The levels of epithelial anchor proteins $\beta$-catenin and zona occludens-1 are altered by E7 of human papillomaviruses 5 and 8

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Infection with viruses of the genus Betapapillomavirus, $\beta$-human papillomaviruses ($\beta$-HPV), is implicated in the development of non-melanoma skin cancer. This was first evidenced for HPV5 and HPV8 in patients with the skin disease epidermodysplasia verruciformis (EV). The relocalization of the junctional bridging proteins $\beta$-catenin and zona occludens-1 (ZO-1) from the adherens and tight junctions are common processes of the epithelial-mesenchymal transition (EMT) associated with tumour invasion. Here, we report that $\beta$-catenin and ZO-1 are strongly upregulated by the E7 oncoproteins of HPV5 and HPV8 in keratinocytes grown in organotypic skin cultures. Although the membrane-tethered form of $\beta$-catenin was elevated, no signs of $\beta$-catenin activity within the canonical Wnt signalling pathway could be detected. The upregulation of $\beta$-catenin and ZO-1 could also be confirmed in the skin of HPV8 transgenic mice as well as in cutaneous squamous cell carcinomas of EV patients. These data provide the first evidence that $\beta$-catenin and ZO-1 are direct targets of E7 of the oncogenic $\beta$-HPV types 5 and 8. The ability to deregulate these epithelial junction proteins may contribute to the oncogenic potential of these viruses in human skin.

INTRODUCTION

Cutaneous squamous cell carcinoma (SCC) is the most common malignancy in organ transplant recipients (OTR), with an approximately 65–250-fold increased incidence compared to immunocompetent people (Nindl & Rösl, 2008). Human papillomaviruses (HPV) of the genus Betapapillomavirus ($\beta$-HPV) are associated with the development of SCC (Akgül et al., 2006). The clinical relevance of $\beta$-HPV infection has clearly been demonstrated in patients suffering from epidermodysplasia verruciformis (EV), a rare inherited disease associated with a high rate of skin cancer (Burger & Itin, 2014; Patel et al., 2010). HPV5 and HPV8 are among the most frequently found types in lesions of EV patients, but many other $\beta$-HPV types have also been identified in such lesions (Akgül et al., 2007b; Cardoso & Calonje, 2011). In the general population, $\beta$-HPV are suspected to have an aetiological role in skin carcinogenesis as well, but this is still a matter of controversial discussion (Howley & Pfister, 2015; Quint et al., 2015; Smola, 2014). The oncogenic potential of HPV8 early proteins has been investigated in mouse models and in vitro organotypic skin cultures (OSC) of primary human keratinocytes (PHK). The expression of HPV8-E7 caused enhanced proliferation and invasion of human keratinocytes (Akgül et al., 2005), and particularly HPV5 and HPV8 E7 expression increased clonogenicity and the number of stem cell-like cells (Hufbauer et al., 2013). In addition, both E7 proteins are also able to overcome p16INK4a induced cell cycle arrest (Westphal et al., 2009) and reduce pRb protein levels (Akgül et al., 2007a; Buitrago-Pérez et al., 2012). Transgenic mice expressing the HPV8 complete early region (CER: ORFs E1/E2/E4/E6/E7) under the control of the human keratin-14 (K14) promoter, developed papillomas with varying degrees of epidermal dysplasia and SCC (Schaper et al., 2005).

The multi-layered, stratified epithelium of the skin is formed by several keratinocyte layers, which express selected types of cellular junctions. Adherens junctions are present in basal, spinous, granular cell layers and tight junctions in the granular cell layer of human epidermis (Pummi et al., 2001).

Changes in intercellular adhesion molecules are linked with the loss of epithelial features occurring in epithelial-mesenchymal transition (EMT) processes associated with conversion of normal keratinocytes to epithelial tumour...
cells. At the molecular level, this involves the reorganization of cell–cell adhesion complexes including adherens and tight junctions responsible for the establishment and maintenance of a polarized cell phenotype (Polette et al., 2007).

Such a reorganization at adherens junctions involves a relocalization of β-catenin, which is important for intercellular adhesion, linking E-cadherin to the actin cytoskeleton. It is also the central mediator of the Wnt/β-catenin signalling pathway, where it acts as a hypo-phosphorylated protein and transcriptional co-factor through interaction with members of the T-cell factor/lymphoid enhancer (TCF/LEF) transcription factors (Morgan et al., 2014). β-catenin thereby regulates several fundamental cellular processes and aberrant accumulation has been implicated in numerous malignancies, including non-melanoma skin cancer (Beronja et al., 2013; Malanchi et al., 2008).

Similarly to adherens junctions, the interaction between occludin and zona occludens-1 (ZO-1) plays a crucial role in maintaining the structure of tight junctions, where occludin is linked to the actin cytoskeleton through ZO-1. Tight junction proteins, e.g. ZO-1, are also found outside of distinct membrane structures in the cytoplasm, suggesting tight junction-structure-dependent and structure-independent functions (Brandner et al., 2015). Both β-catenin and ZO-1 appear to be shuttle molecules, and depending on the cell differentiation and migration state, may be found in different subcellular compartments.

The association between abnormalities of these two proteins and expression of β-HPV oncoproteins has not yet been studied. Thus, in this study, the expression of β-catenin and ZO-1 was examined in monolayer and OSC of β-HPV oncoprotein-expressing human keratinocytes, the skin of HPV8-CER transgenic mice and in skin lesions of EV patients.

**RESULTS**

**HPV8-E7 mediates overexpression of β-catenin in OSC**

A reorganization of cell–cell adhesion complexes by changing β-catenin expression or localization plays a major role in EMT. To investigate whether HPV8 early proteins are able to modulate β-catenin levels, we first analysed the β-catenin expression pattern in de-epidermalized human dermis based OSC repopulated with HPV8-E2, -E6 or -E7 expressing PHK. In the empty vector control culture, staining for β-catenin was restricted to the granular layer of the regenerated epithelium. Staining of the granular layer was found to be absent in the HPV8-E2 and -E6 cultures. In marked contrast, β-catenin was strongly overexpressed in the HPV8-E7 positive regenerated epithelium, showing a diffuse cytoplasmic staining in the lower suprabasal layers and strong membrane-tethered staining in the upper squamous layers (Fig. 1a).

Given that β-catenin was overexpressed in HPV8-E7 positive OSC, we further compared β-catenin expression in OSC with keratinocytes of a second donor, which expressed E7 proteins of HPV1 (μ-HPV) or HPV5, 8, 20 and 38 (β-HPV). In control cultures, in which keratinocytes were transduced with the empty retroviral vector pLXSN, β-catenin expression was restricted to the upper layers of the stratum spinosum and the granular layer of the regenerated epithelium. Compared to the control culture, the pattern of β-catenin localization and staining intensity was not affected by HPV1-E7 and HPV38-E7. Expression of HPV20-E7 led to a reduction in β-catenin levels. In marked contrast, β-catenin was again strongly in HPV8-E7 cultures, showing a diffuse cytoplasmic staining in the lower suprabasal layers and membrane-tethered staining in the upper squamous layers. Interestingly, strong expression of membrane-tethered β-catenin was also found in suprabasal keratinocytes in OSC expressing HPV5-E7 that was comparable to the staining intensity found in HPV8-E7 OSC (Fig. 1b). To analyse whether overexpressed β-catenin influences the actin cytoskeleton, we fluorescently stained the OSC cultures with an anti-actin antibody. Again, only HPV5 and HPV8 E7 cultures showed enhanced membrane-tethered actin immunostaining in the upper squamous cell layers, which was not observed in OSC of the control and HPV1, 20 and 38E7 OSC (Fig. S1, available in the online Supplementary Material). These results demonstrate a direct role of HPV5 and HPV8 E7 on β-catenin and actin localization and expression in differentiating keratinocytes.

**Overexpressed β-catenin is not active in the Wnt signalling pathway**

To verify whether β-catenin is transcriptionally active by translocation into the nucleus, we performed immunohistochemical staining on OSC with the αβC β-catenin antibody. This monoclonal antibody reacts against non-phosphorylated forms of β-catenin, which are active within the canonical Wnt signalling pathway (Staal et al., 2002; van Noort et al., 2002). Non-phospho-β-catenin detection showed a membranous, suprabasal expression pattern in control as well as HPV8-E7 positive OSC. This membranous localization of non-phosphorylated β-catenin overlapped with the staining pattern characterized by the antibody recognizing total β-catenin in Fig. 1. Since no nuclear localization of active β-catenin was observed, we assumed that β-catenin does not act within the canonical Wnt pathway in regenerated epithelia of HPV8-E7 expressing keratinocytes (Fig. 2a). To further confirm that accumulated β-catenin is not active within the Wnt pathway, we co-transfected PHK with expression plasmids for HPV8-E7 and either the β-catenin responsive TCF reporter plasmid (TOP-flash) or the negative control plasmid (FOP-flash) containing mutated TCF binding sites.
Fig. 1. Increase of β-catenin levels correlates with HPV5 and HPV8 E7 expression. (a) Representative immunohistochemical analysis of total β-catenin in OSC expressing the empty retroviral vector pLXSN or HPV8 early proteins E2, E6 or E7. (b) Representative immunohistochemical analysis of total β-catenin in OSC expressing the empty retroviral vector pLXSN or the E7 gene of HPV1, HPV5, HPV8, HPV20 or HPV38. The tissues were counterstained with haematoxylin. Dashed line, basement membrane zone; e, regenerated epithelium; d, dermis; magnification, ×400.
No significant difference in TOP-flash and FOP-flash luciferase activity (\(P > 0.05\), Student’s \(t\)-test) could be measured 48 h post-transfection (Fig. 2b). HPV8-E7 induced \(\beta\)-catenin was also not able to increase luciferase expression from TOP-flash in RTS3b cells (\(P > 0.05\), Student’s \(t\)-test) (Fig. 2c). In addition, no change in mRNA expression of the Wnt/\(\beta\)-catenin target genes c-myc and CD44 was measured in HPV8-E7 positive RTS3b by quantitative reverse transcription PCR (qRT-PCR) (Fig. 2d). As a positive control for the functionality...
of the Wnt signalling pathway in RTS3b, TOP-flash and FOP-flash activity was assessed 6 h after Wnt3a treatment. Promoter activity of TOP-flash, but not of FOP-flash, was significantly stimulated after Wnt3a stimulation (Fig. 2e, right image). Consistent with this we found increased protein expression of the β-catenin target c-myc (Fig. 2e, left image). We thus concluded that despite β-catenin accumulation, the canonical Wnt/β-catenin pathway is not affected in HPV8-E7 positive cells.

**Induction of ZO-1 in OSC of HPV5 and HPV8 E7 expressing PHK**

To determine whether the expression pattern of the junctional bridging-protein ZO-1 is also targeted by HPV E7 proteins in addition to β-catenin, ZO-1 was analysed in the same OSC. In the control as well as the HPV1-E7, HPV20-E7 and HPV38-E7 cultures, ZO-1 expression was restricted to the uppermost layers of the regenerated epithelium. In contrast, ZO-1 was found to be strongly expressed throughout the regenerated differentiating epithelium of HPV5 and HPV8 E7 positive OSC (Fig. 3).

To verify whether increased β-catenin and ZO-1 in HPV8-E7 OSC was due to increased transcription, the mRNA levels of β-catenin, ZO-1 and HPV8-E7 were measured by qRT-PCR. A significant increase in mRNA levels could be found for β-catenin (1.5-fold; \( P < 0.0044 \), Student’s t-test) and ZO-1 (2.5-fold; \( P < 0.0001 \), Student’s t-test), indicative of a correlation with HPV8-E7 mRNA expression within human keratinocytes (Fig. 4a).

To confirm the effect of E7 on β-catenin and ZO-1 expression under differentiating conditions, keratinocytes were exposed to high extracellular calcium concentration (2 mM) for up to 7 days. Whereas differentiation led to a gradual decrease in β-catenin and ZO-1 over time in control cultures, both factors were elevated in differentiating HPV8-E7 positive keratinocytes. Substantially high levels of β-catenin and ZO-1 were found in HPV8-E7 cells at day 7 of calcium treatment (Fig. 4b).

**Fig. 3.** HPV5 and HPV8 E7 expression strongly induces ZO-1 expression. Representative immunohistochemical images of OSC expressing the empty retroviral vector pLXSN or the E7 gene of HPV1, HPV5, HPV8, HPV20 or HPV38 and stained for ZO-1. The tissues were counterstained with haematoxylin. Dashed line, basement membrane zone; e, regenerated epithelium; d, dermis; magnification, \( \times \) 400.
whether siRNA-mediated gene knockdown of β-catenin and ZO-1 has an effect on proliferation or differentiation of HPV8-E7 positive keratinocytes. When compared to si-scramble transfected keratinocytes, knockdown of β-catenin and ZO-1 had no effect on cell proliferation as assessed by Western blotting for proliferating cell nuclear antigen (PCNA). However, in empty vector, as well as HPV8-E7 positive keratinocytes, the knockdown of both proteins strongly inhibited loricrin protein expression, a marker for epidermal differentiation (Fig. 4c).

**Overexpression of β-catenin and ZO-1 in skin of HPV8-CER transgenic mice and EV patients**

To study whether the increase of β-catenin and ZO-1 could also be found in vivo, their expression was analysed by immunohistochemistry in the skin of K14-HPV8-CER mice in comparison to FVB/n-wt skin. In contrast to FVB/n-wt skin, showing weak to moderate staining of β-catenin and ZO-1 in the inter-follicular epidermis, transgenic skin showed strong β-catenin and ZO-1 expression levels in K14 permissive keratinocytes, indicating strong expression under a condition where all HPV8 early genes are expressed simultaneously (Fig. 5a). In humans, normal skin of a donor from the general population showed regular membrane-tethered β-catenin expression, but expression was accentuated at the most superficial cell layers of EV skin lesions (Fig. 5b), similarly to the staining pattern in HPV5 and HPV8 E7 positive OSC. Like β-catenin, elevated levels of ZO-1 were also found to be present throughout the EV-SCC.

**DISCUSSION**

Skin tumours have been associated with abnormal expression of epidermal adhesion proteins, suggesting they have an important role in cutaneous tumorigenesis (Schluter et al., 2007). The role of the Wnt/β-catenin pathway in the development of different types of skin neoplasm has not been fully elucidated yet. Previous research described a relocalization of β-catenin from the plasma membrane to the nucleus in the presence of the E6/E7 oncoproteins of the high-risk α-HPV type 16 (Bello et al., 2015), which is associated with cervical and oropharyngeal cancer (Hübbers & Akgül, 2015). Our results demonstrated that the levels of membrane-tethered β-catenin are elevated in HPV8-E7 positive cutaneous keratinocytes. Our data indicated that the canonical Wnt/β-catenin signalling pathway is not activated by HPV8-E7. In line with our observations, overexpressed β-catenin was found to be localized to the plasma membrane in respiratory papillomas, which are caused by HPV types 6 or 11 (Lucs et al., 2014). Although β-catenin and ZO-1 appeared to be dispensable during proliferation, both factors seem to play a role in the regulation of differentiation-dependent processes of human keratinocytes, independent of HPV oncogene expression. Cells with strong β-catenin and ZO-1 expression were found in the skin of HPV8 transgenic mice and EV lesions, which supports a possible clinical relevance of our data. A broader expression pattern of ZO-1 in epidermal layers has previously been shown in skin SCC, which suggests that ZO-1 contributes to tumour progression, largely in a tight junction independent manner (Brandner et al., 2015; Hintsala et al., 2013; Kirschner & Brandner, 2012; Morita et al., 2004; Rachow et al., 2013). In melanoma cells, an
increase of ZO-1 mRNA and protein levels was observed compared to normal melanocytes. A reduction of ZO-1 in melanoma cells was associated with decreased invasiveness, which suggested its role in cell invasion (Smalley et al., 2005). HPV5 and HPV8 E7 induced ZO-1 levels thus may be involved in cellular processes enabling keratinocyte

**Fig. 5.** β-catenin and ZO-1 are elevated in HPV8 positive murine skin and EV-SCC. Representative immunohistochemical images for total β-catenin and ZO-1 in (a) the skin of FVB/n-wt and K14-HPV8-CER mice as well as in (b) normal human skin and EV-SCC (positive for HPV5, 8, 20, 23, 36, 50). The tissues were counterstained with haematoxylin (magnification, ×400).
invasion. In addition, both E7 proteins may disturb β-catenin functions in the differentiation-dependent anchoring of actin-filaments to cell junctions. In line with this hypothesis, we observed that most of the deregulated proteins observed in HPV8-E7 expressing cells by 2D-DIGE experiments are implicated in the organization of the actin cytoskeleton (Akgül et al., 2009).

In conclusion, our work revealed alterations of β-catenin and ZO-1 in oncogenic β-HPV-positive epithelia, which might disturb adherens and tight junction composition and unbalance cell homeostasis, contributing to HPV5 and HPV8 mediated carcinogenic processes.

METHODS

Tissue samples. OSC expressing HPV8-E2 (Akgül et al., 2011b), -E6 (Leverrier et al., 2007), -E7 (Akgül et al., 2005, 2011a) or a panel of different HPV E7 proteins (Westphal et al., 2009) were previously described. Murine skin was collected from FVB/n-wt controls (described. Murine skin was collected from FVB/n-wt controls (ethical committee at the Medical University of Warsaw. Histopathology results of EV lesions is presented in Table S1, available in the online Supplementary Material). The study was approved by the local ethics committee at the Medical University of Warsaw.

Immunohistochemistry. For immunohistochemistry, 4 μm thick wax sections were de-paraffinized and then microwaved in antigen retrieval solution (0.1 M citrate buffer, pH 6.0) for 4 min. Staining on mouse sections using monoclonal mouse antibodies was performed using a mouse on mouse kit (MOM, Vector Laboratories-Linaris) as previously described (Hufbauer et al., 2015). No antigen retrieval was used for organotypic skin sections stained for β-catenin or ZO-1. The sections were rinsed in PBS and non-specific binding of antibodies was blocked with serum for 30 min at room temperature, before application of the primary antibody. Primary antibodies used in this study were: anti-β-catenin (reacting with total human and mouse β-catenin, BD Biosciences), pAB-β-catenin (reacting with non-phosphorylated Ser-37 and Thr-41, the active forms of β-catenin; Staal et al., 2002; van Noort et al., 2002; clone 8E7, Millipore), anti-ZO-1 (clone 61-7300, Life Technologies) and anti-actin (clone SC, Millipore). For controls, the primary antibody was omitted to verify expression patterns (data not shown). Sections were incubated at room temperature for 90 min, then washed in PBS and treated for 30 min with a biotinylated secondary antibody and processed with a streptavidin-biotin-peroxidase detection system (Vectastain ABC kit, Vector Lab.) as recommended by the manufacturer.

Cell culture. RTS3b keratinocytes were maintained in RM + media (consisting of a 3:1 ratio of DMEM/F12 with 10% FBS, 1% glutamine, 0.4 μg hydrocortisone, cholera toxin 10⁻¹⁰ M, 5 μg transferrin ml⁻¹, liothionine 2 x 10⁻¹¹ M, 5 μg insulin ml⁻¹, 10 ng epidermal growth factor ml⁻¹ and 1 x pen/strep mixture; Akgül et al., 2003). PHK from Caucasian donors were isolated from foreskins and were subsequently passage and maintained in KGM-Gold (Lonzia) containing 0.05 mM calcium. PHK were treated with retroviral supernatants coding for pLXSN or pLXSN-HPV-E7 proteins as described previously. Transduced cells were selected 2 days after infection using G418. Keratinocytes were cultured for up to 7 days in 2 mM calcium containing KGM-Gold to induce cell differentiation.

For transient reporter gene assays, PHK were transiently transfected in duplicate in 6-well dishes using Transfasc (Promega) with 1 μg of luciferase reporter constructs TOP-flash or FOP-flash and 0.5 μg of plLXSN or plLXSN-HPV8-E7. The same DNA amounts were also transfected into RTS3b keratinocytes using FuGene (Promega) transfection reagent. TOP-flash contains two sets (with the second set in the reverse orientation) of of copies of the TCF binding site following by the minimal HSV-1k promoter and luciferase reporter coding sequence, and FOP-flash contains mutated TCF binding sites (kindly provided by Dr David Prowse, University of Hertfordshire, Hatfield, Hertfordshire, UK). Luciferase assays were measured 48 h post-transfection and luciferase values were normalized to protein levels. When necessary, RTS3b cells were stimulated with Wnt3a (50 ng ml⁻¹, dissolved in PBS, R&D Systems) for 6 h prior to harvest.

Silencing of β-catenin and ZO-1 gene expression in plLXSN or plLXSN-HPV8-E7 positive N/TERT keratinocytes was achieved through transfection of siRNA (si-β-catenin: cat. no. SI02662478; si-ZO-1: cat. no. SI02655149; Qiagen) with an RNAiMax kit (Thermo Fisher) according to the manufacturer’s protocol. Cells transfected with a scramble siRNA (cat. no. 1022563; Qiagen) were used as controls.

For total RNA isolation from retrovirally transduced keratinocytes, an RNAeasy kit (Qiagen) was used and cdNA was generated as described previously (Lazic et al., 2011). The primers used for qRT-PCR are as follows:

β-catenin-fw: GCTTTCAGGTGAAGCTGACCA
β-catenin-fw: CAAAGTCCAAGTACAGCTCTC
ZO-1-fw: GAAAGGAGTTGAGCAGGAAATCTA
ZO-1-fw: AGGACTCAGACTGTTTCACC
c-myc-fw: ATCTCGAGAGGAGGACAAAGA
Zc-myc-fw: TGGAGACGTGGCACTCTT
CD44-fw: TGCCGCTTGCAGGTTGAT
CD44-fw: GGGGCTCCGTCACCAGAG
GAPDH-fw: CTCAGTTCAACAGCGCACC
GAPDH-fw: TGCGTGGACCAATCTG
Sample measurements were performed with a plasmid comprising the respective gene fragment, which was used to generate standard dilutions. mRNA levels of target genes were normalized to GAPDH mRNA levels. The relative gene expression levels of the corresponding controls were set as 1. Data are presented as mean ± SD.

Western blot. For Western blot analysis, cells were trypsinized, pelleted by centrifugation and lysed on ice for 30 min in RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Nonidet-P40, 0.5% sodium deoxycholate and 0.1% SDS) supplemented with 1 x protease inhibitor cocktail (Roche Diagnostics). The resulting extracts were sonicated and protein concentration was determined using a Bio-Rad Protein Assay (Bio-Rad). Cell extracts were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. After the membrane was blocked with 5% milk or 5% BSA in TBST (10 mM Tris/HCl, pH 8.0; 150 mM NaCl; 0.05% Tween 20) for 1 h, blots were probed with antibodies for total β-catenin, ZO-1, c-myc (clone 9E10, Thermo Fisher), PCNA (clone C19, Abcam) and loricrin (ab24722, Abcam). GAPDH (mouse monoclonal, Abcam) and tubulin (YLI1/2, Abcam) were used as loading controls. Immunoreactive proteins were
visualized using peroxidase-coupled secondary antibodies and the chemiluminescence system ECL. Plus Western blotting Detection System (GE Healthcare). The nitrocellulose blot was exposed to autoradiographic film.

**Statistical analysis.** All experiments were repeated a minimum of three times. qRT-PCR data were expressed as mean ± SD. The data presented as immunoblots or images of immunohistochemical analysis are from a representative experiment, which was qualitatively similar in the replicate experiments. Statistical significance was determined with unpaired 2-tailed Student’s t-test. The asterisks shown in the figures indicate significant differences between experimental groups (***P<0.001, **P<0.01).

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