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The expression of the transcription factor TAF1 is modified by the HPV16 E2 protein

J. SÁNCHEZ-RAMOS[1], G. POZO-MOLINA[2], E. GARRIDO[1*]

[1]Department of Genetics and Molecular Biology, CINVESTAV-IPN, Mexico City, C.P. 07360, Mexico; [2]Facultad de Estudios Superiores Iztacala-UNAM, Tlalnepantla, Estado de México, C.P. 54090, Mexico

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Summary. – High-risk human papillomaviruses (e.g., HPV16 and 18) are associated with cervical cancer occurrence and development. The early viral gene E2 encodes a protein involved in several key processes in HPV biology, such as replication, genome segregation, and viral gene transcription. E2's presence also affects the expression of a variety of cellular genes involved in a wide range of biological processes, including cell cycle regulation and apoptosis, which are mediated by E2's interaction with cellular proteins. In this report, a lentiviral system was used to express the HPV16 E2 gene in the HPV-negative C-33A cell line for several weeks. E2 expression was measured by RT-qPCR and its biological activity was evaluated using a reporter gene. In HPV16 E2-positive cells, we observed a statistically significant increase in mRNA and protein levels of TAF1 and p27, a basal transcription factor and one of its target genes, respectively. To our knowledge, this is the first study showing that the viral protein HPV16 E2 upregulates TAF1 expression. This suggests that E2's expression promotes a transcriptionally-favorable cellular environment that allows HPV to successfully complete its replication cycle.

Keywords: HPV16; E2 protein; transcription; TAF1 regulation

High-risk human papillomaviruses (e.g., HPV16 and HPV18) are strongly associated with cervical cancer, anogenital cancer, and a growing number of head and neck tumors (Egawa *et al.,* 2015). The early-expressed gene E2 encodes a protein that performs several relevant functions in the viral replication cycle. E2 initiates HPV's genome replication by recruiting the viral helicase E1 and the cell's replication machinery to the viral replication origin. During mitosis, E2 segregates replicated viral genomes into daughter cells through its interaction with chromatin adaptor proteins, such as bromodomain-containing protein 4 (BRD4). E2 also binds to DNA sequence ACCG4NCGGT (E2-binding site (E2BS)) present in the viral LCR to regulate the expression of the viral oncogenes E6 and E7 (Demeret *et al.,* 1997). In addition to regulating viral processes, E2 modifies the expression of several cellular genes that are involved in proliferation, differentiation, apoptosis, senescence, and cell motility (Burns *et al.,* 2010; Ramírez-Salazar *et al.,* 2011; Gauson *et al.,* 2014). Although multiple cellular promoters harbor potential E2BS sequences, E2 binding does not always affect the transcriptional activity of these promoters (Võsa *et al.,* 2012). Therefore, E2 uses an indirect mechanism to modify cellular gene expression, which involves its interaction with cellular proteins (Muller and Demeret, 2012; Jang *et al.,* 2015).

The HPV16 E2 protein interacts with a wide spectrum of cellular proteins involved replication, RNA processing, chromatin remodeling, cell cycle control, and apoptosis. From E2's interaction with TATA binding protein (TBP), Homeobox C9 (HOXC9), transducer of ERBB2 (TOB1) (Muller and Demeret, 2012), P53 (Massimi *et al.,* 1999), p300 (Krüppel *et al.,* 2008), BRD4 (Wu *et al.,* 2016), and TATA-box binding protein associated factor 1 (TAF1) (Centeno *et*

*Corresponding author. E-mail: egarrido@cinvestav.mx; phone: +(52-55)-57473800-5373.

Abbreviations: HPV = human papillomavirus; LCR = long control region; TAF1 = TATA-box binding protein associated factor 1

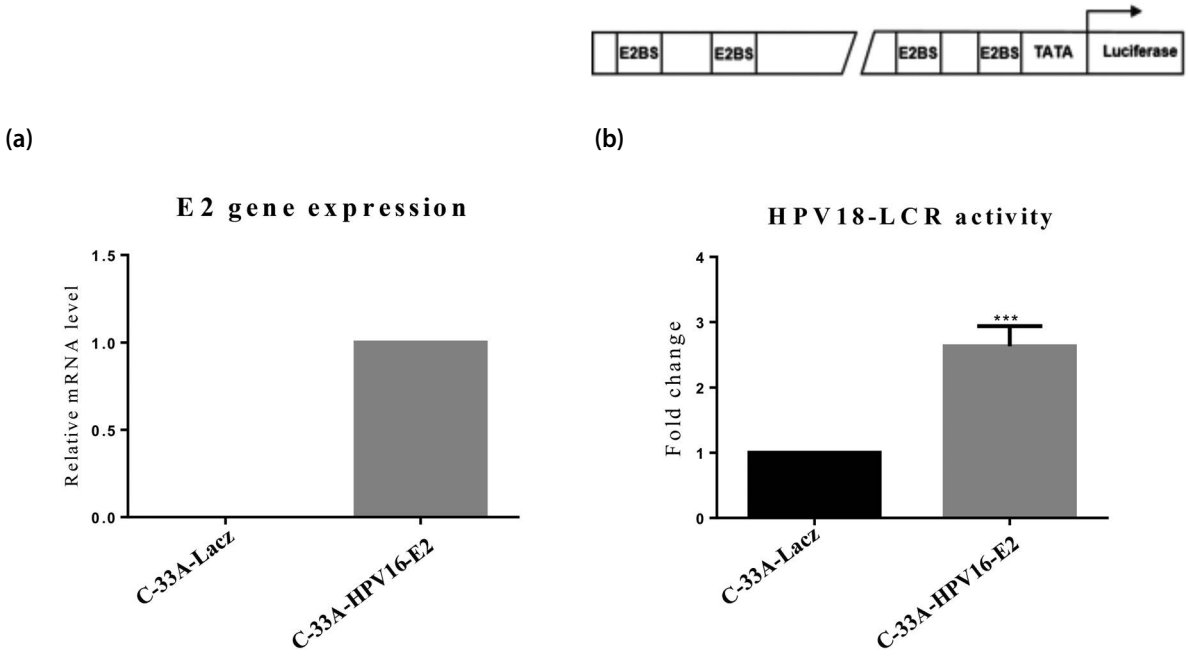

Fig. 1

HPV16 E2 expression and activity

RT-qPCR analysis of HPV16-E2 mRNA levels in C-33A-Lacz and C-33A-HPV16-E2 cells **(a)**. Top: representation of HPV18 LCR cloned into pGL4.10 vector showing four E2-binding sites and a TATA box; bottom: pGL4.10-HPV18 LCR transcriptional activity (E2 responsive promoter) in C-33A-Lacz and C-33A-HPV16-E2 cells **(b)**. Data from three independent experiments presented as mean ± SD (**p <0.05).

al., 2008), it can be deduced that E2 also has a significant participation in transcriptional regulation.

In a previous report, we demonstrated that E2 has a direct protein-protein interaction with TAF1, and this interaction is crucial for the transcriptional regulation of the viral early gene promoter (Centeno *et al.*, 2008), pointing out the importance of TAF1 in transcription of HPV genes. In the present work, we evaluated whether the HPV16 E2 protein affects TAF1 expression and its activity as a transcriptional regulator.

Using a previously reported lentiviral system (Domínguez-Catzín *et al.*, 2017), C-33A cells were infected with Lenti-HPV16-E2 or Lenti-Lacz for 24 h to allow virus adsorption. The viruses where then removed and puromycin selection (0.45 µg/ml) was applied 48 h post infection to obtain C-33A cells that constitutively express HPV16-E2 (C-33A-HPV16-E2 cells) or control C-33A cells that express Lacz (C-33A-Lacz). To prevent loss of the lentiviral vector, cells were permanently maintained in the presence of puromycin. To determine transduction and transgene expression in cells, total RNA was extracted using TRIzol ™ reagent method and treated with RQ1 DNase (Promega, USA) for 2 h at 37°C. Two µg of total RNA were transcribed into cDNA using the enzyme M-MLV RT at 42°C and Oligo-dT15 (Promega, USA). Then, the relative expression of the E2 mRNA was determined by RT-qPCR using a specific pair of primers (Fw: 5' ATTCCGAATTCATGGAGACTCT 3', Rev: 5' TTCGGGATCCTCATATAGACAT 3') that generate a 250 bp amplicon of the HPV16 E2 gene. Untranscribed RNA was used as a no template control (NTC). To assess the biological activity of E2 protein, we cloned the HPV18 LCR (6929nt-88nt from the viral genome) into the 5'-Kpn I/Xho I-3' sites of the pGL4.10-basic® vector (Promega, USA) that encodes the luciferase reporter gene to generate the pGL4.10-LCR18 construct. The HPV18 LCR promoter harbors four E2-BSs. Both cell lines, C-33A-HPV16-E2 and C-33A-Lacz, were transfected with 2 µg the pGL4.10-LCR18 vector using Lipofectamine® 2000 reagent, following the manufacturer's instructions. Luciferase activity was evaluated using the Luciferase assay system (Promega, USA).

The relative levels of TAF1 and p27 mRNA were evaluated after reverse transcription by RT-qPCR with specific primers for TAF1 (Fw: 5' ACAACATCGGGAAGAGATGC 3', Rv: 5' CAGGACGCTCCTTCATTTTC 3'), p27 (Fw: 5' TAACTCT GAGGACACGCAT 3', Rv: 5' TTCTTCTGTTCTGTTGGCTC 3'), and β-actin as a housekeeping endogenous control (Fw: 5' GCGGGAAATCGTGCGTGACATT 3', Rev: 5' GATGGAGTT GAAGGTAGTTTCGTG 3'). As NTC we used untranscribed RNA. The relative expression of these genes was calculated based on $^{\Delta\Delta}$Ct values. The protein level was evalu-

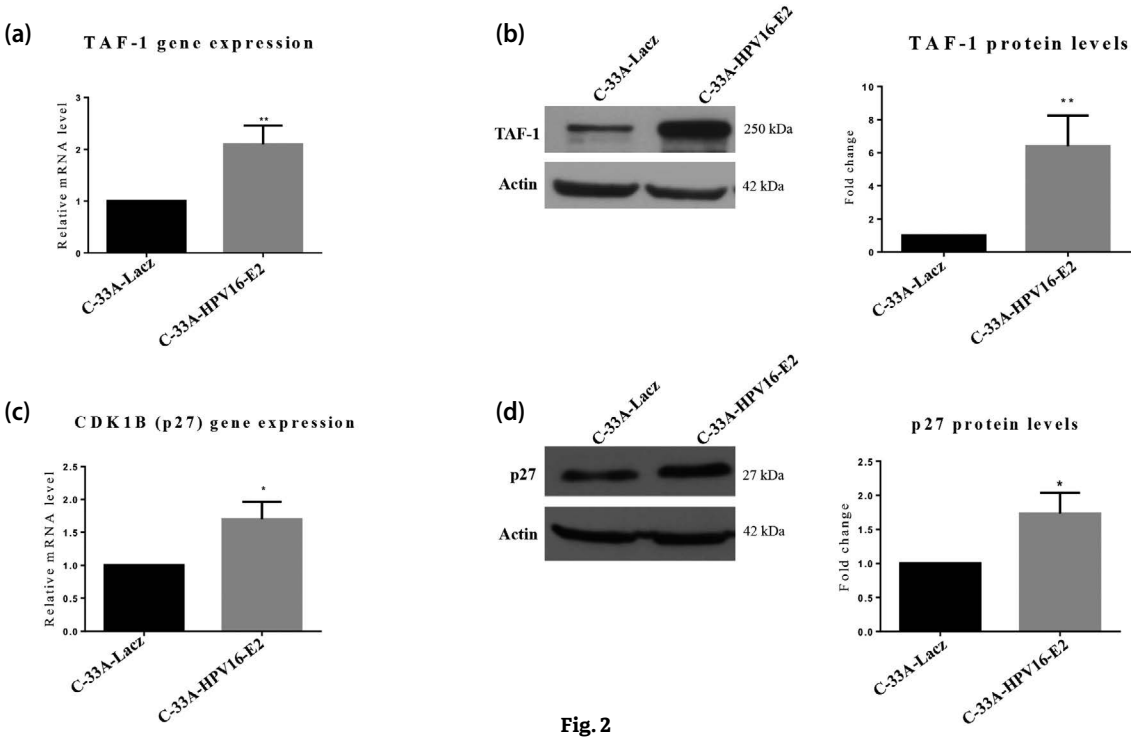

Fig. 2

An increase in TAF1 levels modifies p27 expression levels in C-33A-HPV16-E2 cells

RT-qPCR analysis of TAF1 mRNA levels in C-33A-Lacz and C-33A-HPV16-E2 cells **(a)**. Representative western blot image (left) and densitometric analysis of the TAF1 protein (right) in C-33A-Lacz and C-33A-HPV16-E2 cells **(b)**. RT-qPCR analysis of p27 mRNA levels in C-33A-Lacz and C-33A-HPV16-E2 cells **(c)**. Representative western blot image (left) and densitometric analysis of the p27 protein (right) in C-33A-Lacz and C-33A-HPV16-E2 cells **(d)**. Data from three independent experiments presented as mean ± SD (**p <0.05).

ated by western blot analysis. Briefly, cells were lysed in RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (Complete 1x, Roche Diagnostic, Switzerland). Equal amounts of total proteins (30 µg) from the different samples were separated on a denaturing polyacrylamide gel (SDS-PAGE). Proteins were transferred to a nitrocellulose membrane and blocked with 5% non-fat milk in PBS. Membranes were incubated with anti-TAF1 (1:500) or anti-p27 (1:250) monoclonal antibodies (Santa Cruz Biotechnology, USA), followed by horseradish peroxidase-conjugated goat anti-mouse antibody (Jackson, ImmunoResearch Laboratories, USA). Proteins were observed using SuperSignal™ Western Pico chemiluminescent substrate (Thermo Scientific, USA). The membranes were stripped and re-blotted with anti-actin antibodies (1:100,000) (Sigma, USA). The quantification of the digital images of three independent experiments was performed using ImageJ software. The statistical significance of the difference between intergroup comparisons was obtained using Student's *t*-test. Data were expressed as mean ± SD and were representative of at least three independent experiments. Statistical significance was defined as a p value <0.05.

Figure 1a shows the RNA expression of the HPV16 E2 gene in the transduced cells (C-33A-HPV16-E2 cells). The survival of these cells for several passages indicates that HPV16 E2 is expressed at low levels because it has been reported that high levels of HPV16 E2 induce apoptosis (Demeret *et al.*, 2003). The luciferase activity in C-33A-HPV16-E2 cells transiently transfected with the pGL4.10-LCR18 vector demonstrates that E2 binds HPV18-LCR to promote transcription of the luciferase gene (Fig. 1b). This confirms that our cell-based system is an appropriate model for studying E2's transcriptional activity in the absence of other HPV proteins. In a previous study, we used a recombinant adenoviral system that permitted HPV16 E2 expression in C-33A cells for a short period of time to demonstrate that E2 modifies the mRNA levels of multiple cellular genes, including several TAF1 regulated genes (Ramírez-Salazar *et al.*, 2011).

TAF1 is essential for the assembly of TBP with the rest of the TAFs to create the TFIID complex. TAF1 interacts with transcriptional activators to recruit this complex to particular promoters, thus allowing its transcription (Wassarman and Sauer, 2001). Our results indicate that the constant expression of the viral protein increases TAF1 mRNA levels (Fig. 2a). Increase in TAF1 mRNA levels could

be caused by E2-mediated transcriptional activation of TAF1's promoter. E2 may bind DNA directly or indirectly via interactions with a particular transcription factor or factors. TAF1 protein levels were considerably higher in cells that expressed E2, reaching more than six times the amount observed in control cells (Fig. 2b). To our knowledge, this is the first report that shows evidence of an increase in TAF1 protein levels induced by the presence of a viral protein. However, determining the precise mechanism by which E2 regulates TAF1 overexpression and its biological consequences requires more experiments. The increased expression of TAF1 could have biological consequences in cellular processes that involve HPV16 E2 and/or TAF1. Additionally, the E2-TAF1 protein interaction we described previously (Centeno *et al.,* 2008) could aid in stabilizing TAF1, which is an important factor of the basal transcriptional machinery, and differentially directs its binding to particular gene promoters.

In the cell, TAF1 controls the expression of a high number of genes, including some associated with apoptosis and cell cycle regulation (Kimura *et al.* 2008). The CDKN1B (p27) gene is implicated in both processes (Abbastabar *et al.,* 2018) and is a well-known TAF1 target gene. Using RT-qPCR, we analyzed p27 mRNA levels in C-33A-HPV16-E2 cells, which were about two times higher than in control cells (Fig. 2c). A similar behavior was observed at the protein level, i.e., we detected twice higher amounts of p27 in E2-expressing cells than in control cells (Fig. 2d). These results agree with those reported by Kimura *et al.*, 2008, who demonstrated that TAF1 regulates apoptosis by controlling p27 expression. Our study also suggests that when HPV16 E2 increases TAF1 protein levels, it may modify the expression of other TAF1 target genes.

Increased levels of TAF1 protein in HPV-infected cells might benefit the virus' replication cycle in two points: first, the HPV16 E2-TAF1 interaction impedes E2 from repressing the viral p97 promoter to allow early viral gene expression (Centeno *et al.,* 2008); and second, downstream TAF1-regulated genes could help generate a cellular environment that is favorable for several stages of HPV's replication cycle (Kim *et al.,* 2005).

E2 expression promotes differentiation, which is required for late gene expression and viral maturation. Increased TAF1 levels, and possibly, its interaction with HPV16 E2, could participate in the regulation of many genes involved in differentiation that E2 affects, such as involucrin, filaggrin, CK1, and CK10 (Burns *et al.,* 2010). The transcriptional promoters of some of these genes harbor a TATA box and/or an *Inr* sequence, which could be regulated by TAF1.

In this work, we demonstrated that increased TAF1 levels in C-33A-HPV16-E2 cells correlates with an increase in p27 mRNA levels, suggesting that at least a portion of TAF1 is transcriptionally active to regulate some of its target genes. Preliminary results in our research group indicate that TAF1 binding to the *Inr* sequence in p27's promoter region of p27 is not increased in HPV16 E2-expressing cells, suggesting the existence of alternative transcription start sites in the p27 gene that involve TAF1. TAF1's role in regulating apoptosis was demonstrated using RNA interference to downregulate TAF1, in turn, p27's expression was also decreased and cells became resistant to oxidative stress-induced apoptosis (Kimura *et al.,* 2008). Additionally, p27 downregulates the kinase activity of the cyclin E/cdk2 complex to avoid the G1-S transition in the cell cycle. Because both apoptosis and cell proliferation must be finely controlled by key regulators in response to particular stimuli, the role of TAF1 and p27 in these processes in cells expressing HPV16 E2 should be evaluated in the future.

The results in this work suggest that increasing TAF1 levels might generate a positive feedback system with E2. Initially, E2 upregulates TAF1 expression, and a portion of it interacts with E2 to regulate the viral early gene promoter and particular cellular genes. Subsequently, another portion of TAF1 that is E2-free can regulate a different group of cellular genes.

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