

Human papillomavirus type 16 E7 oncoprotein upregulates the retinoic acid receptor-beta expression in cervical cancer cell lines and K14E7 transgenic mice

Jorge Gutiérrez¹ · Enrique García-Villa¹ · Rodolfo Ocadiz-Delgado¹ · Enoc M. Cortés-Malagón² · Juan Vázquez¹ · Alejandra Roman-Rosales¹ · Elizabeth Alvarez-Rios¹ · Haydar Celik³ · Marta C. Romano⁴ · Aykut Üren³ · Paul F. Lambert⁵ · Patricio Gariglio¹

Received: 11 April 2015 / Accepted: 4 July 2015 / Published online: 15 July 2015
© Springer Science+Business Media New York 2015

Abstract Persistent infection with high-risk human papillomaviruses is the main etiological factor in cervical cancer (CC). The human papillomavirus type 16 (HPV16) E7 oncoprotein alters several cellular processes, regulating the expression of many genes in order to avoid cell cycle control. Retinoic acid receptor beta (RARβ) blocks cell growth, inducing differentiation and apoptosis. This tumor suppressor gene is gradually silenced in late passages of foreskin keratinocytes immortalized with HPV16 and in various tumors, including CC, mainly by epigenetic

modifications. We investigated the effect of E7 oncoprotein on RARβ gene expression. We found that HPV16 E7 increases RARβ mRNA and RARβ protein expression both in vitro and in the cervix of young K14E7 transgenic mice. In E7-expressing cells, RARβ overexpression is further increased in the presence of the tumor suppressor p53 (TP53) R273C mutant. This effect does not change when either C33-A or E7-expressing C33-A cell line is treated with Trichostatin A, suggesting that E7 enhances RARβ expression independently of histone deacetylases inhibition. These findings indicate that RARβ overexpression is part of the early molecular events induced by the E7 oncoprotein.

Electronic supplementary material The online version of this article (doi:10.1007/s11010-015-2504-1) contains supplementary material, which is available to authorized users.

✉ Patricio Gariglio
vidal@cinvestav.mx

- ¹ Departamento de Genética y Biología Molecular, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional (CINVESTAV-IPN), Av. IPN 2508, San Pedro Zacatenco, Gustavo A. Madero, 07360 Ciudad de México, México
- ² Laboratorio de Biología Molecular del Cáncer, Unidad de Investigación, Hospital Juárez de México, Av. IPN 5160, Magdalena de Las Salinas, Gustavo A. Madero, 07760 Ciudad de México, México
- ³ Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC 20057-1469, USA
- ⁴ Departamento de Fisiología, Biofísica y Neurociencias, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional (CINVESTAV-IPN), Av. IPN 2508, San Pedro Zacatenco, Gustavo A. Madero, 07360 Ciudad de México, México
- ⁵ McArdle Laboratory for Cancer Research, University of Wisconsin School of Medicine and Public Health, Madison, WI 53706, USA

Keywords HPV16 E7 oncoprotein · RARβ · TP53 R273C mutant · HDACs · Cervical cancer

Introduction

Cancer is a multistep and multifactorial disease that is characterized by the accumulation of various cell-damaging events such as chromosomal instability, inactivation of cell cycle regulatory proteins, apoptosis inhibition, and epigenetic modifications, among others [1, 2]. Cervical cancer (CC), due to its viral nature, is a paradigm to understand the development of other epithelial cancer types. High-risk human papillomaviruses (HR-HPVs) are the main etiological agents of CC, as they are present in over 95 % of tumors, HPV16 being the most prevalent genotype [3]. However, only a small percentage of women infected with HR-HPVs develop CC, indicating that other cofactors are involved along with HR-HPVs to induce cervical carcinogenesis [4]. These viruses primarily exert their oncogenic potential through the E6 and E7 oncoproteins [5]; the sustained expression of these oncoproteins extends the life-span of

primary human cells and facilitates their immortalization [6]. The E6 and E7 oncoproteins lack specific DNA binding activities but can associate with cellular proteins to induce epigenetic alterations, genomic instability and tumor progression [7]. E6 oncoprotein interacts with the tumor suppressor p53 (TP53) [8], inducing its degradation [9], and consequently inhibiting apoptosis [10]. The primary targets of the E7 oncoprotein are the retinoblastoma protein family, pRb, p107, and p130, and their inactivation leads to cell cycle deregulation [11]. In addition, HPV16 E7 expression causes epigenetic reprogramming of cells at the level of both DNA methylation and histone modifications [12–15]. HPV16 E7 expression reduces global trimethylation of lysine 27 on histone H3 (H3K27me3) mark, releasing polycomb repressive complex (PRC)-mediated repression [14]; E7 can also enhance transcription of several genes by the activation of histone acetyltransferases (HATs) and inhibition of histone deacetylases (HDACs), leading to an increase in global H3 acetylation [7, 12, 15, 16]. All the mechanisms mentioned above are involved in the reprogramming of host epithelial cells to avoid cell cycle control.

The retinoic acid receptor beta (RARβ) gene is expressed in epithelial tissue and is upregulated by treatment with all-trans-retinoic acid (ATRA) or retinoic acid (RA), leading to the growth inhibition of oral cancer and CC cells [17–19]. In normal cervical epithelial cells, the RARβ gene is expressed [20]; however, loss of RARβ expression is dependent on cancer stage, and it has been found with varied frequency and correlated with transcriptional repression of its promoter in different tumors, including CC and head and neck squamous cell carcinomas (HNSCCs) [21–26]. Interestingly, in HPV-positive CC cell lines with wild-type TP53, the RARβ gene is not expressed, whereas the HPV-negative CC cell line (C33-A) with mutant TP53 (TP53 R273C) expresses RARβ [23, 25–27]; furthermore, RARβ promoter methylation in HNSCC cell lines is correlated with loss of RARβ mRNA expression and inversely related to the presence of mutated TP53 [25], suggesting that TP53 status together with the presence of HPV could be important to regulate RARβ transcription during HPV infection and carcinogenesis. This agrees with the observation that some TP53 mutants acquire new oncogenic activities to transactivate genes that can promote proliferation of cancer cells [28]. Supporting this observation, it was reported that cervical carcinomas have TP53 gene mutation frequencies from 9 to 16 % [29, 30], and that these somatic mutations are implicated in the pathogenesis of cervical carcinomas [29, 30].

Here, we report for the first time that the HPV16 E7 oncoprotein induces the overexpression of the tumor suppressor RARβ gene in cell lines and cervix of young K14E7 transgenic mice without cervical neoplasia. RARβ overexpression is even higher in vitro through the

synergistic action of HPV16 E7 and TP53 R273C mutant, and this effect is not dependent on HDACs inhibition in the C33-A cell line.

Materials and methods

Cell culture and treatments

HeLa (HPV18), SiHa and CaSki (HPV16), and C33-A (HPV negative) CC cell lines, as well as primary human foreskin keratinocytes (HFKs) and osteosarcoma Saos2 cell line, were originally obtained from the American Type Culture Collection (ATCC), whereas ViPa and CaLo (HPV18) cell lines were kindly provided by Alberto Monroy (FES-Zaragoza, UNAM, México). These cell lines were cultured in DMEM supplemented with 10 % (v/v) FBS, 2 mM L-glutamine, and 100 U/mL penicillin/streptomycin (Invitrogen). Cell lines were incubated at 37 °C in a humidified 5 % CO₂ environment and were grown to 50–80 % confluence before next passage or further experiments.

Primary HFKs and HPV16 E7-transformed HFKs were cultured in keratinocyte serum-free medium containing recombinant epidermal growth factor and bovine pituitary extract as supplements (Gibco, Life Technologies).

For RA treatment, C33-A cells stably expressing HPV16 E7 oncoprotein were cultured for 24 h; culture medium was replaced and cells were incubated three days with 3 μM of RA (Sigma-Aldrich) and two additional days with RA-containing fresh medium before total RNA extraction. Before adding fresh medium with RA, cells were rinsed with phosphate-buffered saline (PBS). The RA was dissolved in dimethyl sulfoxide (Me₂SO; Merck) as a 10 mM stock solution and kept at –70 °C. Control cell culture was incubated in media containing 0.03 % (v/v) Me₂SO alone. The treatment with Trichostatin A (TSA; Sigma-Aldrich) was done according to Zhang et al. [26]. In the 3-deazaneplanocin A (DZNep) experiments, C33-A cells were treated with 30 μM of DZNep (Sigma-Aldrich) for 48 h.

Transient and stable transfections

Cells were seeded at 5×10^5 cells per 60×15 mm dish, transfected with plasmids pcDNA3 (empty vector), pcDNA3E6, pcDNA3E7, pcDNA3TP53 R273C, and pcDNA3TP53 (wild type), and co-transfected pcDNA3E7 with pcDNA3TP53 R273C, pcDNA3TP53, and SureSilencing TP53 siRNA Plasmid Kit (SABiosciences, Qiagen). Plasmids were constructed by inserting cDNA fragments encoding the HPV16 E6 or E7 oncogenes into the pcDNA3 vector at the unique *Hind*III restriction

enzyme site. Orientation of the inserts was determined by DNA sequencing. Transfection was performed using lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. In order to obtain a stable cell line, transfected cells were selected 2 weeks in a growth media containing 1200 µg/mL of G418 (Invitrogen). To keep clones selection, cells were grown continuously in a medium containing 800 µg/mL of G418. For transient assays, cells were harvested 48 h after transfection.

Cell proliferation assay (MTT assay)

The CC cell line C33-A and C33-A cells stably expressing the E7 oncoprotein were seeded in a 24-well plate at a density of 50,000 cells with 1 mL DMEM medium per well. After attachment of cells during 24 h, RA treatment was made according to Cell culture and Treatments section. Cell viability was determined by the conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich); a MTT solution (0.5 mg/mL in DMEM without serum) was prepared and used immediately; for this, culture medium of the 24-well plate was removed, washed using PBS, and replaced by MTT solution; cells were incubated for 30 min at 37 °C. Formazan dye crystals were solubilized from the removed medium with 500 µL acidified isopropanol, and absorbance was measured in a plate reader at 570 nm wavelength (Sunrise, TECAN). Cell viability was expressed in percentage taking as control the C33-A cells without E7 oncogene or RA treatment.

RT-PCR and reverse-transcription quantitative PCR (RT-qPCR)

Total RNA of the CC cell lines, HFks, and osteosarcoma Saos2 cell line, as well as of cervical tissue from the K14E7 mice (previously homogenized by mortar in liquid nitrogen), was extracted using TRIZOL reagent (Invitrogen); afterwards, it was treated with RNase-free DNase I (Invitrogen) according to the manufacturer's instructions and stored at -70 °C. cDNA was synthesized from 5 µg of RNA using SuperScript II reverse transcriptase (Invitrogen) in a volume of 20 µL, following the manufacturer's recommendations. Gene sequences were obtained from the GenBank database and primers (Table 1) that exclude introns were designed using the Primer Express Software and purchased from Invitrogen. PCR conditions used to determine mRNA levels were the following: 95 °C for 3 min, followed by 40 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s including a final step of 72 °C for 7 min. PCR products were separated on 1.5 % agarose and images were obtained following ethidium bromide staining.

The RT-qPCR experiments were performed using SYBR green Master Mix (Thermo Scientific) in an ABI 7300 Real-Time PCR system (Applied Biosystems), and optimal PCR reactions were established as previously mentioned. Each reaction was performed in triplicate. Human beta-2-microglobulin (B2M) and murine glyceraldehyde-3-phosphate dehydrogenase (Gapdh) were used as reference gene, and the threshold value (C_t) for each sample was used to determine gene relative expression levels ($2^{-\Delta\Delta C_t}$ method). RT-qPCR efficiencies were as follows: B2M ($R^2 = 0.992$, Eff% = 96.459); RARB ($R^2 = 0.882$, Eff% = 97.964); Gapdh ($R^2 = 0.996$, Eff% = 105.177); and Rarb ($R^2 = 0.95$, Eff% = 85.000).

Western blotting

Murine cervical tissue, HFks, and osteosarcoma Saos2 cell line were lysed in lysis buffer containing 1 % triton X-100, 4.9 mM/L MgCl₂, 1 mM/L sodium orthovanadate, 2.1 µg/mL aprotinin, 0.5 µg/mL leupeptin, and 1 mM/mL phenylmethylsulfonyl fluoride (PMSF). Proteins were separated by size on 10 % sodium dodecyl sulfate (SDS) polyacrylamide gels (PAGE) and transferred to nitrocellulose membrane (Millipore) by electroblotting. After 1 h blocking with 5 % non-fat dry milk in PBS, membranes were probed overnight with a mouse primary antibody to Rarb (Santa Cruz Biotechnology, C-19) at 1:500 dilution. TP53 antibody (Santa Cruz Biotechnology, sc-126 HRP) was used at 1:500 dilution. Human RARB antibody (Santa Cruz Biotechnology, sc-552) and HPV16 E7 antibody (Santa Cruz Biotechnology, sc-6981) were used at 1:500 dilution. Equal loading and transfer of the proteins were assessed using a monoclonal antibody against beta-actin and tubulin alpha-1A chain (α -tubulin) used at 1:500 dilution (Sigma-Aldrich). Bands were visualized with an anti-rabbit or anti-mouse antibody conjugated with horseradish peroxidase (Sigma-Aldrich) at 1:10,000 dilution. Signals were developed and visualized by ECL Western blotting reagents (Amersham Biosciences). Protein band intensity was analyzed by ImageJ software (version 1.41).

Animals

Mice were housed and treated according to the American Association of Laboratory Animal Care (AALAC) regulations. All mice procedures were carried out under an animal protocol approved by the Research Unit for Laboratory Animal Care Committee (UPEAL-CINVESTAV-IPN, México; NOM-062-ZOO-1999). 1.5-month-old female K14E7 transgenic and inbred syngeneic FvB control mice were used in this study. K14E7 transgenic mice have been described previously [31]. For RT-qPCR

Table 1 Primers used in this study

Gene ^a	Primer	Sequence (5'-3')
RARA	Forward	CTCTCCACCAAGTGCATCATTAAAG
	Reverse	CAGCCTTGAGGAGGGTGATC
PPARG	Forward	CAGGCCGAGAAGGAGAAGCT
	Reverse	GATCGCCCTCGCCTTTG
NR2F2	Forward	CATAC GGATCTTCCAAGAGCAAGT
	Reverse	CATCTGAGGTGAACAGGACTATGG
RARB	Forward	CTCACAG AGAAGATCCGAAAAGC
	Reverse	AATGCACTTGGTGGCCAGTT
TP53	Forward	GATGCTGTCCCCGGACGA
	Reverse	AGGGGCCGCCGGTGTAG
B2M	Forward	ACCCCACTGAAAAAGATGAG
	Reverse	ATGATGCTGCTTACATGTCTCG
HPV16 E6	Forward	CACAGGAGCGACCCAGAAAAG
	Reverse	TATATAC TATGCATAAAATCCCAGAAAAGC
HPV16 E7	Forward	ATGGAGATACACCTACATTGCATGA
	Reverse	AATGGGCTCTGTCCGGTTCT
Rarb	Forward	ACAAGTCATCGGGCTACCAC
	Reverse	ACTGGCATCGGTTCTTAGTG
Gapdh	Forward	CATCTCCTCCCCTTCTGCC
	Reverse	GTGGTGCAGGATGCATTGC

The gene symbols in uppercase letters correspond to human and the lowercase letters to mouse

^a Official gene symbol by HUGO Gene Nomenclature Committee (HGNC)

analysis, three K14E7 and three FvB control non-transgenic mice were used, whereas for Western blot analysis, four mice from each group were included.

Statistical analysis

Student *t* test was used to evaluate the data statistical significance.

Results

HPV16 E7 oncoprotein increases RARB mRNA expression independently of HDACs inhibition in C33-A cell line and the RA treatment does not inhibit cell proliferation

Complete or partial inhibition of RARB gene expression has been observed in many tumor cell lines and primary human tumors, including cervical cancer (CC) [21–26]. In agreement with previous reports, RARB mRNA expression was absent in all tested HPV-positive cell lines (Fig. S1a). In contrast, RARB mRNA expression is observed in the C33-A cell line, which is HPV negative and contains a mutation in the TP53 gene (TP53 R273C) [27]. Given that RAR-alpha, PPAR-gamma and COUP-TF2 (NR2F2 gene)

have been reported as co-activators on the RARB promoter [20, 32–34], we determined if there was a difference in the mRNA expression of these co-activators between the C33-A cell line and the HPV-positive cell lines. Figure S1b shows that only the PPAR-gamma mRNA level was slightly lower in CaSki cells than in the other cell lines.

In order to determine whether the HPV16 E6 and/or E7 oncoproteins cause changes in the level of RARB gene expression in HPV-containing cervical cell lines, we transiently transfected these viral oncogenes into the C33-A cell line (Fig. 1a). Surprisingly, we found by RT-qPCR that the E7 oncoprotein increased the expression of RARB gene almost fourfold when compared with empty vector transfected cells (Fig. 1b). However, in C33-A cells transfected with E6, the RARB mRNA levels were unchanged, and when both oncoproteins were expressed, an intermediate level of RARB mRNA expression was observed (Fig. 1b). To determine if this result was an effect of the transient transfection or due to the quantity of plasmid, we generated stable C33-A cell transfections increasing the amounts of plasmid encoding the HPV16 E7 oncoprotein. We observed by RT-qPCR that RARB expression was about 10-fold higher at all concentrations of the stably transfected E7 expressing clones compared with empty vector (Fig. 1c), and in a similar experiment employing E6, the level of RARB mRNA was not altered (not shown). In addition, we

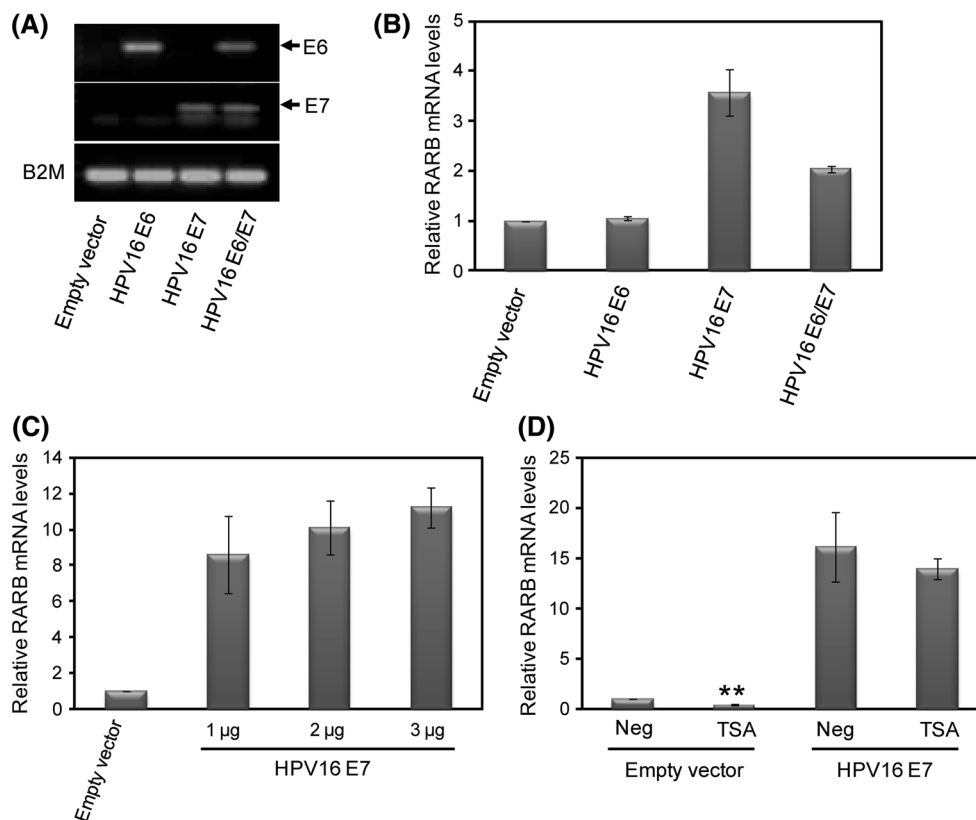


Fig. 1 Exogenous HPV16 E7 increases the level of RARB mRNA expression. C33-A cells were transfected with either pcDNA3 (empty vector) or with plasmids expressing HPV16 E6, E7, or E6/E7 proteins (pcDNA3E6 and pcDNA3E7), as indicated in “Materials and methods” section. **a** Expression of E6 or E7 transcripts in transiently transfected C33-A cells. *Black arrows* indicate the expression either of E6 or E7; additional bands in E7 expression belong to primers used in the RT-PCR reaction as indicated. **b** RT-qPCR for RARB mRNA expression in transiently transfected (1 µg) C33-A cells. **c** RT-qPCR

for RARB mRNA expression in stably transfected C33-A cells with increasing concentrations of HPV16 E7. **d** C33-A cells with stable HPV16 E7 oncoprotein expression were cultured with or without 0.6 µM of TSA, and analyzed by RT-qPCR for RARB mRNA expression. Beta-2-microglobulin (B2M) was used as reference gene. Each point represents the mean of three independent experiments, and *bars* represent \pm standard deviation (SD). Statistically significant changes are indicated by *two asterisks* ($P < 0.001$)

analyzed RARB expression in HPV16 E7-transformed HFKs at passage 5 (RT-qPCR) and passage 7 (Western blotting). The RT-qPCR experiments in HFKs do not show changes on RARB mRNA levels, whereas the Western blotting experiments suggest that in HFKs there is only a slight (1.54-fold) increase in RAR-beta protein levels, due to the presence of the HPV16 E7 oncoprotein (Fig. S2).

Given that RA activates the RARB promoter, we next determined RA effect on the RARB mRNA expression in the C33-A cells expressing E7 by RT-qPCR. In control cells, we obtained a threefold increase in RARB mRNA expression after RA treatment; however, C33-A cells stably expressing the E7 oncoprotein showed a strong (75-fold) RARB mRNA expression after RA treatment (Fig. S3a). It is also known that RARB overexpression promotes growth inhibition of cancer cells [17–19]; but we found through MTT assay that both control C33-A cells and C33-A cells stably expressing the E7 oncoprotein do not show statistically significant changes in proliferation after RA treatment (Fig. S3b; see also Fig. 5 in

“Discussion” section). Moreover, since TSA increases the RARB mRNA expression in SiHa cells through HDACs inhibition [26], we cultured C33-A cells stably expressing E7 in the presence of TSA to determine whether the increased expression of RARB mRNA induced by E7 could be further augmented by HDACs inhibition. C33-A control cells treated with TSA show a slight but significant decrease in RARB mRNA expression; meanwhile, C33-A cells expressing E7 treated with TSA do not show any effect on RARB mRNA expression (Fig. 1d). These results suggest that upregulation of RARB mRNA expression by the E7 oncoprotein in C33-A cells is independent of HDACs inhibition.

To determinate if induction of RARB expression by HPV16 E7 is mediated by a global H3K27me3 mark demethylation, we treated C33-A control cells with 3-Deazaneplanocin A (DZNep), which has been reported to inhibit EZH2 expression and H3K27me3 mark [35]; as part of polycomb repressive complexes, this enzyme is responsible for the trimethylation of H3K27 (H3K27me3) [35]. We

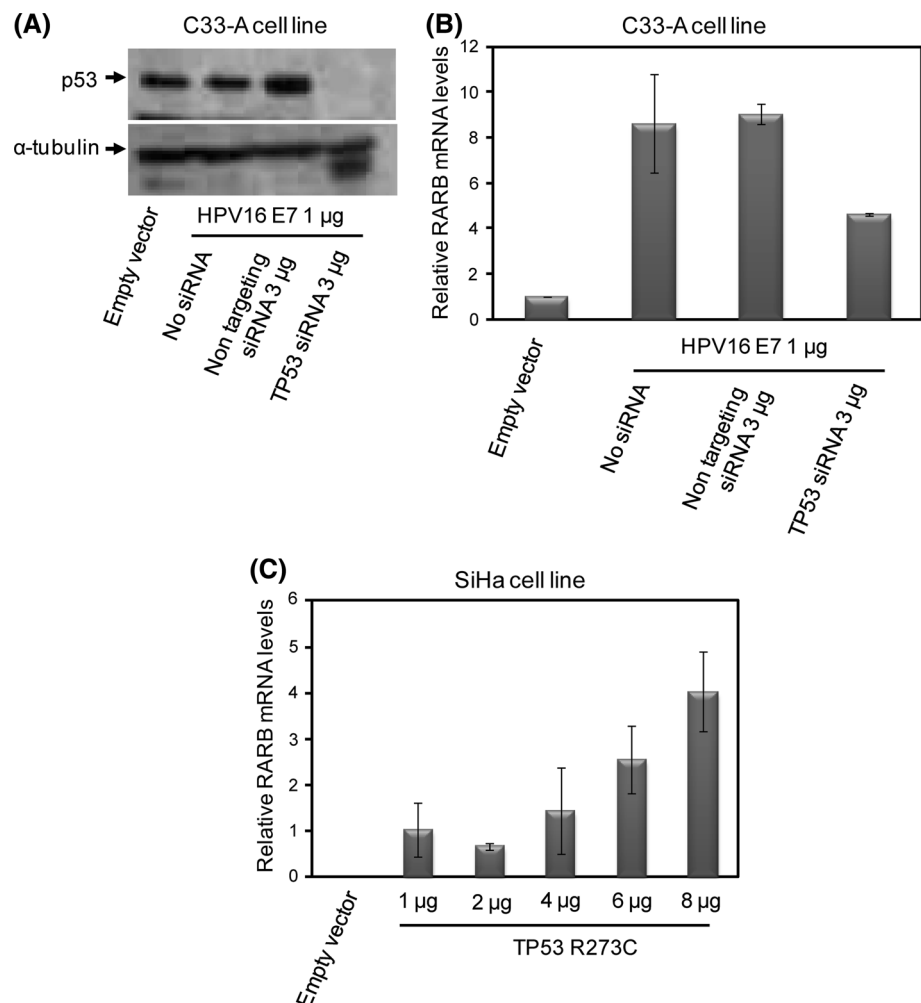
expected that DZNep enhanced RARB mRNA levels mainly in C33-A control cells, but intriguingly, RARB mRNA expression was slightly decreased in both C33-A control cells and C33-A cells expressing E7 (not shown).

The TP53 R273C mutant is involved in the RARB upregulation in cervical cell lines expressing the E7 oncoprotein

Since the C33-A cell line has TP53 mutated, the effect of this mutation was analyzed on RARB expression. Considering that TP53 mutants can transactivate several genes in different tumor cell types [28], it is possible that the TP53 R273C mutant present in C33-A cells could be partially responsible for the increase in RARB mRNA expression (Fig. S1a); hence, we co-transfected C33-A cells stably expressing the E7 plasmid with different amounts of specific TP53 siRNA. We show in Fig. 2a that silencing of mutant TP53 induced a decrease of RARB mRNA levels in C33-A cells; this decrease of RARB mRNA levels was shown only after transfection with 3 μg

of the specific TP53 siRNA, compared with controls without siRNA and with no-targeting siRNA (Fig. 2b); the transfection with less than 3 μg of the specific TP53 siRNA does not silence the TP53 R273C mutant and does not significantly affect RARB mRNA expression (data not shown). To further reinforce the possibility that TP53 R273C mutant is capable to activate the RARB gene, we stably transfected the SiHa cervical cell line (HPV16 positive, with low wild-type TP53 expression) with increasing amounts of plasmid encoding for TP53 R273C mutant or TP53 wild type. Employing RT-qPCR we detected a gradual increase in the expression of RARB mRNA levels in SiHa cells after transfection with increasing amounts of TP53 R273C mutant (Fig. 2c) but not for TP53 wild type (not shown). Considering that SiHa cell line does not express RARB mRNA because its promoter is epigenetically downregulated through an increase in histone deacetylation [26], it is possible that TP53 R273C mutant upregulates RARB expression by a mechanism involving activation of HATs or inhibition of HDACs in SiHa cells.

Fig. 2 TP53 R273C mutant increases RARB mRNA expression in cervical cancer cell lines. **a** Western blot analysis of p53 levels in C33-A and stably E7 transfected C33-A cells co-transfected with TP53 siRNA. Equal amount of proteins [normalized for tubulin alpha-1A chain (α -tubulin)] were loaded onto a SDS PAGE. Three independent experiments were performed. **b** RT-qPCR for the RARB mRNA expression in C33-A cells stably transfected with HPV16 E7 oncogene and silenced for mutant TP53 (TP53 siRNA). **c** SiHa (HPV16-positive) cell line which does not express RARB was stably transfected with increased concentrations of TP53 R273C mutant and analyzed by RT-qPCR for RARB mRNA expression. Beta-2-microglobulin (B2M) was used as reference gene. Each point represents the mean of three independent experiments, and bars represent \pm SD



HPV16 E7 oncoprotein and TP53 R273C mutant synergize to upregulate the RARB mRNA expression

Considering that TP53 R273C mutant together with HPV16 E7 oncoprotein stimulate RARB mRNA expression in the C33-A cell line, we decided to analyze these factors separately in a cell line (Saos2) that do not express the TP53 gene (Fig. S4) or HPV oncoproteins (data not shown). Hence, Saos2 cell line was stably transfected with E7 alone or stably co-transfected with E7 and either wild-type TP53 or TP53 R273C mutant expressing plasmids. As observed in Fig. 3, Saos2 cells stably transfected with TP53 wild type or TP53 R273C mutant plasmids did not substantially modify RARB mRNA expression. On the other hand, HPV16 E7 plasmid alone or together with TP53 R273C mutant increased 6 and 20-fold the RARB mRNA expression level in Saos2 cells, respectively (Fig. 3). Wild-type TP53 had no effect on the RARB mRNA expression when co-transfected with the HPV16 E7 plasmid. These results suggest that *in vitro* TP53 R273C mutant synergizes with the HPV16 E7 oncoprotein to increase the RARB mRNA level.

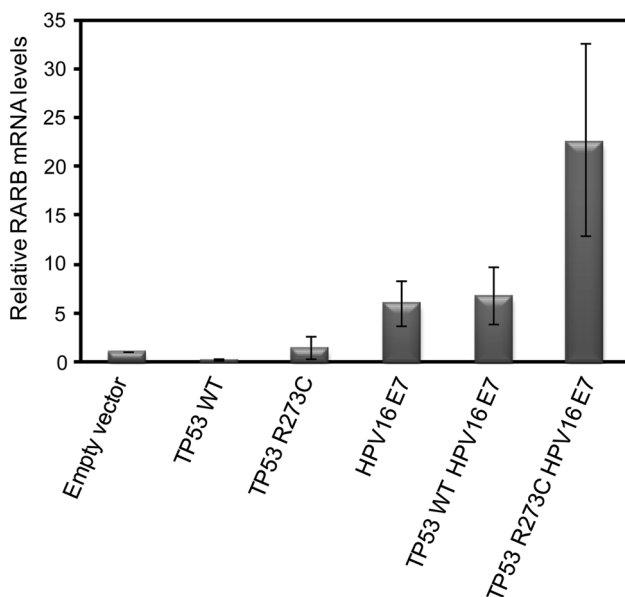


Fig. 3 HPV16 E7 and TP53 R273C mutant synergize to increase the RARB mRNA expression in Saos2 cell line. In the Saos2 cells stably transfected with E7, the RARB expression was increased compared to cells stably transfected with empty vector as determined by RT-qPCR. RARB overexpression was observed with HPV16 E7 alone or stably co-transfected with the TP53 R273C mutant. The Saos2 cells stably transfected with TP53 wild-type (TP53 WT) or TP53 R273C mutant did not alter the RARB expression. Each point represents the mean of three independent experiments and bars represent \pm SD. Beta-2-microglobulin (B2M) was used as reference gene

HPV16 E7 oncoprotein increases Rarb expression in cervical tissue of K14E7 transgenic mice

The K14E7 transgenic mouse expresses the E7 oncoprotein under the human cytokeratin 14 promoter in cervical tissue keratinocytes; this mouse does not develop CC unless chronically treated for 6 months with low doses of 17 β -estradiol [31, 36]; this is a well-validated model to study the effects of the HPV16 E7 oncoprotein and cofactors in cervical carcinogenesis. In order to elucidate the early impact of the HPV16 E7 oncoprotein as a major etiologic factor implicated in cervical carcinogenesis, we determined in young mice the Rarb gene expression by RT-qPCR analysis in cervical tissue obtained from 1.5-month-old K14E7 transgenic mice and FvB control mice. Figure 4a shows that the mRNA level of Rarb was significantly elevated in K14E7 mice when compared with FvB mice. Furthermore, as shown in Fig. 4b, c, RAR-beta protein expression is also increased in 1.5-month-old K14E7 transgenic mice. Thus, we also show *in vivo* that the E7 oncoprotein induces RARB gene overexpression.

Discussion

The present study aimed to determine the effect of the HPV16 E7 oncoprotein on the RARB expression in both cervical cell lines and cervix of young transgenic mice. RARB is the most potent tumor suppressor among retinoic acid receptors; it is involved in cell growth inhibition and blocks AP1 function [17–19, 37]. Moreover, RARB expression is affected in many cancer types, including CC and HNSCC, due to epigenetic modifications (promoter hypermethylation and histone modifications), loss, or structural rearrangements of its sequence [21–24, 26]. For example, in CC development, the RARB transcription decrease is correlated with methylation increase of its promoter, from 11 % in low-grade to 29 % in high-grade lesions and up to 33–63 % in invasive cancers [23, 24]. In this regard, one study reported that transcription of this important gene shows a 1.5-fold increase in benign cervical lesions, while a progressive reduction of RARB expression was seen with advanced stage of the disease [38]. On the other hand, in foreskin keratinocytes immortalized with HPV16 or HPV18, there is a sequential RARB promoter methylation and a decrease at the RARB mRNA level, although this methylation did not become manifested until late passages of these cells [39]. These data suggest that in the presence of HPV, this epigenetic modification on the RARB promoter plays an important role in its transcriptional regulation and that it is dependent on the stage of cervical carcinogenesis. In agreement with these results, we reconfirmed that the RARB gene is not expressed in all

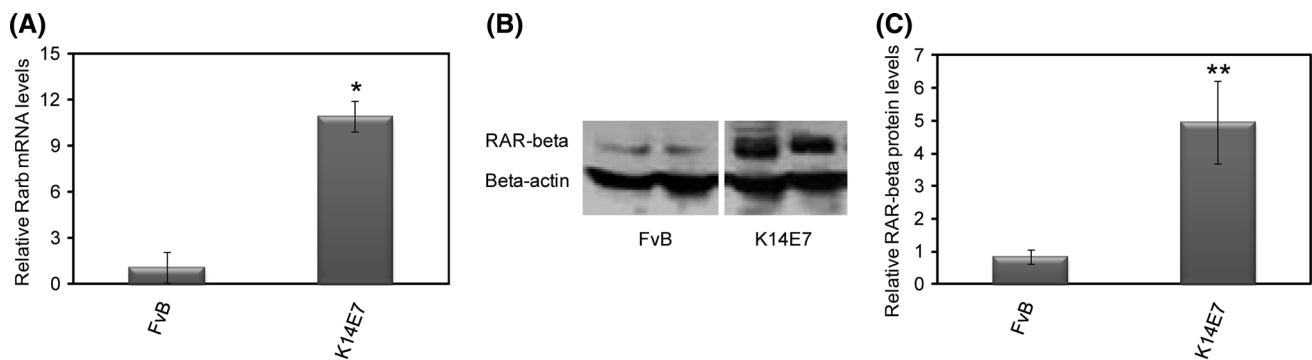


Fig. 4 HPV16 E7 oncoprotein increases Rarb mRNA and RAR-beta protein expression in cervical tissue of 1.5 month-old K14E7 transgenic mice. **a** RT-qPCR of Rarb mRNA expression for indicated cervical samples. Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was used as reference gene, and each bar represents the mean and \pm SD from three independent experiments, each performed in triplicate. **b** Western blot analysis of RAR-beta protein expression

for indicated cervical samples (two different 1.5-month-old FvB or K14E7 transgenic mice), normalized for beta-actin. **c** Densitometry analysis of RAR-beta protein expression with averages \pm SDs of four K14E7 transgenic mice and four FvB control mice was performed at least in triplicate for each mouse. Statistically significant changes are indicated by *one asterisk* ($P < 0.05$) or *two asterisks* ($P < 0.001$)

cancer cell lines tested HPV positive but expressed in the HPV-negative C33-A cell line (Fig. S1a).

Since the HPV16 E7 oncoprotein can repress E-cadherin promoter by augmenting DNA (cytosine-5)-methyltransferase 1 activity (DNMT1) [13, 40], we thought that E7 could be responsible for the RARB silencing in HPV-positive cancer cell lines. Unexpectedly, we found that RARB mRNA expression increased in C33-A cells transiently or stably transfected with the E7 oncoprotein (Fig. 1b, c), respectively. In addition, Saos2 cell line stably transfected with the E7 oncoprotein also increased RARB mRNA levels, as compared with non-transfected Saos2 cells (Fig. 3). These results indicate that the HPV16 E7 oncoprotein does not repress RARB promoter in these cells; on the contrary, it increases RARB mRNA expression.

Unlike cervical keratinocytes of K14E7 transgenic mice, primary HFKs are minimally responsive to the RARB induction by the E7 oncoprotein. One possible explanation may be related with the report of Darwiche [41], indicating that only RARA and RXR transcripts were expressed in the cervical stratified squamous subjunctional epithelium compared to the simple columnar epithelium (highly responsive to vitamin A), which expresses high levels of RARA, RXR, and RARB transcripts [41]. Unfortunately, primary HFKs are obtained from foreskin, formed mainly of stratified squamous epithelium, suggesting that they could be relatively resistant to RARB overexpression induced by the HPV16 E7 oncoprotein (Fig. S2). Besides, it is important to consider that *in vivo*, several microenvironmental factors, as well as some transcriptional cofactors related to the cervical tissue of K14E7 transgenic mice, may contribute to RARB overexpression (Fig. 4). Among these microenvironmental factors, we can include the unaltered mucosa, the presence of stroma and immune system that may affect the outcome in E7-transfected HFK cells.

Because HR-HPV E7 interacts with HDACs to regulate expression of certain genes [12, 16], it is possible to predict that HDACs could be regulating RARB expression; however, when the C33-A cell line and C33-A expressing the E7 oncoprotein were treated with the HDAC inhibitor TSA, the RARB mRNA expression did not change (Fig. 1d), suggesting that in control C33-A and C33-A cells expressing E7, the RARB gene upregulation is not dependent on HDACs inhibition.

Somatic mutations of TP53 gene are found in 9–16 % of CC tumors [29, 30]; since the cervical cancer-derived C33-A cell line contains the TP53 R273C mutant [27], we evaluated its effect on RARB expression. We surprisingly found that this TP53 R273C mutant cooperates with E7 in RARB overexpression; this effect was observed in C33-A, SiHa, and Saos2 cells. Since RARB promoter is silenced by HDACs in the SiHa cell line [26], we postulated that the TP53 R273C mutant in the presence of the E7 oncoprotein might contribute to the RARB activation through a mechanism involving HDACs inhibition in this cell line (Fig. 2c). With respect to this, it was shown that several genes are transactivated by TP53 mutants [28] through histone acetylation at target promoters [42]. Additionally, it has been demonstrated that several genes are upregulated by the TP53 R273H mutant, which is very similar to TP53 R273C mutant (both TP53 mutants enhance proliferation, invasion, and drug resistance, among others) [43, 44]; TP53 R273C mutant is a DNA-contact mutant which contains a missense mutation that commonly makes direct contact with DNA sequences and has negative dominance over wild-type TP53 [28].

Even though the wild-type TP53 degradation is promoted by the E6 oncoprotein [9], some TP53 mutants are resistant to E6-mediated degradation [45]. We observed that the RARB overexpression in C33-A cells stably transfected

with both E7 and E6 oncoproteins is lower compared with E7 alone, suggesting that E6 oncoprotein might partially degrade the TP53 R273C mutant; in line with this result, it was published that HPV16 E6 binds to mutant TP53 R273C protein and promotes its degradation [46].

The *in vitro* results were also confirmed using the *in vivo* model in which E7 is expressed by keratinocytes in the cervical tissue of 1.5-month-old K14E7 transgenic mice without cervical neoplasia (the absence of exogenous estrogen treatment), indicating a remarkable increase in both *Rarb* mRNA and RAR-beta protein levels compared to FvB control mice (Fig. 4). It is important to emphasize that even though E7 induces high genomic instability, this mouse does not develop cancer but only a moderate squamous epithelial hyperplasia in the exocervix [31]. As was mentioned previously, the K14E7 transgenic mice only develops cervical cancer when they are chronically treated for 6 months with 17 β -estradiol; interestingly, it was reported in breast cancer that the estrogen receptors (ER α and ER β), which are activated by 17 β -estradiol repress RARB gene transcription [47]. Based on these findings, one might suggest that in K14E7 transgenic mouse, the increase of *Rarb* expression induced by the E7 oncoprotein is given in the absence of estrogen before the tumor develops.

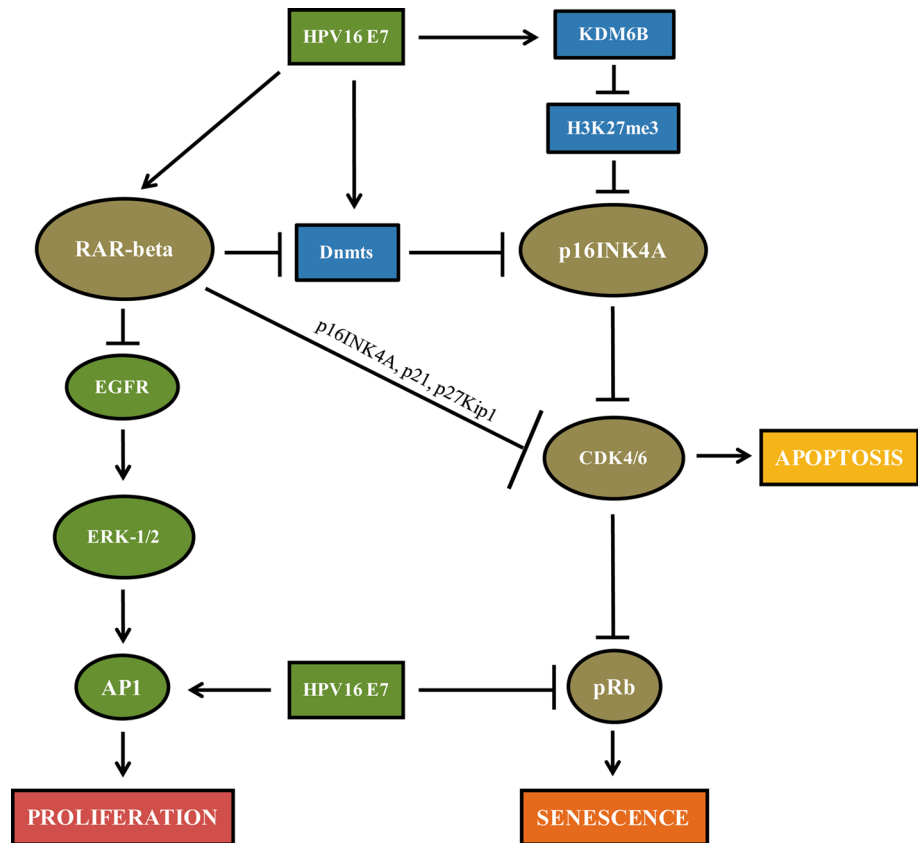
It has been demonstrated that the repressive H3K27me3 mark is decreased in HPV16 E7-expressing cells and in HPV16-positive cervical lesions by transcriptional induction of the KDM6B histone 3 lysine 27-specific demethylase [48]. Even more, the HPV16 E7 oncoprotein induces the overexpression of the tumor suppressor p16INK4A gene through KDM6B activation, which is necessary for survival of cervical carcinoma cell lines [14, 49], indicating that RARB is not the only tumor suppressor gene activated by E7. DZNep inhibits the repressive H3K27me3 mark, inducing reactivation of genes not silenced by methylation of CpG islands on the promoter [35]. It is important to note that RARB promoter is hypomethylated in C33-A cells [26]; interestingly, this gene was not induced in either C33-A control cells or in E7-expressing C33-A cells by DZNep, but instead, there was a slight reduction in RARB mRNA levels in these cells (not shown). One possible explanation for the observed RARB reduction in the presence of DZNep could be related to inhibition of the active histone H3K4me3 mark [35]. It is also tempting to speculate that DZNep treatment induces a RARB repressor, masking the expected result; but we still think that RARB upregulation by E7 may be related to the modification of histone methylation marks. Supporting this, it is known that the H3K27me3 mark is catalyzed by EZH2 as part of the polycomb repressive complex 2 (PRC2), and that the HPV16 E7 oncoprotein reduces these complexes in HPV16 E7-expressing cells [50]. On the other hand, RARB overexpression in HPV16 E7-expressing cells might be similar

to that induced by Adenovirus E1A oncoprotein. This oncoprotein is a structural and functional homologous of the E7 oncoprotein [11], being a cofactor for RARB promoter activation through direct interaction with the RAR-beta protein [51, 52]. According to the structural homology between E1A and E7 oncoproteins, E1A oncoprotein also induces p16INK4A expression in primary human fibroblasts [53]. Thus, it is possible that both p16INK4A and RARB may have oncogenic functions in carcinogenesis. In line with this, it has been published that the presence of RARB in the stromal compartment is essential for the growth of mammary carcinoma [54], and that inactivation of RARB gene inhibits Wnt1-induced mammary tumorigenesis by suppressing epithelial-mesenchymal transitions [55]. According to these findings, RARB expression might be essential in early cervical carcinogenesis.

The observed increase of RARB expression in E7-expressing cells could be required to block Dnmts activity [56] in early carcinogenesis (Fig. 5), favoring p16INK4A gene expression [14]; however, HPV16 E7 expression may also increase Dnmts activity [13]. It has been seen that RARB (activated by RA or ATRA) induces cellular senescence through p16INK4A and p21 activation [56]. Although HPV16 E7 activates p16INK4A [14] and RARB expression (our article), there is no senescence induction because E7 simultaneously inhibits the hypophosphorylated active form of the pRb protein [57], which is a key mediator of p16INK4A-induced senescence [58]. In addition, p16INK4A expression is necessary for survival of HPV E7-expressing cells, and this oncogenic p16INK4A activity is dependent on inhibition of cyclin-dependent kinases (CDKs) [49]. Even though RARB inhibits cell cycle progression by increasing the rate of cyclin D1 proteolytic turnover and the expression of CDK inhibitors such as p21, p27Kip1 and p16INK4A [56, 59–61], it is well known that the HPV16 E7 oncoprotein can override the activities of CDK inhibitors by abrogating their function [62–64]. Another important tumor suppressor activity of RARB is mediated by inhibition of the epidermal growth factor receptor (EGFR) [65], with subsequent decreases in ERK-1/2 phosphorylation and AP1 activity [66]. Interestingly, HPV E7 abrogates AP1 inhibition by directly transactivating “downstream” the AP1 family of transcription factors [67]. Thereby, E7 might avoid unfavorable consequences derived from the upregulation of both p16INK4A and RARB tumor suppressors in similar ways (Fig. 5). Finally, there is a possibility, as was described for p16INK4A [49], that RARB gene might have some unknown function that provides an advantage in early HPV infection.

In conclusion, we demonstrate that under certain *in vitro* cellular backgrounds and in the cervix of young K14E7 transgenic mice, the HPV16 E7 oncoprotein induces the

Fig. 5 Schematic representation for the biological significance of tumor suppressor RARB overexpression induced by the HPV16 E7 oncoprotein. HPV16 E7 upregulates p16INK4A through KDM6B induction, which inhibits the H3K27me3 mark, inducing senescence and inhibition of CDK-dependent apoptosis. The RARB upregulation by the HPV16 E7 oncoprotein might inhibit Dnmts activity and cause hypomethylation of the p16INK4A promoter. Furthermore, tumor suppressor RARB could also enhance the level of CDK inhibitors and block EGFR leading to a decrease in API function. However, the HPV16 E7 oncoprotein could bypass RARB antiproliferative functions by directly activating API family of transcription factors. See “Discussion” section for details and references



RARB gene, as previously observed in benign cervical lesions [38]. The mechanism involved in RARB overexpression induced by the HPV16 E7 oncoprotein needs further investigation.

Acknowledgments The authors would like to thank CONACYT, Lauro Macías, and Gabriela Mora (CINVESTAV-IPN) for technical support.

Compliance with Ethical Standards

Conflict of interest No personal interest to declare.

References

- Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. *Cell* 144:646–674
- Mesri EA, Feitelson MA, Munger K (2014) Human viral oncogenesis: a cancer hallmarks analysis. *Cell Host Microbe* 15:266–282
- Li N, Franceschi S, Howell-Jones R, Snijders PJ, Clifford GM (2011) Human papillomavirus type distribution in 30,848 invasive cervical cancers worldwide: variation by geographical region, histological type and year of publication. *Int J Cancer* 128:927–935
- Gariglio P, Gutierrez J, Cortes E, Vazquez J (2009) The role of retinoid deficiency and estrogens as cofactors in cervical cancer. *Arch Med Res* 40:449–465
- Munger K, Baldwin A, Edwards KM, Hayakawa H, Nguyen CL, Owens M, Grace M, Huh K (2004) Mechanisms of human papillomavirus-induced oncogenesis. *J Virol* 78:11451–11460
- Halbert CL, Demers GW, Galloway DA (1992) The E6 and E7 genes of human papillomavirus type 6 have weak immortalizing activity in human epithelial cells. *J Virol* 66:2125–2134
- McLaughlin-Drubin ME, Munger K (2009) Oncogenic activities of human papillomaviruses. *Virus Res* 143:195–208
- Werness BA, Levine AJ, Howley PM (1990) Association of human papillomavirus types 16 and 18 E6 proteins with p53. *Science* 248:76–79
- Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM (1990) The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* 63:1129–1136
- Lagunas-Martinez A, Madrid-Marina V, Gariglio P (2010) Modulation of apoptosis by early human papillomavirus proteins in cervical cancer. *Biochim Biophys Acta* 1805:6–16
- McLaughlin-Drubin ME, Munger K (2009) The human papillomavirus E7 oncoprotein. *Virology* 384:335–344
- Brehm A, Nielsen SJ, Miska EA, McCance DJ, Reid JL, Banister AJ, Kouzarides T (1999) The E7 oncoprotein associates with Mi2 and histone deacetylase activity to promote cell growth. *EMBO J* 18:2449–2458
- Burgers WA, Blanchon L, Pradhan S, de Launoit Y, Kouzarides T, Fuks F (2007) Viral oncoproteins target the DNA methyltransferases. *Oncogene* 26:1650–1655
- McLaughlin-Drubin ME, Crum CP, Munger K (2011) Human papillomavirus E7 oncoprotein induces KDM6A and KDM6B histone demethylase expression and causes epigenetic reprogramming. *Proc Natl Acad Sci U S A* 108:2130–2135

15. Zhang B, Laribee RN, Klemsz MJ, Roman A (2004) Human papillomavirus type 16 E7 protein increases acetylation of histone H3 in human foreskin keratinocytes. *Virology* 329:189–198
16. Bodily JM, Mehta KP, Laimins LA (2011) Human papillomavirus E7 enhances hypoxia-inducible factor 1-mediated transcription by inhibiting binding of histone deacetylases. *Cancer Res* 71:1187–1195
17. Geisen C, Denk C, Kupper JH, Schwarz E (2000) Growth inhibition of cervical cancer cells by the human retinoic acid receptor beta gene. *Int J Cancer* 85:289–295
18. Hayashi K, Yokozaki H, Naka K, Yasui W, Lotan R, Tahara E (2001) Overexpression of retinoic acid receptor beta induces growth arrest and apoptosis in oral cancer cell lines. *Jpn J Cancer Res* 92:42–50
19. Si SP, Lee X, Tsou HC, Buchsbaum R, Tibaduiza E, Peacocke M (1996) RAR beta 2-mediated growth inhibition in HeLa cells. *Exp Cell Res* 223:102–111
20. Geisen C, Denk C, Gremm B, Baust C, Karger A, Bollag W, Schwarz E (1997) High-level expression of the retinoic acid receptor beta gene in normal cells of the uterine cervix is regulated by the retinoic acid receptor alpha and is abnormally down-regulated in cervical carcinoma cells. *Cancer Res* 57:1460–1467
21. Choi CH, Lee KM, Choi JJ, Kim TJ, Kim WY, Lee JW, Lee SJ, Lee JH, Bae DS, Kim BG (2007) Hypermethylation and loss of heterozygosity of tumor suppressor genes on chromosome 3p in cervical cancer. *Cancer Lett* 255:26–33
22. Ivanova T, Petrenko A, Gritsko T, Vinokourova S, Eshilev E, Kobzeva V, Kisseljov F, Kisseljova N (2002) Methylation and silencing of the retinoic acid receptor-beta 2 gene in cervical cancer. *BMC Cancer* 2:4
23. Narayan G, Arias-Pulido H, Koul S, Vargas H, Zhang FF, Vilella J, Schneider A, Terry MB, Mansukhani M, Murty VV (2003) Frequent promoter methylation of CDH1, DAPK, RARB, and HIC1 genes in carcinoma of cervix uteri: its relationship to clinical outcome. *Mol Cancer* 2:24
24. Xu XC, Mitchell MF, Silva E, Jetten A, Lotan R (1999) Decreased expression of retinoic acid receptors, transforming growth factor beta, involucrin, and cornifin in cervical intraepithelial neoplasia. *Clin Cancer Res* 5:1503–1508
25. Youssef EM, Lotan D, Issa JP, Wakasa K, Fan YH, Mao L, Hassan K, Feng L, Lee JJ, Lippman SM, Hong WK, Lotan R (2004) Hypermethylation of the retinoic acid receptor-beta(2) gene in head and neck carcinogenesis. *Clin Cancer Res* 10:1733–1742
26. Zhang Z, Joh K, Yatsuki H, Zhao W, Soejima H, Higashimoto K, Noguchi M, Yokoyama M, Iwasaka T, Mukai T (2007) Retinoic acid receptor beta2 is epigenetically silenced either by DNA methylation or repressive histone modifications at the promoter in cervical cancer cells. *Cancer Lett* 247:318–327
27. Scheffner M, Munger K, Byrne JC, Howley PM (1991) The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. *Proc Natl Acad Sci U S A* 88:5523–5527
28. Freed-Pastor WA, Prives C (2012) Mutant p53: one name, many proteins. *Genes Dev* 26:1268–1286
29. Ojesina AI, Lichtenstein L, Freeman SS, Pedamallu CS, Imaz-Rosshandler I, Pugh TJ, Cherniack AD, Ambrogio L, Cibulskis K, Bertelsen B, Romero-Cordoba S, Trevino V, Vazquez-Santillan K, Guadarrama AS, Wright AA, Rosenberg MW, Duke F, Kaplan B, Wang R, Nickerson E, Walline HM, Lawrence MS, Stewart C, Carter SL, McKenna A, Rodriguez-Sanchez IP, Espinosa-Castilla M, Woie K, Bjorge L, Wik E, Halle MK, Hoivik EA, Krakstad C, Gabino NB, Gomez-Macias GS, Valdez-Chapa LD, Garza-Rodriguez ML, Maytorena G, Vazquez J, Rodea C, Cravioto A, Cortes ML, Greulich H, Crum CP, Neuberger DS, Hidalgo-Miranda A, Escareno CR, Akslen LA, Carey TE, Vintermyr OK, Gabriel SB, Barrera-Saldana HA, Melendez-Zajgla J, Getz G, Salvesen HB, Meyerson M (2014) Landscape of genomic alterations in cervical carcinomas. *Nature* 506:371–375
30. Tomesello M, Annunziata C, Buonaguro L, Losito S, Gregg S, Buonaguro FM (2014) TP53 and PIK3CA gene mutations in adenocarcinoma, squamous cell carcinoma and high-grade intraepithelial neoplasia of the cervix. *J Transl Med* 12:255
31. Herber R, Liem A, Pitot H, Lambert PF (1996) Squamous epithelial hyperplasia and carcinoma in mice transgenic for the human papillomavirus type 16 E7 oncogene. *J Virol* 70:1873–1881
32. James SY, Lin F, Kolluri SK, Dawson MI, Zhang XK (2003) Regulation of retinoic acid receptor beta expression by peroxisome proliferator-activated receptor gamma ligands in cancer cells. *Cancer Res* 63:3531–3538
33. Kruyt FA, van den Brink CE, Defize LH, Donath MJ, Kastner P, Kruijer W, Chambon P, van der Saag PT (1991) Transcriptional regulation of retinoic acid receptor beta in retinoic acid-sensitive and -resistant P19 embryocarcinoma cells. *Mech Dev* 33:171–178
34. Lin B, Chen GQ, Xiao D, Kolluri SK, Cao X, Su H, Zhang XK (2000) Orphan receptor COUP-TF is required for induction of retinoic acid receptor beta, growth inhibition, and apoptosis by retinoic acid in cancer cells. *Mol Cell Biol* 20:957–970
35. Miranda TB, Cortez CC, Yoo CB, Liang G, Abe M, Kelly TK, Marquez VE, Jones PA (2009) DZNep is a global histone methylation inhibitor that reactivates developmental genes not silenced by DNA methylation. *Mol Cancer Ther* 8:1579–1588
36. Brake T, Lambert PF (2005) Estrogen contributes to the onset, persistence, and malignant progression of cervical cancer in a human papillomavirus-transgenic mouse model. *Proc Natl Acad Sci U S A* 102:2490–2495
37. De-Castro Arce J, Soto U, van Riggelen J, Schwarz E, zur Hausen H, Rosl F (2004) Ectopic expression of nonliganded retinoic acid receptor beta abrogates AP-1 activity by selective degradation of c-Jun in cervical carcinoma cells. *J Biol Chem* 279:45408–45416
38. Abu J, Batuwangala M, Symonds P (2008) Expression of RAR beta2 gene by real-time RT-PCR: differential expression in normal subjects compared to cervical cancer patients normalised against GAPDH as a housekeeping gene. *Eur J Obstet Gynecol Reprod Biol* 140:295–296
39. Henken FE, Wilting SM, Overmeer RM, van Rietschoten JG, Nygren AO, Errami A, Schouten JP, Meijer CJ, Snijders PJ, Steenbergen RD (2007) Sequential gene promoter methylation during HPV-induced cervical carcinogenesis. *Br J Cancer* 97:1457–1464
40. Laurson J, Khan S, Chung R, Cross K, Raj K (2010) Epigenetic repression of E-cadherin by human papillomavirus 16 E7 protein. *Carcinogenesis* 31:918–926
41. Darwiche N, Celli G, De Luca LM (1994) Specificity of retinoid receptor gene expression in mouse cervical epithelia. *Endocrinology* 134:2018–2025
42. Vrba L, Junk DJ, Novak P, Futscher BW (2008) p53 induces distinct epigenetic states at its direct target promoters. *BMC Genom* 9:486
43. Li J, Yang L, Gaur S, Zhang K, Wu X, Yuan YC, Li H, Hu S, Weng Y, Yen Y (2014) Mutants TP53 p. R273H and p.R273C but not p.R273G enhance cancer cell malignancy. *Hum Mutat* 35:575–584
44. Vaughan CA, Singh S, Windle B, Sankala HM, Graves PR, Andrew Yeudall W, Deb SP, Deb S (2012) p53 mutants induce transcription of NF-kappaB2 in H1299 cells through CBP and STAT binding on the NF-kappaB2 promoter and gain of function activity. *Arch Biochem Biophys* 518:79–88
45. Bernard X, Robinson P, Nomine Y, Masson M, Charbonnier S, Ramirez-Ramos JR, Deryckere F, Trave G, Orfanoudakis G (2011) Proteasomal degradation of p53 by human papillomavirus E6 oncoprotein relies on the structural integrity of p53 core domain. *PLoS One* 6:e25981

46. Scheffner M, Takahashi T, Huibregtse JM, Minna JD, Howley PM (1992) Interaction of the human papillomavirus type 16 E6 oncoprotein with wild-type and mutant human p53 proteins. *J Virol* 66:5100–5105
47. Ribeiro MP, Santos AE, Custodio JB (2014) Interplay between estrogen and retinoid signaling in breast cancer—current and future perspectives. *Cancer Lett* 353:17–24
48. McLaughlin-Drubin ME, Munger K (2013) Biochemical and functional interactions of human papillomavirus proteins with polycomb group proteins. *Viruses* 5:1231–1249
49. McLaughlin-Drubin ME, Park D, Munger K (2013) Tumor suppressor p16INK4A is necessary for survival of cervical carcinoma cell lines. *Proc Natl Acad Sci USA* 110:16175–16180
50. McLaughlin-Drubin ME, Huh KW, Munger K (2008) Human papillomavirus type 16 E7 oncoprotein associates with E2F6. *J Virol* 82:8695–8705
51. Folkers GE, van der Saag PT (1995) Adenovirus E1A functions as a cofactor for retinoic acid receptor beta (RAR beta) through direct interaction with RAR beta. *Mol Cell Biol* 15:5868–5878
52. Kruyt FA, Folkers GE, Walhout AJ, van der Leede BJ, van der Saag PT (1993) E1A functions as a coactivator of retinoic acid-dependent retinoic acid receptor-beta 2 promoter activation. *Mol Endocrinol* 7:604–615
53. Mroz EA, Baird AH, Michaud WA, Rocco JW (2008) COOH-terminal binding protein regulates expression of the p16INK4A tumor suppressor and senescence in primary human cells. *Cancer Res* 68:6049–6053
54. Liu X, Nugoli M, Laferrriere J, Saleh SM, Rodrigue-Gervais IG, Saleh M, Park M, Hallett MT, Muller WJ, Giguere V (2011) Stromal retinoic acid receptor beta promotes mammary gland tumorigenesis. *Proc Natl Acad Sci U S A* 108:774–779
55. Liu X, Giguere V (2014) Inactivation of RARbeta inhibits Wnt1-induced mammary tumorigenesis by suppressing epithelial-mesenchymal transitions. *Nucl Recept Signal* 12:e004
56. Lim JS, Park SH, Jang KL (2011) All-trans retinoic acid induces cellular senescence by up-regulating levels of p16 and p21 via promoter hypomethylation. *Biochem Biophys Res Commun* 412:500–505
57. Boyer SN, Wazer DE, Band V (1996) E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteasome pathway. *Cancer Res* 56:4620–4624
58. Ohtani N, Yamakoshi K, Takahashi A, Hara E (2004) The p16INK4a-RB pathway: molecular link between cellular senescence and tumor suppression. *J Med Investig* 51:146–153
59. Li R, Faria TN, Boehm M, Nabel EG, Gudas LJ (2004) Retinoic acid causes cell growth arrest and an increase in p27 in F9 wild type but not in F9 retinoic acid receptor beta2 knockout cells. *Exp Cell Res* 294:290–300
60. Mongan NP, Gudas LJ (2007) Diverse actions of retinoid receptors in cancer prevention and treatment. *Differentiation* 75:853–870
61. Park SH, Lim JS, Jang KL (2011) All-trans retinoic acid induces cellular senescence via upregulation of p16, p21, and p27. *Cancer Lett* 310:232–239
62. Jones DL, Alani RM, Munger K (1997) The human papillomavirus E7 oncoprotein can uncouple cellular differentiation and proliferation in human keratinocytes by abrogating p21Cip1-mediated inhibition of cdk2. *Genes Dev* 11:2101–2111
63. Shin MK, Balsitis S, Brake T, Lambert PF (2009) Human papillomavirus E7 oncoprotein overrides the tumor suppressor activity of p21Cip1 in cervical carcinogenesis. *Cancer Res* 69:5656–5663
64. Zerfass-Thome K, Zwerschke W, Mannhardt B, Tindle R, Botz JW, Jansen-Durr P (1996) Inactivation of the cdk inhibitor p27KIP1 by the human papillomavirus type 16 E7 oncoprotein. *Oncogene* 13:2323–2330
65. Song S, Lippman SM, Zou Y, Ye X, Ajani JA, Xu XC (2005) Induction of cyclooxygenase-2 by benzo[a]pyrene diol epoxide through inhibition of retinoic acid receptor-beta 2 expression. *Oncogene* 24:8268–8276
66. Xu XC (2007) Tumor-suppressive activity of retinoic acid receptor-beta in cancer. *Cancer Lett* 253:14–24
67. Antinore MJ, Birrer MJ, Patel D, Nader L, McCance DJ (1996) The human papillomavirus type 16 E7 gene product interacts with and trans-activates the AP1 family of transcription factors. *EMBO J* 15:1950–1960