The E6 Oncoprotein from HPV16 Enhances the Canonical Wnt/β-Catenin Pathway in Skin Epidermis In Vivo

José Bonilla-Delgado1, Gúlyay Bulut3, Xuefeng Liu4, Enoch M. Cortés-Malagón1, Richard Schlegel4, Catalina Flores-Maldonado2, Rubén G. Contreras2, Sang-Hyuk Chung6, Paul F. Lambert5, Aykut Üren3, and Patricio Gariglio1

Abstract

The contribution of the Wnt signaling pathway to human papilloma virus (HPV)-induced carcinogenesis is poorly understood. In high-grade dysplastic lesions that are caused by high-risk HPVs (HR-HPV), β-catenin is often located in the cell nucleus, which suggests that Wnt pathway may be involved in the development of HPV-related carcinomas. Most of the oncogenic potential of HR-HPVs resides on the PDZ-binding domain of E6 protein. We hypothesized that the PDZ-binding domain of the HPV16-E6 oncoprotein induces the nuclear accumulation of β-catenin due to its capacity to degrade PDZ-containing cellular targets. To test this hypothesis, we evaluated the staining pattern of β-catenin in the skin epidermis of transgenic mice expressing the full-length E6 oncoprotein (K14E6 mice) and measured LacZ gene expression in K14E6 mice that were crossed with a strain expressing LacZ that was knocked into the Axin2 locus (Axin2−−/LacZ mice). Here, we show that the E6 oncoprotein enhances the nuclear accumulation of β-catenin, the accumulation of cellular β-catenin–responsive genes, and the expression of LacZ. None of these effects were observed when a truncated E6 oncoprotein that lacks the PDZ-binding domain was expressed alone (K14E6ΔPDZ mice) or in combination with Axin2−−/LacZ. Conversely, cotransfection with either E6 or E6ΔPDZ similarly enhanced canonical Wnt signaling in short-term in vitro assays that used a luciferase Wnt/β-catenin/TCF-dependent promoter. We propose that the activation of canonical Wnt signaling could be induced by the HPV16-E6 oncoprotein; however, the participation of the E6 PDZ-binding domain seems to be important in in vivo models only.

Introduction

Human papilloma viruses (HPV) are small DNA tumor viruses that cause the formation of warts and papillomas in several stratified epithelia such as those of the skin, genitalia, and upper respiratory tract (1). Epidemiologic and experimental data have shown that the “high-risk” HPVs (HR-HPV), such as type 16 and 18, contribute to HPV-related cancer development (2). These HR-HPVs owe their oncogenic potential to the constitutive expression of the E6 and E7 oncoproteins whose products enhance cell proliferation and perturb cell differentiation through several mechanisms (3). E7 promotes cell proliferation by associating with the tumor suppressor pRb and other cell-cycle–regulatory proteins (4), and E6 binds to and inactivates p53 tumor suppressor protein and several proteins that participate in cell–cell adhesion (5).

The progression of HPV-related cancers clearly involves dysplastic lesions that are caused by the E6 and E7 oncoproteins; nevertheless, some of the molecular events that are regulated by these oncoproteins are not fully understood (3). To date, cell culture experiments have shown that the exogenous expression of HR-E6 proteins induces the immortalization of human mammary epithelial cells (6). Furthermore, transgenic mice that express the HPV16-E6 oncoprotein in the basal stratified epithelium under the control of the human keratin 14 promoter (K14E6 mice) develop papillomas and cancer of the skin epidermis (7). The in vivo tumor capacity of E6 is dramatically reduced when a truncated version of E6 that lacks the PDZ-binding domain (K14E6ΔPDZ, transgenic mice) is expressed in the basal stratified epithelium of transgenic mice (8). Although p53 is
the most studied cellular target of E6, its participation in skin carcinogenesis seems to be minor in comparison with other E6 cellular targets, such as those that contain PDZ domains (7, 8). Recent experimental evidence suggests that the canonical Wnt signaling pathway may play a key role in the oncogenic potential of E6 (9).

Wnt ligands control several differentiation processes during normal tissue homeostasis (10, 11), and they are implicated in pathologic conditions such as in certain types of cancer (12, 13). In the absence of Wnt ligands, β-catenin forms a “degradation complex” with kinases and scaffold proteins, such as glycogen synthase kinase-3β (GSK3β), casein kinase 1 and 2 (CK1 and CK2), adenosomatous polyposis coli (APC), and Axin2. This degradation complex phosphorylates β-catenin at serine and threonine residues inducing its ubiquitination by β-TcRP ubiquitin ligase and degradation. The activation of canonical Wnt signaling induces the phosphorylation of the intracellular Dishevelled (Dvl) protein, which eventually interacts with Axin2 and impedes the formation of the β-catenin degradation complex; this process leads to the accumulation and nuclear translocation of β-catenin. Once it is translocated into the nucleus, β-catenin binds members of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors, of which TCF4 is the best characterized. β-Catenin/TCF4 complexes control the expression of several target genes that regulate cell polarity, proliferation, and differentiation (12) including c-jun (13), c-myc (14), cyclin D1 (15), multidrug resistance 1 (16), matriixine (17), Axin2 (18), and survivin (13) and others (http://www.stanford.edu/~rnusse/pathways/targets.html).

The participation of canonical Wnt pathway in cervical cancer is evident because the nuclear accumulation of β-catenin correlates with tumor progression in human patients (19). Nuclear β-catenin is commonly found in human HPV16-positive invasive carcinoma biopsies, but it is uncommon in early dysplastic lesions (20–22). In addition, recent in vitro evidence supports the hypothesis that HR-E6 alone can enhance canonical Wnt signaling (9).

In this study, we present for the first time in vivo evidence supporting the hypothesis that the HR-E6 protein enhances canonical Wnt signaling. However, the importance of the E6 PDZ-binding domain remains controversial; although it is necessary for Wnt pathway activation in transgenic mice, it is not required in cultured COS7 cells or primary keratinocytes, indicating that other factors present in mice are required for Wnt signaling activation.

Materials and Methods

K14E6, K14E6ΔPDZ, and Axin2+/−/LacZ mice and sample isolation

The construction of transgenic mice containing the full version of the HPV16-E6 oncogene (K14E6), the truncated version lacking the PDZ-binding domain (K14E6Δ146–151 or K14E6ΔPDZ), and the knock-in of Axin2+/−/LacZ has been described previously (7, 8, 23). The mice were housed and treated according to the American Association of Laboratory Animal Care (AALAC) regulations, and the research protocols were approved by the Research Unit for Laboratory Animal Care Committee (UPEAL-CINVESTAV-IPN, Mexico; NOM-062-ZOO-1999). Skin biopsies from the transgenic strains and FvB nontransgenic mice (NTG) were resected, fixed, and paraffin-embedded for the histologic procedures or they were immediately frozen in liquid nitrogen for protein or RNA isolation (TRizol method). For the histologic procedures (immunohistochemistry and immunofluorescence), 5-μm thick transversal sections were mounted on charged microscope slides (Fisher Scientific) for immunohistochemistry or immunofluorescence techniques.

Immunohistochemistry and immunofluorescence procedures

The skin sections were deparaffinized and rehydrated as described previously (7). The protein detection for immunohistochemistry was conducted using the Mouse/Rabbit PolyDetector HRP/DAB Detection System (Bio SB) according to manufacturer’s recommendations. The samples were incubated overnight with primary antibodies against proliferating cell nuclear antigen (PCNA), p53, Dlg1, or Scribble primary antibodies (Santa Cruz Biotechnology) diluted at 1:100. Following the immunohistochemical procedures, the tissues were counterstained with hematoxylin and mounted in GVA-mount reagent (Zymed). For the immunofluorescence procedures, the skin sections were rinsed in 1× PBS and blocked for 2 hours at 4°C with 1× PBS that was supplemented with 0.3% Triton X-100 and 1% bovine serum albumin; they were washed 3 times with 1× PBS and incubated for 1 hour at 37°C with an anti-β-catenin antibody (Santa Cruz Biotechnology). The sections were then incubated with a fluorescein isothiocyanate (FITC)-labeled secondary antibody (Zymed) for 30 minutes at room temperature; they were rinsed above, counterstained with propidium iodide, and mounted in Vectashield (Vector). The preparations were examined by confocal microscopy using an SP2 (Leica Microsystems). Captured images were imported into the ImageJ software program (version 1.37v, NIH, Bethesda, MD) to produce maximum projections, and Adobe Photoshop (Adobe Systems) was used to equalize the brightness and contrast in all of the images. The positive signals in the immunohistochemistry and immunofluorescence images were quantified from 4 different animals per experiment (amplification, 40×; 3 sections per mouse) using the Image-Pro Plus 7.0 software program (Media Cybernetics), and Student t statistical test (*, P<0.05; **, P<0.01; and ***, P<0.001) was conducted using the SPSS 13.0 software package (IBM).

Wnt-responsive Axin2+/−/LacZ mice assays

Axin2+/−/LacZ mice were crossed to NTG, K14E6, or K14E6ΔPDZ mice. Skin tissues were resected from the resulting 7-month-old double transgenic mice and immediately embedded in paraffin for the histologic procedures. The skin sections were fixed (0.5% glutaraldehyde, 1.25 mmol/L EGTA, and 2 mmol/L MgCl2 in 1× PBS, pH = 7.3), washed with 1× PBS that contained detergents (0.01%
sodium deoxycholate and 0.02% Igepal CA-630) and 2 mmol/L MgCl₂, and then incubated in LacZ staining buffer (0.6 mg/mL X-gal, 4 mmol/L potassium ferrocyanide, and 4 mmol/L potassium ferricyanide in wash buffer). The tissues were subsequently counterstained with Nuclear Fast Red and mounted with Permount.

Relative mRNA quantification by real-time quantitative PCR and data analysis using the \(2^{-\Delta\Delta Ct}\) method

Quantitative real-time PCR (qRT-PCR) was carried out using a LightCycler 2.0 apparatus (Roche) and a DNA Master SYBR Green I kit (Roche). The templates were amplified in 45 cycles of a 3-step PCR process, which included 30 seconds of denaturation step at 95°C, a 30-second primer-dependent annealing phase (60°C), and a 30-second template-dependent elongation at 72°C. The amplification of each template was conducted in duplicate in one PCR run. The differential expression of the investigated genes was calculated as a ratio normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression. The data were analyzed using the equation that was previously described by Livak and Schmittgen (amount of target = \(2^{-\Delta\Delta Ct}\); ref. 24).

Luciferase assays

COS7 cells were plated in 12-well tissue culture plates and grown for 24 hours. The cells were then cotransfected with E6, E6ΔPDZ (aa146–151 deleted), E7, Dvl2 (in the pCS2 vector that included a Flag tag and was kindly provided by Dr. Jeff Rubin), β-catenin S37A (pFlagCMV2, which was kindly provided by Dr. Stephen Byers), or the empty vector control alone or together with Super8XTOPFlash (which contains 8 copies of TCF/LEF-binding sites upstream of the luciferase gene and was kindly provided by Dr. Randall Moon) and the Renilla-TK construct (transfection control, which was kindly provided by Dr. Stephen Byers) using FuGENE6 according to the manufacturer's instructions (Roche Applied Science). All of the E6 and E7 gene–related constructs were cloned into pJS55 vector and tagged with an AU1 epitope at the C-terminus. The Dual-Luciferase Reporter Assay was conducted 24 hours after the transfection according to the manufacturer’s protocol (Promega).

Immunoprecipitation

COS7 cells were cotransfected with Dvl2, E6, E6ΔPDZ, or the empty vector alone or together by using FuGENE6. Twenty-four hours after transfection, the cells were lysed with phospho lysis buffer (50 mmol/L HEPES, pH 7.9, 100 mmol/L NaCl, 4 mmol/L NaPP, 10 mmol/L EDTA, 10 mmol/L NaF, 1% Triton X-100, 2 mmol/L fresh vanadate, 1 mmol/L phenylmethylsulfonylfluoride, 2 µg/mL aprotinin, and 2 µg/mL leupeptin). One milligram of the protein lysate protein was subjected to
overnight immunoprecipitation with an anti-Dvl2 antibody (Santa Cruz) and this was followed with a 1-hour incubation with protein G-agarose beads (Invitrogen). The immunoprecipitates were subjected to SDS-PAGE and transferred to an Immobilon-P membrane (Millipore). The membrane was blocked in 5% nonfat dry milk in 1× TBS (20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, and 0.5% Tween-20) for 2 hours, blotted with an anti-Dvl2 (1:1,000) or anti-AU1 (1:1,000; Covance) primary antibody overnight, and then incubated with a horseradish peroxidase (HRP)-linked anti-mouse or anti-rabbit secondary antibody (GE Healthcare) for 1 hour. The membranes were developed using the Millipore Immobilon Western Chemiluminescent HRP Substrate according to the manufacturer’s instructions. Chemiluminescence was detected using a FujiFilm LAS-3000 imaging system.

Results

The PDZ-binding domain of E6 is required to maintain skin hyperplasia in adult transgenic mice

K14E6 and K14E6ΔPDZ mice were previously reported to express the complete and truncated E6 oncoprotein in the basal layer of stratified epithelia, respectively (7, 8). However, of the 2 transgenic strains, only the K14E6 mice developed skin hyperplasia and skin cancer at advanced ages (7, 8). On the basis of this observation, we first compared the cell proliferation pattern in the skin epidermis for the E6 transgenic strains and the NTG mice at juvenile and adult ages (Fig. 1A).

The immunostaining of PCNA, a cell proliferation marker, shows that cell proliferation at a young age was quite similar in the K14E6 and K14E6ΔPDZ mice. However, unlike the K14E6ΔPDZ or NTG mice, only the K14E6 adult mice retained suprabasal cell proliferation (Fig. 1A). It was previously reported that both transgenic E6 strains display equally diminished p53 protein expression in adult animals (8). We also found that both of the transgenic strains can degrade the p53 protein in younger animals after UV light irradiation (Fig. 1B). Therefore, the cell proliferation differences between the 2 transgenic strains are not related to differences in p53 degradation.

To test the functionality of the PDZ-binding domain of E6 in adult mice, we examined the levels of Dlg1 and Scribble (Scrib), which are 2 well-described PDZ cellular targets of HR-E6. Importantly, these targets participate in processes such as cell–cell junction formation and canonical Wnt signaling activation (25). We observed that the K14E6 mice displayed lower levels of both cellular proteins than in the K14E6ΔPDZ counterpart and NTG mice (Fig. 2).

Therefore, it is possible that the oncogenic effects that were observed in the adult skin epidermis of the K14E6 mice could be partially related, in part, to cellular processes regulated by those PDZ-containing targets including the Wnt pathway.
The PDZ-binding domain of E6 induces nuclear β-catenin protein accumulation and enhances Wnt signaling pathway

To evaluate whether canonical Wnt signaling is activated by the E6 PDZ-binding domain, we detected β-catenin in skin slices from mice at different ages. In 8-day-old mice, β-catenin was not apparent in the cell nucleus; however, from the age of 4 months onward, an increased amount of β-catenin level was detected in the nuclei of the K14E6 mice and the most pronounced accumulation was observed in 1-year-old mice (Fig. 3A). We next asked whether the nuclear β-catenin detected in the skin epidermis of adult mice was transcriptionally active. Axin2 is a negative feedback regulator of the Wnt pathway and is expressed in response to Wnt signaling (26). The insertion of the LacZ gene into the Axin2 locus (Axin2+/LacZ mice) mimics the expression pattern of Axin2, but it does not lead to a detectable Axin-deficient phenotype in the heterozygous state (27). Therefore, we crossed Axin2+/LacZ mice with K14E6, K14E6ΔPDZ, or control NTG mice to measure Wnt signaling activity in adult skin samples. LacZ was expressed more strongly in the skin epidermis of the Axin2+/LacZ/K14E6 double transgenic mice than in the Axin2+/LacZ/K14E6ΔPDZ or Axin2+/LacZ/NTG mice (Fig. 3B). We also measured the mRNA levels of 3 well-described β-catenin targets that participate in cell-cycle progression or cell survival such as c-myc, Birc5, and ccnd1 genes in adult skin. As shown in Fig. 4A, the mRNA induction of these target genes was enhanced in adult skin tissue of the K14E6 mice compared with the K14E6ΔPDZ mice. c-Myc expression was also validated by RT-PCR and Western blotting (Fig. 4B). Therefore, we concluded that Wnt signaling may be enhanced in vivo by the HPV16-E6 oncogene and that the PDZ-binding domain plays an important role in this induction.

Figure 3. β-Catenin nuclear cell accumulation and Wnt signaling induction. Skin tissues from the indicated mice were paraffin embedded. A, immunofluorescence detection of the β-catenin protein was conducted in 5-μm thick dorsal skin samples from mice of 3 different ages as indicated. Note that only the K14E6 mice gradually accumulated nuclear β-catenin, and the highest accumulation occurred at 1 year of age. B, Wnt/β-catenin signaling induction was evaluated by the Axin2-dependent expression of LacZ gene in the skin epithelia of 7-month-old double transgenic mice. Note that only the Axin2+/LacZ/K14E6 mice show maximal LacZ expression. A: Green signal, β-catenin; red signal, propidium iodide (PI) counterstaining; yellow signal, merged signal. B: Blue signal, LacZ activity; counterstaining was conducted with Fast Red. Magnification, 40×; Ep, epidermis; dashed line, basal membrane; scale bar, 30 μm; arrows, positive LacZ nuclear signal.
The E6 oncoprotein associates with Dvl2 and induces canonical Wnt signaling in vitro independently of the PDZ-binding domain in vivo

To analyze Wnt signaling induction in an in vitro system, we transiently transfected COS7 cells with a well-established functional assay vector that contains several copies of the TCF-binding site and regulates a luciferase reporter gene (the TOPFLASH construct). The transfection of the HPV16-E6 expression vector alone results in the minimal expression of the TOPFLASH vector; however, the combination of HPV16-E6 and a Wnt signaling inducer, such as β-catenin or Dvl2 (the Dvl isofrom that is preferentially expressed in the skin), clearly enhances TOPFLASH activity (Supplementary Fig. S1). Interestingly, only transfected HPV16-E6, but not HPV16-E7, cooperates with β-catenin or Dvl2 to trigger TOPFLASH induction (Supplementary Fig. S1).

To determine whether the E6 PDZ-binding domain is involved in Wnt pathway induction in vitro, we cotransfected COS7 cells with constructs expressing the complete HPV-E6 protein or a truncated version lacking the last 6 amino acids which comprise the PDZ-binding domain. In contrast to our in vivo observations and consistent with the report of Lichtig and colleagues (9), both versions of the E6 oncoprotein cooperated with β-catenin or Dvl2 and induced Wnt signaling (Fig. 5A). We also carried out these in vitro experiments with primary human foreskin keratinocytes and obtained similar results to those that were observed in the COS7 cells (Supplementary Fig. S2).

Dvl proteins play a central role in the regulation of both canonical and noncanonical Wnt signaling. There are 3 Dvl isofroms in mammals (28), all of them containing PDZ domains (29). Because E6 contains a PDZ-binding domain, it was possible to consider a PDZ connection with Dvl isofroms. To test this possibility, we immunoprecipitated E6 and blotted against the 3 Dvl isofroms (and vice versa) E6 coimmunoprecipitated with the Dvl2 protein but not with the Dvl1 or Dvl3 isofroms (Supplementary Fig. S3) which suggests that the E6 oncoprotein preferentially binds the Dvl isofrom that is expressed in the skin (30). We next tested the ability of E6 to interact with the Dvl2 protein via its PDZ domain using the E6 or E6ΔPDZ expression vectors. Interestingly, both of the E6 oncoproteins coimmunoprecipitated with Dvl2 (Fig. 5B), which suggests the presence of a PDZ-independent interaction. Therefore, E6 proteins that contain or lack the PDZ domain could enhance Wnt signaling and interact with the Dvl2 protein in short-term in vitro assays.

Discussion

The K14E6 mouse strains has been established as a model of papilloma-induced skin cancer (7). In this model, the expression of the HPV16-E6 oncogene was directed to the basal layer of stratified epithelia which includes the skin epidermis, lens, and cervical tissue, among others (7, 31). Although E6 is virtually expressed in all stratified epithelia, it only develops spontaneous dysplasias or tumors in the skin epidermis (not in other tissues such as the cervix; refs. 32, 33), which suggests that tissue-specific factors are critical for mediating the oncogenic potential of E6 (7, 8, 33). In an attempt to explain discrepancies that occur due to tissue type, we recently reported the global gene expression differences in the skin epidermis and cervical tissue in adult K14E6 mice (33). We found that the expression of genes that participate in cell adhesion and Wnt signaling were

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preferentially altered in the skin epidermis compared with the cervical tissue (33). On the basis of these observations, we used the skin epidermis of K14E6 mice as a functional model to study the relationship between the E6 oncoprotein and Wnt signaling.

The in vivo data that were obtained with the K14E6 mouse model suggests that the oncogenic potential of E6 in skin epidermis relies on its PDZ-binding domain (8, 34). In this study, we show that although K14E6 and K14E6ΔPDZ mice have a quite similar hyperproliferative skin phenotype at 8 days of age (Fig. 1A), only K14E6 mice retain the hyperproliferative skin phenotype, at 4 months of age, which suggests that the early hyperproliferative effects of E6 on the skin are independent of the PDZ-binding domain. However, the PDZ-binding domain is indeed required to maintain suprabasal cell proliferation in adult mice (Fig. 1).

Most of the previous studies about HPV-positive cervical lesions support the view that Wnt signaling represents a late-stage step in cervical carcinogenesis and that other factors are needed to activate this pathway (19–22, 35). Unlike its role in cervical tissues, E6 is sufficient to induce skin papillomas and cancer in K14E6 mice (7); however, similarly as to cervical tissue, Wnt signaling occurs only after PDZ-independent hyperproliferation. As shown in Fig. 3A, the adult K14E6 animals accumulated β-catenin in their skin cell nuclei, but the precise mechanism by which E6 enhances Wnt pathway is beyond the scope of this study. A possible mechanism by which E6 induces Wnt pathway activation may involve its effects on cellular targets that contain PDZ domains such as Dlg1 and Scribble. It is well-known that Dlg1 and Scribble proteins (both containing PDZ domains) participate in the zonula adherens formation (36), and they also interact with APC protein in a region necessary for tumor suppressor activity (37, 38). Furthermore, APC-hDlg complexes were shown to negatively regulate cell-cycle progression in NIH3T3 mouse fibroblast (39). Therefore, E6-induced degradation of the Dlg1 and Scribble (5) may lead to decreased APC activity and the nuclear accumulation of β-catenin.
It is difficult to know whether the slight reduction in Dlg1 that was observed in the K14E6 mice (Fig. 2) is sufficient to activate Wnt signaling or whether the partial loss of Dlg1 synergizes with the more sharply reduced Scribble protein levels (Fig. 2). Other evidence suggests that the reduction of Dlg1 alone may not lead to β-catenin nuclear accumulation (39). Therefore, Dlg1 reduction may not be the primary cause of Wnt signaling enhancement by E6. However, considering current data, we cannot completely discount a potential role for Dlg1 in Wnt signaling induction (at least in in vitro models). Importantly, in vitro models may not reflect the mechanisms that occur in vivo. Consistent with these findings, Cavatorta and colleagues also reported the presence of only a slight reduction in the hDlg protein in benign HPV-infected cervical lesions (in which nuclear β-catenin is uncommon), but they reported a more dramatic reduction in cervical cancers (in which nuclear β-catenin is frequent; ref. 40). Therefore, low levels of the hDlg protein and nuclear β-catenin may be considered as late-stage markers in HPV-related carcinogenesis (22, 40).

Short-term in vitro experiments in COS7 cells and primary human foreskin keratinocytes (Fig. 5 and Supplementary Figs. S1 and S2) showed that exogenous expression of the HPV16-E6 oncoprotein alone only weakly induced Wnt signaling, which was indicated by TOPFLASH induction, but it showed a significant effect in combination with exogenous β-catenin or Dvl2 expression. Lichtig and colleagues reported similar results in HEK293T cells, although their short-term experiments were carried out in a Wnt3a-enriched medium. Therefore, the E6 oncoprotein appears to enhance, but not to initiate, Wnt signaling (9). Consistent with this view, we did not observe any nuclear accumulation of β-catenin in the young K14E6 animals (Fig. 3A).

Concerning the role of the E6 PDZ-binding domain, the discrepancies between our in vitro and in vivo observations may be due to the use of transient (as opposed to stable) transfections and by the fact that E6 may alter cellular pathways in younger animals in a PDZ-independent manner. This work shows for the first time that HPV16-E6 can enhance canonical Wnt signaling in vivo and in vitro. However, our paradoxical findings about the PDZ-binding domain of E6 suggest that the interaction between E6 and the Wnt pathway is complex and that it may be dependent on the status of other signaling pathways. The precise mechanism for Wnt signaling activation remains unknown, and more studies are needed to clarify the mechanism by which E6 enhances Wnt signaling.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank Tziriakian Madrigal-Dominguez, Rodolfo Oxáide-Delgado, Enrique García-Villa, and Elizabeth Álvarez-Ríos for their technical support; Drs. Gustavo Acosta-Alamirano and Martín Antonio-Manrique for their financial support; and Gabriela Mora-Macias for her secretarial assistance.

Grant Support

This work was supported by CONACyT (grant numbers: 83597 and 127335). During this work, J. Bonilla-Delgado was the recipient of a fellowship from CONACyT and COMECyT.

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Received June 20, 2011; revised October 31, 2011; accepted November 23, 2011; published OnlineFirst December 7, 2011.
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Mol Cancer Res  Published OnlineFirst December 7, 2011.

Updated version  Access the most recent version of this article at: doi:10.1158/1541-7786.MCR-11-0287

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