, whereas SiHa cells were transfected with 30 nM siRNA against HPV16 E7. In addition, 30 nM siRNA targeting *GAPDH* was transfected as a positive control in both cell lines. Cells subjected to treatment with siPORT™ NeoFX™ Transfection Agent with Opti-MEM® I (cat. no. 31985070; Thermo Fisher Scientific, Inc.) were used as a reference control to obtain the relative values, and untransfected cells were used as a reference control for statistical comparisons. Culture medium supplemented with 12% FBS and no antibiotics was added to make up a final transfection volume of 1.2 ml. Cells were incubated for 48 h at 37˚C with 5% CO_2 and subsequently the extraction of nucleic acids and proteins was performed. Transfection efficiency was determined by measuring the expression of E7 and GAPDH at the mRNA level.

Bisulfite conversion and DNA methylation analysis. Total genomic DNA was isolated from the treated and transfected cell lines using the Wizard® Genomic DNA Purification kit (cat. no. A1120; Promega Corporation) and 1.5 μg genomic DNA was treated with bisulfite according to the manufacturer's protocol of the EZ DNA Methylation-Gold™ kit (cat. no. D5006; Zymo Research Corp.). Methylation of CpG sites at the *CDH1* promoter region was analyzed by the bisulfite sequencing PCR (BSP) protocol (Fig. 1A) (10-12,51,52) or using oligonucleotides for MSP-protocol provided by Dr Alfonso Dueñas-Gonzalez (INCan-UNAM, Mexico City, Mexico); the PCR conditions for MSP-protocol were the same as those used for the BSP protocol. PCR was performed with a total volume of 25 μl, containing 1X PCR Gold Buffer, 1 mM dNTP, 2 mM $MgCl_2$, 10 pMol forward *CDH1*-BSP (5'-TTTTAGTAATTT TAGGTTAGAGGGTTAT-3') and reverse *CDH1*-BSP (5'-AAA CTCACAAATACTTTACAATTCC-3') oligonucleotides, 1 U AmpliTaq Gold® DNA Polymerase (cat. no. 4338856; Applied Biosystems; Thermo Fisher Scientific, Inc.) and 300 ng bisulfite-treated DNA. The thermocycling conditions were as follows: 95˚C for 7 min, followed by 35 cycles of 95˚C for 35 sec, 57˚C for 35 sec and 72˚C for 60 sec, and a final extension at 72˚C for 7 min. PCR products were treated using *Exo*I (cat. no. EN0581; Thermo Fisher Scientific, Inc.) and *SAP* (cat. no. EF0651; Thermo Fisher Scientific, Inc.) enzymes. The treated PCR products were sequenced using the BigDye® v3.1 Cycle Sequencing kit (cat. no. 4337455; Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol in an ABI PRISM™ 3100-Avant Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.). The BSP oligonucleotides were designed by MethPrimer v2.0 software (The Li Lab; PUMCH; Chinese Academy of Medical Sciences) using GenBank sequence DQ335132.1 for the *CDH1* gene (53). The sequencing data obtained from BSP were analyzed using Chromas v2.6.4 software (Technelysium Pty., Ltd.) and Lasergene v7 package (DNASTAR, Inc.).

RNA extraction and reverse transcription-quantitative (RT-q) PCR. Total RNA was extracted from the treated and transfected cells using TRIzol® reagent (cat. no. 15596026; Invitrogen; Thermo Fisher Scientific, Inc.). The RNA was treated with *DNase I* (cat. no. EN0521; Invitrogen; Thermo Fisher Scientific, Inc.), and purified with Direct-zol™ RNA MicroPrep (cat. no. R2060; Zymo Research Corp.) according to the manufacturer's protocol. Complementary DNA (cDNA) was obtained from the purified RNA using the SuperScript™ IV First-Strand Synthesis system (cat. no. 18091050; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The RNA-primer mix was incubated at 65˚C for 5 min and 4˚C for 1 min, and the RT reaction mix was incubated at 55˚C for 15 min and 80˚C for 10 min to inactivate the reaction and placed on ice for subsequent use. Subsequently, 60 ng cDNA was subjected to qPCR to determine the expression levels of the genes of interest using the primers listed in Table I. qPCR conditions were as follows: 95˚C for 10 min, followed by 40 cycles of 95˚C for 20 sec, 60˚C for 30 sec and 72˚C for 35 sec, and a final extension at 72˚C for 7 min. This was followed by a melting curve analysis at 65-95˚C. All qPCR assays were analyzed using Rotor-Gene Q Series v2.1.0 software (Qiagen, Inc.).

To obtain expression levels of *CDH1*, *SNAI1*, *SNAI2*, HPV16 E7 and HPV18 *E7* in the HeLa, SiHa, Ca Ski and

Table I. Primer sequences used for the BSP and qPCR assays.

Gene	Assay	Primer sequences (5'→3')
CDH1	BSP	F: TTTTAGTAATTTTAGGTTAGAGG GTTAT
		R: AAACTCACAAATACTTTACAATT CC
CDH1	qPCR	F: GTCAGTTCAGACTCCAGCCC
		R: AAATTCACTCTGCCCAGGACG
SNAI1	qPCR	F: ACCACTATGCCGCGCTCTT
		R: GGTCGTAGGGCTGCTGGAA
SNAI2	qPCR	F: GACCCTGGTTGCTTCAAGGA
		R: TGTTGCAGTGAGGGCAAGAA
E7 HPV16	qPCR	F: CAGCTCAGAGGAGGAGGATG
		R: TGCCCATTAACAGGTCTTCC
E7 HPV18	qPCR	F: TGAAATTCCGGTTGACCTTC
		R: CACGGACACACAAAGGACAG
GAPDH	qPCR	F: AAGGTCGGAGTCAACGGATTTG
		R: CCATGGGTGGAATCATATTGGAA
HPRT	qPCR	F: GGACTAATTATGGACAGGACTG
		R: GCTCTTCAGTCTGATAAAATCT AC

BSP, bisulfite sequencing PCR; qPCR, quantitative PCR.

HaCaT cells, a commercial sample of RNA extracted from normal cervix negative for HPV (Human Cervix Total RNA; cat. no. AM6992; Ambion; Thermo Fisher Scientific, Inc.) was used as a reference. The ΔCq values for each gene were normalized to the reference gene *GAPDH* using the $2^{-\Delta\Delta Cq}$ method (54). The commercial sample of normal cervix negative for HPV was set as 1, and the results are not presented. For statistical analysis, the HaCaT cell line was used for comparison. For experiments involving the treatment of HeLa and SiHa cells with 5-AzadC and TSA, cells treated with DMSO were used as a reference, but data were not included in the graphs. For experiments involving the transfection of HeLa and SiHa cells with siRNAs, the cells treated with siPORT™ NeoFX™ Transfection Agent with Opti-MEM® I were used as a reference, but results were not included in the graphs. ΔCq values of *CDH1*, *SNAI1*, *SNAI2*, HPV16 E7 and HPV18 *E7* were normalized using the $2^{-\Delta\Delta Cq}$ method (39) with *GAPDH* as reference for 5-AzadC and TSA treatments, and *HPRT* as reference for experiments involving siRNA transfections. For statistical analysis, untreated (Unt) HeLa and SiHa cells were used for comparisons.

Protein extraction and western blot analysis. Proteins were obtained using a lysis buffer containing 5 mM EDTA, 150 mM NaCl, 5 mM Tris-HCl pH 9.0, 1% Nonidet-P40 and 1.2 mg/ml cOmplete™ protease inhibitor cocktail (Roche Applied Science). Protein extracts were forced through a 22-gauge needle 10 times and centrifuged for 10 min at 17,000 x g at 4˚C. Protein concentration was determined using the Bradford method. Subsequently, 30 mg protein was loaded and separated on 12% SDS-PAGE gels followed by transfer to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk in 1X TBS with 0.1% Tween-20 (TBST) at 4˚C for 1 h with gentle agitation and incubated overnight at 4˚C with antibodies against E-cadherin (cat. no. sc-8426; 1:1,000), GAPDH (cat. no. sc-48167; 1:1,000), β-actin (cat. no. sc-1616; 1:1,000; all from Santa Cruz Biotechnology, Inc.), Snai1 (cat. no. L70G2; 1:1,000; Cell Signaling Technology, Inc.) and Snai2 (cat. no. C19G7; 1:1,000; Cell Signaling Technology, Inc.) diluted in TBST with 5% BSA (cat. no. 9998; Cell Signaling Technology, Inc.). Subsequently, membranes were incubated with secondary antibodies for 2 h at room temperature, including goat anti-mouse IgG-horseradish peroxidase (HRP; cat. no. sc-2005; 1;10,000), donkey anti-rabbit IgG-HRP (cat. no. sc-2313; 10,000) and donkey anti-goat IgG-HRP (cat. no. sc-2020; 1:5,000; all from Santa Cruz Biotechnology, Inc.) diluted in TBST with 5% non-fat dry milk. Immobilon Western Chemiluminescent HRP substrate (EMD Millipore) was used for protein detection, and images were acquired using C-DiGit Blot scanner equipment (Li-Cor Biosciences) and processed in the Image Studio™ Lite version 5.2 software (Li-Cor Biosciences).

Nitrocellulose membranes were incubated with two primary antibodies in the following manner: i) Incubation was performed as described against a primary antibody, including E-cadherin, Snai1 or Snai2 with their respective secondary antibody; ii) images were acquired; iii) membranes were washed 3x5 min with TBST at room temperature; iv) membranes were re-probed with a second primary antibody, including GAPDH or β-actin with their respective secondary antibody; and v) images were acquired.

Statistical analysis. Statistical analysis was performed using GraphPad Prism v4.0 (GraphPad Software, Inc.). One-way ANOVA with Turkey's post hoc test were used to evaluate significant differences in gene expression and methylation levels, and results were presented as the mean ± SD. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Different methylation patterns in the CDH1 promoter region are present in HPV16- and HPV18-positive cancer cell lines. A common site-specific methylation pattern in certain CpG islands (-160, -150, -131 and -122) of the *CDH1* promoter region was detected in HeLa and SiHa cells, whereas other CpG islands (-45, -136, -105, -103, -83, -57, -52, -45, -36, -13, +6 and +9) were identified to be methylated in HeLa only (Fig. 1B). Of note, Ca Ski cells did not exhibit methylation of any of the 17 CpG sites of the *CDH1* promoter that were analyzed (Fig. 1B). Quantification of the methylation levels in the *CDH1* promoter region indicated that HeLa presented a methylation frequency of 88.24%, SiHa cells exhibited a methylation frequency of 17.65% and Ca Ski cells demonstrated no methylation (frequency, 0%; Fig. 1C).

Based on previous studies (25-28), initial experiments were conducted using the C33-A, C33-A transfected with pE7/HPV16 (C33-A pE7/HPV16), MCF-7 and MCF-7 transfected with pE6/E7 from HPV18 (MCF-7 pE6/E7) cell lines as HPV-negative cancer models. Validation of C33-A and MCF-7 cell stable clone selection with pE7/HPV16

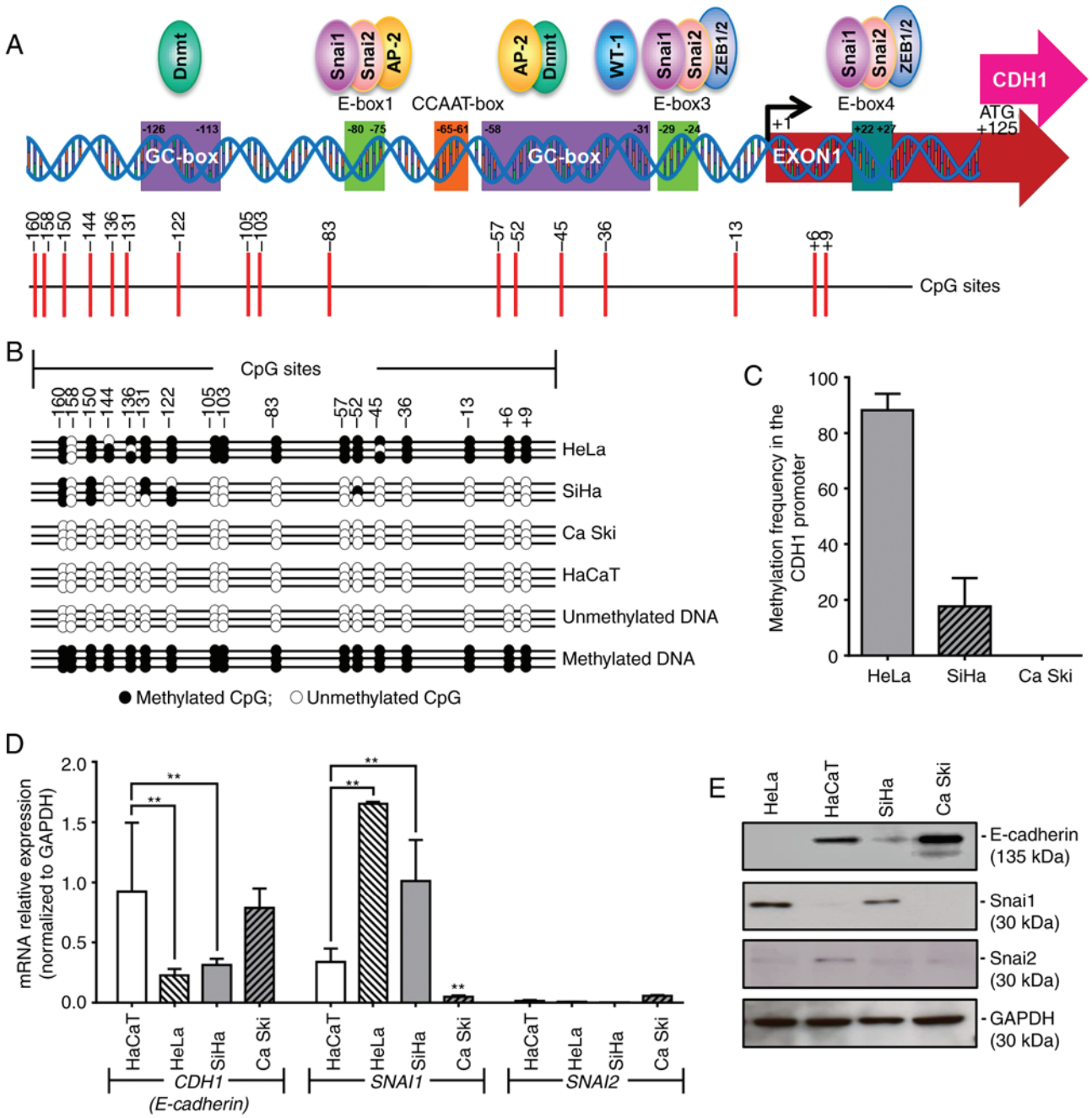

Figure 1. Methylation of the *CDH1* gene promoter region. (A) The location of different elements; the E-box and GC box motifs that regulate transcription of *CDH1*. Red vertical lines indicate each CpG site contained in the promoter region. (B) A lollipop methylation diagram of bisulfite sequencing of the *CDH1* promoter in the HeLa, SiHa, Ca Ski and HaCaT cell lines. Black indicates a methylated CpG site; white indicates an unmethylated CpG site. (C) Quantification of the methylation frequency of the *CDH1* promoter in the HeLa, SiHa and Ca Ski cell lines. (D) Quantitative PCR was performed to measure the expression levels of *CDH1*, *SNAI1* and *SNAI2* genes in the indicated cell lines. *P<0.05 and **P<0.01 vs. HaCaT. (E) Protein expression levels of E-cadherin, Snai1 and Snai2 were assessed by western blot analysis in HPV-positive cell lines. GAPDH was used as a loading control. *CDH1*, cadherin 1; Dmnt1, DNA methyltransferase 1; AP-2, activating protein 2; ZEB1/2, zinc finger E-box-binding homeobox 1/2.

and pE6/E7, respectively, was performed by evaluating E7 mRNA expression by RT-PCR (Fig. S1B). However, C33-A vs. C33-A pE7/HPV16 and MCF-7 vs. MCF-7 pE6/E7 did not exhibit any differences in the methylation of *CDH1* promoter regions (Fig. S1A) or in the expression of CDH1 at mRNA and protein levels (Fig. S1B and C). As the MCF-7 cell line is an adenocarcinoma that derives from the mammary gland and exhibits an epithelial phenotype with a high expression level of CDH1, similar to that observed in Ca Ski cells, and since the C33-A cell line originally does no express CDH1, C33-A and MCF7 cells were eliminated from the study; neither the effect of oncoprotein E7 on the suppression of *CDH1* expression, nor the methylation patterns in the *CDH1* promoter could be evaluated in these cells.

Methylation levels in the CDH1 promoter are associated with the mRNA and protein expression levels of CDH1. Analysis of *CDH1* expression in the different cell lines demonstrated a significant decrease of the *CDH1* mRNA level in the HeLa (P<0.001) and SiHa (P<0.01) cell lines compared

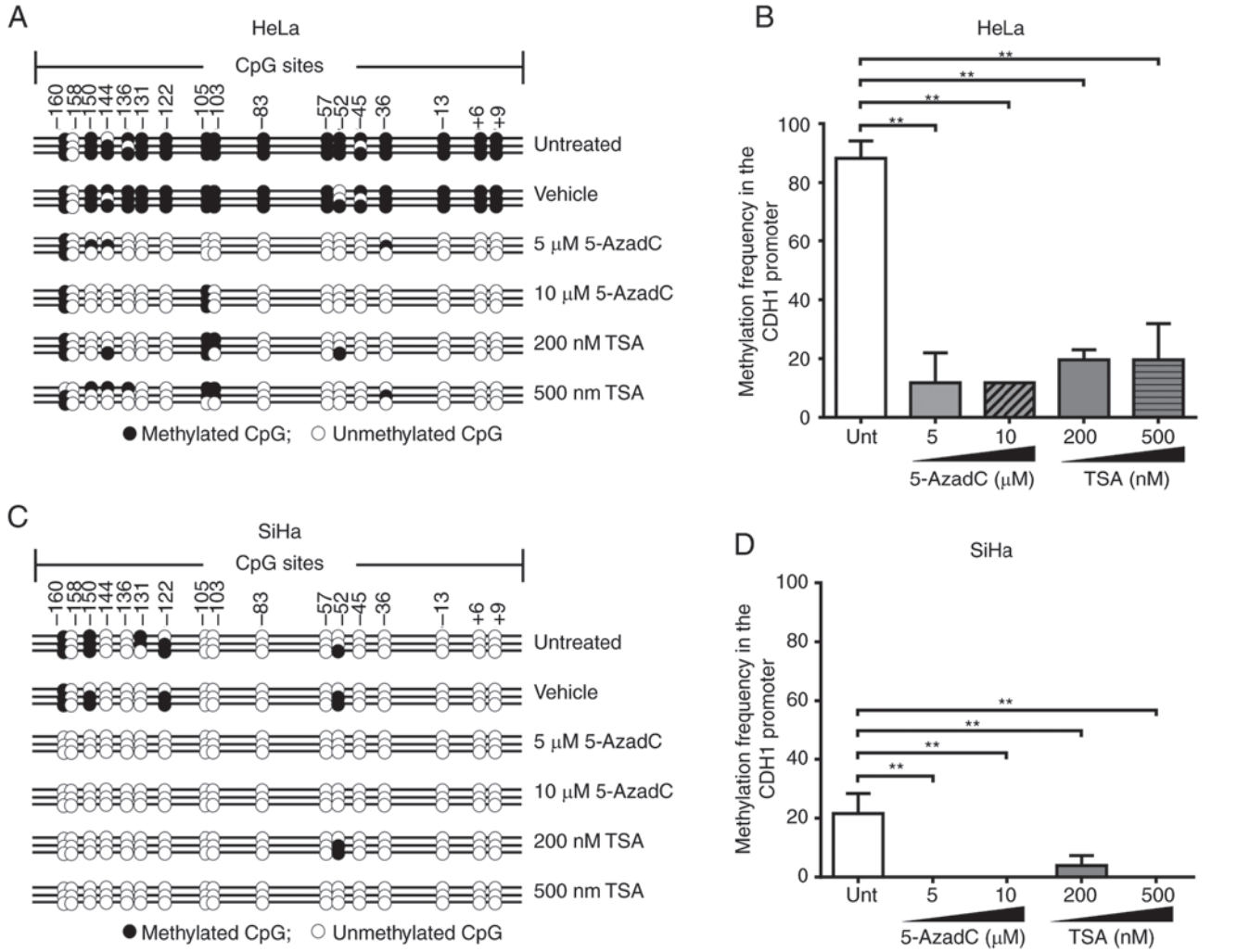

Figure 2. Changes in the methylation pattern of the *CDH1* promoter in HeLa and SiHa cell lines following treatment with 5-AzadC and TSA. (A and C) Lollipop methylation diagrams demonstrating changes in the methylation pattern in CpG sites of the *CDH1* promoter region in (A) HeLa and (C) SiHa cell lines following treatments with 5-AzadC and TSA compared with Unt and vehicle-treated cells. Black indicates a methylated CpG site; white indicates an unmethylated CpG site. (B and D) Quantification of the methylation frequency of the *CDH1* promoter in (B) HeLa and (D) SiHa cell lines following treatment with the indicated concentrations of 5-AzadC and TSA compared with untreated cells. **P<0.01 vs. untreated cells. Unt, untreated; *CDH1*, cadherin 1; 5-AzadC, 5-aza-2'-deoxycytidine; TSA, trichostatin A.

with the HaCaT cell line (Fig. 1D). A similar decrease was observed at the protein level (Fig. 1E). By contrast, the Ca Ski cell line exhibited high *CDH1* expression, similar to the control HaCaT cell line, at the mRNA and protein level (Fig. 1D and E).

CDH1 expression level is associated with the expression level of SNAI1. The present study measured the expression of *SNAI1* and *SNAI2* to test the hypothesis that the expression of *CDH1* is regulated by the transcription factors Snai1 and Snai2, which mediate EMT by negatively regulating the expression of *CDH1* (27,55-57). The results revealed that the mRNA expression level of *SNAI1* is significantly increased in HeLa (P<0.0001) and SiHa cells (P<0.001), but significantly reduced in Ca Ski cells (P<0.01) compared with HaCaT cells (Fig. 1D). This result was also reflected at the protein level (Fig. 1E). No significant differences were observed in the expression of *SNAI2* at the mRNA (Fig. 1D) and protein (Fig. 1E) level among all cell lines. Since the Ca Ski cell line exhibits a non-mesenchymal phenotype, a high expression level of E-cadherin and low expression levels of Snai1 and Snai2, this cell line was excluded from further analysis.

Treatment with 5-AzadC and TSA affect methylation and re-expression of CDH1. HeLa and SiHa cells were treated with different concentrations of 5-AzadC and TSA, followed by analysis of the expression levels of *CDH1*, *SNAI1*, and *SNAI2*. HeLa and SiHa cells treated with the vehicle (DMSO) were used for normalizing the expression values, as well for performing comparisons in statistical analysis.

The results demonstrated that in the HeLa cell line, the methylation pattern was maintained at CpG sites -103, -105, and -160 of the *CDH1* promoter region following treatment with 5-AzadC and TSA. By contrast, in the SiHa cell line, this region was completely demethylated following treatment with 5 or 10 μM 5-AzadC or 500 nM TSA, and only CpG site -52 was methylated following treatment with 200 nM TSA (Fig. 2A and C). Compared with untreated HeLa and SiHa cells, a decrease in methylation of 76.48% was observed following 5 or 10 μM 5-AzadC treatment, whereas following

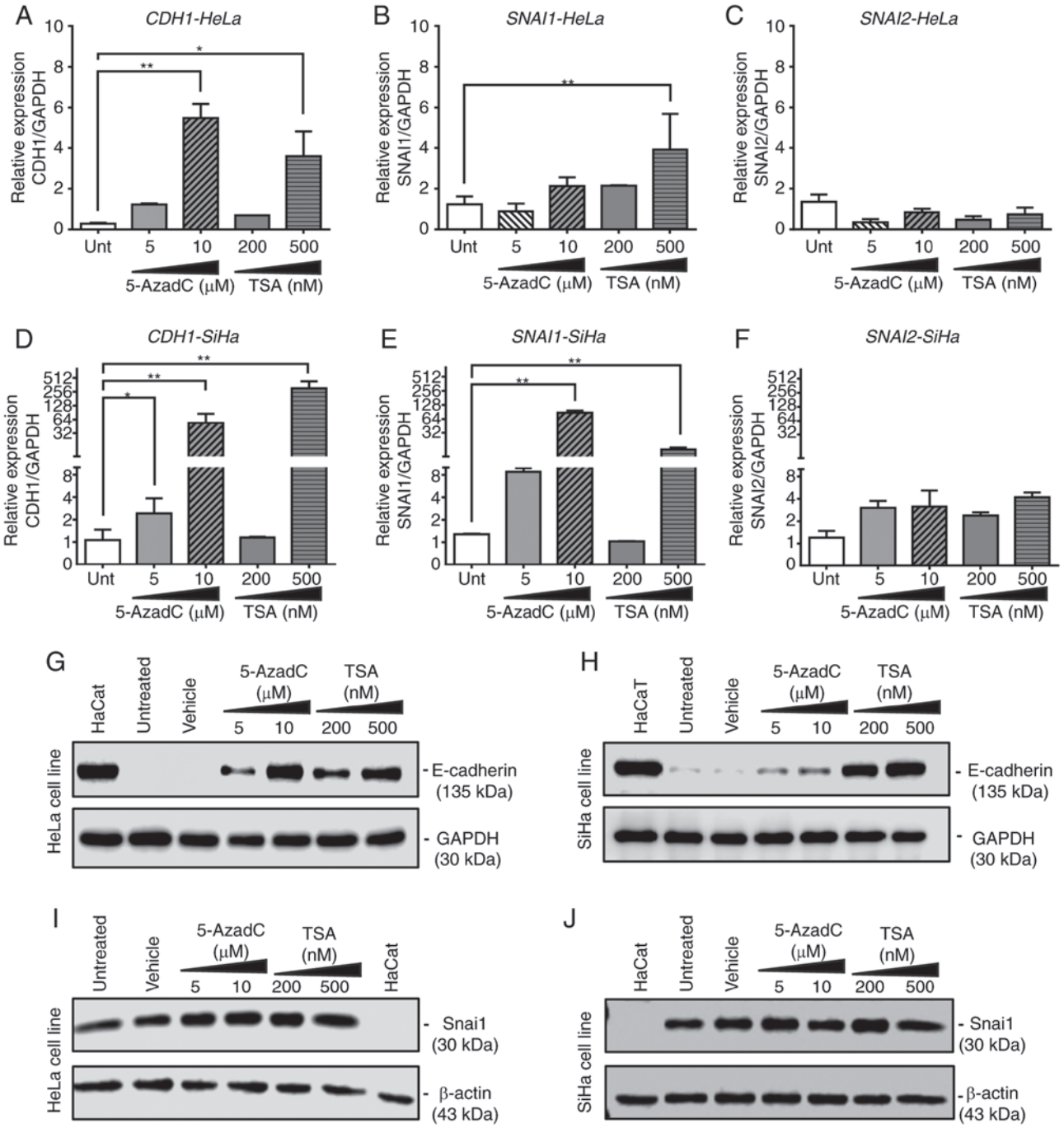

Figure 3. Changes in the expression levels of *CDH1*, *SNAI1* and *SNAI2* in HeLa and SiHa cells following treatment with 5-AzadC and TSA. (A-F) Quantitative PCR analyses of *CDH1, SNAI1* and *SNA2* mRNA expression levels in (A-C) HeLa and (D-F) SiHa cells following treatments with 5-AzadC and TSA compared with untreated cells; *P<0.05 and **P<0.01 vs. untreated cells. (G and H) Protein expression of E-cadherin in (G) HeLa and (H) SiHa cell lines. (I and J) Protein expression of Snai1 in (I) HeLa and (J) SiHa cell lines following the indicated treatments. Unt, untreated; *CDH1*, cadherin 1; 5-AzadC, 5-aza-2'-deoxycytidine; TSA, trichostatin A.

treatment with 200 or 500 nM TSA, a decrease in methylation of 68.63% was observed. By contrast, in SiHa cells, a decrease of 21.57% was observed following treatment with 5 or 10 μM 5-AzadC or 500 nM TSA, whereas following treatment with 200 nM TSA, a 17.65% decrease in methylation was observed. Therefore, it was demonstrated in the two cell lines that treatment with 5-AzadC or TSA significantly diminished the level of *CDH1* methylation (P<0.001; Fig. 2B and D). However, no significant differences were observed between the two treatments in diminishing the methylation levels of *CDH1*.

In the HeLa cell line, treatments with 10 μM 5-AzadC (P=0.004) and 500 nM TSA (P=0.03) significantly increased the expression of *CDH1* mRNA and protein (Fig. 3A and G). In addition, 500 nM TSA significantly increased the mRNA expression of *SNAI1* (P=0.01, Fig. 3B) in HeLa cells without notable changes in protein expression (Fig. 3I). In the SiHa cell line, the mRNA expression of *CDH1* significantly increased following treatment with 5-AzadC at 5 and 10 μM (P<0.05 and P<0.01, respectively) or 500 nM TSA (P=0.004; Fig. 3D); a similar effect was observed at the protein level (Fig. 3H).

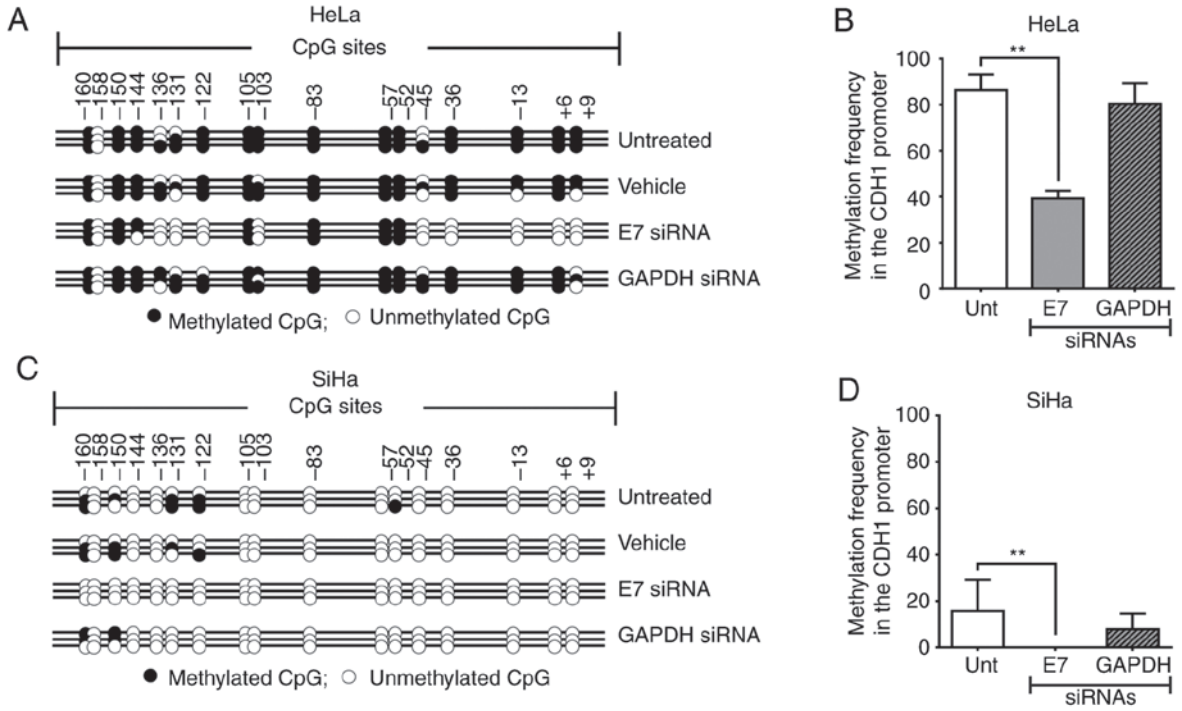

Figure 4. Silencing of E7 from HPV18 and HPV16 modifies the methylation pattern of the *CDH1* promoter in HeLa and SiHa cells. (A and C) Lollipop methylation diagrams demonstrating changes in the methylation pattern in CpG sites of the *CDH1* promoter region in (A) HeLa and (C) SiHa cell lines following transfection with siRNA targeting *E7* and *GAPDH* compared with Unt and vehicle control cells. Black indicates a methylated CpG site; white indicates an unmethylated CpG site. (B and D) Methylation frequency of the *CDH1* promoter in (B) HeLa and (D) SiHa cells following transfection with siRNA targeting *E7* from HPV18, *E7* from HPV16 and *GAPDH* compared with untreated cells. **P<0.01 vs. untreated cells. *CDH1*, cadherin 1; Unt, untreated; siRNA, small interfering RNA; HPV, human papilloma virus.

In addition, the expression of *SNAI1* mRNA was significantly increased following treatment with 10 μM 5-AzadC (P=0.004) or 500 nM TSA (P=0.01) (Fig. 3E) in SiHa cells without notable changes at the protein level (Fig. 3J), similar to that observed in HeLa.

5-AzadC and TSA also increased the mRNA expression level of *SNAI1* in the HeLa (Fig. 3B) and SiHa cell lines (Fig. 3E); however, no changes were observed in SNAI1 protein levels in the two cell lines (Fig. 3I and J). The expression of SNAI2 was not significantly modified at the mRNA level under any treatment condition in the tested cell lines (Fig. 3C and F).

Suppression of E7 by siRNA modifies the methylation patterns of the CDH1 promoter and induces CDH1 expression in HeLa and SiHa cell lines. To examine the involvement of *E7* in the *CDH1* methylation and expression patterns, HeLa and SiHa cell lines were transfected with siRNA against *E7*, which resulted in 57.9 and 42.5% reduction in the *E7* mRNA level, respectively (Fig. 5A and C). In the HeLa cell line, *E7* silencing led to demethylation of *CDH1* CpG promoter sites located between +9 and -45, as well as at -103 and between -122 and -136 (Fig. 4A). In addition, in the SiHa cell line, total demethylation of the CDH1 promoter was observed following *E7* silencing (Fig. 4C). Therefore, partial silencing of *E7* in HeLa and SiHa cells yielded a significant decrease in *CDH1* promoter methylation compared with untreated control cells (P<0.001; Fig. 4B and D). Increased *CDH1* mRNA and protein levels were observed following *E7* silencing in HeLa (Fig. 5B and F) and SiHa (Fig. 5B and G) cells. The use of Silencer® Select *GAPDH* siRNA as a positive control siRNA excluded the possibility that changes in the methylation pattern of the *CDH1* promoter were due to the siRNA transfection conditions as *GAPDH* silencing did not induce significant changes in the methylation pattern of the *CDH1* promoter in HeLa (P=0.7140; Fig. 4B) and SiHa (P=0.3248; Fig. 4D) cells; therefore, the changes in the methylation pattern were likely due to *E7* silencing.

Suppression of E7 inhibits SNAI1 and SNAI2 expression in HeLa and SiHa cells. Silencing of *E7* not only induced the expression of *CDH1*, but also significantly decreased the expression of *SNAI1* and *SNAI2* (P<0.001) in HeLa and SiHa cells (Fig. 5D, E, H and I). This suggested that E7 may be not only involved in suppressing the expression of *CDH1*, but may also regulate the expression of *SNAI1* and *SNAI2*, which negatively regulate CDH1. No significant changes were observed in the mRNA expression of *E7*, *CDH1*, *SNAI1* and *SNAI2* following *GAPDH* silencing in HeLa and SiHa cells (P>0.5; Fig. 5A, B, D and E). However, silencing of *GAPDH* with siRNA resulted in a decrease in the protein expression of Snai1 in HeLa cells (Fig. 5H).

As housekeeping genes *GAPDH* and *HPRT* were used to normalize the expression levels of the genes studied, no significant differences were noted in the reduction of *SNAI1* expression at the mRNA level (Fig. 5D). Further analysis of the expression levels of *GAPDH*, *CDH1* and *SNAI1* genes normalized against β-actin demonstrated that *GAPDH* expression was 2.6-fold higher in HaCaT, 1.6-fold higher in HeLa, 3.3-fold higher in SiHa and 3.8-fold higher in Ca Ski cells

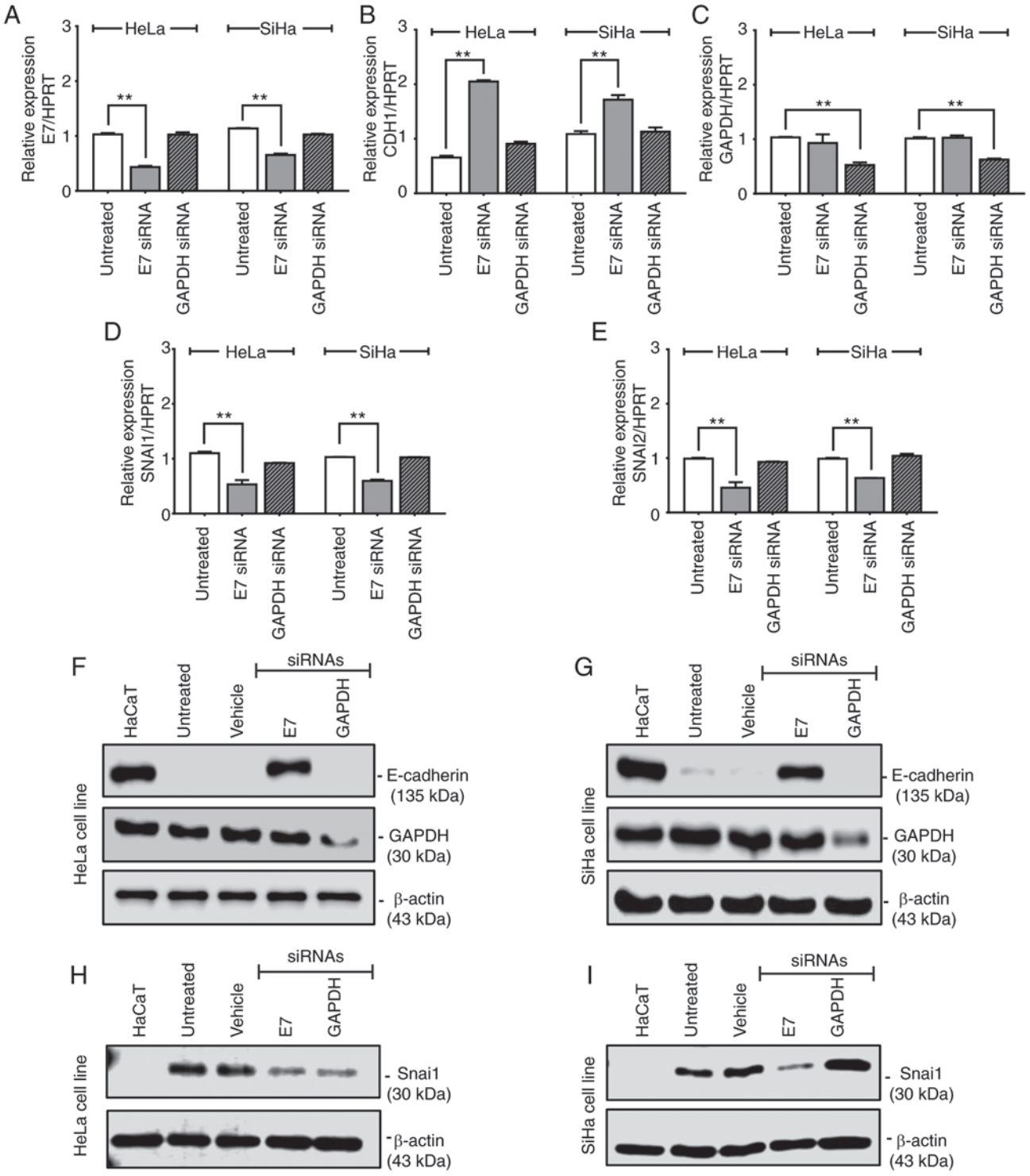

Figure 5. Silencing of E7 from HPV18 and HPV16 modifies the expression of *CDH1* and *SNAI1* genes in HeLa and SiHa cell lines. (A-E) The relative mRNA expression levels of (A) *E7*, (B) *CDH1*, (C) *GAPDH*, (D) *SNAI1* and (E) *SNAI2* in HeLa and SiHa cell lines following transfection with siRNA against *E7* and *GAPDH*. **P<0.01 vs. untreated cells. E-cadherin, Snai1, GAPDH and β-actin protein expression levels in (F and H) HeLa and (G and I) SiHa cells following transfection with siRNA targeting *E7* and *GAPDH*. *CDH1*, cadherin 1; siRNA, small interfering RNA; HPV, human papilloma virus.

compared with a commercial sample of RNA extracted from normal cervical tissue (Fig. S2).

Discussion

HR-HPV activates the cell methylation machinery, which not only methylates its own genome, but also the promoter regions of cellular genes (21). Laurson *et al* (26) have demonstrated that E7 induces the expression of Dnmt1 and suppresses the expression of *CDH1*. Reduction of E-cadherin has been reported to contribute to the persistence of HPV, which is in agreement with reports that E7 interacts with and induces the expression of Dnmt1 and triggers its *de novo* methylation activity (16) (Fig. 6A). The results of the present study agree

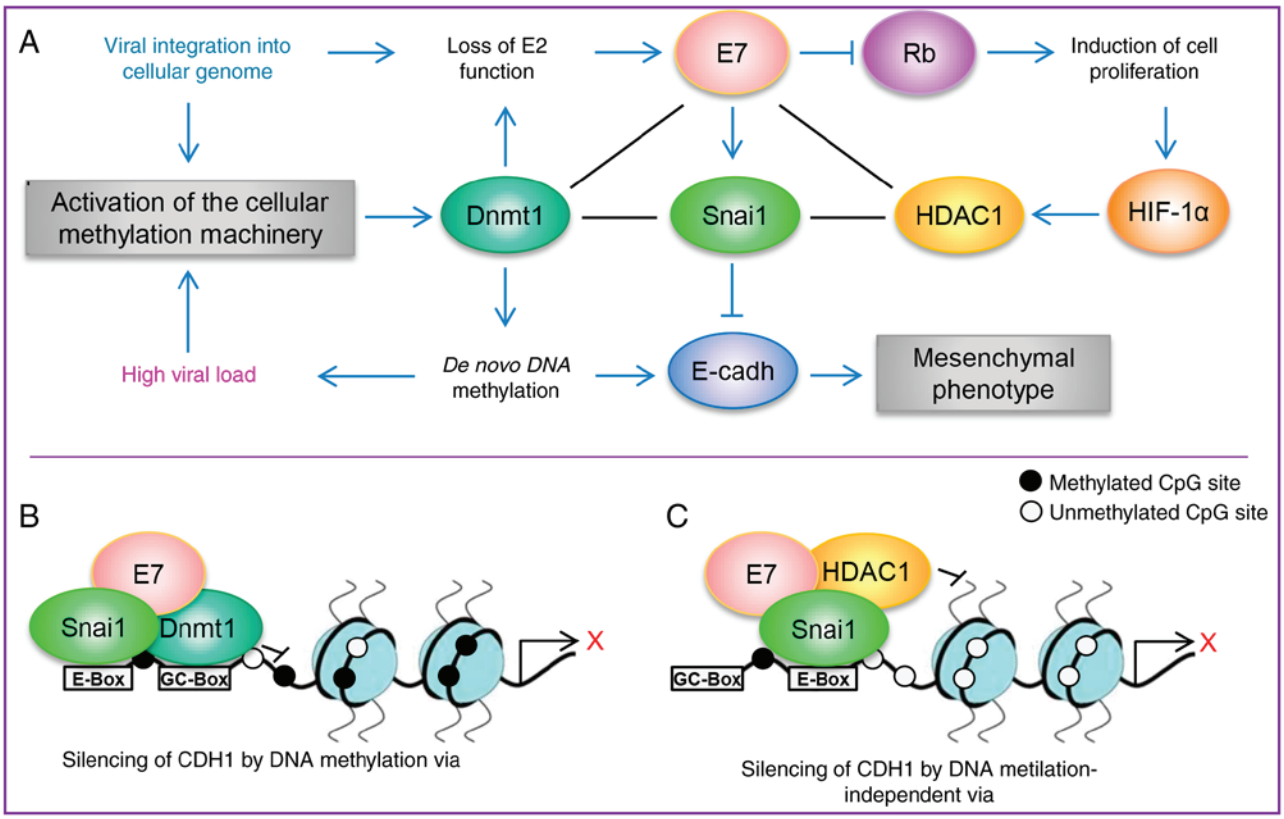

Figure 6. A schematic model demonstrating how high-risk HPV E7 may induce epithelial-mesenchymal transition via Snai1. (A) A schematic model of proteins that interact with or are induced by E7. The loss of E2 function, either from the integration or methylation of the E2 binding sites in the HPV long control region, leads to the dysregulated expression of the oncoproteins E6 and E7. E7 serves an important role in regulating several pathways; E7 induces cellular proliferation via pRB and E7 not only induces the expression of Dnmt1 and HDAC1, but also physically interacts with them, which promotes epigenetic regulation of HPV and cellular genes. The results of the present study indicated that E7 may not only suppresses the expression of *CDH1* through the methylation of its promoter region, but also induce the expression of Snai1, which is a negative regulator of *CDH1* expression. (B and C) Proposed models of how E7 may regulate the expression of *CDH1*. HPV, human papilloma virus; *CDH1*, cadherin 1; Dmnt1, DNA methyltransferase 1; HDAC1, histone deacetylase type 1; HIF-1α, hypoxia-inducible factor 1α; pRB, retinoblastoma protein.

with previous findings by Laurson *et al* (26), in which E7 from HPV16 suppressed the expression of *CDH1*. However, in this previous study, no significant differences were observed in the methylation pattern of the *CDH1* promoter of NIKS cells overexpressing E7, as the 17 CpG sites analyzed were not methylated (23). In addition, treatment with 5-AzadC re-established the expression of *CDH1* at the mRNA and protein levels (26); however, the concentration of 5-AzadC used was not stated and the changes in the expression of other cellular genes were not discussed.

The present study evaluated the re-expression of *CDH1* at the mRNA and protein levels in HeLa and SiHa cell lines treated with 5-AzadC and TSA, as well as changes in the methylation pattern of the promoter region of *CDH1*. Additionally, although TSA is not a demethylating agent, demethylation in the promoter of *CDH1* following treatment with TSA was observed. This result was an agreement with previous studies that have reported that TSA induces DNA demethylation and proposed that a change in chromatin modification, including the deacetylation of histones induced by an HDAC inhibitor, such as TSA, may render a gene susceptible to DNA demethylation (58,59).

In addition, an increase in *SNAI1* mRNA level was observed after treatment with 500 nM TSA in HeLa cells, whereas in SiHa cells an increase in *SNAI1* mRNA level was observed after treatment with 10 μM 5-AzadC and 500 nM TSA; however, no apparent changes were observed in SNAI1 protein level in the two cell lines. This result was in agreement with studies in which such treatments not only re-established the expression of *CDH1*, but also induced upregulation of *SNAI1* and *SNAI2* at the mRNA level (60,61), which was likely due to modifications in the methylation pattern in the promoter regions of *SNAI1* and *SNAI2* (62). The role of *SNAI1* regulation by epigenetic mechanisms is largely unknown. A previous study has demonstrated that the treatment with 5-AzadC in fibroblast cell IMR90, induced pluripotent stem cells from IMR90, BeWo and HTR8/SVneo cell lines induces a greater expression of *SNAI1* and *SNAI2* at the mRNA level and that the regulation of the two genes is mediated by DNA methylation of their first intron and not due to DNA methylation of their promoter region; however, this previous study did not determine the expression of these genes at the protein level (62). The present study did not determine the methylation status of the *SNAI1* promoter region as it is transcriptionally active in HeLa and SiHa cells. The differences observed in the effect on SNAI1 expression at the mRNA and protein levels by treatment with 5-AzadC and TSA indicated that other factors may regulate the expression of SNAI1 in the two cell lines.

On the other hand, the results published by Laurson *et al* (26) suggested that in the NIKS-cell model, suppression of *CDH1*

expression by E7 was independent of the methylation status of the *CDH1* promoter region, which was observed in the SiHa cell line in the current study. The previous study also suggested that repression of *CDH1* may be regulated via *SNAI2* (*SLUG*); however, it was reported that the expression of *SNAI2* was not altered by the presence of E7 (26). The results of the present study revealed that while *SNAI1* mRNA and protein was expressed in HeLa and SiHa cells, *SNAI2* mRNA expression was barely detectable in HaCaT and Ca Ski cells. *SNAI2* has been demonstrated to be upregulated in HaCaT cells during the process of cell motility and wound-healing (63).

The results of the present study demonstrated that following silencing of *E7* from HPV16 and HPV18, *CDH1* expression was recovered in HeLa and SiHa cells, which is in agreement with a previous study by Caberg *et al* (25). This previous study reported that following 24-h transfection of SiHa cells with siRNA against HPV16 *E7*, *CDH1* was upregulated and an increase of the Retinoblastoma protein (pRB), which is responsible for a major G1 check point, blocking S-phase entry and cell growth, and activating protein 2α were detected without changes in the mRNA expression levels of *SNAI1* and *SNAI2* (25). By contrast, the present study demonstrated that following 48-h transfection with siRNA against *E7*, changes in the methylation pattern of the *CDH1* promoter region were observed in HeLa and SiHa cells. In addition, an increase in the mRNA and protein expression levels of *CDH1* were identified, as well as a decrease in the mRNA and protein expression levels of *SNAI1* and *SNAI2*. Therefore, the current results suggested that E7 not only suppressed the expression of *CDH1* via the methylation of its promoter, but also regulated the expression of *SNAI1*, a negative regulator of *CDH1* involved in EMT and associated with metastasis (27,55-57). These observations are also concordant with the mechanism of action reported for other oncogenic viruses, where the X protein of HBV (HBx), core protein of HCV and latent membrane protein 1 (LMP1) of EBV promote EMT and metastasis by inducing the expression *SNAI1* and suppressing *CDH1* expression (64-67).

Following silencing *GAPDH* with siRNA, *SNAI1* expression was partially suppressed in HeLa cells at the protein level, but not at mRNA level. This was consistent with a previous study that demonstrated that the interaction of GAPDH with Sp1 resulted in increased expression of Snai1, which promoted the proliferation and metastasis of cancer cells, and that suppression of *GAPDH* with shRNA resulted in a significant decrease of Snai1 in the HCT116 and LoVo cell lines (68). This suggested that GAPDH may serve a role in the metastasis of cervical adenocarcinoma (HeLa) by affecting EMT through the upregulation of Snai1 expression mediated by Sp1, similar to its role reported in colon cancer (68), but further studies are required to verify this.

It is currently unknown how HPV may activate the expression of *SNAI1*; however, it has been reported that in other cancer types associated with virus, HBx, core and LMPI proteins increased *SNAI1* expression through the activation of the PI3K/Akt and MAPK pathways by transforming growth factor-β (TGF-β) action (64,69-72), which is in agreement with a previous study by Peinado *et al* (73) suggesting that TGF-β induces *SNAI1* transcription through MAPK and PI3K.

In the present study, the signaling pathways involved in regulating *SNAI1* expression were not determined; however, previous studies have reported that HR-HPV infection activates the PI3K/Akt/mTOR pathway (74), E7 from HPV upregulates Akt activity though the pRB protein (75) and TGF-β stimulates EMT and tumor invasion in SiHa cells (76).

In summary, the results of the present demonstrated that HR-HPV E7 may regulate the expression of *CDH1* by two different pathways, in which Snai1 is involved. The first pathway involves hypermethylation of the *CDH1* promoter region and the expression of Snai1, as observed in the HeLa cell line. The second pathway involves hypomethylation of the *CDH1* promoter region with expression of Snai1 as observed in SiHa cell line, suggesting that *CDH1* and *SNAI1* may be considered as biomarkers of metastasis in uterine cervical cancer. Therefore, based on the present results and previous evidence that E7 interacts with Dnmt1 and HDAC1 (15-17), it would be beneficial to determine if E7 from HR-HPV may interact with Snai1 to form a co-repressor complex with either Dnmt1 or HDAC1 in the *CDH1* promoter (Fig. 6B and C), which may explain the suppression of *CDH1* expression during the EMT process.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

PRC performed all experiments, interpreted the data and wrote the manuscript. VAV, GDBO and CCPM performed western blot analysis and interpreted the data. AC and AGC conceived and designed the study, interpreted the data and

edited the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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