

Cutaneous human papillomavirus E7 type-specific effects on differentiation and proliferation of organotypic skin cultures

K. Westphal^{a,*}, B. Akgül^{b,c,*}, A. Storey^b and I. Nindl^{a,**,***}

^a *Department of Dermatology, Venereology and Allergy, Charité, Skin Cancer Center Charité, University Hospital of Berlin, Berlin, Germany*

^b *Cancer Research UK, Skin Tumour Laboratory, ICMS, London, UK*

^c *Institute of Virology, University of Cologne, Cologne, Germany*

Abstract. *Background:* A role for cutaneous human β -papillomavirus (HPV) types as co-factors in the development of non-melanoma skin cancer has been postulated. Here we have investigated the effects of E7 expression on keratinocyte differentiation, proliferation and cell-cycle proteins in organotypic skin cultures.

Methods: Recombinant retroviruses containing the E7 genes from cutaneous HPV types 1, 4, 5, 8, 20, 38 and RTRX7 were produced that include types associated with benign and malignant lesions. Adult human primary keratinocytes were transduced with these recombinant retroviruses and differentiated into skin-equivalents using de-epidermalised human dermis.

Results: Expression patterns of the basal keratinocyte marker cytokeratin 14 (CK14) were not altered by any of the viral E7 types analysed. However, expression of the early and late differentiation markers CK10 and involucrin were markedly altered in HPV 1, 4 and 38 cultures. The highest proliferation rates in basal cell layers, as judged by BrdU and Ki67 staining, were observed in HPV 1, 4 and 38 cultures. Interestingly, co-expression of cyclin E and p16^{INK4a} within the same cell of the suprabasal cell layers was observed only in cultures generated using E7 of HPV 5 or HPV 8.

Conclusion: HPV types associated with either benign or malignant lesions perturb keratinocyte proliferation and differentiation in different ways. Moreover, expression of E7 from HPV 5 or HPV 8 seem able to overcome p16^{INK4a} induced cell cycle arrest in a subset of keratinocytes.

Keywords: β -PV, human papillomavirus (HPV), human primary keratinocytes (HPK), organotypic skin cultures, p16^{INK4a}

1. Introduction

Human papillomaviruses (HPV) are small double-stranded DNA viruses that infect mucosal and cutaneous epithelia. Presently, about 100 HPV types are completely characterised and are classified into mucosal/genital, wart-associated and cutaneous types based on sequence analyses and clinical manifestation. Phylogenetic analysis of complete L1 gene sequences

have been identified a series of taxonomic units such as α -, β -, γ -, μ - and ν -PV [1]. PV diversification results from multiple evolutionary mechanisms such as host-linked evolution, recombination and adaptive radiation [2]. Cutaneous HPV types infecting the skin are particularly found in β - and γ -PV. HPV DNA is present in various epithelial tumors, and a causal correlation between genital high-risk α -HPV with the pathogenesis of cervical cancer has been established [3,4]. The HPV 16 oncogenes E6 and E7 are consistently expressed in cervical cancer, and play a key role during anogenital carcinogenesis, including cellular transformation and immortalisation [5]. Moreover, the inactivation of the cell cycle regulatory proteins p53 and the retinoblastoma protein (pRb) by the α -HPV oncogenes E6 and E7 has been recognised as important pathogenic mechanisms of tumor formation [6]. The E7 protein can

*Both authors contributed equally to this work.

** Corresponding author: I. Nindl, PhD, Department of Dermatology, Venereology and Allergy, Charité, Skin Cancer Center Charité, University Hospital of Berlin, Berlin, Germany. Tel.: +49 6221 42 31 60; Fax: +49 6221 42 31 69; E-mail: i.nindl@dkfz.de.

*** Present address: Division Viral Transformation Mechanisms, Research Group Viral Skin Carcinogenesis, German Cancer Research Center (DKFZ), Heidelberg, Germany.

interact with and promote the proteolytic degradation of pRb [7] through the ubiquitin–proteasome pathway [8], that functions as a negative regulator of entry from the G1- into the S-phase of the cell division cycle [9]. The cyclin-dependent kinase inhibitor p16^{INK4a} is regulated via a negative feedback control by pRb [10,11], and over-expression of p16^{INK4a} was observed in pRb negative-cell lines and in cells with a lack of pRb function through binding of viral E7 with pRb [12]. p16^{INK4a} is over-expressed in high-risk HPV (e.g., HPV 16) infected cervical (pre)cancer [13] due to reduced or lost pRb function. Thus, as the expression level of the E7 protein is very low, p16^{INK4a} expression serves as a surrogate marker of E7 activity.

A role for HPV has also been proposed in other malignancies including non-melanoma skin cancer (NMSC), with particular reference to cutaneous squamous cell carcinoma (SCC) [14–16]. The first evidence for the involvement of specific β -HPV types in cutaneous SCC was reported in patients with *Epidermodysplasia verruciformis* (EV), a very rare hereditary disease that pre-disposes individuals to infection by specific HPV types of the β -genus that were historically referred to as EV/cutaneous types, with the DNA of HPV types 5 and 8 being detected in 90% of cutaneous SCC in EV patients [17,18]. In contrast, other β -HPV types, such as 14, 17, 20 and 47, are rarely found in skin cancers [19]. However, the mechanisms by which cutaneous HPV contribute to the development of NMSC are poorly understood at present. Using monolayer cultures, the first study analysing the ability of wart-associated or cutaneous HPV types to immortalize human foreskin keratinocytes was performed with E6 and E7 of HPV 1 (μ -PV) and HPV 8 [20]. Only HPV 8 E7 showed a weak immortalizing potential whereas HPV 8 E6, HPV 1 E6 or E7 were not able to immortalize human keratinocytes. The E6 and E7 proteins of HPV 38 (β -PV), but not HPV 10 (α -PV) or HPV 20 (β -PV), display transforming activity by increasing the life span of human primary keratinocytes (HPK) [21]. The binding efficiency of HPV 38 E7 to pRb is similar to that of HPV 16 E7, whereas E7 proteins of HPV types 10 and 20 bind much less efficiently [21]. In a further study, the E7 proteins of HPV 1 and HPV 8 were also shown to bind pRb more weakly (66% and 34% respectively) compared to the HPV 16 prototype [20]. Moreover, HPV 8 E7 has been able to reduce pRb expression in non-differentiated HPK, however, the downstream effects on p16^{INK4a} expression were not characterised [22].

Organotypic skin cultures, in which keratinocytes differentiate and stratify into a squamous epithe-

lium [23], is a very powerful technology to investigate the role of HPV genes during skin carcinogenesis because the natural viral life cycle is adapted to the differentiation stage of keratinocytes. A previous study using this skin cultures with neonatal foreskin keratinocytes and a collagen matrix investigated the effects of expression of the E6 and E7 genes of the β -HPV types 5, 12, 15, 17, 20 and 38 [24]. The authors showed that these HPV types had only weak effects on the growth and differentiation of keratinocytes and no invasive phenotype was observed. In contrast, however, we showed that expression of the HPV 8 E7 gene alone induced accelerated differentiation and promoted invasion of keratinocytes into the dermis using an organotypic system with adult HPK on human dermis [25]. This transformed cell phenotype is associated with the disruption of the basement membrane where collagen types IV and VII are degraded, most likely through the over-expression of matrix-metalloproteinases MMP-1, MMP-8 and MT-1-MMP.

In the present study, we investigated the effects of the E7 protein of selected β -, γ - and μ -HPV types on differentiation, proliferation, and cell division cycle using the organotypic skin cultures, generated with human dermis and adult HPK that were transduced with recombinant retroviruses. Our findings implicate the nature of the dermal substrate as being important for the phenotype of the HPV E7-expressing keratinocytes, and highlight differences in the ability of E7 proteins from diverse HPV genera to perturb keratinocyte differentiation and cell cycle.

2. Materials and methods

2.1. Cell cultures

Cells were incubated in a humidified 10% CO₂ atmosphere at 37°C, and the medium was changed every 2 days. The NIH 3T3-derived mouse fibroblast packaging line PT67 (Clontech, Heidelberg, Germany), which was used to generate amphotrophic retroviruses, was maintained in DMEM, supplemented with 10% heat-inactivated FCS and 1% antibiotics. Dermal human primary fibroblasts and HPK were isolated from abdominal tissue of a 21 year old patient and were cultivated as described previously [25]. HPK were isolated and co-cultured with mitomycin C (Sigma, Munich, Germany) treated NIH 3T3 feeder cells, and were grown in keratinocyte culture media (RM+) composed of three parts DMEM and one

part Ham's F12 with 10% FCS and supplements [26]. For the liberation of fibroblasts, dermis was digested with collagenase D (Roche, Lewes, United Kingdom) and cells were passaged in DMEM with 10% FCS and antibiotics. Trypsinised cells were resuspended in FCS with 10% DMSO and stored in liquid nitrogen.

2.2. Cloning of HPV expression vectors and production of recombinant retroviruses

The Moloney murine leukaemia retrovirus vector pLXSN (Clontech) was used to generate recombinant retroviruses containing HPV E7 genes. E7 open reading frames (ORFs) of HPV types 1, 4, 5, 8, 16, 20, 38 and RTRX7 were amplified by HPV type specific PCR (Table 1). Each primer contained a restriction endonuclease site at their 5' ends with the exception of HPV 1 and HPV 16. The E7 PCR amplicon was digested with the corresponding restriction enzymes and inserted into the digested pLXSN vector, downstream of the Moloney murine sarcoma virus 5' long terminal repeat sequence, in order to obtain pLXSN-HPV-E7 (Table 1). All constructs with the E7 genes of these 8 types were sequenced and compared to HPV sequences. All E7 genes revealed HPV prototype sequences of the following accession numbers (HPV 1, NC_001356; HPV 4, NC_001457; HPV 5, NC_001531; HPV 8, M12737; HPV 16, NC_001526; HPV 20, U31778; HPV 38, U31787; and RTRX7, NC_004761). Amphotrophic recombinant retroviruses were generated by transfecting pLXSN-derived plasmid DNA into the packaging cell line PT67 with the transfection reagent FuGENE 6 (Roche) following the manufacturer's instructions. Two days after transfection, cells were transferred into selection media containing 500 µg/ml G418 (Sigma). Resistant transfected cells were grown to confluence, and subsequently retrovirus containing supernatants were collected and stored at -80°C for further infections.

2.3. Infection of epidermal HPK with recombinant retroviruses

Adult HPK at passage 1 were seeded in 'defined keratinocyte serum-free medium' (Gibco; Karlsruhe, Germany) at a cell density of 5×10^4 cells/cm² in 6 cm dishes. After 2 days, retroviral supernatants were mixed with an equal volume of serum-free DMEM in the presence of 5 µg/ml of hexadimethrine bromide

(polybrene, Sigma) and added to the keratinocytes. Infection was performed by centrifugation at $350 \times g$ for 1 h. Subsequently, cells were washed with phosphate buffered saline (PBS) and cultured in defined keratinocyte-serum-free medium. After 2 days, cells were selected with G418 (150 µg/ml) for 4 days after which exclusively infected keratinocytes survived. The survival rate after G418 selection of E7 infected cells for all HPV types was between 80–90%. Keratinocytes were incubated in a humidified 5% CO₂ atmosphere at 37°C, and the medium was changed every two days. The cells were trypsinised before reaching confluency and were used immediately in organotypic skin cultures as described below. From the remaining infected keratinocytes RNA was isolated to control the expression of cutaneous HPV E7 using RT-PCR and quantify the mRNA levels by real-time RT-PCR (Table 1).

2.4. Organotypic skin cultures with de-epidermalised dermis

The organotypic cultures were obtained as previously described [25]. Glycerol-preserved skin (Euro Skin Bank, Beverwijk, The Netherlands) was washed three times in PBS and incubated in PBS containing antibiotics (600 units/ml penicillin-G, 600 µg/ml streptomycin sulfate, 250 µg/ml gentamicin sulfate and 2.5 µg/ml fungizone) at 37°C for up to 10 days. Subsequently, the epidermis was mechanically removed using forceps, and de-epidermalised dermis (DED) was cut into 2 × 2 cm squares and placed in culture plates with the papillary dermal surface on the underside. Stainless steel rings were placed on top of the dermis, and human primary dermal fibroblasts (5×10^5 cells) in RM+ medium were inoculated into the rings on the reticular dermal surface. Following an overnight incubation, DED was inverted for the orientation of the papillary dermal surface on top before the rings were replaced. Retroviral-infected HPK (3.0×10^5 cells) in RM+ medium were seeded inside the rings onto the dermis. After 2 days, the dermis was raised to the air-liquid interface in the same orientation by placing the composites on stainless steel grids and the RM+ medium was refreshed every 3 days. After 10 days the differentiated keratinocytes were incubated with 50 µM BrdU (5-bromo-2-deoxyuridine; Sigma) for 2 h. Subsequently, skin cultures were fixed in 10% buffered formalin and embedded in paraffin for further analysis.

Table 1
Primer sequences used to generate recombinant retroviruses and to examine E7 RNA expression

	Amplification of E7 to generate pLXSN-HPV E7 expression vectors			RT-PCR		Quantitative real-time RT-PCR	
	Forward primer 5' → 3'	Reverse primer 5' → 3'	Restriction site	Forward primer 5' → 3'	Reverse primer 5' → 3'	Forward primer 5' → 3'	Reverse primer 5' → 3'
HPV 1 E7	[39]			TAT GCT GTC GTT GCT TCC TG	CGA TGT TCA AAG ATC GCA GA	TAT GCT GTC GTT GCT TCC TG	CGA TGT TCA AAG ATC GCA GA
HPV 4 E7	GGA ATT CCA ACA ATG AGA GGA GCA GCG C	CGG GAT CCC GCA TTC TGC TTC ATG GAC AAT A	<i>EcoRI/BamHI</i>	GCT GAG TGA GGA GGT CTT G	TGT TCC AAG GTC CGT AGT CC	GGA GTC GGT GGT TCC ATT TA	CGA TGT TCA AAG ATC GCA GA
HPV 5 E7	GGA ATT CCG GAA TCT GTA GGC AGT GTA AGC A	CCG CTC GAGC GGC ACC AAT CAC CAA ACC CTT C	<i>EcoRI/XhoI</i>	GGT TGC AGG AAC TGT GAG GT	GCA GAT CTC CGG TCA GTA GC	GAG GAG CCT GAC AAC GAA AG	TCC GTC ATG TTT GCA GTT TC
HPV 8 E7	AAG CTT GAA TTC GAG TTT GC AGG CTT TGT AAG C	GAA GCT TGG ATC CCC TTC TTT AGA TGT ACT ACC	<i>EcoRI/BamHI</i>	TGC TGT CAG GTC AAG CTA CG	GGC ACT CAG GAC ACA GAA GC	AGG AAA CGG AGG AGG AGC TA	CGT AGC TTG ACC TGA CAG CA
HPV 20 E7	GGA ATT CCG GAA TCT GTA GGC TGT GTA AGC	CCG CTC GAG CGG CAA TCA TCC AAC CCG TCT T	<i>EcoRI/XhoI</i>	CAG CCT GAG GTT CAA CCA GT	TCC TCT CTC TCC TGC TGC TC	GAG CAG CAG GAG AGA GAG GA	GCG ACA GTC AGG ACA CAA CA
HPV 38 E7	GAA GCT TGA ATT CCG GCA TTG CAA AGC AAT AGA A	AGC TTG GAT CCC GCA GCC TTC TTT AGG ATC AGT ACC	<i>EcoRI/BamHI</i>	AGA GGA GGA GCC AGC ATA CA	GGT GGG ACA CAG AAG CCT TA	AGA GGA GGA GCC AGC ATA CA	TGC GAA TGT CTT CTC GAC AG
RTRX7 E7	AAG CTT GAA TTC GAG TGT GCA GGC ACT GTA AGC	GAA GCT TGG ATC CCT TCT TTA GAT GTA CTA CC	<i>EcoRI/BamHI</i>	AGT GAG GTG CAG CCT GAA GT	ACT CCT CCT CCG TTT CCT GT	AGT GAG GTG CAG CCT GAA GT	ACA CGG TGC AAT GAC TTT GA
HPV 16 E7	[30]			GCT CAG AGG AGG AGG ATG AA	AAC CGA AGC GTA GAG TCA CA	–	–
RPS9	–	–	–	ATC CGC CAG CGC CAT A	TCA ATG TGC TTC TGG GAA TCC	–	–
GAPDH	–	–	–	–	–	[40]	–

2.5. RNA isolation and reverse transcription- (RT) PCR

Total RNA was isolated from retroviral infected epidermal HPK using the RNeasy Micro Kit (Qiagen; Hilden, Germany) following manufacturer's instructions. The cells were digested with 10 µl proteinase K (10 mg/ml) (Roth, Karlsruhe, Germany) at 55°C for 15 min. Subsequently, the isolated nucleic acids were digested with DNA-free Dnase I (Invitrogen, Karlsruhe, Germany). Quantification of total RNA was performed by photometrical analysis using Biometra Gene Ray according to the manufacturer's instructions (Biometra, Göttingen, Germany).

After reverse transcription with oligo-dT and the Superscript First-Strand Synthesis system for RT-PCR (Invitrogen), the concentration of cDNA was quantified with an OliGreen ssDNA Quantitation Reagent Kit (Molecular Probes, Karlsruhe, Germany). E7 gene specific primers were designed for a subset of different HPV types (Table 1) using the Primer3 software [27]. PCR was performed using the Eppendorf mastercycler gradient (Eppendorf, Hamburg, Germany). The PCR reaction mixture of 25 µl contained 50 ng cDNA in a standard 'PCR reaction buffer' (Qiagen), 1.5 units Taq polymerase (Qiagen), 1.5 mM MgCl₂, 200 µM dNTPs and 500 nM of each primer. PCR reaction was initiated with 2 min denaturation at 95°C followed by 40 cycles consisting of a denaturation step (95°C for 45 s), a primer annealing step (60°C for 30 s) and an elongation step (72°C for 30 s). The RNA quality and amount of each specimen was controlled by analysing the housekeeping gene 40S ribosomal protein S9 (RPS9). In addition, the PCR amplicons were separated in a 3% agarose gel by electrophoresis (Suppl. Fig. S1: <http://www.qub.ac.uk/isco/JCO>).

2.6. E7 quantification using real-time RT-PCR

cDNA synthesis and quantification was performed as described above. qRT-PCR was conducted using LightCycler technology (Roche) and was performed in duplicate for each sample in three independent experiments. The amplification mix (10 µl) contained 420–450 ng cDNA (1 µl), 1 mM of each E7 specific primer (Table 1) (total 1 µl; 0.5 µl of each 20 mM primer), 2 µl LightCycler® FastStart DNA MasterPLUS SYBR Green I (Roche) and 6 µl DNase/RNase-free water. The qRT-PCR protocol included 10 min initial denaturation at 95°C and 50 cycles: 10 s at 95°C (denaturation step), 5 s at 60°C (annealing step)

and 20 s at 72°C (extension step). Specificity of PCR products was verified by melting curve analysis using temperatures from 65°C to 95°C for 5 s plus 50°C for 15 s. This analysis showed that specific products and not primer dimers were the majority of amplicons. Linear regression analysing a dilution series of 1, 0.1 and 0.01 µl cDNA of each HPV type was performed. Moreover, amplicons were separated in 1% agarose gel to confirm the estimated size of each E7 gene of all HPV types included in this study. Expression levels were determined as the ratio between each E7 gene (as the mean of three independent experiments) and the reference gene GAPDH in order to correct for variation in the mRNA levels (Suppl. Fig. S2: <http://www.qub.ac.uk/isco/JCO>).

2.7. Immunohistochemistry

For immunohistochemistry, 4 µm sections were deparaffinised, rehydrated and rinsed in PBS. Consecutive sections of each skin culture were stained with haematoxylin and eosin for histology. Furthermore, sections were subjected to antigen retrieval with citrate buffer (pH 6.0) at 97°C for 20 min. Unspecific binding of the primary antibody was blocked by the incubation of 10% normal goat serum at room temperature for 30 min. The primary mouse monoclonal antibodies were specific for: cyclin E (clone HE12, 1:30 diluted; Biozol, Eching, Germany), anti-BrdU (clone BMC 9318, 1:50 diluted; Roche), Ki67 (clone Ki-S5, 1:100 diluted; Dako, Hamburg, Germany), cytokeratin 10 (CK10) (clone VIK-10, 1:200 diluted; Acris, Hiddenhausen, Germany), CK14 (clone LL002, 1:200 diluted; Acris), involucrin (clone SY5, 1:200 diluted; Acris) and p16^{INK4a} (using the CINtec(R) histology kit based on antibody clone MTM E6H4, 1:200 diluted; mtm laboratories, Heidelberg, Germany). Sections were incubated at room temperature for 1 hour, washed in PBS, and incubated for 30 min either with a biotinylated secondary goat anti mouse-antibody and processed with a streptavidin-biotinperoxidase detection system (LSAB2; Dako) as recommended by the manufacturer or with a goat anti mouse-IgG-specific fluorescent conjugated secondary antibody (1:4000 diluted; Molecular Probes). To exclude unspecific staining of the secondary antibody and the staining substrate immunohistochemistry was performed without the primary antibody. Double staining of p16^{INK4a} and cyclin E was performed with IgG-specific fluorescent conjugated secondary antibodies as described above. Sections were counterstained with hematoxylin

or DAPI and mounted in aqueous permanent mounting solution or fluorescent anti-fade (Dako) covered with a slip and air-dried. At least two independent consecutive sections of all organotypic cultures were stained and only concordant results were used for further analysis.

3. Results

3.1. Organotypic skin cultures expressing different E7 proteins of cutaneous HPV types showed altered histology

The expression of different E7 proteins from cutaneous HPV types showed diverse effects on keratinocyte differentiation in organotypic skin cultures. Epidermal HPK were infected with recombinant retroviruses expressing a given E7 gene under the control of the MoMuSV retroviral 5'-LTR-promoter, whose activity is insensitive to keratinocyte differentiation, and organotypic cultures generated over a 10 day period. The following HPV types were used: HPV 1 (μ -PV, associated with benign cutaneous warts), HPV 4 (γ -PV, wart-associated and detected in primary cutaneous SCC and metastases [28]), HPV types 5, 8, 20, 38, RTRX7 (β -PV, detected in hairs and cutaneous SCC from (non)immunosuppressed patients and EV patients) and the oncogenic high-risk genital type HPV 16 (α -PV). The presence of E7 mRNA of all HPV types (HPV 1, 4, 5, 8, 20, 38 and RTRX7) in undifferentiated monolayer HPK was confirmed by RT-PCR (Suppl. Fig. S1: <http://www.qub.ac.uk/isco/JCO>). The E7 protein expression was confirmed for HPV 16 by western blot analysis of cells grown in monolayers, detectable by immunocytochemistry in monolayer cells (Fig. 1) but not by immunohistochemistry in organotypic skin cultures.

The histology of the epithelial tissue from skin cultures was compared with human normal skin (Fig. 2). HPK transduced with control empty retrovirus (pLXSN) established a distinct squamous epithelium with typical layers indicating that the empty vector did not influence culture conditions and epithelial differentiation. Overall, organotypic skin cultures generated from epidermal HPK transduced with E7 showed an altered histology compared either to human normal epithelium (non-infected) or vector-only infected HPK (Fig. 2). Hyperkeratosis was observed in E7 infected cultures of all HPV types examined and was strongest with HPV 4, which displayed characteris-

tic morphological features of cutaneous warts such as papillary structures and distinct hyperkeratosis. Cultures infected with HPV 16 and HPV 1 were disorganised and showed dysplastic morphology with dyskeratotic keratinocytes in the granular layer. Furthermore, suprabasal nuclei appeared larger than those in basal layers of HPV 5 skin cultures, which also showed the strongest occurrence of papilla-like structures. Parakeratosis was exclusively observed in HPV 38 infected skin cultures indicated by nuclei in differentiated keratinocytes of the stratum corneum.

3.2. Alteration of keratinocyte late differentiation by E7 of HPV 1, 4 and 38 in organotypic skin cultures

To examine the influence of E7 on the differentiation of keratinocytes, we analysed the protein expression of the basal cell marker CK14, together with early (CK10) and late (involucrin) differentiation markers by immunohistochemistry. A representative experiment is shown in Fig. 3. The CK14 expression pattern observed was not altered in any of the skin cultures, indicating that very early stages of keratinocyte differentiation were not disturbed by any of the HPV E7 types investigated (Suppl. Fig. S3: <http://www.qub.ac.uk/isco/JCO>). HPV 38 E7 expression altered specifically the CK10 expression pattern such that the protein was either absent in some cells, or alternatively localised in aggregates on the cell membrane in other regions of the culture (Fig. 3). In contrast, the other E7 types investigated had only marginal effects on CK10 expression patterns. Involucrin was expressed in the cornified strata of all cultures, except for HPV 1 and HPV 4 E7 cultures where involucrin expression was strongly reduced through the entire upper granular layer indicating a perturbation of late differentiation by these E7 types (Fig. 3).

3.3. Increased proliferation by E7 of HPV 1, 4 and 38, and to a lesser extent of HPV 5, 8 and 20 in organotypic cultures

Proliferation of keratinocytes was analysed using both Ki67 expression and transient BrdU incorporation that specifically labels cells in S-phase. We investigated whether Ki67 was expressed in suprabasal keratinocytes transduced with E7 of different HPV types, since in normal epidermis Ki67 protein should only be found in the nuclei of basal cells. Ki67 expression was detected in basal cells and additionally in suprabasal

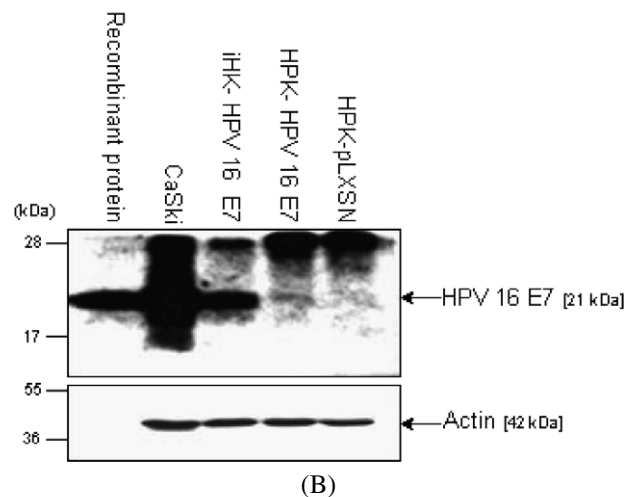
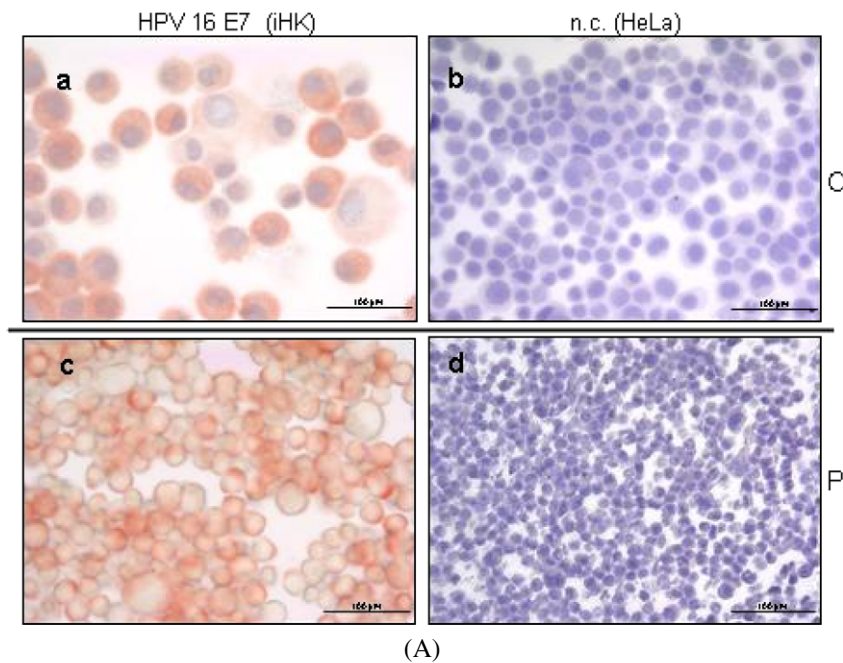


Fig. 1. Detection of HPV 16 E7 protein in transfected human primary keratinocytes (HPK). (A) For immunocytochemistry, either cytopins (a, b) or sections of formalin fixed and paraffin-embedded cells (c, d) were used. Protein expression of HPV 16 E7 in immortalized human foreskin keratinocytes (iHK) in a ratio of 1:1 (HPV 16 E6 and HPV 16 E7) (a, c) or HPV 18 positive HeLa cells (b, d). The HPV 16 E7 protein could be detected in the cytoplasm of both the cytopins and paraffin-embedded iHK using the monoclonal antibody (Zymed, clone 8C9, diluted 1:2). Specificity was shown with HeLa cells, which were negative under these conditions. (B) Western blot analysis was performed with whole protein extracts (100 μ g/lane) of cell lines or 30 μ g purified recombinant HPV 16 E7 protein separated in a 12% polyacrylamid gel. Monoclonal antibodies were used to detect HPV 16 E7 (clone nm2, diluted 1:2,000; kindly provided by M. Müller, DKFZ, Heidelberg) or Actin (MP biomedical, clone C4, diluted 1:40,000). Detection was performed using the enhanced chemiluminescence (ECL) substrate according to the manufacturer's instructions ("super signal west femto maximum sensitivity substrate", Pierce Biotechnology, Rockford, USA). The highest HPV 16 E7 protein amounts were present in CaSki cells, followed by iHK, and only weak expression was observed in retroviral-infected HPK. (n.c., negative control; C, cytopins; P, sections of paraffin-embedded cells.)

keratinocytes expressing E7 of HPV types 1, 4 and 38, and as expected of HPV 16 indicating that proliferation was induced in differentiated keratinocytes that are normally post-mitotic. In all other E7-expressing

cultures Ki67 expression was restricted to the basal cells. To examine host DNA replication in proliferating cells, we analysed BrdU incorporation. In E7 expressing skin cultures, BrdU positive cells were observed

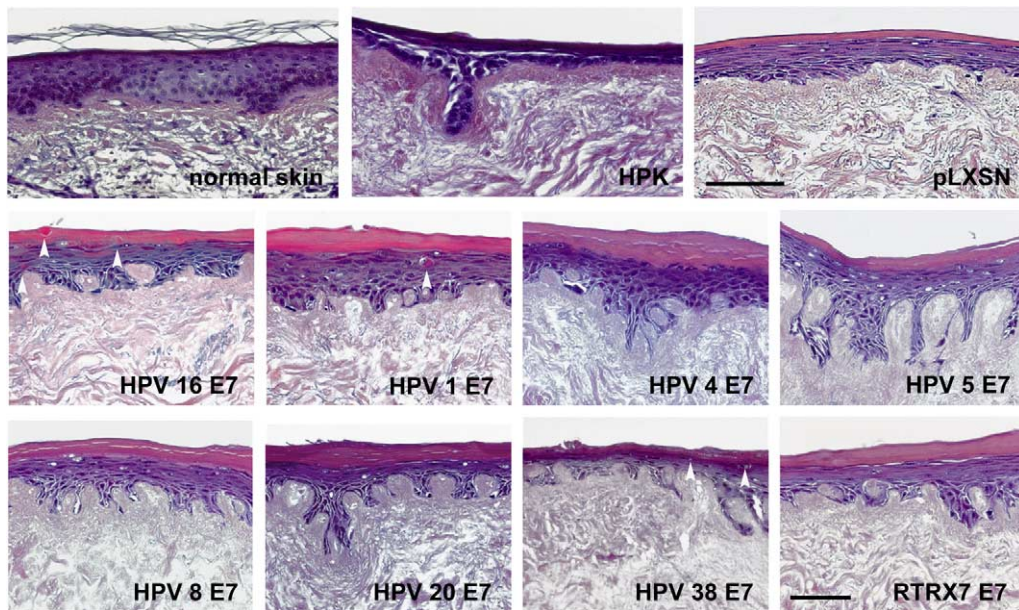


Fig. 2. Altered histology in organotypic skin cultures infected with E7 of cutaneous HPV types. Epidermal human primary keratinocytes (HPK) were non-infected, infected with the control vector pLXSN, or pLXSN-HPV × E7 containing recombinant retroviruses and used in organotypic skin cultures. For histological examination tissue sections were stained with hematoxylin and eosin. Retrovirus infected cultures both with and without HPV E7 sequences showed a distinct hyperkeratosis, which was absent in normal human skin and non-infected HPK. An enhanced hyperkeratosis was observed in E7 infected cultures of any HPV type and the strongest hyperkeratosis was present with HPV 4, which is characteristic for cutaneous warts. Cultures infected with pLXSN showed a normal morphology of the suprabasal layers. The histology of E7 infected skin cultures varied to different degrees depending on the HPV type. The epithelium of HPV 16 and HPV 1 infected HPK were disorganised and showed dysplastic morphology (marked with white arrows). Parakeratosis, which is characteristic of HPV induced warts, was only observed in HPV 38 infected tissues. HPV 5 showed distinct papilla-like structures and the largest nuclei were present in the granular layer and not in the spinous layer. HPV types used to infect HPK are denoted. Original magnification was ×200. Scale bar: 100 µm.

in the stratum basale and stratum spinosum. Four experiments using different sections of each paraffin-embedded skin culture were performed and the mean numbers of BrdU or Ki67 positive cells within 1 cm² were calculated (Fig. 4(B)). BrdU incorporation was restricted to cycling cells of the basal layer in most E7 cultures examined and was comparable to that seen in control cultures. However, expression of E7 of HPV types 1, 4 and 16, resulted in BrdU incorporation in differentiated cell layers that are normally post-mitotic. Expression of HPV 38 E7 also resulted in higher numbers of BrdU positive cells when compared to cultures generated by vector-only, but in contrast to the HPV 1, 4 and 16 E7 cultures the BrdU positive cells were restricted to less differentiated cell layers.

3.4. Co-localisation of p16^{INK4a} with cyclin E in HPV 5 and HPV 8 infected organotypic skin cultures

The p16^{INK4a} pathway modulates an important checkpoint in the cell division cycle of epithelial ker-

atinocytes. We have determined the expression of this protein by immunohistochemistry. This cyclin-dependent kinase inhibitor was present in the nuclei and the cytoplasm in keratinocytes of the skin cultures infected with the control vector pLXSN and all HPV types. The p16^{INK4a} staining pattern was grouped in three categories for analysis: (i) less than 30%, (ii) between 30% and 60%, and (iii) more than 60% of positive keratinocytes. The lowest number of p16^{INK4a} positive keratinocytes was observed in E7 cultures expressing either HPV 1 or HPV 4 cultures (less than 30%), followed by HPV 20 and HPV 38 E7 cultures (30–60%), with the highest p16^{INK4a} staining being seen in cultures derived from expression of E7 from HPV 5, HPV 8, RTRX7 and HPV 16 (more than 60%) (Fig. 5, Suppl. Fig. S4: <http://www.qub.ac.uk/isco/JCO>). Cyclin E is a marker of late G1-/S-phase of cell cycle division and disruption of the G1-/S-transition in HPV 16 E7 expressing human cells is associated with altered regulation of cyclin E [29]. We therefore investigated co-localisation of p16^{INK4a} and the cell cycle marker cyclin E within

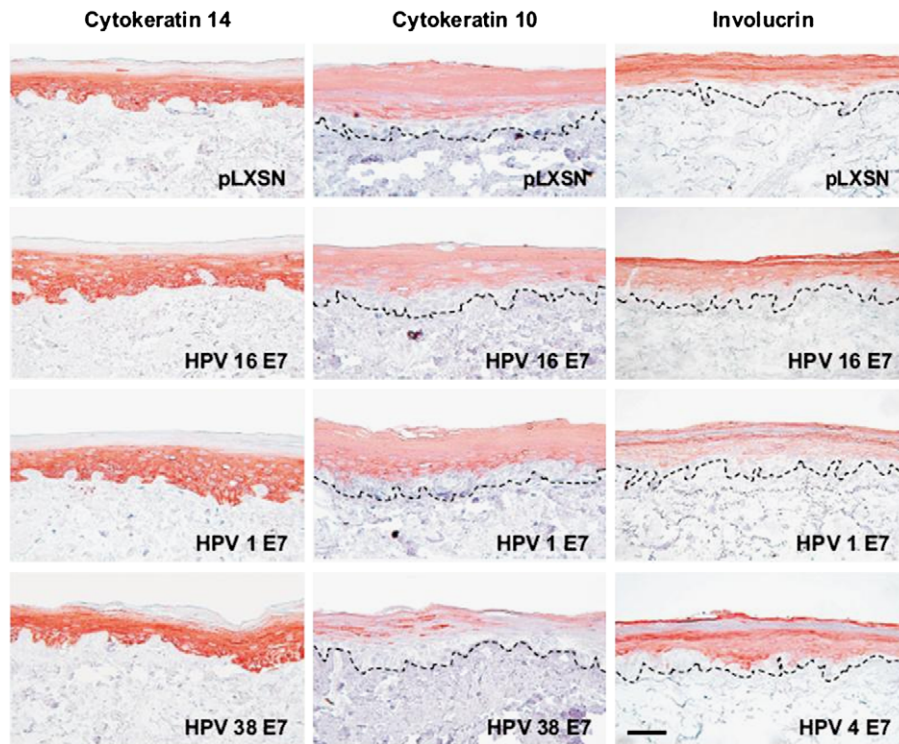


Fig. 3. Perturbed differentiation in HPV 1, HPV 4 and HPV 38 E7 organotypic skin cultures. Paraffin-embedded sections of organotypic skin cultures were stained with antibodies to CK14, CK10 and involucrin. The expression of the basal cell marker CK14 was not altered by any of the HPV types investigated (Suppl. Fig. S3: <http://www.qub.ac.uk/isco/JCO>). HPV 1, HPV 4 and HPV 38 E7 cultures showed a disturbed expression pattern of either the early (CK10) and/or the late (involucrin) differentiation marker. pLXSN, HPK infected with the control vector. Original magnification was $\times 400$. Scale bar: 100 μm .

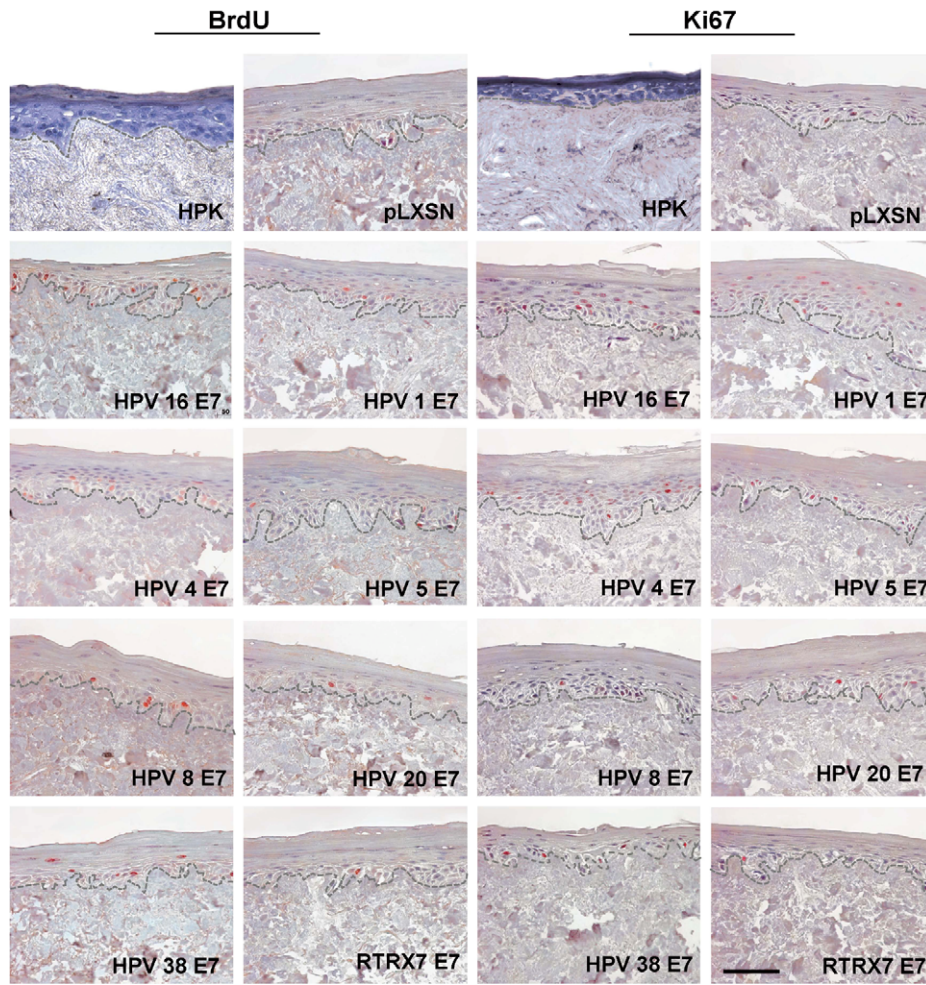
the same cell by double staining using fluorescence immunohistochemistry to determine whether E7 expression was able to overcome p16^{INK4a} induced cell cycle block in post-mitotic cells. Keratinocytes that stained positive for both p16^{INK4a} and cyclin E were observed in suprabasal layers of skin cultures infected with E7 of genital HPV 16 and, interestingly, or the β -HPV types 5 and 8, whereas co-localisation was absent in the negative control (pLXSN) and all other HPV types investigated (Fig. 5). Noteworthy also is the observation that in the HPV 5 and 8 E7 cultures that in the cells doubly positive for cyclin E and p16^{INK4a}, the p16^{INK4a} expression was mis-localised to the cytoplasm.

4. Discussion

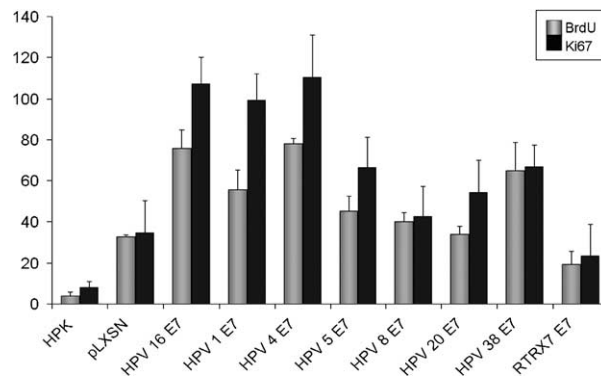
In this study, we examined effects on histology, differentiation, proliferation and cell division cycles in E7 transduced organotypic skin cultures of cutaneous HPV types associated with benign or malignant lesions. The skin cultures showed an altered histology

and differentiation pattern, an increased proliferation rate and differences in p16^{INK4a} expression to a varying degree depending on the HPV type.

Epidermal HPK were infected with recombinant retroviruses using the pLXSN vector designed for retroviral gene delivery. Gene expression was controlled by the 5'LTR promoter, whose activity is independent of the differentiation state of infected keratinocytes in organotypic skin cultures [24,30,31]. In our study, retrovirally infected epithelial HPK were used to generate organotypic skin cultures that showed hyperkeratosis with the control vector alone, most likely due to culture conditions [32], but this hyperkeratosis was enhanced by expression of E7 sequences from all analysed HPV types, but a distinctive and more pronounced hyperkeratosis was seen in HPV 4 infected cultures. The transduction of E7 of different cutaneous HPV types resulted in varying degrees of disorganisation of the epithelial tissue. Interestingly, HPV 16 and HPV 1 induced disorganised and dysplastic morphology with dyskeratotic cells. Of all the HPV types investigated only HPV 38 infected skin cultures



(A)



(B)

Fig. 4. Increased proliferation in organotypic skin cultures infected with E7 of cutaneous HPV types. (A) The presence of the proliferation markers BrdU and Ki67 was investigated by immunohistochemistry. Representative examples of paraffin embedded sections from organotypic skin cultures are shown with non-infected adult human primary keratinocytes (HPK), or infected with recombinant retroviruses (pLXSN, or E7 of HPV types 1, 4, 5, 8, 16, 20, 38 or RTRX7). Original magnification was $\times 200$. Scale bar: 100 μm . (B) BrdU and Ki67 positive cells in the epidermis are given as mean \pm SD within 1 cm^2 of four independent experiments (*y*-axis). The numbers of BrdU or Ki67 positive keratinocytes were determined within 1 cm^2 of the epidermis of each section. The highest proliferation rates indicated by BrdU or Ki67 positive cells was observed with HPV types 1, 4, 16 and 38, and lower rates with HPV types 5, 8 and 20.

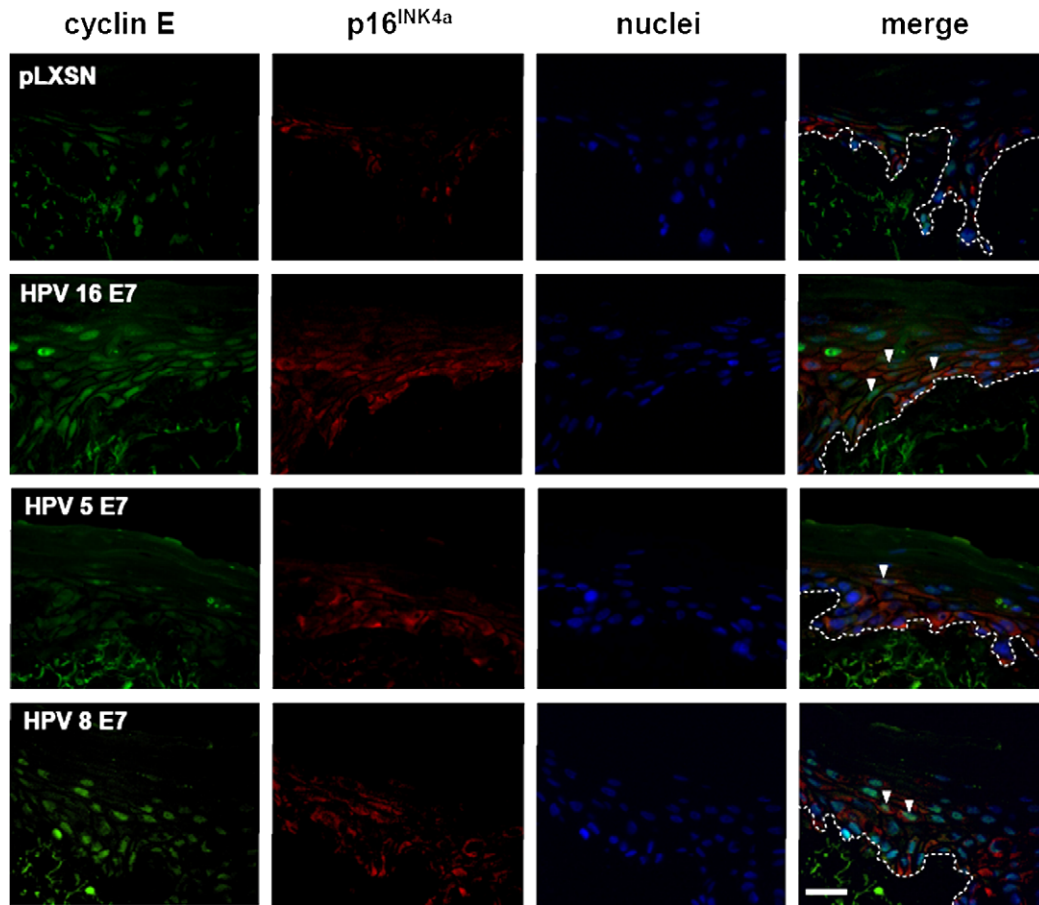


Fig. 5. Suprabasal co-localisation of cyclin E and p16^{INK4a} in organotypic skin cultures infected with E7 of HPV 5, or 8. The presence of cyclin E (green) and p16^{INK4a} (red) was examined by double staining of paraffin embedded organotypic skin culture sections. The nuclei were stained with DAPI (blue). Four representative examples of skin cultures infected with E7 of HPV 5 or 8 (β -PV) or HPV 16 (α -PV, positive control) or the empty vector pLXSN (negative control) are shown. Co-localisation of p16^{INK4a} and the cell cycle marker cyclin E within the same keratinocytes at the suprabasal layer of HPV 5 and HPV 8 are shown indicated by white arrows. Original magnification was $\times 400$. Scale bar: 100 μm . (The colors are visible in the online version of the article.)

showed parakeratosis, which is characteristic for HPV induced human cutaneous warts. Distinct papilla-like structures and larger nuclei in the granular layer were associated with E7 expression of HPV 5, and not in the spinous layer as would normally be expected.

We found neither differences of CK14 expression in the basal layer of control pLXSN cultures nor in any HPV type examined indicating that the expression of E7 does not disturb very early differentiation patterns in our organotypic skin cultures. CK10 is a marker for early differentiation expressed in the lower and upper suprabasal layer. A previous study analysing the effect of E6/E7 of β -HPV types 5, 12, 15, 17, 20 and 38 with an organotypic skin culture system found that CK10 was only expressed in the upper suprabasal layer [24]. In our study, a perturbed

and discontinuous CK10 staining pattern was observed in HPV 38 E7 skin cultures, where CK10 was only present in the upper suprabasal layer and displayed a punctate pattern. In contrast, cultures of HPV types 4, 5, 8, 20, RTRX7 had a normal CK10 staining pattern, with only marginal effects produced by expression of HPV 1 E7. We found no altered CK10 expression with HPV 5 E7 and HPV 20 E7 whereas Boxman and colleagues [24] showed a disturbed early differentiation in HPV 5 and 20 skin cultures expressing both proteins E6 and E7. The differences observed in both studies may be due to the different target cells, neonatal foreskin keratinocytes versus adult HPK used in our study. Another explanation is that the E6 gene rather than the E7 gene, or a combination of both, alters CK10 expression in neonatal foreskin keratinocytes.

The late differentiation marker involucrin is a cytoplasmic protein expressed in epithelial skin [33] and is localised to the upper third of human normal epidermis. In *Verruca vulgaris* involucrin is uniformly expressed in the cornified, granular and spinous layers, but absent or only stains patches of keratinocytes in cutaneous SCC [34]. However, in our study an alteration of late differentiation was only found with the wart-associated types HPV 1 and HPV 4. These cultures showed no involucrin expression in the upper third of the epithelium with the exception of the fully keratinised cells in the cornified layer. Interestingly, HPV types 1 and 4, which altered early and late differentiation, also induced the highest proliferation rates in our organotypic skin cultures with cycling cells being present in suprabasal cell layers. Thus, the establishment of the hyperproliferative lesion by HPV 1 and HPV 4 E7 cultures is at the expense of epithelial differentiation processes.

The cyclin-dependent kinase inhibitor p16^{INK4a} is regulated via a negative feedback control by pRb [10,11], and over-expression of p16^{INK4a} was observed in pRb negative-cell lines and in cells with a lack of pRb function through binding of E7 with pRb [12]. In cervical cancer patients over-expression of p16^{INK4a} was observed only in high-risk but not low-risk genital HPV infected cervical intraepithelial neoplasia (CIN) [13]. However, the direct interactions of E7 of β -HPV types with pRb, and thus indirect interference with p16^{INK4a} expression are by contrast only poorly understood. p16^{INK4a} is over-expressed not in all but in a subset of dysplastic keratinocytes in actinic keratosis and cutaneous SCC [35]. In our study, p16^{INK4a} was expressed in skin cultures either with or without HPV E7 expression. A significant reduction of p16^{INK4a} positive keratinocytes was only observed in HPV 1 and HPV 4 skin cultures. These findings suggest that these viral E7 types cannot overcome the cell cycle inhibitory effects of p16^{INK4a}, and that the high proliferation rates observed in skin cultures generated using these E7 types may depend upon p16^{INK4a} down-regulation. How these E7 types may down-modulate p16^{INK4a} activity is unknown.

Cyclin E expression is necessary for the progression from the G1- to S-phase where it advances the cell cycle through the inactivation of pRb [36], and has been shown to be over-expressed in a number of human tumors [37]. In normal epidermis cyclin E expression is restricted to a minority of basal keratinocytes that are in cell cycle. Since it is possible that the suprabasal cells that were in cell cycle were cyclin E

positive/p16^{INK4a} negative, and vice versa, we investigated whether cyclin E and p16^{INK4a} expression was co-incident in the same cell in cultures of the different HPV E7 proteins. Interestingly, only in HPV 5 and HPV 8 skin cultures we found keratinocytes of the suprabasal layers positive for both cyclin E and p16^{INK4a} suggesting that E7 of these types were able to overcome the inhibitory effects of p16^{INK4a} on the cell cycle. Co-expression of cyclin E together with p16^{INK4a} in the same cell was not seen for any of the other E7 types tested, implying that only a subset of E7 types can overcome p16^{INK4a} activity. The expression status of p16^{INK4a} in cervical lesions has been proposed to be associated with infection of oncogenic anogenital HPV types [38]. Our findings suggest that the E7 of HPV types 5 and 8 may be more associated with the development of malignant lesions.

Skin carcinogenesis is a complex and multifactorial process and at present the possible roles of cutaneous HPV E7 proteins has not yet been defined fully. Our data demonstrate that in organotypic skin cultures the E7 protein of HPV 1, HPV 4 and HPV 38 perturb keratinocyte proliferation, differentiation, and cell cycle markers in different ways that may either contribute towards benign or malignant growth of cells. In contrast, E7 of HPV 5 and HPV 8 seem to be able to overcome p16^{INK4a} induced cell cycle effects in a subset of post-mitotic cells. The uncoupling of normal cell cycle checkpoints and keratinocyte differentiation by HPV types 5 and 8 that results in unscheduled DNA synthesis may in turn lead to genetic instability in these cells, and thereby contribute towards the early stages of cutaneous SCC development.

Acknowledgements

The work of K. Westphal was supported by a fellowship of the Charité, University Hospital of Berlin, Germany, and by a travel allowance of Boehringer Ingelheim Fonds, Heidesheim, Germany. B. Akgül was supported by the Mildred Scheel Stiftung/Deutsche Krebshilfe, Germany, and by the grant PF123/5-1 of the Deutsche Forschungsgemeinschaft.

References

- [1] E.M. de Villiers, C. Fauquet, T.R. Broker, H.U. Bernard and H. zur Hausen, Classification of papillomaviruses, *Virology* **324** (2004), 17–27.

- [2] M. Gottschling, A. Stamatakis, I. Nindl, E. Stockfleth, A. Alonso and I.G. Bravo, Multiple evolutionary mechanisms drive papillomavirus diversification, *Mol. Biol. Evol.* **24** (2007), 1242–1258.
- [3] N. Munoz, F.X. Bosch, S. de Sanjose, R. Herrero, X. Castellsague, K.V. Shah, P.J. Snijders and C.J. Meijer, Epidemiologic classification of human papillomavirus types associated with cervical cancer, *N. Engl. J. Med.* **348** (2003), 518–527.
- [4] International Agency for Research on Cancer, IARC monographs on the evaluation of carcinogenic risks to humans. Human papillomaviruses, *IARC Lyon France* **64** (1995).
- [5] H. zur Hausen, Molecular pathogenesis of cancer of the cervix and its causation by specific human papillomavirus types, *Curr. Top. Microbiol. Immunol.* **186** (1994), 131–156.
- [6] H. zur Hausen, Papillomaviruses and cancer: from basic studies to clinical application, *Nat. Rev. Cancer* **2** (2002), 342–350.
- [7] E. Berezutskaya, B. Yu, A. Morozov, P. Raychaudhuri and S. Bagchi, Differential regulation of the pocket domains of the retinoblastoma family proteins by the HPV16 E7 oncoprotein, *Cell Growth Differ.* **8** (1997), 1277–1286.
- [8] S.N. Boyer, D.E. Wazer and V. Band, E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin–proteasome pathway, *Cancer Res.* **56** (1996), 4620–4624.
- [9] N. Dyson, P.M. Howley, K. Muenger and E. Harlow, The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product, *Science* **243** (1989), 934–937.
- [10] Y. Li, M.A. Nichols, J.W. Shay and Y. Xiong, Transcriptional repression of the D-type cyclin-dependent kinase inhibitor p16 by the retinoblastoma susceptibility gene product pRb, *Cancer Res.* **54** (1994), 6078–6082.
- [11] S.N. Khleif, J. DeGregori, C.L. Yee, G.A. Otterson, F.J. Kaye, J.R. Nevins and P.M. Howley, Inhibition of cyclin D-CDK4/CDK6 activity is associated with an E2F-mediated induction of cyclin kinase inhibitor activity, *Proc. Natl. Acad. Sci. USA* **93** (1996), 4350–4354.
- [12] D. Parry, S. Bates, D.J. Mann and G. Peters, Lack of cyclin D-Cdk complexes in Rb-negative cells correlates with high levels of p16INK4/MTS1 tumour suppressor gene product, *EMBO J.* **14** (1995), 503–511.
- [13] R. Klaes, T. Friedrich, D. Spitkovsky, R. Ridder, W. Rudy, U. Petry, G. Dallenbach-Hellweg, D. Schmidt and D.M. von Knebel Doeberitz, Overexpression of p16(INK4A) as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri, *Int. J. Cancer* **92** (2001), 276–284.
- [14] H. Pfister, Chapter 8: Human papillomavirus and skin cancer, *J. Natl. Cancer Inst. Monogr.* **31** (2003), 52–56.
- [15] B. Akgül, J.C. Cooke and A. Storey, HPV-associated skin disease, *J. Pathol.* **208** (2006), 165–175.
- [16] I. Nindl, M. Gottschling and E. Stockfleth, Human papillomaviruses and non-melanoma skin cancer: Basic virology and clinical manifestations, *Dis. Markers* **23** (2007), 247–259.
- [17] S. Jablonska, J. Dabrowski and K. Jakubowicz, Epidermodysplasia verruciformis as a model in studies on the role of papovaviruses in oncogenesis, *Cancer Res.* **32** (1972), 583–589.
- [18] G. Orth, S. Jablonska, M. Favre, O. Croissant, M. Jarzabek-Chorzelska and G. Rzesza, Characterization of two types of human papillomaviruses in lesions of epidermodysplasia verruciformis, *Proc. Natl. Acad. Sci. USA* **75** (1978), 1537–1541.
- [19] S. Majewski and S. Jablonska, Human papillomavirus-associated tumors of the skin and mucosa, *J. Am. Acad. Dermatol.* **36** (1997), 659–685.
- [20] A. Schmitt, J.B. Harry, B. Rapp, F.O. Wettstein and T. Iftner, Comparison of the properties of the E6 and E7 genes of low- and high-risk cutaneous papillomaviruses reveals strongly transforming and high Rb-binding activity for the E7 protein of the low-risk human papillomavirus type 1, *J. Virol.* **68** (1994), 7051–7059.
- [21] S. Caldeira, I. Zehbe, R. Accardi, I. Malanchi, W. Dong, M. Garre, E.M. de Villiers, R. Filotico, P. Boukamp and M. Tommasino, The E6 and E7 proteins of the cutaneous human papillomavirus type 38 display transforming properties, *J. Virol.* **77** (2003), 2195–2206.
- [22] B. Akgül, L. Ghali, D. Davies, H. Pfister, I.M. Leigh and A. Storey, HPV8 early genes modulate differentiation and cell cycle of primary human adult keratinocytes, *Exp. Dermatol.* **16** (2007), 590–599.
- [23] L.T. Chow and T.R. Broker, *In vitro* experimental systems for HPV: Epithelial raft cultures for investigations of viral reproduction and pathogenesis and for genetic analyses of viral proteins and regulatory sequences, *Clin. Dermatol.* **15** (1997), 217–227.
- [24] I.L. Boxman, L.H. Mulder, F. Noya, V. de Waard, S. Gibbs, T.R. Broker, F. ten Kate, L.T. Chow and J. ter Schegget, Transduction of the E6 and E7 genes of epidermodysplasia-verruciformis-associated human papillomaviruses alters human keratinocyte growth and differentiation in organotypic cultures, *J. Invest. Dermatol.* **117** (2001), 1397–1404.
- [25] B. Akgül, R. Garcia-Escudero, L. Ghali, H.J. Pfister, P.G. Fuchs, H. Navsaria and A. Storey, The E7 protein of cutaneous human papillomavirus type 8 causes invasion of human keratinocytes into the dermis in organotypic cultures of skin, *Cancer Res.* **65** (2005), 2216–2223.
- [26] J.G. Rheinwald, *Methods for Clonal Growth and Serial Cultivation of Normal Human Epidermal Keratinocytes and Mesothelial Cells*, IRL Press, Oxford, 1989, pp. 81–94.
- [27] S. Rozen and H. Skaletsky, Primer3 on the WWW for general users and for biologist programmers, *Methods Mol. Biol.* **132** (2000), 365–386.
- [28] I. Nindl, A. Koehler, T. Meyer, T. Forschner, C.J. Meijer, P.J. Snijders, W. Sterry and E. Stockfleth, Detection of human papillomavirus DNA in primary squamous cell carcinoma and metastases, *Br. J. Dermatol.* **154** (2006), 797–799.
- [29] L.G. Martin, G.W. Demers and D.A. Galloway, Disruption of the G1/S transition in human papillomavirus type 16 E7-expressing human cells is associated with altered regulation of cyclin E, *J. Virol.* **72** (1998), 975–985.
- [30] C.L. Halbert, G.W. Demers and D.A. Galloway, The E6 and E7 genes of human papillomavirus type 6 have weak immortalizing activity in human epithelial cells, *J. Virol.* **66** (1992), 2125–2134.
- [31] W. Zhao, F. Noya, W.Y. Chen, T.M. Townes, L.T. Chow and T.R. Broker, Trichostatin A up-regulates human papillomavirus type 11 upstream regulatory region-E6 promoter activity in undifferentiated primary human keratinocytes, *J. Virol.* **73** (1999), 5026–5033.

- [32] J. Steude, R. Kulke and E. Christophers, Interleukin-1-stimulated secretion of interleukin-8 and growth-related oncogene- α demonstrates greatly enhanced keratinocyte growth in human raft cultured epidermis, *J. Invest. Dermatol.* **119** (2002), 1254–1260.
- [33] R.H. Rice and H. Green, Presence in human epidermal cells of a soluble protein precursor of the cross-linked envelope: activation of the cross-linking by calcium ions, *Cell* **18** (1979), 681–694.
- [34] G.F. Murphy, T.C. Flynn, R.H. Rice and G.S. Pinkus, Involucrin expression in normal and neoplastic human skin: a marker for keratinocyte differentiation, *J. Invest. Dermatol.* **82** (1984), 453–457.
- [35] I. Nindl, T. Meyer, T. Schmook, C. Ulrich, R. Ridder, H. Au-dring, W. Sterry and E. Stockfleth, Human papillomavirus and overexpression of P16^{INK4a} in nonmelanoma skin cancer, *Dermatol. Surg.* **30** (2004), 409–414.
- [36] V. Dulic, E. Lees and S.I. Reed, Association of human cyclin E with a periodic G1-S phase protein kinase, *Science* **257** (1992), 1958–1961.
- [37] R. Donnellan and R. Chetty, Cyclin E in human cancers, *FASEB J.* **13** (1999), 773–780.
- [38] T. Sano, T. Oyama, K. Kashiwabara, T. Fukuda and T. Nakajima, Expression status of p16 protein is associated with human papillomavirus oncogenic potential in cervical and genital lesions, *Am. J. Pathol.* **153** (1998), 1741–1748.
- [39] S. Smola-Hess, J. Pahne, C. Mauch, P. Zigrino, H. Smola and H.J. Pfister, Expression of membrane type 1 matrix metalloproteinase in papillomavirus-positive cells: role of the human papillomavirus (HPV) 16 and HPV8 E7 gene products, *J. Gen. Virol.* **86** (2005), 1291–1296.
- [40] D.J. Griffiths, P.J. Venables, R.A. Weiss and M.T. Boyd, A novel exogenous retrovirus sequence identified in humans, *J. Virol.* **71** (1997), 2866–2872.