Disc large 1 expression is altered by human papillomavirus E6/E7 proteins in organotypic cultures of human keratinocytes

M. Bugnon Valdano,1 A. L. Cavatorta,1 M. G. Morale,2 F. Marziali,1 V. de Souza Lino,3 R. D. M. Steenbergen,4 E. Boccardo3† and D. Gardiol1†

1Instituto de Biología Molecular y Celular de Rosario – CONICET, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, Rosario, Argentina
2Department of Biochemistry, Institute of Chemistry, University of Sao Paulo, Sao Paulo, Brazil
3Department of Microbiology, Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo, Brazil
4Department of Pathology, VU University Medical Center, Amsterdam, The Netherlands

Loss of cell polarity is a fundamental process in cell transformation. Among polarity proteins, we focused on human disc large (DLG1), which is localized mainly at adherens junctions and contributes to the control of cell proliferation. We previously demonstrated that its expression is altered in HPV-associated cervical neoplastic lesions, but the mechanisms beyond this remain unknown. In this study, we analysed the contribution of HPV proteins to the changes in DLG1 expression in the squamous epithelium. We observed tissue and intracellular misdistribution of DLG1 when high-risk HPV-18 E7 or E6/E7 proteins were expressed in organotypic raft cultures. The viral oncoproteins induce the loss of DLG1 from the cell borders and an increase in the level of DLG1 protein, reflecting the pattern observed in cervical lesions. These findings were corroborated in cultures bearing the entire HPV-18 genome. Interestingly, changes in tissue distribution and abundance of DLG1 were also detected in organotypic cultures expressing the low-risk HPV-11 E7 or E6/E7 proteins, suggesting a conserved function among different HPV types. However, for low-risk HPVs, the subcellular localization of DLG1 at cell-to-cell contacts was predominantly maintained. This report offers new evidence, we believe, of the involvement of HPV proteins in DLG1 expression pattern and our data support previous observations regarding DLG1 expression in cervical lesions.

INTRODUCTION

Human disc large 1 (DLG1) is a modular scaffolding protein bearing different protein interaction modules, including the PSD-95/DLG/ZO-1 (PDZ) domains, which allow formation of multiprotein complexes (Lue et al., 1994; Roberts et al., 2012). DLG1 is able to assemble different proteins into signal transduction networks, where DLG1 has structural and signalling functions. DLG1 functions in controlling cell polarity were first shown in Drosophila, where it was also demonstrated to be a regulator of cell proliferation. In addition, functional loss of DLG1 has been associated with neoplastic transformation (Bilder, 2004). In mammalian epithelial cells, DLG1 localizes in the cytoplasm and at sites of cell contacts in association with adherens junctions (Laprise et al., 2004), being part of the Scribble polarity complex, which is crucial for the establishment and maintenance of apicobasal polarity (Assémat et al., 2008).

Most of the biological functions of DLG1 rely on its ability to interact with several regulatory proteins, such as protein 4.1/ERM family members (Lue et al., 1994), several kinases (Gaudet et al., 2000; Sabio et al., 2005; Gaudet et al. 2011) and two important tumour suppressors: phosphatase and tensin homologue (PTEN) and adenomatous polyposis coli (APC) (Sotelo et al., 2012). Remarkably, DLG1 : APC binding is important for the negative regulation of cell growth (Ishidate et al., 2000) and the interaction with PTEN is required for PTEN stability, cooperating with the inactivation of the proliferative pathways (Sotelo et al., 2012; Valiente et al., 2005). DLG1 was shown to have a dual role in the regulation of both cell polarity and proliferation, highlighting that tissue polarity and

†These authors contributed equally to this report.
Three supplementary figures are available with the online Supplementary Material.
Several reports using human biopsies have described changes in DLG1 abundance and distribution during malignant progression (Facciuto et al., 2012). Interestingly, while a marked reduction of DLG1 levels in poorly differentiated tumours was described, overexpression and changes in DLG1 distribution at earlier stages of cervical, colon and breast cancers have been observed by different groups (Watson et al., 2002; Cavatorta et al., 2004; Fujii et al., 2004; Gardiol et al., 2005). Importantly, the loss of DLG1 expression at cell contacts during neoplastic progression has been consistently reported in studies using histological samples. It has been suggested that the intracellular localization of DLG1 is critical for its biological functions, most likely because the precise distribution of DLG1 may define the probable interacting partners, implying the orchestration of different specific signalling pathways.

However, the molecular mechanisms responsible for such alterations, the contribution of high DLG1 expression to tumour prognosis and the precise temporal deregulation of this protein at different stages of cancer progression are not fully understood. In addition, despite the fact that DLG1 has been postulated as a tumour suppressor protein, although to a lesser extent. This suggests a conserved mechanism among HPV types associated with different pathologies. In the case of cultures expressing the low-risk viral proteins, however, DLG1 subcellular localization at cell contacts was predominantly maintained. This report offers new evidence, we believe, concerning the involvement of HPV proteins in DLG1 expression pattern, and our data support previous observations regarding DLG1 expression in cervical lesions.

RESULTS

HPV-18 oncoproteins interfere with the pattern of DLG1 expression in organotypic raft cultures

We first analysed DLG1 expression pattern in the context of the squamous epithelium in the presence or absence of the HPV-18 E7 or E6/E7 oncoproteins. We focused on HPV-18 since DLG1 was shown to be a preferential target for this virus (Thomas et al., 2008). Therefore, we generated organotypic cultures from primary human keratinocytes (PHKs) previously infected with retroviral vectors expressing E7 or E6/E7. Raft cultures obtained from PHKs were used as a control (Fig. 1a). A section of each paraffinized organotypic culture was stained with haematoxylin and eosin, in order to analyse the morphology of the tissue (Fig. 1a). As expected, the presence of E7 or E6/E7 was associated with thickening of the stratified cell layers. Nuclei retention throughout the epithelium, including the uppermost layers, was also observed, as previously reported (Delury et al., 2013). The expression of the HPV-18 genes was determined in the context of the squamous epithelium in the presence or absence of the HPV-18 E7 or E6/E7 oncoproteins. We focused on HPV-18 since DLG1 was shown to be a preferential target for this virus (Thomas et al., 2008). Therefore, we generated organotypic cultures from primary human keratinocytes (PHKs) previously infected with retroviral vectors expressing E7 or E6/E7. Raft cultures obtained from PHKs were used as a control (Fig. 1a). A section of each paraffinized organotypic culture was stained with haematoxylin and eosin, in order to analyse the morphology of the tissue (Fig. 1a). As expected, the presence of E7 or E6/E7 was associated with thickening of the stratified cell layers. Nuclei retention throughout the epithelium, including the uppermost layers, was also observed, as previously reported (Delury et al., 2013). The expression of the HPV-18 genes was determined...
by reverse transcription PCR (RT-PCR) (Fig. 1b). In addition, the functionality of the viral proteins was assessed by evaluating the expression of E7 and E6 cellular targets: retinoblastoma (Rb) and p53. E7 expression induces a reduction of Rb and an increase in p53 protein levels as described previously (Fig. 1c) (Thomas & Laimins, 1998; Seavey et al., 1999; Flores et al., 2000; Münger et al., 2001). p53 expression was slightly decreased in E6/E7-expressing rafts when compared with the E7 rafts and in relation to the loading control (Fig. 1c) (Flores et al., 2000). The effect of the viral proteins was also corroborated by immunohistochemistry on sections of the raft cultures. The results presented in Fig. S1 (available in the online Supplementary Material) clearly indicate the expression and functionality of both viral proteins.

Our data indicate that in control tissues DLG1 was expressed mainly in the basal and parabasal layers of the epithelium but was absent in the uppermost differentiated cellular strata. DLG1 was localized preferentially in the cytoplasm of the basal cells, but in the suprabasal areas it was predominantly present at cell contacts (Fig. 1d). Isolated nuclei of some epithelial cells showed positive staining, especially in the basal layer. This observation is in agreement with the DLG1 pattern previously reported in normal cervical samples (Cavatorta et al., 2004).

For organotypic cultures expressing HPV oncoproteins, the overall intracellular staining for DLG1 was more intense than in control samples. In this case, DLG1 expression was observed throughout the epithelial strata. In addition, DLG1 cell localization was cytoplasmic in the basal as well as in the uppermost layers, where DLG1 at intercellular contacts was reduced (Fig. 1d). This effect was more striking in samples expressing both E6 and E7, where DLG1 expression at the cell borders was greatly diminished; interestingly, an increase in nuclear staining was also observed. Remarkably, these findings are similar to DLG1 expression in SILs (Cavatorta et al., 2004).

Our results demonstrate that HPV-18 E7 can induce changes in DLG1 distribution along the squamous epithelium and in the subcellular localization, these effects being more marked when both HPV-18 E6 and E7 proteins are expressed together.
In order to confirm our results, DLG1 expression pattern was analysed within the context of the whole HPV genome, using raft cultures established from PHKs bearing the full-length HPV-18 genome (FK18B) at passage 27, which morphologically resemble mild dysplasia in vivo (Steenbergen et al., 1998). DLG1 staining was much more intense than for the control, and, in agreement with the data presented for the E6/E7 cultures, DLG1 was expressed throughout the epithelium thickness, with a predominant cytoplasmic distribution and loss from cell contacts (Fig. 2).

**HPV-18 E7 protein induces an increase in the levels of DLG1**

In addition to changes in DLG1 distribution by HPV-18 E7 or E6/E7, a slight change in DLG1 abundance could also be observed (Fig. 1d). In order to apply a more quantitative assay, we analysed DLG1 levels by Western blotting (WB) using protein extracts from the organotypic cultures. HPV-18 E7 and E6/E7 proteins induced an increase in DLG1 levels compared with control samples (Fig. 3a). This rise could be due to the fact that, in tissues expressing the HPV proteins, DLG1 expression extended throughout all the cell strata, while it was only present in the less differentiated layers in the control (Fig. 1d). Considering this, we evaluated whether the presence of E7 could be involved in the upregulation of DLG1 intracellular levels, independently of the tissue context. For this, DLG1 abundance was analysed by WB using protein extracts from HEK293 cells transiently transfected with plasmids expressing HPV-18 proteins. RT-PCR assays were used to confirm viral gene expression (Fig. S2). In these experimental conditions HPV-18 E6 was shown to promote the degradation of DLG1 in a PDZ-binding motif-dependent manner (Gardiol et al., 1999; Pim et al., 2012). However, it is important to understand the regulation of PDZ proteins in the context of HPV infection, where both oncoproteins are expressed together. As observed in Fig. 3(b), E7 alone or together with E6 resulted in a moderate increase in DLG1 protein level, in agreement with the observations obtained using the organotypic cultures and, apparently, overriding the reported effects of E6-mediated DLG1 degradation.

**Expression of low-risk HPV E7 and E6/E7 proteins also alters DLG1 expression in squamous raft cultures**

In several neoplastic lesions, tissue disorganization has been associated with alterations in DLG1 expression pattern (Facciuto et al., 2012). However, there are no data about possible changes in lesions associated with low-risk HPV, which, even linked to benign lesions which rarely progress to malignancy, cause cell hyperproliferation and aberrant differentiation (Jian et al., 1999; Cheng et al., 1995; McCord et al., 2014). We analysed the effects of E7 and E6/E7 proteins derived from low-risk HPV-11 on DLG1 expression in organotypic cultures. PHKs were infected with retroviral vectors expressing HPV-11 E7 or E6/E7. In tissues expressing the HPV-11 proteins, the epithelium was thicker than that in control cultures, owing to an increased number of cell layers in the spinous stratum (Fig. 4a). However, the morphological alterations were less evident than in cultures expressing high-risk HPV proteins (Fig. 1a). The expression of the HPV-11 genes was assessed by RT-PCR (Fig. 4b). Then, DLG1 immunostaining was evaluated and, interestingly, some changes were observed when the low-risk HPV proteins were expressed (Fig. 4c). There was a general increase in DLG1 abundance, and its expression was also detected in the uppermost strata. However, DLG1 was present at high levels at cell borders, especially in the samples were only E7 was expressed. Besides, only a moderate reduction of DLG1 at that precise cell localization was

![Fig. 2. DLG1 pattern of expression is altered in organotypic cultures bearing the full-length HPV-18 genome. Analysis of the expression and localization of DLG1 in control and FK18B (passage 27, p27) raft cultures by immunohistochemistry. Representative paraffin-embedded sections of rafts immunostained with anti-DLG1 (brown) and counterstained with haematoxylin are shown. Blue arrows indicate DLG1 localization at the cell borders in control culture. Red arrows show DLG1 misdistribution from the cell contacts to the cytoplasm in HPV-18 entire genome culture. Scale bars, 20 μm.](image-url)
observed in the E6/E7-expressing samples. In both conditions, an increase in the cytoplasmic DLG1 expression could also be seen.

Our data suggest that low-risk HPVs also induce a redistribution of DLG1 along the squamous epithelium, but they do not markedly alter the subcellular localization at the cell contacts, as when high-risk HPV proteins are expressed.

We next evaluated by WB whether the presence of the HPV-11 E7 and E6/E7 proteins induces a change in DLG1 abundance. As can be seen, the protein extracts derived from organotypic cultures expressing HPV-11 E7 exhibited a slight increase in DLG1, and this effect was more striking for those samples where HPV-11 E6 and E7 were present (Fig. 5a).

In order to study the potential effect of HPV-11 proteins on DLG1 levels within the cell, we performed experiments using transiently transfected HEK293 cells with expression plasmids for the viral proteins. The expression of the viral sequences was tested by RT-PCR (Fig. S2). It is known that the presence of HPV-11 E6 alone does not change DLG1 levels, since low-risk E6 proteins cannot bind and degrade the PDZ DLG1 protein (Gardiol et al., 1999). As can be seen in Fig. 5b, the presence of HPV-11 E7 and E6/E7 also induces an increase in DLG1 levels.

The overall data suggest that low- and high-risk HPV share a conserved mechanism leading to changes in DLG1 expression levels that should be important for virus replication. This idea points out the hypothesis that common functions of E7 or E6/E7 proteins among low- and high-risk HPVs might participate in molecular pathways that induce these changes.

**DISCUSSION**

In this study, we describe the changes in DLG1 expression in the presence of HPV proteins. Several studies have demonstrated alterations in DLG1 abundance and localization during malignant progression, although it is not clear whether these alterations are a cause or a consequence of neoplastic hyperproliferation. In order to better understand the complex pattern of DLG1 expression, we analysed this in a model that is relevant for HPV infection and the associated lesions. As HPV life cycle is entirely dependent on the epithelium differentiation, we set up organotypic cultures that in vitro mimic the tissue structure.

The presence of HPV-18 E7 or E6/E7 proteins induces a change in tissue and cell distribution of DLG1 compared with control cultures. Our results clearly resemble DLG1 expression patterns observed in HPV-associated SILs (Cavatorta et al., 2004). In the presence of viral proteins, DLG1 was expressed throughout the thickness of the epithelium, in contrast to control cultures, where it was...
absent in the uppermost differentiated cells. HPV E7 or E6/E7 expression is expected to alter the epithelium differentiation programme. Therefore, regarding the differentiation status, cells that compose the tissue strata might not be equivalent to those present in the control tissue. HPV E6 and E7 oncogenes have been shown to disturb the differentiation schedule of the host cell (Pei et al., 1998; Zehbe et al., 2009). Moreover, it was recently reported that E6 and/or E7 from high-risk HPVs are able to downregulate the expression of differentiation genes (Gyöngyösi et al., 2012). This would be important for viral cycle, inducing the cellular replication machinery in differentiated keratinocytes. In this sense, HPV-18 E6/E7 induced the expression of the cell cycle and proliferation markers cyclin A and proliferating cell nuclear antigen (PCNA) in the epithelium suprabasal layers (Fig. S3), as described previously (Flores et al., 2000; Wang et al., 2009). Although differences in cell cycle activity may contribute to DLG1 altered expression observed in organotypic cultures, our results do not rule out the involvement of other mechanisms, as discussed below.

One of the interesting features shown in Fig. 1(d) is the clear reduction of DLG1 at cell contacts while the staining increased in the cytoplasm in the presence of HPV-18 proteins. Our results indicate a possible role of the HPV proteins in DLG1 changes observed for HPV-associated neoplasias (Watson et al., 2002; Cavatorta et al., 2004). In fact, the accumulation of DLG1 in the cytoplasm may have an oncogenic significance since it was shown that specific cellular pools of DLG1 in the presence of viral oncoproteins could have oncogenic functions (Frese et al., 2006; Krishna Subbaiah et al., 2012).

Several studies have demonstrated that E6 can interact and induce the proteasome-mediated degradation of DLG1; however, this degradation involves specific subcellular pools. Furthermore, degradation seems to be incomplete since significant levels remain in HPV cancer cell lines, and certain pools of DLG1 are actively maintained by the continuous expression of E6/E7 (Krishna Subbaiah et al., 2012). In a recent study it was shown that the residual cytoskeletal-bound forms of DLG1 influence RhoG activity by forming a complex with SGEF, and this association is maintained by the presence of E6 and E7 oncoproteins. These cooperative functions between E6 and E7, promoting the activity of RhoG, a protein that has been involved in cell proliferation and differentiation, could be useful for

![Fig. 4. HPV-11 E7 and E6/E7 proteins induce a redistribution of DLG1 along the squamous epithelium in organotypic raft cultures. (a) HPV-11 E7 and E6/E7 induce a thickening of the stratified epithelium and mildly modify tissue morphology. Paraffin-embedded sections of the different organotypic raft cultures were stained with haematoxylin and eosin in order to show the morphological details of the tissues. Scale bar, 20 μm. (b) HPV-11 E7 and E6 gene expression was ascertained by RT-PCR with specific primers. Lanes labelled ‘–RT’ demonstrate the absence of residual contaminating viral DNA in DNase-treated mRNA samples. (c) Analysis of the expression and localization of DLG1 in control, HPV-11 E7 and HPV-11 E6/E7 raft cultures by immunohistochemistry. Representative paraffin-embedded sections of rafts immunostained with anti-DLG1 (brown) and counterstained with haematoxylin are shown. Blue arrows indicate DLG1 localization at the cell borders in control, HPV-11 E7 and HPV-11 E6/E7 cultures. Red arrows show cytoplasmic DLG1 expression in HPV-11 E7 and HPV-11 E6/E7 raft culture. Scale bars, 20 μm.](image-url)
virus replication in the tissue context (Krishna Subbaiah et al., 2012). These observations may contribute to explaining the data shown in Fig. 1(d), where a considerable amount of DLG1 was maintained in the cytoplasm, perhaps complexed with cell proteins involved in cell growth. E7 expression in epithelial cells was demonstrated to cause a drop in the levels of the adhesion protein E-cadherin, which is involved in DLG1 localization at cell contacts (Reuver & Garner, 1998; Hellner et al., 2009). This may account for the slight decrease in DLG1 immunostaining at cell borders in the presence of HPV-18 E7. Moreover, DLG1 reduction at cell contacts was more striking in E6/E7 cultures. Therefore, it is possible to speculate that E6 could contribute to DLG1 downregulation, perhaps by preferentially targeting those DLG1 forms for degradation (Massimi et al., 2006).

At present, it is not possible to discard the idea that other mechanisms induced by the presence of the viral oncoproteins could also promote changes in DLG1 protein-binding capacity, stimulating its interaction with different partners which redirect its localization. This is consistent with the hypothesis that diverse DLG1 pools may have different functions and, possibly, opposite activities regarding malignant progression.

It is important to highlight that the presence of E6/E7 also promotes an increase in DLG1 nuclear localization. It was shown that, in conditions where cell polarity can be altered, cell junction proteins are disassembled and can migrate to the nucleus and regulate transcriptional activity (Polette et al., 2005). Moreover, Narayan et al. (2009) have shown that DLG1 nuclear localization is highly dependent on phosphorylation by cycling kinases during cell cycle progression, and high-risk HPV oncoproteins are known to promote cell cycle entry even in differentiated cells (Fig. S3).

Moreover, changes in both DLG1 abundance and localization were also observed in tissues derived from the FK18B passage 27 cells (Fig. 2), highlighting the significance of our findings. These results indicate that expression of viral proteins in the context of the entire HPV-18 genome is responsible for those alterations, and, in view of our previous data, E7 or E6/E7 expression is most likely involved. These results are also relevant in light of DLG1 expression in cervical biopsies. FK-18B passage 27 cultures exhibit morphological alterations suggestive of mild/moderate dysplasia (Steenbergen et al., 1998), and we previously demonstrated that DLG1 was overexpressed and exhibited altered cellular distribution in SIL HPV-positive lesions, with a progressive loss from cell contacts (Cavatorta et al., 2004).

**Fig. 5.** HPV-11 E7 and E6/E7 proteins increase DLG1 expression levels. (a) Protein extracts from epithelial control cultures and from rafts expressing HPV-11 E7 or E6/E7 were analysed by WB for DLG1 expression. (b) HEK293 cells transfected with the corresponding empty vector or expressing HPV-11 E7 or HPV-11 E6/E7 proteins. After 24 h, cells were harvested and protein extracts were assessed by WB for DLG1 levels. Numbers show fold band intensity for DLG1 in rafts (a) and cells (b) expressing viral proteins, with respect to the corresponding control sample (considered as 1). Right panels, densitometry analysis of Western blots for DLG1 (mean ± SD, n=3), showing DLG1 level (DLG1/γ-tubulin ratio) in rafts (a) and cells (b) expressing viral proteins, relative to each control sample, set as 1. The intensity of each band was normalized to γ-tubulin expression, used as loading control.
Most of the previous studies about HPV interference with cell polarity were focused on high-risk HPVs. No data were available about the expression of polarity proteins in general, or specifically for DLG1, in low-risk HPV-associated lesions. Unexpectedly, we also found some changes in DLG1 expression in the presence of HPV-11 proteins. We observed that raft cultures expressing HPV-11 sequences exhibited morphological changes suggesting a low-grade dysplasia, albeit to a lesser extent than for high-risk HPVs (Figs 1a, 4a) (Thomas et al., 2001; Fang et al., 2006). In organotypic cultures expressing HPV-11 E7 or E6/E7, DLG1 immunostaining was altered compared with control cultures (Fig. 4c). DLG1 was observed throughout the tissue strata, possibly due to changes in cellular differentiation status (Thomas et al., 2001; Fang et al., 2006). However, unlike the results described for HPV-18, DLG1 was present at cell borders in cultures expressing HPV-11 proteins, and this may be relevant considering the different capacity of each virus in transforming cells and in promoting malignant progression. DLG1 reduction at cell contacts may contribute to alterations in signal transduction pathways controlling cell proliferation, as part of DLG1 oncosuppressing functions.

Nevertheless, HPV-11 proteins were also capable of inducing an increase in DLG1 levels in both organotypic and monolayer epithelial cell cultures (Fig. 5). It is possible to speculate that this augmentation in DLG1 abundance may be a critical conserved viral activity in order to favour virus replication. Some function conservations between the E6/E7 proteins from high- and low-risk HPV types were shown (Pim & Banks, 2010) and probably E6/E7 from both HPV could induce molecular mechanisms involved in regulation of DLG1 levels. In addition, infections by low-risk HPV types also induce unscheduled host DNA synthesis in a fraction of post-mitotic, differentiated cells (Cheng et al., 1995; Jian et al., 1999; McCord et al., 2014). We observed that HPV-11 E6/E7 induced expression of cyclin A and PCNA proteins in the epithelium suprabasal layer (Fig. S3), although to a lesser extent than observed in HPV-18 cultures. It is possible that DLG1 expression and cell cycle progression may be functionally linked, explaining the differences observed in the different conditions analysed in this study.

How the abundance of low and high-risk HPV proteins could influence DLG1 expression pattern during the viral cycle is still unknown. However, deregulated viral protein expression might have an impact on DLG1 expression. This is supported by the observation that, in cervical cancer samples, where HPV oncoprotein expression is deregulated and no viral replication occurs, there is a dramatic reduction of DLG1 levels, compared with intraepithelial precursor lesions (Cavatorta et al., 2004). Further studies are needed to clarify this important issue and for a complete comprehension of HPV-associated pathogenesis.

In summary, the data from this study demonstrated that E7 and E6/E7 proteins derived from both HPV-11 and HPV-18 induce changes in the distribution and abundance of DLG1 polarity protein in organotypic cultures. The most significant differences were the DLG1 expression at cell contacts, which may reflect the difference in the pathologies associated with low- or high-risk HPV. Moreover, alterations in DLG1 expression were also observed in raft cultures bearing the entire HPV-18 genome. DLG1 protein expression is frequently altered in a variety of human cancers, and the results presented here suggest a role for E7 and E6/E7 proteins in these changes to the HPV-associated neoplastic lesions.

METHODS

Organotypic raft cultures. Organotypic raft cultures were generated as described elsewhere (Boccardo et al., 2010). Low-passage-pooled neonatal foreskin keratinocytes (LonzaWalkersville) were grown in serum-free medium (Invitrogen). Cells were infected with the recombinant retroviruses and after 24 h were selected with the corresponding antibiotic. After 2 days, when 100 % of mock-infected controls were dead, infected cells were used to seed the epithelial raft. Recombinant retroviral vectors containing HPV-11 E7 and E7/E6 genes were kindly provided by Dennis J. McCance (Queen’s University, Belfast, UK) and are described elsewhere (Guess & McCance, 2005). Recombinant retrovirus vectors containing unregulated regulatory region (URR)-E7 or URR-E6/E7 sequences from HPV-18 are described elsewhere (Boccardo et al., 2004). After 10 days, organotypic raft cultures were harvested for protein or RNA analysis or fixed for histological investigation as described below. Organotypic raft cultures from the keratinocyte cell line FK18B, which harbours the HPV-18 full-length genome, were established as previously described (Pinheiro et al., 2014). Briefly, PHKs were transfected with the HPV-18 full-length genome (FK18B) and grown for different numbers of passages before raft-culture seeding (Steerenbergen et al., 1998).

Cell culture, plasmids and transfection. HEK293 epithelial cells were grown in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10 % (v/v) FBS (PAA). The HPV-18 E6 or E7 genes were cloned under the control of the CMV promoter (Facciuto et al., 2014). Plasmids expressing HPV-11 E6 or E7 proteins were kindly provided by Dennis J. McCance (Queen’s University, Belfast, UK; Guess & McCance, 2005). Cells were transfected with the indicated constructions, using calcium phosphate precipitation as described by Matlashewski et al. (1987).

Western blotting. Protein was extracted from organotypic raft cultures as previously reported (Boccardo et al., 2004) using extraction buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 0.5 % NP-40) containing protease inhibitors. HEK293 cells were lysed in ice-cold lysis buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 % NP-40, 0.5 % deoxycholate) containing Halt Protease Inhibitor single-use cocktail (Thermo Scientific Pierce). Subsequently, WB experiments were carried out as described (Gardiol et al., 1999). Equal amounts of proteins were separated by SDS-PAGE and transferred to appropriate membranes. Specific protein levels were determined by immunoblot analysis using mouse monoclonal anti-DLG1 (2D11; Santa Cruz Biotechnology), mouse monoclonal anti-p53 (DO-1; Santa Cruz Biotechnology), rabbit polyclonal anti pRb (M-153; Santa Cruz Biotechnology) and mouse monoclonal anti-γ-Tubulin (T6557; Sigma Aldrich). The secondary antibodies used were anti-mouse (NAX931; Amersham GE) or anti-rabbit (NA934; Amersham GE) coupled to HRP and detected by chemiluminescence, using the SuperSignal West Pico Chemiluminescent Substrate reagent (Thermo Scientific Pierce). γ-Tubulin was measured as a loading control. Protein band intensities were quantified using the ImageJ quantification program.
RNA isolation, cDNA synthesis and RT-PCR. For testing E7 or E6 gene expression, total RNA was extracted using Trizol Reagent (Life Technologies) following the manufacturer’s instructions. cDNA was synthesized from 2 μg RNA using 200 U MuLV RevertAid reverse transcriptase (Fermentas) and oligo(dT) primers. Complementary DNA from each sample was subjected to PCR amplification with specific forward (F) and reverse (R) primers: HPV-11E6 F 5′-TATATAGCTATGGAAGTAAACGTCC-3′, R 5′-TATAACCTTTTTAGTTGAACCACTCCTTCAG-3′; HPV-18E6 F 5′-TTATAGCTATGGAAGTAAACGTCC-3′, R 5′-TATAACCTTTTTAGTTGAACCACTCCTTCAG-3′; HPV-11E7 F 5′-GTCGACAAAAGAAGGACCA-3′, R 5′-TGCGCAAGAATGGCTTTCA-3′; HPV-18E7 F 5′-TGCGCAAGAATGGCTTTCA-3′, R 5′-CTCGTGCGGCTGTTAAGTGT-3′. The human succinate dehydrogenase (SDH) gene, used as housekeeping marker, was amplified with SDH-F (5′-GGCACCCGTGTCCCTTG-3′) and SDH-R (5′-CACGTCGAGCCTGTCA-3′) primers.

Immunohistochemistry. Organotypic raft cultures were harvested, fixed in buffered formalin, embedded in paraffin, cut into 3 μm sections and mounted on pretreated glass slides. Sections were stained with haematoxylin and eosin to observe histology. The immunohistochemistry assays were performed as described previously (Cavatorta et al., 2004; Gardiol et al., 2006). Briefly, the samples were deparaffinized in xylene and rehydrated using a graded alcohol series. Endogenous peroxidase activity was blocked by immersing sections in 3% hydrogen peroxide in methanol for 20 min. Sections were placed in 10 mM Tris–1 mM EDTA buffer (pH 8.3) and heated for 12 min on high power using a conventional microwave oven, to facilitate antigen retrieval. Samples were allowed to cool down. After blocking non-specific binding by addition of normal horse serum (Vectastain ABC kit; Vector) for 40 min, sections were incubated with the respective primary antibody overnight at 4 °C in a humid chamber. The primary antibodies anti-DLG1 (2D11, 1 : 40), anti-Parkin (A), anti-Caspase 9 (B), anti-PCNA (C) and anti-PCNA were purchased from Novocastra and Zymed, respectively. For detection, samples were treated successively with biotinylated secondary antibody for 30 min and with avidin–biotin peroxidase complex for a further 30 min at room temperature (DAKO). The reaction was developed using a diaminobenzidine chromogenic substrate kit for peroxidase (Vector), and sections were counterstained with haematoxylin. Negative controls were processed as described, except that primary antibody was omitted.

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