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“REGULACIÓN DE LA VÍA DE SEÑALIZACIÓN CELULAR WNT/β-CATENINA POR LAS PROTEÍNAS E6 Y E6*I DEL VPH-18 A TRAVÉS DEL FACTOR TRANSCRIPCIONAL TCF-4”

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Mamá,

Papá,

Kenia y Tania,

y María…..

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Índice

Abstract 9
Resumen 10
Introducción 11
Cáncer cervicouterino 11
Virus del Papiloma Humano 11
Ciclo replicativo del VPH 12
Oncoproteínas virales (E6 y E7) 15
Isoformas de E6 (E6*) 17
Funciones de E6*I 19
Vía de señalización celular Wnt/β-catenina 20
Regulación de la vía de Wnt por la oncoproteína E6 23
Justificación 27
Hipótesis 27
Objetivo general 28
Objetivos específicos 28
Materiales y métodos 29
Cultivo de células y transfección 29
Plásmidos 29
Ensayos de actividad transcripcional del TCF-4 30
Reacción en cadena de la polimerasa cuantitativa (qPCR) 30
Western blot 31
Ensayo de inmunoprecipitación 31
Análisis de la estabilidad del TCF-4 32
Ensayo de inmunofluorescencia 32
Extracción y purificación de proteínas de fusión GST-E6 33
Ensayo de fraccionamiento celular 33
Ensayo *in vitro* de interacción de proteínas con el ADN 34
Ensayo de inmunoprecipitación de la cromatina 34
Ensayos de proliferación celular 35
Análisis estadístico 36

Resultados 37
Discusión.. 54
Conclusiones.. 59
Perspectivas.. 59
Referencias 60

Abstract

The Wnt/β-catenin signaling pathway regulates diverse processes associated with cell proliferation and differentiation, and its aberrant activation in cervical cancer has been previously described. Persistent infection with high-risk human papilloma virus (HR-HPV) is the main risk factor for the development of this neoplasia, since HPV viral oncoproteins E6 and E7 alter cellular processes commonly found in cancer. Previously, the E6 oncoprotein of HPV-16 has been associated with the regulation of the Wnt/β-catenin signaling pathway. However the participation of E6 of HPV-18 has not been studied. The aim of this work was to investigate the participation of the E6 and E6*I proteins of HPV-18 in the regulation of the Wnt/β-catenin signaling pathway. In this study, we demonstrate that E6 proteins enhance TCF-4 transcriptional activity and promote overexpression of Wnt/β-catenin target genes. Moreover, it was shown that E6 and E6*I bind to TCF-4 and β-catenin, impacting in stabilization of TCF-4. We found that the E6 and E6*I proteins interact with the Sp5 promoter, *in vitro* and *in vivo*. In addition, although differences were found in the transcriptional activation of TCF-4 among the E6 oncogene intratype variants, no changes were observed in the levels of target genes tested. In addition, our data indicate that the E6 isoforms cooperate with β-catenin to promote cell proliferation. In this work, a novel mechanism of the Wnt/β-catenin pathway activation by the E6 and E6*I of HPV-18 is described for the first time.

Resumen

La vía de señalización de Wnt/β-catenina regula diversos procesos asociados a la proliferación y diferenciación celular y su activación aberrante se ha descrito en cáncer cervical. La infección persistente con el virus del papiloma humano de alto riesgo (VPH-AR) es el factor más importante para el desarrollo de esta neoplasia, ya que las oncoproteínas virales E6 y E7 alteran los procesos celulares y promueven el desarrollo del cáncer cervical. Previamente se ha relacionado la oncoproteína E6 del VPH-16 en la regulación de la vía de señalización de Wnt/β-catenina; sin embargo, la participación de E6 del VPH-18 no se ha estudiado. El objetivo de este trabajo fue investigar la participación de las proteínas E6 y E6*I del VPH-18 en la regulación de la vía de señalización Wnt/β-catenina. En este estudio demostramos que las proteínas E6 potencian la actividad transcripcional de TCF-4 y promueven la sobreexpresión de los genes blanco de la vía de Wnt. Además, se demostró que E6 y E6*I se unen a TCF-4 y β-catenina, lo que afecta a la estabilización de TCF-4. Encontramos que las proteínas E6 y E6*I interactúan con el promotor de Sp5, *in vitro* e *in vivo*. Además, aunque se encontraron diferencias en la activación transcripcional de TCF-4 entre las variantes intratipo del oncogén E6 del VPH-18, no se observaron cambios en los niveles de genes blanco probados. Además, nuestros datos indican que las isoformas de E6 cooperan con β-catenina para promover la proliferación celular. En este trabajo se evidencia por primera vez un mecanismo de regulación de la activación de la vía de Wnt/β-catenina por las proteínas E6 y E6*I del VPH-18.

Introducción

Cáncer cervicouterino

El cáncer cervicouterio (CaCU) es un grave problema de salud pública y ocupa el cuarto lugar en mortalidad e incidencia en neoplasias femeninas en todo el mundo, registrando 311,365 muertes y 569,847 nuevos casos anuales (Bray et al., 2018). En México, para el año 2018 se estimaron 7,869 nuevos casos y 4,121 defunciones (IARC, 2018). La infección persistente por el Virus del Papiloma Humano (VPH) es la causa necesaria más no suficiente para el desarrollo de CaCU, encontrándose la presencia del genoma viral en el 99.7% de los casos (Walboomers et al., 1999). Sin embargo, diversos factores de riesgo se han asociado al establecimiento del CaCU, que pueden ser determinantes para su desarrollo, tales como: el tabaquismo, el uso prolongado de anticonceptivos orales, número de parejas sexuales, multiparidad, edad del inicio de la vida sexual, co-infecciones con otros agentes biológicos como *Chlamydia trachomatis* y Herpes virus tipo 2, entre otros (Chelimo et al., 2013).
Además de la fuerte asociación entre la infección del VPH y el desarrollo del CaCU, estudios epidemiológicos demuestran que otros tipos de cáncer están altamente relacionados con este agente biológico como : el cáncer de vagina (70% de los casos), vulva (43%), pene (50%), ano (88%) y orofaringe (13-56%) (Forman et al., 2012; Li and Xu, 2017).

Virus del Papiloma Humano

Actualmente se han descrito más de 200 tipos de VPH (de Villiers, 2013) capaces de infectar el epitelio escamoso en diferenciación, al estar en contacto con las células basales a través de micro lesiones. De acuerdo con el tipo de epitelio que infectan los tipos virales son clasificados en dos grupos: cutaneotrópicos y mucosotrópicos. La mayoría de los cutáneos pertenecen al género beta y gama, mientras que el género alfa contiene todos los tipos mucosos hasta ahora conocidos y cerca de 40 miembros de este género son capaces de infectar la región anogenital (Bzhalava et al., 2013). Estos tipos de VPH mucosotrópicos son clasificados de acuerdo con su potencial oncogénico en VPH de bajo riesgo (VPH-BR) y de alto

riesgo (VPH-AR), los primeros se asocian al desarrollo de verrugas y displasias benignas, mientras que los segundos se asocian al desarrollo de cáncer. Los tipos de VPH-AR más comunes encontrados en biopsias de cáncer son: 16, 18, 58, 33, 45, 31, 52, 35, 59, 39, 51, y 56, de los cuales los tipos VPH-16 y VPH-18 son los más prevalentes, encontrándose en cerca del 60% y 15% de los casos de cáncer cervical, respectivamente; mientras que dentro de los VPH-BR los más frecuentes en verrugas benignas son los tipos VPH-6 y VPH-11 (Muñoz et al., 2003).

Los VPH son virus pequeños no envueltos que poseen un tamaño de 55 nm, contienen un genoma de DNA de doble cadena circular de aproximadamente 8,000 pares de bases (pb) que contiene más de 10 marcos de lectura abierta (ORF) y generalmente una hebra es transcripcionalmente activa (zur Hausen, 1996). Para su estudio, el genoma del VPH se ha dividido en tres regiones: la región larga de control (LCR), que regula la transcripción y replicación viral, la región temprana, que contiene secuencias de al menos 6 genes comunes (E6,E7,E1, E2, E4 y E5) que regulan funciones, tanto virales como celulares y que se expresan en una infección primaria (Thierry et al., 1987) y una región tardía que contiene ORF que codifican para las proteínas estructurales L1 y L2 involucradas en la formación de la cápside viral (Egawa et al., 2015) (**Figura 1**).

Ciclo replicativo del VPH

El ciclo replicativo del VPH depende de los procesos de diferenciación y replicación de la célula infectada. Se caracteriza por tener dos fases: infección latente, donde el episoma viral se replica y se mantiene, mientras que en la infección productiva, las proteínas tardías son producidas y los viriones son formados (Pinidis et al., 2016).

Dependiendo del tipo viral, múltiples vías de entrada han sido sugeridas. Generalmente, a través de una microlesión, los VPH infectan las células del epitelio basal no diferenciado. Adicionalmente, la accesibilidad de las células que están cerca o forman parte de la unión escamo-columnar incrementa la posibilidad de la infección por el VPH (Doorbar et al., 2012).

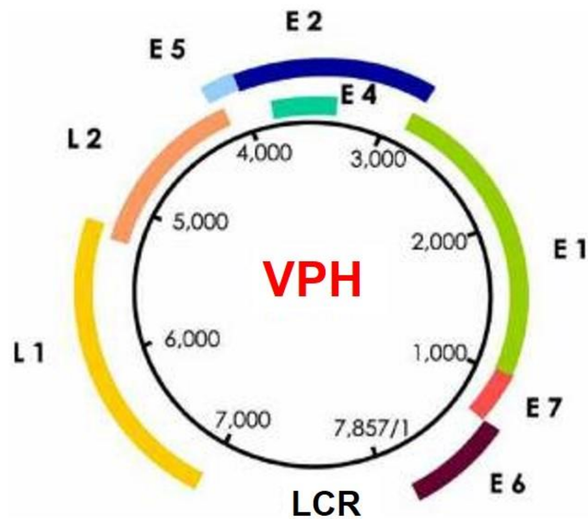


Figura 1. Esquema del genoma del VPH. Se muestra la región larga de control (LCR) y los distintos marcos de lectura abierta (ORFs) que codifican para las proteínas virales, tanto tempranas (E6, E7, E1, E2, E4 y E5) como tardías (L1 y L2) (Modificado de Muñoz N, et al., 2003).

El potencial oncogénico de los VPH-AR principalmente se atribuye a la expresión de las oncoproteínas E6 y E7, las cuales están directamente involucradas en la transformación celular (Riley et al., 2003; Song et al., 1999). Estas proteínas interfieren con reguladores del ciclo celular e inducen inestabilidad genómica, la cual contribuye en la adquisición de un fenotipo maligno.

El mecanismo preciso y los receptores usados por el VPH para infectar las células epiteliales aún no están del todo claro. Sin embargo, se han propuestos modelos que sugieren que la proteína L1 del VPH-16 se une a receptores de proteoglicanos del tipo de Heparan Sulfato (HSPG) (Shafti-Keramat et al., 2003) induciendo de esta manera cambios conformacionales en la cápside y transfiriendo la partícula viral a un segundo receptor de entrada (Cerqueira et al., 2015). Esta transferencia es facilitada por la enzima furina convertasa, la cual realiza un corte proteolítico de la proteína L2 (Day et al., 2008). Sin embargo, algunos estudios utilizando modelos de infección con pseudoviriones de VPH demuestran que los HSPG y el corte de L2

mediado por furina convertasa no son requeridos en todos los tipos de VPH (Cruz and Meyers, 2013; Patterson et al., 2005).

Múltiples receptores secundarios específicos para L1 han sido propuestos para mediar el proceso infeccioso, tales como la α-integrina (Evander et al., 1997), el receptor del factor de crecimiento de queratinocitos (KGFR), el receptor del factor de crecimiento epidermal (EGFR) (Surviladze et al., 2012) y las tetraspaninas (Spoden et al., 2008). Finalmente, la subunidad S100A10 del heterotetrámero de la Annexina A2, se une específicamente a la proteína L2 promoviendo la internalización viral a la célula hospedera (Woodham et al., 2012).

Después de la unión viral con la célula hospedera, la entrada endocítica involucra vías de internalización no canónicas relacionadas con micropinocitosis dependiente de la dinámica de actina (Schelhaas et al., 2012). Sin embargo, los componentes celulares precisos que median la entrada de VPH en las células huésped aún no se conocen claramente.

Tras la entrada del virus, la cápside se une a Sortin Nexin 17 en los compartimentos endosómicos, lo que facilita que el complejo L2-ADN escape del lisosoma (Bergant and Banks, 2013). Finalmente, el genoma viral viaja al núcleo por un transporte mediado por dineína a través de los microtúbulos (Schneider et al., 2011).

En la fase latente, las proteínas de expresión temprana E1, E2, E6 y E7 se expresan en niveles bajos en células basales, donde la diferenciación normal se retarda. Durante esta fase, se produce una tasa de replicación baja de aproximadamente 50–100 episomas virales por célula (Moody, 2017). Por otra parte, en la etapa proliferativa, las proteínas E6 y E7 aumentan su expresión, desde las capas media a superior del epitelio diferenciado (Coupe et al., 2012). La proteína E2 recluta a E1, una helicasa viral, a su sitio de unión en el origen de replicación, lo que facilita la replicación del genoma y la producción de miles de copias por cada célula infectada (McBride, 2013; Pinidis et al., 2016). Por otro lado, la proteína E4 estabiliza a E2 y facilita la localización nuclear de E1, aumentando la amplificación del genoma viral dependiente de E1/E2 (Egawa et al., 2017). Además, E2 actúa como factor transcripcional al unirse con la LCR, promoviendo la activación o represión de genes tempranos virales (McBride, 2013). Finalmente, el ciclo viral se completa con la

síntesis de las proteínas L1 y L2 en la capa superior del epitelio, lo que permite la formación y la liberación de viriones maduros (Doorbar et al., 2012).

La mayoría de las infecciones por VPH son transitorias y eliminadas por el sistema inmunológico en menos de dos años. Cuando se generan lesiones clínicas, la mayoría tiene regresión espontánea (Ho et al., 2011). Se ha propuesto que un factor determinante para la progresión neoplásica es la infección persistente por VPH-AR, que después de mucho tiempo podría conducir a una inestabilidad genómica y a la integración del genoma viral en el genoma del huésped, en esta etapa no se produce progenie (Groves and Coleman, 2015). Cuando el VPH está en su forma episomal, la expresión de los genes tempranos está controlada por E2, pero cuando se produce la integración, la expresión del gen E2 comúnmente se interrumpe, lo que conlleva a un aumento en la expresión de E6 y E7. La formación y el mantenimiento de tumores requiere la expresión constante de las oncoproteínas E6 y E7 (Goodwin and DiMaio, 2000).

En las biopsias de CaCU, el genoma VPH-AR generalmente se encuentra integrado; sin embargo, en una pequeña proporción de los casos, el genoma del VPH permanece en estado episomal, pero con un número alto de copias (Vinokurova et al., 2008). Se ha propuesto que en los episomas, los sitios de unión a E2 en la LCR pueden metilarse, lo que induce la represión transcripcional de E2 y permite la sobreexpresión de las oncoproteínas E6 y E7 (Chaiwongkot et al., 2013). Esto indica que la integración del VPH en algunos casos puede no ser un requisito para la transformación celular.

Oncoproteínas virales (E6 y E7)

Las proteínas de expresión temprana E6 y E7 son consideradas las oncoproteínas virales más importantes, ya que desempeñan un papel crucial en la transformación celular (McLaughlin-Drubin and Münger, 2009). Entre varias interacciones celulares, la oncoproteína E6 se une a la proteína p53, la cual es una proteína supresora de tumores, y a la ubiquitin ligasa de tipo 3 E6AP, promoviendo la degradación de p53 a través del proteosoma y facilitando el daño al ADN y la acumulación de mutaciones (Scheffner et al., 1993). También se conoce que la oncoproteína E7 se

asocia con un complejo formado por Culina 2, una ubiquitin ligasa de tipo 2, lo que impacta en la degradación de la proteína supresora de tumores pRB, lo que promueve la continuidad del ciclo celular (Huh et al., 2007).

El efecto de las proteínas E6 y E7 de los VPH-AR en el establecimiento de la carcinogénesis se ha estudiado extensivamente. Estudios previos han demostrado que la expresión conjunta de E6 y E7 inducen la inmortalización altamente eficiente de cultivos primarios (Sashiyama et al., 2001). Por otra parte, la expresión de E6 y E7 en cultivos organotípicos produce cambios celulares, que son similares a los observados en lesiones intraepiteliales escamosas de alto grado (McCance et al., 1988). En modelos *in vivo*, utilizando ratones transgénicos que expresan E6 y E7 dependientes de estrógenos en bajas dosis, se observó el desarrollo de carcinomas escamosos epiteliales (Arbeit et al., 1996). En este modelo, la expresión sola de E7 es suficiente para inducir lesiones cervicales de alto grado y neoplasia cervical invasiva; sin embargo, cuando se incluye la expresión de E6 se observaron tumores más agresivos. Estos datos demuestran el efecto cooperativo de E6 y E7 en la promoción del desarrollo del cáncer (Riley et al., 2003). Durante la infección por VPH, las proteínas E6 y E7 inducen la proliferación de células suprabasales diferenciadas y no diferenciadas, además de promover la inhibición de la apoptosis. Estas acciones promueven la acumulación de daño en el ADN y mutaciones que pueden resultar en la transformación celular y el desarrollo de cáncer (Moody and Laimins, 2010).

La proteína E6 está constituida de aproximadamente 150 aminoácidos y contiene un motivo LXXLL en la región amino terminal que se requiere para interactuar con distintas proteínas como: E6AP, E6BP, IRF3, Tuberina y Paxillina. Otro motivo crítico en la proteína E6 se encuentra en el extremo carboxilo terminal, llamado PBM (motivo de unión a PDZ), que media la interacción con proteínas celulares que contienen dominios PDZ, especializados en interacciones proteína-proteína (Howie et al., 2009). La interacción de E6 con proteínas que contienen dominios PDZ comúnmente induce su degradación a través del proteosoma (Zhang et al., 2007). Interesantemente se ha observado que el motivo PBM está presente solo en las

proteínas E6 de VPH-AR, lo que sugiere un posible papel para este motivo en la oncogénesis inducida por VPH (Lee and Laimins, 2004).

La proteína E7 está formada por 98 aminoácidos que para su estudio se han dividido en tres regiones conservadas: CR1, CR2 y CR3. La región CR2 incluye un motivo LXCXE conservado que media la unión específica con la proteína pRB (Münger et al., 1989). La región CR3 contiene dos motivos CXXC separados por 29 o 30 residuos, formando un dominio de unión a zinc. Esta región es crítica para la interacción con proteínas celulares como: pRB (Liu et al., 2006), p21 (Funk et al., 1997), p27 (Zerfass-Thome et al., 1996), TBP (Massimi et al., 1997) y E2F (Hwang et al., 2002).

Las oncoproteínas del VPH pueden inducir alteraciones en vías de señalización celular que contribuyen a la carcinogénesis. Tras la infección inicial, esta modulación puede ser necesaria para completar el ciclo replicativo y formar partículas virales infecciosas. Sin embargo, los constantes niveles de expresión de las oncoproteínas virales pueden eventualmente alterar las funciones normales de la célula, desencadenando un proceso de transformación descontrolado. A través de sus diferentes interacciones celulares, E6 y E7 pueden desregular las diferentes vías de señalización celular implicadas en los tipos de cáncer asociados a la infección con el VPH.

Isoformas de E6 (E6*)

En los VPH de bajo riesgo, los genes E6 y E7 se transcriben a partir de dos promotores independientes, mientras que, en los VPH de alto riesgo, estos genes se transcriben del promotor temprano como un pre-ARNm policistrónico. Dependiendo del estado de diferenciación de las capas epiteliales, los promotores virales tempranos o tardíos se activan. Los transcritos producidos son poliadenilados en secuencias específicas, que se ubican rio abajo de cada ARNm policistrónico (Graham, 2017; Zheng and Baker, 2006).

Varios transcritos son generados a lo largo de una infección por VPH mediante el proceso de splicing alternativo, exhibiendo diferentes patrones de expresión de ARNm (Graham and Faizo, 2017). El splicing alternativo dentro de los ORF de E6-

E7 es una característica común de los VPH-AR, a diferencia de los VPH-BR que no presentan este proceso en esta región (Mesplède et al., 2012). La forma completa de E6 se expresa a partir de un ARNm sin splicing dentro del ORF de E6, mientras que E7 se puede transcribir a partir de diferentes ARNm, incluidos aquellos con splicing en E6 (del Moral-Hernández et al., 2010; Tang et al., 2006).

El proceso de splicing produce varios transcritos pequeños de E6 denominados E6*, que se derivan de un sitio donador dentro del ORF de E6 y uno de los diferentes sitios aceptores localizados a lo largo del ARNm de la región temprana (J. Chen et al., 2014). La isoforma más abundante de E6 se denomina E6*I, que es una proteína poco estudiada. E6*I comparte aproximadamente los primeros 44 aminoácidos con la proteína completa de E6 y debido a la remoción del intrón contenido dentro del ARNm de E6, se produce un corrimiento en el marco de lectura, lo que genera aproximadamente 13 aminoácidos nuevos que solo están presentes en la isoforma E6*I (Pim et al., 2009). E6*I conserva solo la mitad del motivo de unión a zinc en la porción amino terminal de E6. Además, la mayoría de las isoformas E6*I, contienen un motivo hidrofóbico (L /M/I)XX (L/I/V)X(L/V/I) que está asociado a la unión con E6 y E6AP (Pim and Banks, 1999) (**Figura 2**).

Dependiendo del tipo de VPH-AR, diferentes transcritos se pueden generar de uno de los sitios donadores contenidos en el ORF de E6 y uno de los sitios aceptores ubicados dentro de los ORF de E7, E2 o E4. El patrón de splicing del pre-ARNm del VPH-16 se ha estudiado exhaustivamente y se han identificado los siguientes transcritos: E6*I, E6*II, E6*III, E6^E7, E6^E7*I, E6^E7*II, E6*IV, E6*V y E6*VI. Mientras que los transcritos descritos para el VPH-18 son: E6*I, E6*II, E6*III, E6^E7 (Figura 3). Adicionalmente, poco se conoce acerca de los transcritos que resultan del splicing del pre-ARNm de E6 de otros tipos de VPH-AR. (Olmedo-Nieva et al., 2018).

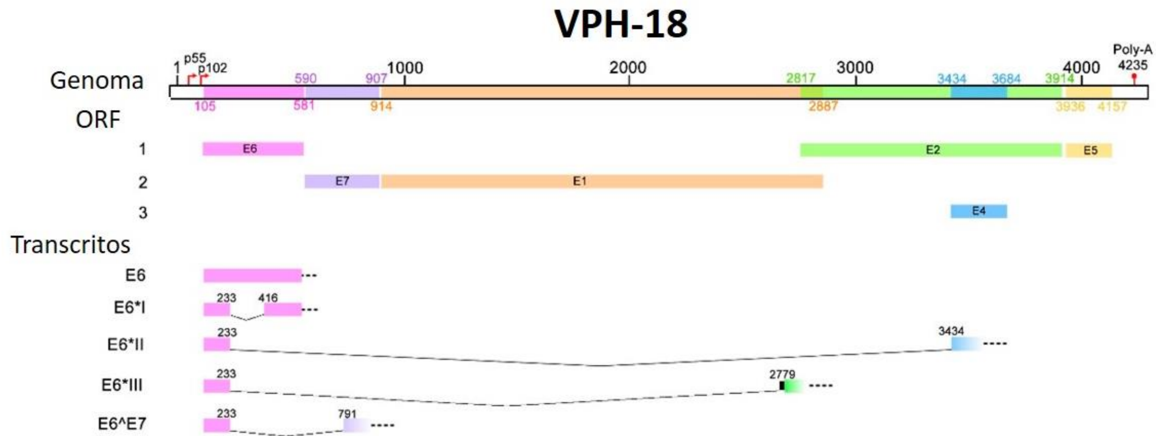


Figura 2. Diferentes transcritos derivados de E6 del VPH-18. Las distintas variantes de E6* se generan a partir de un sitio donador de splicing dentro del ORF de E6 y al menos un sitio aceptor localizado en los ORF de E6, E7, E2 y E4. Hasta el momento solo cinco isoformas de E6 han sido descritas. (Modificado de Olmedo-Nieva, et al., 2018).

Funciones de E6*I

Una de las funciones mejor descritas de E6* es la de facilitar la traducción de la oncoproteína E7, ya que en el transcrito del cual se genera E6* se aumenta el espacio entre el codón de paro de E6 y el codón de inicio de E7, lo que permite un mejor ensamblaje del ribosoma. Sin embargo, estos datos no son del todo concluyentes, ya que otros estudios demuestran que la exclusión del intrón tiene un efecto mínimo o nulo en la traducción de E7, ya que la proteína E7 se traduce principalmente de un ARNm donde E6 no ha sufrido de splicing. Además, otras funciones se han atribuido a las proteínas E6*, principalmente a E6*I, independientemente de facilitar la expresión de E6 y E7 (Olmedo-Nieva et al., 2018). Se ha demostrado que las proteínas E6 de VPH-AR promueven la degradación de p53 a través de la unión con E6AP. Sin embargo, la proteína E6*I interfiere con este proceso, ya que es capaz de unirse con E6AP, E6 y p53, evitando así la degradación proteosomal de p53 (Filippova et al., 2009).

Por otra parte, diversos estudios demuestran que la oncoproteína E6 se une e induce la degradación de proteínas que contienen dominios PDZ. Interesantemente, la proteína E6*I del VPH-18, al igual que E6, induce la degradación de ciertas

proteínas que contienen estos dominios, tales como Dlg, MAGI-1 y h-Scrib. Esta capacidad de promover la degradación de estas proteínas se conserva entre las proteínas E6*I de los VPH-31, 16 y 18; sin embargo, E6*I no es capaz de unirse a ellas. Interesantemente, la única proteína que contiene dominios PDZ y que puede interactuar con E6*I es PATJ, aunque esta interacción no depende de este dominio. Además, este estudio demostró que E6*I del VPH-18 induce la degradación de Akt, a diferencia de E6 que no disminuye los niveles proteicos de esta proteína. Estos datos sugieren que E6*I de VPH-18 podría estar regulando procesos involucrados en la supervivencia, proliferación y el crecimiento celular (Pim et al., 2009; Storrs and Silverstein, 2007).

En otros estudios realizados con análisis proteómicos se observó que E6*I del VPH-16 regula los niveles de proteínas celulares involucradas en una variedad de vías de señalización celular, tales como: cinasa ligada a la integrina (ILK), fosforilación oxidativa y disfunción mitocondrial. El aumento en la disfunción mitocondrial inducida por E6*I promueve una disminución en los niveles de la molécula antioxidante GSH y el subsecuente daño al ADN (Evans et al., 2016.). Por otro lado, se ha demostrado que la proteína E6*I del VPH-16, disminuye los niveles de las enzimas antioxidantes SOD2 y Gpx, lo que lleva a la acumulación de especies reactivas de oxígeno (ROS) y un aumento en el daño al ADN (Williams et al., 2014). Interesantemente, aun cuando el daño al ADN promovido por E6*I podría eventualmente culminar en la apoptosis, algunos datos apoyan la idea de que la inducción del daño al ADN por ROS podría estar relacionada con la amplificación del genoma del VPH o con la integración del VPH al genoma celular, lo que sugiere que E6*I podría participar tanto en el ciclo viral del VPH, como en el establecimiento del cáncer (Olmedo-Nieva et al., 2018).

Vía de señalización celular Wnt/β-catenina

Muchas vías de señalización celular que controlan la proliferación celular y la apoptosis se han encontrado desreguladas en diferentes tipos de cáncer, tales como: PI3K/Akt, ERK/MAPK, Notch y Wnt/β-catenina.

Diferentes alteraciones en la vía de señalización Wnt se han encontrado en diversos tipos de cáncer como: osteosarcoma (Lin et al., 2014), hepatocarcinoma (Takigawa and Brown, 2008), colorectal (Krausova and Korinek, 2014) y el cáncer de mama (Khramtsov et al., 2010). Además, esta vía de señalización también se ha encontrado alterada en tipos de cáncer relacionados con la infección del VPH como: orofaringe (Rampias et al., 2010) y cervical (Clevers and Nusse, 2012; Rodríguez-Sastre et al., 2005).

La vía de señalización Wnt está implicada en diversos procesos celulares como: desarrollo, proliferación (Bilir et al., 2013), diferenciación (Peng et al., 2014), adhesión (Gradl et al., 1999) y polaridad celular (Dollar et al., 2005). Hasta el momento se conocen al menos tres vías de señalización activadas por los ligandos Wnt: la vía de polaridad celular planar (Wnt/PCP), la vía Wnt/Ca^{2+} y la vía canónica Wnt/β-catenina. La activación de las diferentes vías depende del tipo de receptor que se asocia a los diferentes ligandos disponibles. Hasta el momento se han identificado 11 receptores en humanos que son miembros de la familia Frizzled (Fz), dentro de estos receptores se incluyen Fz1 al Fz10 y Smo, así como los co-receptores LRP5/6, y todos estos son responsables de la activación de la señalización Wnt. Adicionalmente, se han descrito 19 ligandos de Wnt para estos receptores: Wnt1, 2, 2b, 3, 3a, 4, 5a, 5b, 6, 7a, 7b, 8a, 8b, 9a, 9b, 10a, 10b, 11 y 16 (Bello et al., 2015).

La vía de señalización canónica Wnt/β-catenina ha sido la más estudiada. En ausencia de ligandos Wnt (estado inactivo), la proteína β-catenina se localiza principalmente en la membrana celular participando en las uniones celulares. Sin embargo, una pequeña cantidad permanece en el citoplasma y se une a un complejo responsable de su degradación a través del proteosoma. Diversas fosforilaciones en β-catenina son necesarias para promover su degradación, la proteína de andamiaje Axina, recluta elementos esenciales para este proceso,
como GSK3β, CK1, APC, YAP/TAZ y β-TrCP. En un inicio, CK1 fosforila a β-catenina en el residuo Ser45, mientras que GSK3β fosforila esta proteína en los Ser33, Ser37 y Thr41. Posteriormente, el complejo YAP/TAZ recluta a la ubiquitin ligasa E3 β-TrCP, que reconoce estas fosforilaciones que permiten la ubiquitinación

de β-catenina y su posterior degradación proteosomal (**Figura 3A**) (Bello et al., 2015).

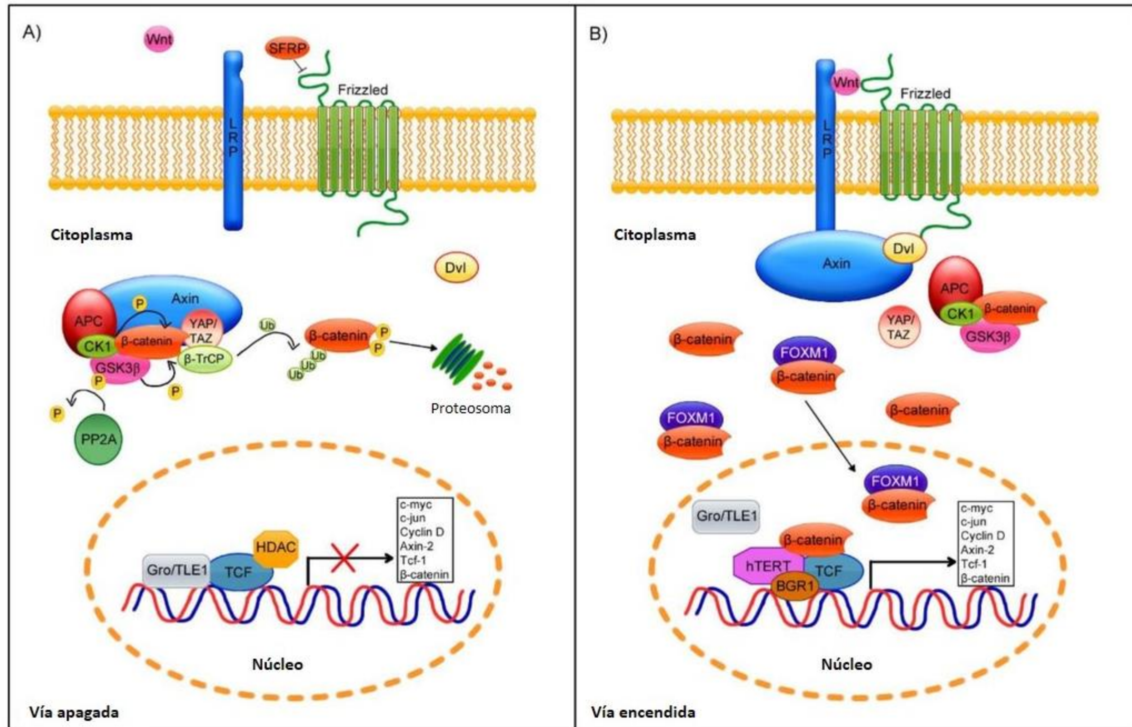


Figura 3. Vía de señalización de Wnt/β-catenina. **A**) En ausencia de los ligandos de Wnt (vía apagada), las proteínas SFRP regulan negativamente al receptor Fz, impidiendo una mayor interacción del receptor con su ligando. En el citoplasma, se forma el complejo de degradación de β-catenina, en el cual esta proteína es fosforilada en residuos específicos por GSK3β y CK1. Estas marcas de fosforilación son reconocidas por βTrCP, que media la degradación proteosomal de β-catenina. En el núcleo, el represor Groucho/TLE se une a factor transcripcional TCF/LEF, evitando su activación. **B**) En presencia de los ligandos de Wnt (vía encendida), el receptor Fz se dimeriza con el co-receptor LRP5/6. Posteriormente, la proteína de andamiaje Axina se une a LRP5/6, mientras que Disheveled (Dvl) interactúa con Fz, de esta manera se favorece el desensamble del complejo de degradación de β-catenina. Finalmente, β-catenina se acumula en el citoplasma, se une a FOXM1 y se transloca al núcleo, donde se une al factor transcripcional TCF/LEF para dar inicio a la transcripción de genes blanco de la vía de Wnt. (Modificado de Bello et al., 2015).

Como consecuencia de la unión del ligando Wnt con el receptor Fz y al co-receptor LPR5/6 (estado activo), β-catenina se deslocaliza de la membrana y se acumula en el citoplasma y el núcleo. Cuando el receptor Fz se dimeriza con el co-receptor

LRP5/6, la porción intracelular del receptor Fz recluta a la proteína Dishevelled (Dvl); en este punto, la cinasa CK1 fosforila al co-receptor LPR5/6, permitiendo que se una a Axina, este mecanismo favorece el desensamble del complejo de destrucción de β-catenina. Como consecuencia, β-catenina se acumula y transloca al núcleo. Además, se ha postulado que la unión de β-catenina con FOXM1 favorece este proceso. En el núcleo, β-catenina se une a factores transcripcionales miembros de la familia TCF/LEF, provocando la disociación de co-represores transcripcionales, como Groucho/TLE. Subsecuentemente, co-activadores como CREPT, FHL2 y CBP/p300 y remodeladores de cromatina como Brg-1 son reclutados para dar inicio a la transcripción de genes blanco como c-Jun, c-Myc, Ciclina D1, Axina 2, Tcf-1, Sp5 y β-catenina (**Figura 3B**) (Bello et al., 2015).

Regulación de la vía de Wnt por la oncoproteína E6

La activación de la vía Wnt/β-catenina se considera un paso crucial en el desarrollo del cáncer asociado a la infección por VPH. Diversos estudios apoyan la participación directa o indirecta de las oncoproteínas de VPH en la desregulación de esta vía.

En líneas celulares de cáncer de orofaringe positivas a VPH-16, se demostró que E6 y E7 regulan la expresión de β-catenina y aumentan la transcripción mediada por TCF-4. Este efecto se atribuyó a una disminución de la proteína Siah-1 (ubiquitin ligasa tipo 3), que al igual que β-TrCP promueven la degradación de la β-catenina. Dado que p53 induce la expresión de Siah-1 (Matsuzawa and Reed, 2001), la degradación de p53 mediada por E6, induce una disminución en el transcrito y los niveles proteicos de Siah-1, evitando de esta manera la degradación de β-catenina. Sin embargo, la activación de la vía de Wnt/β-catenina por la oncoproteína E7 es poco conocida (Rampias et al., 2010).

Un estudio *in vitro* demostró que las proteínas E6 de los VPH-AR y -BR pueden diferencialmente aumentar la actividad transcripcional de TCF-4, con una evidente incremento observado para las proteínas E6 de VPH-AR (Lichtig et al., 2010). Además, en otro estudio realizado por el mismo grupo de trabajo se observó que E6 del VPH-16 promueve la activación de la vía de señalización Wnt/β-catenina, sin

afectar la estabilidad proteica y la expresión de β-catenina. Interesantemente, este efecto fue independiente de la degradación de p53 mediada por E6, del motivo de unión a PDZ contenido en E6, de la actividad del complejo APC/Axina/GSK3β, y de la localización nuclear de β-catenina. Sin embargo, la presencia del complejo E6/E6AP potenció la actividad transcripcional de TCF-4, siendo esté efecto dependiente de la actividad del proteosoma e independiente de los cambios en niveles proteicos de β-catenina (Lichtig et al., 2010).

Por otra parte, otro estudio demostró que E6AP actúa como un nuevo regulador de señalización Wnt en cooperación con E6. Interesantemente, los niveles proteicos de E6 disminuyen considerablemente cuando se induce la activación de la vía Wnt. Sin embargo, los niveles de E6 se recuperan y se estabilizan en presencia de E6AP, lo que sugiere que E6 requiere la presencia de E6AP en aquellas células donde la vía de Wnt se encuentra activa. Asimismo, se observó que la participación de E6AP en la regulación de la actividad transcripcional de TCF-4 es independiente de la actividad catalítica de E6AP. Además, se demostró que E6 y E6AP inducen la estabilización de β-catenina y este proceso requiere la actividad catalítica de E6AP. El mecanismo por el cual E6/E6AP estabiliza a β-catenina no está claro. Hasta la fecha, no se ha demostrado una interacción directa de E6 o E6AP con β-catenina; sin embargo, se ha propuesto que E6 y E6AP podrían participar de alguna manera en la regulación de distintos elementos específicos de la vía de Wnt (Sominsky et al., 2014).

Otra proteína que se ha involucrado recientemente en la regulación de la vía de señalización Wnt es FOXM1. Esta proteína puede inducir la translocación nuclear de β-catenina al unirse directamente a ésta. En células positivas al VPH, la oncoproteína E6 se ha asociado con la sobreexpresión de FOXM1. Esta regulación está mediada por la expresión de los factores transcripcionales MZF1 y NKX2-1. Se ha observado que E6 del VPH-16 induce la expresión MZF1 y este a su vez activa la transcripción de NKX2-1. Indirectamente, E6 induce la transcripción de FOXM1, dado que el promotor de FOXM1 contiene tres sitios putativos para NKX2-1. En estas células donde se expresa E6 de manera exógena, se producen altos niveles de FOXM1 que aumentan la translocación de β-catenina. Este efecto tiene como

consecuencia un incremento en la activación transcripcional de TCF-4 y la expresión de genes blanco de la vía de Wnt/β-catenina como c-Myc y Ciclina D1 (P.-M. Chen et al., 2014).

Otro estudio *in vivo* utilizando ratones transgénicos apoya el papel de E6 del VPH-16 en la regulación de la vía de señalización Wnt. En éste se observó que la acumulación nuclear de β-catenina depende del motivo de unión a dominios PDZ en E6. En este modelo, los genes blanco de la vía de Wnt, tales como MYC, BIRC5 y CCND1 fueron sobre-expresados en presencia de E6, pero no así en aquellos ratones que expresaban la proteína E6 carente del motivo de unión a PDZ. Sin embargo, al evaluar este efecto *in vitro* en células transfectadas con E6, se observó que tanto E6 como E6 sin motivo de unión a PDZ pueden interactuar con Dvl2 e inducir un incremento en la actividad transcripcional de TCF-4. Estos resultados sugieren que la capacidad de E6 para activar la respuesta de este factor de transcripción puede ser dependiente e independiente de la translocación de β-catenina (Bonilla-Delgado et al., 2012).

Los hallazgos actualmente descritos de la regulación de la vía de Wnt mediada por la proteína E6 de VPH-AR se muestran en la **figura 4**.

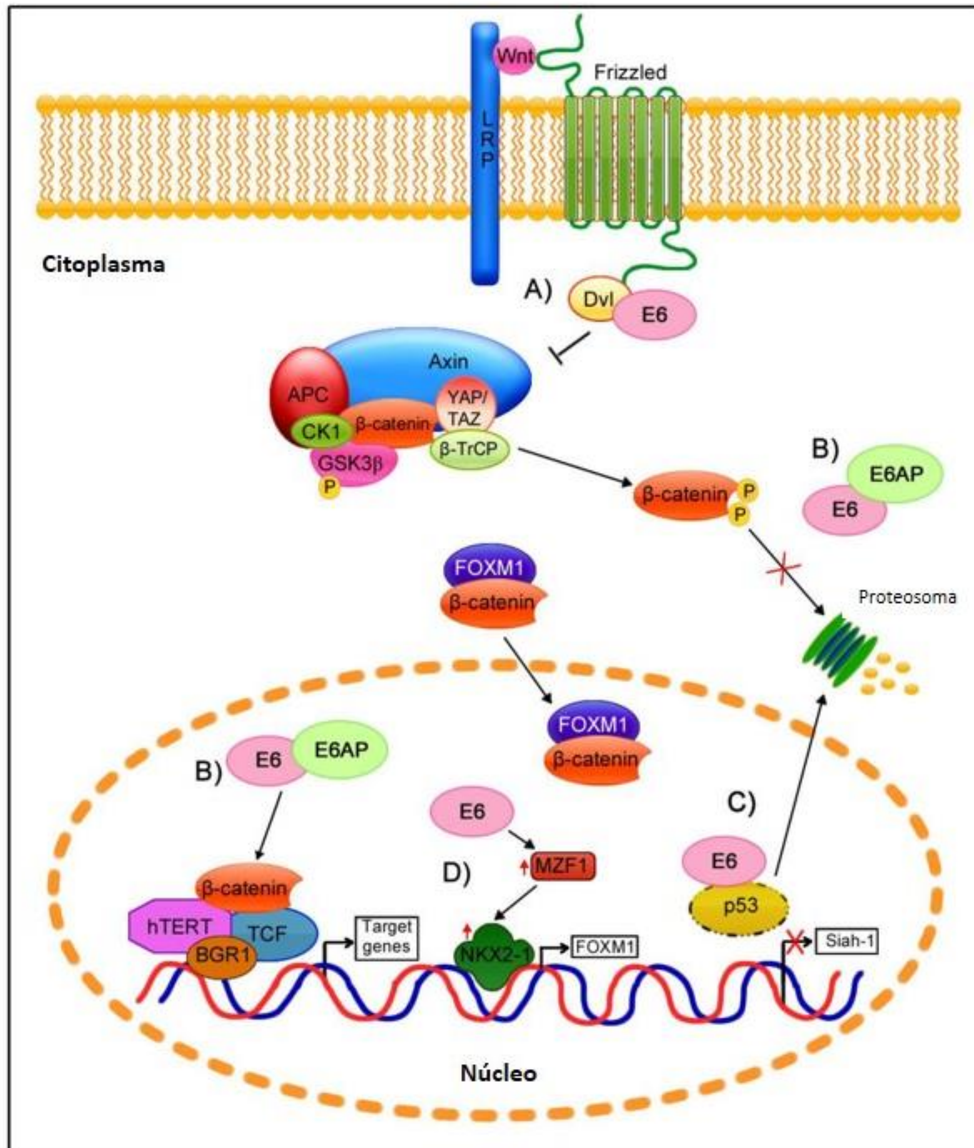


Figura 4. Regulación de la vía de Wnt/β-catenina por la oncoproteína E6 de VPH-AR a través de distintos mecanismos. **A**) La unión de las proteínas E6 y Dvl promueven la liberación de β-catenina del complejo de degradación, favoreciendo su acumulación en el citoplasma. **B**) El complejo E6/E6AP estabiliza a β-catenina, evitando su degradación proteosomal y promoviendo su translocación nuclear y activación transcripcional de TCF-4. **C**) E6 inhibe la expresión de Siah-1 a través de p53, de esta manera se evita la degradación a β-catenina. **D**) E6 induce la expresión de FOXM1 a través del MZF1 y NKX2-1, promoviendo la translocación nuclear de β-catenina y la activación transcripcional de TCF-4 (Modificado de Bello et al., 2015).

Justificación

El esclarecimiento de los procesos involucrados en la carcinogénesis inducida por el VPH resulta un tema de especial atención, dada la alta prevalencia actual de este tipo de tumor y su asociación con el VPH. Múltiples vías de señalización celular se han visto alteradas en cáncer, particularmente, la vía de señalización de Wnt/β-catenina, ha sido descrita como el segundo paso requerido para el establecimiento de la carcinogénesis cervical. Los mecanismos por los cuales el oncogen viral E6 modula la actividad transcripcional de TCF-4 en respuesta a la activación de la vía de señalización celular Wnt/β-catenina aún no son claros, por lo que es de interés evaluar el efecto de E6 y su isoforma E6*I del VPH1-8 en este evento.

Hipótesis

Las proteínas E6 y su isoforma E6*I del VPH-18 regulan la activación de la vía de señalización celular Wnt/β-catenina a través del factor transcripcional TCF-4.

Objetivo general

Evaluar el efecto de las proteínas E6 y E6*I del VPH-18 en la activación de la vía de señalización celular Wnt/β-catenina.

Objetivos específicos

1. Establecer el efecto de E6 y E6*I sobre la actividad transcripcional de TCF-4.
2. Analizar la expresión de genes blanco de la vía Wnt como Ciclina D1 y Axina 2, en células transfectadas con E6 y E6*I del VPH18.
3. Determinar los niveles de proteínas totales de TCF-4 y β-catenina en células transfectadas con E6 y E6*I del VPH-18.
4. Evaluar la localización de β-catenina y TCF-4 en células transfectadas con E6 y E6*I del VPH-18.
5. Medir la vida media de TCF-4 en células que expresan E6 y E6*I del VPH-18.
6. Examinar la interacción de E6 y E6*I del VPH-18 con las proteínas que forman el complejo activador transcripcional de la vía de Wnt (β-catenina y TCF-4).
7. Evaluar la interacción de E6 y E6*I con promotores dependientes de TCF-4 (promotor del gen SP5).
8. Determinar el efecto sobre la proliferación en células co-transfectadas con E6 y E6*I del VPH-18 con β-catenina.
9. Analizar la participación de las variantes de E6 del VPH-18 sobre la actividad transcripcional de TCF-4
10. Evaluar la interacción de las variantes de la proteína E6 del VPH-18 con β-catenina y TCF-4.

Materiales y métodos

Cultivo de células y transfección

Las células C33A se adquirieron de ATCC (American Type Culture Collection) y las células HaCaT fueron proporcionadas por el Dr. Alejandro García-Carrancá (Instituto Nacional de Cancerología, Ciudad de México). El cultivo celular se llevó acabo utilizando el medio DMEM F12 (Dulbecco's modified Eagle medium) suplementado con 10% de suero fetal bovino y mantenido en una incubadora humidificada con 5% de CO_2. Las transfecciones se realizaron utilizando el reactivo Lipofectamine 2000 (Invitrogen). De acuerdo con las instrucciones del fabricante.

Plásmidos

El ORF de 18E6WT (Wild-Type) se amplificó mediante PCR a partir de una biopsia de cáncer cervical positiva al VPH-18, mientras que el ORF de 18E6*I mediante amplificación por RT-PCR de las células HeLa (VPH-18 positivo), y finalmente, se amplificó el ORF de 16E6 por PCR a partir de células CaSki (células positivas al VPH-16). La secuencia de la mutante de splicing de E6 del VPH-18 (18E6SM), se amplificó a partir del plásmido pCAHPV18-E6sm previamente reportado (Pim et al., 2009). La secuencia del inserto del 18E6SM posee una mutación en el sitio donador de splicing (G233A), que favorece solo la expresión de E6. La variante E6Af del VPH-18 (de la rama filogenética africana) y la variante E6AsAi (de la rama filogenética asiático-amerindia), que es la de referencia, se amplificaron por PCR a partir del ADN obtenido previamente de biopsias de tumores (Lizano et al., 1997). Todos estos fragmentos se purificaron y se clonaron en el vector de expresión p3x-FLAG CMV.10 (Sigma Aldrich), este plásmido tiene la característica de que el ORF inicia con secuencias que codifican para el polipéptido FLAG, de este modo las proteínas expresadas de este vector contendrán en su extremo amino terminal el tag FLAG. Todas las construcciones se verificaron mediante secuenciación del ADN. Los plásmidos que expresan β-catenina, pCAHPV18-E6sm y pGW1-18E6-HA (marcados con hemaglutinina) fueron amablemente proporcionados por el Dr. Lawrence Banks (ICGEB, Trieste, Italia). El plásmido reportero de TCF-4

(TOPFLASH) que contiene dos sets de 3 copias de los sitios de unión TCF-4 se utilizó para realizar los ensayos de actividad transcripcional (Merck-Millipore) y el plásmido de pCMV-β-galactosidasa se utilizó para evaluar la eficacia de la transfección (Promega).

Ensayos de actividad transcripcional del TCF-4

Las células C33A se sembraron en una placa de 24 pozos y se transfectaron con 50 ng de los distintos plásmidos de E6, 100 ng de TOPFLASH y 1 ng de plásmido reportero de β-galactosidasa, en co-transfección con 50 ng de vector vacío o el plásmido de β-catenina, según se indica en cada figura. Los extractos celulares se obtuvieron 48 h después de la transfección y se analizaron las actividades de luciferasa y β-galactosidasa (Tropix Inc) utilizando un luminómetro Glomax (Promega). Los datos son representados como veces de incremento, donde se comparan las células que expresan E6 con el vector vacío. En todas las lecturas de luciferasa los valores del vector vacío se ajustaron a 1 después de la normalización con β-galactosidasa. Se realizaron al menos tres experimentos independientes, cada uno por triplicado.

Reacción en cadena de la polimerasa cuantitativa (qPCR)

Las células C33A se sembraron en una placa de cultivo de 60 mm y se transfectaron con 3 μg de cada plásmido de E6. Después de 48 h de la transfección, se realizó la extracción del ARN total utilizando el kit RNeasy mini (Qiagen). El ARN obtenido se trató con el kit DNAse Free DNA removal (Thermo Fisher Scientific). Un total de 400 μg de ARN se retrotranscribieron utilizando oligonucleótidos hexámeros aleatorios con el kit GeneAmp RNA PCR Core (Applied Biosystems) Para la amplificación de Ciclina D1, se utilizaron los oligonucletidos 5'-ACAAACAGATCATCCGCAAACAC-3 'y 5'-TGTTGGGCTCCTCAGGTTC-3'. Para la amplificación de Sp5, 5'-TCGGATATAGGGACCCAGTT-3' y 5′-CTGACGGTGGGAACGGTTTA-3'. Como gen de expresión constitutiva se utilizó el 18S y se amplificó con los oligonucleótidos 5'-AACCCGTTGAACCCATT-3' y 5'-CCATCCAATCGGTAGCG-3'. Para llevar a cabo las reacciones de qPCR se utilizó el kit SYBR Select Master Mix (Applied

Biosystems). Para la amplificación de Axina 2, se utilizaron las sondas Taqman (Applied Biosystems): Axina 2 FAM (Hs00610344_m1) y 18S VIC (Hs99999901_s1), y las reacciones de qPCR se realizaron con el kit Taqman Gene Expression Master Mix (Applied Biosystems). Los resultados se presentan como cuantificación relativa utilizando el método ΔΔCt.

Western blot

Las células C33A se cultivaron en placas de 60 mm y se transfectaron con 3 μg cada plásmido. Después de 48 h de la transfección, las células se lisaron utilizando 300 μl del buffer RIPA (Tris 100 mM, pH 8.0, $NaCl_2$ 50 mM, Nonidet P-40 al 0.5% y cóctel de inhibidor de proteasas (Roche)). Se realizó un corrimiento electroforético con 20 μg de extractos de proteínas celulares utilizando geles de SDS-PAGE (10-12%) y se transfirieron a una membrana de nitrocelulosa de 0.22 μm (Bio-Rad). Las membranas se bloquearon por 1 hora a temperatura ambiente con leche al 10% en TBS-Tween 20 al 0.1%, posteriormente se realizó la incubación con el anticuerpo primario indicado: anti-FLAG M2 (Sigma Aldrich); anti-TCF-4 (Santa Cruz Biotechnologies); anti-β-catenina (Santa Cruz Biotechnologies). Después de lavar tres veces con TBS-Tween 20 al 0.1%, las membranas se incubaron con anticuerpo anti-ratón secundario conjugado con HPR en una dilución 1:10000 (Santa Cruz, Biotechnologies). Las proteínas se visualizaron utilizando el kit Immobilon Western (Millipore) de acuerdo con las instrucciones del fabricante. Los ensayos se realizaron por lo menos tres veces para asegurar la reproducibilidad del resultado.

Ensayo de inmunoprecipitación

Después de 48 h de transfección con los plásmidos indicados, 400 μg de extractos proteicos se incubaron durante toda la noche a 4°C con 1 μg de anticuerpo de anti-β-catenina (Santa Cruz Biotechnologies), Anti-TCF-4 (Santa Cruz Biotechnologies), o con un control de isotipo IgG (Santa Cruz Biotechnologies). Posteriormente, se adicionaron 20 μl de perlas de la proteína G-agarosa (Upstate) a cada muestra y se incubaron a 4°C, durante 3 h. Los complejos se lavaron tres veces con PBS-0.1% NP-40, se resuspendieron en el buffer de carga Laemmli y fueron análizados por

inmunoblot, utilizando los anticuerpos de anti-FLAG M2 (Sigma Aldrich) Anti-β-catenina (Santa Cruz Biotechnologies) y anti-TCF-4 (Santa Cruz Biotechnologies).

Análisis de la estabilidad del TCF-4

Las células C33A se sembraron en placas de 60 mm y se transfectaron con 3 µg de cada plásmido indicado. 48 h después de la transfección, las células se trataron con 200 µg/mL de cicloheximida (un inhibidor de la biosíntesis de proteínas) (Sigma Aldrich). Después de cada 0, 6 y 12 h de incubación, los extractos proteicos se obtuvieron utilizando el buffer de carga Laemmli (Bio-Rad). Se realizaron ensayos de Western blot para analizar la estabilidad de la proteína TCF-4.

Ensayo de inmunofluorescencia

Se sembraron células C33A y HaCaT sobre portaobjetos en placas de 6 pozos y se transfectaron con los plásmidos indicados. Después de 48 h de la transfección, las células se fijaron con PBS-paraformaldehído al 3.7% durante 10 minutos y se permeabilizaron con PBS-Triton X-100 al 0.1%. Posteriormente, las células fueron tratadas con una solución de BSA al 0.3% y se incubaron toda la noche con anticuerpos anti-FLAG M2 (Sigma Aldrich) y anti-β-catenina (Cell Signaling) o anti-TCF-4 (Santa Cruz Biotechnologies). Las células se lavaron extensivamente con PBS y luego se incubaron con anticuerpos secundarios anti-conejo o anti-ratón conjugados con Rhodamina o Alexa-488 (Invitrogen). Los portaobjetos se lavaron extensivamente y fueron montados utilizando el medio Prolong Diamond Antifade Mounting (Molecular Probes). Para el análisis de las laminillas, se utilizó un microscopio confocal (Zeiss LSM 710 DUO), con láseres que proporcionaron líneas de excitación a 488 y 594 nm. Se observaron alrededor de veinte campos para cada tratamiento y se adquirieron imágenes representativas. Los datos de tres experimentos independientes se recopilaron con un objetivo de inmersión en aceite al 63X.

Extracción y purificación de proteínas de fusión GST-E6

Los ORF de E6 y E6*I del VPH-18 se clonaron en el plásmido de expresión pGEX-2T (GE) y la identidad de cada plásmido se verificó mediante secuenciación del ADN. La producción de la proteína de fusión GST se obtuvo utilizando la células *Escherichia coli* cepa DH5-α en medio de cultivo LB adicionado con 10 mM de IPTG. Después de tres horas de inducción, las proteínas se purificaron lisando las células con PBS-Triton X-100 al 1% y separando la fracción insoluble por centrifugación. Las proteínas obtenidas se incubaron con perlas de glutatión sefarosa (Sigma Aldrich). Posteriormente, las perlas se lavaron varias veces y luego se resuspendieron en 1 ml de PBS-Triton X-100 al 0.1% con un cóctel de inhibidor de proteasas (Roche). Una cantidad igual de proteínas de fusión con GST se incubaron durante toda la noche con 40 µg de extracto proteico de las células C33A; posteriormente, las perlas se lavaron varias veces y la proteína unida se analizó mediante Western blot utilizando los anticuerpos anti-TCF-4 y β-catenina.

Ensayo de fraccionamiento celular

Las células C33A se sembraron en una placa de 60 mm y se transfectaron con 3 µg del plásmido indicado. 48 h después de la transfección las células se resuspendieron en 300 µl de buffer de lisis (Tris 10 mm pH 6.5, $Na_2S_2O_5$ 27 mM, Triton X-100 al 1%, $MgCl_2$ 10 mM, sacarosa 25 mM y cóctel de inhibidores de proteasas) y se incubaron durante 10 min a 4ºC en agitación suave. Las muestras se centrifugaron y se obtuvieron los sobrenadantes (fracción citoplasmática). El botón de células fue resuspendido en buffer de extracción (HEPES 10 mM pH 7.9, KCl 10 mM, EDTA 0.1 mMm pH 8.0, EGTA 0.1 mM pH 8.0 y cóctel de inhibidores de proteasas) y se centrifugaron utilizando un gradiente de sacarosa 0.34 M para aislar los núcleos celulares. Posteriormente, estos organelos se resuspendieron en el buffer RIPA (Tris 100 mM pH 8.0, $NaCl_2$ 50 mM, Nonidet P-40 al 0.5% y cóctel de inhibidores de proteasas) (fracción nuclear). Las muestras se analizaron mediante inmunoblot, utilizando los anticuerpos anti-TCF-4 (Santa Cruz Biotechnologies), Anti-Lamina B1 (Abcam), anti-GAPDH (Santa Cruz Biotechnologies) y anti-FLAG M2 (Sigma Aldrich).

Ensayo *in vitro* de interacción de proteínas con el ADN

Se amplificó un fragmento del promotor de Sp5 utilizando el oligonucleótido marcado con biotina, 5' Bio-GGGTCTCCAGGCGGCAAG3', y el 5'-AGCGAAAGCAAAGCAAATCCTTTGAATCC-3'. La sonda se purificó con el kit de purificación de PCR QIAquick (Qiagen) de acuerdo con las instrucciones del fabricante. Las células C33A se sembraron y se transfectaron con 10 µg de cada plásmido como se indica. 48 horas después de la transfección, las células se lisaron utilizando el buffer HKMG (HEPES 10 mM pH 7.9, KCl 100 mM, $MgCl_2$ 5 mM, DTT 1 mM, Na_3VO_4 1 mM, Glicerol al 10%, NP-40 al 0.5% y cóctel inhibidor de proteasa). Posteriormente, se incubó 20 min a 4°C y después fueron pasadas a través de una aguja de calibre 25 unida a una jeringa de 1 ml por 20 veces. Los lisados se centrifugaron y se obtuvieron los sobrenadantes. Los extractos de proteínas se pre-clarearon con 60 µl de perlas de estreptavidina agarosa (Invitrogen), durante 30 minutos a 4°C, después se centrifugaron y se obtuvieron los sobrenadantes. Para cada muestra, se agregaron una cantidad de 4 µg de sondas de biotina y 2.5 µg de Poly dI-dC (Sigma Aldrich) y se incubaron durante toda la noche. Posteriormente, se añadieron 60 µl de perlas de estreptavidina agarosa a cada muestra, se incubaron durante 30 minutos a 4ºC. Las muestras se centrifugaron y los sobrenadantes se descartaron. Las perlas obtenidas se lavaron cinco veces con el buffer HKMG y se resuspendieron con el buffer de carga Laemmli (Bio-Rad). Finalmente, las muestras fueron analizadas utilizando ensayos de Western blot.

Ensayo de inmunoprecipitación de la cromatina

Las células C33A se sembraron en placas de 100 mm y se cotransfectaron con 7 µg de los plásmidos 18E6HA y β-catenina. 48 h después de la transfección, las células se fijaron con medio DMEM-formaldehído al 1% durante 10 minutos y después fueron tratadas con 0.125 M de glicina por 5 minutos. Los lisados celulares se obtuvieron utilizando el buffer de lisis (SDS al 1%, EDTA 10 mM pH 8, Tris HCl 50 mM pH 8 y cóctel inhibidor de proteasa) y fueron sonicados utilizando un Bioruptor Pico (Diagenode), los tamaños de los fragmentos de ADN obtenidos van desde 200 hasta 500 pb. Se utilizó un total de 20 µg de cromatina por muestra y se

diluyó 1:5 con un buffer de dilución (Triton X-100 al 1%, NaCl 150 mM, EDTA 2 mM pH 8, Tris HCl 20 mM pH 8 y cóctel de inhibidores de proteasas). Después, todas las muestras se pre-clarearon con 50 µl de perlas de proteína G agarosa/ADN de esperma de salmón (Millipore) durante 3 horas a 4°C y posteriormente se centrifugaron. Los sobrenadantes se incubaron con los anticuerpos anti-HA (Cell Signaling), anti-TCF-4 (Abcam) o anti-IgG (Santa Cruz Biotechnologies) durante toda la noche a 4°C. Posteriormente, se agregaron 50 µl de perlas de proteína G agarosa/ADN de esperma de salmón (Millipore) y se incubaron durante 3 horas a 4°C. Las muestras se centrifugaron y las perlas se lavaron cuatro veces con el buffer de lavado I (Triton X-100 al 1%, SDS al 0.1%, NaCl 150 mM, EDTA 2 mM pH 8, Tris-HCl 20 mM pH 8 y cóctel inhibidor de proteasas) y una vez con el buffer de lavado II (Triton X-100 al 1%, SDS al 0.1%, NaCl 500 mM, EDTA 2 mM pH 8, Tris-HCl 20 mM pH 8 y cóctel de inhibidores de proteasas). Los complejos inmunoprecipitados se obtuvieron con el buffer de elución (SDS al 1%, $NaHCO_3$ 100 mM) y se trataron con NaCl 200 mM durante 5 horas a 65°C. Todas las muestras se trataron con ARNasa (200 µg) y Proteinasa K (160 µg). Los fragmentos de ADN se obtuvieron utilizando la técnica fenol/cloroformo. Después, se realizaron ensayos de qPCR para evaluar la interacción de las proteínas con el promotor de Sp5 utilizando los oligonucleótidos: 5'GGTCTCCAGGCGGCAAG3' y 5'AAGCGAAAGCAAAGCAAATCCTTTGAATCC3'. Para analizar los datos, se realizó el método de veces de enriquecimiento (ΔΔCt).

Ensayos de proliferación celular

Las células C33A se sembraron en placas de 60 mm y se transfectaron con 3 µg de plásmidos de E6 y β-catenina, como se indica. Después de 24 h de la transfección, las células se recogieron y se sembraron en una placa de 96 pozos durante 72 h. Los ensayos de MTS se realizaron utilizando el kit CellTiter 96 Aqueous One Solution Cell Proliferation (Promega), siguiendo las instrucciones del fabricante.

Para los ensayos de cristal violeta, las células se fijaron con PBS-formol al 10% durante 30 minutos a temperatura ambiente. Posteriormente, las células se tiñeron con cristal violeta/PBS durante 15 min. Después de varios lavados, las células se

trataron con ácido acético/PBS y la solución obtenida fue medida a 490 nm. Los datos se graficaron para determinar el porcentaje de proliferación celular para cada condición.

Análisis estadístico

Los datos que muestran los efectos de VPH-18 E6 y E6*I y VPH-16 E6 en los diferentes ensayos, son presentados como promedios y ± SD. El valor de *p* se calculó mediante la prueba *t* de Student o con la prueba ANOVA con el análisis post hoc de Tukey. Las diferencias fueron consideradas significativas con el valor de $p \leq 0.05$.

Resultados

Las proteínas E6 y E6*I del VPH-18 potencian la actividad transcripcional mediada por TCF-4

Las células C33A se transfectaron de forma transitoria con un plásmido reportero de luciferasa dependiente de TCF-4 (TOPFLASH) y con los plásmidos que expresan 18E6WT, 18E6SM, 18E6*I o 16E6 marcados en la porción N-terminal con el polipéptido FLAG. Todos los experimentos se realizaron co-transfectando el vector vacío (control negativo) o el plásmido que expresa β-catenina (control positivo), como se indica. Después de 48 h de transfección, se realizaron ensayos de Western blot para evidenciar la expresión de las proteínas E6 (**Figura 5A**). En este ensayo se observa que la relación de la expresión de la proteína de E6 y E6*I en las células transfectadas con 18E6WT es de alrededor del 20% y 80%, respectivamente; mientras que en las células transfectadas con el plásmido 18E6SM, dicha relación se invierte, siendo de aproximadamente el 80% para E6 y 20% para E6*I. Este efecto se debe a que el inserto del 18E6SM contiene una mutación en la posición A233G, que es el sitio donador de splicing que promueve una disminución en la expresión de E6*I. Por lo tanto, se utilizó este plásmido para comparar una condición con una expresión más alta de E6. La expresión exógena de 18E6WT y 18E6*I aumentó 1.5 veces la actividad transcripcional de TCF-4 (**Figura 5B**), en comparación con el vector vacío. En las células transfectadas con el plásmido 18E6SM se encontró un efecto similar en la activación transcripcional de TCF-4, aunque éste no fue estadísticamente significativo. Además, se observó una inducción de 2.9 veces de la actividad de TCF-4 en las células transfectadas con 16E6, similar al efecto obtenido cuando β-catenina fue sobre expresada. Posteriormente, cuando la vía de Wnt se sobre activó con la co-transfección de β-catenina y los plásmidos que expresan E6, se produjo un aumento de la actividad de TCF-4 en todas las condiciones probadas, por encima de la respuesta de la transfección solo con β-catenina (alrededor de 1.6 veces). Como se muestra en la **Figura 5B**, tanto E6 como E6*I del VPH-18 seguían mostrando un aumento en la respuesta de TCF, sin embargo, 16E6 alcanzaba un mayor efecto (3 veces más).

Por otra parte, se evaluó la activación de genes celulares con respuesta a TCF-4, tales como: Axina 2 y Ciclina D1, los cuales fueron evaluados por qPCR. Como se observa en la **Figura 5C** y **D**, las proteínas E6 promovieron la expresión de Ciclina D1 (hasta 2 veces en comparación con el vector vacío), mientras que Axina 2 alcanzó un aumento de hasta 1.5 veces; sin embargo, la proteína 18E6*I no mostró ningún efecto en la expresión de Axina 2. En conjunto, estos hallazgos sugieren que E6 y E6*I del VPH-18 promueven la activación de la vía de señalización canónica de Wnt.

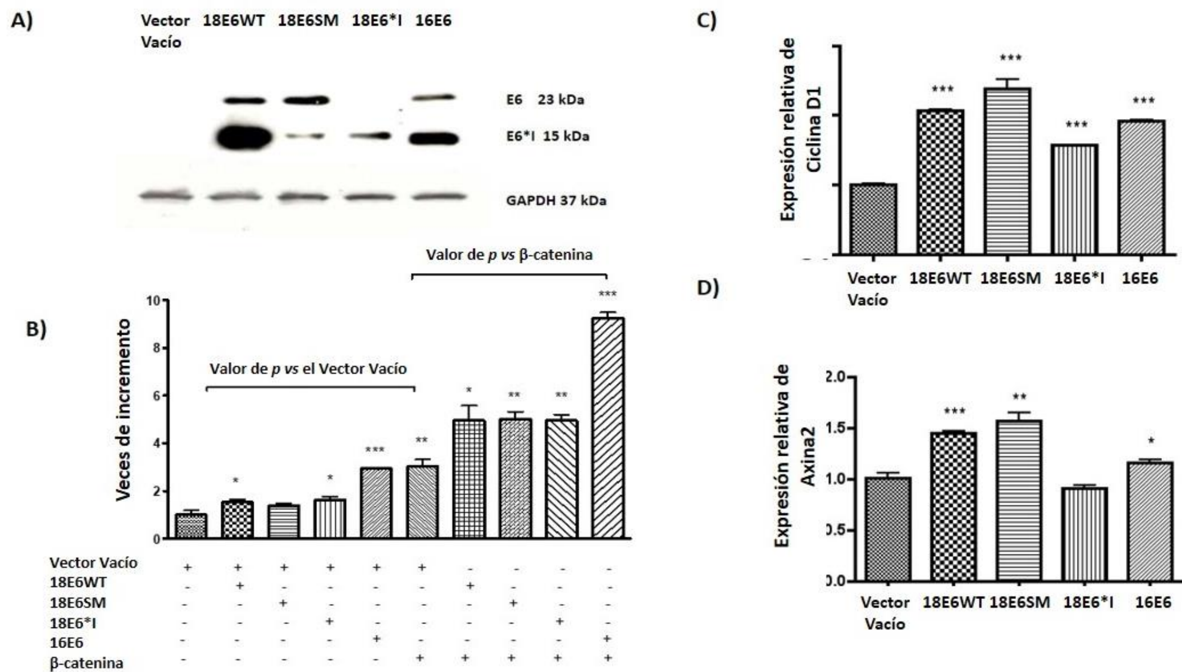


Figura 5. Las proteínas E6 y E6*I inducen la actividad transcripcional de TCF-4. (**A**) La expresión de las proteínas E6 y E6*I se analizó 48 h después de la transfección en células C33A, mediante Western blot. (**B**) Los plásmidos 18E6WT, 18E6SM, 18E6*I, 16E6 y β-catenina se transfectaron como se indica, con TOPFLASH (plásmido reportero de TCF-4) y los plásmidos reporteros de β-galactosidasa en células C33A. La actividad del reportero de luciferasa se midió 48 h después de la transfección. Las actividades de la luciferasa se compararon con el vector vacío o el plásmido de β-catenina, como se muestra. La expresión de Ciclina D1 (**C**) y Axina 2 (**D**) se evaluó mediante qPCR en células transfectadas con los distintos plásmidos de E6. Los promedios ± D.E. de tres experimentos independientes fueron representados en cada gráfico. La prueba *t* de Student se realizó para evaluar las diferencias significativas, los valores de *p* se representan como * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (Modificado de Muñoz-Bello JO et al., 2018).

Las proteínas E6 y E6*I del VPH-18 aumentan los niveles de las proteínas β-catenina y TCF-4, pero no alteran su localización subcelular

Para determinar el efecto de las proteínas E6 en los niveles de β-catenina y TCF-4, se realizaron ensayos inmunoblot utilizando lisados celulares totales. Observamos que tanto las proteínas E6 como E6*I aumentan significativamente los niveles proteicos de β-catenina (**Figura 6A, B**) y TCF-4 (**Figura 6C, D**).

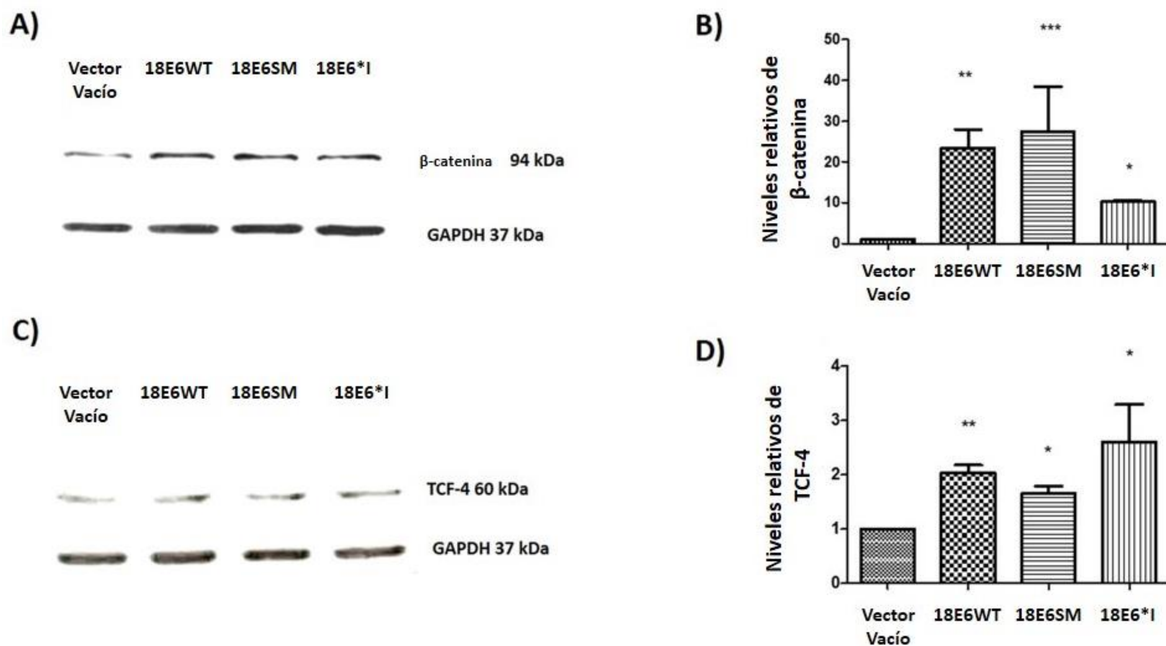
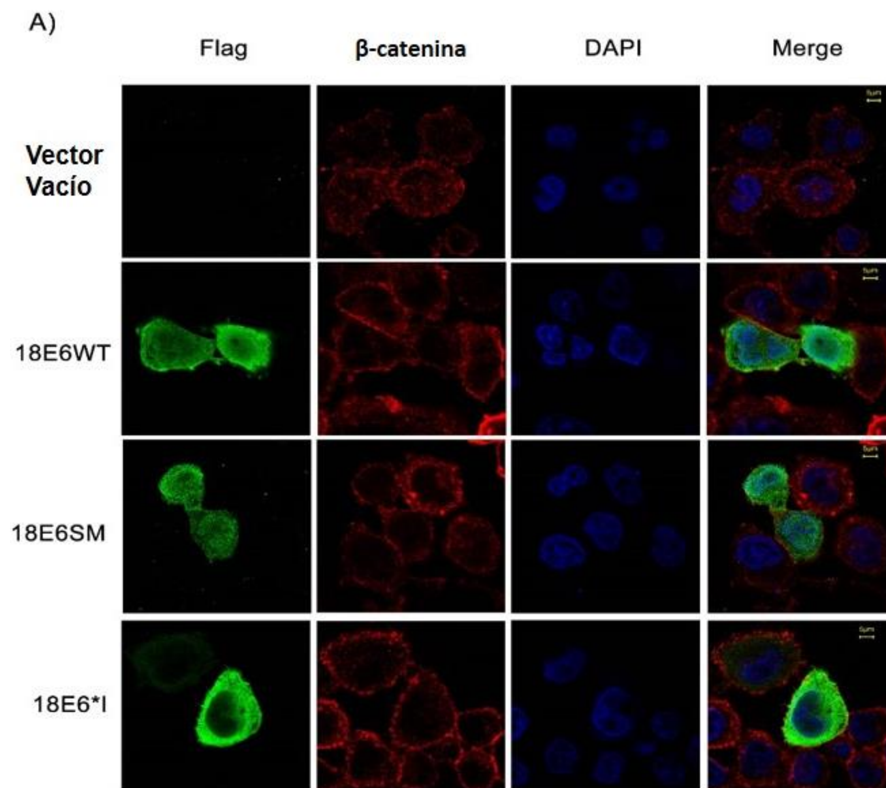
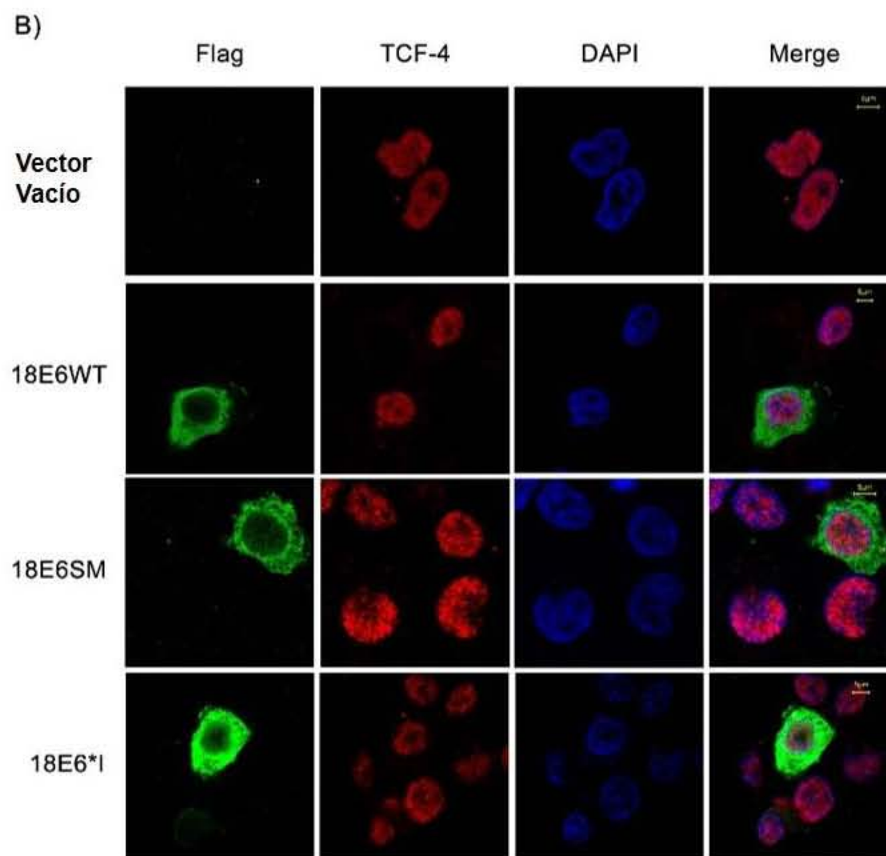


Figura 6. Las proteínas E6 y E6*I del VPH-18 aumentan los niveles proteicos de β-catenina y TCF-4. Las células C33A se transfectaron con plásmidos que expresan E6WT, E6SM y E6*I. Después de 48 h de la transfección, los lisados celulares se analizaron mediante Western blot. (**A**) Western blot de β-catenina y (**B**) grafica del análisis densitométrico; (**C**) Western blot de TCF-4; y (**D**) grafica del análisis densitométrico. Los datos de tres experimentos independientes fueron recolectados y graficados mostrando la media ± D.E. Se realizó el análisis estadístico con la prueba *t* de Student, * $p<0.05$, ** $p<0.01$ y *** $p<0.001$ *vs* valores de vector vacío (Modificado de Muñoz-Bello JO et al., 2018).

Dado que este incremento en los niveles de TCF-4 y β-catenina podría repercutir en el cambio de la localización subcelular de estas proteínas, se realizaron ensayos de inmunofluorescencia. Las células C33A se transfectaron con los plásmidos de expresión 18E6WT, 18E6SM o 18E6*I y después de 48 h, las células fueron

analizadas. Como se muestra en la **Figura 7A** y **B**, todas las isoformas de E6 se detectaron en el citoplasma y el núcleo. Por otro lado, β-catenina se encontró ubicada principalmente en la membrana celular y el citoplasma, sin un aparente efecto en su localización por la presencia de las isoformas de E6 (**Figura 7A**). Resultados similares se obtuvieron cuando se utilizó un modelo derivado de queratinocitos (células HaCaT) transfectados con E6 (**Figura 7C**). En concordancia con estudios previos realizados con la proteína E6 del VPH-16 (Lichtig et al., 2010), observamos que E6 y E6*I de VPH-18 no alteran la distribución subcelular de β-catenina. Como se muestra en la **Figura 7B**, la localización subcelular de TCF-4 tampoco se modificó en presencia de las isoformas E6.

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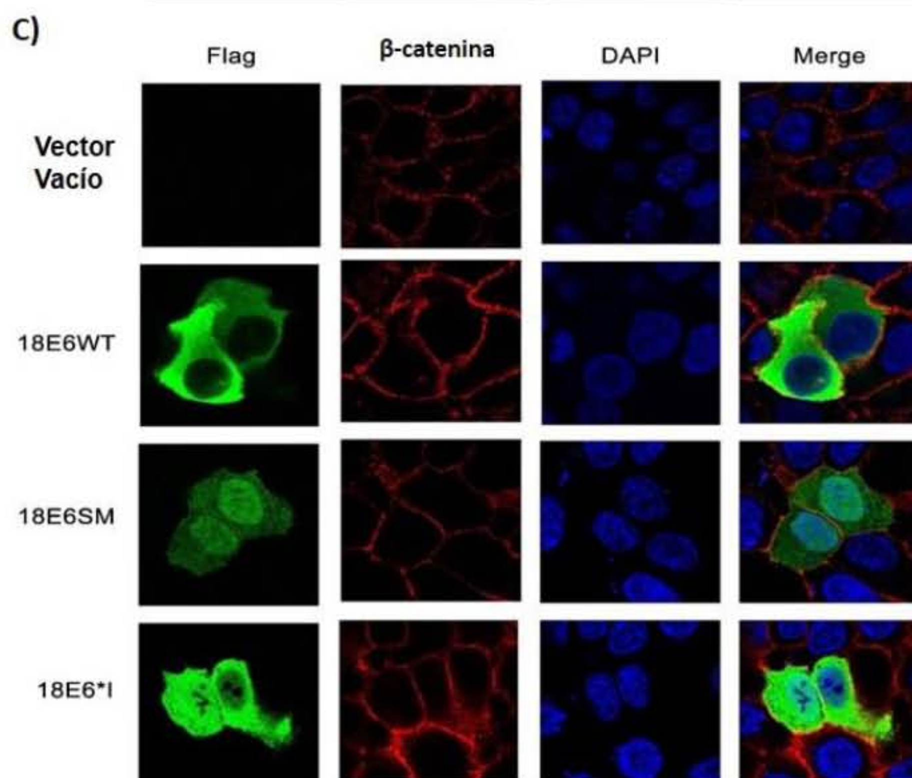

C)
Flag
β-catenina
DAPI
Merge
Vector Vacío
18E6WT
18E6SM
18E6*I

Figura 7. Las proteínas E6 no alteran la distribución subcelular de β-catenina y TCF-4. Las células C33A se transfectaron con plásmidos de expresión 18E6WT, 18E6SM o 18E6*I como se indica. Después de 48 h de la transfección se realizó una tinción de inmunofluorescencia utilizando: (**A**) anticuerpos específicos contra β-catenina; o (**B**) anticuerpos contra TCF-4 (rojo) y FLAG (verde). (**C**) En otro modelo, usando células HaCat se observa que E6 y E6*I (verde) no afecta la localización de β-catenina (rojo) en queratinocitos inmortalizados. Todas las células adicionalmente se tiñeron con DAPI (azul) para visualizar los núcleos. Las imágenes fueron adquiridas mediante microscopía confocal con un objetivo de inmersión de 63X (Modificado de Muñoz-Bello JO et al., 2018).

Las proteínas E6 interactúan con el complejo de activación de Wnt *in vivo* e *in vitro*

Estudios previos han demostrado que E6 del VPH-16 interactúa con miembros de la vía de señalización Wnt, tales como Dvl2 (Bonilla-Delgado et al., 2012). Para investigar una interacción con β-catenina y TCF-4, se realizaron ensayos de inmunoprecipitación en células C33A transfectadas con los diferentes plásmidos de expresión de E6. Después de 48 h de la transfección, se obtuvieron los lisados proteicos y se incubaron con anticuerpos específicos de anti-β-catenina o anti-TCF-4. Posteriormente, se realizó un Western blot utilizando el anticuerpo anti-FLAG para evaluar la unión de las proteínas de E6 con β-catenina o TCF-4. Como se observa en la **Figura 8A** y **B**, las proteínas E6 del VPH-16, E6 y E6*I del VPH-18 fueron capaces de unirse a β-catenina y a TCF-4.

Estos resultados fueron confirmados cuando se realizaron ensayos de interacción *in vitro* utilizando lisados de células C33A y proteínas de fusión GST-E6 (**Figura 8C**), donde las proteínas recombinantes fueron capaces de unirse a β-catenina y TCF-4. Estos datos sugieren que las proteínas E6 regulan la vía de señalización Wnt β-catenina a través de la interacción con el complejo de activación transcripcional de TCF-4.

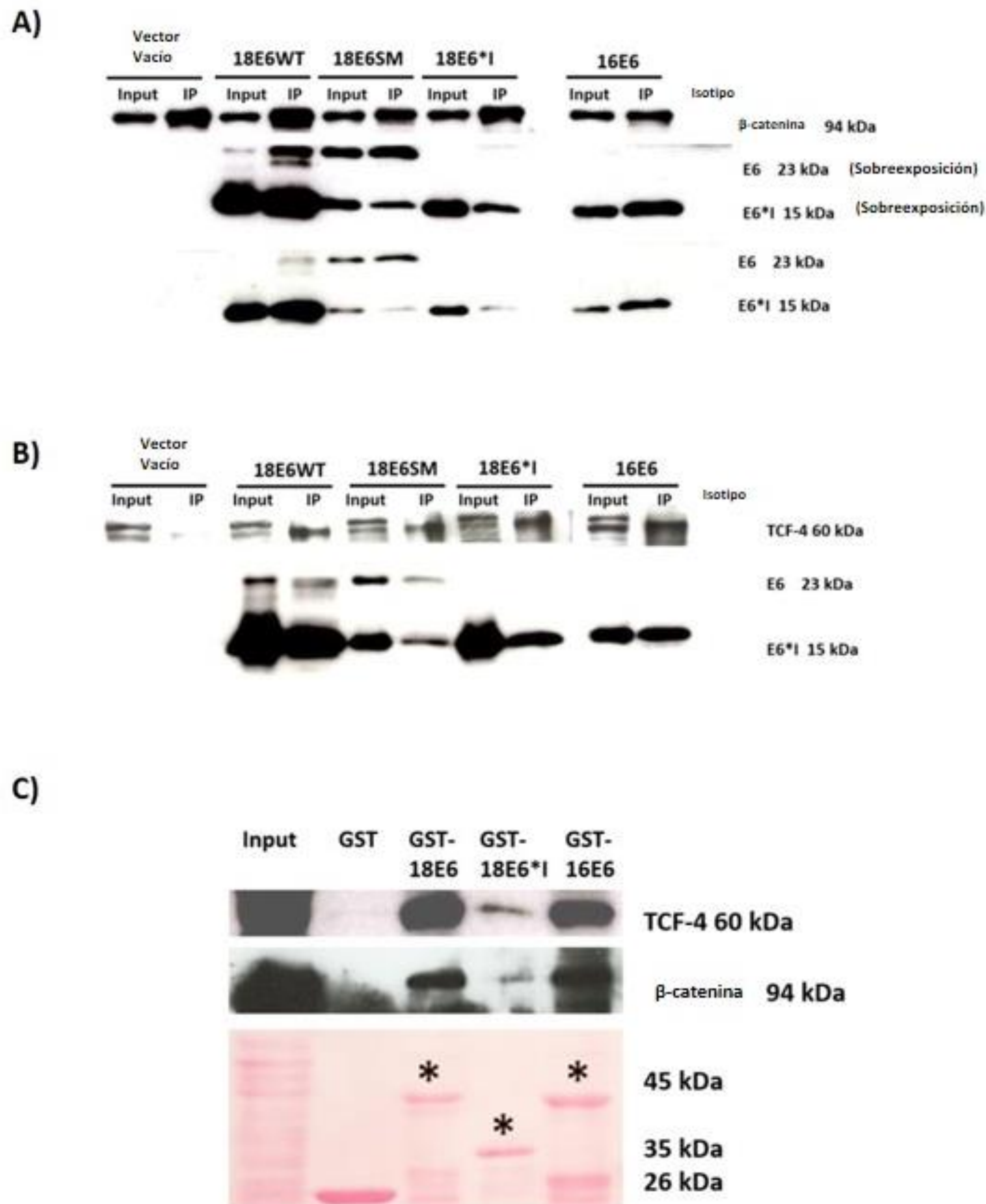


Figura 8. Las proteínas E6 y E6*I interactúan con las proteínas β-catenina y TCF-4, *in vivo* e *in vitro*. Las células C33A se transfectaron con diferentes vectores de expresión de E6, 48 h después de la transfección se obtuvieron los lisados proteicos. Las proteínas (**A**) β-catenina y (**B**) TCF-4 se inmunoprecipitaron con los anticuerpos específicos. Los inmunocomplejos se analizaron mediante Western blot usando los anticuerpos anti-β-catenina y anti-TCF-4 para detectar la proteína inmunoprecipitada y con un anti-FLAG para detectar las proteínas de E6. La imagen muestra un experimento representativo de tres realizados. En comparación, se muestra el 10% de la proteína utilizada para la inmunoprecipitación (input) y la precipitación con un anticuerpo IgG irrelevante

(isotipo). Una sobre exposición de proteínas E6 se muestra en el panel A. (**C**) Las proteínas recombinantes GST-18E6, GST-18E6*I y GST-16E6 se incubaron con extractos proteicos de células C33A, mientras que solo la proteína purificada GST se usó como control. Los inmunoblots se realizaron utilizando anticuerpos anti-β-catenina y anti-TCF-4. Se usó 10% de extracto de proteína como input. El panel inferior muestra la tinción con rojo Ponceau S de una membrana de nitrocelulosa representativa para visualizar las proteínas recombinantes. Los asteriscos (*) muestran las proteínas recombinantes de E6 (Modificado de Muñoz-Bello JO et al., 2018).

Las proteínas E6 y E6*I del VPH-18 aumentan la estabilidad de TCF-4

Para determinar el efecto de la interacción de las proteinas E6 y E6*I del VPH-18 con TCF-4, se evaluó la estabilidad de TCF-4 mediante ensayos de vida media. Las células C33A se transfectaron con plásmidos que expresan E6 o E6*I; después de 48 h de la transfección, las células se trataron con 200 µg/mL de cicloheximida y la tasa de degradación de TCF-4 se evaluó a las 0, 6 y 12 h después del tratamiento, se observó que después de 6 y 12 h los niveles de TCF-4 disminuyeron considerablemente en las células transfectadas con el vector vacío (**Figura 9A** y **B**). Curiosamente, los niveles de esta proteína se mantuvieron en presencia de las proteínas E6 y E6*I después de 6 h de tratamiento, alcanzando 4.37 a 7.25 veces de cambio, en comparación con las células transfectadas con el vector vacío. Finalmente, los niveles de proteína TCF-4 fueron mayores a las 12 h únicamente en las células que expresaban el plásmido 18E6WT. Estos datos sugieren fuertemente que TCF-4 se estabiliza en presencia de E6 y E6*I y a su vez, que tal efecto podría explicarse por la interacción que existe entre E6 y E6*I con TCF-4.

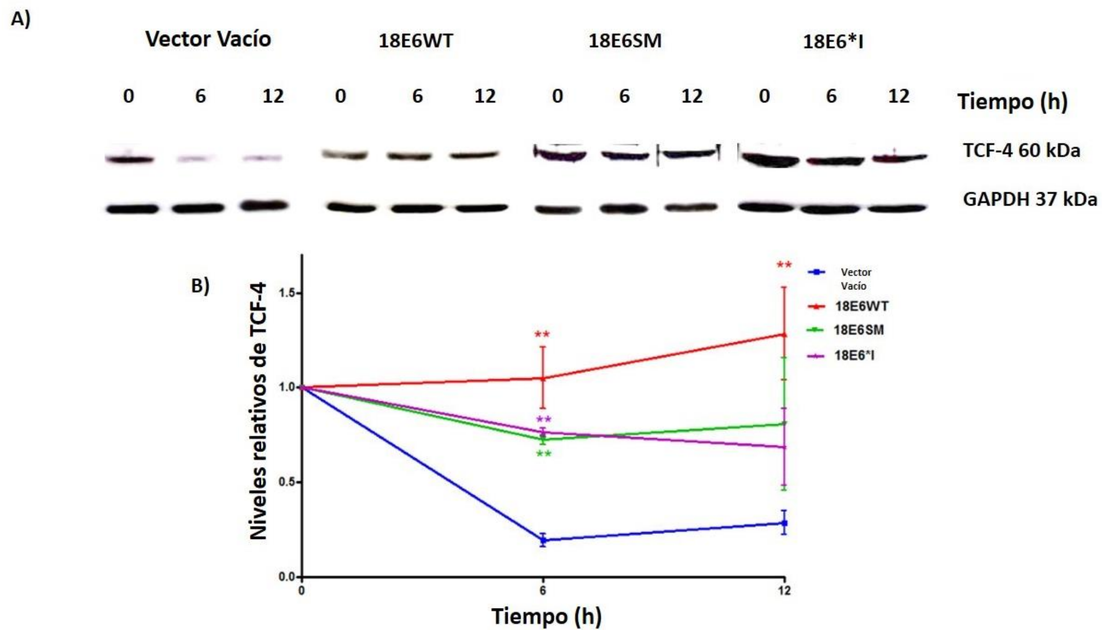


Figura 9. Las proteínas E6 del VPH-18 aumentan la estabilidad de TCF-4. Las células C33A se transfectaron con plásmidos que expresan las distintas isoformas de E6. Después de 48 h post-transfección, se adicionaron 200 μg/mL de cicloheximida al medio de cultivo. Los extractos proteicos se obtuvieron a las 0, 6 y 12 h después del tratamiento. (**A**) Se muestra un inmunoblot representativo con los diferentes tratamientos. En células transfectadas con el vector vacío, los niveles de TCF-4 disminuyeron a las 6 y 12 h después del tratamiento, en contraste con las células que expresan las diferentes isoformas de E6, donde los niveles de TCF-4 permanecieron sin cambios a las 6 y 12 h. (**B**) el gráfico muestra los datos representados en promedios ± D.E. de tres experimentos independientes. Se realizó la prueba estadística ANOVA de una vía y una prueba post hoc de Tukey, ** $p < 0.001$ comparado con el valor del vector vacío (Modificado de Muñoz-Bello JO et al., 2018).

Las isoformas de E6 del VPH-18 aumentan los niveles nucleares de la proteína TCF-4

Para determinar el efecto de las proteínas E6 en los niveles nucleares de TCF-4, se realizó un ensayo de fraccionamiento celular. Como se muestra en la **Figura 10A**, la proteína E6 se localiza principalmente en el núcleo, mientras que la isoforma E6*I se encuentra tanto en el núcleo como en el citoplasma. Curiosamente, los niveles

de TCF-4 aumentaron significativamente en el núcleo en células que expresan a E6 y E6*I (**Figura 10A** y **B**). Estos resultados se relacionan con los datos obtenidos previamente ya que el aumento nuclear de TCF-4 podría estar determinado por el incremento de la vida media de TCF-4.

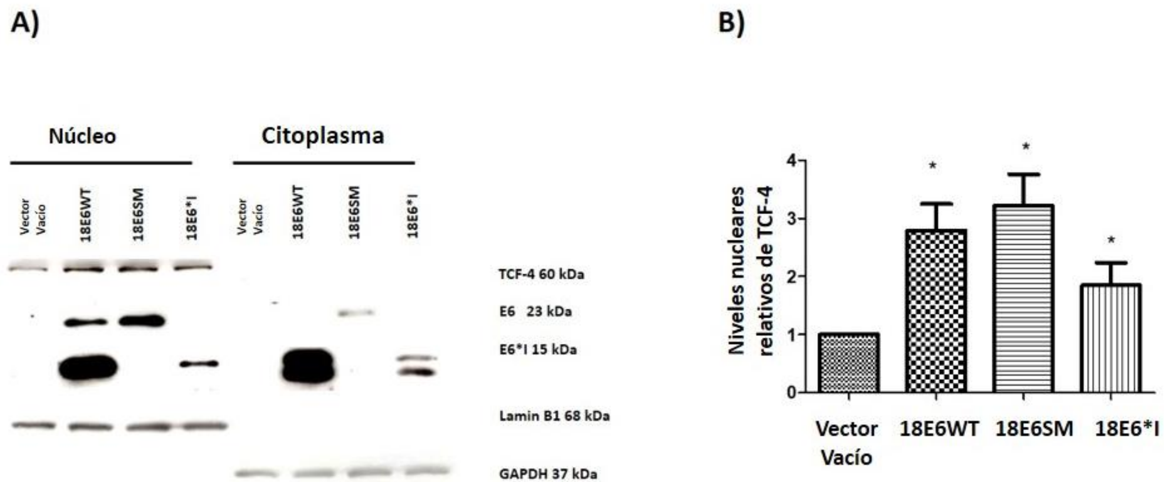


Figura 10. Las isoformas de E6 del VPH-18 aumentan los niveles nucleares de TCF-4. (**A**) Inmunoblot representativo de las proteínas TCF-4 y E6 en fracciones solubles nucleares y citoplasmáticas de células C33A transfectadas con plásmidos de expresión de E6. Las proteínas Lamin B1 y GAPDH se utilizaron como controles de carga nuclear y citoplásmica, respectivamente. (**B**) Se muestra la gráfica de análisis densitométrico de los niveles nucleares relativos de TCF-4 en presencia de las proteínas E6. Los datos de tres experimentos independientes fueron recolectados y graficados, mostrando la media y ± D.E. Se realizó el análisis estadístico utilizando la prueba *t* de Student, el valor de * $p<0.05$ se muestra en comparación con el vector vacío (Modificado de Muñoz-Bello JO et al., 2018).

Las proteínas E6 y E6*I del VPH-18 se unen a un promotor dependiente de TCF-4 *in vivo* e *in vitro*

Previamente otros estudios habían demostrado que TCF-4 se une a una secuencia conservada, A-C/G-A/T-C-A-A-A-G, que se encuentra en los promotores dependientes de TCF-4/β-catenina, como en el caso del promotor del gen Sp5 (Hatzis et al., 2008; Takahashi et al., 2005). Se realizaron ensayos de qPCR para determinar el efecto de las proteínas de E6 en la regulación de un gen con elementos de respuesta a TCF-4, como el gen Sp5. En la figura 11A se observa que

las proteínas E6 aumentan los niveles del ARNm de Sp5 en células C33A co-transfectadas con β-catenina. Debido a este efecto y con los hallazgos previamente obtenidos de la unión de E6 y E6*I con TCF-4 *in vivo* e *in vitro*, se analizó si las proteínas E6 podrían interactuar con el promotor de Sp5. Se diseñaron oligonucleótidos para amplificar una secuencia que se localiza en los nucleótidos -326 al -129 del promotor de Sp5, ya que esta región contiene dos sitios de unión de TCF-4 ubicados en la posición -303 al -296 y del -149 al -142. Estos sitios de unión fueron confirmados cuando se analizó el fragmento *in silico* mediante el uso de la herramienta informática Tfsitescan. Las células C33A se co-transfectaron con los plásmidos E6 del VPH-18 marcado con un polipéptido HA (hemaglutinina) y β-catenina. Posteriormente, se realizaron ensayos de inmunoprecipitación de la cromatina (ChIP) y se observó que E6 del VPH-18 se une al promotor de Sp5 *in vivo* (Figura 11B). Como era de esperarse, el factor transcripcional TCF-4 también se unió a este promotor, tanto en las células transfectadas con el vector vacío como en aquellas con el plásmido 18E6-HA. Vale la pena mencionar que la unión de E6 del VPH-18 al promotor Sp5 es contundente, ya que aparentemente no se observa ninguna amplificación cuando la inmunoprecipitación se lleva a cabo con el anticuerpo anti-HA en células transfectadas con el vector vacío.

Para confirmar los resultados obtenidos, las células C33A se transfectaron con los plásmidos 18E6WT, 18E6SM o 18E6*I, y 48 h después de la transfección, se realizó el ensayo de interacción *in vitro* de proteínas con el ADN. Como se esperaba, TCF-4 fue capaz de interactuar con el promotor de Sp5 en todas las muestras analizadas (**Figura 11C**). Interesantemente, las proteínas E6 y E6*I también son capaces de interactuar con el promotor de Sp5. Además, como control negativo se utilizó la proteína p53, ya que este factor transcripcional no tiene elementos de respuesta dentro del promotor de Sp5. Es importante mencionar que, aunque p53 se une a E6 y E6*I no se encontró formando un complejo dentro del fragmento del promotor de Sp5 analizado (**Figura 11C**). Estos resultados indican que las proteínas E6 y E6*I del VPH-18 interactúan con un promotor dependiente de TCF-4 y que esto puede deberse a la interacción de TCF-4 con las proteínas E6 y E6*I. Estos resultados

muestran un nuevo mecanismo por el cual el oncogén E6 y su isoforma E6*I regulan la activación de la vía de señalización celular de Wnt/β-catenina (**Figura 11D**).

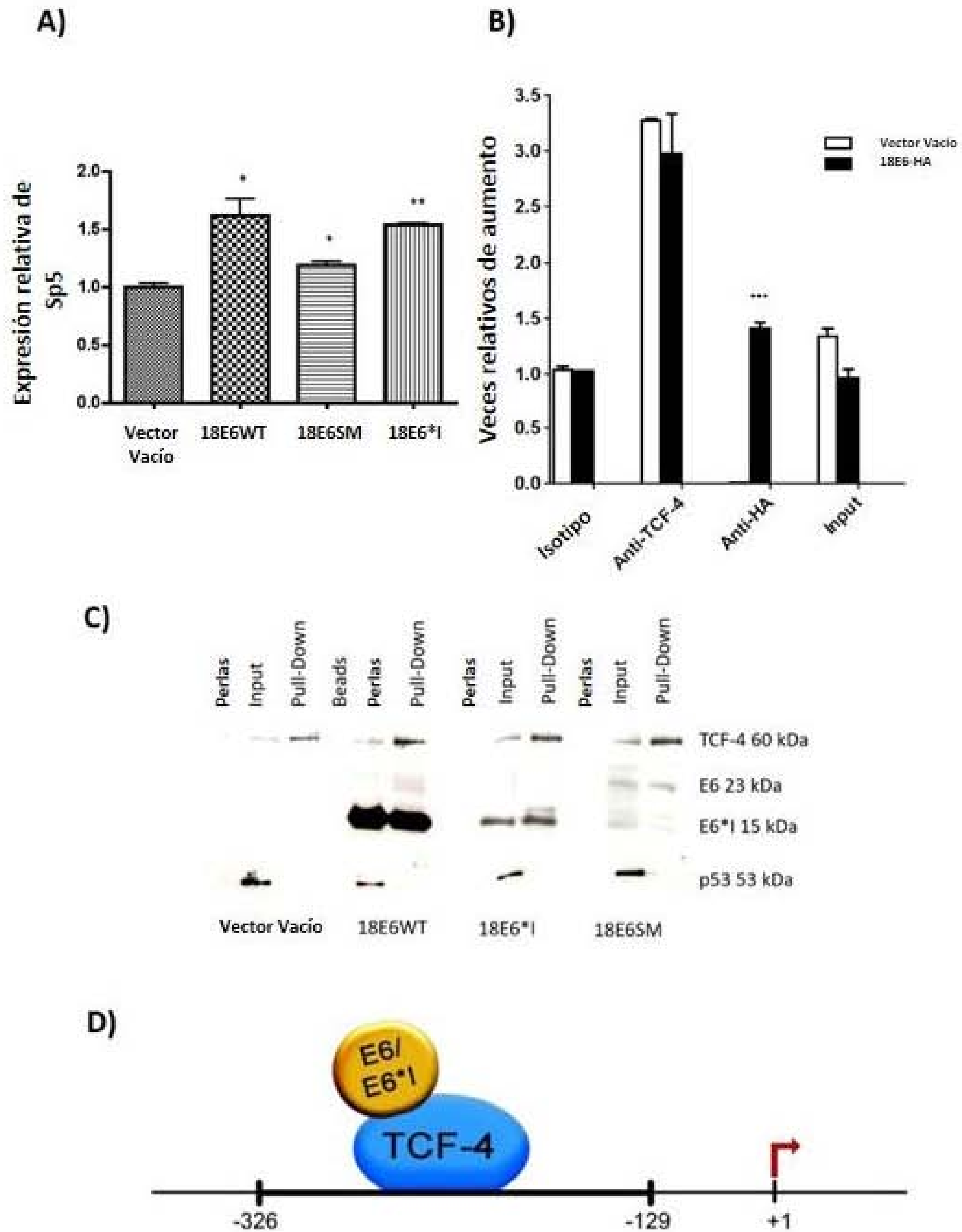


Figura 11. Las proteínas E6 y E6*I del VPH-18 se unen con el promotor de Sp5. Las células C33A se co-transfectaron con los plásmidos de expresión de β-catenina y de E6 como se indica: (**A**) las isoformas de E6 aumentan la expresión relativa de Sp5 como se muestra en el análisis de qPCR; * $p<0.05$, ** $p<0.01$, comparado con el vector vacío; (**B**) El ensayo de inmunoprecipitación de la cromatina (ChIP) muestra que E6 del VPH-18 se une al promotor de Sp5 *in vivo*. El anticuerpo anti-HA se usó para detectar la proteína E6-HA, y los anticuerpos anti-TCF-4 y anti-IgG se usaron como controles positivo e isotipo, respectivamente. Se analizó el 10% de la cromatina como input. *** $p<0.001$ E6-HA comparado con el vector vacío. (**C**) Las células C33A se transfectaron con los plásmidos de 18E6WT, 18E6SM o 18E6*I y 48 h después de la transfección, se realizó un ensayo interacción *in vitro* de proteínas con DNA. Como se esperaba, el factor transcripcional TCF-4 interactúa con la sonda del promotor de Sp5. Interesantemente, se observó que tanto E6 como E6*I interactuaban con el promotor de Sp5 *in vitro*. Además, el factor de transcripción p53 se usó como control negativo a estos sitios analizados. (**D**) En el esquema se muestran las interacciones sugeridas de E6 y E6*I de VPH-18 con el promotor dependiente de TCF-4, proponiendo un posible mecanismo de regulación de la vía de señalización Wnt por las isoformas de E6 (Modificado de Muñoz-Bello JO et al., 2018).

Las proteínas E6 y E6*I del VPH-18 en cooperación con β-catenina inducen un incremento en la proliferación celular

Finalmente, para determinar la contribución de las proteínas E6 en la vía de señalización de Wnt/β-catenina, se realizaron ensayos de proliferación celular en células C33A co-transfectadas con β-catenina utilizando los métodos de cristal violeta y MTS. Como se muestra en la **Figura 12A** y **B**, en aquellas células donde se transfectaron solo los plásmidos de E6, se observó un aumento en la proliferación de entre un 70 y 85%, evaluado por ensayos de MTS, mientras que en los ensayos de cristal violeta se observó un aumento del 20 al 30% en comparación con el vector vacío. Adicionalmente, la sola transfección de β-catenina mostró un aumento en la proliferación del 97% en los ensayos de MTS y del 51% en los ensayos de cristal violeta. Por otro lado, cuando E6 y E6*I se expresaron con β-catenina se observó un aumento adicional en la proliferación del 40 al 50% en los ensayos de MTS, en comparación con la sola expresión de β-catenina, mientras que el aumento encontrado en el ensayo de cristal violeta fue del 15 al 30% (**Figura 12A** y **B**). Esto sugiere que las isoformas de E6 cooperan con β-catenina para promover la proliferación celular.

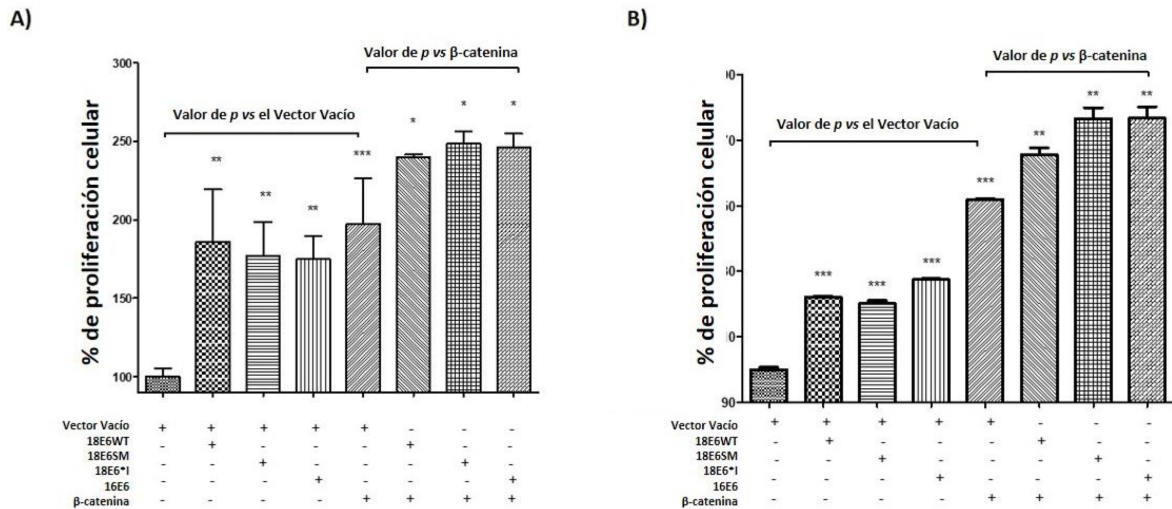


Figura 12. Las proteínas E6 y E6*I del VPH-18 aumentan la proliferación celular en cooperación con β-catenina. Las células C33A se transfectaron con los plásmidos que se indican y 24 h después de la transfección las células fueron resembradas en una placa de 96 pozos. Posteriormente, los experimentos fueron evaluados después de 72 h, ya sea mediante ensayos de (**A**) MTS o (**B**) Cristal violeta. Los datos de tres experimentos independientes fueron recolectados y graficados mostrando la media ± D.E. Se realizó el análisis de *t* de Student para analizar la significancia, los valores de * $p<0.05$, ** $p<0.001$ y *** $p<0.0001$ se muestran comparados con valores de vector vacío y β-catenina como se indica (Modificado de Muñoz-Bello JO et al., 2018).

Las variantes de E6 del VPH-18 regulan diferencialmente la transcripción mediada por TCF-4

Se ha propuesto que las variantes intratipo del VPH pueden presentar distintos comportamientos biológicos que les confieren diferentes riesgos patogénicos que pudieran determinar el desenlace de la infección (De la Cruz-Hernández et al., 2005; Lizano et al., 2006, 1997). Para determinar si las variantes de E6 del VPH-18 inducen diferencialmente a la actividad transcripcional de TCF-4, se analizó la variante E6Af que pertenece a la rama filogenética africana del VPH-18 y alberga variaciones genómicas que conducen a cambios dentro de la secuencia aminoacídica, y difieren en ésta con la variante de referencia, la E6AsAi, que en

este estudio también se muestra como 18E6WT (De la Cruz-Hernández et al., 2005). Las células C33A se transfectaron con los plásmidos E6Af y E6AsAi del VPH-18 y se co-transfectaron con el plásmido reportero de luciferasa (TOPFLASH), el reportero de β-galactosidasa y, en ciertos casos, con plásmidos de β-catenina, según se indica. La expresión de las proteínas de las variantes de E6 se evaluó mediante inmunoblot como se muestra en la **Figura 13A**. Interesantemente, en los ensayos de luciferasa, la variante E6Af fue capaz de aumentar la actividad transcripcional de TCF-4 hasta 2.8 veces; este aumento fue mayor que la observada para la variante E6AsAi que obtuvo un incremento de 1.5 veces. Estos efectos fueron más evidentes cuando se co-transfectaron las células C33A con el plásmido de β-catenina, donde la variante E6Af alcanzó una actividad de hasta 2.25 veces, mientras que E6AsAi mostró una de 1.6 veces en comparación con β-catenina. Estos resultados muestran que las variantes de E6 del VPH-18 inducen diferencialmente la activación transcripcional de TCF-4 (**Figura 13B**). Sin embargo, cuando se midió la expresión de los genes responsivos a TCF-4 por qPCR, ambas variantes de E6 fueron capaces de inducir la expresión de Axina 2 y Ciclina D1, este efecto se muestra en la **figura 12C** y **D**.

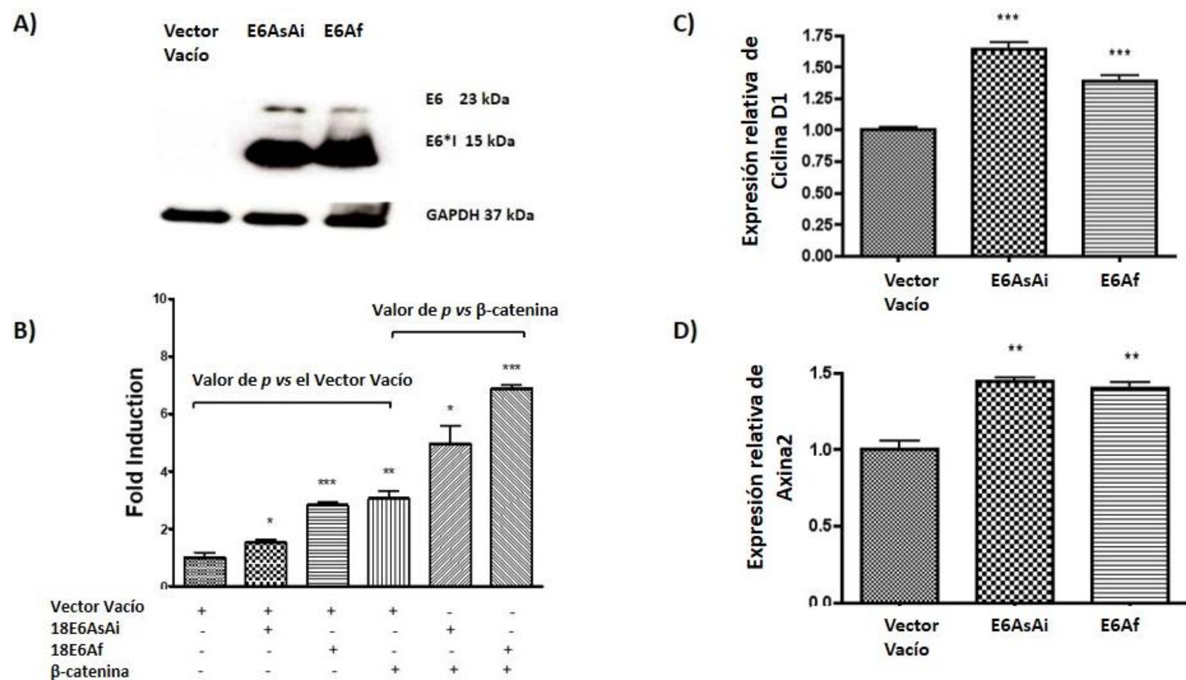
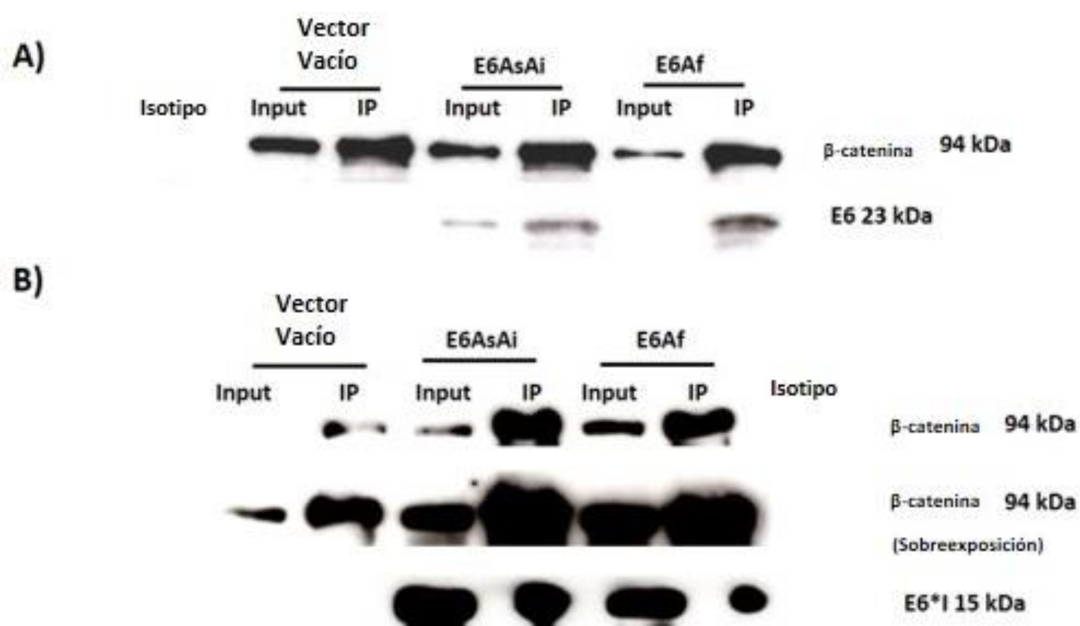


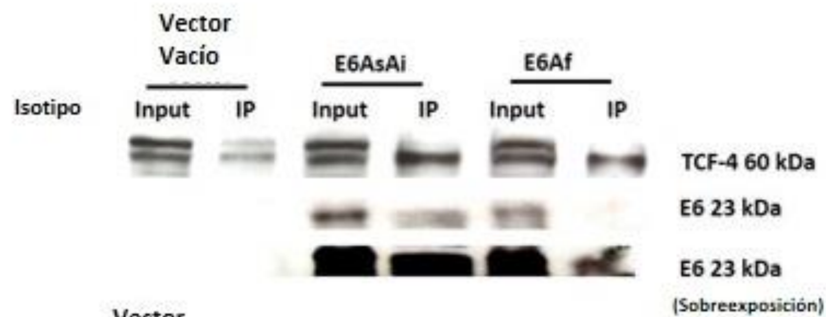
Figura 13. Las variantes E6AsAi y E6Af del VPH-18 modulan la actividad transcripcional de TCF4. (**A**) La expresión de las proteínas E6AsAi y E6Af de VPH-18 fueron evaluadas por inmunoblot. (**B**) Las células C33A se transfectaron con los plásmidos de E6AsAi y E6Af solas o en combinación con el plásmido de β-catenina, y se cotransfectaron con el plásmido reporteros TOPFLASH y de β-galactosidasa según se indica. La expresión génica de Ciclina D1 (**C**) y Axina 2 (**D**) se analizó mediante qPCR. Los promedios ± D.E. de tres experimentos independientes se representan en cada gráfico. La prueba *t* de Student se realizó para evaluar las diferencias significativas, los valores se representan como * p<0.05, **p <0.01, *** p <0.001 (Modificado de Muñoz-Bello JO et al., 2018).

Las variantes intratipo del VPH-18 interactúan con β-catenina y TCF-4.

Para demostrar la interacción de las proteínas β-catenina y TCF-4 con las variantes E6AsAi y E6Af, se realizaron ensayos de inmunoprecipitación. Las células C33A se transfectaron con plásmidos que expresan E6AsAi y E6Af. Después de 48 h de la transfección se lisaron las células y se incubaron con anticuerpos anti-TCF-4 y anti-β-catenina. El análisis de Western blot mostró que ambas variantes de E6 podían interactuar con β-catenina (**Figura 14A** y **B**) y con TCF-4 (**Figura 14C** y **D**), respectivamente. Por lo tanto, aunque las variantes de E6 probadas mostraron un efecto diferencial en otras vías celulares previamente analizadas en nuestro grupo de trabajo (Contreras-Paredes et al., 2009), no se observaron cambios que repercutieran en la capacidad de unirse a TCF-4 o a β-catenina ni en la expresión de los genes probados.



C)



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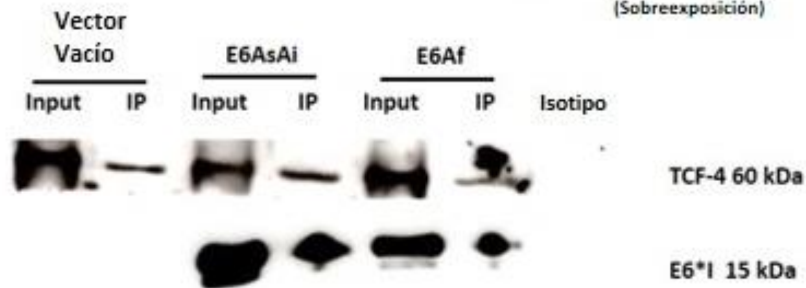

Figura 14. Las variantes de E6 del VPH-18 interactúan *in vivo* con β-catenina y TCF-4. Las células C33A se transfectaron con los plásmidos de las variantes de E6. 48 h después de la transfección, se lisaron las células y se inmunoprecipitaron las proteínas utilizando los anticuerpos anti-β-catenina (**A** y **B**) y anti-TCF-4 (**C** y **D**). Los inmunoblots muestran la interacción de las variantes de E6 (A y C) y la isoforma E6*I (**B** y **D**) con ambas proteínas. Para una mejor apreciación se muestran las sobreexposiciones de β-catenina y E6 en el panel **B** y **C**, respectivamente. Las imágenes representativas de tres experimentos realizados son mostradas en cada ensayo. El 10% de la proteína utilizada para la inmunoprecipitación se indica como input y se utilizó un anticuerpo irrelevante (isotipo) (Modificado de Muñoz-Bello JO et al., 2018).

Discusión

La expresión continua de las oncoproteínas E6 y E7 es necesaria para la inmortalización y transformación celular en los tipos de cáncer relacionados con la infección del VPH. La oncoproteína E6 contribuye a la progresión maligna al regular un conjunto de proteínas celulares implicados en los procesos antes mencionados (Moody and Laimins, 2010). Una característica común del ARNm de E6-E7 de los VPH-AR es producir pequeñas isoformas denominadas E6*, cuyas funciones celulares son poco conocidas (Olmedo-Nieva et al., 2018). Se ha propuesto que la proteína E6*I del VPH-18 antagoniza los efectos de E6 (Pim et al., 1997), mientras que algunos estudios muestran que E6* tiene funciones propias independientes de E6 (Evans et al., n.d.; Manzo-Merino et al., 2014; Pim et al., 2009; Tungteakkhun et al., 2010). Se ha observado que E6* interactúa con E6 y E6AP (Pim et al., 1997) y que también disminuye los niveles proteicos de las proteínas que contienen los dominios PDZ, como: hDlg, Scrib, MAGI-1 y MAGI-2 (Pim et al., 2009). También se ha demostrado que E6* modula proteínas relacionadas con la apoptosis, por ejemplo, se une a la procaspasa 8 afectando su estabilidad. Previamente, nuestro grupo de trabajo demostró que E6*I induce la activación y la translocación nuclear de la procaspasa 8, sin inducir la muerte celular (Manzo-Merino et al., 2014). Estos hallazgos sugieren que las isoformas E6* pueden, de alguna manera que aún no se ha descrito, participar con E6 para favorecer la progresión maligna.

Las vías de señalización son los mecanismos por los cuales las células se comunican con otras células y su entorno. La unión de los ligandos a los receptores celulares puede activar las cascadas de proteínas y, en consecuencia, afectar los niveles de transcripción génica. A través de estos procesos complejos, las células transforman los estímulos externos en señales bioquímicas que controlan los efectos biológicos, como la proliferación, la diferenciación y la muerte.

Previamente, se ha descrito la activación aberrante de la vía de señalización celular Wnt durante la carcinogénesis cervical (Ramos-Solano et al., 2015; Rodríguez-Sastre et al., 2005; Uren et al., 2005) y se ha propuesto que la oncoproteína E6 del VPH-16 juega un papel importante en esta activación en diversos modelos celulares (Kuslansky et al., 2016; Lichtig et al., 2010; Rampias et al., 2010; Sominsky et al.,

2014). Sin embargo, el efecto de la oncoproteína E6 del VPH-18 y su isoforma derivada del splicing alternativo E6*I no se ha analizado en esta vía de señalización celular.

En este estudio, demostramos que E6*I puede inducir la activación de la vía de señalización celular Wnt/β-catenina. Observamos que, E6*I del VPH-18 por sí solo potenció la actividad transcripcional de TCF-4 en células C33A, alcanzando hasta 1.5 veces en comparación con el control. Adicionalmente, se observó un efecto similar cuando se expresó 18E6WT. Esto sugiere que E6*I coopera con E6 en la activación transcripcional mediada por TCF-4. En un intento por expresar únicamente la isoforma completa E6, utilizamos el plásmido 18E6SM (mutante en el sito donador de splicing G233A en el inserto de E6). La mutación dentro del plásmido 18E6SM permite la expresión de una mayor cantidad de E6, aunque todavía se produce E6*I en bajos niveles; esto podría deberse al posible uso de otro sitio donador de splicing aún no descrito. Cuando el plásmido 18E6SM fue transfectado en células C33A se observó un ligero incremento en la regulación transcripcional de TCF-4, aunque no significativa, en relación con el vector control. Este resultado nos sugiere que el aumento en la actividad transcripcional de TCF-4 es preferencialmente mediado por la isoforma E6*I.

Interesantemente, cuando la vía de señalización celular Wnt se sobre activó con la co-transfección de β-catenina, la activación transcripcional de TCF-4 se potenció en presencia de proteínas E6 o E6*I, sugiriendo que las isoformas E6*I y E6 colaboran directamente en la activación de la vía Wnt/β- catenina. También encontramos que 16E6 promovió una respuesta más alta en la activación de TCF-4, alcanzando una inducción de hasta 2.5 veces y este efecto se incrementó ligeramente en presencia de β-catenina exógena (3 veces). Nuestros resultados concuerdan con estudios previos, donde se observó tres veces de incremento en la actividad de TCF-4 en células HEK293T que expresan ectópicamente las proteínas E6 del VPH-16, el receptor Fz1 y el ligando Wnt3a (Lichtig et al., 2010). Adicionalmente, el grupo de trabajo de Bonilla-Delgado et al. (2012) reportó una inducción de 50 veces de la activación transcripcional de TCF-4 en células COS-7 donde se transfectó E6 del VPH-16 en combinación con Dvl-2 y β-catenina. Estos datos sugieren que el

contexto celular y/o el método utilizado para la activación de la vía Wnt desempeñan un papel importante en el efecto producido por la oncoproteína E6.

Adicionalmente, se evaluó la expresión de genes blanco de TCF-4 en presencia de E6 y E6*I, estos ensayos se observó un aumento en la expresión de Ciclina D1, Axina 2 y Sp5. Estos datos concuerdan con un estudio previo donde E6 de VPH-16 fue capaz de activar el promotor de Ciclina D1 (Lichtig et al., 2010). Además, en otro modelo utilizando ratones transgénicos que expresan la proteína E6 del VPH-16, la expresión de Ciclina D1 también se encontraba sobre regulada (Bonilla-Delgado et al., 2012). Dentro de los genes blanco de la vía de Wnt cuya expresión está regulada por TCF-4, se encuentra el gen Axina 2. Este gen está considerado un supresor de tumores y las mutaciones dentro de este gen están asociadas al desarrollo del cáncer (Koch et al., 2007; Liu et al., 2000). Axina 2 está involucrada en la regulación negativa de la vía canónica de Wnt, ya que puede limitar la intensidad de la duración o la propagación de la señal (Leung et al., 2002; Lustig et al., 2002). Es interesante que a pesar de que E6*I promueve un incremento de la expresión de Ciclina D1, no logró promover una sobreexpresión de Axina 2. Es posible que E6 reclute co-reguladores que sean distintos de aquellos reclutados por E6*I que podrían ser necesarios para la expresión de Axina 2.

Se ha demostrado previamente que E6 del VPH-16 induce la activación transcripcional de TCF-4 sin afectar la localización de la β-catenina (Lichtig et al., 2010). De acuerdo con este resultado, nuestros hallazgos revelaron que, si bien E6 y E6*I aumentan los niveles proteicos de β-catenina y TCF-4, no alteran la localización subcelular de estos. Además, nuestros resultados demuestran que tanto E6 como E6*I son capaces de interactuar con β-catenina y TCF-4 *in vivo* e *in vitro*, que son las principales proteínas involucradas en la activación transcripcional dependiente de TCF-4. Por otro lado, observamos que E6 y E6* del VPH-18 también inducen la estabilización de TCF-4. Más estudios son necesarios para dilucidar si la interacción de TCF-4 con las proteínas E6 es responsable directo de la estabilización de TCF-4. Estudios previos han demostrado que la proteína E6 del VPH-16 E6 en colaboración con E6AP inducen la estabilización de otros miembros de la vía canónica de Wnt, como β-catenina, sin embargo la sola expresión de la

oncoproteína E6 del VPH-16 no es suficiente para llevar acabo la estabilización de β-catenina (Sominsky et al., 2014).

Por otra parte, cuando se activa la vía de señalización Wnt, β-catenina se transloca al núcleo y se une con el factor transcripcional TCF-4, promoviendo su actividad (Shitashige et al., 2008). Nuestros resultados demuestran que tanto E6 como E6*I aumentaron los niveles de proteína TCF-4 en el núcleo, lo que podría impactar directamente en la activación transcripcional de TCF-4. Sorprendentemente, los resultados de la inmunoprecipitación de la cromatina y el ensayo de interacción de proteínas con el ADN revelaron que E6 y E6*I interactúan con un promotor dependiente de TCF-4. Esta interacción podría explicarse a través de complejos formados por las proteínas E6, E6*I y TCF-4 que reconocen secuencias específicas ubicadas en el promotor Sp5 u otros promotores de respuesta a TCF-4. Además, en concordancia con estudios previos (Ben et al., 2015; Wang et al., 2013), encontramos que en células que expresan ectópicamente E6 y E6*I inducen un aumento en la proliferación de estas. Curiosamente, esta proliferación se ve incrementada cuando E6 y E6*I se expresan juntamente con β-catenina. Por lo tanto, nuestros resultados muestran un nuevo mecanismo mediante el cual E6 y E6*I regulan la vía de Wnt/β-catenina.

Dado que se ha propuesto que las variaciones intratipo del VPH afectan el comportamiento biológico del VPH, nos interesó también determinar si las variantes de E6 del VPH-18 podrían afectar diferencialmente a la vía de señalización celular de Wnt/β-catenina. Las variantes intratipo de VPH se definen como aquellas que contienen cambios de nucleótidos en menos del 1% en las regiones codificantes (Burk et al., 2013; Lizano et al., 2009). Nuestro grupo ha reportado previamente que las variantes del VPH-18 exhiben diferencias en las proporciones de los transcritos de E6/E6*I, lo que afecta directamente a los niveles de p53 (De la Cruz-Hernández et al., 2005). Además, se ha observado que las variantes de E6 modulan diferencialmente la vía de señalización Akt/PI3K (Contreras-Paredes et al., 2009). Estudios previos demostraron que las variantes E6 del VPH-16 regulan de manera diferencial la activación de la vía de señalización Wnt/β-catenina (Zehbe et al., 2011). Los ensayos de luciferasa descritos en este trabajo mostraron que las

variantes de E6 del VPH-18 tienen una capacidad diferencial para aumentar la actividad transcripcional de TCF-4, donde se observó que la variante E6Af promovió una inducción de 2.8 veces en comparación con 1.5 veces de la observada por la variante E6AsAi. Además, cuando las variantes de E6 se co-transfectaron con β-catenina, E6-Af y E6AsAi alcanzaron una activación de TCF-4 de hasta 2.25 veces y 1.6 veces, respectivamente, en comparación con la obtenida con la expresión exógena de β-catenina. Sin embargo, a pesar de que las variantes de E6 mostraron diferentes niveles de activación de TCF-4, tanto E6Af como E6AsAi promovieron la expresión de Axina 2 y Ciclina D1, sin diferencias significativas entre ellas. Este efecto podría deberse a una capacidad distinta de las variantes de E6 para regular o interactuar con otras proteínas no probadas en este estudio que se encuentran involucradas en la activación de la vía de señalización Wnt. Sin embargo, observamos que ambas variantes de E6 del VPH-18 interactúan con TCF-4 y β-catenina de una manera similar, lo que sugiere que los cambios aminoacídicos en las variantes E6 no afectan este proceso.

En este estudio, demostramos que no solo E6, sino también E6*I del VPH-18 son capaces de regular la vía de señalización celular de Wnt/β-catenina, a través de su interacción con el complejo de activación TCF-4. Sin embargo, es necesario analizar los efectos sobre otros miembros de la vía de Wnt para determinar la contribución específica de E6 y la isoforma E6*I en la transformación celular inducida por la vía Wnt/β-catenina.

Este trabajo aporta información que ayudará a entender la participación de los oncogenes virales, en la regulación de vías de señalización que participan en procesos relacionados al establecimiento y mantenimiento del cáncer, permitiendo que en un futuro se identifiquen moléculas que puedan utilizarse como un posible blanco terapéutico.

Conclusiones

- Las proteínas E6 y E6*I del VPH-18 inducen la activación transcripcional mediada por TCF-4
- Las proteínas E6 y E6*I del VPH-18 aumentan los niveles proteicos de las proteínas β-catenina y TCF-4 sin alterar su distribución subcelular
- Las proteínas E6 del VPH-18 interactúan con el complejo de activación de Wnt (TCF-4 y β-catenina) *in vivo* e *in vitro*
- E6 y E6*I del VPH-18 incrementan la vida media de TCF-4
- Las isoformas de E6 del VPH-18 incrementan los niveles nucleares de la proteína TCF-4
- Las proteínas E6 y E6*I del VPH-18 se unen a un promotor dependiente de TCF-4 *in vivo* e *in vitro*
- Las proteínas E6 y E6*I del VPH-18 en cooperación con β-catenina inducen un incremento en la proliferación celular
- Las variantes E6AsAi y E6Af del VPH-18 regulan diferencialmente la transcripción mediada por TCF-4
- Las variantes intratipo del VPH-18 interactúan con β-catenina y TCF-4

Perspectivas

- Analizar si ocurre una unión directa de E6 y E6*I con TCF-4 y su implicación en el aumento de la vida media de este factor de transcripción.
- Analizar la unión de E6 con otros promotores dependientes de TCF-4 y determinar si dicha unión se da de manera directa.
- Evaluar el significado biológico de la interacción de las variantes del VPH-18 con β-catenina y TCF-4.

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Anexos:

Artículos publicados

1. **Muñoz-Bello JO**, Olmedo-Nieva L, Castro-Muñoz LJ, Manzo-Merino J, Contreras-Paredes A, González-Espinosa C, López-Saavedra A, Lizano M. HPV-18 E6 Oncoprotein and Its Spliced Isoform E6*I Regulate the Wnt/β-Catenin Cell Signaling Pathway through the TCF-4 Transcriptional Factor. Int J Mol Sci. 2018 Oct 13;19(10).
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HPV-18 E6 Oncoprotein and Its Spliced Isoform E6*I Regulate the Wnt/β-Catenin Cell Signaling Pathway through the TCF-4 Transcriptional Factor

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Abstract: The Wnt/β-catenin signaling pathway regulates cell proliferation and differentiation and its aberrant activation in cervical cancer has been described. Persistent infection with high risk human papillomavirus (HR-HPV) is the most important factor for the development of this neoplasia, since E6 and E7 viral oncoproteins alter cellular processes, promoting cervical cancer development. A role of HPV-16 E6 in Wnt/β-catenin signaling has been proposed, although the participation of HPV-18 E6 has not been previously studied. The aim of this work was to investigate the participation of HPV-18 E6 and E6*I, in the regulation of the Wnt/β-catenin signaling pathway. Here, we show that E6 proteins up-regulate TCF-4 transcriptional activity and promote overexpression of Wnt target genes. In addition, it was demonstrated that E6 and E6*I bind to the TCF-4 (T cell factor 4) and β-catenin, impacting TCF-4 stabilization. We found that both E6 and E6*I proteins interact with the promoter of *Sp5*, in vitro and in vivo. Moreover, although differences in TCF-4 transcriptional activation were found among E6 intratype variants, no changes were observed in the levels of regulated genes. Furthermore, our data support that E6 proteins cooperate with β-catenin to promote cell proliferation.

Keywords: HPV-18 E6; HPV-18 E6*I; TCF-4 transcription factor; Wnt/β-catenin signaling

1. Introduction

The Wnt signaling pathway regulates a variety of processes, including cell proliferation and differentiation [1]. Briefly, in the off-state of the canonical pathway, the effector protein, β-catenin, is associated to a multiprotein complex that promotes its phosphorylation in specific residues in a GSK3β (Glycogen synthase kinase 3β) and CK1 (Casein kinase 1) kinase-dependent fashion. Those residues are recognized by the ubiquitin-ligase β-TrCP, allowing β-catenin ubiquitylation and subsequent degradation via the proteasome, whilst in the nucleus, the co-repressor, Groucho/TLE

(Transducin-like enhancer), suppresses transcriptional activation through the inhibition of TCF/LEF (Lymphoid enhancer binding factor) transcriptional factors. In the on-state, the Wnt ligands bind to the Frizzled receptor and the LRP 5/6 co-receptor, which dimerize, leading to disassembly of the destruction complex. Subsequently, β-catenin is released in the cytoplasm and translocated into the nucleus, where it binds to TCF/LEF and replaces the repressor protein, Groucho/TLE [2]. This event induces TCF/LEF transcriptional activation and expression of genes, such as *Axin2*, *Jun*, *Myc*, *Ccnd1*, and *Sp5* (Specificity protein transcription factor 5) [3]. It has been demonstrated that alterations in the Wnt cell signaling pathway contribute to the development of several types of cancer [4], including colorectal [5], hepatocarcinoma [6], breast [7], and HPV-related cancers [8–10].

The persistent infection with high risk human papillomavirus (HR-HPV) is the main risk factor associated to cervical cancer development [11]. HPV-16 and HPV-18 are the most prevalent types, found in almost 70% of cervical cancer cases worldwide [12]. HR-HPV transformation capacity is mainly due to the overexpression of the E6 and E7 viral oncoproteins, which interact with many cellular proteins, thus affecting their functions [13]. E6 is implicated in the modulation of several cell signaling pathways involved in cell adhesion, proliferation, and apoptosis, such as RAF (Rapidly accelerated fibrosarcoma)/MEK (MAPK/ERK kinase)/ERK (Extracellular signal-regulated kinase) and PI3K (Phosphoinositide 3 kinase), among others [14].

The E6–E7 open reading frames (ORFs) contain spliced donor and acceptor sites, highly conserved among the HR-HPV. Those sites are recognized by the spliceosome complex, promoting the removal of a small intron and the generation of a premature stop codon, giving place to short forms of E6, termed E6*. In HPV-16, at least four isoforms of E6* (I–IV) have been identified, whereas in HPV-18, only one has been reported hitherto, termed E6*I [15]. Although these E6 small isoforms are highly expressed in premalignant lesions and cervical cancer biopsies [16,17], their functions are poorly understood [18].

The abnormal activation of the Wnt cell signaling pathway has been reported in HPV-related tumors [8,19,20]. In cervical tumor biopsies and HPV positive cell lines, β-catenin is mainly located in the cytoplasm and nucleus, while in normal tissue, it is mainly distributed at the cell membrane [9,20]. In vitro assays have demonstrated that HPV-16 E6 induces TCF-4 transcriptional activation, whereas β-catenin is not stabilized. Moreover, E6AP ubiquitin ligase contributes to the increase in the TCF transcriptional activation mediated by E6, in a proteasome-dependent manner, without affecting β-catenin levels [21,22].

No interactions of HPV-18 E6 protein with members of the Wnt activation complex (TCF-4, β-catenin) have been identified so far, and the mechanisms by which E6 induces TCF transcriptional activation are poorly understood. Moreover, the effect of E6* proteins in this pathway remains unknown. Therefore, the aim of this study was to investigate the role of HPV-18 E6 and E6*I proteins in the Wnt/β-catenin signaling regulation. Through a TCF-4-dependent luciferase reporter plasmid, we show that E6 and E6*I up-regulate TCF-4 transcriptional activity, which is enhanced with the expression of exogenous β-catenin. Moreover, Wnt target genes are overexpressed in E6 and E6*I transfected cells. We also found that E6 and E6*I increase β-catenin and TCF-4 protein levels, but they do not alter their subcellular distribution. Immunoprecipitation and pull-down assays revealed an interaction of TCF-4 and β-catenin with E6 and E6*I proteins and those interactions impact in TCF-4 stabilization. We found that E6 and E6*I interact with the *Sp5* gene promoter, in vivo and in vitro. Furthermore, proliferation induced by β-catenin is enhanced by E6 and E6*I proteins. Finally, although E6 intratype variants differentially affected TCF-4 transcriptional activation, no differences appeared in their ability to bind TCF-4 or β-catenin.

2. Results

2.1. HPV-18 E6 and E6*I Proteins Enhance β-Catenin/TCF-4 Transcription

C33A cells were transiently transfected with a TCF-4-dependent luciferase reporter plasmid (TOPFLASH) and FLAG-tagged versions of 18E6WT, 18E6SM, 18E6*I, or 16E6 expressing plasmids. All experiments were performed co-transfecting the empty vector (p3X) or the β-catenin expressing plasmid, as indicated. After 48 h of transfection, immunoblot assays were performed for each experiment, confirming the expression of FLAG-tagged E6 proteins (Figure 1A). It is worth mentioning that the relation of protein expression of E6 full length and E6*I in the 18E6WT transfected cells is around 20% and 80%, respectively; while in 18E6SM transfected cells, such a relation is inverted, being around 80% and 20%, respectively. This effect is because 18E6SM harbors an A233G mutation in the donor splicing site that promotes a decrease in the expression of E6*I. Therefore, 18E6SM was used to compare a condition with a higher expression of E6 full length. Ectopic expression of both 18E6WT and 18E6*I increased 1.5-fold TCF-4 transcriptional activity (Figure 1B), compared with the empty vector. 18E6SM showed a similar effect in the TCF4 transcriptional activation as observed for the other E6 expressing plasmids, although non-significant. A 2.9-fold induction of TCF-4 activity was observed in 16E6 transfected cells, similar to the effect of ectopically expressed β-catenin. Subsequently, when the Wnt pathway was over activated through the co-transfection of β-catenin and E6 expressing plasmids, an enhancement of TCF-4 activity occurred in all tested conditions, above the β-catenin response (around 1.6-fold). As shown in Figure 1B, the 18E6 full-length or E6*I continued showing an increase in TCF response, with a consistent higher effect for 16E6 (3-fold).

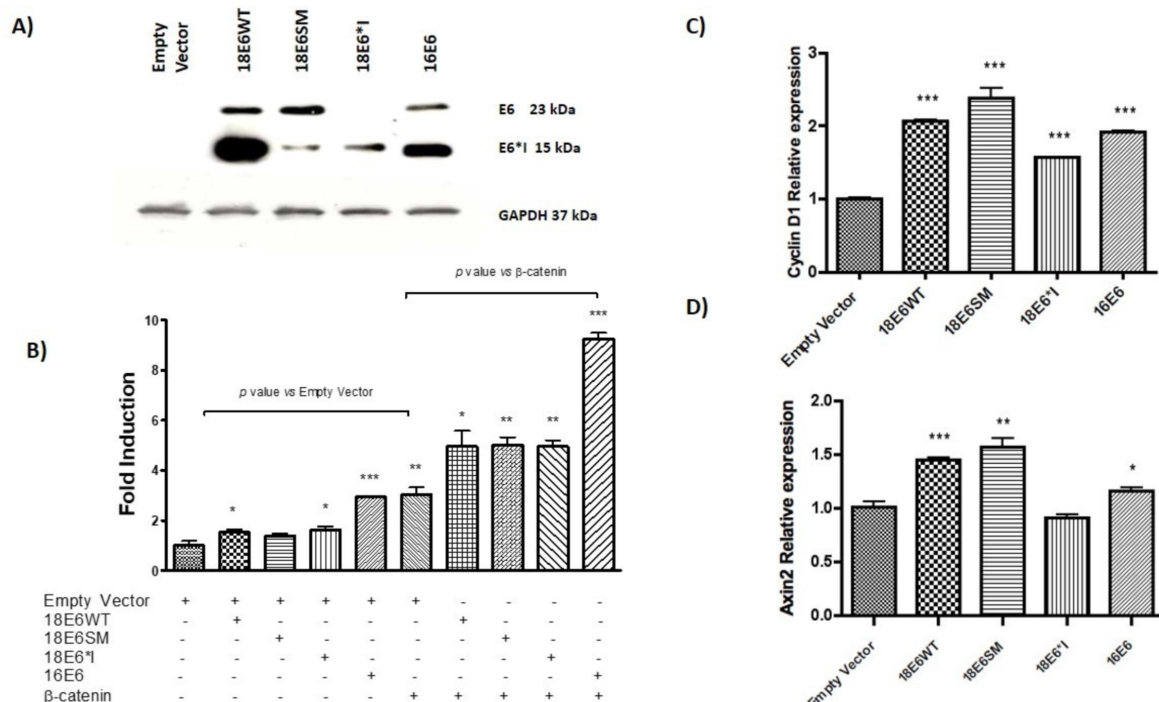

Figure 1. E6 and E6*I proteins induce TCF-4 transcriptional activity. (**A**) Expression of E6 and E6*I proteins was analyzed 48 h post-transfection in C33A cells, by western blot. (**B**) 18E6WT, 18E6SM, 18E6*I, 16E6, and β-catenin expressing vectors were transfected as indicated, with TOPFLASH (TCF-4 reporter plasmid) and β-galactosidase reporter plasmids in C33A cells. Luciferase reporter activity was measured 48 h post-transfection. Luciferase activities were compared with the empty vector or β-catenin plasmid. (**C**) *Cyclin D1* and (**D**) *Axin2* gene expression was evaluated by qPCR in E6 transfected cells. The means and ±SD of three independent experiments are depicted in each graph. Student t test was performed to evaluate the significant differences, the values are represented as * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$.

Overexpression of E6 proteins also stimulate native promoters containing TCF-4 responsive elements, as evidenced by the expression of the Wnt target genes, *Axin2* and *Cyclin D1*, evaluated by qPCR assay. As observed in Figure 1C,D, E6 proteins enhanced the expression of *Cyclin D1* (up to 2-fold, compared to the control vector), while *Axin2* reached up to a 1.5-fold increase; although 18E6*I had no effect on *Axin2* expression. Taken together, these findings suggest that HPV-18 E6 and E6*I cooperate in the activation of the canonical Wnt/β-catenin cell signaling.

2.2. E6 Proteins Increase β-Catenin and TCF-4 Protein Levels, But Do Not Alter Their Subcellular Localization

To determine the effect of E6 proteins on β-catenin and TCF-4 levels, we performed immunoblot assays using total cell lysates. We observed that both E6 and E6*I proteins significantly increase β-catenin (Figure 2A,B) and TCF-4 (Figure 2C,D) levels. Therefore, immunofluorescence assays were performed to investigate whether HPV-18 E6 full length and E6*I proteins alter β-catenin or TCF-4 localization. C33A cells were transfected with 18E6WT, 18E6SM, or 18E6*I expressing plasmids and after 48 h, cells were fixed and analyzed. As shown in Figure 3A,B, all the E6 proteins were detected in the cytosol and nuclei. On the other hand, β-catenin was found mainly at the cellular membrane and cytosol, which was unaffected by the presence of E6 proteins (Figure 3A). Similar results were obtained in HaCaT E6-transfected cells, a keratinocyte-derived model (Figure S1). Concordantly with previous reports carried out with 16E6, we observed that E6 and E6*I of HPV-18 do not alter β-catenin subcellular distribution [21]. As shown in Figure 3B, TCF-4 subcellular localization was also unaltered in the presence of the transfected E6 isoforms.

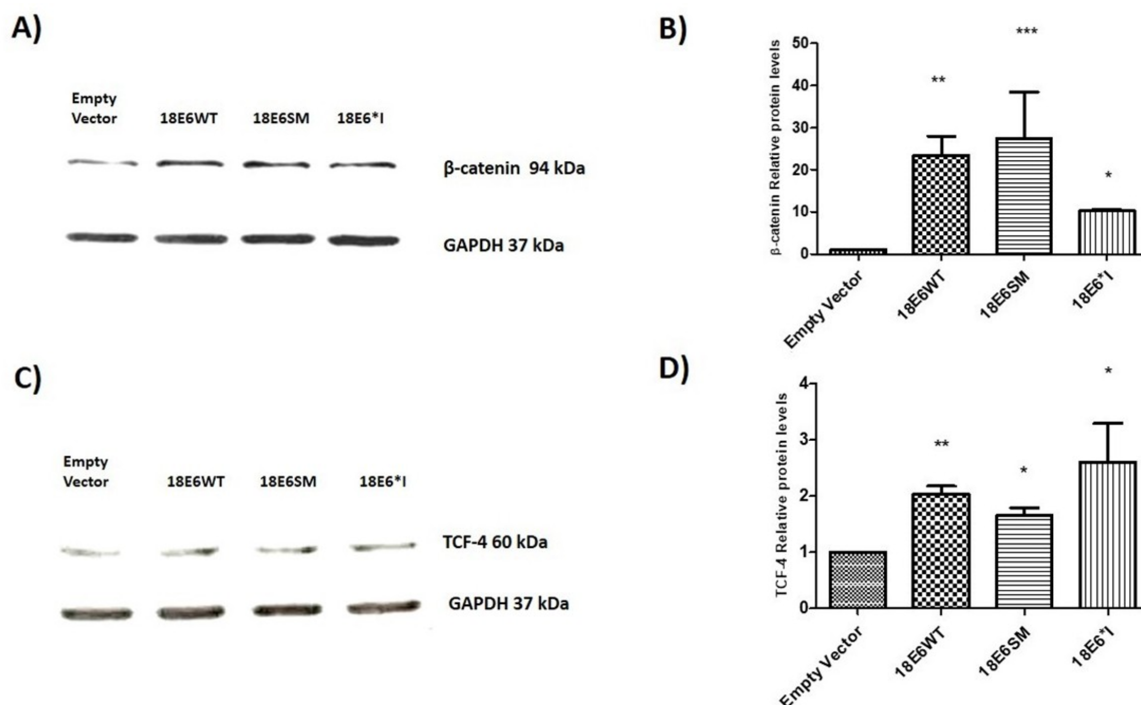

Figure 2. HPV-18 E6 and E6*I increase β-catenin and TCF-4 total protein levels. C33A cells were transfected with E6WT, E6SM, and E6*I expressing vectors. 48 h post-transfection, total cell lysates were analyzed by western blot. (**A**) β-catenin immunoblot and (**B**) densitometric analysis; (**C**) TCF-4 immunoblot; and (**D**) densitometric analysis. Data from three independent experiments were collected and graphed showing the mean and ±SD. *t* student analysis was performed, * $p < 0.05$, ** $p < 0.001$ and *** $p < 0.0001$ vs. empty vector values.

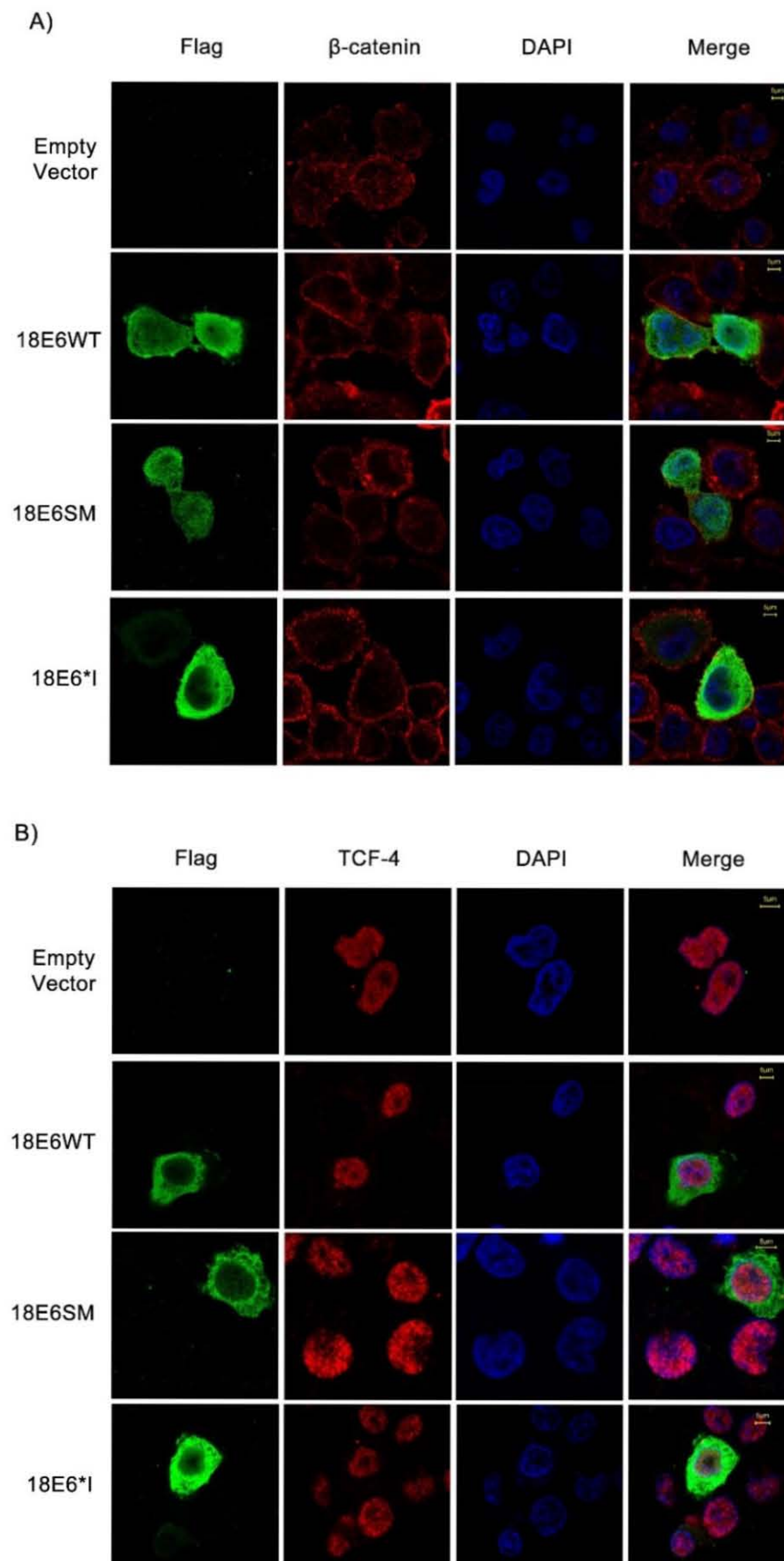

Figure 3. E6 proteins do not alter subcellular distribution of β-catenin or TCF-4. C33A cells were transfected with plasmids encoding 18E6WT, 18E6SM, or 18E6*I as indicated. 48 h post-transfection cells were fixed, and immunofluorescence stain was performed using specific antibodies against β-catenin (**A**) or TCF-4 (**B**) (Red) and FLAG (Green). Cells were also stained with DAPI (Blue) to visualize the nuclei. Images were acquired by confocal microscope. Data from three independent experiments were collected with a 63× objective oil immersion lens. Scale bar size 5 µm.

Figure 3. E6 proteins do not alter subcellular distribution of β-catenin or TCF-4. C33A cells were transfected with plasmids encoding 18E6WT, 18E6SM, or 18E6*I as indicated. 48 h post-transfection cells were fixed, and immunofluorescence stain was performed using specific antibodies against β-catenin (**A**) or TCF-4 (**B**) (Red) and FLAG (Green). Cells were also stained with DAPI (Blue) to visualize the nuclei. Images were acquired by confocal microscope. Data from three independent experiments were collected with a 63× objective oil immersion lens. Scale bar size 5 µm.

2.3. E6 Proteins Interact with the Wnt Activation Complex In Vivo and In Vitro

Previous reports demonstrate that HPV-16 E6 interacts with members of the Wnt signaling pathway, such as Dvl2 (Dishevelled Segment Polarity Protein 2) [23]. To investigate a further interaction with β-catenin and TCF-4, immunoprecipitation, assays were performed in C33A cells transfected with the different E6 expressing plasmids. After 48 h post-transfection, cell protein lysates were obtained and incubated with anti-β-catenin or anti-TCF-4 specific antibodies to immunoprecipitate these proteins. Afterwards, a western blot with anti-FLAG antibody was performed to assess the binding of the E6 proteins with β-catenin or TCF-4. As observed in Figure 4A,B, the immunoblot revealed that 16E6, 18E6 full-length, and 18E6*I proteins were able to bind to β-catenin (94 kDa band) and TCF-4 (60 kDa band), respectively.

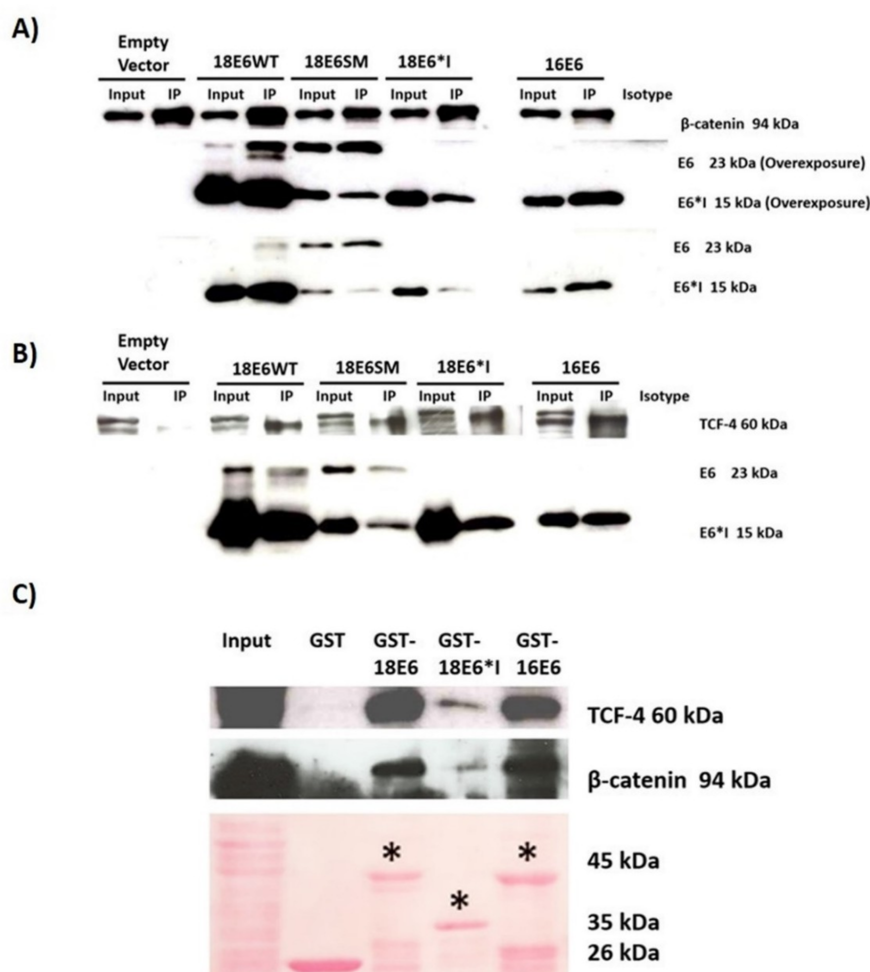

Figure 4. E6 and E6*I proteins interact with β-catenin and the transcriptional factor, TCF-4, in vivo and in vitro. C33A cells were transfected with different E6 expressing vectors, and 48 h post-transfection, protein lysates were obtained. (**A**) β-catenin and (**B**) TCF-4 were immunoprecipitated with the appropriate antibodies. The immuno-complexes were analyzed by Western blot using anti-β-catenin and anti-TCF-4 antibodies to detect the immunoprecipitated protein, and with an anti-FLAG to detect E6 proteins. Image shows a representative experiment of three performed. For comparison, 10% of protein used for immunoprecipitation (input) and the precipitation with an irrelevant IgG antibody (isotype) are shown. An overexposure of E6 proteins in shown in panel A. (**C**) Purified GST-18E6, GST-18E6*I, and GST-16E6 recombinant proteins were incubated with C33A protein extracts, while GST purified protein was used as a control. Immunoblots were performed using anti-β-catenin and anti-TCF-4 antibodies. 10% of protein extract was used as input. Lower panel shows Ponceau S red staining of a representative nitrocellulose membrane. Asterisks (*) show the E6 recombinant proteins.

These results were further confirmed by GST pull down assays using C33A lysates and GST-E6 fusion proteins (Figure 4C), where recombinant proteins bound to β-catenin and TCF-4. These data suggest that the E6 proteins regulate the Wnt/β-catenin cell signaling pathway through the interaction with the TCF-4 activation complex.

2.4. E6 and E6*I from HPV-18 Increase TCF-4 Protein Stability

To further determine the effect of the interaction of HPV-18 E6 and E6*I with TCF-4, we evaluated the TCF-4 stability through half-life determination assay. C33A cells were transfected with E6 or E6*I expressing plasmids, and 48 h post-transfection, cells were treated with 200 µg/mL of cycloheximide and the TCF-4 degradation rate was evaluated at 0, 6, and 12 h post-treatment. Overtime, it was observed that TCF-4 protein levels decreased considerably in E6 non-transfected cells after 6 and 12 h (Figure 5A,B). Interestingly, TCF-4 protein levels were maintained in the presence of E6 and E6*I proteins after 6 h, reaching up to 4.37- to 7.25-fold, compared to cells transfected with the empty vector. Finally, TCF-4 protein levels were higher at 12 h in 18E6WT expressing cells compared to those transfected with the control vector. These data strongly suggest that the half-life of TCF-4 is elongated in E6 and E6*I expressing cells, and that such an effect could be explained through the E6/E6*I-TCF-4 interaction.

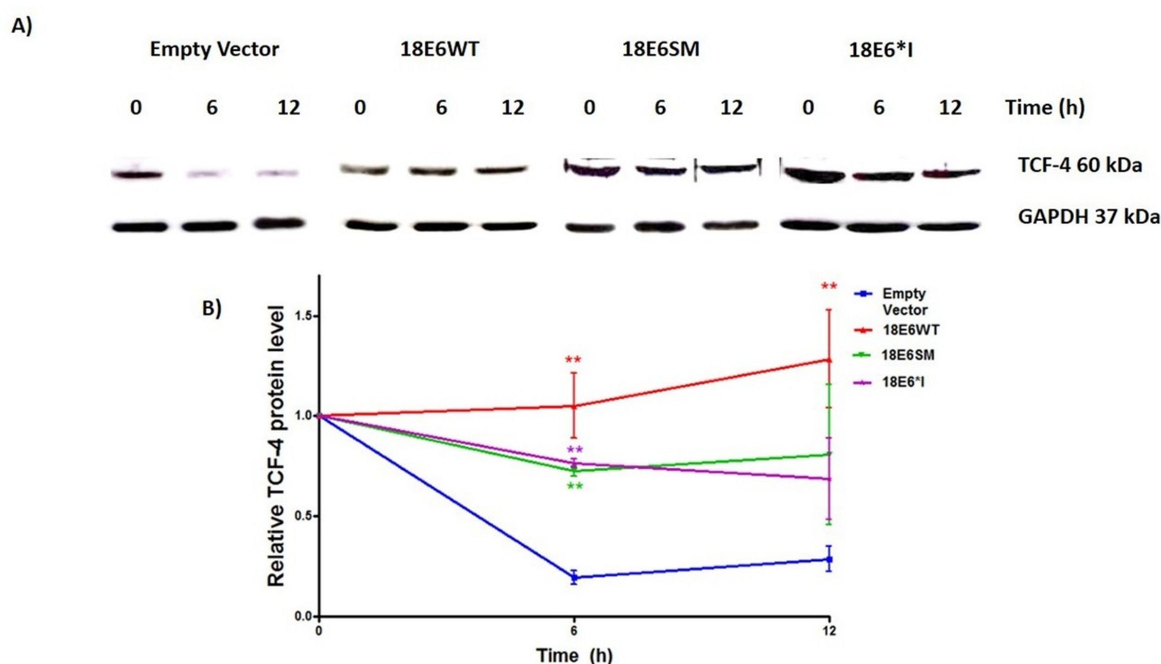

Figure 5. HPV-18 E6 proteins augment TCF-4 stability. C33A cells were transfected with E6 expressing plasmids. 48 h post-transfection, 200 µg/mL of cycloheximide was added to the culture medium. Protein extracts were obtained at 0, 6, and 12 h after treatment. (**A**) A representative immunoblot is shown with the different treatments. In non-E6 transfected cells, the TCF-4 levels were diminished at 6 and 12 h post-treatment, in contrast to E6 expressing cells, where TCF-4 levels remained without change at 6 and 12 h. (**B**) Graph showing the data as the mean and ± SD of three independent experiments. One-way ANOVA and a Tukey's post-hoc test, ** $p < 0.001$ versus empty vector values.

2.5. HPV-18 E6 and E6*I Increase Nuclear TCF-4 Protein Levels

To further determine the effect of E6 proteins on nuclear TCF-4 levels, soluble cellular fractionation was performed. As is shown in Figure 6A, full-length E6 is mainly located in the nucleus while E6*I is found in both the nucleus and cytoplasm. Interestingly, TCF-4 was significantly increased in the nucleus in both E6 and E6*I expressing cells (Figure 6A,B). This supports our data showing an increase in TCF-4 stability, which may lead to an enrichment of nuclear TCF-4.

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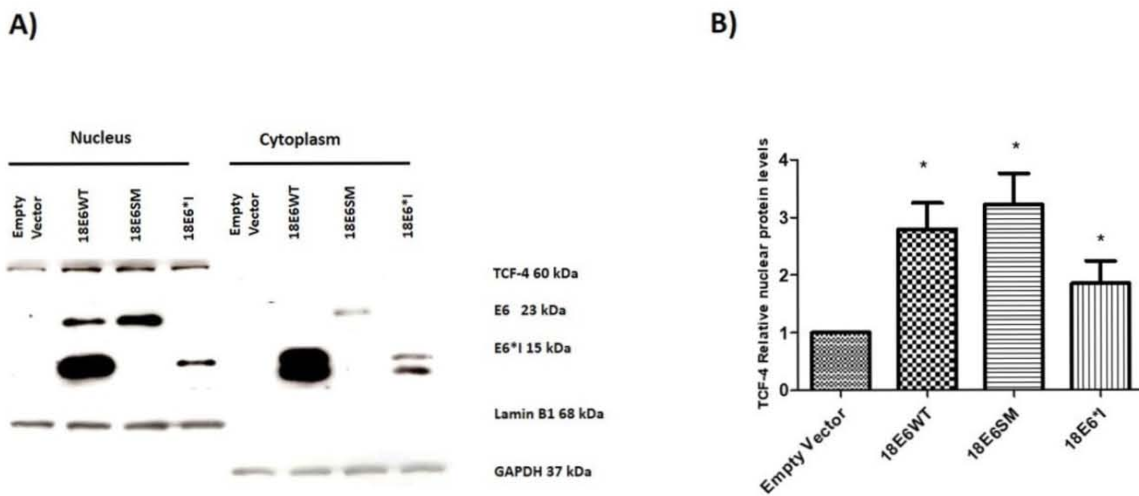

Figure 6. E6 proteins increase nuclear TCF-4 protein levels. (**A**) Representative immunoblot of TCF-4 and E6 proteins in nuclear and cytoplasmic soluble fractions of C33A cells transfected with E6 expressing plasmids. Lamin BI and GAPDH proteins were used as nuclear and cytoplasmic load controls, respectively. (**B**) A densitometric analysis of relative nuclear TCF-4 levels shows an increase of TCF-4 protein levels in the presence of the E6 proteins. Data from three independent experiments were collected and graphed showing the mean and ± SD. *t*-student analysis was performed, * $p < 0.05$ vs. empty vector values.

2.6. HPV-18 E6 and E6*I Proteins Bins to a TCF-4 Dependent Promoter In Vivo and In Vitro

The effect of E6 proteins in *Sp5* expression was analyzed by qPCR. Figure 7A demonstrates that E6 proteins increase *Sp5* mRNA levels in C33A cells co-transfected with β-catenin. Therefore, we further analyzed whether E6 proteins could interact with the *Sp5* promoter, which is TCF-4-dependent. It was previously demonstrated that TCF-4 binds to a conserved sequence, A-C/G-A/T-T-C-A-A-A-G, found in TCF-4/β-catenin-dependent promoters, such as the *Sp5* gene promoter [24,25]. We used a pair of primers to amplify a *Sp5* promoter sequence flanking the -326 to -129 nucleotides, since this region contains two TCF-4 binding sites located at nucleotides, -303 to -296, and -149 to -142. Those binding sites were confirmed when the fragment was analyzed in silico using the informatics tool, Tfsitescan [26]. Co-transfections of E6-HA-tagged and β-catenin plasmids were performed in C33A cells. Chromatin immunoprecipitation assay (ChIP) revealed that 18E6 binds to the *Sp5* promoter (Figure 7B). TCF-4 also bound to this promoter, either in cells with the empty vector or 18E6-HA transfected cells. It is worth mentioning that the binding of 18E6 to the *Sp5* promoter is overwhelming, since virtually no amplification is seen when the immunoprecipitation is carried out with anti-HA in cells transfected with the empty vector.

In order to confirm the obtained results, C33A cells were transfected with 18E6WT, 18E6SM, or 18E6*I expressing plasmids, and 48 h post-transfection, the DNA pull-down assay was performed. As expected, TCF-4 interacted with the *Sp5* promoter in all the tested samples (Figure 7C). Interestingly, E6 full-length as well as E6*I were also able to interact with the *Sp5* promoter. As a negative control, we used the p53 transcription factor, which has no response elements within the *Sp5* promoter. It is worth mentioning that even though p53 binds to E6 and E6*I, p53 was not found forming a complex in the *Sp5* fragment (Figure 7D). Taken together these results indicate indicates that E6 and E6*I proteins from HPV-18 interact with a TCF-4 dependent promoter, suggesting that such binding could be performed through TCF-4/E6/E6*I interactions, which may allow the up-regulation of the Wnt/β-catenin signaling pathway (Figure 7D).

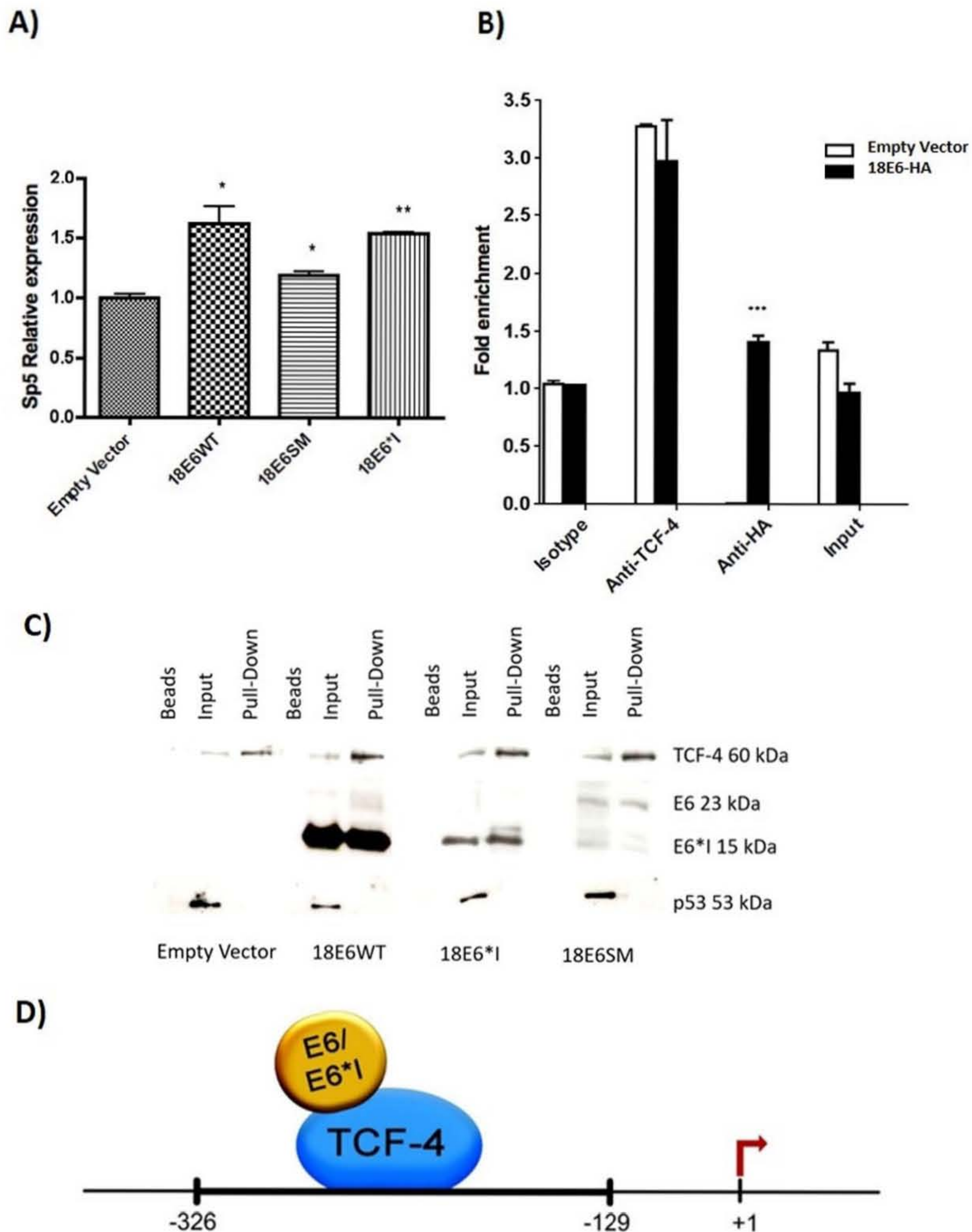

Figure 7. E6 and E6*I of HPV-18 interact with the *Sp5* promoter. C33A cells were co-transfected with β-catenin and E6 expressing plasmids as indicated: (**A**) E6 proteins increase *Sp5* relative expression as shown by qPCR analysis; * $p < 0.05$, ** $p < 0.01$, compared to the empty vector; (**B**) Chromatin immunoprecipitation assay (ChIP) shows that 18E6 binds to the *Sp5* promoter in vivo. Anti-HA antibody was used to detect E6-HA tagged protein, and anti-TCF-4 and anti-IgG antibodies were used as positive and isotype controls, respectively. 10% of input was analyzed. *** $p < 0.001$, of E6-HA compared to the empty vector. (**C**) C33A cells were transfected with 18E6WT, 18E6SM, or 18E6*I expressing plasmids, and 48 h post-transfection, DNA pull-down was performed. As expected, in all the samples, TCF-4 interacted with the *Sp5* promoter probe; interestingly, both E6 full-length and E6*I form a complex with the *Sp5* promoter. The p53 transcription factor was used as a negative control since it does not bind to the *Sp5* promoter. (**D**) Scheme showing the suggested interactions of E6 and E6*I of HPV-18 with the TCF-4 dependent promoter, proposing a possible mechanism of Wnt cell signaling regulation by E6 proteins.

*2.7. HPV-18 E6 and E6*I Proteins Induce Cell Proliferation in Cooperation with β-Catenin Overexpression*

2.7. HPV-18 E6 and E6*I Proteins Induce Cell Proliferation in Cooperation with β-Catenin Overexpression

Finally, to determine the contribution of E6 proteins in the Wnt/β catenin signaling pathway, MTS and crystal violet proliferation assays were performed in C33A cells co-transfected with β-catenin. As shown in Figure 8A,B, when E6 proteins were transfected alone, there was an increase in proliferation of between 70 and 85% in MTS assays, while crystal violet assays showed an increase of only 20–30% in relation to the empty vector. Additionally, transfection of β-catenin alone showed an increase in proliferation of 97% in MTS assays and 51% in crystal violet assays. Furthermore, when both E6 and E6*I and β-catenin were co-transfected, there was a further increase in proliferation of 40–50% in MTS assays, as compared to β-catenin alone, while the increase found using crystal violet was of 15–30%. This suggests that E6 proteins cooperate with β-catenin to promote the proliferation of these cells.

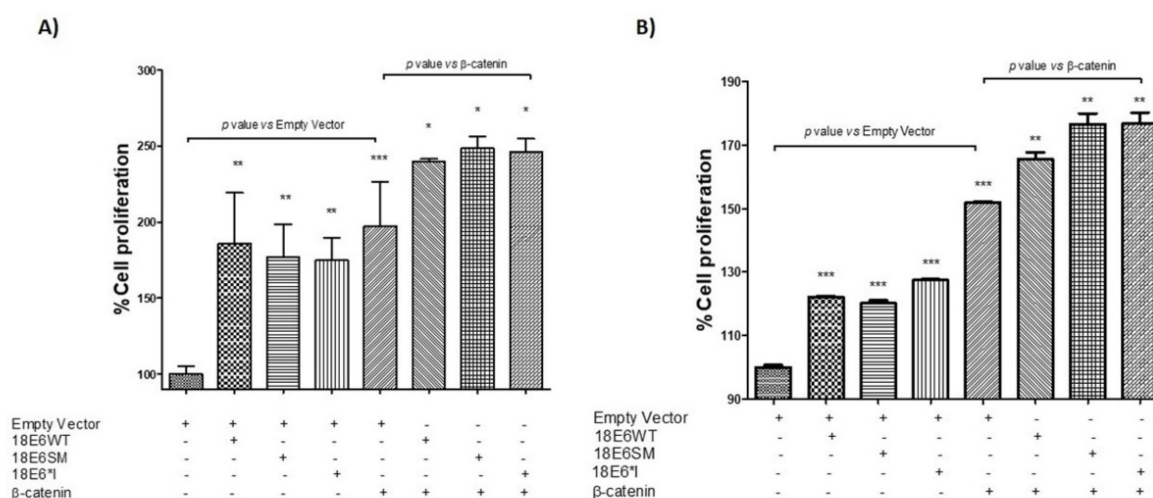

Figure 8. HPV-18 E6 and E6*I alone or in combination with β-catenin increase cell proliferation. C33A cells were transfected with the indicated plasmids, and 24 h post-transfection were seeded into a 96 well plate. Then, experiments were assessed after 72 h either by (**A**) MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) or (**B**) Crystal violet assays. Data from three independent experiments were collected and graphed showing the mean and ± SD. *t* student analysis was performed, * $p < 0.05$, ** $p < 0.001$ and *** $p < 0.0001$ vs. empty vector values.

2.8. HPV-18 E6 Variants Differentially Modulate TCF-4-Mediated Transcription

It has been proposed that HPV variants of the same type may present distinct biological behaviors conferring different pathogenic risks [17,27,28]. To determine whether HPV-18 E6 variations differentially induce TCF-4 transcriptional activity, we tested the E6Af variant belonging to the African phylogenetic branch and harbors genomic variations that lead to amino acidic changes, compared to the reference variant, E6AsAi, that in this study, is also shown as 18E6WT [17]. HPV-18 E6Af and E6AsAi expressing plasmids were transfected in C33A cells and co-transfected with the TCF-4-dependent luciferase reporter plasmid (TOPFLASH), β-galactosidase reporter, and, in some cases, with β-catenin plasmids, as indicated. Protein expression of E6 variants was evaluated by immunoblot as shown in Figure 9A. E6Af was able to augment TCF-4 transcriptional activity up to 2.8-fold, higher than the 1.5-fold induction observed for E6AsAi. These effects were also evident when β-catenin was added, where E6Af reached up to 2.25-fold induction, while E6AsAi showed a 1.6-fold induction above exogenous β-catenin. These results show that HPV-18 E6 variants differentially induce TCF-4 transcriptional activation (Figure 9B). However, when the levels of Wnt target native genes were analyzed by qPCR, both E6 variants were able to enhance the expression of *Axin2* and *Cyclin D1*, with no significant differences among them, as shown in (Figure 9C,D).

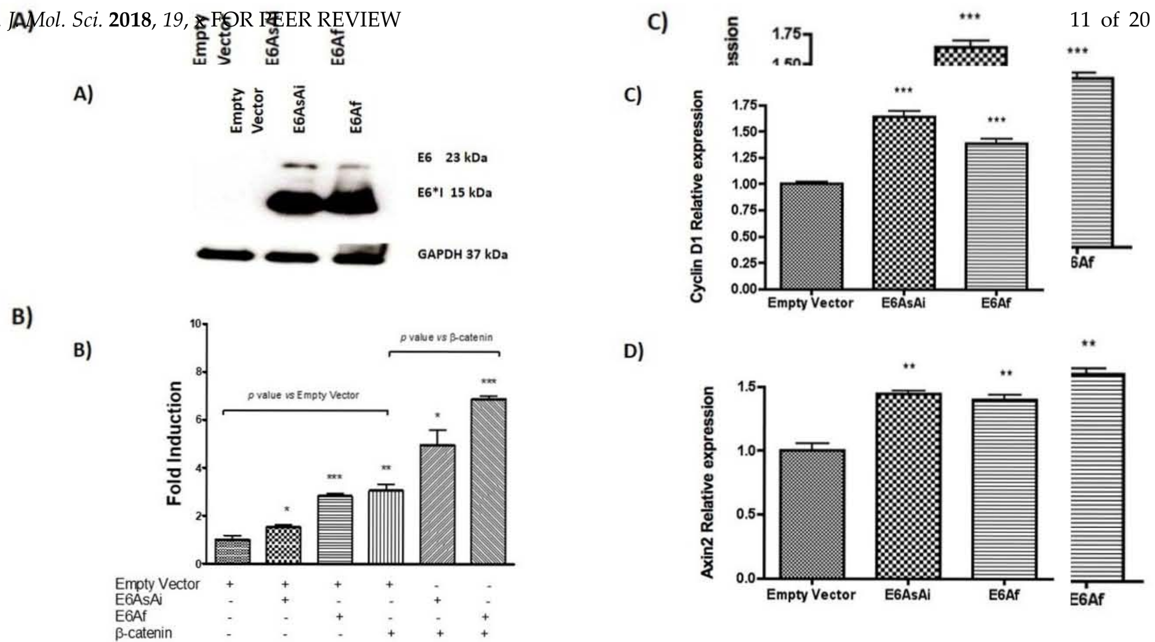

Figure 9. HPV-18 E6AsAi and E6Af variants modulate TCF4 transcriptional activity. (**A**) HPV-18 E6AsAi and E6Af protein expression in C33A transfected cells. (**B**) C33A cells were transfected with E6AsAi, E6Af, alone or combined with β-catenin expressing plasmids, and co-transfected with TCF-4 transcriptional reporter plasmid (TOPFLASH) and β-galactosidase reporter vector as indicated. (**C**) *Cyclin D1* and (**D**) *Axin2* gene expression was analyzed by qPCR. The means and ±SD of three independent experiments are depicted in each graph. Student t test was performed to evaluate the significant differences, the values are represented as * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$.

2.9. HPV-18 Intratype Variants Interact with β-Catenin and TCF-4

In order to demonstrate the interaction of β-catenin and TCF-4 proteins with E6AsAi or E6Af variants, immunoprecipitation assays were done. C33A cells were transfected with E6AsAi and E6Af expressing plasmids, and 48 h post-transfection cells were lysed and incubated with anti-TCF-4 and anti-β-catenin specific antibodies. Immunoblot analysis revealed that both E6 variants were able to interact with both β-catenin (Figure 10A,B) and TCF-4 (Figure 10C,D), respectively. Therefore, although the tested E6 variants showed a differential effect in other cellular pathways [29], no changes were observed in the ability to bind TCF-4/β-catenin nor in the levels of the regulated genes.

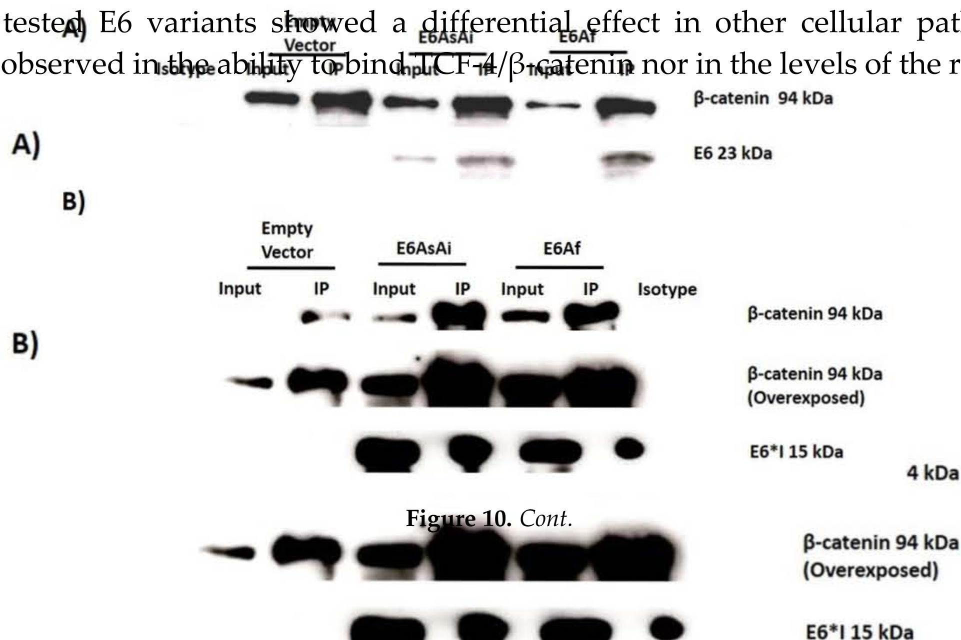

Figure 10. *Cont.*

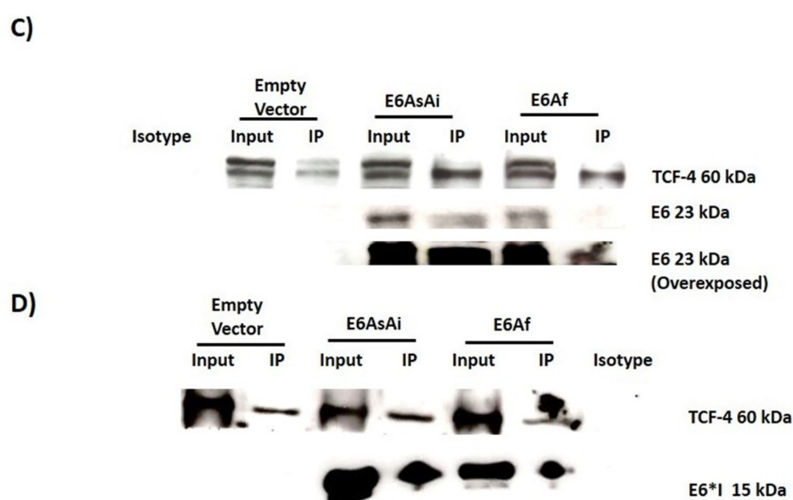

Figure 10. HPV-18 E6 variants interact in vivo with β-catenin and TCF-4. C33A cells were transfected with the E6 variant plasmids. 48 h post-transfection, cell lysates were collected and immunoprecipitated with anti-β-catenin (**A**,**B**) and anti-TCF-4 (**C**,**D**). Immunoblots show an interaction of E6 (**A**,**C**) and E6*I (**B**,**D**) with both proteins. Overexposures of β-catenin and E6 proteins are shown in panel B and C, respectively. Representative images are shown from three experiments performed. 10% of protein used for immunoprecipitation is indicated as input, and an irrelevant antibody was used (isotype).

3. Discussion

The continuous expression of the E6 and E7 oncoproteins is necessary for cellular transformation and immortalization in HPV-related cancers. E6 oncoprotein contributes to malignant progression through targeting a set of cellular proteins [30]. A common feature of E6-E7 mRNA from HR-HPV is to produce small isoforms termed E6*, whose cellular functions are poorly understood [18]. It is proposed that HPV-18 E6*I antagonizes the effects of full-length E6 [31], while some studies show that E6* has proper E6-independent functions [32–35]. E6* interacts with E6 and E6AP [31], and also downregulates PDZ domain containing proteins, such as hDlg, Scrib, MAGI-1, and MAGI-2 [32]. It has also been demonstrated that E6* modulates apoptosis related-proteins, since it binds to procaspase 8, affecting its stability [36]. Our research group previously showed that E6* induces the activation and nuclear translocation of the procaspase 8, without inducing cell death [33]. These findings suggest that E6* isoforms may, in a way that has not yet been described, cooperate with E6 in the malignant progression.

An aberrant activation of the Wnt cell signaling pathway has been described during cervical carcinogenesis [9,20,37] and a role of HPV-16 E6 in this activation has been proposed in cellular models [8,21,22,38]. Nevertheless, the effect of HPV-18 E6 and E6*I in this pathway has not yet been analyzed.

In the present study, we demonstrate that E6*I hyperactivates the Wnt/β-catenin pathway. HPV-18 E6*I by itself enhanced the TCF-4 transcriptional activity in C33A-transfected cells in 1.5-fold compared to the control, and a similar effect was observed when 18E6WT was expressed. This suggests that E6*I cooperates with E6 full length in the TCF-4 response. In an attempt to increase the expression of E6 over E6*I, we used an E6 expressing plasmid whose expression is enriched with full-length E6 (18E6SM). The mutation in the 18E6SM plasmid allows the expression of higher amounts of E6 full-length, although some E6*I is still produced since another donor site not yet described could be used during the splicing process. When transfecting this plasmid there was a slight increment in the TCF-4 transcriptional regulation, although non-significant, in relation to the control vector.

Interestingly, when the Wnt pathway was over activated with the co-transfection of β-catenin, TCF-4 transcriptional activation was enhanced in the presence of E6 or E6*I proteins, supporting that E6*I and E6 collaborate in Wnt/β-catenin pathway activation. We also found that 16E6 induced a

higher response in TCF-4 activation, reaching up to a 2.5-fold induction, and this effect was slightly enhanced in the presence of exogenous β-catenin (3-fold). Our results are in agreement with previous reports, where three-fold-induction of the TCF-4 luciferase reporter was obtained in HEK293T cells ectopically expressing HPV-16 E6, the Wnt receptor HFz1, and the Wnt3a ligand [21]. Additionally, Bonilla-Delgado et al. (2012) [23] reported a 50-fold induction of the TCF-4-dependent response in COS-7 cells where HPV-16 E6 in combination with Dvl-2 and β-catenin were expressed. These data suggest that the cellular context and/or the method used for Wnt pathway activation play an important role in the effect induced by HPV E6.

The expression of TCF-4 target genes was evaluated in the presence of E6 and E6*I. In HPV-18, E6WT and E6SM expressing cells increased *Cyclin D1*, *Axin2*, and *Sp5* expression, in agreement with a previous report where E6 from HPV-16 was able to activate the Cyclin D1 promoter [21]. Moreover, in a transgenic mice model expressing HPV-16 E6, *Cyclin D1* was also up-regulated [23].

Among the Wnt target genes, *Axin2* is considered a tumor suppressor and mutations within this gene are associated to cancer development [39,40]. Axin2 is involved in a Wnt negative feedback loop that may limit the duration intensity or the spread of Wnt signaling [41,42]. It is interesting that even though E6*I increased *Cyclin D1* expression, it failed in promoting an up-regulation of *Axin2*. It is possible that E6 recruits co-regulators that are distinct to those recruited by E6*I that could be necessary for *Axin2* expression.

It has been previously demonstrated that HPV-16 E6 induces TCF-4 transcriptional activation without affecting β-catenin localization [21]. Consistent with this result, our findings revealed that although E6 and E6*I increase β-catenin and TCF-4 protein levels, they do not alter their subcellular localization. Moreover, our results demonstrate that both E6 and E6*I are able to complex with β-catenin and TCF-4 in vivo and in vitro, which are the main proteins involved in the TCF-4-dependent transcriptional activation.

We demonstrate that E6 and E6*I from HPV-18 not only interact with TCF-4, but are also able to induce the TCF-4 stabilization. Further studies are needed to elucidate whether the interaction of TCF-4 with E6 proteins is responsible for TCF-4 stabilization. Previous studies have shown that HPV-16 E6 together with the E3 ubiquitin ligase E6AP, and induce stabilization of other members of the canonical Wnt pathway, such as β-catenin, impacting in the activation of the pathway [22].

Furthermore, it is well known that when Wnt signaling is activated, β-catenin complexes with TCF-4 in the nucleus, inducing the TCF-4 transcriptional response [43]. Our findings revealed that both E6 and E6*I increased nuclear TCF-4 protein levels, which may directly impact in TCF-4 transcriptional activation. Remarkably, the DNA pull-down as well as the ChIP results revealed that both E6 and E6*I interact with a TCF-4 dependent promoter. This interaction could be explained through complexes formed by E6/E6*I and TCF-4 that recognize specific sequences located at the *Sp5* promoter or other TCF-4 response promoters. Additionally, in concordance with previous studies [44,45], we found an enhancement in proliferation in E6 expressing cells. Interestingly, proliferation enhanced by β-catenin was increased when E6 and E6*I were co-transfected. Therefore, our findings support a new mechanism by which E6 and also E6*I could modulate the Wnt/β-catenin pathway.

Since HPV intratype variations have been proposed to affect the HPV biological behavior, we were also interested in determining if HPV-18 E6 variants could differentially affect the Wnt/β-catenin signaling pathway. HPV intratype variants are defined as those containing less than 1% of nucleotide changes in coding regions [46,47]. Our group has previously reported that HPV-18 variants exhibit differences in E6 full-length/E6*I transcript proportions, impacting on p53 levels [17]. Moreover, E6 variants differentially modulate the Akt/PI3K signaling pathway [29]. Previous studies demonstrated that E6 variants from HPV-16 exert different abilities in the activation of the Wnt/β-catenin signaling pathway [48]. Luciferase assays described herein showed that HPV-18 E6 variants have a different ability to augment TCF-4 dependent transcription, showing that E6Af promoted at least a 2.8-fold induction of the reporter gene transcription compared with 1.5-fold of E6AsAi. In addition, when E6 variants where co-transfected with β-catenin, E6-Af and E6AsAi reached

up to a 2.25-fold and a 1.6- fold TCF-4 induction, respectively, compared to that obtained with β-catenin exogenous expression. Nevertheless, even though E6 variants displayed different levels of TCF-4 activation, both E6Af and E6AsAi enhanced the expression of *Axin2* and *Cyclin D1*, with no differences among them. This effect could be due to a distinct capacity of E6 variants to regulate or interact with other untested proteins involved in the activation of the Wnt signaling pathway. However, we observed that both E6 variants interact with TCF-4 and β-catenin in a similar manner, which reveals that aminoacidic changes in E6 variants do not influence at least in such a binding capacity.

In this study, we demonstrated that not only E6, but also E6*I from HPV-18 are able to up-regulate Wnt/β-catenin signaling, involving their interaction with the TCF-4 activation complex. Additional effects on members of the Wnt pathway should be analyzed in order to determine the specific contribution of E6 and the spliced isoform E6*I in cell transformation induced by the Wnt/β-catenin pathway.

4. Materials and Methods

4.1. Cell Culture and Transfection

C33A epithelial cells were acquired from ATCC and HaCaT were kindly provided by A. García-Carrancá (Instituto Nacional de Cancerología, Mexico City, Mexico) and were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% of fetal bovine serum (FBS) in a humidified incubator with 5% CO_2. Transfections were performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

4.2. Plasmids

18E6WT Open Reading Frame (ORF) was obtained and PCR-amplified from an HPV-18 positive cervical cancer biopsy, 18E6*I ORF was obtained by RT-PCR amplification from HeLa cells (HPV-18 positive), and 16E6 ORF was amplified by PCR from CaSki cells (HPV-16 positive). The HPV-18 E6 spliced mutant (18E6SM) sequence was amplified from the plasmid, pCAHPV18-E6sm [32]. 18E6SM harbors a mutation at the donor splicing site (G233A), favoring the expression of the E6 full-length. The HPV-18 E6Af variant (from African phylogenetic branch) and the E6AsAi variant (from Asian-Amerindian phylogenetic branch), which is the canonical reference variant, were PCR-amplified from DNA previously obtained from tumor biopsies [27]. All these fragments were purified and cloned into the p3x-FLAG CMV.10 expression vector (Sigma Aldrich, Sant Louis, MO, USA) Constructs were verified by DNA-sequencing. β-catenin, pCAHPV18-E6sm, and pGW1-18E6-HA (hemagglutinin-tagged) expressing plasmids were kindly provided by Lawrence Banks (ICGEB, Trieste, Italy). The TCF-4 reporter plasmid (TOPFLASH) containing two sets of 3 copies of TCF-4 binding sites upstream of Thymidine Kinase minimal promoter and luciferase ORF (Merck-Millipore, Burlington, MA, USA) was used to perform luciferase assays, and pCMV-β-galactosidase plasmid (Promega, Madison, WI, USA) was used to evaluate the efficiency of transfection.

4.3. Luciferase Reporter Activity Assays

C33A cells were seeded in a 24 well plate and transfected with a mix containing 50 ng of appropriate E6 expressing plasmid, 100 ng of TOPFLASH, and 1 ng of β-galactosidase reporter plasmid, either with 50 ng of empty vector or β-catenin, as indicated. Cell extracts were obtained 48 h post-transfection and assayed for luciferase and β-galactosidase activities (Tropix Inc, Bedford, MA, USA) using a Glomax 96-well plate luminometer (Promega, Madison, WI, USA) The presented data are shown as relative luciferase readouts comparing the E6 expressing cells vs the control vector, where luciferase readouts in empty vector condition were adjusted to 1 after normalizing with β-galactosidase activity. At least three independent experiments were performed, each by triplicate.

4.4. Quantitative Polymerase Chain Reaction (qPCR)

C33A cells were seeded in a 60 mm culture dish and transfected with 3 μg of each E6 plasmid. After 48 h post-transfection, cells were collected, and total RNA extraction was performed using the RNeasy mini kit (Qiagen, Hilden, Germany). The isolated RNA was treated with the DNAse Free DNA removal kit (Thermo Fisher Scientific, Waltham, MA, USA) and 400 μg of RNA was reverse-transcribed with random hexamers utilizing the GeneAmp RNA PCR Core Kit (Applied Biosystems, Foster City, CA, USA) For the *Cyclin D1* amplification, forward 5′-ACAAACAGATCATCCGCAAACAC-3′ and reverse 5′-TGTTGGGGCTCCTCAGGTTC-3′ primers were used. For *Sp5* amplification, forward 5′-TCGGACATAGGGACCCAGTT-3′ and reverse 5′-CTGACGGTGGGAACGGTTTA-3′. As a house keeping control, 18S mRNA was amplified with forward 5′-AACCCGTTGAACCCATT-3′ and reverse 5′-CCATCCAATCGGTAGTAGCG-3′ primers. SYBER select Master Mix (Applied Biosystems, Foster City, CA, USA) was utilized for qPCR reactions. For Axin2 amplification, Taqman probes were used (Applied Biosystems, Foster City, CA, USA): *Axin2* FAM (Hs00610344_m1) and 18S VIC (Hs99999901_s1) probes, with Taqman Gene Expression Master Mix for qPCR analysis (Applied Biosystems, Foster City, CA, USA). The results are presented as relative quantification using the $^{\Delta\Delta Ct}$ method.

4.5. Western Blotting

C33A cells were cultured in 60 mm dishes and transfected with 3 μg of the indicated plasmid. 48 h post-transfection, cells were lysed using 300 μL of RIPA buffer (100 mM Tris pH 8.0, 50 mM $NaCl_2$, 0.5% Nonidet P-40, and protease inhibitor cocktail (Roche, Basel, Switzerland)). 20 μg of cell protein extracts were analyzed by SDS-PAGE gels (10–12%) and transferred in a 0.22 μm nitrocellulose membrane (Bio-Rad). Membranes were blocked with 10% skimmed milk in TBS-0.1% Tween 20 per 1 h at room temperature, followed by incubation with the indicated primary antibody diluted 1:1000: anti-FLAG M2 (Sigma Aldrich, Sant Louis, MO, USA); anti-TCF-4 (Santa Cruz Biotechnologies, Dallas, TX, USA); anti-β-catenin (Santa Cruz Biotechnologies, Dallas, TX, USA). After washing three times with TBS-0.1% Tween 20, membranes were incubated with HPR-conjugated secondary anti-mouse antibody in a dilution 1:10000 (Santa Cruz, Biotechnologies, Dallas, TX, USA). Proteins were visualized utilizing the Immobilon Western (Millipore) according to the manufacturer′s instructions. Western blots were performed at least three times each to assure result reproducibility.

4.6. Immunoprecipitation Assay

After 48 h of transfection with the indicated plasmid, 400 μg of protein extracts were incubated with 1μg of anti-β-catenin (Santa Cruz Biotechnologies, Dallas, TX, USA), anti-TCF-4 (Santa Cruz Biotechnologies, Dallas, TX, USA) antibodies, or IgG isotype control (Santa Cruz Biotechnologies, Dallas, TX, USA) overnight at 4 °C. A total of 20 μL of protein G-agarose beads (Upstate) were added to each sample and incubated at 4 °C, for 3 h. Complexes were washed three times with PBS-0.1% NP-40, resuspended in Laemmli sample buffer, and submitted to immunoblot analysis with anti-FLAG M2 (Sigma Aldrich, Sant Louis, MO, USA), anti-β-catenin (Santa Cruz Biotechnologies, Dallas, TX, USA), and anti-TCF-4 antibodies (Santa Cruz Biotechnologies, Dallas, TX, USA).

4.7. Analysis of TCF-4 Stability

C33A cells were seeded in 60 mm dishes and transfected with 3 μg of the indicated plasmid. 48 h post-transfection, cells were treated with 200 μg/mL of cycloheximide (an inhibitor of protein biosynthesis) (Sigma Aldrich, Sant Louis, MO, USA). After 0, 6, and 12 h post-treatment protein extracts were isolated using 2× Laemmli sample buffer (Bio-Rad, Hercules, CA, USA). Western blot assays were carried out in order to analyze the TCF-4 protein stability.

4.8. Immunofluorescence Staining and Cell Imaging

C33A and HaCaT cells were seeded over slides in 6 well plates and transfected with the indicated plasmids. After 48 h post-transfection cells were fixed with 3.7% paraformaldehyde in PBS for 10 min and permeabilized with PBS-0.1% Triton X-100. Then, cells were incubated with anti-FLAG M2 (Sigma Aldrich, Sant Louis, MO, USA) and anti-β-catenin (Cell Signaling) or anti-TCF-4 (Santa Cruz Biotechnologies, Dallas, TX, USA) antibodies overnight at 4 °C, after blocking with a 0.3% BSA solution. Cells were washed extensively with PBS and later incubated with anti-rabbit or anti-mouse antibodies conjugated to Rhodamine or Alexa-488 (Invitrogen, Carlsbad, CA, USA), respectively. Slides were washed and mounted with Prolong Diamond Antifade Mounting (Molecular Probes, Eugene, OR, USA) and then analyzed with a confocal microscope (Zeiss LSM 710 DUO, Oberkochen, Germany), with lasers giving excitation lines at 488 and 594 nm. Around twenty fields were observed for each treatment and representative images were acquired. The data of three independent experiments were collected with a 63× objective oil immersion lens.

4.9. GST- Fusion Protein Purification

E6 coding sequences were cloned into the pGEX-2T (GE) expression plasmid and the identity of each plasmid was verified by DNA-sequencing. GST-fusion protein production was induced in DH5-α *E. coli* strain with 10 mM IPTG. After three hours of induction, proteins were purified by lysing the cells using 1% triton/PBS and separating the insoluble fraction by centrifugation. Supernatant was then incubated with glutathione sepharose beads (Sigma Aldrich, Sant Louis, MO, USA), washed several times, and then re-suspended in 1 mL of 1% triton/PBS and analyzed by SDS-PAGE. Similar amounts of GST-fusion proteins were incubated overnight with 40 μg of C33A cellular protein extract, beads were then washed several times, and bound protein was analyzed by western blot using anti-TCF-4 and β-catenin antibodies.

4.10. Soluble Cell Fractionation Assay

C33A cells were seeded into a 60 mm dish and transfected with 3 μg of the indicated plasmid. 48 h post-transfection, cells were pelleted and washed with PBS (Phosphate-Buffered Saline). Cells were resuspended in 300 μL of lysis buffer (10 mm Tris pH 6.5, 27 mM $Na_2S_2O_5$, 1% Triton X-100, 10 mM $MgCl_2$, 25 mM Sucrose, and protease inhibitor cocktail) and incubated for 10 min at 4 °C with gentle agitation. The samples were centrifuged and the supernatants were collected (Cytoplasmic fraction). The pellets were resuspended in extraction buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA pH 8.0, 0.1 mM EGTA pH 8.0, and protease inhibitor cocktail) and centrifuged through a 0.34 M sucrose gradient. Then, the pellets were resuspended in RIPA buffer (100 mM Tris pH 8.0, 50 mM $NaCl_2$, 0.5% Nonidet P-40, and protease inhibitor cocktail (Roche, Basel, Switzerland)) (Nuclear fraction). The samples were analysed by immunoblot using anti-TCF-4 (Santa Cruz Biotechnologies, Dallas, TX, USA), anti-Lamin B1 (Abcam, Cambridge, UK), anti-GAPDH (Santa Cruz Biotechnologies, Dallas, TX, USA), and anti-FLAG M2 (Sigma Aldrich, Sant Louis, MO, USA) antibodies.

4.11. DNA Pull-Down Assay

A fragment of the *Sp5* promoter was amplified using the biotin labelled forward primer, 5′Bio-GGGTCTCCAGGCGGCAAG3′, and reverse specific primer, 5′-AGCGAAAGCAAATCCTTTGAATCC-3′. The probe was purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. C33A cells were seeded and transfected with 10 μg of each plasmid as indicated. 48 h post-transfection cells were lysed using the HKMG buffer (10mM HEPES pH 7.9, 100 mM KCl, 5 mM $MgCl_2$,1 mM DTT, 1 mM Na_3VO_4, 10% glycerol, 0.5% NP-40, and protease inhibitor cocktail), incubated 20 min at 4 °C, and then passed through a 25-gauge needle attached to a 1 mL syringe for 20 times. The lysates were centrifuged, and the supernatant were collected. The protein extracts were pre-cleared with 60 μL of Streptavidin

agarose beads (Invitrogen, Carlsbad, CA, USA) during 30 min at 4 °C and then were centrifuged and the supernatants were collected. For each sample, a total amount of 4 µg of biotin probes and 2.5 µg of Poly dI-dC (Sigma Aldrich, Sant Louis, MO, USA) were added and incubated overnight. 60 µL of Streptavidin agarose beads were added to each sample and incubated during 30 min at 4 °C. The samples were centrifuged, and the supernatants were discarded. The obtained beads were washed five times with the HKMG buffer and finally resuspended with 2× Laemmli sample buffer (Bio-Rad, Hercules, CA, USA). Then, Western blot assays were carried out.

4.12. Chromatin Immunoprecipitation Assay

C33A cells were seeded in a 100 mm plates and co-transfected with 7 µg of each 18E6HA and β-catenin expressing plasmids. 48 h post-transfection, cells were cross-linked with 1% of formaldehyde and quenched with 0.125 M of glycine. Cell lysates were obtained using a Lysis Buffer (1% SDS, 10 mM EDTA pH 8, 50 mM Tris HCl pH 8, and protease inhibitor cocktail) and sonicated with a Bioruptor Pico (Diagenode, Denville, NJ, USA), obtaining DNA fragments ranging from 200 to 500 bp. A total of 20 µg of chromatin per sample was used and diluted 1:5 with a Dilution Buffer (1% Triton X-100, 150 mM NaCl, 2 mM EDTA pH 8, 20 mM Tris HCl pH 8, and protease inhibitor cocktail). Then, all samples were precleared with 50 µL of protein G agarose/Salmon Sperm DNA beads (Millipore) during 3 h at 4 °C and centrifuged. Supernatants were incubated using anti-HA (Cell Signaling), anti-TCF-4 (Abcam, Cambridge, UK), or anti-IgG (Santa Cruz Biotechnologies, Dallas, TX, USA) rabbit antibodies overnight at 4 °C. Further, 50 µL of protein G agarose/Salmon Sperm DNA (Millipore) was added and incubated during 3 h at 4 °C. Samples were centrifuged, and the beads were washed four times with Wash Buffer I (1% Triton X-100, 0.1% SDS, 150 mM NaCl, 2 mM EDTA pH 8, 20 mM Tris-HCl pH 8, and protease inhibitor cocktail) and once with Wash Buffer II (1% Triton X-100, 0.1% SDS, 500 mM NaCl, 2 mM EDTA pH 8, 20 mM Tris-HCl pH 8, and protease inhibitor cocktail). Immunoprecipitated complexes were eluted with the Elution Buffer (1% SDS, 100 mM $NaHCO_3$) and de-crosslinked with 200 mM NaCl for 5 h at 65 °C. All samples were treated with RNAse (200 µg) and Proteinase K (160 µg). DNA fragments were obtained using the phenol/chloroform protocol. Further, qPCR was performed to evaluated proteins interaction with *Sp5* promoter using specific primers: Forward 5′-GGGTCTCCAGGCGGCAAG-3′ and Reverse 5′-AGCGAAAGCAAATCCTTTGAATCC-3′. To analyze the data, the fold enrichment method was performed.

4.13. Proliferation Assays

C33A cells were seeded in a 60 mm dishes and transfected with 3 µg of E6 and β-catenin expressing plasmids, as indicated. After 24 h post-transfection, cells were harvested and seeded in a 96-well plate for 72 h. The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt) assays were performed using the CellTiter 96 Aqueous One Solution Cell Proliferation kit (Promega, Madison, WI, USA), according to the manufacturer's instructions.

For crystal violet assays, cells were fixed with 10% of formol/PBS for 30 min at room temperature while shaking. Cells were then stained with crystal violet/PBS for 15 min. After several washes, cells were treated with acetic acid/PBS and then measured at 490 nm. The data were graphed to determine the percentage of cell proliferation for each assay condition.

4.14. Statistical Analysis

Data showing the effects of HPV-18 E6 and E6*I and HPV-16 E6 on TCF-4 in the different assays are presented as mean ± SD. p was calculated by Student's t-test or ANOVA Tuckey's post-hoc analysis. Significance differences were accepted at $p \leq 0.05$, as indicated.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/19/10/3153/s1.

Author Contributions: J.O.M.-B., L.O.-N., L.J.C.-M. carried out the experiments and analyzed the data. A.L.-S. performed the confocal microscopy acquisition and analysis of the images. A.C.-P., J.M.-M. and C.G.-E. critically revised the manuscript and participated in data interpretation. M.L. conceived and designed the study, directed and wrote the manuscript.

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Review

Regulation of the Wnt/β-Catenin Signaling Pathway by Human Papillomavirus E6 and E7 Oncoproteins

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Abstract: Cell signaling pathways are the mechanisms by which cells transduce external stimuli, which control the transcription of genes, to regulate diverse biological effects. In cancer, distinct signaling pathways, such as the Wnt/β-catenin pathway, have been implicated in the deregulation of critical molecular processes that affect cell proliferation and differentiation. For example, changes in β-catenin localization have been identified in Human Papillomavirus (HPV)-related cancers as the lesion progresses. Specifically, β-catenin relocates from the membrane/cytoplasm to the nucleus, suggesting that this transcription regulator participates in cervical carcinogenesis. The E6 and E7 oncoproteins are responsible for the transforming activity of HPV, and some studies have implicated these viral oncoproteins in the regulation of the Wnt/β-catenin pathway. Nevertheless, new interactions of HPV oncoproteins with cellular proteins are emerging, and the study of the biological effects of such interactions will help to understand HPV-related carcinogenesis.

This review addresses the accumulated evidence of the involvement of the HPV E6 and E7 oncoproteins in the activation of the Wnt/β-catenin pathway.

Keywords: Wnt/β-catenin; HPV E6 and E7 oncoproteins; HPV-related cancers

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1. Introduction

Signaling pathways are the mechanisms by which cells decide their fate and communicate with other cells and their environment. The binding of ligands to cell receptors can activate protein cascades and consequently affect gene transcription levels. Via these complex processes, cells transform external stimuli into biochemical signals that control biological effects, such as proliferation, differentiation, and death.

Many signaling pathways have been identified as being deregulated in cancer. Consequently, numerous elements targeting these pathways have been proposed as therapeutic targets. Consistent alterations of some important pathways controlling cell proliferation and apoptosis, such as PI3K/Akt, ERK/MAPK, Notch, and Wnt/β-catenin, have been identified in different types of cancer. In particular, the activation of the Wnt signaling pathway has been implicated in osteosarcoma [1], hepatocellular carcinoma [2], colorectal cancer [3], and breast cancer [4]. More recently, this signaling pathway was also implicated in oral cavity, oropharyngeal [5], and cervical cancers [6,7].

Cervical cancer is the fourth most common cancer in women worldwide and is one of the leading causes of cancer death in women in developing countries [8]. Persistent infection with Human Papillomavirus (HPV) is a necessary factor for cervical cancer development [9]. HPV is also associated with other pathologies, such as head and neck [10] and anal cancers [11]. HPV types linked to cancer are those classified as high-risk HPV (HR-HPV), whose viral oncogenes interact and regulate the function of several cellular proteins.

This review addresses the participation of the Wnt/β-catenin signaling pathway in HPV-related cancers and the possible mechanisms by which HPV E6 and E7 oncoproteins induce the activation of this pathway.

2. Wnt/β-Catenin Cell Signaling Pathway

The Wnt signaling pathway is involved in development, proliferation [12], differentiation [13], adhesion [14], and cellular polarity [15]. The term Wnt, which was adopted in 1991, includes a family of genes that encode secretory glycoproteins. Wnt is an acronym of homologous wingless (wg) and Int-1, which had been described in the fly and mouse, respectively [16]. In 1982, Nusse and Varmus found that the mouse mammary tumor virus (MMTV) promotes mammary carcinogenesis in mice by inserting itself in a specific gene of the host genome [17]. They called this gene Int-1, and its nucleotide and amino acid sequences were obtained in 1984 [18]. Later, in 1987, the wingless gene in *Drosophila melanogaster* proved to be a homologue of Int-1 [19].

Currently, 11 receptors that are members of the Frizzled (Fz) family have been identified in humans. These receptors include Fz1 to Fz10 and Smo, as well as the two co-receptors LRP 5 and 6, and all

of these receptors are responsible for Wnt signaling activation. Moreover, 19 Wnt ligands have been described for these receptors: Wnt1, 2, 2b, 3, 3a, 4, 5a, 5b, 6, 7a, 7b, 8a, 8b, 9a, 9b, 10a, 10b, 11, and 16 [20].

At least three signal transduction pathways activated by Wnt ligands are known, namely the canonical Wnt/β-catenin pathway and two non-canonical pathways: the planar cell polarity pathway (Wnt/PCP) and the Wnt/Ca^{2+} pathway. Moreover, the activation of the different pathways is ligand-specific, and the primary ligands that activate the canonical pathway are Wnt1, 2 [21], 3, 3a [22], 7a [23], 8 [24], and 10b [25,26]. The activation of the non-canonical pathways is mediated by Wnt4 [27], 5a [28,29], and 11 [30] ligands. However, diverse Wnt ligands have been shown to elicit various effects when binding to the same Fz receptor [31].

The non-canonical Wnt/PCP, also known as the Wnt/JNK pathway, is important in various processes including wound healing [32], the correct development of the neural tube [33], motility, and the modulation of cellular morphology [34]. These events are all generated by the reorganization of the actin cytoskeleton. Some of the main proteins involved in the transduction of the extracellular signal generated by Wnt/PCP are vangl2, celsr1-3 [35], Dvl, JNK, PKC [36], Rac, and RhoA [37].

In the Wnt/Ca^{2+} pathway, secondary messengers, such as IP3 and DAG, liberate calcium ions from the endoplasmic reticulum [29] and subsequently activate CaMKII [38] and PKC [39].

The processes that are triggered by the activation of this non-canonical pathway include the following: the regulation of convergent extension movements [40], the reorganization of the actin cytoskeleton [41], the modulation of cell motility [42], and the contribution to the inflammatory response [43].

The Wnt canonical signaling pathway is the best understood Wnt signaling cascade. In the absence of Wnt ligands (OFF-STATE), β-catenin is mainly located at cellular junctions. Nevertheless, a small amount remains in the cytoplasm and binds to a complex responsible for the degradation of β-catenin via the proteasome. This degradation complex consists of the scaffold protein Axin which recruits essential elements during this process such as GSK3β [44], CK1 [45], APC [46], YAP/TAZ, and β-TrCP [47]. CK1 phosphorylates β-catenin at the Ser45 residue, whereas GSK3β phosphorylates this protein at the Ser33, Ser37, and Thr41 residues [48,49]. Moreover, APC impedes the β-catenin dephosphorylation mediated by PP2A phosphatase [50]. Subsequently, the YAP/TAZ complex recruits the E3 ubiquitin ligase β-TrCP, which recognizes Ser/Thr phosphorylation, to promote β-catenin ubiquitination and its subsequent proteosomal degradation [47,51] (Figure 1A).

As a consequence of the Wnt ligand binding to the Fz receptor and LPR5/6 co-receptor [52] (ON-STATE), β-catenin delocalizes, accumulating in the cytoplasm [22] and nucleus [53,54]. When the Fz receptor dimerizes with the LRP5/6 co-receptor, the intracellular motifs of the Fz receptor recruits Disheveled (Dvl) protein [55], whereas CK1 phosphorylates LPR5/6 to allow Axin binding [56,57], which results in the disassembly of the β-catenin destruction complex. This process permits the accumulation and translocation of β-catenin to the nucleus. Moreover, the binding of FOXM1, a member of the Forkhead box (Fox) transcription factor family, to β-catenin promotes its nuclear translocation [58]. In the nucleus, β-catenin binds to transcriptional factors members of the TCF/LEF family [53,59], inducing the dissociation of co-repressors, such as Groucho/TLE [60], which allows the interaction with co-activators including CREPT [61], FHL2, and CBP/p300 [62,63] and remodelers of chromatin such as Brg-1 [64] (Figure 1B). These interactions with β-catenin promote the expression

of diverse genes that regulate cellular polarity, proliferation, and differentiation, such as c-jun, c-myc, Cyclin D1, Axin-2, Tcf-1 [20], and β-catenin itself [65].

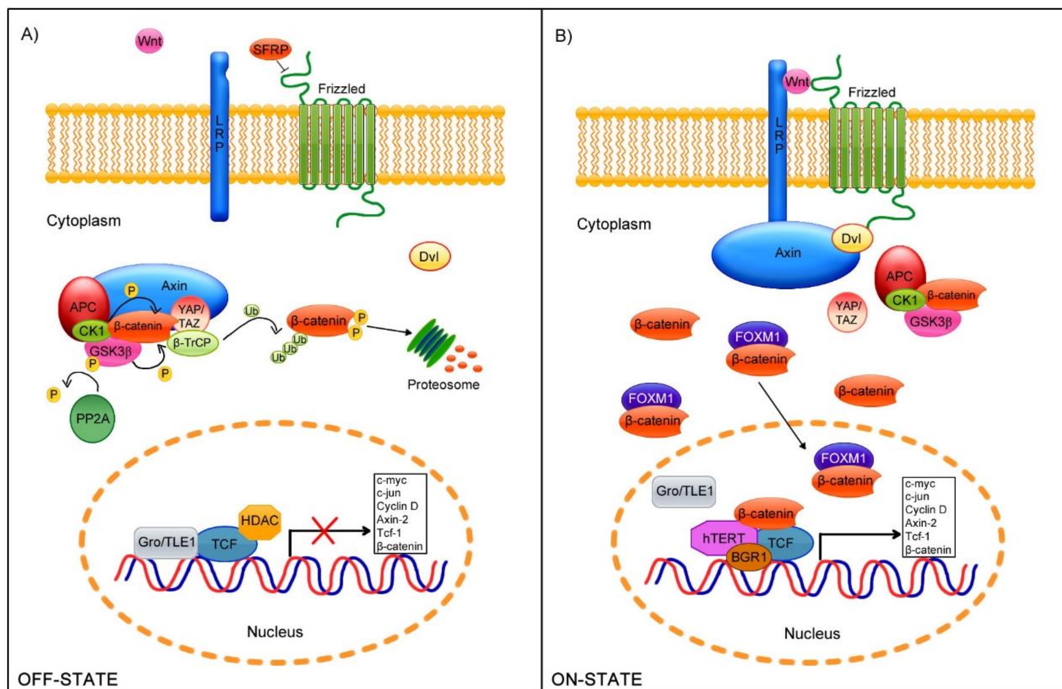

Figure 1. Wnt/β-catenin cell signaling pathway. (**A**) In the absence of stimuli (OFF-STATE), the Fz receptors are regulated by a group of antagonist proteins, such as SFRP, which prevent further receptor-ligand interaction. In the cytoplasm, a degradation complex is formed, to which β-catenin is recruited and phosphorylated at specific residues by the GSK3β and CK1 kinases. These phosphorylated sites are recognized by βTrCP ubiquitin ligase, which mediates β-catenin proteosomal degradation. In the nucleus, the Groucho/TLE repressor binds to TCF/LEF, avoiding its transcriptional activation; (**B**) In the presence of Wnt ligands (ON-STATE), LRP5/6 and Fz dimerize; subsequently, Axin binds to LRP5/6, whereas Disheveled (Dvl) interacts with Fz, allowing Axin-Dvl binding and the disassembly of the β-catenin degradation complex. Finally, β-catenin is released in the cytoplasm and translocated to the nucleus, aided by its binding partner FOXM1, where it binds to TCF/LEF and detaches the Groucho/TLE repressor.

3. Human Papillomavirus

Persistent infection with Human Papillomaviruses (HPVs) has been implicated in the carcinogenesis of the uterine cervix [9]. Oropharyngeal [10] and anal cancers have also been related to HPV [11]. In fact, almost 70% of cervical cancer cases are associated with HPV16 and HPV18 [66].

The carcinogenic potential of HPV is mainly due to the expression of E6 and E7 viral proteins, which are directly involved in cellular transformation [67,68]. The E6 and E7 oncoproteins interfere with cell cycle regulators and induce genomic instability, which results in a malignant phenotype.

More than 170 HPV types have been identified [69]. HPVs can infect the differentiating squamous epithelium and are classified in two main groups: cutaneotropic and mucosotropic. The majority of

cutaneous HPVs belong to the beta and gamma genus, whereas the alpha genus contains all known mucosal HPV types, and at least 40 members of this genus infect the anogenital region [70]. The mucosal HPVs are further divided according to the outcome of infection into low-risk HPVs (LR-HPVs), which are associated with benign and self-limiting benign warts, and high-risk HPVs (HR-HPVs), which are linked to pre-malignant lesions (low- and high-grade cervical intraepithelial neoplasia) and cancer. The most frequent HR-HPV types are: 16, 18, 58, 33, 45, 31, 52, 35, 59, 39, 51, and 56 [71].

Persistent HR-HPV infection is a crucial event in cellular transformation, but additional events are required to complete the malignant phenotype. Other mechanisms implicated in HPV-related cancers include the activation of multiple cellular pathways such as the Hedgehog [72], Erk/MAPK [73], Notch [74], and Wnt signaling pathways [75], which are involved in embryonic processes, differentiation, survival, proliferation, cell cycle progression, and self-renewal in stem cells.

HPV Genome

The HPV genome consists of a double-stranded circular DNA of approximately 8000 bp that contains genes that are expressed early (E) or late (L) during the viral life cycle and whose transcription and replication are mediated by the long control region (LCR) [76].

L1 and L2 are the HPV structural proteins [77,78]. Specifically, L1 is the major capsid protein and constitutes approximately 80% of the viral capsid [77].

E1 is the viral DNA helicase, and E2 a transcriptional activator and repressor that also complexes with E1 as a critical component of the HPV replisome [79]. E2 protein plays a crucial role in the HPV life cycle due to its ability to regulate viral DNA replication and the transcription of E6 and E7 oncogenes [80].

The E4 coding sequence is contained within the E2 open reading frame (ORF). Although E4 is located in the early region, it is expressed as a late gene and is regulated by a promoter that is responsive to differentiation transcription factors. Moreover, the properties of E4 have not been fully characterized, but several studies implicate E4 in virion release via its association with keratin filaments [81].

E6 and E7 are considered the most important viral oncoproteins: they play a clear role in cellular transformation [82]. Among several cellular interactions, E6 oncoprotein binds to the tumor suppressor protein p53 and to the E3-ubiquitin ligase E6AP, promoting p53 degradation via the proteasome and facilitating DNA damage and mutation [83]. Furthermore, E7 oncoprotein associates with a complex that contains Cullin 2, an E2 ubiquitin ligase, leading to the degradation of the tumor suppressor pRB, to promote cell cycle progression [84].

During the normal viral life cycle, the HPV genome exists in host cells as an episome. However, the viral genome may be incorporated into the host genome in rare cases. Viral genome integration is closely tied to the development of cancer because most HPV-induced cervical cancer cases contain an integrated form of the HPV genome. Viral episome rupture during integration frequently occurs in a zone that includes E1 and E2. The consequent loss of E2 causes the uncontrolled expression of the E6 and E7 oncoproteins, which increases the likelihood of HPV-induced carcinogenesis [85,86].

4. E6 and E7 in Cellular Transformation

E6 and E7 are small proteins that localize to the nucleus and cytoplasm and the interaction of both E6 and E7 immortalizes primary cells in a highly efficient manner [87].

Moreover, the expression of E6 and E7 in organotypic raft cultures results in cellular changes that are similar to those observed in high-grade squamous intraepithelial lesions [88]. Accordingly, transgenic mice expressing HR-HPV E6 and E7 developed basal epithelial squamous carcinomas upon low-dose estrogen treatment [89]. In this model, E7 alone is sufficient to induce high-grade cervical lesions and invasive cervical neoplasia; nevertheless, the inclusion of E6 resulted in larger and more extended tumors. These data demonstrate the cooperative effect of E6 and E7 in promoting the development of cancer [67]. Even when E6 and E7 can immortalize cells in culture, these cells do not form tumors in nude mice models in which the co-expression of supplementary oncogenes, such as v-ras [90] or v-fos [91], is required for tumorigenesis [92].

During HPV infection, E6 and E7 induce the proliferation of undifferentiated and differentiated suprabasal cells and also inhibit apoptosis. These actions promote the accumulation of DNA damage and mutations that can result in cell transformation and the development of cancer [92]. Table 1 summarizes the known E6 and E7 cellular targets and their biological consequences.

Table 1. Cell biological effects induced by HPV E6 and E7 oncoproteins via interactions with cellular elements.

E6-Interactions	Biological Effects
Protein PDZ-domain	Degradation of proteins harboring PDZ domains, with a loss of cell architecture and polarity [93].
E6AP	Degradation of targets such as p53 [83]. Activation of hTERT transcription, inducing immortalization [94].
Bak, FADD Procaspase 8	Induction of respective protein degradation, suppressing apoptosis [95,96].
BRCA1	Activation of estrogen receptor ER signaling pathway [97].
Tyk2	Impairment of Tyk2 activation thereby inhibiting IFN-induced signaling [98].
CBP/p300	Down-regulation of p53 activity by targeting the transcriptional coactivator CBP [99].
NFX1-91	Degradation of NFX1-91 and activation of hTERT [100].
c-Myc	Increased hTERT gene expression [101].
Dvl2	β-catenin stabilization and Wnt signaling activation [102].
E7-Interactions	**Biological effects**
pRb family proteins	Disruption of pRb-E2F complexes thereby initiating the E2F-mediated transcription [103].
AP1	Transactivation of members of AP1 family [104].
Cyclin A/CDK2	Regulation of cell cycle [105].
Cyclin E/CDK2	Regulation of cell cycle (binding through p107) [106].
p21	Inactivation of p21, modulating CDK and PCNA inhibitory functions [107].
MPP2	Enhancement of MPP2-specific transcriptional activity [108].
p600	Contribution to anchorage-independent growth and transformation [109].
Mi2	Complexes with HDAC to promote the E2F2-mediated transcription [110].
IRF1	Abrogation of transactivation function of IRF1 [111].
p48	Down-regulation of IFN α-mediated signal transduction [112].
p27	Abolishment of p27's cell cycle inhibitory function, which endows the cell with invasive properties [113].
PP2A	Inhibition of PP2A catalytic activity [114].

The E6 protein consists of approximately 150 amino acids and contains an LXLL motif in the amino terminal region that is required to interact with the ubiquitin ligase E6AP. Moreover, several proteins also bind to E6 via its LXLL motif, such as E6BP, IRF3, Tuberin, and Paxillin. Another critical E6 motif found in the carboxyl terminus is the S/TXV PBM (PDZ-binding motif), which mediates the interaction with specific domains on cellular proteins known as PDZ domains, specialized in protein-protein interactions [115]. E6 interactions with PDZ-containing proteins commonly induce their proteasome-mediated degradation [116]. PBM is present only in E6 of HR-HPV, suggesting a possible role for this motif in HPV-induced oncogenesis [117] (See Table 1).

The E7 protein consists of 98 amino acids separated in three conserved regions: CR1, CR2, and CR3. CR2 includes a conserved LXCXE motif that mediates high-affinity binding to pRB [118]. The CR3 region contains two CXXC motifs separated by 29 or 30 residues, forming a zinc-binding domain. This region is critical for interaction with cellular proteins, including pRB [119], p21 [107], p27 [120], TBP [121], and E2F [122] (See Table 1).

Growing evidence suggests that HPV oncoproteins can modulate cell signaling pathways to contribute to the carcinogenesis. Upon initial infection, this modulation may be necessary to complete the viral cell cycle and form infective viral particles. Nevertheless, the consistent high-level expression of viral oncoproteins may eventually alter the normal functions of the cell, triggering an uncontrolled transformation process. Via their different cellular interactions, E6 and E7 may be deregulating the different cell signaling pathways implicated in HPV-related cancers. Some of these pathways are involved in cell proliferation and apoptosis, such as PI3K/Akt, Ras/Raf, Notch, and Wnt/β-catenin [123].

5. Wnt/β-Catenin Signaling in HPV-Related Cancers

Several mutations in different components of the Wnt/β-catenin pathway have been described in various types of cancer [124]. In contrast, in HPV-related neoplasias, mutations in Wnt pathway members such as the CTNNB1 and AXIN1 genes are uncommon [125]. However, in cervical cancer biopsies and oropharyngeal squamous carcinoma cells, membrane β-catenin is lost, whereas cytoplasmic and nuclear β-catenin accumulation is observed during cancer progression [6,75]. Some studies have shown that LGR5, a member of the G protein-coupled receptor family, is progressively expressed in cervical neoplasia, promoting the proliferation and tumorigenesis of cervical cancer cells via the activation of the Wnt/β-catenin pathway [126]. Furthermore, post-transcriptional modifications have been identified in components that negatively regulate the pathway; for instance, in cervical cancer samples, GSK3β is inactivated by the phosphorylation of its Ser9 residue, inducing the over-activation of the Wnt signaling pathway [127].

Furthermore, patients with oral and lung cancers that express high levels of the β-catenin-binding partner FOXM1 exhibit worse overall and relapse-free survival than patients with tumors that express low levels of FOXM1; interestingly, this effect is significantly enhanced by the presence of HPV DNA sequences [128]. Therefore, alterations in Wnt cell signaling pathway regulatory elements are associated with cancer progression and poor prognosis in HPV-related cancers.

Epigenetic changes that suppress the activity of the negative regulator of the Wnt pathway have also been identified. Specifically, methylation markers in the APC and SFRP3 promoters have been found in ovarian cancer samples, but only in cases in which HR-HPV genomic sequences were detected [129].

Moreover, in cervical cancer samples, methylation markers have been found in the SRFP2 and DKK3 promoters [130].

Microarray expression studies of cervical cancer-derived tumors and cell lines have identified the over-expression of genes involved in Wnt pathway maintenance and regulation, such as JUN, MYC, FZD2, RAC1, GSK3β, Dvl-1, and CTNNB1 [131,132]. Specifically, Wnt/β-catenin elements are differentially expressed in HPV-positive cervical cancer cell lines (HeLa and SiHa) compared with a non-tumorigenic immortalized cell line (HaCaT) [133]. In this study, 38 genes were identified to be deregulated. Specifically, 15 genes were up-regulated (including CCND3, LRP5, TCF7, and FDZ9), and 23 gene were down-regulated (including CCND2, WNT10A, WNT7A, TCF3, WNT1, FZD4, and BTRC). Because these authors found that WNT7A expression was also significantly reduced in cervical cancer samples, they restored WNT7A expression in HeLa cells, which resulted in a strong decrease in cell viability, proliferation, and migration. In addition, aberrant hypermethylation in the CpG islands within the WNT7A promoter was found in HeLa and SiHa cells but not in HaCaT cells; this event suggests as a possible mechanism by which WNT7A is repressed.

Additionally, a systematic study in cervical cancer samples showed an alteration in the expression of miRNAs involved in Wnt/β-catenin pathway modulation [134]. In this study, miR-21-5p, miR-34c, and miR-96a were up-regulated, whereas miR-99b, miR-497-5p, and miR-617 were down-regulated. Although functional analysis was not performed, these expression patterns were hypothesized to modify the levels of their targets, *i.e.*, WNT5A, FZD1, FAS, MYC, FZD6, CCND1, and PDGFRA, to facilitate cell proliferation and invasion.

Clear evidence indicates that the Wnt pathway is hyperactivated in HPV-related cancers. Currently, HPV oncoproteins are known to bind and alter the function of several cellular targets associated with Wnt pathway regulation, including hTERT, p53, p300/CBP, Dvl, and PP2A (see Table 1), and information about the possible viral regulatory mechanisms in this pathway is emerging.

6. Wnt/β-Catenin Cell Signaling Regulation by E6 and E7 Oncoproteins

The activation of the canonical Wnt pathway represents a second requirement for the malignant transformation of the HPV-infected epithelium [75,135]. Specifically, several findings support the direct or indirect participation of HPV oncoproteins in this pathway.

In HPV-positive oropharyngeal cells, β-catenin expression is strongly localized in the cytoplasm and nucleus, whereas β-catenin is mainly detected in the membranes of HPV-negative cells [5].

In these HPV16-positive oropharyngeal cancer cell lines, E6 and E7 repression was shown to significantly decrease the β-catenin cytoplasmic and nuclear protein levels as well as the β-catenin mRNA levels. Moreover, both E6 and E7 expression were confirmed to up-regulate β-catenin expression and to enhance TCF-mediated transcription. This effect was attributed to a decrease in the ubiquitin ligase type 3 Siah-1 protein (seven in absentia homologue-1 protein), which acts as β-TrCP to induce β-catenin degradation. Because p53 mediates Siah-1 transcriptional activation [136], the down-regulation of p53 induces a decrease in the Siah-1 mRNA and protein levels in HPV-positive cells that are E6-mediated, avoiding β-catenin degradation. However, the activation of Wnt/β-catenin by the E7 oncoprotein is currently poorly understood [5].

An *in vitro* study showed that the HR and LR-HPV E6 proteins can distinctly augment the TCF response, with the highest activity observed for the HR-HPV E6 proteins [137]. In contrast to the previous research, E6 augmented the Wnt/β-catenin/TCF signaling response, although it did not significantly alter β-catenin stability and expression. This process did not depend on p53 degradation, the E6 PDZ-binding motif, APC/Axin/GSK3β complex activity, or β-catenin nuclear localization; instead, the presence of the E6/E6AP complex enhanced the TCF transcriptional activity mediated by the proteasome, independent of changes in β-catenin levels.

Subsequently, E6AP was confirmed to act as a novel Wnt signaling regulator that cooperates with E6 [138]. Specifically, the levels of E6 decrease in a proteasome-dependent manner in cells in which the Wnt pathway is activated; however, E6 is restored and stabilized in the presence of E6AP, suggesting that E6 requires E6AP to function in Wnt-activated cells. The participation of E6AP in the induction of the TCF response is independent of its catalytic activity. In contrast, β-catenin is stabilized by E6/E6AP, a process that requires the E6AP catalytic domain. Subsequently, β-catenin nuclear accumulation depends on its phosphorylation by GSK3β [138]. The mechanism by which E6/E6AP stabilizes β-catenin is not clear. To date, a direct interaction of E6 or E6AP with β-catenin has not been proven, but the E6/E6AP complex could alternatively participate in the sequestration of a negative regulator of the Wnt pathway.

Another element participating in the Wnt signaling pathway is FOXM1, which is also regulated by the HPV E6 oncogene. FOXM1 can induce β-catenin nuclear translocation by directly binding to β-catenin [58]. In cells harboring the HPV genome, E6 but not E7 oncoprotein was associated with FOXM1 overexpression [128]. This regulation is mediated by the MZF1/NKX2-1 transcriptional factors axis. E6 induces MZF1 expression, and MZF1 consequently activates NKX2-1 transcription. Because the FOXM1 promoter contains three putative sites for NKX2-1, E6 indirectly enhances FOXM1 transcription. In E6-expressing cells, the high levels of FOXM1 increase β-catenin translocation, which promotes TCF transcriptional activation and the expression of Wnt/β-catenin targets such as c-Myc and Cyclin D1 and stemness genes such as Nanog and Oct4. Thus, via the MZF1/NKX2-1 axis, E6 is responsible for metastasis, invasiveness, and stemness induced by the FOXM1-mediated activation of the Wnt/β-catenin pathway.

In vivo studies of transgenic mice support the role of the HPV E6 oncogene in the Wnt signaling pathway. In the K14E6 transgenic mice, the nuclear accumulation of β-catenin depends on the E6 PDZ-binding motif [102]. In this model, Wnt target genes (MYC, BIRC5, and CCND1) were up-regulated in the presence of full-length E6, but these genes were not up-regulated in mice expressing a truncated version of E6 lacking the PDZ-binding motif. Nevertheless, *in vitro* studies showed that both E6 forms enhanced TCF transcriptional activity due to the interaction of E6 with Dvl2, which is responsible for the disassembly of the β-catenin degradation complex [102]. These results suggest that the ability of E6 to activate the TCF response can be both dependent and independent of β-catenin translocation.

Other assays of double-transgenic mice expressing E7 and a constitutively active β-catenin indicated that the co-expression of both proteins promotes invasive cervical cancer, supporting that the activation of the Wnt/β-catenin pathway in premalignant lesions may be partly due to HPV [135].

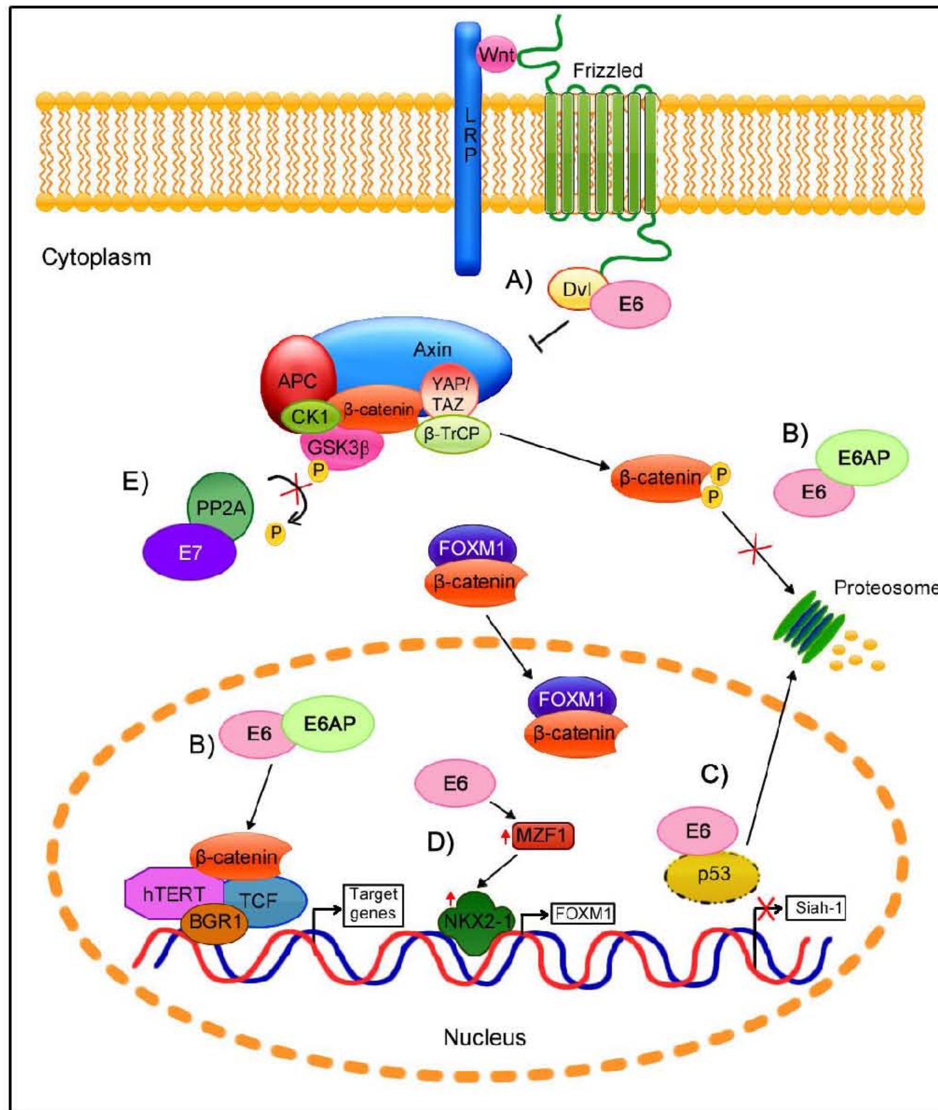

Figure 2. Participation of HPV oncogenes at different levels of Wnt/β-catenin cell signaling regulation. (**A**) The binding of E6-Dvl can disrupt the β-catenin degradation complex, releasing β-catenin which then accumulates in the cytoplasm; (**B**) The E6/E6AP complex stabilizes β-catenin, avoiding its proteasomal degradation and promoting its nuclear translocation, which results in an increase in TCF transcriptional activity; (**C**) E6-induced p53 degradation inhibits Siah-1 expression, which reduces β-catenin degradation; (**D**) E6 induces FOXM1 expression via the MZF1/NKX2-1 axis, which promotes FOXM1/β-catenin nuclear translocation and TCF transcriptional activation; (**E**) E7 binds to PP2A in the structural and catalytic domain, which may avoid the GSK3β activation and consequently β-catenin is stabilized.

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Although the role of the E7 oncoprotein in the regulation of Wnt signaling has not been studied as well as that of E6, some findings suggest that this protein is involved in this pathway. Specifically, PP2A participates as a negative regulator of Wnt signaling. This phosphatase induces GSK3β activation via its dephosphorylation at the Ser 9 residue, which results in β-catenin degradation [139]. In a model based on primary human foreskin keratinocytes immortalized with E6 and E7 oncoproteins and transformed

with SV40 small T antigen (smt), the smt antigen directly binds to the PP2A catalytic domain, preventing its activation to consequently induce Wnt signaling [75]. Moreover, cell line studies have demonstrated that the functions of E7 and smt are similar: they both strongly bind to the catalytic subunit of PP2A to inhibits its activity [114]. This role of E7 may contribute to β-catenin stabilization in the cytoplasm.

Notably, the above-described findings strongly support a role for HPV oncoproteins in the Wnt canonical pathway. Nevertheless, HPV has not been conclusively linked to the Wnt non-canonical pathway regulation, although some E6 targets, such as WNT7A and Dvl, are known to participate in the activation of Wnt non-canonical pathways.

Evidence supporting the role of HPV in the modulation of the Wnt signaling pathway is shown in Figure 2, which depicts the possible contribution of HPV oncoproteins at different levels in the activation of Wnt signaling.

7. Conclusions

Persistent infection with high-risk HPV types is clearly a main factor in cervical cancer development, and such infections are also implicated in the development of other types of cancer. HPV infection and the activation of diverse cellular processes such as signaling pathways are required to induce a malignant phenotype. Moreover, the Wnt/β-catenin pathway is deregulated in various neoplasias, and has been implicated in HPV-related cancers.

Several studies support the role of HPV oncoproteins in the activation of the canonical Wnt/β-catenin pathway, which may be involved in the onset, progression and maintenance of transformed cells.

Deciphering the precise mechanisms by which HPV oncogenes participate in Wnt/β-catenin modulation will help to elucidate HPV-related carcinogenesis. This information could eventually aid in identifying biomarkers of prognosis and contribute to the design of more effective targeted therapeutics.

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Author Contributions

J.O.M.B., L.O.N., A.C.P., and A.M.F.G. performed the bibliographic review and wrote the manuscript; L.R.Z. reviewed the manuscript; and M.L. directed and wrote the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Review

The Role of E6 Spliced Isoforms (E6*) in Human Papillomavirus-Induced Carcinogenesis

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Abstract: Persistent infections with High Risk Human Papillomaviruses (HR-HPVs) are the main cause of cervical cancer development. The E6 and E7 oncoproteins of HR-HPVs are derived from a polycistronic pre-mRNA transcribed from an HPV early promoter. Through alternative splicing, this pre-mRNA produces a variety of E6 spliced transcripts termed E6*. In pre-malignant lesions and HPV-related cancers, different E6/E6* transcriptional patterns have been found, although they have not been clearly associated to cancer development. Moreover, there is a controversy about the participation of E6* proteins in cancer progression. This review addresses the regulation of E6 splicing and the different functions that have been found for E6* proteins, as well as their possible role in HPV-induced carcinogenesis.

Keywords: HPV; E6; splicing; E6*; spliceosome

1. Introduction

Cervical cancer continues to be a major public health problem, being the fourth cause of cancer mortality among women worldwide [1]. The persistent infection with High-Risk Human Papillomavirus (HR-HPV) is the main risk factor associated with cervical cancer development [2]. HPV sequences have been detected in almost 99% of the analyzed cervical cancer biopsies [3,4]. Moreover, HPV has also been linked to other anogenital [5,6] and oropharyngeal cancers [7].

Hitherto, more than 200 HPV types have been identified [8,9], which differ in more than 10% of nucleotide sequences within the L1 gene [10]. Commonly, HPVs infect basal layer cells of epithelia and are classified as cutaneous or mucosal types, being the infections with mucosal HPVs the most frequent sexually transmitted diseases worldwide [11]. From approximately 40 HPV types that infect the anogenital mucosal epithelium, 15 types are the most commonly found in cancer biopsies and thus, have been classified as HR-HPVs: HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82. Low-Risk HPV (LR-HPV) types are mainly related to mild dysplasia or genital warts [12]. HR-HPV16 and 18 are the most prevalent HR types, found in close to 60% and 15% of cervical cancer cases, respectively. LR-HPV6 and 11 are the most frequent types found in warts [13].

Human Papillomaviruses are small non-enveloped viruses of 55 nm, containing a circular double-stranded DNA of approximately 8 kb in length. The HPV genome is divided into three regions: the long control region (LCR) that regulates transcription and replication, the early region harboring nucleotide sequences of six common genes (E6, E7, E1, E2, E4 and E5) expressed in a primary

infection [14] and the late region that contains open reading frames (ORFs) encoding the L1 and L2 structural proteins involved in viral encapsidation [15].

The main viral oncoproteins E6 and E7 regulate cell cycle progression by promoting the degradation of the tumor suppressor proteins p53 and pRb, respectively. These and other interactions affect cellular pathways leading to malignant transformation [16,17].

Multiple HPV genes are expressed in a polycistronic pre-mRNA from a single strand. Depending on the state of differentiation of the epithelial layers, early or late promoters are activated leading the transcription of the early and late regions as polycistronic mRNAs. These transcripts are polyadenylated at sequences termed early and late polyadenylation sites, which are located downstream of each polycistronic mRNA [18,19].

Several transcripts are produced throughout an HPV infection by alternative splicing, which generates different mRNA expression patterns [20]. Alternative splicing within E6-E7 ORFs is a common feature of HR-HPVs, while no splicing in this region has been detected among LR-HPVs [21]. Full-length E6 from HR-HPV types is expressed from mRNAs with no splicing within E6 ORF, while E7 can be transcribed from different mRNAs including those with splicing in E6 [22,23]. The splicing process produces several transcripts containing E6 truncated mRNAs named E6*, which are derived from a donor splicing site within the E6 ORF and one of the different acceptor sites located in the early mRNA [24]. The most abundant E6 truncated mRNA is termed E6*I, which is a poorly studied protein. E6*I shares approximately the first 44 aa with the E6 full-length protein (E6) and the intron removal promotes a change in the E6 ORF adding approximately 13 aa that are only contained in E6*I isoform and generating a new stop codon [25].

This review focusses on the transcription patterns of E6/E6* and their regulation in different models, as well as the controversial roles of E6* proteins that affect cellular processes. The evidences and controversies represent an opportunity for the study of E6* proteins in order to establish their participation in the HPV life cycle and/or in the initiation or progression of cancer.

2. HPV Life Cycle

The HPV life cycle depends on differentiation and replication of the host-infected cells and is characterized by having two phases: latent infection, where the episome is replicated and maintained and productive infection, where the late proteins are produced and virions are formed [26].

Depending on HPV type, multiple entry pathways have been suggested. Generally, HPVs infect the undifferentiated basal cells of the epithelium through a micro-wound. Additionally, the accessibility of cells that are close to the squamo-columnar junction increases the possibility of HPV infection of this single cell layer [27]. The precise mechanism and receptors used by HPV to infect the epithelial cells are poorly known. The most accepted models for HPV16 suggest that the HPV L1 capsid protein attaches to heparan sulfate proteoglycans (HSPGs) [28] inducing conformational changes in the capsid and transferring the viral particle to a secondary non-HSPG entry receptor [29]. This transfer is facilitated through cleavage of the L2 protein by the convertase furin [30]. In contrast to the use of pseudovirus models, some analyses with native viruses have shown that the heparan sulfate receptor and furin cleavage activity are not required for all HPV types [31–33].

Several secondary L1-specific receptors have been proposed to mediate the infection, such as α-6 integrin [34], keratinocyte growth factor receptor (KGFR), epidermal growth factor receptor (EGFR) [35] and tetraspanins [36]. Finally, an L2-specific receptor, the S100A10 subunit of the annexin A2 heterotetramer, is thought to be involved in promoting viral entry [37].

After viral attachment to the host cell, the endocytic uptake of HPV implies a non-canonical internalization pathway related to micropinocytosis dependent on actin dynamics [38]; however, the precise cellular components mediating HPV uptake into host cells remain unknown.

Following virus entry, the viral capsid binds to Sortin nexin 17 at the endosomal compartments, which seems to help the L2-DNA complex to escape from the lysosome [39] and finally travel to the nucleus via dynein-mediated transport along microtubules [40].

In the latent phase, low levels of E1, E2, E6 and E7 are expressed in undifferentiated basal cells, where normal differentiation is retarded. During this phase, low replication rate occurs generating approximately 50–100 viral genomes per cell [41]. Further, in the proliferative phase, E6 and E7 are highly expressed from the middle to the upper layers of the differentiating epithelium [42]. The E2 protein recruits E1, a viral DNA helicase, to its binding site in the viral origin of replication, facilitating viral DNA replication and leading to the production of thousands of viral genome copies per cell in differentiated keratinocytes [26,43]. E4 stabilizes E2 and facilitates nuclear localization of E1, increasing E1/E2 dependent viral genome amplification [44]. Moreover, E2 acts as transcriptional factor controlling the expression of viral genes, through the recruitment of cellular factors to the LCR, promoting the activation or repression of viral transcription [43].

Finally, the viral life cycle is completed by the synthesis of L1 and L2 proteins in the uppermost layer of the epithelium, allowing the encapsidation of newly replicated genomes and the release of mature virions [27].

Most of the HPV infections are transient and cleared by the immune system in less than two years. Furthermore, when clinical lesions are generated, the majority undergo spontaneous regression [45]. It has been proposed that a determinant key to neoplastic progression is the persistent infection by HR-HPVs, which after a long time could lead to genomic instability and to viral genome integration into the host genome, at this stage, no viral progeny is produced [46]. As an episome, viral early gene expression is controlled by E2 but when integration occurs, E2 gene expression is commonly disrupted, leading to an increase in the expression of E6 and E7. The formation and maintenance of tumors needs the constant expression of E6 and E7 oncoproteins [47].

In cervical cancer biopsies, the HR-HPV genome is commonly found integrated, although in a small proportion of the cases HPV-DNA remains as an episome but at a high copy number [48]. It has been proposed that in HPV episomes, E2 binding sites contained in the LCR can be methylated preventing the E2 transcriptional repression and allowing the overexpression of E6 and E7 oncoproteins [49]. This indicates that HPV integration in some cases may not be a requirement for cellular transformation.

3. The Splicing Process

The splicing process is an essential mechanism that regulates gene expression and contributes to cell proteomic diversity. During transcription, RNA polymerase II generates a pre-mRNA that harbors exonic and intronic *cis* regulatory elements, able to recruit the spliceosome complex. The spliceosome regulates the exon-exon junction, generated when the intronic sequences are released, which is a crucial step in the maturation of the pre-mRNA [50,51]. The spliceosome is formed by a variety of small nuclear RNAs (U1, U2, U4/U6 and U5) organized in small nuclear ribonucleoproteins (snRPNs), complexed to several regulatory proteins [52,53]. The spliceosome complex assembly is directed by consensus sequences that flank exon-intron joints at the 5′ donor site ((C/A)AGGU(A/G)AGU) and 3′ acceptor site ((C/U)AG) of the pre-mRNA, in addition to intronic sequences termed branch points ((C/U)NC/U)U(A/G)A(C/U)) and a polypyrimidine tract [54]. Moreover, the pre-mRNA harbors auxiliary *cis*-acting elements termed exonic/intronic splicing enhancers (ESEs and ISEs, respectively) and exonic/intronic splicing silencers (ESSs and ISSs, respectively) that regulate splicing through the binding with regulatory proteins that stimulate or repress the spliceosome complex assembly [54].

Briefly, the U1 small nuclear ribonucleoprotein (snRNP) binds to the 5′ splice site, allowing the binding of the splicing factor 1/mammalian branch point binding protein (SF1/mBBP) to the branch point and the interaction of the U2 Auxiliary Factor (U2AF) with the polypyrimidine tract, forming the E complex which approaches the 5′ and 3′ splicing sites. Then, the U2 snRNP associates with the branch point and induces the displacement of SF1/mBBP, leading to the formation of the A complex. Later, the pre-assembled complex, U4/U6/U5 tri-snRNP, is recruited, generating the pre-catalytic B complex. In this step, all snRNPs are catalytically inactive and require other rearrangements to induce the first splicing reaction. Afterwards, U1 and U4 are removed from the B complex while U2, U5 and

U6 are rearranged, forming the active B complex. This complex is then catalytically activated by the DEAH (Asp-Glu-Ala-His)-box RNA helicase Prp2 (catalytically activated B complex). In this step, the phosphodiester bond at the 5′ splice site (exon-intron joint) is attacked and broken by the 2′-OH of the adenosine at the branch point, which creates a new bond between the 5′ side of the intron and the adenosine, forming the lariat structure. At this point the C complex is formed, which induces the catalysis of the second bond between the 3′-OH of the first exon and the 5′ acceptor site of the second exon (exon-exon joint). Finally, the intronic sequences are discarded, the exons come together and the spliceosome is disassembled [20,54,55] (Figure 1).

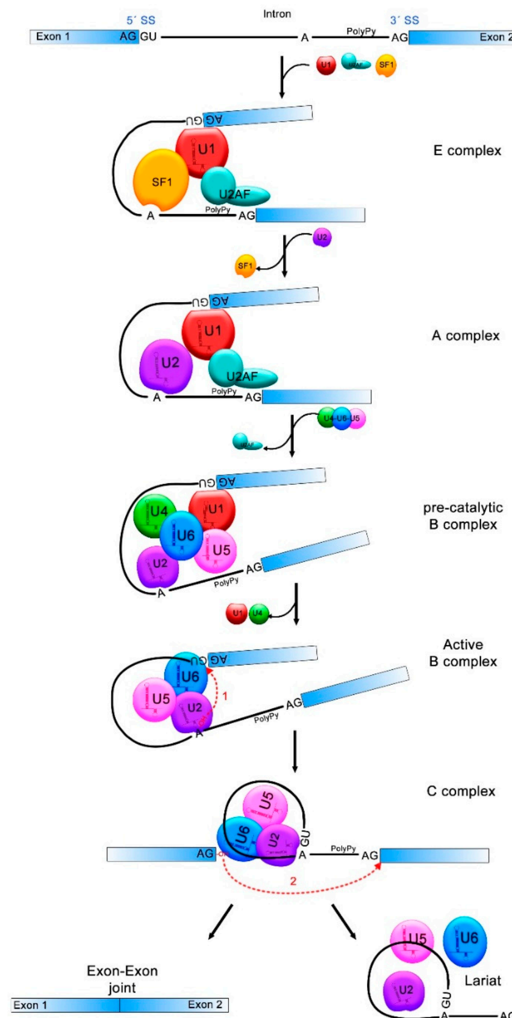

Figure 1. pre-mRNA splicing process. Donor and acceptor splicing sites (5′SS and 3′SS) in the exon-intron junctions, the branch point (A) and the polypyrimidine tract (PolyPy) are contained in the pre-mRNA. In the E complex, the U1 small nuclear ribonucleoprotein (snRNP) binds to the 5′SS, the splicing factor 1 (SF1) to the branch point and the U2 Auxiliary Factor (U2AF) to the PolyPy, approaching the 5′SS and 3′SS. In the A complex, U2 associates to the branch point and SF1 is disassembled. U4/U6/U5 complex binds and U2AF is released from the spliceosome in the pre-catalytic B complex. The active B complex is formed when U1 and U4 exit from the spliceosome and structure rearrangements induce the first splicing reaction where the phosphodiester bond at the 5′SS is attacked by the 2′-OH of the A forming a lariat structure. In the C complex, the second reaction forms a bond between the 3′-OH of the first exon and the 5′-P of the second exon. The intronic sequences are discarded and exons 1 and 2 come together. The transitions between one and other splicing complex are indicated with black solid arrows and the two splicing reactions are indicated with red dotted arrows.

Since the consensus sequences in splicing sites are not well conserved, the nucleotide combinations increase the possibility of multiple choices of splice sites within the pre-mRNAs, which leads to selective intron and exon removal, allowing the expression of a great variety of isoforms derived from a single pre-mRNA. This process is termed alternative splicing [20].

In addition, exonic and intronic splicing enhancers (ESEs and ISEs) and/or silencers (ESSs and ISSs) are required to regulate the splicing process: negatively, by the interaction with the heterogeneous ribonucleoproteins (hnRNPs) and positively, with serine/arginine-rich protein (SR). The hnRNPs (i.e., hnRNPA1 and hnRNPA2) bind mainly to the silencer elements, blocking the recognition of the exon-intron junctions by elements of the spliceosome. In contrast, the SR proteins (SRF1-12) usually bind to the enhancer sequences, acting as general activators of exon definition. The contribution of the SR and hnRNP proteins defines the overall recognition potential of an exon and/or the affinity for the spliceosome [20,52,56,57] (Figure 2A).

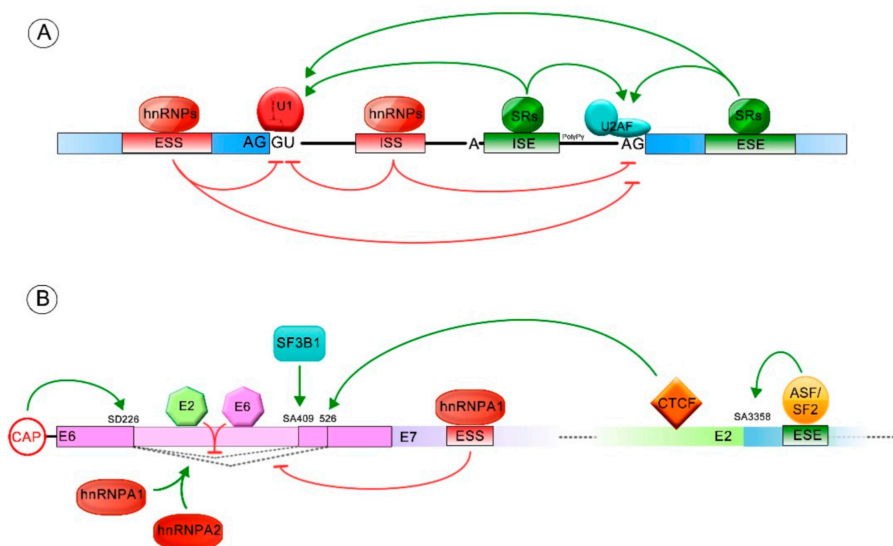

Figure 2. Splicing regulation. Green arrows indicate positive splicing regulation, while red arrows represent negative splicing regulation. (**A**) General regulation mediated by *cis* and/or *trans* elements is shown. The exonic and intronic splicing enhancers (ESE and ISE) frequently stimulate the splicing process by binding to serine/arginine-rich proteins (SR proteins). The exonic and intronic splicing silencers (ESS and ISS) commonly repress the splicing process, through binding with heterogeneous ribonucleoproteins (hnRNP) regulatory proteins; (**B**) Splicing regulated by *cis* and *trans* acting elements, allowing formation of different E6/E6* transcript patterns. The ESS and ESE sequences (exonic splicing silencer and enhancer, respectively) and the splicing donor (SD) and acceptor (SA) sites involved in E6 splicing regulation are also shown.

4. Splicing within HR-HPV E6

The LCR contains cellular and viral transcription factor binding sites, as well as transcriptional enhancers, a replication origin, a late polyadenylation site and late regulatory elements [19,27]. The early promoter is located upstream of the E6 ORF (p105 for HPV18 and p97 for HPV16) and is responsible for early gene transcription. The late promoter that resides inside of the E7 ORF, drives E4, L1 and L2 gene expression. Other sequences that could act as possible promoters have been described but their functions are not clearly understood.

In low-risk HPVs the E6 and E7 genes are transcribed from two independent promoters, while in high-risk HPVs those genes are transcribed as a single polycistronic pre-mRNA from the early promoters. A common feature of high-risk HPVs is that the E6/E7 polycistronic mRNA contains at least one donor and one acceptor splicing site that can trigger the alternative splicing process, inducing

the expression of a variety of E6 spliced transcripts termed E6* [18,58]. In contrast, low-risk HPVs and beta-papillomavirus types do not undergo splicing in this region [21].

Depending on the HR-HPV type, different transcripts are derived from one of the donor splicing sites contained in the E6 ORF and one of the acceptor splicing sites located within E7, E2 or E4 ORFs. The splicing pattern of HPV type 16 has been thoroughly studied and the following spliced transcripts have been identified: E6*I, E6*II, E6*III, E6^E7, E6^E7*I, E6^E7*II, E6*IV, E6*V and E6*VI [18,59–62]. Conversely, the described transcripts for HPV18 are: E6*I, E6*II, E6*III, E6^E7 [60,63,64]. Less is known about transcripts resulting from splicing in the E6 pre-mRNA of other HR-HPV types, such as HPV31 having E6*I and E6^E4; HPV33 with E6*I, E6*II and E6*III; and HPV58 with E6*I and E6*II [65–68]. For other HPV types only the E6*I transcript has been detected, although the existence of other E6 spliced transcripts cannot be discarded [21,69]. Donor and acceptor sites for the identified different transcripts are depicted in Table 1.

Table 1. Transcripts derived from alternative splicing within the E6 open reading frame (ORF). The table summarizes the E6* isoforms for 23 HPV types where alternative splicing has been observed. The detailed donor and acceptor splicing sites for each E6 truncated transcript are enlisted below.

HPV Type	E6* Transcripts	Donor-Acceptor Splicing Sites (Nucleotide Position)	References
16	E6*I	226–409	[59]
	E6*II	226–526	[70]
	E6*III	226–3358	[59]
	E6*IV	226–2709	[71]
	E6*V	221–409	[61]
	E6*VI	191–409	[61]
	E6^E7 (E6*X)	226–742	[60]
	E6^E7*I	174–718	[62]
	E6^E7*II	221–850	[62]
18	E6*I	233–416	[63,64]
	E6*II	233–3434	[63,64]
	E6*III	233–2779	[64]
	E6^E7	233–791	[60,64]
26	E6*I	173–406	[21]
30	E6*I	229–420	[21]
31	E6*I	210–413	[65,68]
	E6^E4 (E6*III)	210–3295	[65]
33	E6*I	231–509	[66]
	E6*II	231–785	[66]
	E6*III	231–3351	[66]
34	E6*I	223–414	[21]
35	E6*I	228–419	[21]
39	E6*I	231–420	[21]
45	E6*I	230–413	[69]
51	E6*I	173–406	[21]
52	E6*I	224–502	[69]
53	E6*I	236–419	[69]
56	E6*I	157–420	[21]
58	E6*I	232–510	[67]
	E6*II	232–3355	[67]
59	E6*I	183–582	[69]
66	E6*I	157–420	[21]
67	E6*I	224–502	[69]
68b	E6*I	232–415	[69]
69	E6*I	178–411	[21]
70	E6*I	231–422	[21]
73	E6*I	227–410	[69]
82	E6*I	178–411	[21]

Interestingly, it has been proposed that E6 nucleotides 226 and 409 (donor and acceptor sites, respectively) from HPV16 are preferentially selected among other splicing sites, leading to the release of intron I, generating E6*I [61]. A suboptimal branch point sequence was previously identified within intron I of HPV16 (AGUGAGU) which contains the 328G instead of the typical adenosine [72]. An optimal branch point was further discovered within the same intron (AACAAAC), proposed to be the preferred branch point sequence, where 385A allows the selection of E6*I and expression of E7 [61].

5. E6/E6* Transcription Patterns

Many studies have described the E6/E6* patterns found in cervical cancer cell lines with endogenous expression of HPV or in cells with ectopic expression of HPV sequences. These patterns have also been identified in HPV infected biopsies with normal or altered cytology and in HPV-related cancers. Most of those studies are focused on the expression patterns of HPV16 and HVP18; although, information is available for other HR-HPV types such as, 31, 33 and 58 (Figure 3) [65–67].

The donor and acceptor splicing sites necessary to generate E6*I were described for the first time in the HPV16 positive CaSki cell line [73]; however, this isoform was first named E6* in a study performed using the HPV18 positive HeLa cell line [74].

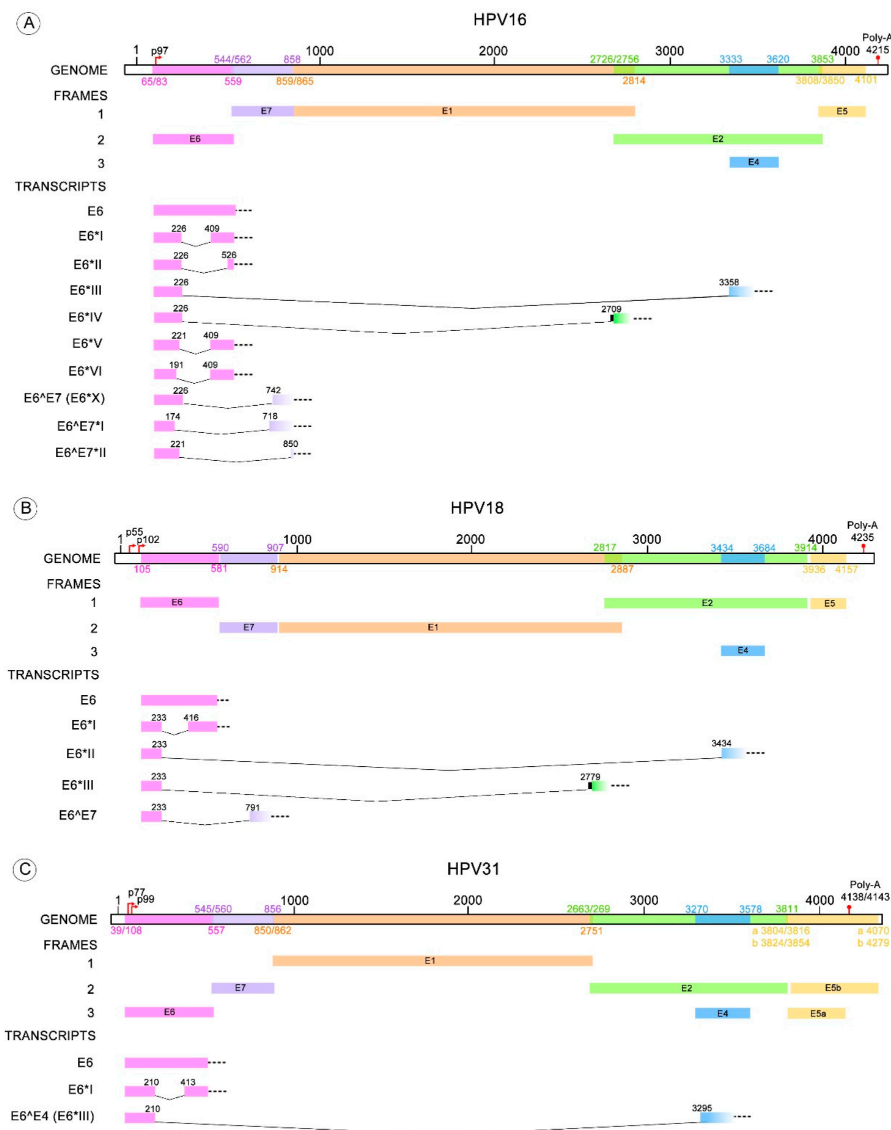

Figure 3. *Cont.*

Viruses **2018**, *10*, 45 8 of 20

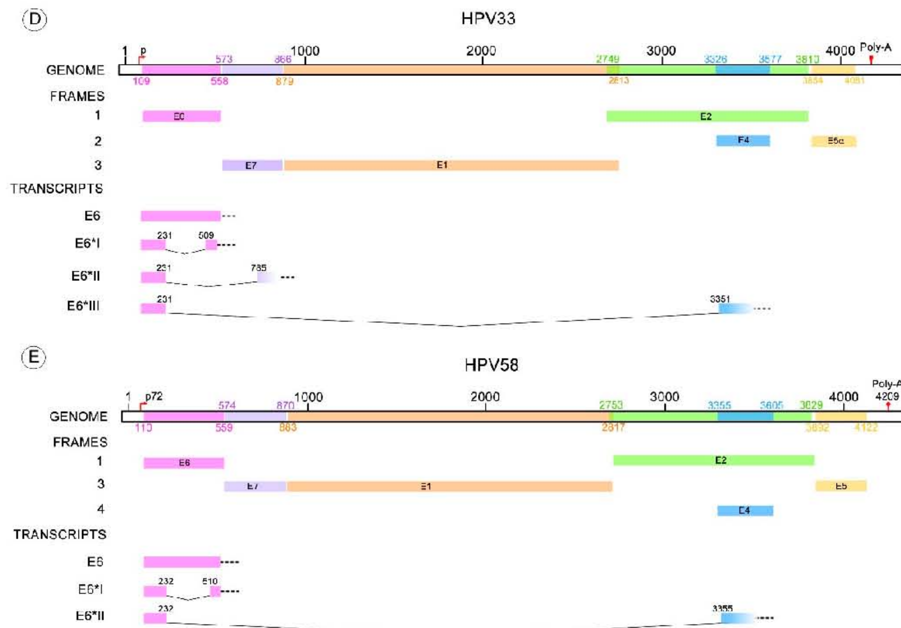

Figure 3. HR-HPV E6 alternative RNA splicing. The donor and acceptor splicing sites for the E6 truncated transcripts of different HPV types that possess more than one variant of E6* mRNA. (**A**) The E6* transcripts identified in HPV16 are E6*I to VI, E6^E7, E6^E7*I and E6^E7*II; (**B**) Four E6* transcripts have been described for HPV18 termed E6*I to III and E6^E7; (**C**) E6*I and E6^E4 transcripts are known for HPV31; (**D**) E6*I to III for HPV33; (**E**) E6*I and II transcripts for HPV58. All these transcript variants contain a donor splicing site within the E6 open reading frame (ORF), while the acceptor splicing site is contained through the early pre-mRNA (E6, E7, E2 or E4 ORFs). The nucleotide positions of early promoters (p) and early polyadenylation (Poly-A) sequences, as well as the positions of early genes, were obtained from Papillomavirus episteme [8]. The early promoter of HPV58 was obtained from Li Y. et al. 2013 [67]. All the donor and acceptor splicing sites are listed in Table 1.

E6/E7 splicing patterns have been recognized by different methods in a variety of HR-HPV containing cell lines and those studies consistently reveal the presence of higher amounts of E6*I mRNA compared to the E6 transcript [74–77]. In addition, the E6*II transcript is usually present in higher amounts than E6 but at lower levels than E6*I [75].

It has been demonstrated that E6*I is highly expressed in a model of HPV primary infection, where the replication cycle of HPV18 is efficient [63], suggesting that the expression of E6*I could have an important role in the first stages of viral infection.

Moreover, studies in W12—cells derived from a low grade cervical lesion with episomal HPV16—showed that while E6 mRNA was not detected, E6*I and E6*III were expressed [59]. In further studies, different subclones were isolated from the W12 cell line, generating a W12-derived model of cervical tumor progression. Such clones contain different physical states of the HPV16 genome (episomal or integrated), exhibiting different biological outcomes: differentiated non-tumorigenic, less differentiated non-tumorigenic, tumorigenic and invasive cells. Interestingly, all of these cell lines express E6, E6*I and E6*II transcripts but the carcinogenic clones showed a significant increase in the expression of all E6 transcripts, in addition to the expression of the E6*X [70]. These findings suggest that the E6/E6* expression patterns could be independent of the physical state of the HPV genome but dependent on the lesion grade.

The E6/E6*I transcription patterns were evaluated in 12 oncogenic and 11 possibly-oncogenic HPVs, where E6/E6*I were found to be expressed in the majority of those HPV types, although with different patterns. In contrast to several studies, this report shows that E6*I transcript from HPV16 and 18 were present in lower amounts than E6 [21]. It was previously demonstrated that the distance

between the 5′ Cap site and the intron is rate limiting for E6 RNA splicing [78]. Therefore, changes in the proportion of E6/E6* observed in different studies could be partially explained by the 5′ added nucleotide sequences in the E6 expressing vectors, which increase the distance between the E6 intron and the 5′ Cap in the pre-mRNA.

E6 and E6-spliced mRNAs have been investigated in patient samples, aiming to find a correlation with different stages during transformation. Many studies show that premalignant or malignant cervical and oropharyngeal lesions positive for HPV16 genomes, exhibit higher amounts of E6*I than E6, similar to the results described in cell lines [77,79,80]. Other studies detected E6*I and E6*III transcripts in cervical cancer, as well as in low and high-grade lesions, where no E6 mRNA was identified, maybe due to the different sensitivities of the technical approaches used [81]. In HPV16 positive cervical cancer biopsies, the proportion of E6, E6*I, E6*II and E6^E7 transcripts varies but E6^E7 is consistently present at lower levels, while the expression of E6*I is the highest [60]. Furthermore, the levels of HPV16 E6, E6*I and E6*II mRNAs are higher in cervical cancer samples compared to those in oropharyngeal cancer [82], suggesting that cellular contexts could be involved in the expression of HPV sequences.

Through RNA-seq quantitative sequencing, the proportion of HPV transcripts in cervical samples has been determined. In Cervical Intraepithelial Neoplasia grade 3 (CIN3) and Squamous Cervical Cancer (SCC), low levels of E6 transcripts were found, representing less than 5% of all HPV transcripts in each sample; conversely, E6*I represented close to 5%, 40% and 50% of all HPV mRNAs in Cervical Intraepithelial Neoplasia grade 2 (CIN2), CIN3 and SCC samples, respectively [24].

Controversial results about the association between the expression of E6*I/E6*II and the grade of cervical lesions have been reported. A positive association between higher concentrations of E6*I and E6*II transcripts and high-grade cervical lesions, as well as cervical cancer, was found, being E6*I the most abundant of these mRNAs [82,83]. In contrast, another study did not reveal differences in E6*I levels in the different lesion grades but a significant decrease of E6*II was observed in high-grade lesions [84]. Moreover, some studies have proposed E6*II expression as an indicator of cervical neoplasia severity [85]. These results show that the association between E6*I/E6*II patterns and lesion grade cannot be confirmed at this moment.

E6* expression has also been studied in uncommon HPV-related cancers. In squamous cell scrotal cancer samples, HPV16 E6*I transcripts were found [86]. Additionally, in breast tumor samples infected with HPV16, E6*I, E6*II, E6^E7 (E6*X), E6^E7*I and E6^E7*II transcripts were detected [62]. These results suggest that HPV is transcriptionally active in those tumor samples.

It is worth mentioning that variations in 2 to 5% of genomic sequences within the same HPV type are defined as intra-type variants, which have been associated with distinct biological outcomes of HPV infections [87]. It has been reported that nucleotide changes within HPV18 E6 variant genes (Asian-Amerindian, European and African phylogenetic branches) result in different E6/E6*I splicing patterns in MCF-7 cells and cervical tumor biopsies. Interestingly, the cells and tumors harboring the Asian-Amerindian variant of E6 expressed higher levels of E6 than E6*I, while those with the African variant exhibited a higher proportion of E6*I [88,89]. Furthermore, European variants of HPV16 do not exhibit differences in E6/E6* splicing patterns [90].

In conclusion, even when E6/E6* patterns differ in pre-malignant lesions and cancer, E6*I is the transcript present in higher amounts. Moreover, it seems that all transcript levels increase as the lesions progress to cancer. This effect could be related to an increase in HPV transcription and/or replication rates, which might allow the detection of those spliced transcripts found at low levels. However, further studies are needed to confirm this statement.

6. Regulation of E6/E6* Patterns

Alternative splicing of HPV transcripts increases the complexity of viral gene expression. The E6/E6* patterns change through the cell cycle, being the E6*I transcript more abundant than E6 during G2/M phase [91]. Several regulators have been identified that control transcription, splicing

and polyadenylation of early and late mRNAs. However, few *cis* and *trans* acting regulators have been found to modulate E6/E6* splicing patterns (Figure 2B) [19,20,92,93].

The serine/arginine-rich splicing factor 1, 2 and 3 (SRSF1, 2 and 3) are augmented in HPV16 positive cervical cancer cell lines compared with HPV16 positive non-tumorigenic cells. These proteins increase E6/E7 mRNA stability and protect E6 transcript from decay. Interestingly, E6/E6* splicing is not affected by the SRSF overexpression [70].

Using a raft culture model, it has been shown that CCCTC-Binding Factor (CTCF) can bind to E2 ORF of HR-HPV types and induce an increase of E6*II mRNA without affecting other E6 spliced transcripts [94].

The ASF/SF2 splicing factor interacts with an HPV16 splicing enhancer located downstream of the SA3358 site, promoting splicing particularly at this acceptor site. SA3358 site allows the production of E6*III if the SD226 site is selected but can also produce other E6* mRNAs with the SD880, promoting an increase in all of the E6 spliced transcripts [95,96].

The SF3B1 splicing factor has also been reported to increase HPV16 E6 mRNA splicing, favoring the E6*I isoform [97]. Head and neck cancer cells positive for HPV16 were treated with meayamycin B, a potent inhibitor of SF3B1, showing a decrease in the levels of E6*I mRNA with an increase of the full-length E6 transcript. When SF3B1 was knocked down, similar effects were observed, demonstrating that the biogenesis of E6*I is influenced by SF3B.

The splicing at the SD226 site is favored when E6/E7 mRNAs are capped through the interaction with Cap binding factors. When the distance from 5′mRNA Cap to the SD226 is increased, the levels of E6 are higher, while a distance less than 307 nucleotides seems to be optimal to promote the splicing at SD226, facilitating E6*I expression [78].

Together, hnRNPA1 and hnRNPA2 promote splicing of E6 HPV16 mRNA. In contrast to hnRNPA1 only, that in the presence of Epidermal Growth Factor (EGF) induces an increase in un-spliced E6 mRNA [98]. This evidence could be associated to the exonic splicing silencer (ESS) within the E7 ORF, which contains an hnRNPA1 binding motif that reduces 233ˆ416 splicing (E6*I) and induces E6 expression in HPV18-transfected or -infected cells [64].

Upon activation of EGFR and Erk1/2 MAPK by EGF, E6/E7 splicing is reduced. Although the exact mechanism has not yet been described, it is proposed that this effect could be mediated through regulators controlled by growth factor pathways, such as Brm and Sam68, which increase the levels of E6/E7 mRNA in the presence of EGF [98].

Interestingly, HPV proteins also modulate E6/E7 mRNA splicing by acting as RNA binding proteins. E2 and E6 proteins bind to intron 226–409 and might interfere with the cellular splicing machinery, decreasing the levels of E6*I transcript in HPV16 infected cells. This reduction could be carried out by SR proteins through their interaction with E2 and E6 viral proteins [99]. Therefore, expression of E6, E6* and E7 can be affected by the different splicing regulatory proteins, depending on their availability during cellular differentiation or cancer progression.

7. E6* Related Functions

One of the most characterized E6* transcript functions is to facilitate translation of the E7 oncoprotein by increasing the space in the mRNA between the E6 stop codon and the E7 start codon, allowing better ribosome assembly [23,78,100]. However, other studies demonstrate that intron exclusion has a minimal or no effect on E7 translation, since the E7 protein is mainly translated from E6 non-spliced mRNA [22,101]. Moreover, other functions have been attributed to E6* proteins, mainly to E6*I, independent of E6 and E7 expression (Figure 4).

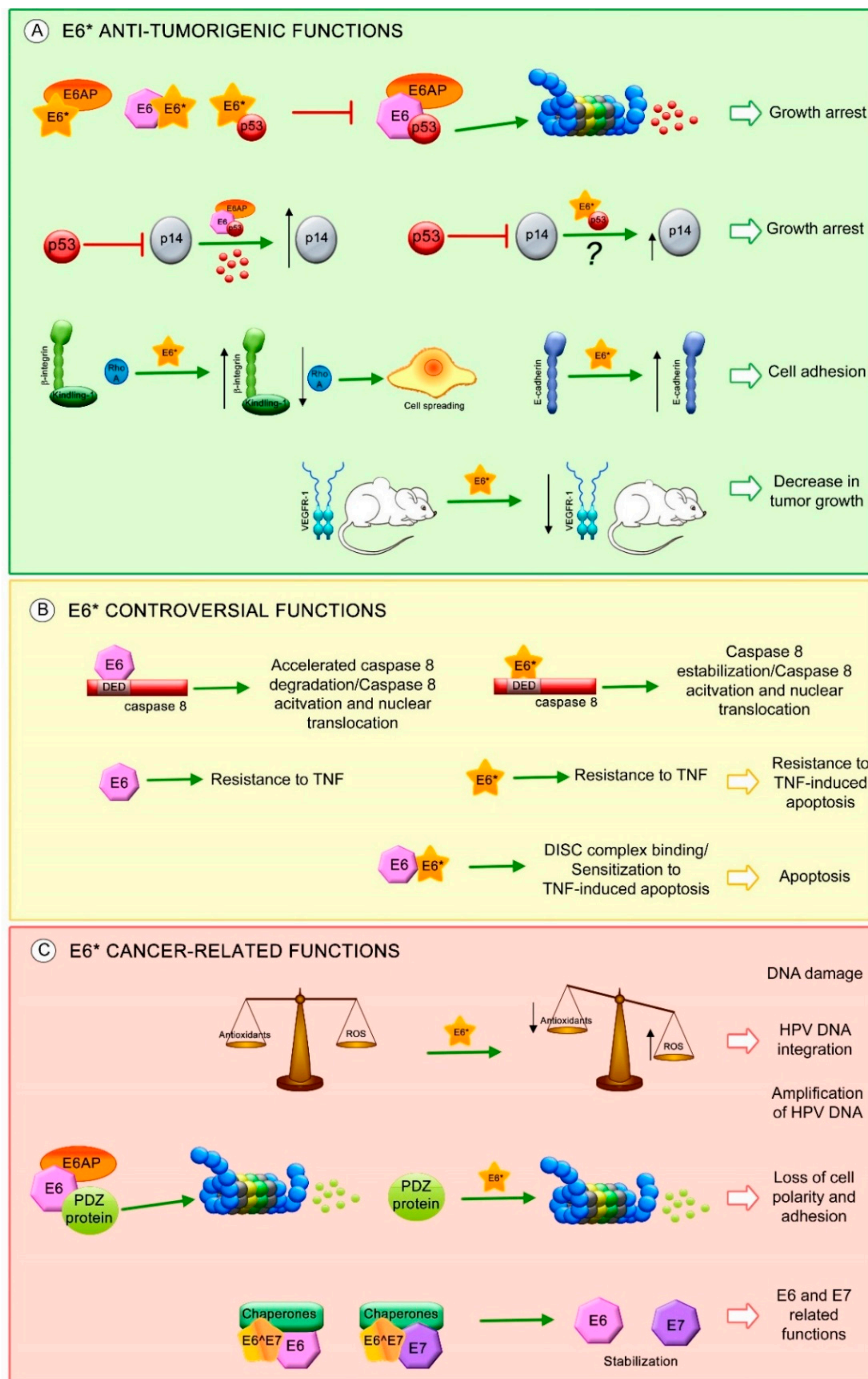

Figure 4. E6* isoform-related functions. Proposed E6* functions involved in (**A**) anti-tumorigenic effects, such as: increase in growth arrest through inhibition of E6-mediated p53 degradation and increase in p14 protein levels possibly through E6*/p53 interaction (?), increase in cell adhesion by the activation of β-integrin signaling and overexpression of E-cadherin, decrease in tumor growth associated with a reduction in VEGFR-1; (**B**) Controversial effects of E6* in apoptosis regulation; and (**C**) Carcinogenic characteristics, such as: promotion of DNA damage by ROS, which may allow HPV DNA amplification and integration into the host genome, degradation of postsynaptic density-95/discs large/zonula occludens-1 domain (PDZ) containing proteins involved in cell polarity and adhesion and stabilization of E6 and E7 oncoproteins. The red T-bars indicate inhibition, the green arrows show induction of the related process, the black arrows depict an increment or a reduction in protein levels.

E6*I protein was detected for the first time in 1987 in CaSki cells [102] and like E6*II, displays both nuclear and cytoplasmic localization; conversely, E6 is mostly found in the cell nucleus [91,103,104].

E6*I HPV18 is a polypeptide of 57 aa that shares the first 44 aa of its N-terminal domain with E6 and contains 13 aa derived from the change in the E6 open reading frame after the splicing sites [25]. Due to different donor and acceptor splicing sites contained in HR-HPVs, the predicted E6*I proteins differ from E6, in size, by approximately 50 to 55 aa for HPV16, 18, 30, 33, 34, 36, 35, 39, 68 and 70; and 29 to 36 aa for HPV26, 31, 51, 56, 66, 69 and 82 [21].

The specific structure of E6*I has not been well characterized due to the difficulty in acquiring a compact monomeric fold in such a small polypeptide. However, α-helix or β-sheet conformations, depending on experimental conditions, have been suggested [105]. E6*I conserves only half of the N-terminal zinc binding motif present in E6. Moreover, most of the HR-HPV E6*I, excepting HPV56 and 66, contain a hydrophobic motif (L/M/I)XX(L/I/V)X(L/V/I) which is associated to E6 and E6AP binding [106].

It has been widely demonstrated that the HR-HPV E6 proteins promote p53 degradation through binding with the E3 ubiquitin ligase E6AP [107,108]. Furthermore, E6*I protein interferes with E6-mediated degradation of p53 by its binding to E6AP, E6 and to p53, although with lower affinity [21,91,109,110].

Furthermore, it has been shown that HPV18 E6 increases the levels of p14ARF through p53 degradation, while HPV18 E6*I over-expression only induces a moderate increase of the p14ARF [88]. This result shows that E6*I may have a direct effect over p14ARF, independent of E6, possibly through E6*I and p53 interaction, preventing p14ARF regulation by p53. However, more evidence is still needed.

Additionally, it has been demonstrated that HPV16 E6*I does not increase keratinocyte immortalization and proliferation [100]. HPV18 E6*I decreases cell proliferation in HPV16 positive cancer cells, while HPV18 E6*I overexpression in p53 null cancer cells does not exhibit this anti-proliferative effect, indicating that this effect could be attributed to protection of p53 by E6*I [110].

Anti-tumorigenic features have been associated with E6*I expression. The β-integrin pathway that regulates cytoskeleton rearrangements, cellular shape and mobility was evaluated in SiHa cells. The levels of β-integrin and its co-stimulatory molecule kindling-1, increased in the presence of E6*I, while a reduction in RhoA levels was observed, promoting cell morphological changes related to cell spreading. Moreover, this study found a decrease in Alkaline phosphatase activity in those cells transfected with HPV16 E6*I, which is related to loss of both pluripotency and undifferentiated cell phenotype [111].

Furthermore, in a study performed in SiHa cells, HPV16 E6*I promoted the overexpression of E-cadherin protein, a biological marker related to cell adhesion and epithelial phenotype. However, in C33A cells, this effect was not observed. Interestingly, a xenograft mouse model using SiHa and C33A cells transfected with HPV16 E6*I, showed an evident decrease in tumor size with a decrease in VEGFR-1 levels, a biological marker for angiogenesis [112].

Since E6*I does not induce immortalization and cell proliferation, it has been postulated that it could be regulating pathways involved in cell death, such as apoptosis. Different studies showed that both E6 and E6*I of HPV16 and HPV18 bind to the dead effector domain (DED) of procaspase 8 via different sites [113–115]; however, only HPV16 E6 can bind to Fas-associated protein with death domain (FADD) DED [116]. One of the studies showed that HPV16 E6*I stabilizes procaspase 8 while E6 has the opposite effect [114]; however, a further study demonstrates that neither HPV18 E6 nor E6*I induces procaspase 8 stabilization. Nevertheless, these viral proteins increase the levels of active caspase 8 and induce its nuclear translocation without inducing apoptosis [113].

Additionally, it has been shown that HPV16 E6 and E6*I exert different effects in apoptosis either together or alone. Both viral proteins independently expressed, promote resistance to TNF-induced apoptosis; in contrast, when they are expressed together they promote TNF-dependent apoptosis [109]. Furthermore, it has been demonstrated that overexpression of HPV16 E6*I but not E6*II, sensitizes oropharyngeal squamous cell carcinoma cell lines to radiation, promoting cell death [117]. Recent

studies suggest that this effect could be dependent on cellular context, since it is not observed in non-head and neck cancer cell lines. Together, these facts indicate that the regulation of apoptosis by E6*I and E6 is a complicated mechanism and that the E6/E6* expression patterns and cellular contexts could play an important role.

A proteomic analysis comparing HPV16 positive and negative cell lines revealed that HPV16 E6*I modifies the expression of cellular proteins involved in a variety of cellular signaling pathways such as: integrin-linked kinase (ILK), oxidative phosphorylation and mitochondrial dysfunction. HPV16 E6*I promotes an increase in mitochondrial dysfunction in HPV positive and negative cells, which then induces a decrease in the levels of the antioxidant molecule GSH and subsequent DNA damage [111]. These data correlate with results observed in HPV16 positive cells, where HPV16 E6*I protein but not E6, decreases the levels of the antioxidant enzymes SOD2 and Gpx, leading to the accumulation of reactive oxygen species (ROS) and an increase in DNA damage [118]. Even when the DNA damage promoted by E6*I could eventually culminate in apoptosis, some data support the idea that the induction of DNA damage by ROS could be related to the amplification of HPV DNA, which would require different regulators of the homologous recombination DNA repair system [119] or to HPV genome integration [120], suggesting that E6*I could be participating in the HPV viral cycle, as well as in cancer establishment.

It is well known that E6 targets PDZ (postsynaptic density-95/discs large/zonula occludens-1 domain) containing proteins, inducing their degradation. Moreover, HPV18 E6*I protein, as well as E6, induces the degradation of PDZ containing proteins such as Dlg (Drosophila disc-large), MAGI-1 and h-Scrib. The ability to promote Dlg degradation is conserved among HPV31, 16 and 81 E6*I proteins; however, E6*I cannot bind to this protein. Currently, there is only one PDZ containing protein shown to interact with E6*I, allowing its degradation. This protein, termed PATJ can interact with E6*I in a PDZ binding motif (PBM)-independent manner or through other cell proteins that allow this interaction. In addition, this study demonstrated that HPV18 E6*I induces the degradation of Akt, in contrast to E6, which is not able to decrease Akt levels. This suggests that E6*I of HPV18 could be regulating processes involved in survival and cell growth [25,121].

Very little is known about the functions of other E6 spliced isoforms. HPV16 E6^E7 is a predominantly cytoplasmic protein that contains 41 aa of E6 in its N-terminal half and 38 aa of E7 in its C-terminal half. It has been shown that E6^E7 binds to the cellular chaperones HSP90α, HSP90β and Glucose-regulated protein 78 (GRP78) but only HSP90β and GRP78 induce E6^E7, E6 and E6*I stabilization. In addition, E6, E7 and E6*I proteins are stabilized by E6^E7, in a manner dependent on the endogenous chaperones [60].

8. Conclusions

The sustained higher proportion of E6*I compared to E6 mRNA observed in different lesions and tumors, suggests that the generation of E6* isoforms has an important role in cancer development.

Alternative splicing within the E6 ORF could be mediated by donor and acceptor splicing site sequences and surrounding fragments, which regulate the most efficient recruitment of the spliceosome elements. Discrepancies found in E6 splice patterns in diverse study models could be due to the presence of specific regulatory factors depending of the cell context or to differences in the physical state of the HPV genome during the progression of an HPV infection to cancer. The loss of E2 protein due to viral genome integration [122] could also affect the splicing process, since E2 is a mRNA binding protein which regulates E6 splicing [99]. Moreover, since HPV genome integration occurs at distinct sites in the host genome [75], it cannot be discarded that in some cases host genes involved in splicing regulation could be disrupted and therefore change the splicing patterns. Until now, little is known about the specific mechanisms regarding the modulation of E6 splicing patterns but all the evidence suggests that the presence of E6 spliced transcripts is a common event in cervical carcinogenesis.

It is worth mentioning that comparing the E6 splicing patterns among biological models analyzed with different methodological tools is a difficult task. The variations in results among different studies may be due to the choice of different techniques.

In studies using RT-PCR, the selection of primers commonly leads to the amplification of splice variants just within the E6/E7 ORFs, excluding some of the spliced transcripts involving the early HPV mRNA. In contrast, studies using deep sequencing techniques describe the splice forms extensively, allowing a robust analysis of the transcripts. Although this technology gives us a better approach to the diversity and quantity of E6 transcripts, more information is still needed to associate these transcripts with cancer progression. Moreover, it is difficult to achieve an adequate comparison between observations obtained through diverse methods that present different sensitivity. Nevertheless, the quality of the studies has increased over the time, permitting the detection of transcripts that are present in very low concentrations, such as E6^E7 (E6*X).

Some authors argue that while E6* transcripts can be abundant in some models, E6* proteins cannot be detected [59,101,123]. Nevertheless, other researchers have clearly identified E6* proteins, supporting that E6* transcripts can produce at least one E6* protein [91,109,114,118,124]. Anti-oncogenic effects have been attributed to E6* proteins [112], although other effects, such as promotion of DNA damage [118,120], degradation of PDZ containing proteins related to cellular polarity [25] and stabilization of E6 and E7 oncogenic proteins [60] are effects involved in cancer development, clearly demonstrated for E6* proteins . In addition, E6* proteins could have different effects depending on the cellular context where different E6* protein conformations could be generated [105], promoting distinct interactions with cellular binding partners.

The different splicing patterns for E6/E6* observed among tumors or during the different stages in cancer progression could provide a wide variety of E6 isoforms with an impact on biological processes. Nevertheless, oncogenic and/or non-oncogenic functions reported for E6* proteins, make it difficult to sort them out as tumor suppressor or oncoproteins in HPV-related tumors. Currently, the possibility that E6* proteins contribute to the HPV transformation process has gained attention and much data has been generated that has opened a window of opportunities in the study of these proteins regarding their participation in the HPV life cycle and/or in cancer establishment.

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ORIGINAL ARTICLE

Intratype variants of the E2 protein from human papillomavirus type 18 induce different gene expression profiles associated with apoptosis and cell proliferation

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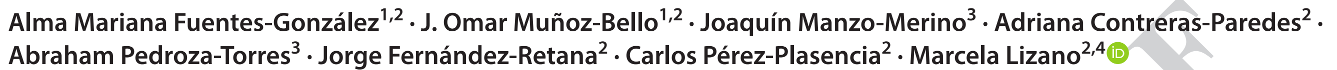

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Abstract

Persistent infections with high-risk human papillomaviruses (HR-HPVs) are linked to development of cervical cancer due to a deregulation of the productive viral cycle in the host cell, leading to cell transformation. The E2 viral protein is expressed early during an HPV infection and regulates viral replication and transcription. Other functions have been attributed to E2, such as the promotion of apoptosis that are independent of its role in the regulation of the expression of E6 and E7 viral oncogenes. Moreover, it has been shown that the HPV16 E2 protein has regulatory effects on cellular gene expression, suggesting that it participates in the modulation of different cellular processes. Intratype genomic variations within high-risk HPV types have an impact on the prognosis of HPV-related lesions. Nevertheless, the biological significance of HPV18 E2 intratype variations has not been analysed previously. The aim of this study was to determine whether HPV18 E2 intratype variations differentially modulate gene expression and whether cell-death-related genes are affected by variations in E2. We demonstrate that HPV18 E2 intratype Asian Amerindian (AsAi) and African (Af) variants differentially affect gene expression profiles. Although the E2-AsAi variant was found to modulate a larger number of cellular genes, both E2 variants affected similar cellular processes. Nevertheless, E2-AsAi and E2-Af variants showed differences in their ability to induce apoptosis, where E2-Af had a stronger effect. The differences in gene expression profiles in cells harbouring E2 intratype variants suggest a possible effect on diverse cellular signalling pathways, and this might suggest an approach for identifying biological processes regulated by HPV18 E2 intratype variants.

Abbreviations

HPV	Human papillomavirus
HR	High-risk
CC	Cervical cancer
DMEM	Dulbecco´s modified Eagle´s medium
RT-qPCR	Quantitative reverse transcription PCR
ΔΔ Ct	Double delta Ct

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Introduction

Cervical cancer has been linked to persistent infection with high-risk human papillomaviruses (HR-HPVs), with HPV16 and 18 being the most prevalent types [1]. HPV intratype variants have been identified for several HR-HPV types, which differ in 2 to 5% of their nucleotide sequences when compared to the reference genome sequence [2]. It has been proposed that HPV intratype variations may confer different pathogenic risks associated with distinct biological effects [3–7]. In previous work, we identified HPV18 DNA isolates in cervical cancer cases in a Mexican population, belonging to the Asian-Amerindian (AsAi), European (E) and African (Af) phylogenetic branches [7]. The distribution of those variants in different histological types of cervical cancer suggested that HPV18 variations may have an impact on cancer prognosis [6, 7]. We have also demonstrated a functional significance of HPV intratype variations, mostly through the analysis of activities relevant to the carcinogenic potential of E6. In particular, we demonstrated that HPV18

Journal : Large 705 | Article No : 4124 | Pages : 13 | MS Code : ARVI-D-18-00632 | Dispatch : 11-12-2018

51 E6 oncogene variants exhibited different biological effects related to proliferation and induction of tumour growth [5, 8].

The early-expressed E1 and E2 viral proteins are responsible for viral transcriptional regulation and replication and participate in the long-term maintenance of the viral genome in dividing cells [9]. Although the main role of E2 is the regulation of the virus replicative cycle, its participation in cell transformation has also been reported [10, 11] .

Additionally, some studies have demonstrated various interactions of E2 with cellular proteins involved in regulation of transcription, RNA processing, intracellular trafficking, disruption of the mitochondrial respiratory chain, and apoptosis [12–17]. Moreover, it has been shown that E2 regulates host gene expression by interacting with several transcriptional factors [18–21]. Through the analysis of expression profiles, the effect of HPV16 E2 in biological processes has also been studied, showing that this protein alters several cellular pathways that are involved in the regulation of cell death, proliferation and differentiation [22, 23].

Previously, our group identified nucleotide sequence variations within the HPV18 E2 gene that resulted in changes in the amino acid sequence (Table S1). Such variations in HPV18 E2 did not alter viral transcriptional activity [24], but their biological significance for cell death or proliferation was not analysed.

The aim of this study was to determine whether HPV18 E2 intratype variations differentially modulate gene expression profiles. We found that different E2 variants affect different cellular mRNA profiles but nevertheless affect the same cellular processes. Most of the effects observed were associated with cell-death-related genes. Our findings show that E2 variants differentially regulate apoptosis and cell viability and that the E2-Af variant showed a stronger effect than the E2-AsAi variant. These results may help in understanding differences in viral biological behaviour between HPV-induced neoplasias.

Materials and methods

Plasmids

HPV18 E2 ORFs of Asian-Amerindian (AsAi) and African (Af) variants were obtained from cervical cancer biopsy samples as described previously [7, 24]. Nucleotide and amino acid changes within those E2 variants are summarized in Fig. S1. The E2 coding regions were amplified by PCR with primers flanked by XbaI and BamHI restriction sites for cloning into the pCG cloning vector: E2-5´, 5’-GGGTCT AGAATGCAGACACCGAAGGAAACC-3’, E2-3´, 5’-AAA GGATCCTTACATTGTCATGTATCCC-3’. The correct cloning of the E2 variants was verified by sequencing.

Cell culture and transfection

Human cell lines that were negative for HPV sequences – C33A (squamous cervical cancer, HPV negative), HEK293 (embryonic kidney) and MCF7 (breast cancer) – were obtained from the American Type Culture Collection (ATCC, USA). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM-F12) (Gibco-BRL, USA) supplemented with 10% foetal bovine serum (Gibco-BRL, USA) and incubated at 37 °C in a 5% CO_2 atmosphere.

Transfections with the HPV18 E2 intratype variant-expressing plasmids AsAi, Af or pCG Empty Vector (EV) were performed using Lipofectamine 2000 Reagent (Invitrogen, USA) as recommended by the manufacturer’s instructions.

Transfection efficiency assay

C33A cells were seeded onto slides in 6-well plates and transfected with 5 µg of the appropriate E2-expression plasmid. At 24 h post-transfection, the cells were washed with PBS to remove dead cells, and the live (attached) cells were fixed with 3.7% paraformaldehyde in PBS for 10 min and permeabilized with PBS-0.1% Triton X-100. Then, the cells were blocked with a 0.3% BSA solution and incubated overnight at 4 °C with an anti-HPV18 E2 antibody (Santa Cruz Biotechnologies, USA). The cells were washed extensively with PBS and then incubated with anti-goat antibody conjugated to Alexa Flour 488 (Invitrogen, USA). The slides were washed and mounted with Prolong Diamond Antifade Mountant (Molecular Probes, Eugene, Oregon, USA). Images were acquired using an EVOS FL microscope (Thermo Fisher Scientific, Waltham, MA, USA). Around 20 fields were examined for each treatment.

RNA isolation and expression analysis

For the analysis of gene expression profiles, HPV18 E2-AsAi and -Af variant genes were expressed in C33A, MCF7 and HEK293 cells, used as a biological triplicate as described previously [25]. Cells were transfected with 5 µg of each plasmid. At 24 h post-transfection, total RNA was purified using an RNeasy Mini Kit (QIAGEN, Germany) according to the manufacturer’s instructions. Three independent transfections were performed, and extracted mRNAs from each cell line were pooled and used for microarrays. Then, the microarray data were analysed considering the three cell lines a biological triplicate. The samples were treated with 1 U of DNase I (Gibco-BRL, USA) to ensure RNA purity.

For cDNA preparation, 1 µg of total RNA was reverse transcribed with random hexamers using a GeneAmp RNA

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PCR Kit system for RT-PCR (Applied Biosystems, USA). Part of the resulting cDNA was used to verify the expression of the E2 variants in the transfected cells lines. Independent experiments were set up to obtain cDNA, which was used as template for further qPCR validation analysis.

Gene expression profiles

First, 250 ng of total RNA from the pooled transfections was amplified using a Whole Transcriptome Amplification Kit 2 (Sigma-Aldrich, USA). Then, 4 µg of Cy3-labeled cDNA was used to hybridize to the high-density array NimbleGen 12X135 K Microarray (Mannheim, Germany). Microarray images were acquired using a NimbleGen MS200 scanner and analysed using the NimbleScan v2.5 Software (Mannheim, Germany).

Microarray images were processed using GenePix 4.0 software. After aligning and collecting intensity values, a robust multi-array background correction and quantile normalization was performed. Normalized data for each of the experimental conditions, HPV-18 E2-AsAi and -Af, were compared to those for the control condition (empty vector). Experimental replicates were averaged among the three cell lines before calculating fold change values. Differential expression of the experimental groups E2-AsAi and -Af variants versus the empty vector were assessed by *t*-tests, and the statistical significance for each gene was determined by the empirical Bayes method implemented in the limma package in R software. Significant genes were identified using a Benjamini-Hochberg multiple hypothesis correction ($p < 0.05$) with a minimum twofold expression change (Tables S3 and S4).

Ontological analysis was performed with selected significant genes using WebGestalt (WEB-based GEne SeT AnaLysis Toolkit) [26]. Only enriched GOsets (hypergeometric, $p < 0.05$) containing two or more genes were included in the subsequent analyses. GO analysis for the three domains was performed. Biological processes, molecular functions, and cellular components were analyzed using advanced correction factors, including Elim and Weight elimination methods that take into account the hierarchical relationships and provide more-significant results. We also performed impact analysis using the iPathwayGuide tool for gene and protein expression analysis, which uses a systems biology approach to identify significantly affected pathways.

Quantitative reverse transcription PCR (RT-qPCR)

The qPCR assay was performed using SYBER Select Master Mix (Applied Biosystems, USA) (primer sequences in Supplementary Table S1). The expression of the housekeeping gene 18S mRNA was used as a normalizing control. Relative quantification was evaluated by the double delta Ct (ΔΔCt) method. For validation experiments, freshly prepared RNA samples from three different transfections were used independently of the microarray material. qPCR assays were performed for C33A, MCF7 and HEK293 cells.

Immunoblot assay

A total of 40 µg of protein of each cell lysate from C33A transfected cells was loaded onto an SDS-PAGE gel. The proteins were separated by electrophoresis and transferred to a nitrocellulose membrane (Hybond-C, Amersham Biosciences). The membrane was blocked with blocking solution (TBS, 10% skimmed milk and 0.1% Tween 20), followed by incubation with anti-E2 primary antibody (Abcam ab100969, UK). For constitutive protein detection, α-actinin (Santa Cruz Biotechnology, USA) was assessed. Membranes were incubated with peroxidase coupled to secondary antibodies, and the levels of the corresponding proteins were visualized utilizing the ECL system (Amersham, Biosciences, UK). Western blots were performed three times each to assure reproducibility and to perform densitometric analysis. Densitometric analysis was performed using the ImageJ program ver.1.48h3 (National Institutes of Health [NIH]).

Cell viability assay

C33A cells were transfected with 5 µg of each plasmid, and at 24 h post-transfection, the cells were washed with PBS to remove dead cells, and the live (attached) cells were fixed with 10% formaldehyde in PBS, washed several times, and stained using 10% crystal violet reagent diluted in PBS. After several washes, the plates were dried and eluted with 500 µL of 10% acetic acid in PBS. Absorbance was measured at 595 nm using a Titertek Multiskan MCC Reader Type 340 (California, USA), data were collected, and statistical analysis was performed.

Annexin V

Transient transfections with expression plasmids for producing HPV18 E2 intratype variants were performed using the C33A cell line. Twenty-four hours after transfection, live (attached) cells were harvested and mixed with detached cells (dead cells) contained in the media. The cells were centrifuged, and the pellet was stained using an Annexin-V-FLUOS/FITC Kit (Roche, Linscott) according to the manufacturer's instructions. Then, 20,000 events for each treatment were analysed by cytometry using a BD FACSCanto II Flow Cytometer. As a positive control, cells were treated with chelerythrine (10 µM) (Sigma-Aldrich, USA) as an apoptotic inductor.

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Journal : Large 705 | Article No : 4124 | Pages : 13 | MS Code : ARVI-D-18-00632 | Dispatch : 11-12-2018

241 ## Statistical analysis

242 All of the experiments were analysed using GraphPad
243 software (Version 7.03). Cell viability, cell death and RT-
244 qPCR data were analysed using either Student's *t*-test or an
245 ANOVA test with Tukey's post-hoc test. The effects were
246 considered significantly different when p was less than 0.05.
247 The microarray data analysis was carried out using the
248 limma package from Bioconductor (Version 3.6). Con-
249 fidence intervals and *p*-values for fold changes were also
250 calculated using a 2-sided Welch modified 2-sample *t*-test.
251 *p*-values of 0.05 or less were considered significant. In this
252 study, only results with a $\pm$2.0-fold change between empty
253 vector vs. HPV18 E2-AsAi or E2-Af were considered dif-
254 ferentially expressed.

255 ## Results

256 ### HPV18 E2 variants differentially affect transcription
257 ### profiles

258 C33A, HEK293 and MCF-7 cells were transiently trans-
259 fected with plasmids encoding E2-AsAi and E2-Af genes.
260 The pCG vector was used as a control vector (empty vector,
261 EV). Similar transfection efficiencies were observed with
262 all of the cell lines transfected with each of the E2 vari-
263 ants. The transfection efficiencies determined in living cells
264 with the E2-AsAi and E2-Af variants were 51-53% in C33A
265 cells, 50-52% in HEK293 cells, and 42-43% in MCF7 cells
266 (data not shown). Statistical analysis revealed no differences
267 among the experiments regarding the transfection efficiency.
268 At 24 h post-transfection, cells were harvested, and total
269 RNA and protein were extracted. RT-PCR analysis showed
270 that the E2 mRNA was successfully expressed in all of the
cell lines tested (Fig. 1A). The mRNA expression profiles 271
of HPV18 E2-AsAi- and HPV18 E2-Af-transfected cells, 272
vs. the control vector were obtained by mRNA microarray 273
assay. 274

A supervised analysis approach based on expression val- 275
ues for each gene comparing E2 variants with the control 276
vector (EV), was used to determine the molecular profile for 277
HPV18 E2-AsAi and E2-Af variants. Genes with a twofold 278
or higher increase in expression were considered upregu- 279
lated, and those with a twofold or higher decrease in expres- 280
sion were considered downregulated. All of the changes 281
were statistically significant ($p < 0.05$). Supplementary 282
Table S2 for HPV18 E2-AsAi and Supplementary Table S3 283
for HPV18 E2-Af show the regulated genes for each variant. 284
Data analysis indicated that 515 genes were upregulated and 285
846 were downregulated by the HPV18 E2-AsAi variant, 286
while 138 genes were upregulated and 97 were downregu- 287
lated by the HPV18 E2-Af. Figure 1B illustrates the total 288
number of cellular genes modulated by HPV18 E2-AsAi and 289
HPV18 E2-Af variants. Interestingly, 34 genes were shared 290
among the E2 variants tested, whereas the other genes were 291
exclusively regulated by one variant. These results indicate 292
that HPV18 E2 intratype variants differentially affect cel- 293
lular gene expression. 294

Differentially expressed genes were classified according 295
to biological processes using the WebGestalt software pack- 296
age on the basis of hypergeometric tests. We characterized 297
genes with significant GO terms ($\boldsymbol{p} < 0.05$) in biological pro- 298
cesses using the web-based GO analysis toolkit. The GO 299
annotation of upregulated and downregulated genes involved 300
in cellular processes in E2-AsAi- and E2-Af- expressing 301
cells is shown in Figure 2A and 2B, respectively. Differ- 302
ences were found in the number of cellular genes affected by 303
E2-AsAi and E2-Af variants that are involved in various pro- 304
cesses such as metabolic regulation (523 vs. 73 deregulated 305

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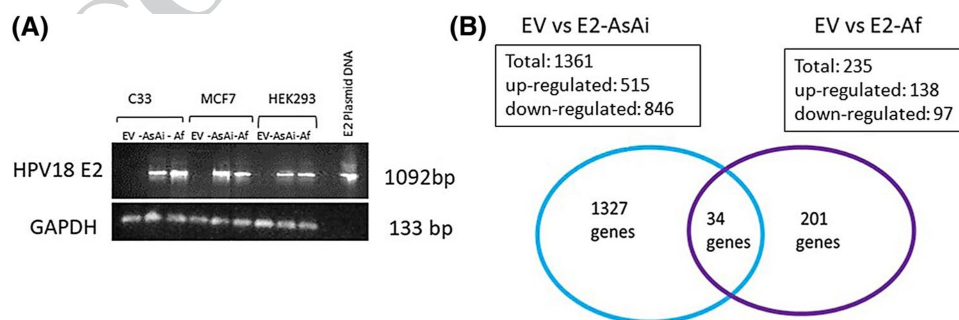

Fig. 1 HPV18 E2 variants differentially affect transcription profiles. C33A, MCF7 and HEK293 cells were transfected with plasmids for expression of HPV18 E2 variants (AsAi, Af) or the control vector (Empty Vector, EV). A) At 24 h after transfection, E2 mRNA expression was analysed by RT-PCR. GAPDH was amplified by PCR as a control, and DNA from the E2 expression plasmid was amplified as a positive control (E2+). B) Venn diagrams indicating genes modulated in cells transfected with HPV18 E2-AsAi (1361 genes) and HPV18 E2-Af variants (235 genes). The number of up- and downregulated genes is indicated. Thirty-four genes were found to be modulated by both the E2-AsAi and E2-Af variants. The number of deregulated genes exclusive for cells expressing E2-AsAi was 1327 (blue), and for cells expressing E2-Af, it was 201 (purple)

Journal : Large 705	Article No : 4124	Pages : 13	MS Code : ARVI-D-18-00632	Dispatch : 11-12-2018

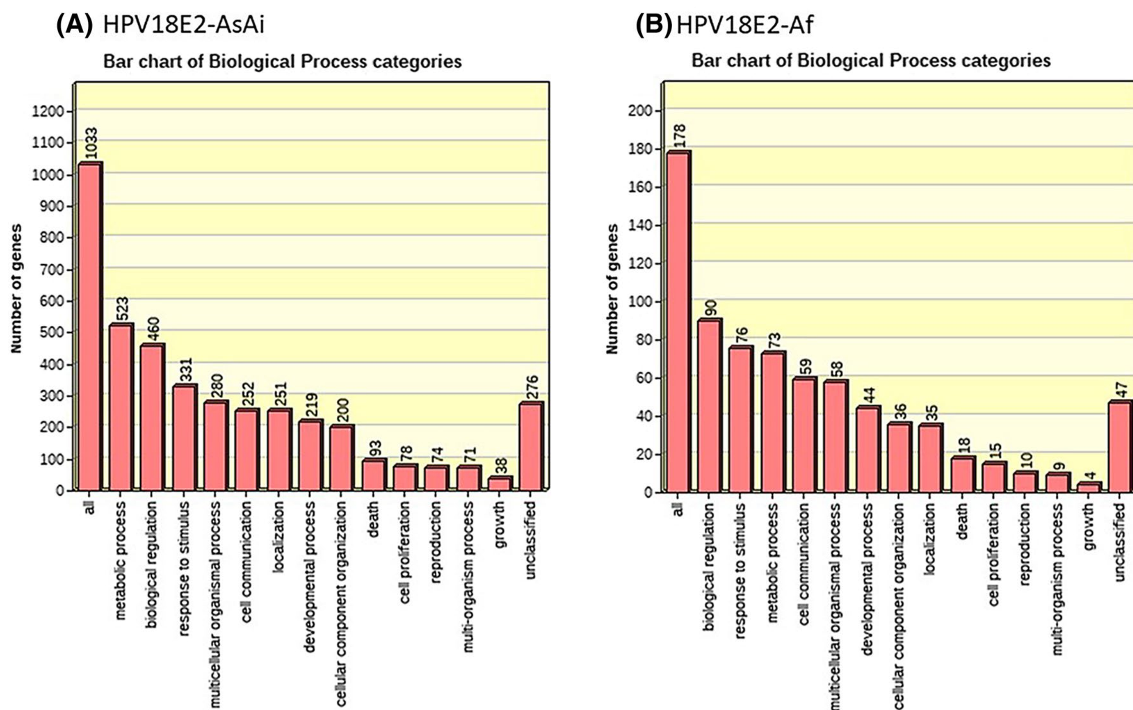

Fig. 2 Biological processes regulated by E2-AsAi and E2-Af intratypic variants. Analysis of biological processes up- and down-modulated by A) HPV18 E2-AsAi and B) HPV18 E2-Af. The identified genes were analysed according to Gene Ontology (GO) enrichment using WebGestalt (WEB-based GEne SeT Analysis Toolkit)

genes, respectively), cell proliferation (78 vs. 15 deregulated genes, respectively), cell communication (252 vs. 59 deregulated genes, respectively), and death (93 vs. 18 deregulated genes, respectively).

To confirm the microarray results, C33A, HEK293 and MCF7 cells were transiently transfected with plasmids expressing the HPV18 E2 variants, and after 24 hours, RT-qPCR was performed to identify differentially regulated genes involved in cell death and proliferation (FOSB, EPS15, CASP4, EPHA2, RAB4B and TRAF1). In concordance with the microarray data, FOSB expression was significantly upregulated, showing an enrichment for the E2-AsAi and E2-Af variants of 7.12- and 14.24-fold in C33A, 1.23- and 2.07-fold in HEK293, and 1.98- and 2.87-fold in MCF-7, respectively (Fig. 3A). These data suggest that the FOSB gene is deregulated by both E2 variants, independently of the cell context. Subsequently, it was found that E2-AsAi consistently upregulated EPS15 with an enrichment of 26.32-fold in C33A, 2.66-fold in HEK293 and 3.37-fold in MCF-7 cells (Fig. 3B); CASP4, with an enrichment of 48.41-fold in C33A, 1.38-fold in HEK293, and 1.19-fold in MCF-7 cells (Fig. 3C); EPHA2, with an enrichment of 15.28-fold in C33A, 1.55-fold in HEK293 and 1.12-fold in MCF-7 cells (Fig. 3D); and RAB4B, with an enrichment of 23.40-fold in C33A, 1.39-fold in HEK293, and 1.33-fold in MCF-7 cells (Fig. 3E). Meanwhile in E2-Af-expressing cells, TRAF1 was upregulated, attaining an enrichment of 1.52-fold in C33A, 1.69-fold in HEK293, and 3.37-fold in MCF-7 cells (Fig. 3F). These results show that the effect of E2 variants on gene regulation was maintained in different cell contexts. However, in some of the genes that were evaluated, the effects differed according to the cell context. For instance, CASP4 was downregulated in E2-Af-expressing C33A and MCF7cells (Fig. 3C), while its expression was upregulated in E2-Af-expressing HEK293 cells. Therefore, the analysis of microarrays in different cellular contexts shows the most pronounced effect with the E2 variants, although some effects in a particular cellular context may not be obvious.

It has been demonstrated previously that expression of the HPV18 E2 protein affects cellular processes such as proliferation, cell death, and oxidative stress [27–29]; therefore, we were interested in examining differences between E2 variants, particularly in the transcriptional modulation of genes involved in cell death. Data obtained from microarray assays were analysed using the iPathwayGuide program for Next-gen pathway analysis, and Advaita's proprietary "Impact Analysis" method was applied. Then, the GO analysis allowed us to identify genes involved in apoptosis that were differentially expressed between cells containing E2-AsAi or E2-Af proteins. All of the genes identified in this analysis are listed in Supplementary Tables S4 and S5. These analyses showed that cells expressing the E2-AsAi variant had a

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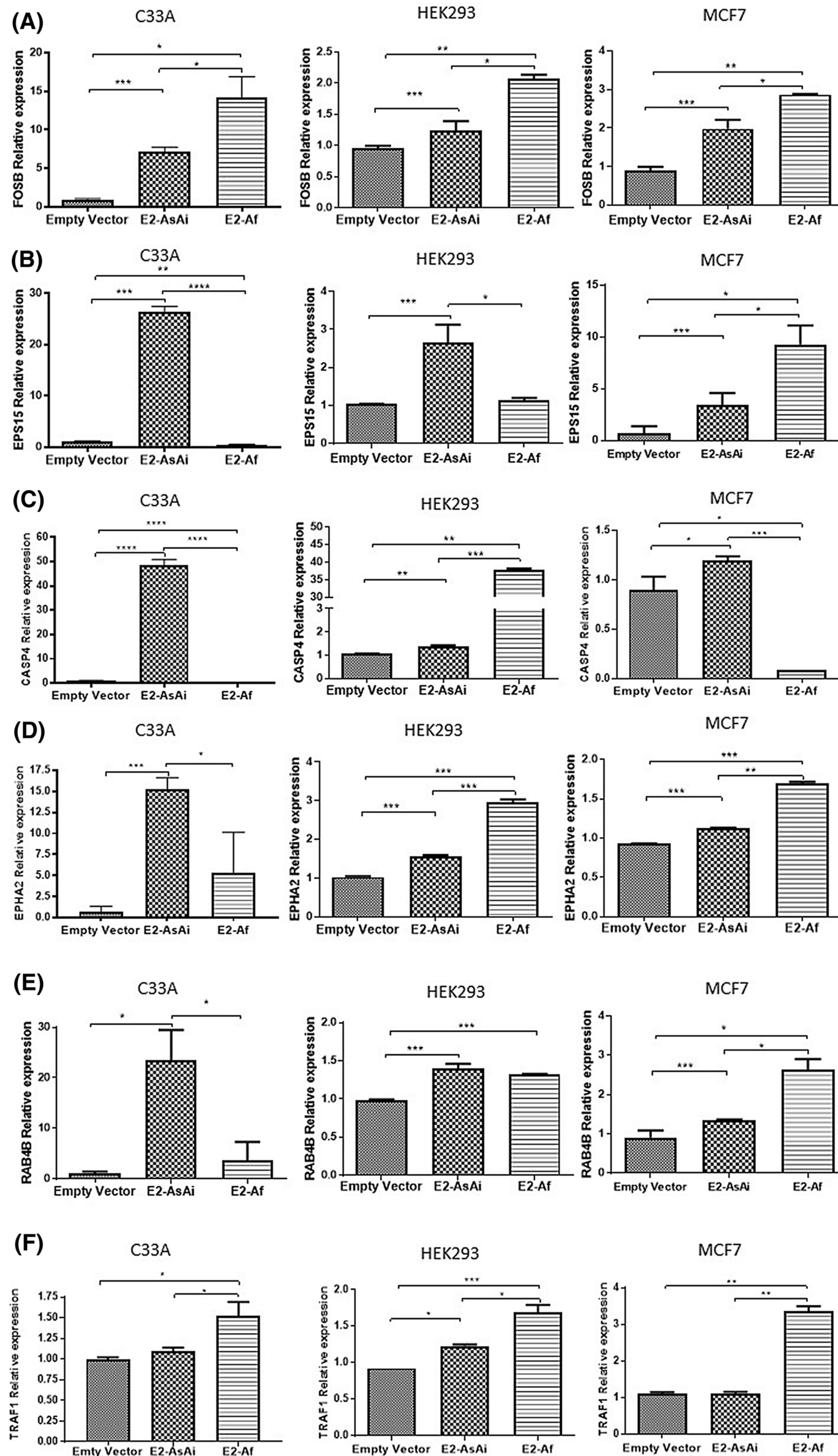

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◂**Fig. 3** Confirmation of differential regulation of genes by HPV18 E2-AsAi and E2-Af variants. qPCR assays were carried out in E2-variant-expressing cells to evaluate relative gene expression of FOSB (A), EPS15 (B), CASP4 (C), EPHA2 (D), RAB4B (E), and TRAF1 (F) in C33A, HEK293 and MCF7 cells as indicated. The 18 s mRNA housekeeping gene was used to normalize gene expression. The relative expression analysis was performed using the ΔΔCt method and depicted in each graph. The experiments were carried out in triplicate. Data are expressed as the mean ± SD. Student's *t*-test was performed to determine the statistical significance among E2 variants and the empty vector. *, $p<0.05$, **, $p<0.001$ ***, $p<0.0001$ and ****, $p<0.00001$

358 larger number of deregulated death-related genes, with 39 genes upregulated and 56 downregulated (Supplementary Fig. S2A), while for the E2-Af variant, only seven of those genes were upregulated and eight were downregulated (Supplementary Fig. S2B).

363 To visualize the impact of E2 variant proteins on cell signalling pathways, we used the KEGG-Path v.3. bioinformatics software. With this analysis, we identified some of the genes involved in the canonical apoptotic pathway that were differentially affected by the E2 variants, as illustrated in Fig. 4. E2 variants modulate the expression of different genes related to cell death, and the data suggest that E2-AsAi may favour the extrinsic apoptotic pathway, while E2-Af may favour the intrinsic apoptotic pathway. These results suggest that the HPV18 E2 intratype variants promote different expression profiles affecting common cell signalling pathways.

375 HPV18 E2 variants differentially affect cell viability and apoptosis

377 Induction of apoptosis by the HPV E2 proteins has been demonstrated previously [15–17, 27, 29]. To investigate whether the E2-AsAi and the E2-Af variants might differentially modulate biological processes, C33A cells were chosen as a cervical cancer model to evaluate the effect of such variants in cell viability and apoptosis. Western blot analysis showed that E2 variant proteins are expressed at similar levels in C33A cells (Fig. 5A and B).

385 Cell viability was assessed in E2-transfected cells through crystal violet dye assay. The results show a significant reduction in the viability of C33A cells expressing the E2 variants compared with those transfected with the control vector (EV) (Fig. 5C), with mean percentages of viable cells of 77.63% for E2-AsAi (**, $p<0.01$) and 46.27% for E2-Af (***, $p<0.001$). Interestingly, a more evident decrease in cell viability was observed in cells harbouring E2-Af compared to cells with the E2-AsAi variant (***, $p<0.001$). These results demonstrate that the E2 variants that were tested differentially reduce cell viability. We then used an annexin V-FITC assay to determine if this result could be associated with different effects on apoptosis. Figure 5D shows the flow cytometry results obtained from a single representative experiment, where both E2 protein variants induce apoptosis compared to the control (EV). The mean number of annexin-positive cells obtained from three independent experiments is shown in Figure 5E, where annexin V 29.4% of the cells transfected with E2-AsAi were positive (*, $p<0.05$) and 53.3% of the cells expressing E2-Af were positive (*, $p<0.05$), which was similar to the number that were positive when treated with chelerythrine (50.5%) as apoptotic inductor. Interestingly, we found that the African E2 variant induced apoptosis more strongly than the AsAi variant (*, $p<0.05$), which agrees with the observed decrease in cell viability (Fig. 5C). Taken together, these results suggest that HPV18 E2-AsAi and E2-Af variants differentially affect apoptosis, which could be related to differences found in gene expression. 413

Discussion 414

415 The HPV E2 protein plays an important role in replication, transcription, and genome segregation during the viral life cycle in productive and persistent infections [9]. During the carcinogenic process induced by HPV, E2 expression is commonly lost [30]. Moreover, it has been demonstrated that E2 is present in premalignant cervical lesions, but its expression is reduced in cervical cancer [31].

422 It has been demonstrated that E2 is involved in the regulation of cellular processes through protein-protein interactions, some of which are involved in transcriptional regulation, highlighting the role of E2 as a transcription factor [13, 22]. The ability of E2 to regulate transcription has been demonstrated by different approaches [18, 20, 21, 32].

428 Among the different risk factors associated with cancer progression, intratype variations have been proposed to influence the clinical outcome of HPV infections [33]. Variations in the HPV genome have been identified in several HR-HPV types, which differ in 2 to 5% of their nucleotide sequences along the complete genome [2]. The distribution of intratype variants of the most prevalent HPV types has been extensively addressed in epidemiological studies [34–36], leading to the conclusion that intratype variations of HPV16 and HPV18 may be predictors of progression to high-grade cervical lesions [6, 37]. Additionally, an epidemiological study found an association of variations in the E6 and E2 genes with the persistence of HPV16 infections [38].

441 Some studies have indicated that HPV intratype variations differentially impact biological processes, such as cell proliferation, angiogenesis, cell adhesion and apoptosis [5, 8, 39–41]. In particular, it has been demonstrated that variations in E2 differentially affect viral transcription [24, 42, 43], although their impact on cellular processes has not been analysed previously. 447

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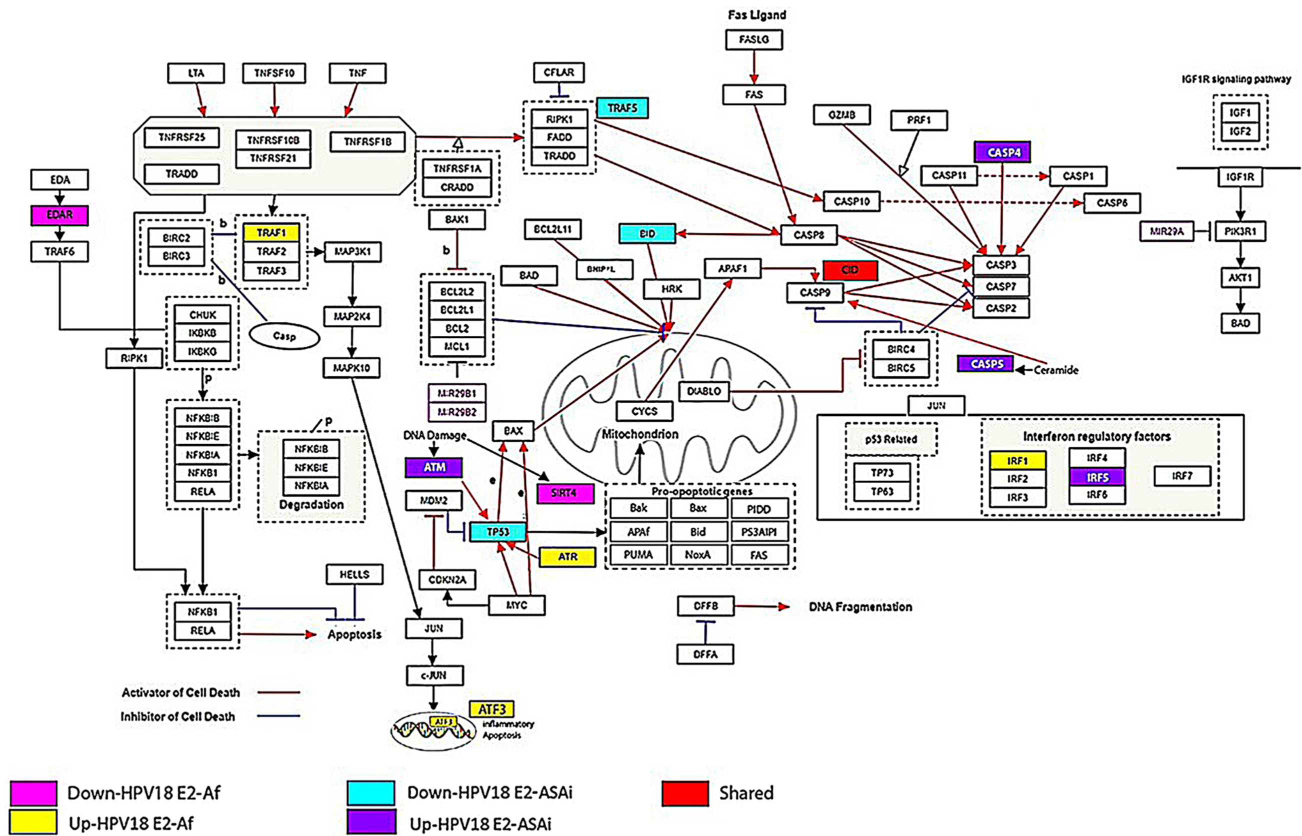

Fig. 4 Cell death elements regulated by the HPV18 E2 variants. Enrichment analysis of genes associated with apoptosis. Upregulated transcripts induced by the Asian-Amerindian E2 variant are depicted in purple boxes on the map, while downregulated genes are shown in aqua boxes. Cellular upregulated transcripts induced by the African E2 variant are shown in yellow boxes, and those that are downregulated are shown in pink boxes. Genes shared by both intratype variants are indicated in orange

It has been demonstrated that the expression of E2 affects cellular processes such as cellular proliferation or death [15, 27, 29, 44]. Several studies have shown that the E2 proteins from high-risk HPV types can induce apoptosis independently of their repressive activity on E6 and E7 expression [29, 44]. For instance, the HPV 16 E2 protein has been shown to induce apoptosis in a number of HPV-negative cell lines [29]. Moreover, some authors have suggested that a direct interaction of HPV16 E2 protein with p53 participates in induction of apoptosis, since mutations preventing HPV16 E2-p53 binding fail to induce apoptosis in non-HPV-transformed cell lines [44, 45]. Our results indicate that both HPV18 E2 variants used in this study induced apoptosis in the HPV-negative C33A cell line, which contains a mutated p53 that lacks DNA-binding capability [46]. Therefore, this effect is independent of p53 function. Our results also agree with a previous report that demonstrated that the HPV18 E2 protein can induce apoptosis in the HPV-negative cell line Saos-2 in the absence of p53 [27]. Previously, we demonstrated that HPV18 E2-AsAi and E2-Af variants do not differ in their ability to regulate LCR transcriptional activity [24]. However, in the present work, the ability to induce apoptosis was higher for the E2-Af variant, which could be related to a less-aggressive phenotype.

Different mechanisms of E2 apoptotic activity have been proposed. For instance, HPV16 E2 binds to the anti-apoptotic regulator c-FLIP, abrogating its function and sensitizing the cells to apoptotic signals [16]. Additionally, it has been demonstrated that HPV18 E2 interacts directly with caspase 8, leading to its activation [15].

In the present study, we identified cellular genes and biological processes that are differentially regulated by intratypic variants of HPV18 E2. Our results show that the E2-AsAi and E2-Af variants both significantly decreased cell viability and induced apoptosis in C33A cells, although the E2-Af variant had a stronger effect (Fig. 5C-E). We previously reported that the variant E2-Af and -AsAi proteins differ in their amino acid sequence [24] (Fig. S1), which could be responsible for their biological behaviour. Since those changes occur throughout the entire E2 protein, their effects on protein conformation could differentially impact both the induction apoptosis and gene expression profiles. Nevertheless, further studies are needed to determine whether those transcription profiles directly affect apoptosis. The

Journal : Large 705 | Article No : 4124 | Pages : 13 | MS Code : ARVI-D-18-00632 | Dispatch : 11-12-2018

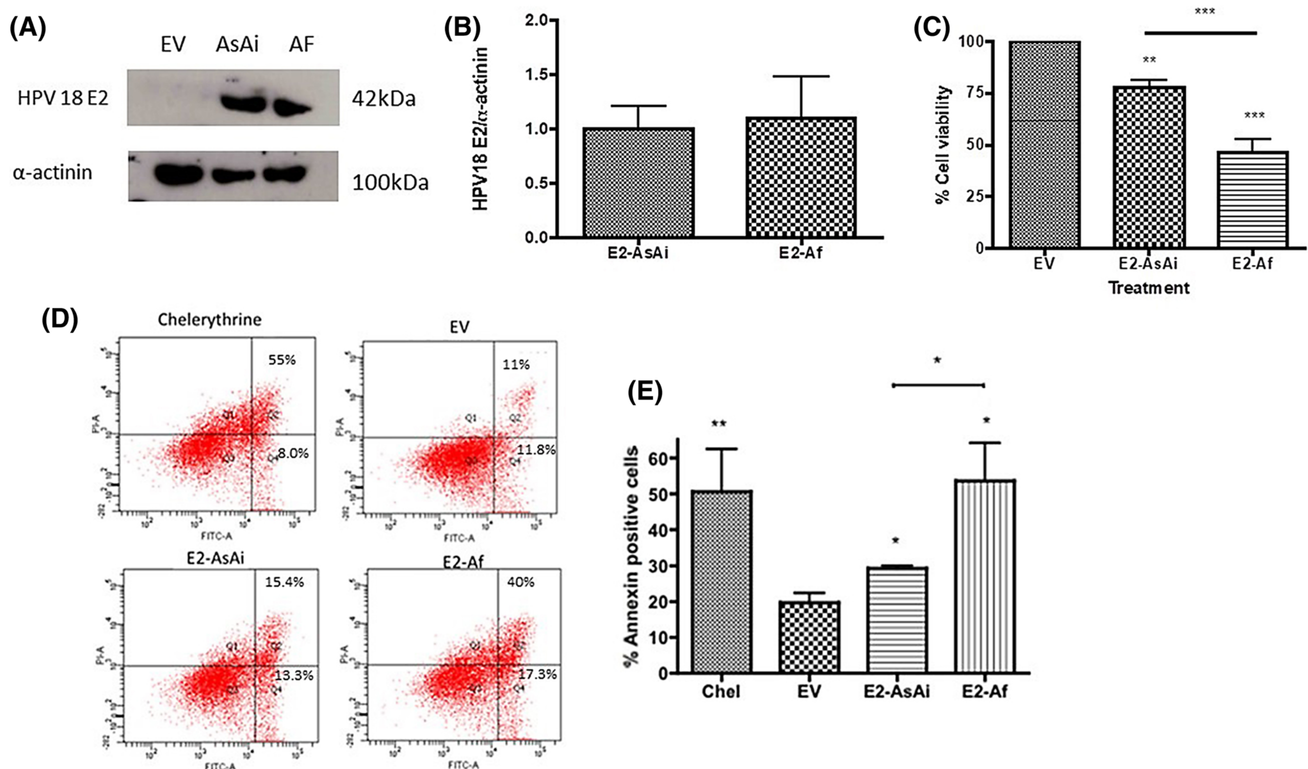

Fig. 5 HPV18 E2 variants differentially affect cell viability and apoptosis. A) Representative immunoblot and B) densitometry analysis of protein levels of HPV18 E2 variants expressed in C33A cells. No differences were observed in the levels of E2 variant proteins. C) Cell viability decrease in the presence of HPV18 E2 variants. Analysis was performed using the ANOVA test followed by Tukey's *post hoc* test. Empty vector (EV) was compared to all of the treatments (**, $p<0.001$ EV vs. E2-AsAi; ***, $p<0.0001$ EV vs. E2-Af, ***, $p<0.0001$ E2-AsAi vs. E2-Af). D) Flow cytometric analysis of annexin-V-FITC-positive cells of one representative experiment and E) percentage of the mean (±SD) of three independent experiments of annexin-V-FITC positive cells for HPV18 E2-variant-expressing cells, control vector (EV), and as apoptotic positive control, chelerythrine-treated cells (Chel). Student's *t*-test showed a significant difference between E2-Af and E2-AsAi (*, $p<0.05$) and both with the empty vector; while the *p*-value for *EV* vs. Chel was <0.01

individual contribution of deregulated genes to the apoptotic effect would be an interesting issue to evaluate, but it should be considered that the biological effect of E2 could be due to the sum of effects of several deregulated genes.

Previous results showed that infection with HPV18 belonging to the Af branch was mostly associated with normal cervical epithelia and premalignant lesions, suggesting a less aggressive outcome for lesions harbouring this variant, which hints that altered functions of the HPV18-Af variant proteins may influence its potential in viral carcinogenesis [6]. It would be interesting to investigate whether premalignant cervical lesions or cervical cancer biopsies harbouring the HPV18-Af variant exhibit differences in clinical and pathological features, including apoptotic rate, compared to non-Af HPV18 variants. Thus, identifying possible functional differences between variant HPV18 proteins could help to understand the reason for different outcomes of HPV infections with the same viral type.

It has been demonstrated previously that E2 interacts with several cellular factors [18, 20, 21, 32, 47]. For instance, many E2-interacting proteins are transcription, replication, or chromatin-remodelling factors or histone-modifying enzymes [13, 19], and such binding could affect cellular gene expression.

The participation of E2 in induction of apoptosis has been well described [16, 27, 48–50], and we were therefore interested in determining if the intratype variations in HPV18 E2 proteins modified transcriptional profiles associated with apoptosis pathways. In this work, we demonstrated that cells expressing Asian-Amerindian and African intratype E2 variants from HPV18 produced different mRNA profiles, affecting many cellular pathways such as metabolic regulation, cell proliferation, and death, among others. Some of those genes were confirmed by qPCR, and as expected, the results showed agreement with the microarray data (Fig. 3). The use of biological triplicates in analysis of microarrays allowed us to identify the most pronounced effects of the E2 variants in different cellular contexts, although some of the effects in a particular cellular context may be hidden in this type of analysis. Nevertheless, significant information

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is obtained using this kind of analysis, and our results show that significant common changes were induced in all three cell lines expressing E2 variants, were confirmed using a sensitive qPCR assay. This approach is less stringent than using a single cell type. Our data could help to understand common pathways in HPV infections at different anatomical sites and in different physiological contexts.

Our microarray data are in agreement with those of previous studies, where the HPV16 E2 protein has been shown to regulate the expression of genes involved in apoptosis and cell proliferation [22]. The authors observed that the expression of a large number of genes participating in the Wnt pathway was disturbed by HPV16 E2. Interestingly, we found that HPV18 E2-AsAi downregulates the expression of Wnt5A, which is involved in the activation of the non-canonical Wnt pathway; while E2-Af downregulates Wnt4, which is related to the activation of Wnt-β-catenin, indicating that regulation of the Wnt pathways could be a common feature of HR-HPV types.

It has also been observed that RAB family genes are deregulated by HR-HPV types. Ramirez-Salazar *et al.* [24] found several members, such as RAB25, RAB31 and RAB38, in HPV16-E2-expressing cells, while our study showed that RAB23 and RAB25 were deregulated by E2-AsAi and that RAB41 was deregulated by E2-Af. It is worth motioning that those genes have been associated previously with cancer [51]. It is possible that some actions of HPV E2 proteins are shared among the different HPV types, although there are several cellular processes that can be differentially affected.

We also found a large number of cellular genes that were exclusively downregulated by the expression of the HPV18 E2-AsAi variant (Table S3), including PRKCB, EPS15, BID, RNF38, ATP1B1, CTPS2, which are involved in oxidative stress, apoptosis and proliferation – processes related to cancer development [52–54]. We also found that the HPV18 E2-AsAi variant induced the overexpression of CASP4, RAB4B, PRSS22, SCD5, CLCF1, TMEM173 (STING), which are involved in cell apoptosis, vesicular trafficking, and cell immunity [55, 56]. In contrast, those genes were not affected by the presence of the E2-Af variant.

Moreover, HPV18 E2-AsAi upregulated IRF5, which participates in the host immune and autoimmune responses and plays a role in cell growth and apoptosis [57]. Meanwhile, HPV18 E2-Af upregulated IRF1, which is known for its tumour-suppressing activity and inductor of apoptosis [58]. Particularly, the E2-AsAi variant regulates the expression of CASP4, IRF5, TRAF5, CID, CASP5, EPHA2 and Bid genes, which are regulators of the canonical apoptotic pathway, whereas the E2-Af protein regulates the genes TRAF1, ATF3, ATR, CAMK2B, CID, and IRF1, which are also involved in apoptotic pathways as well as DNA damage repair. Therefore, gene profiles induced by the HPV18 E2 variants could explain the differences observed in apoptosis and cell viability, although both variants participate in the same processes.

In the tight regulation of the course of a natural HPV infection, different processes are affected by HPV proteins whose expression varies among the different stages of the productive cycle [59]. The capacity of HPV proteins to modulate cellular processes in an antagonistic manner has been studied extensively [60–62]. For instance, the activation of apoptotic molecules such as caspases is associated with a higher viral replication rate [30]. Moreover, although E6 and the E2 proteins promote caspase activation, only E2 induces apoptosis [63, 64]. Notably, the E6 oncoprotein protects against cell death not only by degrading p53 [65] but also by promoting cell proliferation [66]. However, overexpression of E7 sensitizes primary keratinocytes to apoptosis in response to TNF stimulation, while this effect is not observed in E7-transfected human fibroblasts, even when treated with TNF [63], suggesting that the induction of apoptosis by E7 may be related to the cell context. It is important to consider that the apoptotic process could be inhibited or induced at different stages during an HPV infection to complete the viral life cycle, and such regulation could rely on the amount of viral proteins available. Further studies are needed to clarify this.

So far, it is not clear whether E2-induced apoptosis favours the viral replicative cycle or, conversely, participates in the establishment of cancer. It has been suggested that through the induction of apoptosis, E2 could contribute to the viral replication process and stimulate the cellular immune response, inducing spontaneous regression of HPV-related lesions [21]. In this case, an E2 variant with a stronger ability to induce apoptosis, as is shown for E2-Af (Fig. 5), might affect the clearance of a persistent infection. Thus, HPV variants may contribute to differences in clinical outcomes of HPV infections through their ability to regulate cellular processes such as proliferation or cell death. More studies should be performed to determine the causes of the differential effect on apoptosis observed with different HPV18 E2 variants.

Conclusions

Our results show that HPV18 E2 intratype variants differentially modulate gene expression profiles. Ontological analysis indicates that several pathways and cellular processes are altered by the expression of HPV18 E2 intratypic variants that significantly affect apoptotic pathways. Importantly, our results suggest a correlation between differences in E2-induced gene profiles and E2-induced apoptosis. The E2-Af variant protein showed the strongest effect on induction of apoptosis. These results suggest that HPV18

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E2 intratypic variations could be associated with different outcomes of HPV18 infections.

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Compliance with ethical standards

Conflict of interest There are no commercial or financial conflicts of interest to declare.

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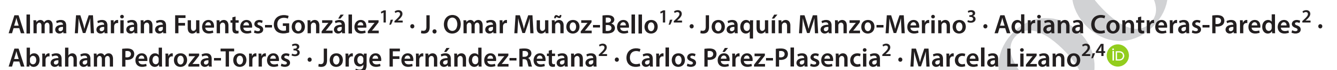
Affiliations



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