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Epigenetic repression of E-cadherin by human papillomavirus 16 E7 protein

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A common feature shared between several human cancer-associated viruses, such as Epstein-Barr virus, Hepatitis B virus and Hepatitis C virus, and Human papillomavirus (HPV) is the ability to reduce the expression of cellular E-cadherin. Since E-cadherin is used by Langerhans cells to move through the stratified epithelium, its reduction may affect the efficiency by which the immune system responds to HPV infection and the length of persistent HPV infections. We observed that the E7 protein of this virus (HPV16) is most efficient at reducing E-cadherin levels. This E7 activity is independent of retinoblastoma protein or AP-2α degradation. Instead it is associated with augmentation of cellular DNA methyltransferase I (Dnmt1) activity. Significantly, inhibition of Dnmt activity re-established E-cadherin levels of the cells, presenting the possibility that similar epigenetic intervention clinically may be a way to re-establish the influx of Langerhans cells into infected epithelium to counteract HPV persistence.

Introduction

Infection of the cervical epithelium by high-risk human papillomaviruses (HPVs) can predispose this tissue to subsequent development of cancer (1,2). Activities of HPV proteins that can cause this have been elucidated and they include the degradation of p53 tumour suppressor protein, expression of the catalytic subunit of telomerase and inactivation or destruction of the retinoblastoma protein (pRb) protein. The first two activities are carried out by the viral E6 protein, whereas activities against the pRb are elicited by the E7 protein (3–7). While the potential contribution of E6 and E7 proteins to the development of cervical cancer is clear, infection by high-risk HPVs do not automatically result in cancers, as these viruses are usually cleared from the tissue after some time. Failure to clear the virus, however, would allow HPV to persist in the cervical epithelium and increase the likelihood of the eventual development of cervical cancer (8–11). Clearance of HPV may be elicited in part by the natural differentiation, migration and exfoliation of infected keratinocytes from the cervical epithelium. In addition to this, the host's immune system also has an important role to play in viral clearance (12,13). Langerhans cells infiltrate and move within the epithelium and participate in detecting, processing and presenting foreign antigens to other immunocytes of the host (14,15). Interestingly, numerous independent investigations have revealed that HPV-infected cervical epithelia possess fewer Langerhans cells than the uninfected neighbouring tissues, suggesting that HPV creates a defined zone that is refractive to Langerhans cells influx within an otherwise healthy epithelium (16–27). Migration of Langerhans cells is influenced by cytokines such as tumour necrosis factor α (28,29), granulocyte-macrophage colony stimulating factor (30,31) and interleukin-10 (32) and chemokines including RANTES and MIP3α (33,34), which are produced by keratinocytes (35). HPV-containing cells were reported to have reduced expression of granulocyte-macrophage colony stimulating factor (31) and clinical lesions containing HPVs are reported to have altered levels of various cytokines which is unfavourable for the activation of the immune system (36). In addition to stimulatory signals, migration of Langerhans cells requires E-cadherin proteins to be present on the membrane of keratinocytes (21,29,37,38). Hence it is of particular significance that the amount of E-cadherin protein in HPV-infected tissues is significantly reduced (21,39–49) or its distribution altered (21,39–42,44,45,48–53).

Using the keratinocyte cell line NIKS, which was derived from human foreskin (54), we generated cells that harboured replicating HPV16 episomes that were able to persist in culture (reminiscent of a persistent infection) (55). Analyses of these cells revealed that the level of their E-cadherin protein was indeed reduced, as is seen in naturally infected cells of the cervical epithelium. However, cells that harboured HPV16 DNA mutant episomes, which do not express E7 protein (as a result of a stop codon within the E7 region) did not exhibit any significant reduction in the E-cadherin protein levels, demonstrating that it is the E7 protein that is predominantly responsible for this. The E7 protein does not target E-cadherin proteins for proteolytic degradation, and its ability to degrade pRb and AP-2α are not associated with E-cadherin reduction. Instead, the E7 protein augments the amount and activity of Dnmt1 in the cell, and this in turn causes a reduction in the transcription of the E-cadherin gene. These observations uncover yet another activity of HPV16 E7, which in this instance is one that works at the epigenetic level to contribute to the successful persistence of HPV in the infected epithelium. Importantly, inhibition of Dnmt activity re-established the level of E-cadherin expression of the host cell, suggesting that such epigenetic intervention to recover E-cadherin expression may encourage re-infiltration of Langerhans cells back into HPV-infected regions of the epithelium.

Abbreviations: Dnmt1, DNA methyltransferase I; EBV, Epstein-Barr virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HPV, human papillomavirus; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; pRb, retinoblastoma protein.

Materials and methods

Cell culture

NIKS cells provided by Dr Paul Lambert were cultured in F-medium (three parts F-12 Ham:1 part Dulbecco's modified Eagle's medium, 5% foetal calf serum, 24 μg/ml Adenine, 8.4 ng/ml cholera toxin, 5 μg/ml insulin, 0.4 μg/ml hydrocortison and 10 ng/ml epidermal growth factor). 3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum. Briefly, NIKS were grown in 10 cm plates containing 2 million gamma-irradiated J2-3T3 cells. To generate NIKS cells harbouring episomal HPV16 DNA, NIKS cells plated the day before at 0.5 million in a well of a six-well plate were transfected with 0.8 μg of recircularized HPV16 DNA and 0.2 μg of pCDNA6A (Invitrogen, Carlsbad, CA). The next day, the transfectants were passed into 10 cm plates with 2 million irradiated blasticidin-resistant J2-3T3. After attachment of cells on the plate, blasticidin was added into the medium to a final concentration of 8 μg/ml. Antibiotics selection was stopped when all the untransfected cells in the control plate were dead.

Plasmids, recircularization of HPV16 DNA and in vitro mutagenesis

The pSPW12 plasmid, from which recircularized HPV16 DNA was generated, was provided by Dr Margaret Stanley. Five micrograms of pSPW12 was digested with BamHI to release the full-length HPV16 DNA, followed by a ligation reaction with 2000 U of New England Biolab's T4DNA Ligase in

a volume of 2 ml at 16°C overnight to recircularize the viral DNA. The recircularized DNA was purified and concentrated using the QIAGEN mini-prep kit according to the protocol provided. Mutagenesis to generate HPV16 DNA that cannot express the E7 protein was carried out using the Pfu Ultra polymerase (Stratagene, La Jolla, CA) according to the protocol provided by the manufacturer. The mutagenesis primers used were forward primer acattg-catgaatattagttagatttgcaacc and reverse primer ggttgcaaatctaactaatattcatgcaatgt were used to introduce a stop codon at nucleotide position 595 of the HPV16 genome. The whole mutant viral DNA was sequenced to verify the integrity of the entire HPV16 sequence.

Retrovirus production

Retrovirus vectors (LXSN, LXSN16E6, LXSN16E7 and LXSN16E6/7) were kindly provided by Dr Denise Galloway. To produce retroviruses, these vectors were transfected into Phoenix A cells (kindly provided by Dr Nolan), and the medium of the cells harvested 48 h later and filtered through 0.2 μ filters. Aliquots were prepared and stored in −80°C until use. For infection, viruses were mixed with polybrene at a concentration of 10 μg/ml and layered onto the cell monolayer. Neomycin was used at 500 μg/ml concentration for selection.

DNA extraction and Southern blotting

DNA from cells was extracted using the QIAamp kit from QIAGEN (Valencia, CA) according to the protocol provided by the manufacturer. The eluted DNA was separated on a 1% agarose gel and blot transferred to nylon filter and probed with ^{32}P-labelled HPV16 DNA probe. After hybridization, the filter was washed and exposed to X-ray film or a phosphorimager screen and analysed.

RNA extraction, reverse transcription and quantitative polymerase chain reaction

J2-3T3 feeder cells were dislodged from the plate by forceful squirting with phosphate-buffered saline (PBS). Keratinocytes were trypsinized and the cell pellet treated according to the protocol supplied by the RNeasy kit from QIAGEN. The quantity of the resulting nucleic acids were measured and 4 μg were transferred into a tube for DNaseI digestion according to the protocol supplied with the Ambion DNase away kit, The resulting RNA were reverse transcribed with Superscript polymerase (Invitrogen) and subjected to quantitative polymerase chain reaction (PCR) analyses using primers to the E-cadherin complementary DNA (forward GGTTATTCCTCCCATCAGCT and reverse CTTGGCTGAG-GATGGTGTA) and beta-actin complementary DNA (forward TGGGCATGGGT-CAGAAGGAT and reverse CGGCCAGAGGCGTACAGGGA).

Protein extraction and western blotting

Prior to harvesting NIKS cells for proteins, the irradiated J2-3T3 cells on the plate were dislodged by squirting PBS on them. After verification of their removal by inverted microscopy, the remaining NIKS cells were scraped off the plate into a 1.5 ml vial and centrifuged at 6000 r.p.m. (4°C) on a bench top microcentrifuge for 2 min. The supernatant was removed and the cell pellet was resuspended in ice-cold 200 μl of Reporter Lysis Buffer (Promega, Madison, WI) supplemented with protease inhibitor cocktail (Bio-Rad, Hercules, CA). The resuspended cells were sonicated at 30% power for 10 s from a Branson sonicator. After 15 min incubation on ice, followed by centrifugation at 15 000 r.p.m. at 4°C for 20 min, the clarified cell lysate was collected and protein was assayed by the Bradford method and stored at −70°C. Proteins were separated on 8, 10 or 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred onto polyvinylidene difluoride membrane, blocked in 5% milk in PBS–0.5% Tween 20 and probed with appropriate primary and secondary antibodies.

Dnmt assay

Feeder cells were removed from the plate by squirting PBS on them. Cells were then trypsinized, washed once with PBS and resuspended in 1–2 vol of lysis buffer (50 mM Tris pH 7.8, 1 mM ethylenediaminetetraacetic acid, 20 mM beta-mercaptoethanol, 10% glycerol, 1% Tween 80 and 60 μg/ml phenylmethylsulfonyl fluoride) followed by three rounds of freeze-thawing and sonication at 35% intensity for 10 s. After centrifugation at 13 000 r.p.m. in a microcentrifuge at 4°C, the clarified cell lysate was collected and the protein concentration measured by the Bradford method. Five micrograms of protein lysate was used in the assay which contained 0.5 μg poly(dI-dC) and 3 μCi of *S*-adenosyl-L-[methyl-^{3}H] methionine in a final volume of 20 μl. The mixture was incubated for 2 h at 37°C, after which the reaction was stopped by placing the tubes in ice water. Two microlitres of 5 mg/ml Herring sperm DNA was added and the whole mixture subjected to purification with QIAGEN mini-prep kit according to the protocol provided. DNA was eluted in 50 μl of elution buffer, followed by ethanol precipitation of the DNA. The resulting DNA pellet was washed twice with 1 ml 70% ethanol and finally resuspended in 20 μl of water and spotted onto GF/C filter. After addition of 10 ml of scintillation fluid, the samples were counted in a scintillation counter.

Bisulphite conversion and sequencing of the E-cadherin promoter region

DNA from cells was extracted using QIAmp kit from QIAGEN according to the protocol provided by the manufacturer. Five hundred nanograms of DNA was bisulphite converted using EZ DNA Methylation-Gold Kit from Zymo Research (Orange, CA). DNA was mixed with CT conversion reagent containing sodium hydroxide for DNA denaturation and sodium bisulphite for sulphonation of C6 of the pyrimidine ring. Reaction was carried out in a thermal cycler at 98°C for 10 min, followed by 64°C for 2.5 h. The converted samples were loaded into the provided columns that were prepared with M-binding buffer containing guanidine hydrochloride to act as a chaotropic reagent. The column was washed with M-Wash buffer and then incubated with M-Desulphonation Buffer (containing sodium hydroxide to provide hydroxide ions for removal of the sulphite group from the pyrimidine ring) for 15 min at room temperature. The column was then washed another two times and DNA was eluted in 10 μl and stored at −70°C. Seventy-five nanograms of DNA was used for the first PCR with the following primers whose sequences were based on the E-cadherin promoter sequence in Genebank with the accession number of L34545. The positions of these primer sequences were based on those reported by Reinhold *et al.* (56). Some modifications were introduced to the reported sequences in order to minimize difference in the annealing temperatures between the primers: forward primer, TTGATTTTAGGTTTTAGT-GAGTT (sequence position −399 to −377) and reverse primer, AACTCCAAAAACCCATAACTAA (sequence position −6 to +16). These primers were used to amplify a fragment of 415 bp. The PCR cycles were 94°C for 2 min followed by 35 cycles of 94°C for 1 min, 55°C 1 min and 72°C for 1 min and ended with 72°C for 2 min. One microlitre of the first PCR was then removed and used for nested PCR using the following primers: forward nested primer, GTAAAACGACGGCCAGTTATTTAGATTTTAGTAATTTT (sequence position −319 to −299) and reverse nested primer, GGAAACAGC-TATGACCATGAACTCCAAAAACCCATAACTAA, which annealed to the 3' terminus of the first PCR product. The resulting nested PCR product contains all 25 CpGs in the minimal E-cadherin promoter region (56) and an additional four CpGs in the 3′end. The PCR product was separated on a 1% agarose gel and the expected DNA fragment of ∼335 bp was excised from the gel and purified using QIAquick Gel Extraction kit from QIAGEN according to the protocol provided by the manufacturer. The PCR product was then ligated into the pGEM-T Easy vector system from Promega and transformed into XL1-Blue Supercompetent Cells from Stratagene. The cells were then plated onto agar containing ampicillin and X-gal overnight and single white colonies were picked for culture in Luria–Bertani broth containing ampicillin. QIAprep Spin Miniprep kit from QIAGEN was used to isolate the plasmid DNA. The DNA preparations were digested with EcoRI to release the cloned PCR fragment and separated on an agarose gel to verify fragment size. Individual plasmid clones obtained from NIKS and NIKS + HPV16 were sent to Geneservice for sequencing using T7 primers. Secondary structure protocol sequencing was required in order to obtain complete sequences of the samples. The sequences were aligned using SeqMan software and all cytosines were analyzed for conversion status. Assuming that CpA, CpC and CpT (collectively referred to here as CpN) are not methylated, they are expected to be converted by the bisulphite reaction. As such, efficiency of the conversion reaction can be ascertained by dividing the number of converted CpN by the total number of CpN within the sequence, and this gave conversion rates of 98.8% for NIKS and 99.3% for NIKS + HPV16 sequences. Furthermore, we ascertained that unconverted DNA (not put through the bisulphite reaction) could not be amplified with the primers used above. Conversely, primers designed to amplify unconverted DNA from the exact region of the E-cadherin promoter above cannot amplify bisulphite converted DNA (data not shown). The efficiency and completeness of the bisulphite conversion reaction is demonstrated by the fact that DNA sequencing of the converted and cloned PCR fragments revealed that virtually all (98.8 and 99.3%) the cytosines of CpNs were converted to thymines in the individual clones. To control for the absence of over-conversion, we methylated the CpGs of the DNA *in vitro* with SssI methyltransferase and then subjected it to bisulphite conversion in parallel with the test reactions and the protocol described above. Sequencing of these DNA revealed that while the cytosines of CpN were converted, those of CpGs were not (data not shown). Collectively, these controls demonstrate that the bisulphite conversion was efficient and specific (with no over-conversion).

Results

Persistence of HPV16wt episomes and HPV16E7-null episomes in NIKS cells

In order to generate an *in vitro* culture system to study long term HPV persistence, NIKS cells, which were derived from foreskin

keratinocytes, were employed as they are capable of supporting stable HPV DNA replication (54,55). HPV16 DNAs excised from plasmids were recircularized and co-transfected into these cells with pcDNA6 plasmids, which express blasticidin-resistance protein. Following antibiotics selection, cells were passaged at least 10 times and at each passage, DNAs were extracted from an aliquot of cells for analyses by Southern blotting. As shown in Figure 1a HPV16 episomes persisted in these cells without any apparent loss or gross reduction of copy number through all the passages. In parallel, recircularized HPV16 genomes with a stop codon inserted in either the E6 or E7 open reading frame were also put through the process described above. While the E6-null HPV genome failed to persist (data not shown), the E7-null HPV16 genome readily persisted in these cells (Figure 1b). The unexplained requirement for E6 in HPV16 persistence is consistent with similar observations in several previous reports (57,58). These lines of cells allowed us to address, *in vitro*, the above-mentioned phenomenon of reduction of E-cadherin levels in HPV-containing epithelial lesions.

Differences in E-cadherin protein levels in NIKS, NIKS HPV16wt and NIKS HPV16E7null cells

When protein lysates from NIKS cells and those that harboured wild-type HPV16 episomes were analyzed, it was apparent that the level of E-cadherin was significantly reduced in the latter, whether they were in a state of confluence or not (Figure 2a). This is reminiscent of HPV-containing cervical lesions, which were reported to have reduced expression of E-cadherin (21,39,42,44,45,48,49). However, when extracts from two independently generated lines of NIKS cells bearing HPV16E7-null episomes were tested, no appreciable reduction of the E-cadherin levels was apparent (Figure 2b). Detection of E7 protein exclusively in cells harbouring the wild-type HPV genomes attests to the fact that the E7-null mutant genomes were truly not expressing E7 proteins. The reduced levels of p53 protein in cells bearing wild-type or E7-null viral DNA is indicative of E6 expression, as HPV16 E6 is able to target p53 protein for proteasome-mediated degradation. These analyses confirmed that wild-type HPV16 DNA expressed both, the E6 and E7 proteins, whereas the HPV16 E7-null episomes expressed only the E6 protein. As such, these results show that HPV16 E7 protein was responsible for the reduction of E-cadherin levels in cells that harboured HPV16 episomes.

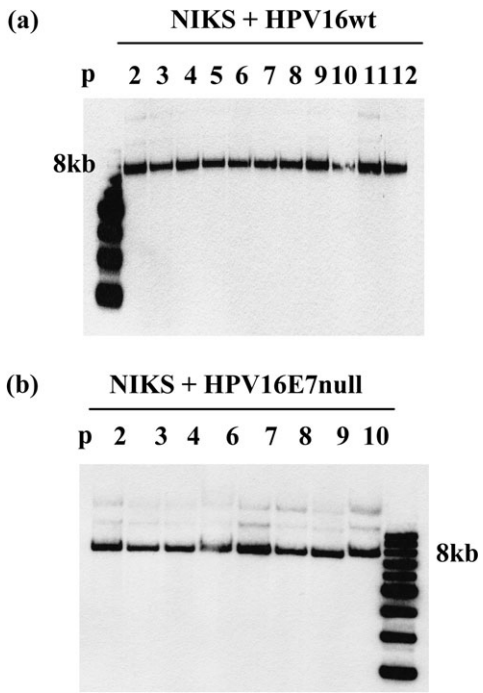

Fig. 1. Southern blot analyses of DNA extracted from various passages (p) of (**a**) NIKS cells that harbour wild-type HPV16 DNA and (**b**) NIKS cells that harbour E7null HPV16 episomes. The blots were probed with full-length HPV16 DNA. The size marker used was a 1 kb supercoiled DNA ladder.

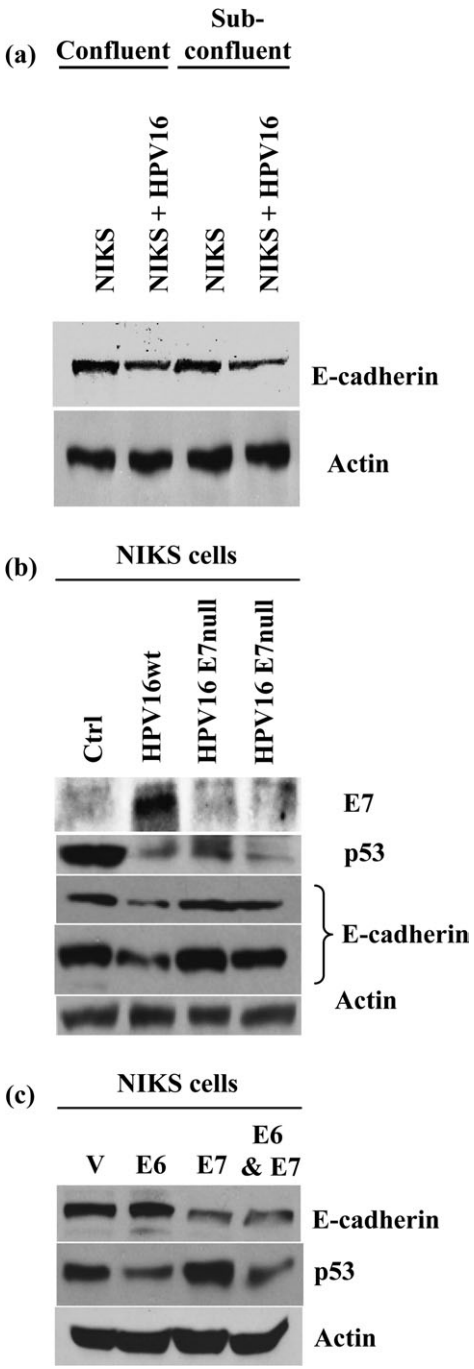

Fig. 2. (**a**) Western blot of proteins extracted from NIKS and NIKS-bearing episomal HPV16 DNA. Lysates were prepared from either confluent or subconfluent cells. The membrane was probed with antibodies against E-cadherin or actin. (**b**) Lysates of NIKS cell, NIKS carrying wild-type episomal HPV16 DNA and two independently derived NIKS lines harbouring E7null HPV16 episomes were analysed by western blotting using antibodies against HPV16 E7, p53, E-cadherin and actin. Two independent analyses of E-cadherin of these cells are shown. (**c**) Western blots of protein lysates from NIKS cells that were infected with LXSN retrovirus (vector), LXSN E6, LXSN E7 or LXSN E6/7, after selection with neomycin. Antibodies against E-cadherin, p53 and actin were used.

HPV16 E7 alone is responsible for the reduction of E-cadherin protein level in cells

While the evidence is sufficiently compelling to suggest that the E7 protein is responsible for the reduction of E-cadherin protein level, it does not exclude the possibility that the E6 protein might also be required for this activity. This possibility is particularly pertinent in view of a report that demonstrated HPV16 E6 to reduce E-cadherin levels (41). In this light, it is conceivable that E6 and E7 proteins may act together to bring about this effect. To test this, NIKS cells were infected with retroviruses bearing E6, E7 or E6 plus E7 (E6/7) genes. Infected cells were selected with neomycin and the surviving infectants were harvested and their E-cadherin proteins analyzed (Figure 2c). The E-cadherin level in E6-expressing cells was comparable with that of control cells. However, E7 and E6/7 infectants exhibited markedly reduced levels of E-cadherin protein. Consistent with what has been reported regarding the effects of E6 and E7 proteins on p53; the p53 protein level in E6-expressing cells was reduced (5), whereas that in E7-expressing cells was increased (59). These results show that HPV16 E7 alone is able to reduce E-cadherin protein levels, and the absence or presence of E6 has no perceivable impact on this activity. It must be noted that in the multiple repeats of these experiments, there were occasions when the E6 protein alone was seen to slightly reduce E-cadherin protein level. However, unlike E7-induced reduction of E-cadherin, the E6-induced reduction is modest and curiously inconsistent. This erratic effect of E6 is apparent in some results in the following sections below.

HPV16 E7 protein does not target E-cadherin protein for degradation

To ascertain how HPV16 reduces E-cadherin level, we first tested the possibility that E-cadherin may be targeted for proteasomal degradation. Cells were either mock treated or treated with proteasome inhibitor ALLN, and the proteins from these cells were analyzed by western blotting (Figure 3a). While the E-cadherin protein levels in NIKS cells were augmented upon proteasome inhibition, E-cadherin protein level in cells that harboured HPV16 DNA was not altered. That the proteasome was indeed inhibited was attested by the fact that ALLN treatment increased the p53 protein levels in all the cells as p53's degradation is proteasome mediated. This result suggests that HPV16 does not reduce E-cadherin levels in cells by channelling E-cadherin protein for degradation by proteasome.

HPV16 E7 suppresses transcription of E-cadherin gene

We set out to test the alternative possibility that HPV16 may repress the expression of the E-cadherin gene instead. RNA isolated from NIKS cells, NIKS bearing HPV16DNA and NIKS cells infected either with control or E7 retroviral vectors were reverse transcribed and the complementary DNA of E-cadherin and that of beta-actin were measured using quantitative PCR. The results in Figure 3b show that the amount of E-cadherin transcripts (normalized against beta-actin transcripts) in cells harbouring HPV16 episomes or expressing E7 was markedly reduced. Although this result does not exclude the possible involvement of non-proteasomal degradation of E-cadherin, it nevertheless demonstrates that repression of gene expression is a means by which HPV16 reduces E-cadherin levels in the cell.

Reduction of E-cadherin protein level by HPV16 E7 is independent of pRb degradation or AP-2α downregulation

The best-characterized activity of HPV16 E7 is pRb degradation. As pRb protein has a profound effect on the expression of many cellular genes, it is possible that E7's repressive effect on E-cadherin expression is mediated via pRb degradation. Indeed, a recent report alluded to the possibility that degradation of pRb and AP-2α is responsible for repressing E-cadherin expression (60). Immunoblotting with antibodies against pRb and AP-2α revealed that while pRb level was clearly reduced in HPV16-containing cells, the level of AP-2α protein remained unchanged between NIKS and NIKS + HPV16wt, in spite of a very clear reduction of E-cadherin level in the latter cells (Figure 4a). This observation excludes AP-2α degradation as a means

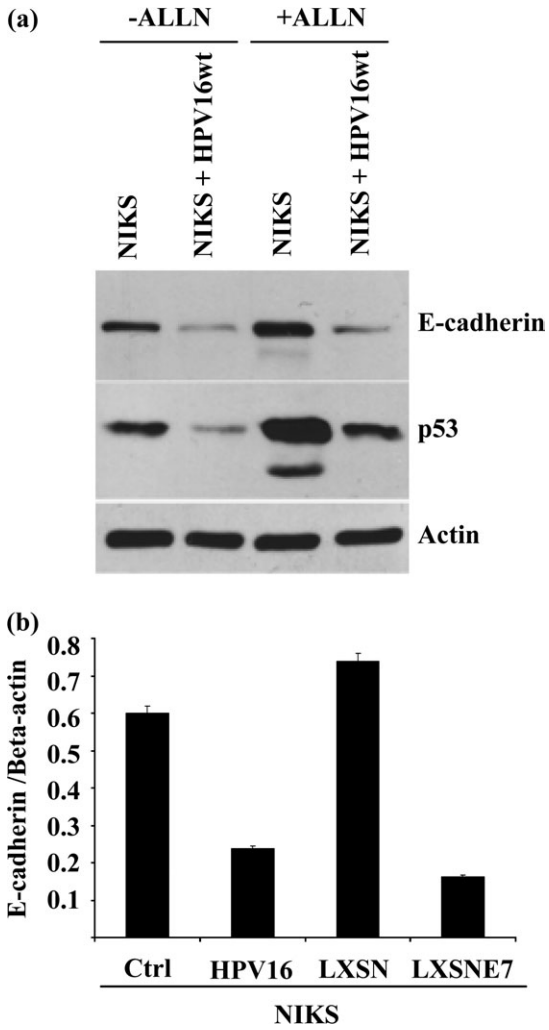

Fig. 3. (**a**) Analyses of E-cadherin protein in NIKS and NIKS harbouring wild-type HPV16 episomes, with (+) and without (−) treatment with the proteasome inhibitor, ALLN. Actin served as loading control and p53 served to indicate that the proteasome was indeed inhibited by the compound. (**b**) Quantitative PCR measurements of E-cadherin transcripts in NIKS (ctrl), NIKS containing HPV16 episomes (HPV16), NIKS infected with empty retroviral vector (LXSN) or E7-contaning retroviral vector (LXSNE7). E-cadherin transcripts are normalized against beta-actin transcript levels.

by which E7 represses E-cadherin expression in our experimental system. To test whether pRb degradation by itself could reduce E-cadherin levels, we infected NIKS cells with recombinant retroviruses expressing short hairpin RNA against pRb. From Figure 4b, it is clear that although short hairpin RNA against pRb reduced the pRb protein level markedly, the level of E-cadherin was unchanged, indicating that degradation of pRb is not the means by which E7 represses E-cadherin expression.

HPV16 E7 augments the level of Dnmt1 in cells

In considering how E-cadherin expression can be suppressed by HPV E7, it is of particular interest to note how other viruses affect similar repression on the expression of this gene. In particular, the hepatitis B virus (HBV) also reduces E-cadherin expression in cells (61–63). This is achieved by the HBV X protein, which augments the level and activity of Dnmt1, which goes on to repress E-cadherin expression. As such we considered the possibility that the HPV16 E7 may also do likewise. Analyses of the Dnmt1 protein of NIKS cells and those that harbour HPV16 episomes revealed that HPV16 does indeed augment greatly the level of Dnmt1 (Figure 4c). Dnmt3a level was also

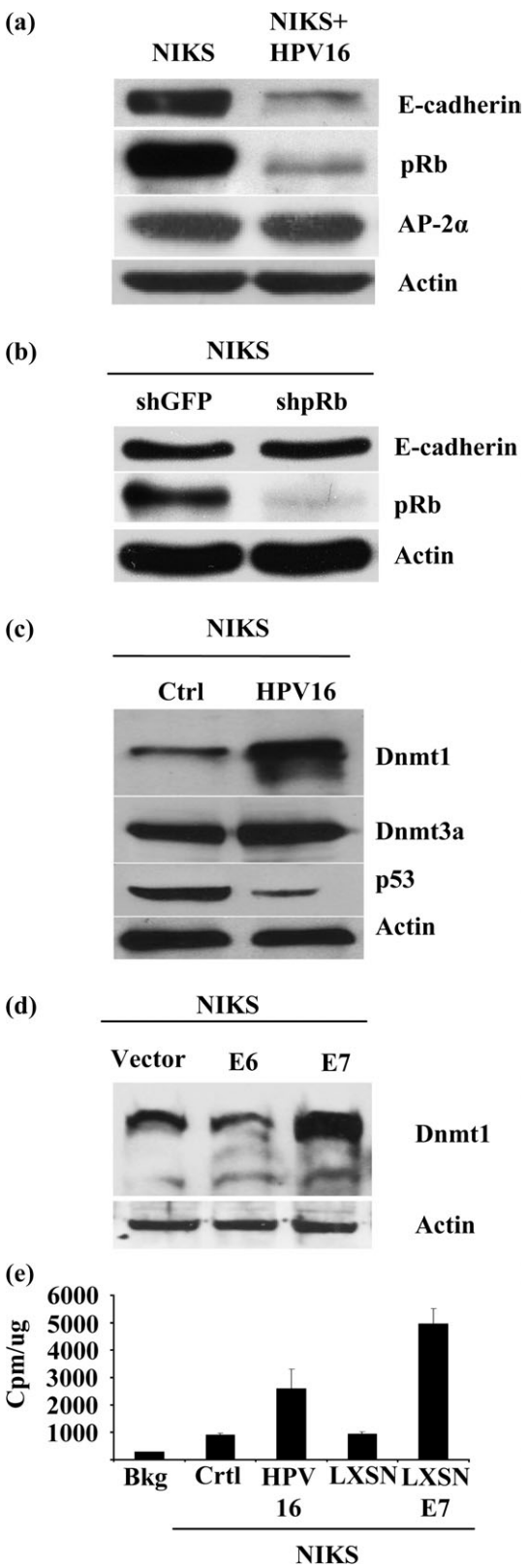

Fig. 4. (**a**) Western blot analyses of E-cadherin, pRb and AP-2α proteins from NIKS and NIKS + HPV16wt DNA. (**b**) Analyses of E-cadherin and pRb in cells infected with retrovirus-expressing shGFP or retrovirus-expressing short hairpin RNA against pRb (shpRb). Beta-actin was used as loading control. (**c**) Western blot analyses of Dnmt1, Dnmt3a, p53 and actin in protein extracts of NIKS (Ctrl) and NIKS harbouring episomal HPV16 DNA. (**d**) Analyses of Dnmt1 protein in extracts from NIKS cells infected with LXSN (vector), LXSNE6 or LXSNE7 retroviruses, after selection with neomycin. Actin serves as loading control. (**e**) DNA methyltransferase assay of proteins extracted from NIKS cells (Ctrl), NIKS cells harbouring HPV16 episomes, NIKS cells infected with empty retroviral vector (LXSN) or E7-bearing retroviruses (LXSNE7). Bkg is the background counts of the assay.

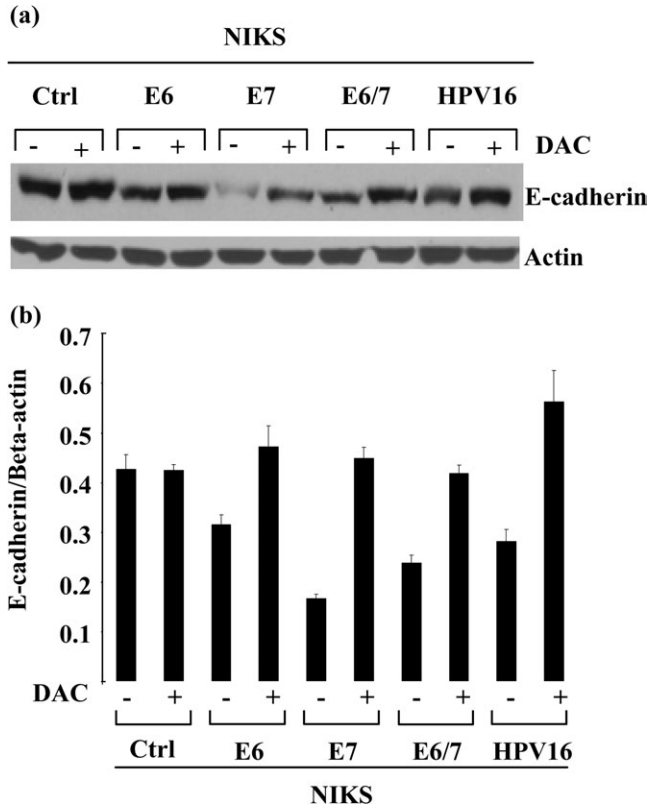

Fig. 5. Treatment of NIKS and various NIKS-derived cells with inhibitor of DNA methyltransferase, 5-aza-deoxycytidine (DAC). (**a**) Protein lysates were prepared and analysed on a western blot with antibodies against E-cadherin and beta-actin (as loading control). (**b**) RNAs were extracted from treated (+) or untreated (−) cells and E-cadherin and beta-actin transcripts were extracted and their relative amounts measured using quantitative PCR and normalized against beta-actin transcripts.

marginally increased, whereas the presence of Dnmt3b and Dnmt3L were below the level of detection (data not shown). By immunoblotting NIKS cells that were infected with either empty retroviral construct or those bearing the E6 or E7 genes, we observed that the E7 protein increased greatly the protein level of Dnmt1 (Figure 4d). To test whether the augmented levels of Dnmt1 protein affects Dnmt activity, we assayed the lysates of these cells and observed that HPV16 or HPV16E7 protein alone, both increased the activity of Dnmt in the cells (as shown in Figure 4e).

Inhibition of Dnmt activity allows recovery of E-cadherin levels

Augmentation of Dnmt activity by HPV16 is particularly relevant in the context of repression of E-cadherin expression. Many cellular promoters, including that of E-cadherin, are silenced when methylated by Dnmts. Indeed it is through such a mode of repression that E-cadherin levels are diminished by HBV (61–63), Hepatitis C virus (HCV) (64,65) and Epstein-Barr virus (EBV) (66–69). If augmenting Dnmt activity is relevant to HPV-mediated repression of E-cadherin, then inhibition of Dnmt would be expected to derepress E-cadherin expression in cells containing HPV DNA or HPV oncogenes. To test this, 5-aza-deoxycytidine or DAC, an inhibitor of Dnmt was fed to the cells. This resulted in the recovery of the E-cadherin protein levels in cells containing HPV16 DNA or expressing E7 protein (Figure 5a). This recovery was also evident at the transcription level when the RNA of

E-cadherin was measured (Figure 5b). Importantly, cells devoid of HPV or its oncogenes were refractive to 5-aza-deoxycytidine, confirming that the HPV-induced augmentation of Dnmt activity does indeed cause the repression of E-cadherin expression. The observations described hitherto demonstrate that HPV increases cellular Dnmt activity and this in turn is responsible for decreasing the E-cadherin gene expression. It does not, however, mean that Dnmt acts directly on the E-cadherin promoter. To test this, we subjected DNA from NIKS and NIKS + HPV16 to bisulphite reaction which converts cytosines to thymines, unless the cytosines were methylated. This was followed by PCR cloning and sequencing of the E-cadherin promoter. We observed that NIKS cells harbouring HPV16 episomes did not exhibit any convincing differences in the CpG methylation state of their E-cadherin promoters from those of cells devoid of the virus. In fact the E-cadherin promoters in these cells (with or without HPV16) were almost fully unmethylated (Figure 6). This observation means that while HPV-induced augmentation of Dnmt activity is responsible for repressing E-cadherin gene expression, this repression is not brought about by methylation of the E-cadherin promoter. Instead, the results suggest that the E7-increased Dnmt activity targets a cellular genes whose protein products impinge on the E-cadherin promoter. One potential candidate is Slug, the repressor of E-cadherin expression (70,71). We tested to see if the protein level of Slug is altered in these cells. Western blotting revealed that the level of Slug was comparable in all the cells tested (data not shown), hence excluding it as the mediator of E-cadherin repression by E7. At this point in time, we do not know the Dnmt target that is responsible for the HPV-induced reduction of E-cadherin. This notwithstanding, it is clear that HPV, like the other cancer-associated viruses (HBV, HCV and EBV) is able to modulate through epigenetic means, expression of the cellular E-cadherin gene. While this results in direct methylation of the E-cadherin promoter by the other viruses, HPV-induced augmentation of Dnmt activity results in the indirect repression of the E-cadherin promoter.

Discussion

Observations from numerous independent analyses of clinical samples have consistently revealed that HPV-containing lesions have markedly reduced levels of E-cadherin protein. This study and those of Matthews *et al.* (41), Caberg *et al.* (60) and Hellner *et al.* (72) collectively show that this is not a secondary feature that is acquired by the cell independently of HPV action. Instead HPV directly reduces cellular E-cadherin expression. In the experiments described above, we show that the E7 protein is capable of reducing E-cadherin levels independently of any other viral proteins. As such we conclude that the E7 protein is the major instigator of E-cadherin reduction. However, it is noteworthy that the E6 protein may also contribute to this activity *in vivo*. We are mindful that in the context of the tissue, the relative expression and contribution of E6 and E7 to reducing E-cadherin level may be different or synergistic. This notwithstanding, it is clear that the E7 protein is able to do this on its own as expression of E7 alone or in the context of E6 resulted in comparable reduction of E-cadherin. While it would have been ideal to generate a NIKS line that harbour HPV16E6null DNA, we and others have not been able to generate such a cell line beyond the second passage (highlighting the requirement of E6 for HPV persistence in replicating cells) (57,58). Hence recombinant retroviruses were used to address this question instead. Although we can be confident that reduction of E-cadherin is not brought about via degradation by the proteasome system, we cannot exclude the possibility that E-cadherin could be channelled by E7 for degradation via another cellular proteolytic system. What is clear, however, is that E7 does reduce the expression of E-cadherin at the transcription level as the quantity of E-cadherin RNA was clearly reduced in cells with HPV16 DNA or HPV16E7 protein. The report by Caberg *et al.* (60) suggests that E7 does this by the combined reduction of pRb and AP-2α. From the outset, this did not appear to be case in our cells as while pRb was reduced by E7, the

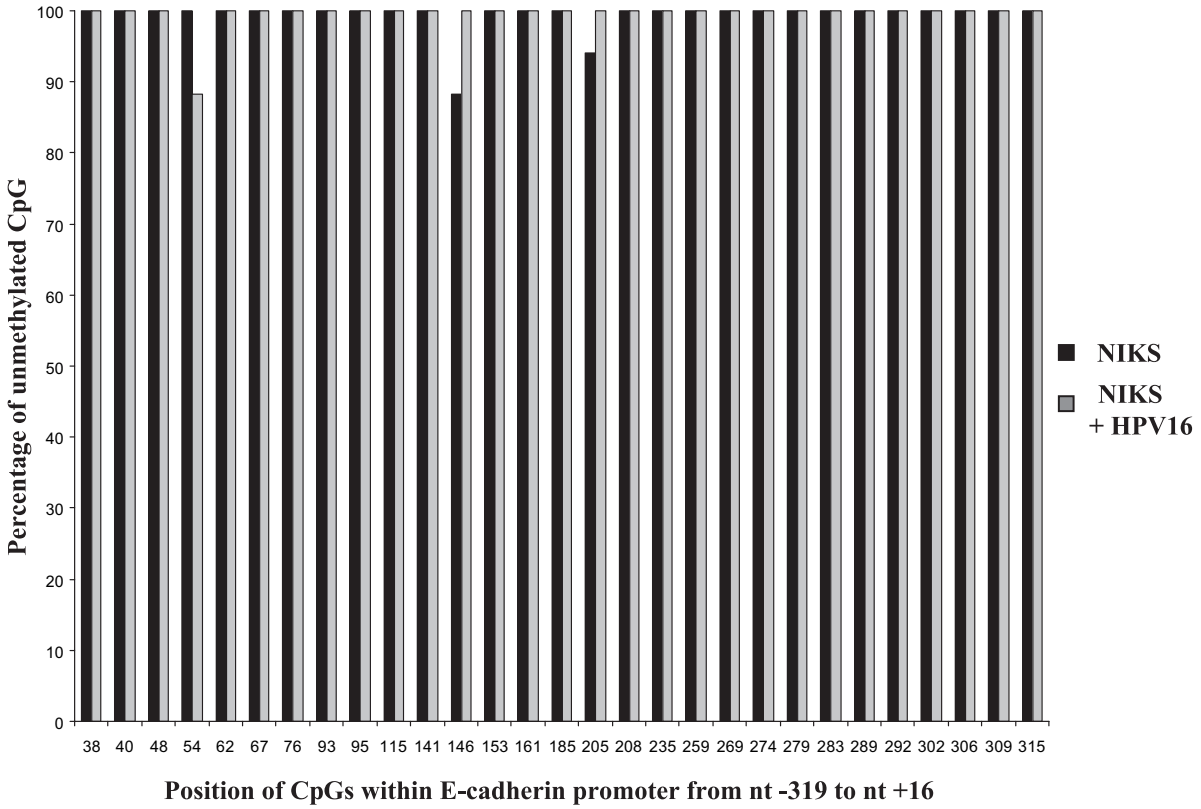

Fig. 6. Methylation status of CpGs within the E-cadherin promoter. The methylation state of each CpG within the E-cadherin promoter (position −319 to +16) was analyzed and obtained from nucleotide sequences of 17 individual clones each of bisulphite-converted DNA that were isolated from NIKS cells and NIKS cells harbouring HPV16DNA.

level of AP-2α was not. This is consistent with a previous report that showed that although reduction of AP-2α can occur, as it does in SiHa cells used by Caberg *et al.*, it is actually a rare event in cervical cancers (73). Furthermore, knock down of pRb levels using shRNA did not reduce E-cadherin levels. Our attention was turned to the interesting fact that several other cancer-associated viruses (HBV, HCV and EBV) also reduce E-cadherin level by reducing expression of this gene. They all did this by repressing the E-cadherin promoter. HBV, via its X protein, activates Dnmt1 expression and causes the repression of E-cadherin expression (61–63). Likewise, EBV latent membrane protein 1 protein activates Dnmt1 expression via C-Jun-N-terminal kinase-Activator protein 1 pathway and as a consequence represses E-cadherin expression (66–69). HCV core protein represses E-cadherin also by activating Dnmt1 and Dnmt3b (64,65). It is very likely that these viruses (including HPV) derive the common advantage of immune evasion by this method. Interestingly all these viruses (HBV, HCV and EBV) including HPV16 repress E-cadherin expression via activation of Dnmt1. For HPV16, this was brought about by E7-mediated augmentation of Dnmt1 protein and activity. This characteristic of E7 was also observed by Burgers *et al.* (74), who showed that E7 associates directly and physically with the Dnmt1 protein. This association contributes to the stability of the Dnmt1 protein (74). Mutation of pRb-interaction region of the E7 protein did not obliterate the ability of E7 to activate Dnmt activity, demonstrating that E7 increases the levels and activity of Dnmt primarily by direct association with the protein (74) and independently of pRb degradation as observed by us. The relevance of Dnmt1 increase and activation in regards to E-cadherin repression was made clear when inhibition of Dnmt by 5-aza-deoxycytidine re-established RNA and protein levels of E-cadherin in cells containing HPV16 DNA or expressing HPV proteins but not in control cells that do not contain HPV DNA.

Up to this point, the mechanism by which HPV16 repressed E-cadherin appears to parallel those of HBV, HCV and EBV. However, while the other viruses instigated a Dnmt1-mediated methylation of the E-cadherin promoter, neither HPV16 nor E7 protein appeared to do this. Instead the promoter of E-cadherin remained largely unmethylated whether or not HPV was present. This suggests that E7-augmented Dnmt1 activity is targeted to a cellular gene/genes whose protein acts on the E-cadherin promoter. Testing revealed that although the protein Slug was a prime candidate to be such a mediator (71,75,76), neither its level nor localization was altered by E7. As yet we have not identified what the cellular factors are. The difference between HPV and the other cancer-associated viruses is interesting when we consider that while cancers (hepatocellular carcinomas and lymphomas) that are associated with the other viruses (HBV, HCV and EBV) usually have their p16 promoter repressed by DNA methylation (presumably owed to virus-mediated increase of Dnmt activity), cervical lesions containing HPV do not have their p16 promoters methylated. This stark difference suggests that while the other viruses increase Dnmt activity more generally, HPV does not. How this fine control is elicited by HPV is not known but it may be linked to the fact that not only does E7 increase Dnmt1 activity, but it also binds to the Dnmt1 protein (74). It is possible that this interaction allows E7 to target Dnmt1 to specific sites as opposed to allowing Dnmt1 to act more liberally. Interestingly a precedent for this, from yet another virus, was presented by Shamay *et al.* (77) who reported that the LANA protein of the Kaposi's sarcoma-associated herpes virus physically associates with Dnmt3a and recruits the latter to specific sites on the cellular DNA to affect gene expression. This site-specific epigenetic modification may be the way by which HPV can modulate expression of some cellular genes without causing a general change in the epigenetic state of the host genome. The testing of this hypothesis should prove interesting as it may reveal further activities of E7 and uncover pathways that regulate E-cadherin expression that are currently unknown.

In summary, our observations outlined above demonstrate that the HPV16 E7 is capable of reducing the levels of E-cadherin in the cell via a mechanism that is partially similar to that employed by other human cancer-associated viruses (e.g. HBV, EBV and HCV). Mechanism aside, the reduction of E-cadherin itself is of significant importance. Work from Hellner *et al.* (72) suggests that the reduction of E-cadherin is a consequence of HPV16E7-mediated epithelial-mesenchymal transition, although HPV16 E6 and E7 have been reported by Azzimonti *et al.* (78) to prevent epithelial-mesenchymal transition instead. Whatever the case may be, it does not detract from the fact that reduced level of E-cadherin on the cell surface will reduce the efficiency of immune surveillance of the epithelium. This point is supported by a recent report by Hubert *et al.* (21), which showed that ectopic expression of E-cadherin induced migration of Langerhans cells into stratified layers of SiHa (HPV16 containing) cells in an organotypic culture. As such the reduction of E-cadherin levels in cervical lesions (21,39–42,44,45,48–53) may be one of the biggest contributors to the depletion of Langerhans cell number in HPV-infected epithelium, as reported by numerous investigators (16–27,79). In the case of HPV-infected epithelia, the reduced ability of Langerhans cells to infiltrate into virus-infected area of the tissue would aid the persistence of the virus in the host. Such persistence is thought to be the prelude to the eventual development of cervical cancer. The observed recovery of E-cadherin levels upon inhibition of Dnmt activity provides some optimism to the notion that such epigenetic targeting may be effective in clearing persistence of HPV in the infected tissue.

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References

1. Howley,P.M. (1987) The role of papillomaviruses in human cancer. *Important Adv. Oncol.*, 55–73.
2. zur Hausen,H. *et al*. (1984) Viruses in the etiology of human genital cancer. *Prog. Med. Virol.*, **30**, 170–186.
3. Huibregtse,J.M. *et al*. (1991) A cellular protein mediates association of p53 with the E6 oncoprotein of human papillomavirus types 16 or 18. *EMBO J.*, **10**, 4129–4135.
4. Huibregtse,J.M. *et al*. (1994) E6-AP directs the HPV E6-dependent inactivation of p53 and is representative of a family of structurally and functionally related proteins. *Cold Spring Harb. Symp. Quant. Biol.*, **59**, 237–245.
5. Scheffner,M. *et al*. (1990) The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell*, **63**, 1129–1136.
6. Scheffner,M. *et al*. (1991) The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. *Proc. Natl Acad. Sci. USA*, **88**, 5523–5527.
7. Scheffner,M. *et al*. (1992) Targeted degradation of the retinoblastoma protein by human papillomavirus E7-E6 fusion proteins. *EMBO J.*, **11**, 2425–2431.
8. Brown,D.R. *et al*. (2005) A longitudinal study of genital human papillomavirus infection in a cohort of closely followed adolescent women. *J. Infect. Dis.*, **191**, 182–192.
9. Dalstein,V. *et al*. (2003) Persistence and load of high-risk HPV are predictors for development of high-grade cervical lesions: a longitudinal French cohort study. *Int. J. Cancer*, **106**, 396–403.
10. Rodriguez,A.C. *et al*. (2008) Rapid clearance of human papillomavirus and implications for clinical focus on persistent infections. *J. Natl Cancer Inst.*, **100**, 513–517.
11. Plummer,M. *et al*. (2007) A 2-year prospective study of human papillomavirus persistence among women with a cytological diagnosis of atypical squamous cells of undetermined significance or low-grade squamous intraepithelial lesion. *J. Infect. Dis.*, **195**, 1582–1589.
12. Giannini,S.L. *et al*. (2002) Influence of the mucosal epithelium microenvironment on Langerhans cells: implications for the development

of squamous intraepithelial lesions of the cervix. *Int. J. Cancer*, **97**, 654–659.
13. Petry,K.U. *et al*. (1994) Cellular immunodeficiency enhances the progression of human papillomavirus-associated cervical lesions. *Int. J. Cancer*, **57**, 836–840.
14. Tindle,R.W. (2002) Immune evasion in human papillomavirus-associated cervical cancer. *Nat. Rev. Cancer*, **2**, 59–65.
15. Romani,N. *et al*. (1989) Presentation of exogenous protein antigens by dendritic cells to T cell clones. Intact protein is presented best by immature, epidermal Langerhans cells. *J. Exp. Med.*, **169**, 1169–1178.
16. al-Saleh,W. *et al*. (1995) Inverse modulation of intraepithelial Langerhans' cells and stromal macrophage/dendrocyte populations in human papillomavirus-associated squamous intraepithelial lesions of the cervix. *Virchows Arch.*, **427**, 41–48.
17. Connor,J.P. *et al*. (1999) Evaluation of Langerhans' cells in the cervical epithelium of women with cervical intraepithelial neoplasia. *Gynecol. Oncol.*, **75**, 130–135.
18. Fukuda,K. *et al*. (1993) Local immune response in persistent cervical dysplasia. *Obstet. Gynecol.*, **82**, 941–945.
19. Guess,J.C. *et al*. (2005) Decreased migration of Langerhans precursor-like cells in response to human keratinocytes expressing human papillomavirus type 16 E6/E7 is related to reduced macrophage inflammatory protein-3alpha production. *J. Virol.*, **79**, 14852–14862.
20. Hawthorn,R.J. *et al*. (1988) Langerhans' cells and subtypes of human papillomavirus in cervical intraepithelial neoplasia. *Br. Med. J.*, **297**, 643–646.
21. Hubert,P. *et al*. (2005) E-cadherin-dependent adhesion of dendritic and Langerhans cells to keratinocytes is defective in cervical human papillomavirus-associated (pre)neoplastic lesions. *J. Pathol.*, **206**, 346–355.
22. Hughes,R.G. *et al*. (1988) Expression of major histocompatibility class II antigens by Langerhans' cells in cervical intraepithelial neoplasia. *J. Clin. Pathol.*, **41**, 253–259.
23. McArdle,J.P. *et al*. (1986) Quantitative assessment of Langerhans' cells in human cervical intraepithelial neoplasia and wart virus infection. *Am. J. Obstet. Gynecol.*, **154**, 509–515.
24. Morelli,A.E. *et al*. (1993) Relationship between types of human papillomavirus and Langerhans' cells in cervical condyloma and intraepithelial neoplasia. *Am. J. Clin. Pathol.*, **99**, 200–206.
25. Morris,H.H. *et al*. (1983) Langerhans' cells in human cervical epithelium: an immunohistological study. *Br. J. Obstet. Gynaecol.*, **90**, 400–411.
26. Tay,S.K. *et al*. (1989) Langerhans cell population in early invasive squamous cell carcinoma of the uterine cervix. *Aust. N. Z. J. Obstet. Gynaecol.*, **29**, 38–40.
27. Viac,J. *et al*. (1990) Langerhans cells and epithelial cell modifications in cervical intraepithelial neoplasia: correlation with human papillomavirus infection. *Immunobiology*, **180**, 328–338.
28. Bhushan,M. *et al*. (2002) Tumour necrosis factor-alpha-induced migration of human Langerhans cells: the influence of ageing. *Br. J. Dermatol.*, **146**, 32–40.
29. Jakob,T. *et al*. (1998) Regulation of E-cadherin-mediated adhesion in Langerhans cell-like dendritic cells by inflammatory mediators that mobilize Langerhans cells *in vivo*. *J. Immunol.*, **160**, 4067–4073.
30. Rupec,R. *et al*. (1996) Granulocyte/macrophage-colony-stimulating factor induces the migration of human epidermal Langerhans cells *in vitro*. *Exp. Dermatol.*, **5**, 115–119.
31. Hubert,P. *et al*. (1999) Colonization of *in vitro*-formed cervical human papillomavirus- associated (pre)neoplastic lesions with dendritic cells: role of granulocyte/macrophage colony-stimulating factor. *Am. J. Pathol.*, **154**, 775–784.
32. Halliday,G.M. *et al*. (2001) Transforming growth factor-beta produced by progressor tumors inhibits, while IL-10 produced by regressor tumors enhances, Langerhans cell migration from skin. *Int. Immunol.*, **13**, 1147–1154.
33. Charbonnier,A.S. *et al*. (1999) Macrophage inflammatory protein 3alpha is involved in the constitutive trafficking of epidermal langerhans cells. *J. Exp. Med.*, **190**, 1755–1768.
34. Caberg,J.H. *et al*. (2009) Increased migration of Langerhans cells in response to HPV16 E6 and E7 oncogene silencing: role of CCL20. *Cancer Immunol. Immunother.*, **58**, 39–47.
35. Wang,B. *et al*. (1999) Role of cytokines in epidermal Langerhans cell migration. *J. Leukoc. Biol.*, **66**, 33–39.
36. Mota,F. *et al*. (1999) The antigen-presenting environment in normal and human papillomavirus (HPV)-related premalignant cervical epithelium. *Clin. Exp. Immunol.*, **116**, 33–40.
37. Tang,A. *et al*. (1993) Adhesion of epidermal Langerhans cells to keratinocytes mediated by E-cadherin. *Nature*, **361**, 82–85.
38. Udey,M.C. (1997) Cadherins and Langerhans cell immunobiology. *Clin. Exp. Immunol.*, **107** (suppl. 1), 6–8.
39. Denk,C. *et al*. (1997) Reduced gene expression of E-cadherin and associated catenins in human cervical carcinoma cell lines. *Cancer Lett.*, **120**, 185–193.
40. Chen,C.L. *et al*. (2003) E-cadherin expression is silenced by DNA methylation in cervical cancer cell lines and tumours. *Eur. J. Cancer*, **39**, 517–523.
41. Matthews,K. *et al*. (2003) Depletion of Langerhans cells in human papillomavirus type 16-infected skin is associated with E6-mediated down regulation of E-cadherin. *J. Virol.*, **77**, 8378–8385.
42. Fujimoto,J. *et al*. (1997) Expression of E-cadherin and alpha- and beta-catenin mRNAs in uterine cervical cancers. *Tumour Biol.*, **18**, 206–212.
43. de Boer,C.J. *et al*. (1999) Changing roles of cadherins and catenins during progression of squamous intraepithelial lesions in the uterine cervix. *Am. J. Pathol.*, **155**, 505–515.
44. Van de Putte,G. *et al*. (2004) E-cadherin and catenins in early squamous cervical carcinoma. *Gynecol. Oncol.*, **94**, 521–527.
45. Yaldizl,M. *et al*. (2005) Expression of E-cadherin in squamous cell carcinomas of the cervix with correlations to clinicopathological features. *Eur. J. Gynaecol. Oncol.*, **26**, 95–98.
46. Sun,H. *et al*. (2000) [E-cadherin expression and its clinical significance in cervical cancer]. *Zhonghua Zhong Liu Za Zhi*, **22**, 496–498.
47. Daniel,B. *et al*. (1997) The link between integration and expression of human papillomavirus type 16 genomes and cellular changes in the evolution of cervical intraepithelial neoplastic lesions. *J. Gen. Virol.*, **78**, (Pt 5)1095–1101.
48. Branca,M. *et al*. (2006) Down-regulation of E-cadherin is closely associated with progression of cervical intraepithelial neoplasia (CIN), but not with high-risk human papillomavirus (HPV) or disease outcome in cervical cancer. *Eur. J. Gynaecol. Oncol.*, **27**, 215–223.
49. Kaplanis,K. *et al*. (2005) E-cadherin expression during progression of squamous intraepithelial lesions in the uterine cervix. *Eur. J. Gynaecol. Oncol.*, **26**, 608–610.
50. Faleiro-Rodrigues,C. *et al*. (2004) E-cadherin, CD44 and CD44v6 in squamous intraepithelial lesions and invasive carcinomas of the uterine cervix: an immunohistochemical study. *Pathobiology*, **71**, 329–336.
51. Jeffers,M.D. *et al*. (1997) E-cadherin and integrin cell adhesion molecule expression in invasive and in situ carcinoma of the cervix. *Gynecol. Oncol.*, **64**, 481–486.
52. Rodriguez-Sastre,M.A. *et al*. (2005) Abnormal distribution of E-cadherin and beta-catenin in different histologic types of cancer of the uterine cervix. *Gynecol. Oncol.*, **97**, 330–336.
53. Vessey,C.J. *et al*. (1995) Altered expression and function of E-cadherin in cervical intraepithelial neoplasia and invasive squamous cell carcinoma. *J. Pathol.*, **176**, 151–159.
54. Allen-Hoffmann,B.L. *et al*. (2000) Normal growth and differentiation in a spontaneously immortalized near-diploid human keratinocyte cell line, NIKS. *J. Invest. Dermatol.*, **114**, 444–455.
55. Hoffmann,R. *et al*. (2006) Different modes of human papillomavirus DNA replication during maintenance. *J. Virol.*, **80**, 4431–4439.
56. Reinhold,W.C. *et al*. (2007) Detailed methylation profiles of the E-cadherin promoter in the NCI-60 cancer cells. *Mol. Cancer Therapy*, **6**, 391–403.
57. Park,R.B. *et al*. (2002) Genetic analysis of high-risk e6 in episomal maintenance of human papillomavirus genomes in primary human keratinocytes. *J. Virol.*, **76**, 11359–11364.
58. Thomas,J.T. *et al*. (1999) Human papillomavirus type 31 oncoproteins E6 and E7 are required for the maintenance of episomes during the viral life cycle in normal human keratinocytes. *Proc. Natl Acad. Sci. USA*, **96**, 8449–8454.
59. Demers,G.W. *et al*. (1994) Elevated wild-type p53 protein levels in human epithelial cell lines immortalized by the human papillomavirus type 16 E7 gene. *Virology*, **198**, 169–174.
60. Caberg,J.H. *et al*. (2008) Silencing of E7 oncogene restores functional E-cadherin expression in human papillomavirus 16-transformed keratinocytes. *Carcinogenesis*, **29**, 1441–1447.
61. Jung,J.K. *et al*. (2007) Expression of DNA methyltransferase 1 is activated by hepatitis B virus X protein via a regulatory circuit involving the p16INK4a-cyclin D1-CDK 4/6-pRb-E2F1 pathway. *Cancer Res.*, **67**, 5771–5778.
62. Lee,J.O. *et al*. (2005) Hepatitis B virus X protein represses E-cadherin expression via activation of DNA methyltransferase 1. *Oncogene*, **24**, 6617–6625.
63. Liu,J. *et al*. (2006) Downregulation of E-cadherin by hepatitis B virus X antigen in hepatocellullar carcinoma. *Oncogene*, **25**, 1008–1017.
64. Iso,Y. *et al*. (2005) Loss of E-cadherin mRNA and gain of osteopontin mRNA are useful markers for detecting early recurrence of HCV-related hepatocellular carcinoma. *J. Surg. Oncol.*, **92**, 304–311.

65. Arora,P. *et al*. (2008) Hepatitis C virus core protein downregulates E-cadherin expression via activation of DNA methyltransferase 1 and 3b. *Cancer Lett.*, **261**, 244–252.
66. Fahraeus,R. *et al*. (1992) Decreased expression of E-cadherin and increased invasive capacity in EBV-LMP-transfected human epithelial and murine adenocarcinoma cells. *Int. J. Cancer*, **52**, 834–838.
67. Krishna,S.M. *et al*. (2005) Down regulation of adhesion protein E-cadherin in Epstein-Barr virus infected nasopharyngeal carcinomas. *Cancer Biomark.*, **1**, 271–277.
68. Tsai,C.N. *et al*. (2002) The Epstein-Barr virus oncogene product, latent membrane protein 1, induces the downregulation of E-cadherin gene expression via activation of DNA methyltransferases. *Proc. Natl Acad. Sci. USA*, **99**, 10084–10089.
69. Tsai,C.L. *et al*. (2006) Activation of DNA methyltransferase 1 by EBV LMP1 Involves c-Jun NH(2)-terminal kinase signaling. *Cancer Res.*, **66**, 11668–11676.
70. Hajra,K.M. *et al*. (2002) The SLUG zinc-finger protein represses E-cadherin in breast cancer. *Cancer Res.*, **62**, 1613–1618.
71. Park,S.H. *et al*. (2008) Estrogen regulates Snail and Slug in the down-regulation of E-cadherin and induces metastatic potential of ovarian cancer cells through estrogen receptor alpha. *Mol. Endocrinol.*, **22**, 2085–2098.
72. Hellner,K. *et al*. (2009) HPV16 E7 oncogene expression in normal human epithelial cells causes molecular changes indicative of an epithelial to mesenchymal transition. *Virology*, **391**, 57–63.
73. Beger,M. *et al*. (2001) Expression pattern of AP-2 transcription factors in cervical cancer cells and analysis of their influence on human papillomavirus oncogene transcription. *J. Mol. Med.*, **79**, 314–320.
74. Burgers,W.A. *et al*. (2007) Viral oncoproteins target the DNA methyltransferases. *Oncogene*, **26**, 1650–1655.
75. Leong,K.G. *et al*. (2007) Jagged1-mediated Notch activation induces epithelial-to-mesenchymal transition through Slug-induced repression of E-cadherin. *J. Exp. Med.*, **204**, 2935–2948.
76. Herfs,M. *et al*. (2008) Transforming growth factor-beta1-mediated Slug and Snail transcription factor up-regulation reduces the density of Langerhans cells in epithelial metaplasia by affecting E-cadherin expression. *Am. J. Pathol.*, **172**, 1391–1402.
77. Shamay,M. *et al*. (2006) Recruitment of the de novo DNA methyltransferase Dnmt3a by Kaposi's sarcoma-associated herpesvirus LANA. *Proc. Natl Acad. Sci. USA*, **103**, 14554–14559.
78. Azzimonti,B. *et al*. (2009) The epithelial-mesenchymal transition induced by keratinocyte growth conditions is overcome by E6 and E7 from HPV16, but not HPV8 and HPV38: characterization of global transcription profiles. *Virology*, **388**, 260–269.
79. Morris,H.H. *et al*. (1983) Langerhans' cells in human cervical epithelium: effects of wart virus infection and intraepithelial neoplasia. *Br. J. Obstet. Gynaecol.*, **90**, 412–420.

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