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Methylation-mediated repression of PRDM14 contributes to apoptosis evasion in HPV-positive cancers

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Promoter methylation of the transcription factor PRDM14 (PRDI-BF1 and RIZ domain containing 14) represents a highly frequent event in human papillomavirus (HPV)-induced cervical cancers and cancer precursor lesions. Here, we aimed to assess the functional consequences of PRDM14 promoter methylation in HPV-induced carcinogenesis. PRDM14 promoter methylation, expression and consequences of ectopic PRDM14 expression were studied in HPV16-positive cervical and oral cancer cell lines (SiHa, CaSki and 93VU147T), human embryonic kidney 293 (HEK293T) cells and primary human foreskin keratinocytes (HFK). PRDM14 mRNA expression was restricted to HEK293T and HFK cells, and could be upregulated in SiHa cells upon DNA methylation inhibition. Ectopic expression of PRDM14 in SiHa, CaSki and 93VU147T cells resulted in significantly more apoptotic cells, as measured by annexin V labelling, compared to HEK293T and HFK cells. MRNA profiling of 41 apoptosis regulators identified NOXA and PUMA as candidate target genes involved in PRDM14-mediated apoptosis induction. Full-length PRDM14 transactivated both NOXA and PUMA promoters. Transactivation was abolished upon deletion of the PRDM14 DNA binding domain. This suggests that NOXA and PUMA expression is directly regulated by PRDM14, which in case of NOXA was linked to a consensus PRDM14 binding motif in the promoter region. Taken together, these results suggest that PRDM14 acts as a regulator of NOXA and PUMA-mediated apoptosis induction, thereby providing evidence for a tumour suppressive role in HPV-induced carcinogenesis. The contribution of methylation-mediated gene silencing of PRDM14 to apoptosis evasion in HPV-positive cancer cells offers novel therapeutic options for HPV-induced cancers.

Introduction

Cervical cancer is the third most common cancer in women worldwide (1). Persistent infection with high-risk human papillomavirus (hrHPV) types is causally involved in the development of both squamous cell carcinoma (SCC) and adenocarcinoma [AdCA (2,3)]. These tumours evolve from hrHPV-positive non-invasive precursor lesions, that is cervical intraepithelial neoplasia (CIN) and adenocarcinoma in situ (ACIS), respectively (4–7). Sustained overexpression of the viral genes is necessary, but not sufficient, for the development of cervical cancer and its high-grade precursor lesions. This process requires additional (epi)genetic aberrations in host cell DNA (8,9). Several studies indicated methylation-mediated repression of tumour suppressor genes to be involved in the process of cervical carcinogenesis. For instance, the gene promoters of CADM1, MAL and miR-124 are frequently methylated in high-grade CIN and cervical carcinomas, which was associated with gene silencing. Re-expression of these genes in HPV-transformed cells suppressed proliferation, anchorage dependent growth, tumourgenicity and/or migration capacity, thereby supporting their tumor suppressive role in cervical carcinogenesis (8,10,11). In a recent genome wide methylation screen on HPV16E6E7-transduced human foreskin keratinocytes we identified the PRDI-BF1 and RIZ domain containing 14 (PRDM14) gene as one of the top methylation targets (12). PRDM14 encodes a 65 kDa protein belonging to the PRDM transcription-family, whose members can act both as tumour suppressors and oncogenes (13). PRDM14 promoter methylation was subsequently detected in 98% of cervical SCC and 96% of cervical AdCA, as well as in a major subset of cervical cancer precursor lesions (12). The observed significant increase in PRDM14 methylation levels with progression of cervical disease, suggests that PRDM14 might play a tumour suppressive role in HPV-induced carcinogenesis. PRDM14 is a protein, which contains a PRDI-BF1 and RIZ homology (PR)-domain, that is related to the Su(var)3–9, Enhancer of Zeste, Trithorax (SET) methyltransferase domain, and six zinc-fingers that mediate sequence-specific DNA binding (14,15). PRDM14 is a critical regulator of cell fate during early mammalian embryogenesis and has been implicated in epigenetic reprogramming. For example, PRDM14 can drive global DNA demethylation in primordial germ cells by downregulation of the DNA methyltransferases (DNMTs) 3A and 3B giving rise to embryonic germ cells (16,17). The possible biological relevance of PRDM14 methylation in HPV-induced carcinogenesis is still unknown. Here, we examined the potential functional role of PRDM14 methylation in HPV-induced carcinogenesis.

Abbreviations: DNMT, DNA methyltransferase; HEK293T, human embryonic kidney 293; HFK, human foreskin keratinocytes; HPV, human papillomavirus; PI, propidium iodide.

Materials and methods

Cell culture, DAC treatment and transfections

Primary human foreskin keratinocytes (HFK) were isolated from foreskin and cultured as described previously (18). The human cervical carcinoma cell lines SiHa, CaSki and human embryonic kidney 293 (HEK293T) were obtained from the American Type Culture Collection (Manassas, VA). Establishment and culture of the HPV16 positive oral cancer cell line 93VU147T has been described previously (19). SiHa, CaSki, 93VU147T and HEK293T cells were cultured in Dulbecco's modified Eagle Medium (Life Technologies, Breda, The Netherlands) supplemented with 10% fetal calf serum, penicillin at 100 U/ml, streptomycin at 100 µg/ml, and L-glutamine at 2 mmol/l (all from Life Technologies). Incubation of SiHa with 5 µm 5′-aza-2′-deoxycytidine (DAC; Sigma Chemical Co, St Louis, MO), was performed as described previously (8). Transfections were performed using LipofectamineTM 2000 (Life Technologies) for cDNA according to the manufacturers recommendations. Human PRDM14 cDNA cloned in the pCMV6-AC-GFP vector and the empty vector pCMV6-AC-GFP were obtained from Origene (Rockville, MD). pCAG-PRDM14, pCAG-deltaDBD and empty vector pCAG were kindly provided by Dr. H.-H. Ng (15). In initial experiments transfected cells were selected using 800 µg/ml G418 (Sigma Chemical Co).

DNA extraction and bisulfite modification

Genomic DNA from cell lines was extracted by proteinase K digestion followed by standard phenol-chloroform extraction as described previously (20). DNAs were bisulfite treated using the EZ DNA Methylation Kit™ (Zymo Research, Orange, CA).

Quantitative MSP (qMSP) analysis

For the amplification reaction 2.5 µl bisulfite treated DNA (50 ng) was added to 10 µl amplification mix containing 1× Quantitect Probe mix (Qiagen, Leusden, The Netherlands), primers (417 nM each) and probe (208 nM). Sequences of primers and hydrolysis probes are listed in Table I. Amplification and real-time measurement was performed in the 7500Fast ABI system (Applied Biosystems, Foster City, CA), using the following conditions; 15 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. All samples were tested in duplicate. Methylation values were normalized to the reference gene β-actin (ACTB) using the comparative Ct method [$2^{-\Delta C_t}$ (21)].

Table I. Primers and probes sequences for qMSP and qRT-PCR

Gene	Primers/Probes (5'–3')	Size (bp)	Tm (°C)
qMSP			
ACTB	F: TGGTGATGGAGGAGGTTTAGTAAGT	133	58.2
	R: AACCAATAAAACCTACTCCTCCCTTAA		58.9
	P: FAM - ACCACCACCCAACACACAATAACAAACACA - TAMRA		68.9
PRDM14	F: TTATTAGCGGGTTAGACGTCGTTT	84	59.1
	R: CGTCCCGAAATCGAACCC		59.8
	P: YY - CTTACTCTCCGCTCCCAATTCGAAAAATCC - BHQ1		69.1
(q)RT-PCR			
snRNP	F: TCCTCACCAACCTGCCAGA	71	58.8
	R: TGAAGCCAGGGAACTGATTGA		59.3
	P: FAM - AGACCAACGAGCTCATGCTGTCCATG - TAMRA		68.2
PRDM14	F: GTGAAGTGAAGACCTACGGAGACAA	162	59.7
	R: GGGAAGCGGGCACAGTT		58.2
	P: FAM - ACATAGGACATCCAGTTCCCCGTACCTCC - BHQ1		68.9
NOXA	F: GAAGAAGGCGCGCAAGAAC	97	59.7
	R: TTTGTCTCCAAATCTCCTGAGTTG		58.4
	P: FAM - AGTAGCACACTCGACTTCCAGCTCTGCTG - BHQ1		68.5
PUMA	F: GGACGACCTCAACGCACAGTA	90	59.8
	R: ATGAGATTGTACAGGACCCTCCA		58.5
	P: FAM - CTGCTCCTCTTGTCTCCGCCGCT - BHQ1		68.8
HPV16E6	F: GAGCGACCCAGAAAGTTACCA	87	57.9
	R: TTGCTTGCAGTACACACATTCTAAT		56.9
HPV16E7	F: CGGACAGAGCCCATTACAA	109	55.7
	R: AACAGGTCTTCCAAAGTACGA		52.8

F, forward; R, reverse; P, hydrolysis probe; YY, Yakima Yellow; BHQ, Black Hole Quencher.

Quantitative RT-PCR

Total RNA was isolated with TRIzol® reagent (Life Technologies). Two hundred nanograms of RNA was reverse transcribed into cDNA using specific reverse primers with AMV reverse transcriptase (Promega, Leiden, The Netherlands). RT-PCR primer sequences are listed in Table I. Quantitative RT-PCR (qRT-PCR) was performed in a total reaction volume of 25 µl, containing 25 ng cDNA, 1× TaqMan® Universal PCR Master Mix (Applied Biosystems), 400 nM of each primer and 200 nM probe. Amplification and real-time measurement was performed on the 7900HT Fast ABI system (Applied Biosystems), using the following conditions; 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. All samples were tested in duplicate. Universal Human Reference RNA (Stratagene, La Jolla, CA) and reactions without reverse transcriptase were used as positive and negative control, respectively. SnRNP was used as a reference gene (22) and the relative expression was determined using the comparative C_t method ($2^{-\Delta C_t}$ (21)). In the semi-quantitative RT-PCR for HPV16 E6 and E7, 25 ng of cDNA was amplified with FastStart Taq PCR buffer (Roche, Woerden, The Netherlands) supplemented with 2 mM $MgCl_2$, 500 nM of each primer, each dNTP at 200 µM, and 0.625 U of FastStart Taq DNA polymerase in a total volume of 25 µl. Amplification reaction was carried out in a GeneAmp® PCR system 9800 (Applied Biosystems) using the following conditions: 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 45 s, with a final extension of 72°C for 4 min. PCR products were analysed by electrophoresis on 2% agarose gels.

AnnexinV-propidium iodide FACS analysis

Apoptosis was studied using AnnexinV-propidium iodide (PI) staining, as described by Vermes *et al*. (23). Cells grown to subconfluency were harvested and washed with annexin-buffer [10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 2.1 mM $MgCl_2$ and glucose (1 mg/ml), pH 7.4]. Cells were incubated with AnnexinV-APC (1:200; IQ Products, Groningen, The Netherlands) in the dark for 20 min at 4°C and washed with annexin-buffer. Pellets were resuspended in 100 µl annexin-buffer and 5 µl of PI (1 mg/ml) was added, followed by fluorescence-activated cell sorting using BD FACSCalibur (BD Biosciences, Breda, The Netherlands).

Reverse transcriptase-multiplex ligation-dependent probe amplification analysis

GFP-positive SiHa, CaSki and HEK293T cells were FACS-sorted using BD FACSAria (BD Biosciences) and RNA samples were prepared using RNbee solution (Tel-test, Friendswood, TX) according to the manufacturers recommendations. Total RNA was analysed by reverse transcriptase-multiplex ligation-dependent probe amplification (RT-MLPA) using SALSA MLPA KIT R011 Apoptosis mRNA from MRC-Holland (Amsterdam, The Netherlands) for the simultaneous detection of 44 mRNA molecules, including three internal references (24). Data were analysed with Genotype and GeneScan software (Applied Biosystems). As internal reference, the housekeeping gene β-glucuronidase (GUS-B) was used to minimize possible effects of unequal amounts of mRNA.

Luciferase assay

The reporter plasmids used in this study, pGL4.18-NOXA and pGL3-PUMA were kindly provided by Dr. Y. Tsujimoto and Dr. G.P. Zambetti, respectively (25,26). SiHa cells seeded on 12-well plates were transfected with 1.6 µg of expression vector, 500 ng of pGL4.18 or pGL3 reporter plasmid, and 25 ng Renilla luciferase reporter vector (Promega) for 5 h at 37°C. Firefly and Renilla activity were measured with Luciferase Assay System (Promega) and Renilla Luciferase Assay System (Promega), respectively, using a Lumat LB9507 luminometer (EG&G Berthold, Bad Wildbad, Germany). The relative luciferase activity was calculated by dividing Firefly luciferase by Renilla luciferase activity.

Site-directed mutagenesis

Nucleotide substitutions were introduced into the putative PRDM14 binding sequence (15) using the Q5 site-directed mutagenesis kit (New England Biolabs, Ipswich, MA) according to the manufacturers recommendations. Nucleotide substitutions were introduced in the consensus PRDM14 binding site (5′-GGTCTCTAA-3′) to generate pGL4.18-NOXA-mut which carried eight mutations (5′-TGAGAGCC-3′). Substitutions were confirmed by DNA sequencing. The wild-type (pGL4.18-NOXA) and the mutant reporter construct (pGL4.18-NOXA-mut) were used for measuring promoter activity.

Statistical analysis

Statistical analysis was performed using SPSS (version 20). All experiments were performed in triplicate and mean values ± standard deviation (SD) were calculated. The independent sample *t*-test was used to compare variables. A two-sided *P*-value of <0.05 was considered statistically significant.

Results

PRDM14 expression is inversely correlated with promoter methylation

In the HPV16-containing cervical cancer cell lines SiHa and CaSki, and the HPV16-positive oral cancer cell line 93VU147T no PRDM14 mRNA was detected. HEK293T cells and primary HFK did show PRDM14 mRNA expression, albeit the latter at relatively lower quantities (Figure 1A). QMSP analysis revealed high PRDM14 promoter methylation levels in SiHa, CaSki and 93VU147T cells. Lower levels

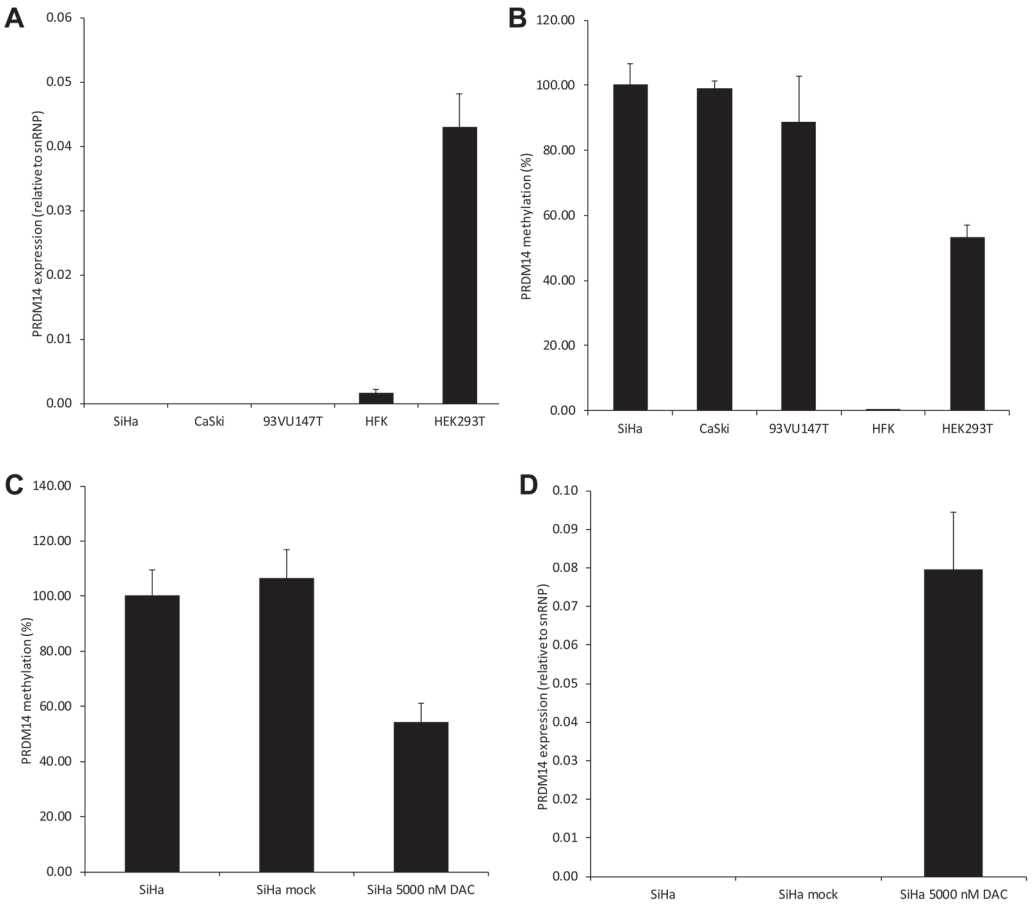

Fig. 1. PRDM14 expression and methylation in SiHa, CaSki, 93VU147T, HEK293T and HFK cells. (**A**) In none of the HPV16-positive cancer cell lines PRDM14 expression was detected. In HEK293T cells and HFK expression of PRDM14 was detected. (**B**) SiHa, CaSki and 93VU147T show high levels of PRDM14 methylation. The level of methylation of PRDM14 in HEK293T cells was approximately 45% lower compared to SiHa cells, for which the level was set to 100%. PRDM14 was unmethylated in HFK cells. (**C**) The PRDM14 methylation levels were reduced by more than 45% in 5000 nM DAC treated SiHa cells. Methylation levels of untreated SiHa cells were set to 100%. (**D**) Untreated and mock (PBS) treated SiHa cells showed no PRDM14 expression, while SiHa cells treated with 5000 nM DAC expression of PRDM14 was detected.

of PRDM14 promoter methylation levels were found in HEK293T cells, whereas PRDM14 methylation was undetectable in HFK (Figure 1B). The apparent discrepancy in PRDM14 mRNA versus DNA methylation levels in HEK293T compared to HFK cells, indicates that other regulatory factors, such as histone modifications and transcription factors are also involved in PRDM14 expression regulation. Treatment of SiHa cells with the demethylating agent DAC resulted in a decrease of >45% in PRDM14 promoter methylation and an increase in PRDM14 mRNA expression compared to untreated and mock-treated SiHa cells (PBS) (Figure 1C and D). The inverse correlation between PRDM14 methylation levels and PRDM14 mRNA expression suggests that PRDM14 downregulation in SiHa cells is mediated by promoter methylation.

PRDM14 induces apoptosis in HPV16-positive cancer cell lines

To investigate the potential functional role of PRDM14 silencing in HPV-induced carcinogenesis, SiHa, CaSki, 93VU147T, HFK and HEK293T cells were transfected with a PRDM14-GFP fusion construct and a GFP+ control vector. Ectopic PRDM14 expression was confirmed by qRT-PCR (Figure 2A). In contrast to control transfectants, none of the HPV16-positive cancer cell lines transfected with PRDM14 survived neomycin selection, while ectopic PRDM14 and GFP expression was observed at 24 h post-transfection. To substantiate that this phenomenon resulted from a strong induction of cell death by PRDM14, we subsequently monitored GFP+ cells only. By flow cytometry a strong decline in the number of GFP+ cells was measured in the three cancer cell lines 24, 48 and 72 h post-PRDM14 transfection. In contrast, in control transfectants as well as PRDM14 and control-vector transfected HFK and HEK293T cells the number of GFP+ cells 24–72 h post-transfection remained the same (Figure 2B–F). The overall reduced number of GFP+ cells in PRDM14 transfected cells compared to the control-vector transfected cells is most likely explained by differences in transfection efficiency, which is dependent on plasmid size (27). To determine whether cell death could be attributed to apoptosis induction, flow cytometry analysis of living cells double labelled with annexin V and PI was performed 24, 48 and 72 h after transfection. Annexin V+/PI- cells indicate early apoptosis and annexin V+/PI+ cells indicate late apoptosis (28). The GFP+ populations were gated and analysed for annexin V/PI positivity. After 24 h, PRDM14 transfected 93VU147T cells showed significantly ($P < 0.05$) more annexin V+ cells (sum of annexin V+/PI− and annexin V+/PI+) compared to controls and PRDM14 transfected HEK293T and HFK cells (Figure 2G). Forty-eight and seventy-two hours post-PRDM14 transfection all HPV16-positive cancer cell lines revealed significantly higher annexin V positivity compared to HEK293T and HFK cells. These results imply that overexpression of PRDM14 specifically induces apoptosis in HPV16-positive cancer cell lines and not in HPV-negative non-malignant cells.

PRDM14 induces expression of the pro-apoptotic genes NOXA and PUMA in HPV16-positive cancer cells

To obtain further insight in the biology underlying PRDM14 induced apoptosis in HPV16-positive cancer cells, mRNA expression levels of 41 apoptosis-regulating genes were analysed by RT-MLPA.

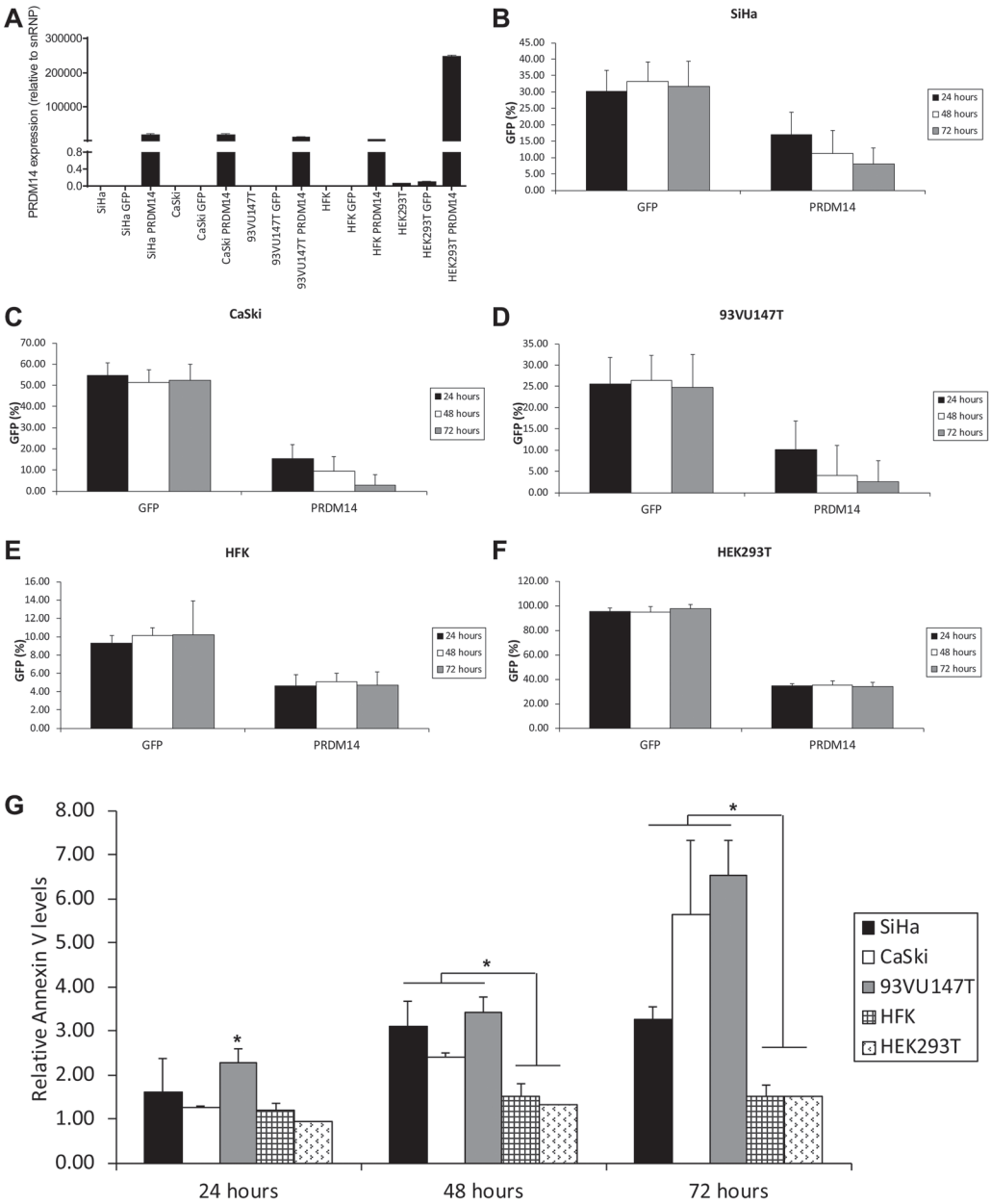

Fig. 2. Ectopic expression of PRDM14 in SiHa, CaSki, 93VU147T, HFK and HEK293T cells. (**A**) In untransfected SiHa, CaSki and 93VU147T and empty vector (GFP) transfected SiHa, CaSki and 93VU147T cells no PRDM14 mRNA was detectable. High PRDM14 mRNA expression was detected in PRDM14 transfected SiHa, Caski and 93VU147T cells. HFK and HEK293T cells showed low PRDM14 expression, which was increased in PRDM14 transfected cells. (**B**–**F**) Percentage of GFP-positive cells at 24, 48 and 72 h post-transfection. The percentage of GFP-positive SiHa (B), CaSki (C) and 93VU147T (D) cells declined over time in the PRDM14 transfected cells, while that of the empty vector (GFP) transfected and PRDM14 transfected HFK (E) and HEK293T (F) cells remained the same. (**G**) Flow cytometry analysis of GFP positive SiHa, CaSki, 93VU147T, HFK and HEK293T cells double labelled with annexin V and PI. The number of apoptotic cells was measured 24, 48 and 72 h post-transfection. The relative annexin V levels were calculated by dividing the number of annexin V positive cells in the PRDM14 transfected cells, by those with the empty vector transfected cells (*$P < 0.05$).

Transfected SiHa, CaSki and HEK293T cells were FACS-sorted and RNA was isolated from the GFP+ cell population. RT-MLPA showed a significant increase in mRNA expression of the pro-apoptotic regulators NOXA (also known as PMAIP1) and PUMA (also known as BBC3) 48 h post-PRDM14 transfection in SiHa and CaSki cells (Figure 3A and B). Increased NOXA and PUMA mRNA levels were not observed in PRDM14 transfected HEK293T compared to control transfectants (Figure 3C). Upregulation of NOXA and PUMA mRNA expression upon PRDM14 overexpression in SiHa cells was confirmed by qRT-PCR analysis (Figure 3D and E). These results suggest that PRDM14 induced apoptosis can be ascribed to increased expression of the pro-apoptotic modulators NOXA and PUMA. It remains to be determined whether increased mRNA expression of NOXA and PUMA is translated into increased protein levels, for which insufficient GFP+ cells were available. By semi-quantitative RT-PCR no major changes in E6 and E7 mRNA expression were seen upon PRDM14 overexpression in SiHa cells (Figure 3F).

PRDM14 can directly upregulate the expression of NOXA and PUMA

As PRDM14 is a transcription factor, we examined whether PRDM14 is a direct transcriptional regulator of the NOXA and PUMA genes. SiHa cells were cotransfected with PRDM14 or empty vector control (pCMV6-AC-GFP) and the respective luciferase reporter constructs

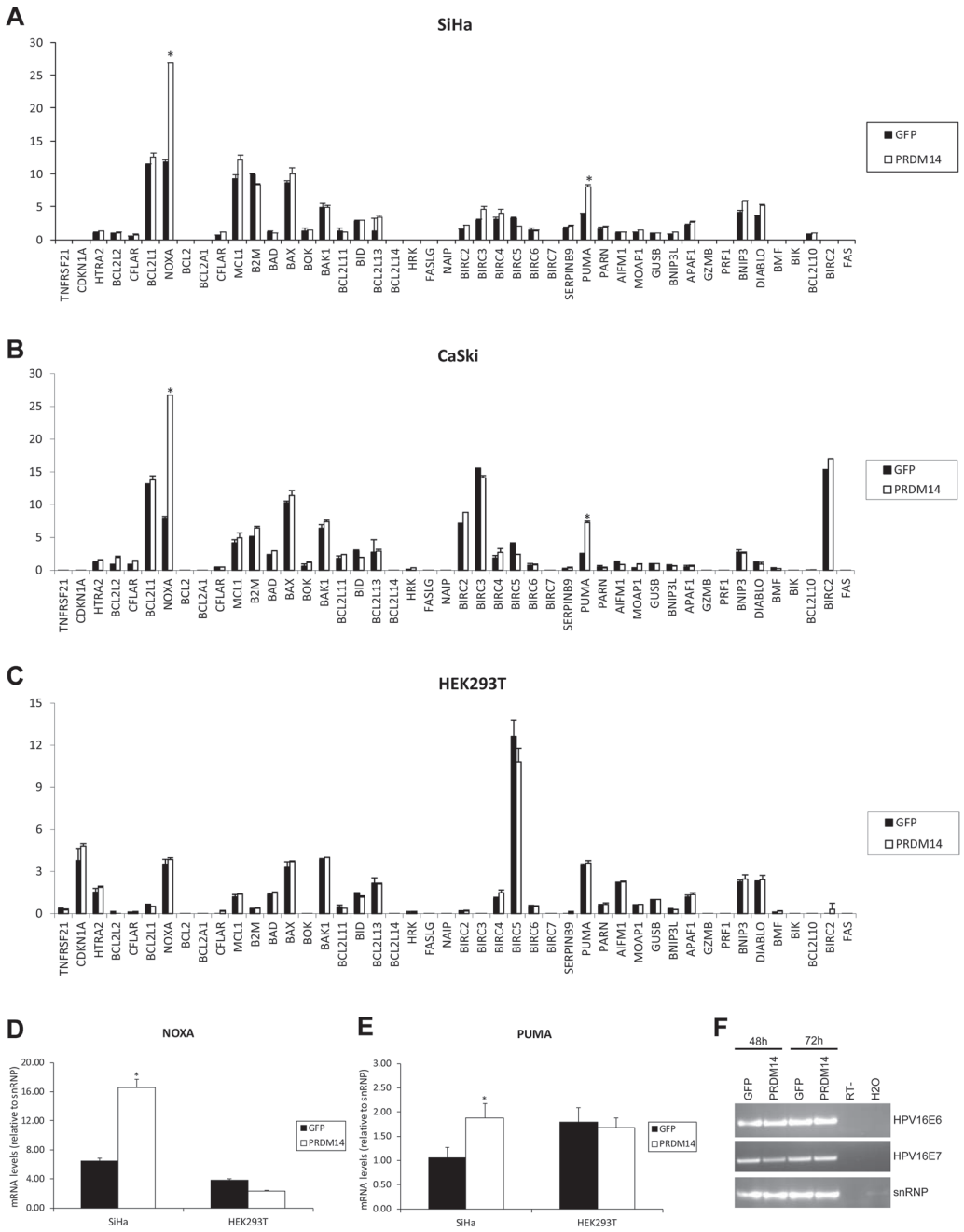

Fig. 3. RT-MLPA results. (**A**) SiHa (**B**) CaSki and (**C**) HEK293T cells. SiHa and CaSki cells transfected with PRDM14 showed a significant increase in mRNA expression of the pro-apoptotic regulators NOXA and PUMA compared to empty vector (GFP) transfected cells 48 h post-transfection. In PRDM14 and empty vector transfected HEK293T cells this increase in NOXA and PUMA mRNA levels was not seen. The increased mRNA levels of NOXA (**D**) and PUMA (**E**) detected in PRDM14 transfected SiHa cells were confirmed by qRT-PCR. (**F**) HVP16E6 and HPV16E7 mRNA expression in FACS-sorted SiHa cells 48 and 72 h post-transfection. SnRNP was used as a reference. (*$P < 0.05$).

containing either the NOXA or PUMA promoter. Both NOXA and PUMA promoters were strongly transactivated by PRDM14 compared to the empty vector control (Figure 4A). As deletion of the carboxy-terminal zinc finger region of PRDM14 has previously been demonstrated to abolish DNA binding (15), SiHa cells were also cotransfected with full-length PRDM14 or a PRDM14 deletion construct lacking its zinc-fingers (deltaDBD; pCAG) with the luciferase reporter constructs. The strong transactivation of NOXA and PUMA promoters as observed with full-length PRDM14 was absent in cotransfectants with the PRDM14-deltaDBD mutant (Figure 4B). These results indicate that PRDM14 can directly activate transcription of NOXA and PUMA by binding to respective promoter sequences.

Interestingly, NOXA has been identified as of one of the candidate genes bound by PRDM14 and we found a consensus PRDM14 DNA binding motif (i.e. 5′–GGTCTCTAA-3′) in the NOXA promoter (15). To determine whether the putative PRDM14 binding site mediates transactivation of the NOXA promoter by PRDM14, eight single nucleotide substitution mutations were introduced to eliminate its potential recognition by PRDM14 (Figure 4C). Whereas the wild-type NOXA promoter region was strongly transactivated, transactivation of the mutant NOXA promoter by wild-type PRDM14 was largely reduced (Figure 4D). These results demonstrate that NOXA is a direct target for PRDM14 transactivation through a PRDM14 binding site within the NOXA promoter region.

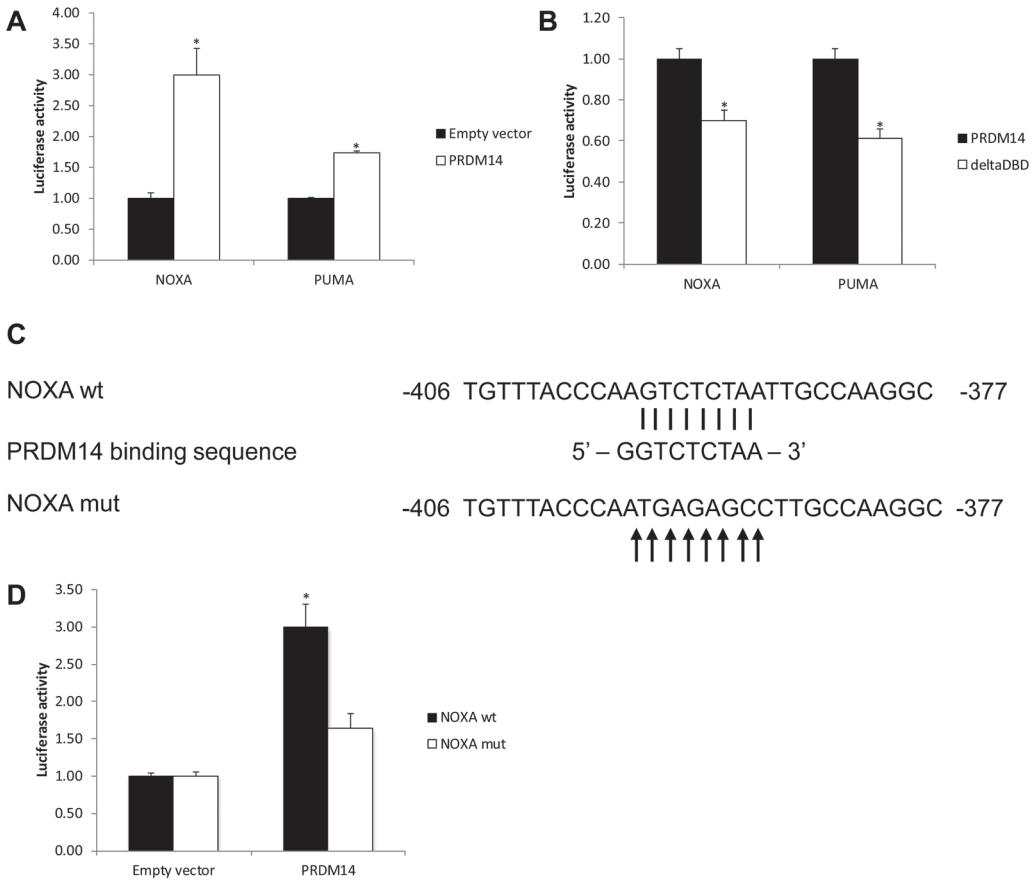

Fig. 4. Luciferase reporter assays. (**A**) PRDM14 transactivated both the NOXA and PUMA promoters. The luciferase activities of empty vector transfected SiHa cells were set to one. (**B**) Ablation of the carboxy-terminal zinc finger of PRDM14 (deltaDBD) reduced transactivation of the NOXA and PUMA promoters compared to full-length PRDM14. The luciferase activities of full-length PRDM14 transfected SiHa cells were set to one. (**C**) Sequence alignment of the putative PRDM14 binding sequence with wild-type NOXA promoter (NOXA wt) is shown. The arrows indicate the mutant nucleotides introduced (NOXA mut). (**D**) Whereas PRDM14 transactivated the wild-type NOXA promoter, transactivation of the mutant NOXA promoter by wild-type PRDM14 was largely reduced (*$P < 0.05$).

Discussion

The present study showed that DNA methylation of the PRDM14 promoter is inversely correlated with its expression in HPV16-positive cancer cells. Re-expression of PRDM14 in HPV16-positive cancer cell lines induced apoptosis, which could be attributed to a direct upregulation of NOXA and PUMA by PRDM14. In case of NOXA this was mediated through a consensus PRDM14-binding site in its promoter. NOXA and PUMA, also known as PMAIP1 and BBC3, respectively, are pro-apoptotic regulators of the intrinsic apoptosis pathway. These results indicate that methylation-mediated silencing of PRDM14 results in apoptosis-resistance in HPV-transformed cells, pointing to a tumour suppressive role for PRDM14 in HPV-induced cancers. In contrast, PRDM14 has been indicated as an oncogenic transcriptional activator in human breast cancer and lymphoid leukaemia in mice (29,30). This discrepancy in oncogenic versus tumour suppressive properties in different cancer types is not an uncommon phenomenon. For example, CADM1 functions as a tumour suppressor in several carcinomas such as lung and cervical cancer (8,10), but as oncoprotein in primary adult T-cell leukaemia/lymphoma (31). p16INK4A on the other hand is a tumour suppressor in various cancers, including colorectal and melanomas (32–34), whereas p16INK4A expression appears necessary for the survival of cervical cancer cells (35). These data indicate that oncogenic or tumour suppressive activities are dependent on the cellular context. The observed tumour suppressive properties of PRDM14 in this study could be dependent on HPV expression. The HPV oncogenes E6 and E7 interact with multiple proteins, thereby altering the cellular context (36–39). One of the HPV16 E6 targets is CARM1, a type-I arginine histone methyltransferase [HMT (40)]. CARM1 methylates histones at p53-responsive promoters resulting in gene activation (41). In addition to directly targeting p53 for degradation through the E6AP pathway, E6 can also target chromatin-bound p53 and repress the activities of the p53 co-activator CARM1. Recently, it has been shown that also PRDM14 can interact with CARM1, thereby regulating chromatin function (42). It may be speculated that the observed PRDM14-mediated upregulation of NOXA and PUMA, both of which are downstream targets of p53, in HPV16-positive cancer cells results from targeting of CARM1 by PRDM14, thereby circumventing E6-mediated CARM1 repression. Next to CARM1, other PRDM14 interacting proteins as well as alternative PRDM14 mRNA transcripts are likely to determine whether or not PRDM14 acts as a transcriptional activator or repressor. Two PRDM14 transcripts have been annotated, one of which encodes the full-length PRDM14 and one short PRDM14 transcript derived from an alternative transcription start-site (43). The latter lacks the PR-domain, which in other PRDM family members has been linked to an oncogenic role [reviewed in (13,44)]. Recently, an un-annotated shorter isoform of PRDM14 was identified in which exon 2 of PRDM14 is skipped (45). Although the PR-domain (encoded by exon 4–5) is retained in this novel PRDM14 isoform, the activity to induce transcription was abolished. It is presently unclear whether this novel PRDM14 isoform is expressed in cancers, and how the alternative PRDM14 transcripts relate to the tumour suppressive function of PRDM14 in HPV-associated cancers. Our data suggest that methylation-mediated silencing of PRDM14 represents a novel mechanism of apoptosis evasion, which may contribute to chemoradiation resistance in HPV-induced cervical cancers and

HPV-positive head-and neck cancers. Cisplatin-based chemotherapy induces apoptosis via the intrinsic pathway and present data indicate a disruption of this pathway by PRDM14 methylation. Additionally, both E6 and E7 of HPV16 are known to contribute to apoptosis evasion by interference with the intrinsic apoptosis pathway as well as the extrinsic pathway via amongst others inhibition of death inducing signalling complex formation (46). A more profound insight into the mechanisms underlying PRDM14-mediated apoptosis regulation in the context of viral oncogene expression is needed to translate current findings into more effective therapeutic strategies for patients with HPV-associated cancers. As epigenetic alterations are reversible, changes in DNA methylation are attractive candidates for cancer therapy (47). Re-expression of PRDM14 may be conferred by treatment with DNA methylation inhibitors such as 5′-aza-2′deoxycytidine. However, their incorporation into nucleic acids limits their specificity of action, which causes hematologic toxicity. Moreover, their half-life in aqueous solution is very short (48). The use of non-nucleoside DNMT inhibitors may offer an alternative approach to reactivate silenced tumour suppressor genes. These inhibitors are not incorporated into DNA and are thought be less toxic and better tolerated in clinical settings. However, the potency of these DNMT inhibitors is much lower than that of 5′-aza-2′-deoxycytidine, and for this reason, they have not yet been used in clinics. Combinatorial therapies of non-nucleoside DNMT inhibitors with chemical drugs may offer more effective treatment options (49). In summary, methylation-mediated silencing of PRDM14 is shown to contribute to apoptosis evasion in HPV-positive oral and cervical cancer cell lines. PRDM14 was identified as a transcriptional regulator of the pro-apoptotic modulators NOXA and PUMA. Given the fact that apoptosis evasion constitutes a major barrier to effective cancer treatment, PRDM14 may serve as a novel therapeutic target in HPV-induced cancers.

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Conflict of Interest Statement: R.D.M.S., P.J.F.S. and C.J.L.M. are shareholders of Self-Screen BV, a spin-off company of VU University Medical Center. All other authors declare no conflicts of interest.

References

1. Ferlay,J. *et al*. (2010) Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int. J. Cancer*, **127**, 2893–2917.
2. Bosch,F.X. *et al*. (2002) The causal relation between human papillomavirus and cervical cancer. *J. Clin. Pathol*., **55**, 244–265.
3. Walboomers,J.M. *et al*. (1999) Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J. Pathol*., **189**, 12–19.
4. McCredie,M.R. *et al*. (2008) Natural history of cervical neoplasia and risk of invasive cancer in women with cervical intraepithelial neoplasia 3: a retrospective cohort study. *Lancet. Oncol*., **9**, 425–434.
5. Wallin,K.L. *et al*. (1999) Type-specific persistence of human papillomavirus DNA before the development of invasive cervical cancer. *N. Engl. J. Med*., **341**, 1633–1638.
6. Winer,R.L. *et al*. (2005) Development and duration of human papillomavirus lesions, after initial infection. *J. Infect. Dis*., **191**, 731–738.
7. Zielinski,G.D. *et al*. (2001) HPV presence precedes abnormal cytology in women developing cervical cancer and signals false negative smears. *Br. J. Cancer*, **85**, 398–404.
8. Steenbergen,R.D. *et al*. (2004) TSLC1 gene silencing in cervical cancer cell lines and cervical neoplasia. *J. Natl. Cancer Inst*., **96**, 294–305.
9. Chen,T.M. *et al*. (1993) Genetic analysis of *in vitro* progression of human papillomavirus-transfected human cervical cells. *Cancer Res*., **53**, 1167–1171.
10. Overmeer,R.M. *et al*. (2008) Association between dense CADM1 promoter methylation and reduced protein expression in high-grade CIN and cervical SCC. *J. Pathol*., **215**, 388–397.
11. Wilting,S.M. *et al*. (2010) Methylation-mediated silencing and tumour suppressive function of hsa-miR-124 in cervical cancer. *Mol. Cancer*, **9**, 167.
12. Steenbergen,R.D. *et al*. (2013) Methylation-specific digital karyotyping of HPV16E6E7-expressing human keratinocytes identifies novel methylation events in cervical carcinogenesis. *J. Pathol*., **231**, 53–62.
13. Fog,C.K. *et al*. (2012) PRDM proteins: important players in differentiation and disease. *Bioessays*, **34**, 50–60.
14. Ma,Z. *et al*. (2011) Sequence-specific regulator Prdm14 safeguards mouse ESCs from entering extraembryonic endoderm fates. *Nat. Struct. Mol. Biol*., **18**, 120–127.
15. Chia,N.Y. *et al*. (2010) A genome-wide RNAi screen reveals determinants of human embryonic stem cell identity. *Nature*, **468**, 316–320.
16. Leitch,H.G. *et al*. (2013) Naive pluripotency is associated with global DNA hypomethylation. *Nat. Struct. Mol. Biol*., **20**, 311–316.
17. Yamaji,M. *et al*. (2013) PRDM14 ensures naive pluripotency through dual regulation of signaling and epigenetic pathways in mouse embryonic stem cells. *Cell Stem Cell*, **12**, 368–382.
18. Steenbergen,R.D. *et al*. (1996) Transition of human papillomavirus type 16 and 18 transfected human foreskin keratinocytes towards immortality: activation of telomerase and allele losses at 3p, 10p, 11q and/or 18q. *Oncogene*, **13**, 1249–1257.
19. Steenbergen,R.D. *et al*. (1995) Integrated human papillomavirus type 16 and loss of heterozygosity at 11q22 and 18q21 in an oral carcinoma and its derivative cell line. *Cancer Res*., **55**, 5465–5471.
20. van Zeeburg,H.J. *et al*. (2005) Generation and molecular characterization of head and neck squamous cell lines of fanconi anemia patients. *Cancer Res*., **65**, 1271–1276.
21. Schmittgen,T.D. *et al*. (2008) Analyzing real-time PCR data by the comparative C(T) method. *Nat. Protoc*., **3**, 1101–1108.
22. de Wilde,J. *et al*. (2008) Alterations in AP-1 and AP-1 regulatory genes during HPV-induced carcinogenesis. *Cell. Oncol*., **30**, 77–87.
23. Vermes,I. *et al*. (1995) A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J. Immunol. Methods*, **184**, 39–51.
24. Hess,C.J. *et al*. (2004) Gene expression profiling of minimal residual disease in acute myeloid leukaemia by novel multiplex-PCR-based method. *Leukemia*, **18**, 1981–1988.
25. Aikawa,T. *et al*. (2010) Noxa is necessary for hydrogen peroxide-induced caspase-dependent cell death. *FEBS Lett*., **584**, 681–688.
26. Han,J. *et al*. (2001) Expression of bbc3, a pro-apoptotic BH3-only gene, is regulated by diverse cell death and survival signals. *Proc. Natl. Acad. Sci. U. S. A*., **98**, 11318–11323.
27. Kreiss,P. *et al*. (1999) Plasmid DNA size does not affect the physicochemical properties of lipoplexes but modulates gene transfer efficiency. *Nucleic Acids Res*., **27**, 3792–3798.
28. Fadok,V.A. *et al*. (1992) Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J. Immunol*., **148**, 2207–2216.
29. Dettman,E.J. *et al*. (2011) Prdm14 initiates lymphoblastic leukemia after expanding a population of cells resembling common lymphoid progenitors. *Oncogene*, **30**, 2859–2873.
30. Nishikawa,N. *et al*. (2007) Gene amplification and overexpression of PRDM14 in breast cancers. *Cancer Res*., **67**, 9649–9657.
31. Masuda,M. *et al*. (2010) CADM1 interacts with Tiam1 and promotes invasive phenotype of human T-cell leukemia virus type I-transformed cells and adult T-cell leukemia cells. *J. Biol. Chem*., **285**, 15511–15522.
32. Voorhoeve,P.M. *et al*. (2003) The tumor-suppressive functions of the human INK4A locus. *Cancer Cell*, **4**, 311–319.
33. Merlo,A. *et al*. (1995) 5′ CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. *Nat. Med*., **1**, 686–692.
34. Esteller,M. (2008) Epigenetics in cancer. *N. Engl. J. Med*., **358**, 1148–1159.
35. McLaughlin-Drubin,M.E. *et al*. (2013) Tumor suppressor p16INK4A is necessary for survival of cervical carcinoma cell lines. *Proc. Natl. Acad. Sci. U. S. A*., **110**, 16175–16180.
36. Boyer,S.N. *et al*. (1996) E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteasome pathway. *Cancer Res*., **56**, 4620–4624.
37. 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.
38. zur Hausen,H. (2002) Papillomaviruses and cancer: from basic studies to clinical application. *Nat. Rev. Cancer*, **2**, 342–350.

39. Steenbergen,R.D. *et al*. (2005) HPV-mediated transformation of the anogenital tract. *J. Clin. Virol*., **32 (suppl. 1)**, S25–S33.
40. Hsu,C.H. *et al*. (2012) The HPV E6 oncoprotein targets histone methyltransferases for modulating specific gene transcription. *Oncogene*, **31**, 2335–2349.
41. An,W. *et al*. (2004) Ordered cooperative functions of PRMT1, p300, and CARM1 in transcriptional activation by p53. *Cell*, **117**, 735–748.
42. Burton,A. *et al*. (2013) Single-cell profiling of epigenetic modifiers identifies PRDM14 as an inducer of cell fate in the mammalian embryo. *Cell Rep*., **5**, 687–701.
43. Wilming,L.G. *et al*. (2008) The vertebrate genome annotation (Vega) database. *Nucleic Acids Res*., **36**(Database issue), D753–D760.
44. Jiang,G.L. *et al*. (2000) The yin-yang of PR-domain family genes in tumorigenesis. *Histol. Histopathol*., **15**, 109–117.
45. Lu,X. *et al*. (2013) SON connects the splicing-regulatory network with pluripotency in human embryonic stem cells. *Nat. Cell Biol*., **15**, 1141–1152.
46. Fuentes-González,A.M. *et al*. (2013) The modulation of apoptosis by oncogenic viruses. *Virol. J*., **10**, 182.
47. Jones,P.A. *et al*. (2007) The epigenomics of cancer. *Cell*, **128**, 683–692.
48. Amatori,S. *et al*. (2011) Premature senescence induced by DNA demethylating agent (Decitabine) as therapeutic option for malignant pleural mesothelioma. *Lung Cancer*, **71**, 113–115.
49. Huang,J. *et al*. (2011) Cancer chemoprevention by targeting the epigenome. *Curr. Drug Targets*, **12**, 1925–1956.

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