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Beta Human Papillomavirus Infection and Skin Cancer in Immunocompromised Host

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INDEX

A Lirio, nonno e maestro…

SUMMARY

Human papillomaviruses (HPVs) are small non-enveloped viruses with a double-stranded DNA genome that infect cutaneous or mucosal squamous stratified epithelia of different body sites. They can establish aysintomatic infections that usually remain latent in healthy individuals but may also be responsible for the development of benign or neoplastic proliferative lesions, depending on the specific oncogenic properties of the different type of virus involved.

To date, more than 150 HPV types have been completely sequenced and classified into five genera based on phylogenetic analyses. These display a variety of different epithelial tropisms and life-cycle strategies, of which, the Alpha genus (α-HPVs) is the best characterized and comprises mucosa-tropic types associated with the development of genital cancer (e.g. HPV 16, 18).
HPVs belonging to the skin-tropic Beta genus (β-HPVs, e.g. HPV 5 and 8) appear to cause widespread unapparent infections without associated disease in the general population.

In patients with epidermodysplasia verruciformis (EV; an inherited primary immunodeficiency characterized by a high susceptibility to active β-HPV infection), β-HPVs replicate very efficiently and reveal their full transforming potential, inducing disseminated wart-like lesions and driving their progression to non-melanoma skin cancer (NMSC). Their involvement in skin carcinogenesis in both immunocompetent and non-EV immunocompromised patients is unclear, as both epidemiological and molecular evidences often conflict and are inconclusive. However, it is thought that, at least in conditions of impaired immune function (resulting from either primary or acquired immunodeficiency) β-HPVs might reactivate and contribute to the pathogenesis of NMSC, whose incidence is greatly increased in immunosuppressed individuals compared to the general population (Doorbar et al. 2012; Akgül et al. 2006; Aldabagh et al. 2013)

Solid organ transplantation is a treatment offered to an increasing number of patients with end-stage organ diseases; although life-saving, it is associated with an increased risk of second malignancies, presumably as result of the long-term post-transplant iatrogenic immunosuppression. Most of these (cancers such as Epstein-Barr virus-associated B-cell lymphomas, Human Herpesvirus 8-associated Kaposi's sarcoma, and Merkel cell carcinoma of the skin, associated with Merkel cell polyomavirus) result from reactivated viruses whose oncogenic potential is suppressed by immunological reactions in healthy individuals. The most frequent type of malignancy arising in

organ transplant recipients (OTRs) is NMSC, and in particular the squamous cell carcinoma (SCC). The incidence of SCC in OTRs is estimated to be 60-250-fold as great as in the general population, and these post-transplant tumours are often found as multiples (commonly more then 10) and are highly aggressive.

Almost all the available studies are aimed to investigate the association between β-HPV infection and NMSC are mostly based on the detection of the presence of viral DNA in tumor tissues or positive antibody responses. Very few studies have addressed whether the viruses are actually localized in malignant cells or whether they are replicating and transcriptionally active in the context of the lesions. Confirmation of the presence of active virus cancerous lesions would greatly strengthen the evidence for an involvement of β-HPVs in the pathogenesis of NMSC.

It has been shown that detection of viral gene products and viral genome amplification by tissue staining procedures can be helpful for the visualization of active HPV infections in associated lesions, and that different expression patterns of viral lifecycle markers can be correlated to different stages of disease progression. This has been particularly noted in the well characterized α-HPV infections, and a recent study on skin tumors from EV patients has suggested that expression of the viral protein E4 and detection of viral DNA may be exploited as markers of viral activity during β-HPV associated skin cancer progression (Aldabagh *et al*., 2013; Pfister, 2003; Feltkamp *et al*., 2008; Doorbar, 2007; Doorbar *et al*., 2012; Middleton *et al*., 2003; Borgogna *et al*., 2012).

To date a number of studies have attempted to clarify the mechanism that leads to HPV becoming persistent infection and to subsequent lesion formation. The general hypothesis has been that lesion formation begins with the infection of a basal stem cell (rather than a basal transiently amplifying cell) and that the longevity of the stem cells is a key factor in the formation of a persistent lesion, with these cells being a reservoir for the infection. High-risk α-HPV chronic infections, characterized by long persistence in the slow-cycling epithelial stem cells in the absence of apparent disease, is a condition *sine qua non* for the development of HPV-associated malignancy.

Several studies have reported the ubiquitous presence of these β-HPV types on the body surface in immunosuppressed individuals and in the general population. Viral burden was greater in sun-exposed sites (e.g. forehead) than in sun-protected skin when its presence was monitored in plucked hairs and skin swabs. HPV appear to establish a reservoir of infection in the follicular stem cells of hair bulbs, which are thought to be an immune privileged site protecting them from immune clearance and therefore enabling the persistence of multiple β-HPV types.

The purpose of this study was to extend observations already made in EV patients to other groups of subjects at high risk of developing NMSC. A series of skin lesions from kidney transplant recipients were systematically analyzed for the presence of βHPV infection, both at the DNA and at the protein level, looking for viral markers of transcriptional activity and replication. Using a combination of antibodies raised against the βHPV E4 and L1 proteins, it was possible to visualize the completion of the viral lifecycle in some precancerous lesions such as actinic keratosis or at the periphery of more advanced disease, in organ transplant recipient.

With the aim of investigating the influence of viral gene expression on keratinocyte differentiation, this study took advantage of a transgenic mice model expressing the complete early region of βHPV8. A specific protocol, for characterization of the keratinocyte stem cell populations has been established on epidermal whole mount samples of both wild type and transgenic mice. Keratinocyte stem cell markers CD34, K15, LGR6, LGR5 and Lrig1 were analysed in correlation with the proliferation marker Ki67.

These data presented in this study demostrates that β-HPVs are transcriptionally active and replicating at site of skin transformation and further reinforces the evidence that these viruses are involved in the process of skin carcinogenesis in patients who have undergone organ transplantation. Detection of viral markers in premalignant lesions and in the adjacent pathological epithelium of high-grade tumors supports the idea that β-HPVs may play a role in the early stages of skin transforming processes, probably enhancing the carcinogenic potential of UV damage rather than in progression and maintenance of neoplastic disease (Akgul *et al*., 2006; Feltkamp *et al*., 2008; Pfister, 2003; Aldabagh *et al*., 2013). Furthermore the analysis of epidermal whole mount samples has shown the expansion of the Lrig1+ area in the hair follicles of the transgenic mice compared with the wild type littermates. Hair follicle keratinocyte stem cell proliferation was demonstrated to be elevated within the Lrig1+ cells, compared to the other keratinocytes stem cells populations in βHPV8 transgenic mice. Intriguingly, these data suggest that the presence of the viral gene may influence normal keratinocyte differentiation, and identifies the specific keratinocyte stem cell population that is positive for the Lrig1 marker in the β-HPV8 *in vivo* model as a possible target. .

Publications:

Borgogna C*, Lanfredini S*, Peretti A, De Andrea M, Zavattaro E, Colombo E, Quaglia M, Boldorini R, Miglio U, Doorbar J, Bouwes Bavinck JN, Quint KD, de Koning MN, Landolfo S, Gariglio M.

"Improved detection reveals active β-papillomavirus infection in skin lesions from kidney transplant recipients."

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*Equal contribution

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"Characterization of skin lesions induced by skin-tropic α- and β-papillomaviruses in a patient with epidermodysplasia verruciformis."
Br J Dermatol. 2014 Dec;171(6):1550-1554. doi: 10.1111/bjd.13156. Epub 2014 Nov 30

The data obtained from the study of the keratinocytes stem cell population in the β-HPV8 transgenic mice were achieved in the laboratory of Dr G. Patel (European Cancer Stem Cell Research Institute, Cardiff University, UK) as a visiting scientist, over a period of 7 months as part of the Ph.D program.

INTRODUCTION

Papillomaviruses: general characteristics

Papillomaviruses are small, unenveloped, double-stranded, circular DNA viruses exhibiting icosahedral symmetry. The papillomavirus genome ranges from 6953 bp [Chelonia mydas papillomavirus type 1 (CmPV1)] to 8607 bp [Canine papillomavirus type 1 (CPV1)] in length, and have been isolated and characterized from reptiles, birds, marsupials, and multiple other mammalian. This wide spectrum in the host species suggests an evolutionary history spanning more than 300 million years. They are species-specific and exhibit a strict tropism for the squamous stratified epithelia where they can induce cutaneous and mucosal hyperplastic lesions; some PVs have also been implicated in the development of epithelial malignancies, especially cancer of the uterine cervix and other tumors of the urogenital tract.

Human Papillomaviruses (HPV) can infect different body sites, such as the upper respiratory, oral and genital mucosae and the skin according to the specific tropism of the different viral species. They typically establish persistent infections which can either remain in an unapparent silent status or either cause clinical manifestations after a latency period of variable duration. The various types of epithelial disease that HPVs cause appear linked to their different strategies of transmission and propagation within the epithelium, and probably also to their different interactions with the immune system. HPVs can be responsible not only for benign hyperproliferative lesions (papillomas) in mucosal and cutaneous sites, but also for malignancies (especially squamous cell carcinoma, SCC). The specific association of HPV type 5 (HPV-5) with skin cancer of epidermodysplasia verruciformis (EV), a rare genetic disease, provided the first molecular evidence for the role of HPVs in human cancer.

HPVs have been implicated in cancers at several sites; in particular, the best documented HPV oncogenic activity concerns cervical cancer, on the basis of a meta-analysis of 1 million women with normal cervical cytology, around 291 million women worldwide are estimated to have human papillomavirus infection of the cervix at a given point, corresponding to an average prevalence of 10·4%, though prevalence is higher in women younger than 25 years (16·9%). Human papillomavirus types 16 and 18 account for roughly 70% of all cervical cancer.

Although the causal association of HPV infection with cervical carcinogenesis is epidemiologically and experimentally ascertained, its implication in the development of precancerous skin lesions that can evolve to high-grade tumors of epithelial origin – comprehensively grouped into the histological classification of non-melanoma skin cancer (NMSC) – has been much less clear to date; such uncertainties partly come from the fact that cutaneous HPVs have been shown to be ubiquitously present in the skin in the general healthy population. Nevertheless, several epidemiologic and experimental evidences are in favor of their potential oncogenic role in the insurgence of skin lesions (Knipe and Howley 2007; Doorbar 2005; Doorbar et al. 2012; Akgül, Cooke, and Storey 2006; Pfister 2003; Burk 2014; Van Doorslaer 2013)

Classification and tropism

Historically, PVs were classified together with the polyomaviruses as a single family, the Papovaviridae. This grouping arose because, although PV genomes and capsids are larger than those of polyomaviruses, the viruses share many features, including a double-stranded circular DNA genome, an icosahedral capsid composed of 72 pentamers, a nonenveloped virion, and the nucleus as the site of viral replication and virion assembly. Sequencing of PV genomes, however, indicated that, although PV share a common genetic organization, they differ from that of polyomaviruses and have no major sequence homology to polyomaviruses, and PV transcription is unidirectional, in contrast to the bidirectional transcription of polyomaviruses. Recognition of these differences, and others, have led to PV being designated as a separate family, the Papillomaviridae, by the International Committee on the Taxonomy of Viruses (Ethel-michele De Villiers et al. 2004; Bernard 2013).

After many years of research and genomic sequencing of thousands of PV isolates, PV classification was based on phylogenetic criteria following hierarchical taxonomic levels (family, genus, species, types, subtypes and variants). Two PV genomes are recognized as distinct types (genotypes) if they share less then 90% identical nucleotides in the most conserved genomic region L1 ORF, encoding the major capsid protein. Types were designated by a number, following the chronological order of their characterization. The PV taxonomy recently adopted is based on the phylogenetic relationship of the L1 ORF of animal and HPVs. Higher-order assemblages are considered as a genera (identified by Greek alphabetical prefixes) and lower-order assemblage as species (each species identified by the number of the best known type). Genera share less than 60% nucleotide identity in the L1 ORF, species within a genus share 60-70%, and types within a species

share 71-89%. By this approach, more than 200 PV types have been discovered to date, encompassing more than 150 HPV types and 103 animal PV types (Ethel-michele De Villiers et al. 2004; Mahy and Van Regenmortel 2008; Bernard 2013).

Historically, viral evolution has mainly been considered from a predator–prey perspective. Under this model, viral fitness (and thus its evolutionary success) is measured by the viral capacity to cause disease in its host. However, papillomaviruses cause benign, mostly unapparent, persistent infections in their hosts. In addition, papillomaviruses are highly host-restricted, and cause abortive infections in non-host species. In fact, the only exceptions to strict species specificity were described in mammalian hosts known to hybridize, thereby challenging the hosts' species definition. The observation that papillomaviruses cause benign infections unable to cross the hosts' species-barrier has led to the hypothesis of "host-linked evolution".

The traditional (orthogenetic) definition of co-evolution states that parasites of closely related host species should be closely related themselves and cluster together in the parasite phylogenetic tree. Furthermore, dates associated with parasite divergence should coincide with the host-species divergence. Therefore, any incongruence between both trees should be considered as evidence that parasite and host did not co-evolve.

With an increase in the number of papillomavirus sequences (and their associated hosts), it became clear that papillomaviruses and their hosts did not follow an identical evolutionary path. Several violations of strict co-evolution can be observed in the phylogenetic tree in Figure 1. For example, human papillomaviruses can be found in five different genera dispersed throughout the phylogenetic tree. Also, strict co-evolution would place the non-human primate papillomaviruses basal to human papillomaviruses, not intermingled as is observed.

Evolutionary events such as cross-species infection, recombination and virus duplication (e.g. following ecological niche adaptation) have been suggested to explain the observed conflicts

Because of the absence of cross-species infections, it is unlikely that horizontal gene transfer played any role in the evolution of the Papillomaviridae. In fact, a study specifically looking at the influence of horizontal gene transfer identified only a single potential cross-species transmission event. This event involved ancestors of a porcupine (EdPV1) and human (HPV41) papilloma- virus. These two viruses are the only members of a divergent genus (Nupapillomavirus).

A more recent version of the co-evolution theory was initially proposed in the early 1960s. This updated theory states that the evolution of parasites follows the evolution of host resources, not the evolution of the host species per-se. The shape of the papillomavirus phylogenetic tree could potentially be explained using this interpretation of co-evolution. Under this model, specific events

in the evolution of hosts (e.g. presence/absence of fur, evolution of sweat glands, etc.) created new ecological niches for papillomaviruses to adopt. Therefore, the data suggests a model in which a generalist ancestral papillomavirus diverged into four or five increasingly specialized viruses (reflected in the 4–5 major clades of the phylogenetic tree). Following these niche adaptation events, the virus evolved alongside its hosts. Throughout the co-evolutionary process, the availability of new niches would in turn drive viral radiation, followed by further co-speciation.
In conclusion, the papillomavirus phylogenetic tree cannot be explained solely by co-evolution. However, initial niche sorting followed by virus–host linked speciation was a key determinant of the papillomavirus evolutionary history (Van Doorslaer 2013).

On the basis of L1 nucleotide sequences, HPVs are distributed in five of the 16 PV genera (Figure 1). The *Alphapapillomavirus* (α-HPV), comprises types differing by their genital, oral cutaneous tropism and by their pathogenicity (low-risk types associated with benign proliferations and high-risk types associated with invasive carcinomas). The genera *Gammapapillomavirus* (γ-HPV), *Mupapillomavirus* (μ-HPV), and *Nupapillomavirus* (ν-HPV) comprise types associated with cutaneous warts, whereas the types usually associated with EV belongs to the genus *Betapapillomavirus* (β-HPV).

The Alpha genus is the most represented one and comprises both mucosal and cutaneous species. Infections by mucosal α-HPVs are more common than those by cutaneous α-HPVs and the majority of them are asymptomatic. Based on their oncogenic potential and association with the development of malignancies, mucosal α-HPVs are further subdivided into low-risk and high-risk groups.
The World Health Organization has defined 12 mucosal α-HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59) as being high-risk cancer-causing types; they are mainly responsible for the development of cervical cancer and can also be associated with cancers at other sites with much lower incidence (head and neck carcinomas such as oropharyngeal cancers, and cancers of the penis, anus, vagina and vulva). Nevertheless, high-risk α-HPVs do not cause cancer in the vast majority of the individuals they infect.
Low-risk α-HPVs cause benign mucosal lesions. Certain types (e.g. HPV 6 and 11) are associated with the development of respiratory papillomatosis (especially HPV 11), which is a laryngeal disease often occurring in children, and with benign external ano-genital warts (especially HPV 6); these types are sometimes occasionally found to be associated with cancers in these sites especially in individuals with immune defects, where such infections are more difficult to manage. Other low-

risk α-HPV types (e.g. HPV 13 and 32) are responsible for the development of oral papillomas as it occurs in the case of oral focal epithelial hyperplasia.

Cutaneous α-HPVs (e.g. HPV 2, 3, 7, 10, 27, 28, 57) are associated with the development of different types of benign skin warts arising on various sites of the hands, face, elbows and knees.

The Beta genus comprises to date 43 types, which exhibit cutaneous tropism (e.g. HPV 5, 8, 9, 14, 17, 20, 21, 23, 36, 38, 47, 49). β-HPVs are evolutionarily distinct from the Alpha genus and establish widespread unapparent asymptomatic infections that remain latent without any clinical manifestation in the general healthy population. In subjects with impaired immune function, it seems that these viruses can spread unchecked and they have been implicated in the development of NMSC. In particular, in individuals suffering from epidermodysplasia verruciformis (EV), a primary immunodeficiency associated with abnormal susceptibility to β-HPV infection, they are responsible for the development of disseminated wart-like lesions that often undergo malignant progression; there is also increasing body of evidence for their involvement in the onset of precancerous skin lesions with potential to evolve to NMSC in other immunosuppressed populations (e.g. OTRs, organ transplant recipients). Their involvement in the pathogenesis of NMSC in immunocompetent individuals is still unclear.

Gamma (e.g. HPV 4), Mu (e.g. HPV 1) and Nu (HPV 41) genera comprise a smaller number of types, exhibiting cutaneous tropism; they are associated with the development of benign palmar and plantar skin warts (Ethel-michele De Villiers et al. 2004; Akgül, Cooke, and Storey 2006; Pfister 2003; Doorbar 2007; Doorbar et al. 2012; Cubie 2013).

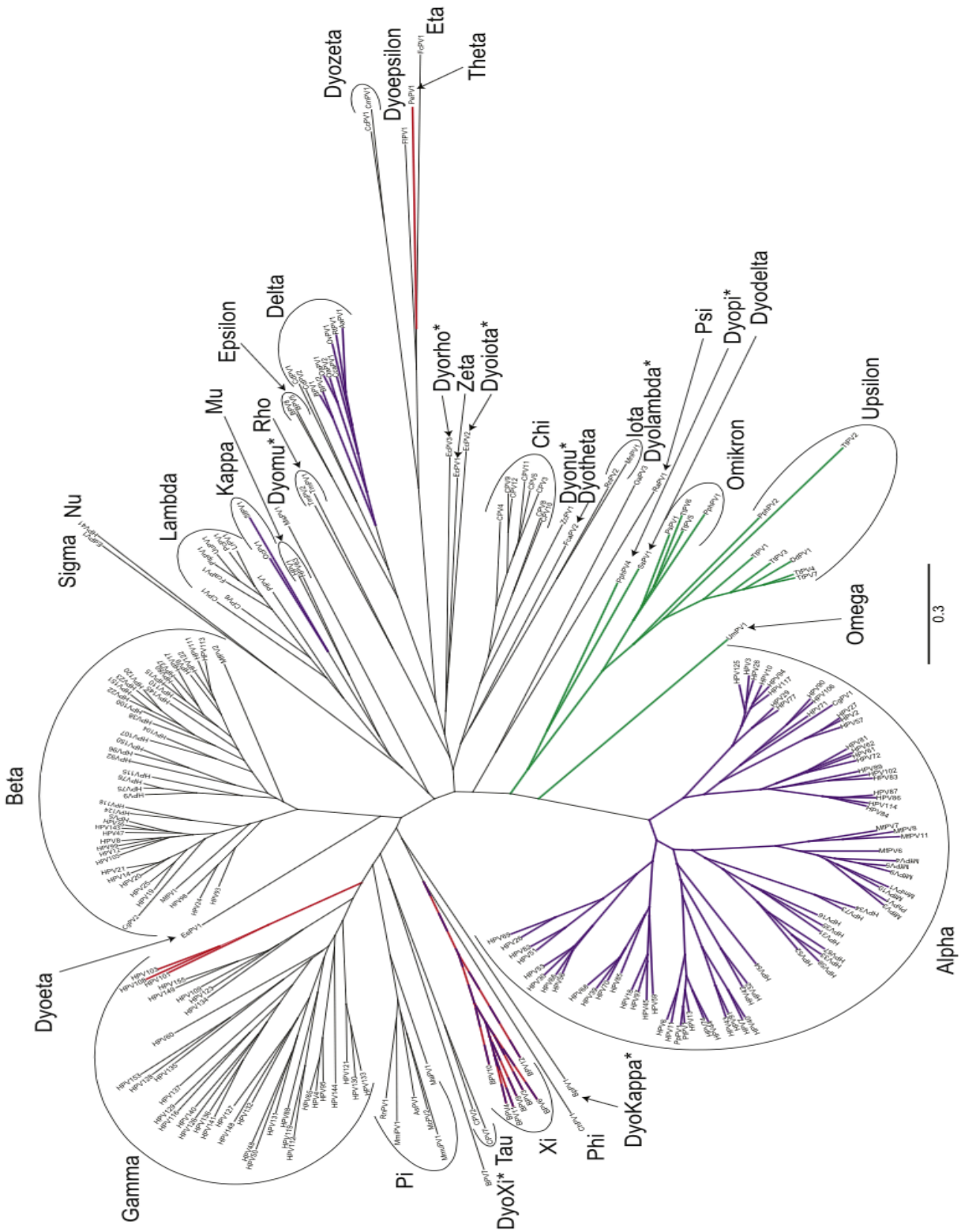

Figure 1. Papillomavirus phylogenetic tree. The DNA sequence coding for E1, E2, L1 and L2 for all 241 papillomaviruses currently on PaVE were downloaded and aligned. A partitioned gene alignment was used as the base for a maximum likelihood reconstruction of the phylogenetic treeGenera marked with an asterisk have been proposed to the ICTV, and are awaiting official recognition (http://talk. ictvonline.org/files/proposals/taxonomy_proposals_vertebrate1/m/vert01/4244.aspx). The tree is color-coded according to presence/absence of the "adaptive proteins". Red clades lack an E6 ORF. The viruses highlighted in green do not code for an E7 protein. The purple clades code for a hydrophobic E5 protein. The Xipapillomaviruses lack an E6 (red), but contain an E5 (purple)(Van Doorslaer 2013).

HPV structure, genomic organization and gene products

The genomes of many of the human and animal papillomaviruses have been sequenced in their entirety, and the genomic organization of each of the PVs is similar. HPVs are characterized by small (52-55 nm diameter), nonenveloped, icosahedral capsid composed of 72 pentameric capsomeres. Their genome is in a double-stranded, covalently closed, circular DNA molecule of 7500-8000 bp. The viral DNA is associated with cellular histones to form a chromatin-like structure. Genomic organization is highly conserved among all HPV family members, with 8-9 open reading frames (ORFs) on a single transcriptionally active DNA strand encoding a larger number of gene products as a result of mRNA splicing (Figure 2). The viral genome is divided into three regions:

- The early region coding for functional proteins (E1, E2, E4, E5, E6 and E7) expressed in all the phases of the viral life cycle, which are responsible for the persistence of the viral genome in a cell, its replication and the stimulation of cell proliferation necessary to support viral replication itself;
- The late region, coding for the structural coat proteins L1 and L2 that are expressed in the final phases of the productive viral life cycle;
- The long control region (LCR), a non-coding fragment regulating the expression and replication of the viral genome (Mahy and Van Regenmortel 2008).

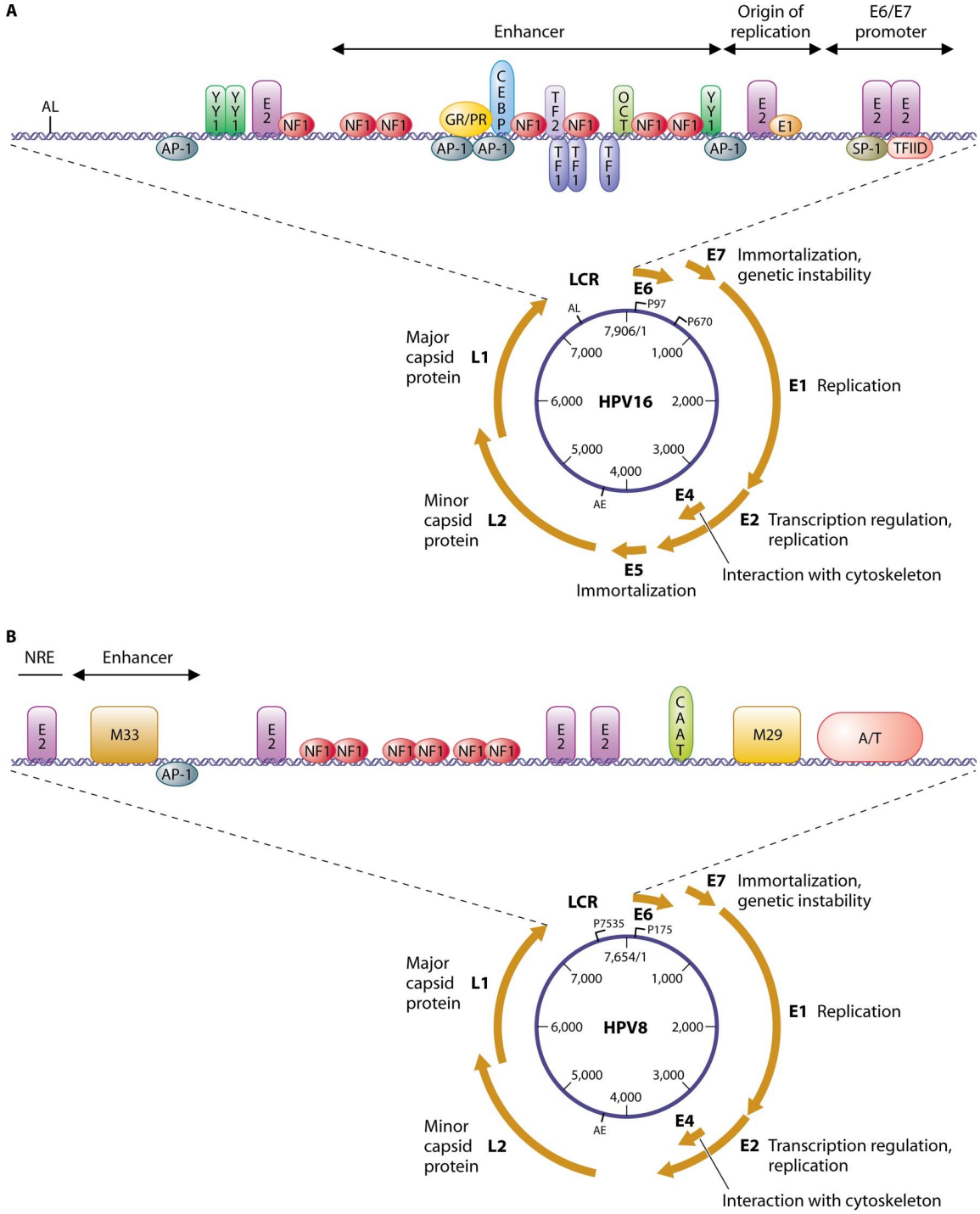

Figure 2. The genome organization of the high-risk α-type HPV16 and β-type HPV8. The HPV genome comprises a long control region (LCR) and eight genes that are necessary for different stages of the virus life cycle. These genes encode a larger number of gene products as a result of mRNA splicing. The LCR contains binding sites for cellular transcription factors (e.g., SP1, AP1, Oct1), as well as for the viral E1 and E2 proteins that control viral replication and gene expression. The best characterized HPV types have two promoter elements (P97 and P670) known as early promoter (PE) and late promoter (PL) that regulate the expression of differentially-spliced mRNAs during epithelial differentiation. Specific conserved motifs (M33 and M29) among the beta-HPVs are shown in the LCR of HPV8 (Lazarczyk et al. 2009).

The LCR is a significant non-coding fragment of viral DNA located between the L1 and E6 ORFs, accounting for about 10% of the entire genome. It contains an origin of replication and cis-responsive elements for regulatory transcription and replication factors of viral genetic material; in detail, there are binding sites for cellular transcription factors (e.g. SP1, AP1, Oct1), as well as for the viral E1 and E2 proteins that control viral replication and gene expression (Figure 2). All the LCR regions contain enhancers that provide the virus with specific tropism to the stratified squamous epithelial cells. Other regulatory enhancer elements are positioned within genes.
The LCR harbors one of the two well-characterized promoter elements, namely the early promoter upstream of the E6 ORF, while in many HPV types the late promoter is located within the E7 ORF (Figure 2). These promoters regulate the expression of differentially spliced mostly polycistronic mRNAs, being trans-activated in a timely and coordinated differentiation-dependent fashion in specific epithelial layers where the different phases of the viral life cycle take place. The early promoter mainly drives E6 and E7 expression, while the late promoter regulates the expression of all the other viral genes.

The mRNA species that encode E1, E2 and E4 terminate at the early polyadenylation site and for many α-HPVs include E5 as their second or third ORF; transcripts encoding L1 and L2 terminate at the late polyadenylation site. The E4 ORF is contained within the E2 ORF, with the primary E4 gene product (E1^E4) being translated from a spliced mRNA including the E1 initiation codon and adjacent sequences. Upon mRNA splicing involving a small portion within the E1 ORF and most of the E2 ORF, some high-risk α-HPV types produce the additional gene product E8^E2C. In addition, for many HPV types several other spliced transcripts have been reported, encoding variants of the early proteins such as N-terminal truncated forms of E2 and E6 (Ciesielska et al. 2012; Doorbar et al. 2012; Doorbar 2007; Tommasino 2014; Doorbar 2013).

The papillomavirus major capsid protein, L1, is a ~55 kD protein with the ability to spontaneously self-assemble into virus-like particles (VLPs). These VLPs present an exterior surface essentially indistinguishable from the native 60 nm non-enveloped papillomavirus virion. Purified recombinant L1 proteins can achieve this complex assembly reaction in the absence of any chaperones (Schiller and Lowy 2012). Assembled VLPs are potent immunogens, likely due to innate B-cell recognition of the regular icosahedrally displayed spacing of surface epitopes (Bachmann et al. 1993). These discoveries laid the foundation for the development of the current VLP-based vaccines that offer highly effective protection against infection with the cancer-causing human papillomavirus (HPV) types 16 and 18. The assembled VLP immunogens used in current

HPV vaccines represent the state of L1 in only one phase of the viral life cycle–namely, the rigid mature form of the virion during its transmission from one cell to another. In contrast to the mature virion state, during the process of virion assembly L1 interactions must be flexible enough to allow selective uptake of the viral genomic DNA into the virion lumen. Transmission of some papillomavirus species is thought to occur via deposition on environmental surfaces, such that the initially fragile immature virion must gain the ability remain infectious in a desiccated state for days or longer to achieve transmission (Roden, Lowy, and Schiller 1997). This high degree of stability is achieved through a maturation process in which the flexible immature virion gradually achieves a more rigid state that is stabilized by disulfide crosslinks between neighboring L1 molecules. Since L1 forms the entire exterior surface of the stabilized mature virion, it obviously must mediate initial attachment to host tissues or cells. After attachment to cells, L1 must again become pliable enough to ultimately allow release of the viral genome into a new target cell (Buck, Day, and Trus 2013).

Similar to L1 the minor capsid protein L2 is a 55KDa protein, and plays major roles in both papillomavirus assembly and the infectious process. While L1 forms the majority of the capsid and can self-assemble into empty virus-like particles (VLPs), L2 is a minor capsid component and lacks the capacity to form VLPs. However, L2 co-assembles with L1 into VLPs, enhancing their assembly. L2 also facilitates encapsidation of the ~8kbp circular and nucleosome-bound viral genome during assembly of the non-enveloped T=7 d virions in the nucleus of terminally differentiated epithelial cells, although, like L1, L2 is not detectably expressed in infected basal cells. With respect to infection, L2 is not required for particles to bind and enter in the cells.
Much of the studies and analysis about L2 are based on HPV16 L2's primary sequence and these mapped domains because unfortunately very little information exists on its higher order structure (Wang and Roden 2013).

The E1 protein, an ATP-dependent DNA helicase, is the only enzyme encoded by the PV genome. It is tightly regulated in vivo, in particular by post-translational modifications that restrict its accumulation in the nucleus. E1 is essential for replication and amplification of the viral episome in the nucleus of infected cells. It does so by interacting with cellular DNA replication factors and assembling with E2 to the origin of replication to trigger viral DNA replication (Bergvall, Melendy, and Archambault 2013).

The E2 proteins function primarily by recruiting cellular factors to the viral genomes, which activate or repress transcriptional processes. The E2 proteins bind specifically to sequence motifs in the viral genome and can activate or repress transcription, depending on the context of these binding

sites and nature of the associated cellular factors. In particular, it negatively modulates the expression of E6 and E7 by down-regulating the activity of the early promoter. Furthermore, E2 recruits E1 to a specific E1-binding motif in the origin of replication and is implicated in the maintenance of the viral genome in its episomal (extrachromosomal) form. It seems to be also involved in the regulation of accurate genome partitioning during basal cell division by associating to the pericentromeric regions of mitotic chromosomes to drive the tethering of viral episomes to the cellular chromatin during mitosis (McBride 2013).

The E4 open reading frame (ORF) lies within the larger E2 ORF, and varies considerably in size between papillomavirus types. In human papillomaviruses, the primary E4 gene product is expressed from a spliced mRNA (the E1∧E4 message). It is the most abundant viral gene product and is expressed before L2 and L1. The structure and function being modified, first by kinases as the infected cell progresses through the S and G2 cell cycle phases, but also by proteases as the cell exits the cell cycle and undergoes true terminal differentiation. E4 is thought to be involved, in association with E2, in viral genome amplification and suppression of cellular proliferation in the late phases of the productive life cycle; The accumulation to very high levels in the upper epithelial layers of the host seems also to be able to disrupt the cellular keratin network facilitating the extracellular release of new viral particles (Doorbar 2013).

In the HPVs genome are presents three proteins with transforming properties: the E5, E6 and E7 oncoproteins. Through combined and cooperative action, they abrogate the activity of tumor suppressor factors, promoting evasion of all cell cycle checkpoints and inducing a deregulated progression of the cell cycle; they mostly function by associating with and functionally reprogramming key components of cellular signal transduction networks. Their activity allows the maintenance of S-phase competence and a forced replicative status in the host cell, which is essential to the viral life cycle; the result is a stimulation of cell growth, survival and proliferation and a delay of terminal cell differentiation. Because of such properties, HPV oncoproteins can trigger the initiation and progression of epithelial cancers; in particular, these transforming activities are stronger in the case of high-risk α-HPVs, underlying their involvement in the development of malignancies.

E5 is the smallest oncoprotein which functionally resembles a viroporin (Venuti et al. 2011). It is an 84-aminoacids viral replication protein involved in the early stages of HPV infection. The hydrophobic E5 protein localizes to cell membranes (including Golgi, endoplasmic reticulum, nuclear membrane) where it may dimerize and trigger cell fusion (Hu et al. 2009). This phenotype

is associated with mitogenic signal intensification via E5-inhibited degradation of epidermal growth factor receptors (EGFR). However, E5 from dermatotropic α-viruses such as HPV6 or EV- type β-viruses such as HPV5 does not cause cell fusion, yet retains even stronger affinity for growth factor receptors (EGFR, HER2 or platelet-derived growth factor b-receptors) than does HPV16 E5. E5 also exerts immunosuppressive effects mediated via inhibition of host MHC class II complexes and/or reduced cytotoxic T cell recognition of HPV (Campo et al. 2010). These actions could be pertinent to the tumorigenicity of HPV16 in the immune-activated niche of the lymphoid tonsil, plausibly reducing immune surveillance to the lower levels of the cervix or post-transplant skin. It is also possible that the stimulatory effects of E5 on keratinocyte EGFR signaling contribute to the epidermotropism of β-HPVs, consistent with the reactivation of E5 signalling seen in some skin SCCs containing multiple HPVs (Orth 2006). Still another tumorigenic effect of E5 is to reduce expression of the epithelial adhesion molecule E-cadherin, increasing the invasiveness of infected cells while driving the epithelial–mesenchymal transition (EMT) (Boulenouar et al. 2010).

E6 and E7 are small proteins, approximately 18 and 13 kDa in size, respectively, and are localized in the nucleus. The E6 proteins are also found in the cytoplasm and some studies have suggested that E7 also has a cytoplasmic. Are multimeric proteins with potential to associate with multiple cellular partners. Such functional differences contribute to the respective transforming abilities of various HPV species and types.

About E7 protein it has been shown to bind zinc and is phosphorylated by casein kinase II (CK II). In the best-characterized HPV species, E7 binds and targets for ubiquitin-dependent proteasomal degradation the hypophosphorylated form of members of the retinoblastoma tumor-suppressor family (pRb, p107, p130). In uninfected epithelium, cell cycle entry and cell division in the basal and parabasal cell layers is controlled by growth factors that stimulate the activity of G1 cyclins including cyclinD/Cdk, which phosphorylates Rb family members and displace them from transcriptional activators of the E2F family allowing the trans-activation of genes necessary for S-phase progression. The continual stimulation of these cells physiologically allows renewal of the epithelium as surface cells exfoliate. As part the regulated stimulation of this cell cycle entry, p16ink4a is up-regulated and forms a compensatory negative feedback loop that suppresses cyclinD/Cdk activity, so preventing the over-expression of itself and other E2F-activated genes (e.g. MCM, PCNA, Ki67). E7 binds to Rb proteins and displaces them and transcriptional repressors of the E2F family from target promoters required for S-phase gene expression without the need for Rb phosphorylation and growth factor stimulation; E2F1, 2 and 3 can then occupy these vacant sites

and stimulate expression of the host genes necessary for DNA replication and cell cycle progression.

In certain HPV species and types, E7 is necessary for the stable maintenance of HPV episomes in epithelial cells. It can stimulate cell cycle progression and subsequent hyperproliferation, induce genomic instability, exert antiapoptotic activities, reprogram host cell metabolism and contribute to evasion of the local antiviral host immune responses to different extents by a number of other mechanisms depending on the specific oncogenic potential.

The HPV E6 proteins are approximately 150 amino acids in size and contain four Cys-X-X-Cys motifs, which form two unusually big zinc finger domains of 29-30 AA, respectively, which are important for protein stability and activity. The E6 proteins from the low risk and high risk HPV appear to have similar transcriptional activation properties.

E6 interferes with DNA damage repair, growth arrest and apoptosis. The function of E6 complements that of E7. The efficient binding of Rb proteins by E7 can lead to inhibited cell growth and apoptosis through a p53-dependent pathway; as a result, E6 proteins of many HPV types have evolved to target the tumor suppressor p53 for ubiquitin-dependent proteasomal degradation or other forms of inactivation, resulting in the abrogation of its activity that plays an essential role in protecting genomic integrity. In uninfected epithelium, p53 expression increases in response to cellular stress such as DNA damage and induces the expression of p21, which allows cell cycle arrest at G1-S checkpoint until errors in DNA replication can be repaired; if DNA damage cannot be repaired, p21 activates the expression of proapoptotic genes to prevent the replication of damaged DNA. In addition, p53 can also trigger apoptosis upon DNA damage through pathways unrelated to its transcriptional activity The consequence of E6 action is the abrogation of all these p53-dependent tumor suppressor functions in the infected cells.

In certain HPV species and types, E6 can inhibit apoptosis by interfering with p53-dependent or p53-independent apoptotic pathways through other mechanisms to different extents depending on the specific oncogenic potential.

In any case, E6 inactivates key mediators of stress-induced programmed cell death more or less strongly depending on the transforming properties of specific HPV species and types. As a consequence, cell cycle arrest and apoptosis in response to E7-mediated cell cycle entry are prevented and DNA replication followed by cellular proliferation is promoted in cells with damaged DNA because of the disruption of the DNA repair process in the host cells; this events, which are more evident in oncogenic HPVs, increase the frequency of spontaneous mutations in the cellular genome during replication, favoring the occurrence of genomic instability underlying transforming processes.

Another important way how E6 proteins of some oncogenic HPVs contribute to transformation is the activation of the human telomerase reverse transcriptase promoter, which controls the transcription of the catalytic telomerase subunit.
In high risk HPVs, E6 can also exert transforming activities by inducing host cell immortalization, loss of host cell polarity and anchorage-independent growth and altering host cell differentiation and metabolism; finally, E6 can facilitate viral escape of the antiviral immune surveillance mechanisms (Knipe and Howley 2007; Moody and Laimins 2010; Doorbar et al. 2012; Doorbar 2007; Akgül, Cooke, and Storey 2006; Ciesielska et al. 2012; McLaughlin-Drubin, Meyers, and Munger 2012).

HPV life cycle

Irrespective of their evolutionary origin, all papillomaviruses must complete their life cycle in the epithelial tissue that they infect (summarized in Figure 3), and produce infectious particles that are eventually secreted from the epithelial surface. Human papillomavirus infects cells in the basal layer of the epithelium, probably via microabrasions in the epithelial surface. It capitalises on the lateral extension of basal cells that accompanies wound healing to gain entry to the cell.
Infectious internalization takes several hours, after which viral DNA is released from the capsid and transported into the nucleus as free genetic material or extrachromosomal episomes. The synthesis of new virions occurs only after the infected cell has undergone mitosis and one of the infected daughter cells has differentiated.

HPV replication requires the timely and coordinated expression of the different viral gene products as the infected cell moves towards the epithelial surface; this highly regulated pattern of gene expression allows the different stages of the life cycle to be completed appropriately. For this reason, HPV life cycle takes 2-3 weeks – the time necessary for an epithelial cell to migrate from the basal to most superficial layers, mature, undergo senescence and die. Whether a productive life cycle is or is not completed depends on the nature of the epithelial site where infection occurs, as well as on the presence of external factors such as hormones and cytokines.
Due to the clear association of high-risk α-HPV types with human carcinogenesis, most of the biological studies so far have been focused on these types (Doorbar et al. 2012; Doorbar 2007; Tommasino 2014; Crosbie et al. 2013; Moody and Laimins 2010).

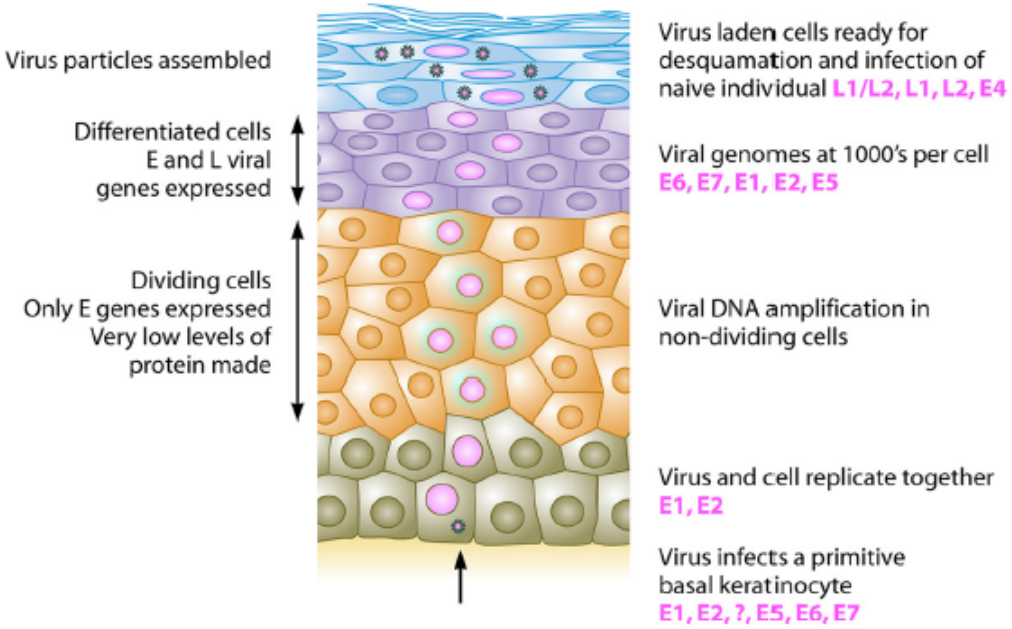

Figure 3. Productive HPV life cycle. HPVs replicate only in fully differentiating squamous epithelia. The life cycle involves both temporal and spatial separation of viral protein expression. The virus first infects a cell in the basal layer of the epithelium where access is naturally facilitated (e.g. microtrauma, epithelial transitional zones, hair follicles). In the lower proliferative compartments of the epithelium, there is a phase of viral episome maintenance al low copy number, in which viral and cellular DNA replicate together. As long as the cell is dividing, HPVs control the expression of their viral proteins very tightly; the E6 and E7 oncogenes are thus expressed at very low levels, along with the genes coding for E1 and E2 replication factors. When the host cells stop dividing and begin to differentiate into mature epithelial cells, this provides a signal to the virus to activate all of its genes to amplify the viral genome copy number to the thousands. In the top layers of the epithelium, all of the viral genes, including those encoding the L1 and L2 proteins, are expressed, and many thousands of viral genomes are encapsidated; finally, the newly assembled infectious viral particles exit the cells in the context of epithelial surface desquamation. The time taken from infection to the generation of new virions is at least 2-3 weeks (Stanley, 2012).

Experimental models suggest that infection requires access of virus particles to the basal lamina and the interaction with heparin-sulphate proteoglycans and possibly also laminin. Structural changes in the virion capsid, which includes furin cleavage of L2, facilitate transfer to a secondary receptor on the basal keratinocyte, which is necessary for virus internalization and subsequent transfer of the viral genome to the nucleus; although the α-6 integrin and growth factor receptors have been implicated in this process, the precise nature of the entry receptor remains somewhat controversial.

Once internalized, virions undergo endosomal transport, uncoating and cellular sorting. The L2 protein-DNA complex ensures the correct nuclear entry of the viral genomes, while the L1 protein is retained in the endosome and ultimately subjected to lysosomal degradation.

In many cases, infection is thought to require epithelial wounding or micro-wounding to allow access of the virus to the basal lamina, and a role for the wound healing response in simulating the lateral expansion of the infected cells has been suggested. Indeed, active cell division, as would occur during wound healing, is thought to be necessary for entry of the virus genome into the nucleus, and it has been proposed that lesion formation requires the initial infection of a mitotically active cell. Given the diversity of HPV types and HPV-associated diseases, is not always possible make such broad generalizations regarding the route of infection, as multiple entry pathways have been invoked depending on the virus type under study. Some HPV species are thought to infect sites where access to the basal layer is naturally facilitated, such as the base of the hair follicle for cutaneous HPVs, or sites where columnar and stratified squamous epithelial cells meet each other, such as the cervical or anal squamo-columnar junctions for high-risk α-HPVs. For some time now, the general hypothesis has been that lesion formation begins with the infection of a basal stem cell (rather than a basal transiently amplifying cell) and that the longevity of the stem cells is a key factor in the formation of a persistent lesion, with these cells being a reservoir for the infection (Doorbar 2007; Doorbar et al. 2012).

Infection of the basal stem cell is followed by initial phase of genome amplification and then by maintenance of the viral episome al low copy number (100-200 copies per cell). In benign oral papillomas in animals, the basal copy number has been quantified using laser capture methods as 50 to 100 copies per cell, but it is likely that there will be variation from lesion to lesion and between different sites.

Due to the lacking of the enzyme machinery necessary for viral DNA replication, the viral genome amplification depends on the presence of the cellular DNA replication machinery. Thus the viral DNA replication follows cellular DNA replication as the cells progress through S-phase.

The viral replication proteins E1 and E2 are thought to be essential for this initial amplification phase, which are expressed from the late promoter and ensure viral DNA replication and transcription. It has been proposed that the use of a viral DNA helicase (i.e., E1), which is distinct from the cellular replication helicases (MCM proteins), allows viral DNA replication to be disconnected from cellular DNA replication during genome establishment and amplification; however, at this stage the viral genomes are replicated in synchrony with cellular DNA replication. E2 also regulates accurate genome partitioning during basal cell division.

The precise role of the E6 and E7 proteins in infected basal cells is uncertain, particularly for the low-risk α-HPVs and cutaneous HPVs. In these HPV types, the role of the wound healing response in driving the initial proliferation of the infected cell(s) may well be critical. In the case of the high-

risk α-HPV types there is a clear role of the viral E6 and E7 proteins in driving cellular proliferation in the basal and parabasal cell layers, where an expansion in lesion size is facilitated.

Viral proteins are not readily detectable prior to the onset of genome amplification during normal productive infection; the low level expression of viral proteins in the basal layer is thought to reflect, at least in part, the need of the virus to avoid detection by the host's immune system (Doorbar 2007; Doorbar et al. 2012).

The maintenance of viral genome in the proliferating basal cells, and in cells in the lower epithelium, is a common feature of all papillomaviruses.

The late productive stage of the life cycle, starting with vegetative viral genome amplification (around 1000 copies per cell), is triggered as the infected cells differentiates and are pushed towards the epithelial surface by the division of the cell beneath. The specific events that induce the onset of genome amplification are not well understood, but depend in part on changes in the cellular environment as the infected cells move to the upper epithelial layers; of key importance is the up-regulation of the late promoter, whose activation in cells expressing E6 and E7 leads to an increase in the levels of viral proteins necessary for replication, including E1, E2, E4 and E5. After an initial increase in viral copy number, the infected 'differentiating' cells move from an S-like to a G2-like phase, with viral genome amplification occurring primarily in G2 after cellular DNA replication has been completed; the virus at this point fully recruits the cellular DNA replication machinery for its own replication.

A key function of the E6 and E7 proteins in most HPVs is not to promote basal cell proliferation, but rather to stimulate cell cycle re-entry in the mid-upper epithelial layers in order to allow genome amplification. These differentiating cells normally would not be replication–competent, since they do not naturally express the replicative machinery that the virus depends on; the expression of the E6 and E7 proteins in these layers allows the infected cells to aberrantly re-enter S-phase and for viral genome copy-number to rise. In high-risk α-HPVs, the stimulation of cell cycle entry occurs in the basal layer and above; for unclear reasons, high-risk oncoproteins can also promote the proliferation of the infected basal cells, which is important for the induction of neoplasia.

There is also a need for the viral replication proteins E1 and E2, which increase in abundance following the up-regulation of the late promoter.

In addition to E1 and E2, it is thought that the E4 and E5 proteins contribute indirectly to genome amplification success by modifying the cellular environment, with E5 also being involved in koilocyte formation.

Abundant high-risk E4 can inhibit cell cycle progression during G2-phase, and this leads to cell cycle arrest at the G2-M boundary by sequestering these proteins in the cytoplasm. It has been suggested that continued expression of E7 in a cell containing abundant E4 might lead to the maintenance of an S-phase environment, which allows accumulation of the viral genomes. The increase in E2 that is thought to accompany E4 must eventually down-regulate the viral early promoter that induces E7 production and entry into S-phase; the steady rise in E2 may act both to initiate genome amplification, and in a timely manner, to turn it off once amplification is complete. E5 is also thought to make an important contribution to genome amplification success through its ability to stabilize EGFR and to enhance EGF signaling and MAPK activity, thus allowing the maintenance of a replication-competent environment (Doorbar 2007; Doorbar et al. 2012).

The late productive stage of the life cycle takes place in the infected cells that undergo terminal differentiation in the most superficial epithelial layers. The completion of the life cycle ultimately involves the expression of the minor coat protein L2, the exit of the cell from the cell cycle and the expression of the major coat protein L1 to allow genome packaging. L1 and L2 are expresses only in cells that have already undergone genome amplification and which contain elevate concentration of E4 protein in their cytoplasm. This seems to require a change in splice site usage rather than promoter activation, leading to transcripts initiated at the late promoter that terminate at the late polyadenylation site rather than the early site, an event that is aided by high levels of E2 expression and by the presence of negative regulatory elements that destabilize late transcripts and allow the preferential synthesis of early transcripts in proliferating cells; furthermore, the pattern of codon usage within the late genes is distinct from that of the host cells, and this may further contribute to the suppression of L1 expression in the lower epithelial layers. It is thought that the accumulation of virion structural proteins to high level is retarded until the cells reach the superficial layers in order to limit detection by the host immune response.

Genome encapsidation involves the recruitment of L2 to regions of replication via E2, prior to the expression of L1 and the assembly of the icosohedral capsid in the nucleus. Virus maturation occurs in the most superficial, dying keratinocytes, leading to the production of extremely stable infectious particles. Although not precisely defined, the abundant E4 protein is thought to contribute to virion release and infectivity in the upper epithelial layers, as it organizes into amyloid-like fibres that disrupt keratin structure and compromise the normal assembly of the cornified envelope; new infectious particles are eventually shed from the epithelial surface as the infected cells exfoliate through desquamation (Doorbar et al. 2012; Doorbar 2007).

α-HPVs and cervical carcinogenesis

Cervical cancer is the second most common malignancy among women worldwide, with approximately 500,000 newly diagnosed cases each year and about 275,000 deaths annually. Despite its worldwide distribution, the frequency of cervical cancer varies considerably, being about ten times more common in some countries than in others. About 80% of cervical cancer occurs in developing countries, where it is frequently the most common cancer of women, accounting for as many as one quarter of female cancers. It occurs less frequently in developed countries.

The frequency of high-risk α-HPV-associated cervical cancers is of around 30-40 people per 100000 and over 99% of cervical lesions harbor viral sequences, although the proportion associated with specific high-risk α-HPV types is different in different countries and shows demographic, ethnic and socio-economic variation. Genital α-HPV infection has a peak of incidence in young individuals between 15-25 years of age following the onset of sexual activity and a progressive fall with age; over 80% of sexually active women become infected at some stage in their life with one or several high-risk and low-risk α-HPV types.

HPV 16 and 18 cause approximately 50% and 20% of the cases of cervical SCC - arising in the stratified squamous cells of the ectocervix - respectively, and both types are equally associated with around 35% of the cases of cervical adenocarcinoma – arising in the columnar glandular cells of the endocervix and more aggressive.

HPV associated pre-cancers present as intraepithelial neoplasia and are named after the site: cervical intraepithelial neoplasia (CIN), VIN and VAIN (vulval and vaginal intraepithelial neoplasia respectively), PIN (penile intraepithelial neoplasia) and AIN (anal intraepithelial neoplasia). Cervical intraepithelial neoplasia and are histologically classified for diagnostic purposes according to the grade of dysplasia: CIN1 (mild dysplasia), CIN2 (moderate dysplasia), CIN3 (in situ carcinoma); CIN1 lesions are low-grade lesions (LSIL, low-grade squamous intraepithelial lesions), while CIN2 and CIN3 lesions are high-grade lesions (HSIL, high-grade squamous intraepithelial lesions). The accurate identification of lesion grade has prognostic significance, as it has been estimated that around 20% of CIN1 will progress to CIN2, and that around 30% of CIN2 will progress to CIN3 if left untreated; CIN3 are generally considered to be the direct precursors of cervical cancer, and it has been suggested that around 40% of CIN3 lesions will progress to cervical cancer in the absence of intervention. In general, more regions with different histological grade can be found in the context of a cervical lesion; different high-risk α-HPV types are usually associated with discrete areas of disease except at junction regions (where lesions abut or are in close proximity) where more than one type may be detected.

Most cervical cancers develop in the transformation zone, corresponding to the squamo-columnar junction; the transitional site where the columnar glandular cells of the endocervical canal meet the stratified squamous cells of the ectocervix. The particular susceptibility of the transformation zone to cancer onset and progression may also be linked to the increased accessibility and proliferation of the basal cell layers at this metaplastic epithelial site, particularly around the time of puberty and the onset of sexual activity. In this case, we can hypothesize that the primary preferential target cells for high-risk α-HPV infection may be cells close to the squamo-columnar junction, such as the epithelial reserve cells, which lie immediately underneath the columnar epithelium of the endocervix, and eventually form the stratified epithelial layers of the transformation zone as the cervix matures. Early acquisition of high-risk α-HPV infections, also facilitated by the low levels in antigen-presenting cells in this site, can disturb the metaplastic changes occurring at this time in the transformation zone and increase the risk of cervical cancer in the future (Yang et al. 2004; Cubie 2013; Doorbar 2007; Doorbar et al. 2012; Tommasino 2014; Moody and Laimins 2010).

Viral life cycle deregulation and cancer progression

The ordered expression of viral gene products that leads to virus particle production is disrupted in HPV-associated neoplasia. In cervical disease, where most research has been done, it is generally thought that the levels of E6 and E7 expression increase from CIN1 to CIN3; this deregulation of early gene expression is accompanied by a concomitant failure to trigger late events until the cells are close to the epithelial surface, and in CIN3 genome amplification is often confined to small pockets at the most superficial layers. Changes in the timing of viral gene expression are accompanied by changes in the levels of the viral proteins, and this is likely to have a key influence in determining lesion grade.

In this scheme, CIN1 lesions typically retain the ability to complete the life cycle and release viral particles, showing a lower level of cell proliferation in the basal and parabasal layers. High-grade lesions such as CIN2 and CIN3 are characterized by the persistence of cycling cells into upper layers, and in CIN3 such cells are detectable at the epithelial surface; such lesions represent abortive infections in which viral gene expression is not properly controlled, and late events in the virus life cycle are not properly supported.

The elevation of E6 and E7 expression in high-risk α-HPV infection that leads to the CIN2+ phenotype predisposes the cell to the accumulation of genetic changes, which increasingly contribute to cancer progression. According to this hypothesis, the relatively low levels of E6 and E7 present in CIN1 do not compromise the functions of their cellular targets sufficiently to facilitate

cancer progression. The viral deregulation seen in CIN2/3+ is also thought to facilitate integration of the viral episome into the host cell chromosome, which can further deregulate the expression of E6 and E7.

It is not clear exactly how gene expression from the viral episome can become deregulated in early CIN. The viral gene expression may be deregulated by changes in cell signaling as can be brought about by hormonal changes, or epigenetic modifications such as viral DNA methylation, which may depend on the nature of the infected epithelial cells. The HPV16 LCR contains hormone response elements that can be stimulated by estrogen, and there is ample evidence of cooperation between estrogen and HPV in the development of cervical cancer. In CIN, it has been reported that the LCR is differentially methylated according to disease severity, which suggests that epigenetic changes may also regulate gene expression (Doorbar 2007; Doorbar et al. 2012).

In early CIN most HPV genomes persist in an episomal state, whereas in many HSIL they are found integrated into the host cell genome (Figure 4). Several studies have shown that the frequency of viral DNA integration increases with the severity of the cervical lesion, indicating that this event is implicated in the progression of the disease.

The majority of cervical cancers contain one or many HPV genome copies, integrated more or less randomly into the host cell genome in a monoclonal fashion, with the viral integration site frequently lying within the regulatory E1 or E2 genes; this event often leads to the disruption of E2 and adjacent ORFs (E4, E5, L2), and as a consequence to the loss of expression of full-length E1, E2, E4, E5 and late genes (Figure 4). The key event is the loss of E2, which is a virally-encoded transcription factor that normally regulates E6/E7 abundance by binding to and silencing the early promoter within the LCR as part of a viral regulatory mechanism.

It is thought that integration occurs in HSIL, and that once this occurs, the already deregulated expression of E6 and E7 can increase still further or else be maintained at a constitutive level. Moreover E6 and E7 transcripts expressed from integrated copies show increased stability, and this was attributed to the longer half-life of transcripts derived from integrated HPV DNA, mediated by 3'-cellular sequences of the fusion transcripts. Furthermore integration imparts a selective growth advantage over cells that harbor only episomal copies of viral DNA. Usually only a segment of the viral genome is integrated containing the LCR and truncated parts of the early and late region (Figure 4); multiple tandem repeats of such viral DNA fragments - from a single to several hundred copies – are often found integrated at random locations in the cellular genome. Upon integration the host cells contain an insignificant amount of viral DNA and proteins other than E6 and E7; the E5 protein might be a weak cofactor in the development of malignancy, mostly in early stages by

driving cellular proliferation cooperating with E6 and E7. This is evident in high-risk α-HPV-positive cervical cancer-derived cell lines, where viral DNA is found to be randomly integrated in the host genome leading to the disruption of several viral ORFs and the preservation of E6 and E7, which are the only actively transcribed genes.

Another significant consequence of the integration is the deregulated expression of E6/E7, which leads to persistent stimulation of S-phase entry, and the loss of the cellular p53 tumor suppressor protein. Thus promoting the emergence of a clonal population of cells with a growth advantage and an increased propensity for transformation and malignant progression; this tumorigenic pathway requires episome loss, and if viral episomes persist, E2 expression continues to block transcription of integrated E6/E7. Noteworthy, the key kinases implicated in sensing and repairing DNA damage, ATM – induced in response to double-stranded DNA breaks - and ATR – induced on the appearance of single-stranded DNA breaks – whose pathways also involve p53 in downstream signaling, which leads to the amplification of integrated HPV sequences and the flanking cellular sequences. This combination of events predisposes to the accumulation of secondary genetic changes in the host cell genome, and compromises the ability of the cell to effectively repair damaged DNA; the consequence is the induction of a condition of genomic and genetic instability which further favors progression towards malignancy.

Experimental models have shown that sustained E6/E7 expression is necessary for the maintenance of the transformed phenotype; however, this condition is necessary but not sufficient for malignant progression. The accumulation of alterations in the host cell genome, particularly in genes controlling the cell cycle, is an important and necessary event in the development of cervical cancer, and can appear over time in women who carry persistent active infections. Such mutations can occur by chance, or can arise as a result of environmental events. Tobacco metabolites, which are present in the cervical secretions of women who smoke, are thought to act as co-factors in the development of cervical cancer. Multiparity and the long-term use of oral contraceptives are also associated with increased risk, as is the presence of additional genital infections such as Chlamydia; hormonal contraception is associated with increased risk due to the effects of estrogens and progesterone, which stimulate transcription and cell proliferation inducing also E6/E7 over-expression.

Cervical cancer can arise from cells containing exclusively episomes, and for HPV 16, around 30% of cervical cancers develop in this way; around 70% of HPV16-associated cervical cancers contain integrated HPV16 sequences, while for HPV18, the viral genome is almost exclusively integrated. Predominantly “episomal” tumors appear characterized by a more favorable natural history than “integrated” tumors, correlating with a stronger immune response to L1/L2 coat

proteins in the former group. In both cases, however, it is the long-term expression, and in particular, the deregulated over-expression of E6 and E7 and the accumulation of genetic errors, which are ultimately important in the progression from CIN3 to cervical cancer (Connolly et al. 2014; Crosbie et al. 2013; Doorbar 2007; Doorbar et al. 2012; Moody and Laimins 2010; Tommasino 2014; Wentzensen, Vinokurova, and Doeberitz 2004).

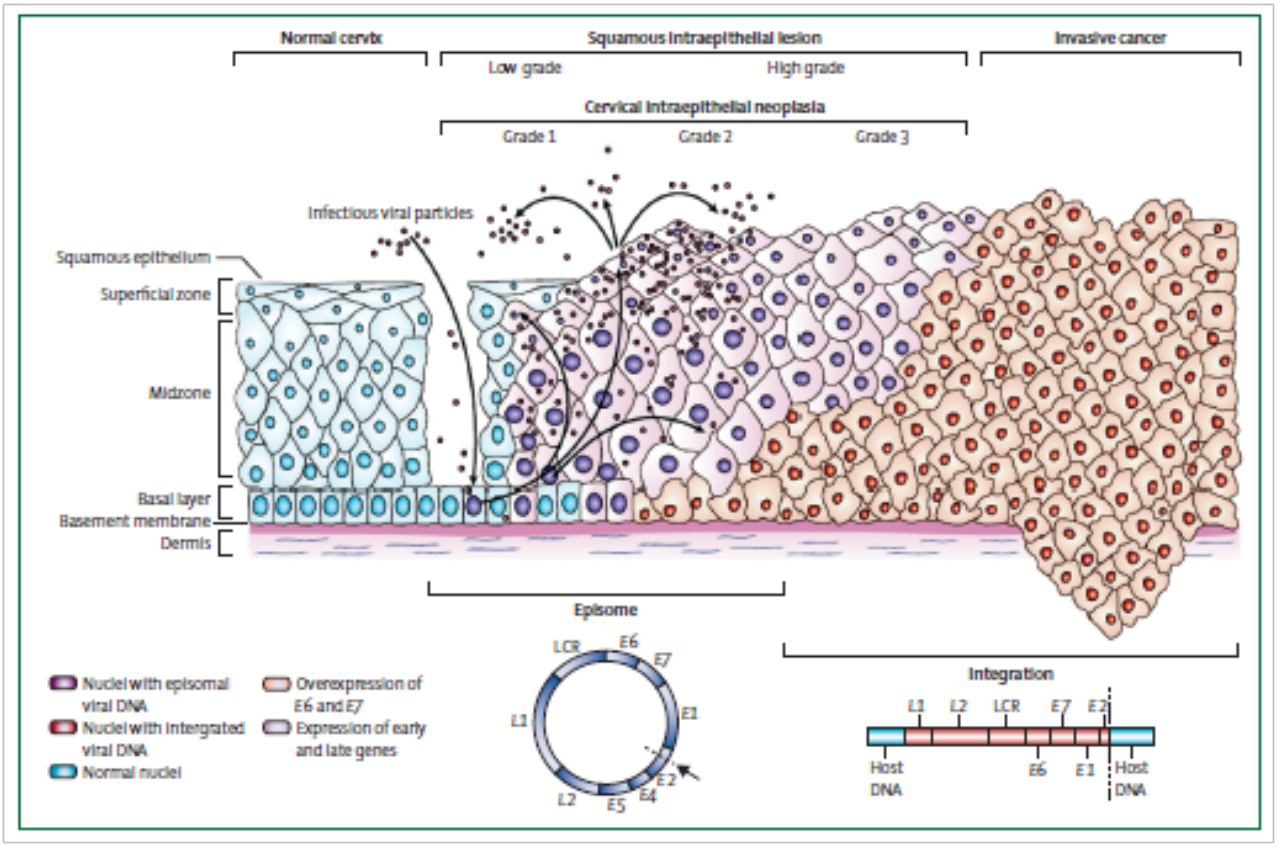

Figure 4. Low grade squamous intraepithelial lesions (CIN1) support productive viral replication. Progression of untreated lesions to high-grade squamous intraepithelial neoplasia (CIN2/3) and invasive cervical cancer is associated with abortive infection and random integration of the viral genome into host chromosomes, often characterized by the loss or disruption of E2 and its adjacent ORFs and the preservation of the E6 and E7 oncogenes with subsequent deregulation of their expression (Crosbie et al. 2013).

Cervical high-risk α-HPV infection: possible outcome

High-risk α-HPV infection of the uterine cervix can have a number of different outcomes. Following infection, expression from the viral genome can sometimes be suppressed (e.g. by genome methylation), leading to a silent infection in which the viral episomes are retained in the basal epithelial layer without a preceding productive stage and overt disease; such unapparent infections can be resolved or persistently remain as sites of viral latency. Infection may alternatively establish an ordered pattern of viral gene expression leading to virus synthesis and release from the upper epithelial layers, with the formation of a productive CIN1 lesion that in most cases regresses

upon clearance of the virus by the host immune system and only in a subgroup of cases evolves to HSIL and consequent cervical cancer.

Despite the high incidence of high-risk α-HPV infection of the genital tract among sexually active individuals, the majority (80-90%) of new infections at any age are cleared without any apparent clinical disease. The regression is the result of an immune response mainly mediated by T-cells and NK, and the infection do not persist long enough for deregulated gene expression and the accumulation of secondary genetic errors to occur. The majority of those who develop productive CIN1 lesions eventually mount an effective cell-mediated immune response and the lesions spontaneously regress; infections are usually resolved in a short term period (12-18 months), with re-infection by the same HPV type being uncommon.

The failure to develop effective cell-mediated immunity to clear or control infection results in persistent infection and in an increased probability of progression to HSIL and invasive carcinoma. It has been suggested that HPV 16 and 18 may be more persistent than other high-risk α-HPV types, explaining the great proportion of cervical cancers caused by these two types compared to the others and therefore their higher oncogenic potential.

Effective evasion of the innate immune recognition seems to be the hallmark of high-risk α-HPV infections. HPV globally down-regulates the innate immune signaling pathways in the infected cells through multiple ways: interference with attractive chemokines; down-regulation of the expression and signaling of pro-inflammatory cytokines; down-regulation of the antiviral IFN response; up-regulation of anti-inflammatory cytokines that prevent migration of immune cells to the sites of infection; attraction and retention of suppressive regulatory T cells; down-regulation of TLR expression; reduction in the activation of antigen-presenting cells and in peptide presentation abilities. Many of the mechanisms of immune evasion by high-risk α-HPVs have been established and seem to be driven by specific functions of E5, E6 and E7 oncoproteins.

The fact that HPV exhibit an exclusively intraepithelial life cycle avoiding cell lysis and necrosis or viraemic phase that would trigger inflammatory responses, and that viral proteins are massively expressed only in the upper epithelial layers, well away from circulating immune cells, may further restrict detection by the host immune system. The viral early proteins, which are expressed also in the lower epithelial layers, are thought to be produced at low levels below those required to effectively trigger an immune response. In contrast to lytic viruses which are rapidly detected by the host immune system causing host cell destruction at the terminal stage of infection, antibodies directed against HPV capsid proteins arise as late as 6-12 months following the infection.

When lesion regression does occur, viral gene expression is shut off in the presence of the infiltrating lymphocytes (possibly as a result of cytokine signaling), but the viral genomes may not

be effectively cleared from the basal layer being still persistently present in a subset of epithelial stem cells in a latency status. The lesion is cleared by the replacement of actively infected cells with "apparently normal cells" as the basal cells continue to divide; these "apparently normal cells" may still contain viral genomes with very low-level expression of viral gene products and without completion of the life cycle – only some occasional bursts of virus production that may explain the frequent detection of viral DNA in the absence of disease. Latent infection is thought to require E1 and E2 to allow basal cell replication of viral episomes, but is not thought to depend on the expression of E6 and E7.

It has been suggested that the life cycle may become reactivated subsequently following changes in hormone levels or in presence of immunosuppression; individuals with immune function defects, such as OTRs or HIV patients, are affected by pathological conditions associated with reduced T-cell function, leading to an impairment of antiviral immune surveillance that may be responsible for the rise in viral genome copy number at the regressed sites. Studies on immunocompromised patients, such as OTRs or HIV-positive subjects, have shown an increased prevalence of detectable single or multiple high-risk HPV infections and associated lesions, compared with healthy people. Accordingly, in such individuals the incidence of HPV-related cervical cancers and other genital cancers is significantly higher compared with the general population.

For cancer to develop, it's well known that the virus has to evade immune detection over a prolonged period to lead the accumulation of genomic and genetic abnormalities. High-risk α-HPV chronic infections characterized by long persistence in the slow-cycling epithelial stem cells in the absence of apparent disease is a condition sine qua non for the development of HPV-associated malignancy; although it is clear that high-risk E6 and E7 target cellular pathways related to innate and adaptive immunity, host and environmental factors significantly contribute to the chronicity of high-risk α-HPV infection. Cancer progression typically occurs over years of decades in patients that do not effectively resolve their initial infection as their immune system fails to clear the infected basal stem cells and the virus; this long time is required for the acquisition of secondary genetic changes.

All these issues suggest that even if high-risk α-HPV infection is decisive for the process, on its own it is not sufficient to induce the development of a malignant tumor; a number of other factors are decisive for the neoplastic transformation of the epithelial cells of the uterine cervix, including age, health and habits of the infected individual, the duration of the infection and environmental factors (Cid Arregui et al. 2012; Ciesielska et al. 2012; Doorbar 2007; Doorbar et al. 2012; Moody and Laimins 2010; Tommasino 2014).

The high-risk α-HPV oncoproteins

The development of cervical cancers involves a coordinated targeting of multiple pathways by high-risk α-HPV oncoproteins, with each pathway having a distinct role in malignant progression. These viral proteins disrupt or usurp multiple cellular signalling pathways to maintain infected cells in a proliferative state to facilitate viral replication. One important consequence of this, however, is the accumulation of mutations in cellular genes and increased genomic instability, which results in full transformation. In addition, high-risk oncoproteins also enable immune escape in order to allow viral persistence in the host. The primary viral factors responsible for altering these pathways and mediating progression to malignancy are the E5, E6 and E7 proteins, with E6 and E7 playing a central role in these processes and acting in a cooperative manner (Moody and Laimins 2010).

It is becoming clear that both mucosal E6 and E7 interfere with a broad spectrum of cellular targets, and that the identity of these substrates differs between HPV types of the same high-risk clade, as well as between the high-risk and low-risk groupings themselves, conferring oncogenic properties to various extents depending on the specific mucosal α-HPV species and types; this is due to different biochemical properties of high-risk and low-risk E6 and E7, with aminoacid substitutions differentially affecting their activity. The high-risk and low-risk α-HPVs also have significant differences in promoter positioning and regulation, as well as in patterns of mRNA splicing. These differences affect expression from the E6 and E7 genes. It is thought that different patterns of viral gene expression, as well as different protein functions, play a major role in determining disease phenotype following infection (Doorbar et al. 2012).

The mucosal E6 and E7 proteins are typically expressed together in infected cells, from a common early promoter. There is a value, however, in dissecting their specific functions by expressing them individually.

The expression of high-risk E7 proteins by themselves can immortalize cultured human keratinocytes – which are known to have a short replicative lifespan in vitro - at a low frequency but high-risk E6 has no such activity, since it can only extend the lifespan of keratinocytes. The combination of high-risk E6 and E7, however, is highly efficient at immortalizing most types of primary cells in culture. Low-risk E6 and E7, on the contrary, do not readily extend the lifespan of keratinocytes, with occasional subpopulation of cells emerging that are immortal. The growth of keratinocytes expressing both high-risk E6 and E7 in organotypic raft cultures, which faithfully duplicate epithelial stratification and differentiation, results in cellular changes identical to those observed in HSIL in vivo, further demonstrating the importance of these factors in HPV pathogenesis.

In addition, transgenic mice that express high-risk E6 and E7 in basal epithelial cells exhibit epidermal hyperplasia and develop squamous carcinomas on low-dose oestrogen treatment or chemical carcinogens. In these mouse models, the phenotype correlated with high-grade cervical dysplasia and invasive cervical malignancies is primarily due to E7 expression. On the contrary, high-risk E6 alone has modest transforming functions when expressed as a transgene from a keratin-specific promoter in the epithelium of mice. The co-expression of high-risk E6 and E7 results in larger and more extensive cervical cancers, highlighting their cooperative activity in promoting tumorigenesis (Moody and Laimins 2010; Vande Pol and Klingelhutz 2013; Tommasino 2014).

The high-risk and low-risk E7 proteins exhibit differential ability to associate with members of the Retinoblastoma (Rb) protein (pRb) family. The high-risk E7 proteins are able to bind and target for ubiquitin-dependent degradation - through interaction with ubiquitin-ligases such as Cul2 and UBR4 - both p105 and p107, which control cell cycle entry in the basal epithelial layer, as well as p130, which is involved in cell cycle re-entry in the mid-upper epithelial layers. The low-risk E7 proteins generally appear to have a lower affinity for p105 and p107 than high-risk E7, but can associate with and degrade p130 in order to create a replication-competent environment in the mid-epithelial layers that is suitable for genome amplification.

An unfortunate characteristic of the high-risk E7 proteins is their ability to stimulate host genome instability, particularly through deregulation of the centrosome cycle in the proliferating basal epithelial cells. E7 induce multiple rounds of centrosome synthesis in a single S-phase through the formation of multiple immature centrioles from a single maternal centriole. This may be due to the binding of E7 to γ-tubulin, a centrosome regulator. This interaction seems to be independent of the Rb binding ad result in removal of γ-tubulin from the mitotic spindle, which may lead to abnormal centrosome synthesis. An abnormal centrosome number can bring to an accumulation of mitotic abnormalities such as chromosomal missegregation. Further the development of aneuploidy that may increase the chance of genetic errors during each round of host cell division. Indeed, most HPV-associated malignancies have numerous chromosomal imbalances, including aneuploidy and chromosomal rearrangements.

High-risk E7 in cooperation with E6 can also stimulate genomic instability through the induction of DNA damage and the activation of the ATM-ATR pathway; such activation of the cellular DNA repair machinery is harnessed for viral replication in differentiated keratinocytes and can lead to an increase in the frequency of foreign DNA integration into the host genome. An important aspect is also that E7 induce the proteolytic degradation of claspin, a key regulator of the ATR DNA damage signaling pathway that is activated in response to replication stress; the accelerated degradation of

claspin by high-risk E7 in G2-M checkpoint may "trick" host cells into initiating checkpoint recovery, allowing aberrant mitotic entry in the presence of DNA damage and potentially leading to genomic instability through defective DNA repair.

In addition to RB destabilization, E7 contributes to immortalization through interaction with key proteins that control cell cycle progression.

The high-risk E7 proteins are able to bind the CDK inhibitors p21 and p27, which are important regulators of growth arrest during epithelial differentiation whose major target is CDK2, important for G1-S transition and cell cycle progression. As a consequence, the p21 and p27 repressor effects on the cell cycle are neutralized and CDK2 activity remains high in E7-expressing cells despite high levels of p21. E7-mediated p21 inactivation is one of the ways through which high-risk α-HPVs inhibit p53 signaling in the infected cells. Low-risk E7 proteins can also bind p21 but with a greatly reduced efficiency and a decreased ability to abrogate its inhibitory effects.

In addition, high-risk and low-risk E7 can bind indirectly to cyclin-CDK2 complexes through pRB, p107 or p130, as well as directly to CDK2 and/or cyclin subunits, allowing sustained CDK2 activity. High-risk E7 has further been shown to increase the levels of the CDC25A phosphatase, which can induce tyrosine dephosphorylation of CDK2, promoting its activation.

All these E7-dependent mechanisms of CDK2 abnormal activation allow G1-S checkpoint to be bypassed, as it occurs upon E7-mediated abrogation of Rb protein activity.

The high-risk E7 proteins promote premature S phase entry and DNA synthesis with the disruption of the Rb-E2F complex resulting in the constitutive expression of E2F responsive genes. E7 interact also with histone-deacetylases, a class of enzymes that act as co-repressors of gene transcription and directly bind and down-regulate the E2F promoter, and with E2F transcriptional repressors; as a result, the suppressive activity of these factors is abrogated and the transcription of genes involved in G0/G1-S transition is promoted, further stimulating host cell proliferation.

Another mechanism by which the high-risk E7 proteins contribute to host cell transformation is the ability to induce histone code modifications (e.g. demethylation), leading to epigenetic reprogramming, as demonstrated by the aberrant expression of homeobox genes – which control cell fate and identity – in host cells.

The high-risk E7 proteins have also been demostrated to induce autophagy in primary human epithelial cells under conditions of metabolic stress, and to promote the Warburg effect, a reprogramming of the cellular metabolism to aerobic fermentation and a general hallmark of human tumor cells.

Finally, high-risk proteins E7 interact with components of the interferon (IFN) response, contributing to escape from immune surveillance and the establishment of a persistent infection. They bind to and inactivate IRF1 and IRF9, inhibiting their ability to stimulate IFN transcription and therefore blocking the IFN response that is normally activated following viral infections; high-risk E7 in cooperation with E6 down-regulate STAT1, which is a key transcription factor that regulates the IFN response and its repression may be crucial for the inhibition of this activity. High-risk E7 can also repress the expression of the adhesion molecule E-cadherin, which is thought to underlie the lower abundance of antigen-presenting cells in the vicinity of the lesions; this function may increase the invasiveness of the infected cells as well. Furthermore, high-risk E7 reduce the expression of genes involved in peptide presentation and activation of the antigen-presenting cells (Ciesielska et al. 2012; Crosbie et al. 2013; Doorbar et al. 2012; McLaughlin-Drubin, Meyers, and Munger 2012; Moody and Laimins 2010; Roman and Munger 2013).

Another characteristic of both high-risk and low-risk E6 proteins bind to and inactivate p53, but only high-risk E6 stimulate its proteolytic degradation. High-risk E6 recruit the cellular ubiquitin-ligase E6AP and mediate the ubiquitination and proteasomal degradation of p53 through the formation of a strong tripartite complex E6-E6AP-p53. Low-risk E6 can also associate with E6AP but surprisingly this does not result in p53 degradation, suggesting that other cellular factors are targets for the low-risk E6-E6AP complex. The transcriptional activity of p53 could be inhibited through direct binding by low-risk E6, and this may be the predominant mechanism by which low-risk HPVs inhibit the growth-suppressive effects of p53.

In addition, high-risk and low-risk E6 interfere with p53 function by suppressing the activity of different histone-acetyltransferases (e.g. CBP/p300 and Ada3), blocking the ability of these factors to acetylate p53 and therefore increase its stability; the result is a destabilization of p53 transcription complexes and their conversion from co-activators to repressors.

In a persistent infection, all these p53-targeting high-risk E6 functions compromise the effectiveness of the p53-mediated cellular DNA damage response in cells that are driven into S-phase by E7 over years or decades, and allow the accumulation of secondary mutations to go unchecked, eventually leading to cancer.

The high-risk E6 proteins exhibit a conserved PDZ-domain-binding motif located at the C-terminus; this motif allows interaction with several PDZ targets, in particular proteins of the MAGUK family localized in the cell membrane, which are generally believed to be associated with the cellular membrane and affect such processes as cell polarity, maintaining cell-to-cell interactions, and mediating signals from membrane. E6/MAGUK interaction, leads to degradation of the cellular protein; the consequence is the abrogation of the functions of all these proteins: loss

of cell-cell contacts upon degradation of proteins forming intercellular junctions, leading to loss of cell polarity and anchorage-independent cell growth. All these conditions are important in the development of malignancies.

The high-risk E6 proteins balance the E7-induced metabolic stress by activating mTORC1, a complex controlling protein synthesis and functioning as a nutrient, energy and redox sensor; the result is a state of blissful ignorance of cellular nutrient supply, and a stimulation of protein synthesis facilitating viral replication in terminally differentiating cells even with low nutrient supply. Increased mTORC1 activity has been detected in HPV-associated pre-malignant lesions and cancers.

Another characteristic of the high-risk E6 proteins is their capacity to up-regulate telomerase activity and maintain telomere integrity during repeated cell divisions. In somatic cells every consecutive division of a cell results in abbreviated telomeres. Telomere length reflects the age of the cells. Elevated telomerase activity, which adds additional telomere sequences, has been documented in human neoplastic cells, and it has been suggested that this may be of significance in the development of tumors. High-risk E6 activate the catalytic subunit of telomerase (hTERT) through E6AP by interacting with myc and modulating the activity of repressors (USF, NFX-91 which is targeted for degradation) and activators (myc-max, SP1, and histone-acetyltransferases) that bind to and induce the hTERT promoter. E6-mediated hTERT activation stimulates the addition of hexamer repeats to the telomeric ends of chromosomes, allowing maintenance of their length and indefinite proliferation, which is expected to inhibit cell senescence and extend the lifespan of cells harboring viral genomes; such immortalizing effects may predispose to persistent infection.

It has been reported that high-risk E6 proteins can induce down-regulation of specific genes that are involved in keratinocyte differentiation; this might delay host cell differentiation until enough genomes have been replicated for subsequent production of infectious virions. Through unknown mechanisms probably involving interactions with transcription factors and signaling proteins, high-risk E6 also affects the expression of miRNAs involved in targeting cell cycle control genes and in the regulation of cell migration. This activity of modulation of the expression of specific cellular genes may contribute to tumor undifferentiation, growth and invasion.

The high-risk E6 and E7 proteins also interfere with the effects of various growth inhibitory cytokines that are induced following infection and can activate apoptotic pathway. In response to viral entry, cells produce inflammatory mediators such as TNFα, which is a potent inhibitor of keratinocyte proliferation. Inflammatory cytokines can activate the extrinsic apoptotic pathway through transmembrane cell surface death receptors of the TNF receptor family, such as TNFR1, FAS and TRAIL receptors. High-risk E6 block TNFα-induced apoptosis by directly binding to

TNFR1, which inhibits the formation of the death-inducing signaling complex and consequent transduction of apoptotic signals. It can also interact with the adaptor protein FADD and caspase 8 to block cell death in response to FAS and TRAIL.

High-risk E6 can also interfere with induction of mitochondrial apoptotic pathways through interactions with the pro-apoptotic Bcl2 members Bak and Bax, inducing their ubiquitin-dependent proteasomal degradation via E6AP; in addition, they can induce up-regulation of inhibitors of apoptosis such as IAP2 and survivin, which can occur through E6-mediated activation of NF-κB. It is unclear if low-risk E6 function in a similar manner; they have been reported to be able to induce Bak proteolytic degradation.

Anoikis is another apoptotic pathway targeted by high-risk E6, which is an essential and conserved mechanism found in multicellular organisms that functions to prevent the abnormal detachment of cells in the host. High-risk E6 have been reported to bind to paxillin, which is a key downstream mediator in the induction of this process. These interactions promote resistance to anoikis and allow HPV-immortalized cells to proliferate, establishing anchorage-independent cell growth, fundamental characteristic in tumor progression and invasion.

Like high-risk E7, E6 can interfere with the IFN antiviral response and facilitate viral escape from immune surveillance.

Both the high-risk and the low-risk E6 proteins repress the transcription of many IFN-inducible genes, including Stat1. High-risk E6 directly interact with components of the IFN response, such as IRF3, resulting in inhibition of IFN transcription.

In addition, high-risk E6 overcome the growth-suppressive effects of IFN through the formation of a tripartite complex with p53 and CBP/p300 (which is the downstream mediator of the p53-dependent IFN antiproliferative response).

This interaction prevents the acetylation of p53 and blocks the activation of p53-target genes, allowing continued viral replication in the presence of IFN. Importantly, the binding of E6 to CBP/p300 may further facilitate viral escape from immune surveillance and establish long-term persistence; p300/CBP is a co-activator of the NF-κB transcription factor, which controls among other things the promoters of certain interleukins which are down-regulated as a consequence of E6 action.

Like high-risk E7, high-risk E6 can also contribute to immune escape by reducing the ability of epithelial cells and antigen-presenting cells to present viral antigens and to activate, affecting the expression of key proteins in this process; in addition, they reduce E-cadherin expression with similar effects to its E7-mediated repression. Finally, another mechanism by which both high-risk and low-risk E6 can locally down-regulate host immune responses is the binding and inactivation of

Tyk2 kinase of the Jak-Stat pathway, which is critical in cytokine signaling (Ciesielska et al. 2012; Crosbie et al. 2013; Vande Pol and Klingelhutz 2013; Tommasino 2014; Doorbar 2007; Doorbar et al. 2012; Moody and Laimins 2010; McLaughlin-Drubin, Meyers, and Munger 2012).

Although E6 and E7 provide the primary transforming activities of high-risk α-HPVs, E5 can augment their function and contribute to tumor progression. In general E5 proteins alone have weaker transforming activity compared to E6 and E7, in contrast some few genotypes show strong transformation activity. Cellular and mouse models have shown that the high level expression of high-risk E5 induce spontaneous tumours formation; suggesting that in some cases they seems to be able to act as oncoproteins. They are thought to enhance the transforming activity of E6 and E7 by promoting host cell hyperproliferation through their mitogenic and anti-apoptotic actions in early stages of the tumorigenic processes, before the occurrence of viral DNA integration in cervical HSIL.

Dimeric complexes of E5 protein are able to trigger cell fusion, yielding binucleate polypolid cells via endoreduplication (koilocytes) which are typical of α-HPV-associated lesions. Another reported high-risk E5 activity is the reduction in E-cadherin expression, which is a function also mediated by E6 and E7; in addition, high-risk E5 can inhibit the host MHC class II complexes, thus compromising the display of viral peptides by the infected epithelial cells and contributing to viral immune escape (Moody and Laimins 2010; Connolly et al. 2014).

Papillomavirus vaccine and cervical screening

Understanding of the ubiquitous role of high-risk α-HPV infection in all CIN lesions and cervical cancer combined with an understanding of HPV natural history has led to the development of the first prophylactic cancer vaccines. These vaccines contain L1 self-assembling recombinant non-infectious virus-like particles, which closely resemble the structure of native virions and are highly immunogenic leading to strong neutralizing specific antibody responses against HPV infection. These antibodies are thought to block the HPV virions before they gain access to the proliferating basal cell layer of the epithelial surface.

Two vaccines are available - the quadrivalent vaccine (Gardasil, Merck), which contains virus-like particles to HPV 6, 11, 16 and 18, and the bivalent vaccine (Cervarix, GlaxoSmithKline), which contains virus-like particles to HPV 16 and 18. Both vaccines protect against precancerous lesions associated with the high-risk types HPV 16 and 18. The quadrivalent one is also able to confer protection against benign mucosal lesions associated with the low-risk types HPV 6 and 11.

Efficacy is close to 100% according to different trials. These vaccines seem also to provide a sort of cross-protection to non-vaccine HPV types, associated with immune responses against HPV types that are phylogenetically related to HPV 16 and 18 respectively (e.g. HPV 31).

The two HPV vaccines were not designed as therapeutic vaccines and have little, if any, prophylactic effectiveness in people that have been previously exposed to the vaccine HPV types; although they block initial infection with the vaccine HPV types, they do not induce regression of ongoing lesions and cannot prevent the progression to cancer in already infected individuals.

To manage a wider range of on-request vaccinations, many countries have included a catch-up range for vaccination in HPV-naive individuals, which overlaps with the typical age of onset of sexual activity. Although the primary focus of HPV vaccination programs has been on women and girls - who bear the greatest burden of HPV-associated cancers - recent clinical trial data showing efficacy of vaccination in men and the potential for herd immunity have led to vaccination of adolescent boys to be recommended in some developed regions; indeed, the HPV vaccines have recently been approved for use in men to prevent the development of HPV-associated penile cancers.

However, the comprehensive preventive cervical screening programs remain the only strategy that has avoided a corresponding epidemic of cervical cancer. Thus, it is important for women to continue to undergo cervical screening, as infections with other high-risk α-HPV types not targeted by the vaccines can still occur. Cervical screening can be performed either by traditional cytological analysis – Papanicolau smears (PAP smears), allowing to detect cervical disease and predict its severity from the examination of exfoliated cells scraped from the epithelial surface – or by HPV DNA/RNA testing. Pap smear screening programs can identify most premalignant lesions. Appropriate follow-up of women with these abnormalities, together with appropriate treatment, can thereby prevent the development of most cases of cervical cancer. In countries with screening programs, however, the incidence of adenocarcinoma and adenosquamous cell carcinoma has been increasing, suggesting that Pap smear screening may be less effective in identifying the precursors to these tumor types. DNA/RNA testing detects HPV infection that is far more common than prevalent disease but is also a biomarker of increased future risk. Widespread HPV vaccination and cervical screening are absolutely required to reach a significant decrease in incidence of HPV-induced cervical disease, especially in developing countries where cervical cancer burden is still considerable. Since high-risk α-HPVs cause tumors years of decades after the initial infection, the currently available prophylactic vaccines will not have a measurable impact on HPV-associated cancer rates for two or three decades (Crosbie et al. 2013; McLaughlin-Drubin, Meyers, and Munger 2012; Moody and Laimins 2010; Knipe and Howley 2007).

β-HPVs and skin carcinogenesis

While the causative relationship between HPV infection and genital SCCs is well established, the role of HPV in the development of cutaneous malignancy is, as yet, unclear. However, there is increasing evidence showing the involvement of cutaneous β-HPV in the development of Non Melanoma Skin Cancer (NMSC). To demonstrate that a pathogen causes cancer, convincing epidemiologic evidence of association and a plausible biological mechanism for oncogenesis are fundamental; while this has been achieved for high-risk α-HPVs and cervical cancer, such requirements have not yet been satisfied for β-HPVs and NMSC, as there is great lack of consistency among epidemiological studies and no molecular role for these viruses in cutaneous tumorigenesis has been certainly proven so far.

NMSC is the most common cancer type among Caucasians, where it accounts for around 30% of all malignancies and its rates are increasing by 4–8% yearly. Although the majority of NMSC cases can be treated surgically and do not usually exhibit high aggressiveness, these cancers are associated with high morbidity and represent a significant burden on the healthcare system. Immunosuppression and UV exposure are the main risk factors for NMSC – these tumors mostly arise in sun-exposed skin sites.

The major histological types of NMSC are basal cell carcinoma (BCC) and cutaneous squamous cell carcinoma (cuSCC), associated with different underlying mutational patterns. BCC is more common in fair-skinned individuals and originates from transformation of basal epidermal cells. cuSCC, which is more aggressive than BCC, is more common among darker skin population and arises from transformation of squamous epidermal cells; it is usually preceded by precancerous lesions, namely actinic keratosis (AK), with high potential to undergo malignant progression; other forms of skin tumors that are histologically related to cuSCC are keratoacanthoma – a low-grade cuSCC subtype originating from the hair follicles, able to regress spontaneously and representing a midpoint between a benign wart and invasive cuSCC – and Bowen's disease – an early stage in situ intraepidermal form of cuSCC (Akgül, Cooke, and Storey 2006; Aldabagh et al. 2013; Ingo Nindl, Gottschling, and Stockfleth 2007; Knipe and Howley 2007; Pfister 2003; E-m de Villiers 1998).

Has been shown that cutaneous β-HPVs are ubiquitously widespread and cause asymptomatic latent infections without apparent disease in the general healthy population. Thus the epidemiological demonstration of a possible causal link between infection and NMSC development is complicated; it is thought that everybody is positive for multiple β-HPV types. With increasing age, the number of infecting β-HPV types seem to rise; β-HPV persistence with the same types is common, and family members often share the same types ("β-HPV signature"); more than half of

the HPV types detected in the specimens from the babies were also isolated from the respective parents, even although only one sample per parent was obtained and analyzed.

Different studies using different sampling techniques have shown that the most commonly detected β-HPV types are HPV 5, 8 and 23; different ethnicities may harbor different β-HPV types, with HPV5 being the most prevalent type and the only type common to all countries studied.

Several studies have reported the ubiquitously presence of these viruses on the body surface, with greater carriage in sun-exposed sites (e.g. forehead) than is sun-protected skin when its presence was monitored in plucked hairs and skin swabs; they appear to establish a reservoir of infection in the follicular stem cells of hair bulbs, which are thought to be an immune privileged site protecting them from immune clearance and therefore enabling the persistence of multiple β-HPV types. However, viral DNA in normal skin is usually detected at very low genome copy numbers, rarely being higher than one genome copy per cell.

β-HPV infection seems to be acquired during or shortly after birth; the presence of the viruses on the skin surface of the mother and other people in close contact with the newborn is the most likely source of infection. Transmission of β-HPV probably takes place via direct contact with infected skin or derivate thereof such as scales and dandruff; the likelihood of a prenatal vertical transmission from mother to fetus has also been proposed. Transmission in later stages of postnatal life is thought to be limited; this may be due to the fact that in the first postpartum days, the thinner horny layer facilitates access to the basal layer, or to the fact that after the sites available to infection have been occupied, the settlement of other β-HPV types is more difficult because of the occurrence of a cross-reactive antiviral response following initial infection. In immunocopetent individuals, this early infection seems to persist in a latency status and without clinical manifestations (Akgül, Cooke, and Storey 2006; Aldabagh et al. 2013; Bouwes Bavinck, Plasmeijer, and Feltkamp 2008; Harwood and Proby 2002; Feltkamp et al. 2008; Pfister 2003).

Epidermodysplasia verruciformis

Epidermodysplasia verruciformis (EV), which is considered to be a primary immunodeficiency (PID), is the first evidence for the involvement of HPV infection in the development of skin cancer. To date, is the only setting where the association between β-HPV infection and skin carcinogenesis has been ascertained.

It is a rare autosomal recessive condition, in which selective depletion of specific T-cell clones - although the immunophenotype might be normal in some patients - is associated with an abnormal susceptibility to persistent infection restricted to a subset of about 20 β-HPV types. Multiple

infections are more frequent rather than the presence of one infecting type. These patients usually develop warts in childhood, which become widespread, do not tend to regress, and, in some instances, may progress to squamous cell cancers. Two predominant types of lesions are seen, which can occur in the same patient. Some lesions have the appearance of flat warts, whereas others are flat, scaly, red-brown macules. The plane warts are usually associated with the cutaneous α-HPV types HPV 3 and 10 and occurring mainly on the trunk, neck and extremities, those persist lifelong. The flat warts are caused by the same HPV type that induces flat warts in the general population, usually α-HPV 3 or 10. The scaly lesions are associated with EV-specific HPV types, most frequently β-HPV 5 and 8; often with more than one β-HPV type being detected, and contain high β-HPV episome copy numbers per cell – indicating a high level of viral replication – and abundant E6 and E7 transcripts – indicating viral gene expression in the lesional tissue.
In the fourth decade of life, around 30-60% of EV patients develop malignant tumors in sun-exposed sites, which are usually low grade in situ carcinomas with some features in common with Bowen's disease, but others are more aggressive cuSCC. HPV 5 and 8 account for 90% of the tumors with HPV 14, 17, 20 and 47 accounting for the remainder. Extrachromosomial β-HPV DNA can reach 100-300 genome copies per cell, even though in a limited number of tumor cells. Unlike cancers caused by high-risk α-HPVs, β-HPV-induced EV skin tumors are associated with productive infections. The high level of viral replication and subsequent active oncogene expression underlies skin carcinogenesis in these patients.

Most of EV patient (approximately 75%) present homozygous truncating mutations in EVER1 or EVER2 genes; these two genes encode transmembrane proteins expressed in a variety of cells types, including hemopoietich cells. These protein form a complex localizing to the endoplasmic reticulum, where they seem to control intracellular zinc homeostasis and in particular to decrease the nuclear zinc concentration, acting as negative regulator of the activity of zinc-regulated transcription factors necessary for viral gene expression (e.g. AP1); thus, the EVER proteins may act as restriction factors for β-HPV gene expression and replication in keratinocytes, even though the exact underlying mechanisms are still unclear. EVER mutations disrupt zinc transport between cell compartments; it is thought that this leads to up-regulation of transcription factors that can induce β-HPV transcription and stimulate keratinocyte proliferation, which in turn further amplifies β-HPV oncoprotein expression. Other HPV species may counteract EVER protein function through different mechanisms in order to facilitate viral gene expression; HPV16 E5 has been reported to be able to bind and inhibit the EVER complex.

However, in a substantial proportion of patients clinically diagnosed with EV, EVER genes are not mutated; suggesting that unknown mutations affecting other genes are involved in the genetic background of these patients.

More recently, mutations in other two genes, the ras homolog gene family member H (RHOH) encoding a Rho-GTPase expressed predominantly in hematopoietic cells involved in intracellular transduction form T-cell and B-cell receptors, and MST1 encoding a kinase involved in many biological processes in various cell types, including apoptosis, negative regulation of cell growth and proliferation, and differentiation. These to mutation have been identified in PID patients associated with high susceptibility to β-HPV infections exhibiting an EV-like phenotype. Both RHOH and MST1 deficiencies lead to T-cells defects, which probably play a role in the pathogenesis of chronic active β-HPV infections (Akgül, Cooke, and Storey 2006; S Tuttleton Arron et al. 2011; Crequer et al. 2012; Crequer, Troeger, and Patin 2012; Cubie 2013; Leiding and Holland 2012; Pfister et al. 2003).

Epidemiological approaches to investigate β-HPV infection and association with NMSC

The DNA by PCR-based methods and genotyping in skin biopsies, skin swabs or plucked hairs, serological analyses for the detection of anti-β-HPV antibodies are the principal approaches that have been performed for the epidemiological investigation of β-HPV infection and the analysis of a possible involvement of β-HPVs in the pathogenesis of NMSC in non-EV patients.

Skin biopsy is considered the gold standard in sampling for β-HPV testing. Skin biopsies are the most clinically adequate samples for this intention and can yield information about β-HPV in epidermal layers deep to the horny layer, which may reveal virus presence in the stem cells of the hair follicle or the eccrine ducts. Nonetheless, it is not known whether β-HPV DNA detection represents viral particles or superficial or deep infection. Moreover biopsy are minimal invasive but requires performer skill and moderate cost, making it an impractical approach for large cohort studies.

Skin swabs are a more convenient sampling method, allowing for quick, noninvasive, painless sampling that can be repeated multiple times with little risk. The disadvantage is that it is difficult to determine whether a positive sample represents contamination, carriage, or transient or persistent infection. Presumably, the presence of the same β-HPV types in multiple samples over a period of time would be more likely to be because of persistent infection.

Plucked hairs represent another tissue specimen that may be tested for β-HPV, being hair follicles a reservoir site for β-HPV infection. This sampling method is noninvasive, comport little risk and can

be performed multiple times like in the case of skin swabbing; in addition, it should have a lower risk of surface contamination than skin swabs.

Finally, blood samples can be used to identify serum antibodies to specific β-HPV types. Drawing blood is simple and quick and can be repeated multiple times; antibody responses are often very durable and can serve both as markers of present infection and as a fossil record of infection, even if tissue specimens do not ultimately contain viral DNA sequences. Disadvantages of the seroepidemiological analysis are: not all hosts mount an antibody response to HPV, seroconversion may only appear months afterwards the infection, cross-reactive antibodies between different β-HPV types may complicate the picture and individual studies of known viral serotypes are required; in addition, information gathered is obviously limited to serology.

The major limitation of all these approaches is that they just aim at the detection of viral DNA or host antibody responses without providing any direct evidence for active β-HPV infection. Due to the widespread asymptomatic infection in the general population, more biologically relevant techniques allowing to detect and monitor viral activity in terms of replication and gene expression rather than infection per se – that might be only due to transient or latent carriage or surface contamination - should be used to get deeper insight into any possible implication of β-HPVs in the pathogenesis of NMSC; indeed, the difference between cases and healthy individuals may not lie either in the presence or absence of infection or in the amount of viral DNA, but rather in the extent of viral replication and gene expression. In addition, all the epidemiological studies performed to date have used multiple sampling and detection methods, small sample sizes (with some exceptions) and detection restricted to specific β-HPV types (Aldabagh et al. 2013; Feltkamp et al. 2008; Pfister 2003; Schiller and Buck 2011).

Epidemiological evidence in the general population

In the first place, the frequency of detection of HPV strictly depends on the HPV detection system and the geographic location where the study has been performed. In the general population β-HPV seroprevalence has been estimated to be around 30-80% according to different seroepidemiological studies; viral DNA prevalence detected in PCR-based analyses is higher, over 80-85% in most studies with differences depending on the type of samples investigated.

Numerous studies have examined the presence of β-HPV DNA or anti-β-HPV antibodies in sporadic NMSC, premalignant and benign lesions, as well as normal, uninvolved skin in immunocompetent individuals. Beside the wide number of studies, the role of β-HPV in the development of NMSC is, as yet, unclear.

Indication in favor of an association between β-HPV infection and sporadic NMSC:

- Greater β-HPV DNA carriage, even with multiple infecting types, in cuSCC and AK (to a lesser extent also BCC and benign lesions) comparing with healthy skin, furthermore 50% of cuSCC are β-HPV DNA-positive
- According to different studies, β-HPV DNA is more associated with sun exposure in both lesions and healthy skin
- Positive correlation between history of NMSC and higher rate of β-HPV DNA detection in normal skin has been demonstrated
- Some studies have shown β-HPV DNA to be more common and with higher load in precursor AK lesions than cuSCC, BCC, benign lesions and normal skin
- Most seroepidemiological studies have found a positive relationship between antibody response to β-HPV (whether against a single or specific type or multiple types) and cuSCC (but not BCC) development; antibodies have been detected close to or at the time of cuSCC occurrence; antibodies against certain types correlated with β-HPV DNA presence in hair bulbs; individuals with cuSCC on chronically sun-exposed skin sites have turned out to be more likely to be positive for anti-β-HPV antibodies than those with cuSCC on sun-protected sites.

Indication against an association between β-HPV infection and sporadic NMSC:

- In contrast with what mentioned before, some studies have not found any differences in β-HPV DNA detection rates among NMSC, precancerous lesions, benign lesions and healthy skin
- There is ample inconsistency among the β-HPV species and types found to be more associated with skin lesions, leading to an impossibility to correlate specific β-HPVs with NMSC or precancerous lesions
- While high-risk α-HPV-associated cervical tumors harbor at least one genome copy per cell, viral load in β-HPV DNA-positive NMSC has been estimated to be around one genome copy per 20-5000 cells, indicating that not every tumor cell contains β-HPV genome, in according with what obtained from quantitative β-HPV DNA analyses in normal skin
- Newer studies examining viral mRNA levels, have not supported a transcriptional mechanism to link HPV with cuSCC.
- Not all seroepidemiological studies have shown a positive correlation between the presence of anti-β-HPV antibodies and NMSC (similar seroprevalences in cases vs controls), and in addition the presence of HPV DNA in tissue and antibodies to the same HPV type in the serum were not significantly correlated (Akgül, Cooke, and Storey 2006; Aldabagh et al. 2013; Meyer and Christophers 2000; Pfister 2003).

Epidemiological evidence in immunocompromised individuals

In the case of primary immunodeficiency, the association of cutaneous HPV infection with the development of skin lesions is well established. A certain oncogenic role of β-HPVs has been clarified only in the EV setting, and patients with RHOH and MST1 deficiency, who exhibit an EV-like phenotype, have recently been found to display high susceptibility to β-HPV infection.

Other PID syndromes (e.g. SCID, WHIM), whose genetic backgrounds have been described and affect various aspects of immune function, are characterized by recurrent severe bacterial and viral infections since infancy and early childhood. As a result, these patients have a number of subsequent clinical manifestations, among which the predominant features are HPV-associated warts and also carcinomas. The detection of β-HPV infections in EV-like skin lesions from these individuals and their possible implication in the onset and development of these lesions, leaving open the possibility that these viruses play a role in cutaneous carcinogenesis affecting many of these subjects.

Patients with acquired T-cell immunodeficencies, whether secondary (e.g. hematological malignancies, AIDS) or iatrogenic (e.g. OTRs undergoing long-term immunosuppressive therapy to prevent graft rejection) are all at increased risk of developing extensive, persistent and recurrent HPV-associated warty and keratotic skin lesions predominantly on sun-exposed sites; this is likely due to their impaired cell-based immunity to viral infections, including HPV. The persistence of HPV-associated wart-like lesions is a major issue for effective clinical management of these subjects and can affect their quality of life significantly.

The consequence of acquired immunosuppression and the inability to clear viral infections can be far reaching because solid organ transplantation and HIV positivity increase the risk of secondary malignancies with a well documented viral etiology (e.g. Kaposi's sarcoma associated with Human Herpesvirus 8 infection or post-transplant lymphoproliferative disorders associated with Epstein-Barr Virus infection), that can arise following de novo viral infections or reactivation of latent viruses; Although this increase is presumed to be from the host's inadequate control of what might otherwise be a harmless infection. (Aldabagh et al. 2013; Leiding and Holland 2012; Cubie 2013).

Organ transplant recipients

Organ Transplantation is consider a modern medical miracle, and today there are over seventy thousand transplant a year worldwide and currently the 10 years survival it's over the 80%. This increased survival rate has lead to the realization that the lifelong immunosuppressive treatment and the resulting modification of the immune system is associated with an increased risk of developing various cancers, with NMSC being the most common post-transplant malignancy. Over 30 different cancer types increase in incidence following post-transplant immunosuppressive therapy, with NMSC being the most frequent. In addition, the natural history of cancer in OTRs tends to be more aggressive than in the general population and these second tumors are the major cause of death in such patients. Although suppression of T-cell immunity and immunosuppressant drugs per se can exert carcinogenic effects, the most obvious mechanism of post-transplant tumorigenesis is that iatrogenic down-regulation of cell-mediated immunity unmasks the transforming activity of infectious agents resulting in the development of virus-related lesions

Resembling an EV-like phenotype, OTRs develop long-lasting multiple wart-like and precancerous skin lesions such as AK with peculiar dysplastic features that are evident even in more benign lesions, and potential to evolve to NMSC; the prevalence of these lesions increases steadily each year after transplantation, and they can serve as early warning signs of cuSCC as they appear sooner after transplant. While the general population more commonly develops BCC as compared to the more invasive cuSCC (1:3) this ratio is inverted to a 4:1 ratio in favor of cuSCC in OTRs; cuSCC represents the most common de novo malignancy in the OTR setting, who exhibit a risk 65-250-fold as great as that of immunocompetent subjects with a cuSCC frequency over 50% at 10 years and over 80% at 20 years post-transplantation – while BCC is increased only 10-fold.

Furthermore, cuSCC arising in OTRs can be higher aggressive compare with one developed by an immunocompetent individual. cuSCC increased rates of recurrence (13%), and the risk of lymph node metastasis developed earlier (mean 1.4 years). Almost 80% develop a second cuSCC within 3 years, mostly developed in sun-exposed sites.

The high burden of post-transplant cuSCC has a considerable impact on the quality of life, treatment costs and survival of OTRs overall.

These lesion co-localizing with other wart-like and precancerous lesions, suggesting that their persistence favors malignant progression, and affect large skin areas in a process called field cancerization. Recent studies suggest a role for β-HPVs in the development of early premalignant lesions and NMSC due to an increased viral replication and gene expression in the context of a reactivation of previously unapparent β-HPV infections kept under control by cell-mediated immunity rather than increased de novo β-HPV infections.

Has been shown that OTRs have a β-HPV carriage even higher than the immunocompetent population, with frequent mixed co-infections and greater persistence especially in sun-exposed areas; as with the immunocompetent, certain types have been found to be the most prevalent on OTR skin (HPV 5, 20, 23, 24). Despite that, other studies have not found consistent differences in β-HPV DNA prevalence between OTRs and healthy subjects in both normal and lesional skin, and seroprevalence in this population is similar to that in immunocompetent individuals.
Has shown for the general population, the evidence implicating β-HPVs in the pathogenesis of post-transplant NMSC is mixed as well; several studies have linked β-HPV infection with cuSCC, but many of these have been small and others uncontrolled.

Evidences in favor of an association between β-HPV infection and post-transplant NMSC:

- Has been found a positive correlation between the detection of β-HPV DNA and anti-β-HPV antibodies (with type concordance) and the higher risk of developing cSCC
- According to different studies, almost 90% of cuSCC and premalignant lesions are β-HPV DNA-positive.
- higher prevalence of β-HPV DNA in sun-exposed areas has been reported to be associated with history of NSMC
- β-HPV DNA has been detected more frequently in post-transplant cuSCC, and also BCC and premalignant lesions, and with more frequent mixed co-infections, compared to the immunocompetent counterpart
- HPV 5 and 8 have turned out to be the most frequent types in post-transplant and occur more often in post-transplant cuSCC than in sporadic cuSCC
- β-HPV DNA load has been shown to be higher in post-transplant cuSCC (but not BCC) than in sporadic cuSCC, with a positive correlation between viral load and time since transplantation
- Few studies based on in situ hybridization have detected β-HPV DNA and RNA in cuSCC, suggesting active infection in the lesional tissue.

Evidences against an association between β-HPV infection and post-transplant NMSC:

- Two studies indicated a similar HPV DNA load in OTR cuSCC, BCC, pre-malignant, and benign lesions.
- Low viral load and transcriptional activity have been found and in some other studies mRNA transcriptome high-throughput deep sequencing has failed to identify β-HPV expression in cuSCC (as in the immunocompetent counterpart)

- Some studies examining normal skin in individuals with and without history of NMSC have not found any significant difference in β-HPV DNA positivity between the two groups
- For the most part seroprevalence in OTRs is similar to the immunocompetent, with the only difference being that reactivity to HPV 8 is increased in the immunosuppressed. (Akgül, Cooke, and Storey 2006; Aldabagh et al. 2013; Connolly et al. 2014; Dubina and Goldenberg 2009; Feltkamp et al. 2008; Harwood and Proby 2002; Hofbauer, Bouwes Bavinck, and Euvrard 2010; I Nindl and Rösl 2008; Pfister 2003; Oberyszyn 2008).

Molecular mechanisms: the β-HPV oncoproteins

Comparing High-risk α-HPV and β-HPV, the molecular mechanisms through which β-HPVs might participate in skin carcinogenic seems to differ from those underlying high-risk α-HPV-induced cervical tumorigenesis; it appears that the functioning of β-HPV oncoproteins - E6 and E7, as E5 is not encoded by most β-HPV types – is more similar to that of low-risk α-HPV oncoproteins, with a great variability among different β-HPV species and types in the specific activities and efficiencies in interference with the functions of their cellular targets (E-m de Villiers 1998).

The β-HPV exhibit a weaker transforming potential, in contrast the high-risk α-HPV, of which oncoproteins expression overtly exert transforming and oncogenic activities in cellular and mouse models. E6 and E7 of most β-HPV types prolong of the lifespan of fibroblasts and primary human keratinocytes, either in monolayer or in organotypic raft cultures mimicking the structure of the epidermis, without efficient immortalization, inducing a moderate increase in proliferation and anchorage-independent growth and perturbing the normal cellular differentiation pattern; these effects are seen mainly when E6 and E7 are co-expressed and to different extents in different β-HPV species and types. The β-HPV types displaying the highest transforming capabilities on the basis of in vitro and in vivo evidences are HPV 8 and 38. Experiments involving the expression of the HPV8 oncoproteins in primary human keratinocytes shown that the activity of these proteins is not sufficient for immortalization; however, E6 seems to be the dominant oncogene and is able to extend cell lifespan, induce anchorage-independent growth and alter cellular morphology, while E7 causes cell hyperproliferation and abnormal keratinization.

With the purpose to study the capability of HPV gene to perturb the keratinocyte differentiation and cell cycle, different transgenic mice models have been developed. Transgenic mice expressing the complete early region of HPV8 under the control of a keratinocyte-specific promoter spontaneously develop benign/premalignant skin lesions and some of them develop cuSCC; skin lesions arise only

arise in mice transgenic for E2, E6 and E6/E7 but not E7, suggesting that E7 alone is not sufficient to induce tumor growth having less oncogenic properties.

Curiously E2 is so far not regarded as an oncogene of HPV. On the contrary, overexpression of genus-a HPV16- and HPV18-E2 results in cell-cycle arrest and apoptosis depending on the cell type. HPV11-E2- transgenic mice showed no histological changes in the skin. In contrast, HPV8-E2 seems to act as an oncogene as E2-transgenic mice also develop skin lesions, even though to a lower extent, more slowly and with less severe malignant features; in vitro evidences have shown that HPV8 E2 can abrogate anoikis and promote anchorage-independent growth. The apparent tumorigenic properties of HPV8 E2 could be due to the ability of E2 to interact with cellular proteins and modulate the activity of host genes, with potential to affect the expression of critical cellular factors and thus favor transforming processes; Has seen also that in E2-transgenic mice, after a single solar-simulated UVA and UVB exposure, tumor growth thus started much faster and more frequently than in non-irradiated transgenics. This might have led to an upregulation of E2 expression levels due to an activation of the keratin promoter, resulting in faster accumulation of E2 effects. E2 might also contribute to tumor progression by interfering with the UV-damage response pathway and impeding cell-cycle arrest and apoptosis. This hypothesis is supported by the fact that E2 of HPV8 and/or other papillomaviruses interacts with several cellular proteins involved in DNA-damage response.

Co-expression of E6 and E7 from HPV 38 and 49 can immortalize primary human keratinocytes. HPV 38 E6/E7 transgenic mice spontaneously develop epidermal dysplasia and, after treatment with chemical carcinogens or chronic UV irradiation, precancerous lesions and malignant tumors (Akgül, Cooke, and Storey 2006; Akgül et al. 2007; Feltkamp et al. 2008; Pfister 2003; I Nindl and Rösl 2008; Schaper et al. 2005; Pfefferle et al. 2008; McLaughlin-Drubin, Meyers, and Munger 2012; Tommasino 2014; Roman and Munger 2013).

Has been recently investigated that E7 seems able to interact with Rb proteins in a large variation degree among different β-HPV species and types. However, the binding efficiency is lower than that of high-risk α-HPV E7 and only few β-HPVs, e.g. HPV38, are able to target Rb proteins for proteolytic degradation; in some β-HPV types it does, or at least destabilizes Rb proteins. In any case, E7 often fails to activate E2F-responsive genes, or it does very modestly.

In human keratinocyte, HPV38 E7 also is able to disrupt the cell cycle regulation through the altered expression of p53-regulated genes. In particular, it promotes via an unknown mechanism nuclear translocation of the IκB kinase beta (IKKβ) kinase complex, which in turn associates with and phosphorylates ΔNp73α leading to its stabilization. ΔNp73α and IKKβ are part of an inhibitory

complex, together with two epigenetic enzymes, namely DNA methyltransferase 1 (DNMT1) and enhancer of zeste homolog 2 (EZH2), which are recruited to a subset of p53-regulated promoters and suppress their activation. The activity of HPV38 E7 therefore enhances the down-regulation of p53-dependent promoters, indirectly abrogating the p53 response upon UV damage and favoring the replication of cells harboring UV-induced genetic errors. ΔNp73α seems also to positively regulate hTERT promoter activity; this might account for an indirect E7-mediated telomerase up-regulation underlying the immortalization of keratinocytes by HPV38 E6/E7.

In both monolayer and organotypic cultures, HPV8 and HPV38 E7 have been shown to up-regulate lipocalin-2, which bind and transport small essential factor or provide growth factor effects, thus modulating cellular response such as differentiation and proliferation. Deregulated expression of lipocalin-2 has been reported in several malignant conditions. HPV8 E7 can also enhance the expression of certain matrix-metalloproteases that can degrade components of basement membranes and extracellular matrix, possibly favoring tumor invasiveness (Akgül, Cooke, and Storey 2006; Harwood and Proby 2002; Tommasino 2014; Bauer et al. 2011; Roman and Munger 2013).

Recent studies have been shown that only HPV49 E6 has the same ability as high-risk α-HPV E6 to target p53 for ubiquitin-dependent proteasomal degradation via E6AP, otherwise β-HPV E6 seem to bypass G1-S checkpoint through different strategies.

The β-HPV E6 proteins have evolved various mechanisms to subvert DNA damage-induced apoptosis and also to interact with different pro-apoptotic factors as the down regulation of kinase family member with a key role in the repair UVB DNA damage. Indeed, E6 can interfere with other components of p53-dependent pathways or selectively inhibit p53-induced pro-apoptotic mediators, and repress the expression of pro-apoptotic factors involved in other pathways (e.g. Fas); like E6 from certain α-HPVs, β-HPV E6 can abrogate the anti-apoptotic function of Bak through its proteolytic degradation and resulting in an inhibition of UV-induced apoptosis.

Interestingly, E6 from some β-HPV types can also interfere with DNA repair factors; for instance, E6 from HPV 8 and 1 abrogates the function of XRCC1 that is involved in repair of single-strand DNA breaks.

HPV5 and HPV8 E6 have been shown to promote destabilization and proteolytic degradation of the histone-acetyltransferase p300, a critical co-activator for many transcriptional programs including p53; a role for this is implicated inhibition of differentiation and abrogation of the ATR/p53 response to UV-induced DNA damage through a strong decrease in both mRNA and protein level of ATR, leading to the accumulation of UV-induced genetic alterations such as thymine dimers and

DNA double-strand breaks. In the case of HPV38 E6, the interaction with p300 result in the prevention of p53 acetylation and inhibition of the p53-dependent transcriptional response similarly to α-HPV E6. Furthermore this interaction appears to be essential for HPV38 E6/E7-mediated keratinocyte immortalization.

A stable interaction of E6 protein and E6AP has been observed in certain β-HPV types (HPV 5, 8, 38), and seem to be able to activate telomerase, but the underlying mechanisms may be different from those of high-risk α-HPV E6-mediated telomerase up-regulation.

A recently discovered interaction between β-HPVs E6 protein and co-activators of Notch signaling has been shown to disrupt epithelial differentiation; notch signaling drives terminal differentiation of keratinocytes and acts as tumors suppressor in epithelial cancers. Notch signaling between adjacent cells affects the developmental fates of those cells, linking the differentiation fate of a given cell to that of its adjacent neighbor. Notch1 and Notch2 genes are expressed in the first spinous layer and the Notch ligand, Jagged2, is expressed in the basal layer; Notch signaling occurs both at the basal-spinous cell junction and in the upper spinous layer where it drives terminal differentiation of the granular layer. Upon canonical Notch signaling, the Notch receptor is cleaved by the intramembranous γ-secretase protease, liberating the Notch intracellular domain that forms a complex with the RBP-J DNA-binding protein and p300; this complex then translocates to the nucleus, and this displaces a repressor histone-deacetylase complex and recruits the MAML1 co-activator, thus converting the RBP-J complex from a transcriptional repressor to an activator.

β-HPV E6 interact with MAML1, resulting in the repression of Notch-dependent transcriptional activation and the disruption of epithelial differentiation, which may favor β-HPV replication and β-HPV-driven transformation of host cells.

There is also evidence that both HPV38 E6 and E7 can activate NF-κB, leading to the up-regulation of the inhibitor of apoptosis cIAP2 – as in the case of high-risk α-HPV E6 - which would be expected to confer some resistance to certain DNA damaging agents such as UV radiation and therefore increase survival of DNA damaged-cells. The consequences of NF-κB activation by E6 are apparently complex and may depend on cell type; e. g. a recent study of high-risk α-HPV E6 indicated that NF-κB activation by E6 in ectocervical cells increases proliferation, whereas it may be inhibitory to growth of cells that are derived from the transformative zone, where most cervical cancers are developed.

HPV8 has been shown to up-regulate certain oncogenic miRNAs and down-regulate tumor suppressive miRNAs in mice transgenic for the complete early region, suggesting that it may interfere with miRNA-mediated gene regulation to induce tumorigenesis (Akgül, Cooke, and Storey 2006; Harwood and Proby 2002; McLaughlin-Drubin, Meyers, and Munger 2012; Wallace et al.

2012; Tommasino 2014; Vande Pol and Klingelhutz 2013; Hufbauer et al. 2011; Vandermark et al. 2012).

The "hit-and-run" model of β-HPV-associated skin carcinogenesis

Based on the epidemiological and molecular data obtained so far, different paper support the so-called "hit-and-run" mechanism of β-HPV-induced skin carcinogenesis. With this model is suggested a role of the viruses in early stages of the pathogenesis of NMSC - particularly cuSCC, which appear to be the NMSC type with stronger association to β-HPV infection - as cofactor and possibly transient carcinogens, being involved in tumor initiation in the presence of additional risk factors rather than in malignant progression or in the maintenance of the transformed phenotype as on the contrary occurs in high-risk α-HPV-driven cervical tumorigenesis. This hypothesis is supported by a large number of evidence. In the first place in non-Ev tumor tissues the viral load detected is low with a minimal gene expression, even in EV cuSCC the higher amount of viral DNA and gene expression is confined to few tumor cells.

β-HPV oncoproteins present a lower transforming potential of compared to high-risk α-HPV counterpart. Another evidence is the impossibility to derive β-HPV DNA-positive cell lines from EV cuSCC because of the early loss of viral DNA (also in organotypic raft cultures), unlike the case of high-risk α-HPV DNA-positive cervical cancer-derived cell lines. Indeed the lack of evidence for β-HPV DNA integration in tumor cells, which on the contrary is a pivotal event in high-risk α-HPV-induced cervical cancer.

In this context, β-HPV oncogene expression could be a condition facilitating a transforming process previously triggered and subsequently carried on by other tumorigenic factors such as UV exposure. In individuals with a predisposing background characterized by the pre-existence of UV-dependent genetic and epigenetic alterations in sun-exposed skin (e.g. loss-of-heterozygosity in tumor suppressor genes), β-HPVs persistently present in epidermal basal stem cells are likely to stimulate cell proliferation and inhibit DNA damage-induced apoptosis, therefore enhancing the propensity to accumulate further UV-induced genetic alterations – such as missense mutations of the of the gatekeeper suppressor gene p53 and activating mutations of the H-Ras proto-oncogene, both of which predispone to cuSCC; the subsequent malignant progression could thus only be due to additional DNA damage events independent on viral oncoprotein activity. In this scheme, could be no longer required the β-HPV persistence when cells are already initiated toward malignancy, and in more advanced stages of the carcinogenic process the viruses might finally be lost.

(Akgül, Cooke, and Storey 2006; Connolly et al. 2014; Harwood and Proby 2002; Pfister 2003).

The link between UV radiation and NMSC was clearly highlighted in several studies. UV radiation plays and important aetiological role in skin carcinogenesis, underlying the pathogenesis of both NMSC and melanoma. UV exposure induces DNA damage by causing mutation in key genes. Specifically UV-B irradiation is able to induce the formation of pyrimidine dimers and other types of photoproducts, which through mainly p53-mediated responses normally either provoke cell cycle arrest and efficient DNA repair or induce an apoptotic program resulting in the eradication of deleterious cells. If DNA damage exceeds the capacity of host intrinsic repair, the accumulation of gene mutation in key cellular pathways is the force driving tumourigenesis in UV-induced skin cancer.

Although the most important environmental risk factor for cuSCC is cumulative sun exposure, according to the hit-and-run model it appears that β-HPVs may act as co-carcinogenic factors. The synergy between UV light and active β-HPV infection is clear in the case of EV patients, who develop β-HPV-associated tumors in sun-exposed skin sites. The relative contribution of β-HPV might be greater in geographical areas characterized by lower sun-exposure.

A weigh of data suggesting that β-HPVs, especially by means of the E6 oncoprotein, can inhibit both apoptotic pathways triggered by UV-induced DNA damage and repair of DNA damage itself, favoring a condition of genetic instability that may lead to the accumulation of mutations in proto-oncogenes and tumor suppressor genes and subsequently to host cell transformation. UVB mediated apoptosis preferentially deleted DNA-damaged unmutated cells, allowing the β-HPV-infected keratinocytes to selectively proliferate in to the vacant niche. This might enable the amplification of apoptosis-refractory cells, which then could acquire further UV-induced somatic mutations (e.g. p53 and H-Ras mutations) that cannot eliminate because of the interference of E6 with the DNA repair machinery. The expansion of this cell population can result in the tumor progression, in which the viruses are likely not to be involved any longer (Figure 5).

UV light has been observed to promote viral gene expression and life cycle progression, in particular in the case of HPV8 and 5. As results we have a high-level UV-responsive oncogene expression, which in turn is likely to amplify the deleterious effects due to UV radiation itself.

Moreover, repetitive painful sunburns followed by epidermal regeneration of the skin from the underlying hair bulb could result in β-HPV amplification in individuals carrying latent infections, which then might drive the onset of transforming processes through high-level oncogene expression following viral life cycle reactivation.

UV radiation not only have impact on the cell cycle directly; UV light is known to promote local immunosuppression disabling the tumor surveillance mechanisms that would otherwise recognize highly antigenic cutaneous lesions; leading the suppression of the local cell-mediated immunity and

interfering with the antigen presentation. One reasonable hypothesis is that UV-induced local immunosuppression promotes permissiveness to β-HPV infection; this would be consistent with the higher infection rates and viral loads in sun-exposed skin sites found by several epidemiological studies and could further account for a synergistic interplay between sun-exposure and β-HPV activity (Akgül, Cooke, and Storey 2006; Aldabagh et al. 2013; Bouwes Bavinck, Plasmeijer, and Feltkamp 2008; Feltkamp et al. 2008; Pfister 2003).

It is important consider that all these proposed mechanisms may not have a relevant role in the above epidermal layers where cells have a short lifespan, but such effects could be significant in the follicular basal stem cells. Indeed, the persistent localization of β-HPVs in these cells – which may be the source of skin cancers – is likely to allow the acquisition of a condition of genetic instability that might convert them into cancers stem cells (Akgül, Cooke, and Storey 2006).

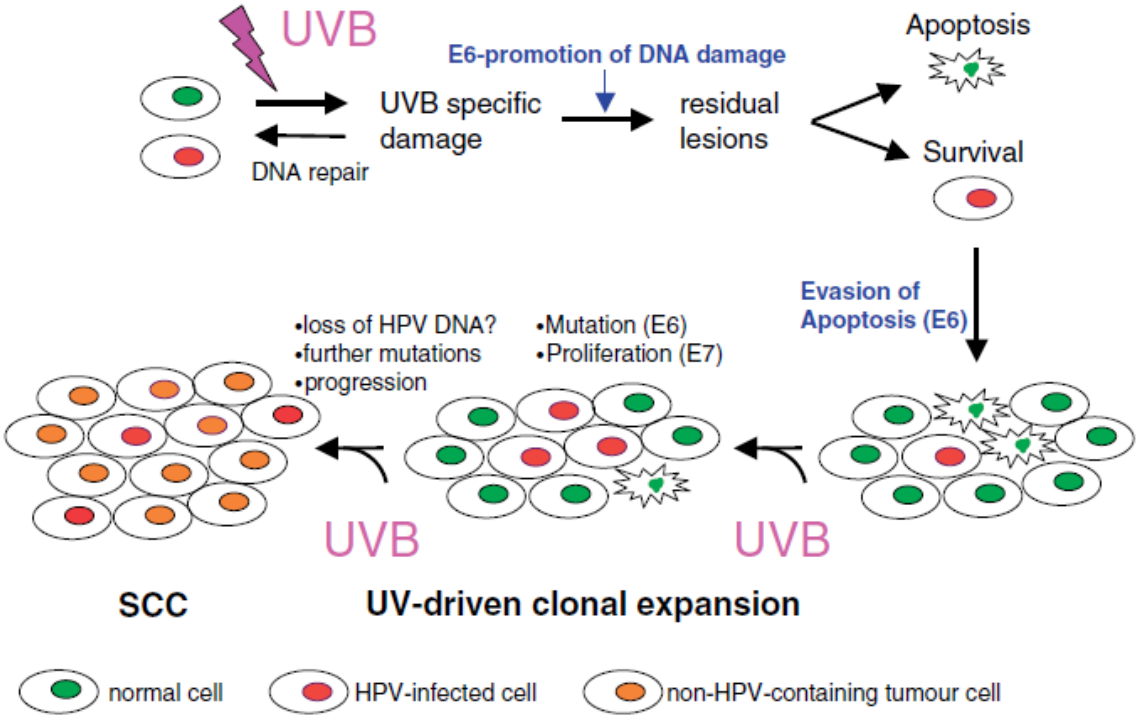

Figure 5. Model of the roles of UV radiation and β-HPV infection in skin carcinogenesis. β-HPV infection may act as an initiator mechanism for cuSCC development, in combination with UV light, on sun-exposed body sites. UV exposure leads to DNA damage in skin cells. In β-HPV-infected cells, E6 is likely to promote retention of DNA damage and protect cells from UV-induced apoptosis. Normal cells that are fully competent for apoptosis might be preferentially deleted following UV damage, allowing clonal expansion of β-HPV-containing cells. Accumulation of genetic damage would then allow cells to progress to cuSCC independently of the proliferative and mutagenic effects of β-HPV infection (Akgül, Cooke, and Storey 2006).

Future implications

Therefore, future epidemiological studies are needed to resolve the conflicting evidence on the association between β-HPV infection and cuSCC development; and also further investigation on the relation between keratinocyte stem cells and β-HPV are needed to understand if these viruses play an active role the skin carcinogenesis and which is the underlying molecular mechanisms.

On the basis of the epidemiological and biological data obtained so far, it appears that the β-HPV types with greater oncogenic properties are HPV 5, 8 and 38; HPV 5 and 8 are the only β-HPV types currently regarded as possible carcinogens driving the development of cuSCC, even though this evidence is linked to the setting of EV only.

Resolving the conflicting evidence on the β-HPV involvement in the pathogenesis of cuSCC, along with the identification of high-risk β-HPV types, will have important implications for screening, prognosis and the development of adequate therapeutic and preventive strategies; e. g. the development of direct antiviral agents or small molecule inhibitors of viral proteins to prevent interaction with and disruption of cell cycle regulatory proteins. Developing new immunosuppressive strategies to address simultaneously the risk of allograft rejection and prevention of cancer in the OTR.

Furthermore, early vaccination against certain types may decrease future disease burden, especially in at-risk patients like OTRs where no specific treatments for HPV-associated wart-like lesions are currently available; some studies for the development of vaccines against the putative high-risk β-HPV types such as HPV 5 and 8 are already ongoing in Australia.

In case that β-HPVs are necessary for skin carcinogenic processes or act just as weak cofactors, the clarification of any viral mechanisms in cuSCC development would lead to enhanced treatment options based on new molecular targets for intervention and a reduction of the significant healthcare costs associated with these cancer types (S Tuttleton Arron et al. 2011; Aldabagh et al. 2013) .

HPV life cycle biomarkers for the visualization of active infection patterns

The highly regulated pattern of gene expression that characterizes HPV life cycle provides a basis for the selection of biomarkers that may be a useful tool to visualize the presence of active infections in lesional tissues through immunostaining procedures, with possible diagnostic applications. This has been deeply investigated for α-HPV types associated with lesions; in particular, the analysis of the expression patterns of certain viral gene products may allow the severity of the underlying disease to be predicted, and the detection of those products might improve cervical screening.

The key life-cycle markers used for the visualization of the HPV infection are E4, E6/E7 and the coat proteins L1 and L2. These proteins are usually co-visualized with the products of E2F-activated genes such as MCM, Ki-67, and PCNA; also used as surrogate markers of E7 expression. All these gene products are normally restricted to the basal layer in uninfected epithelium and are markers of cell cycle entry rather than cell division. Usually associated with E4 expression, DNA FISH analysis is another methodic used to detect the viral genome amplification.

Although E1, E2 and E5 expression is thought to accompany the expression of E4, these proteins have proved difficult to detect in HPV-induced lesions. E1 is thought to be a very minor gene product, as codon usage within the E1 ORF is sub-optimal and E1 transcripts are difficult to detect. E2 by contrast, is thought to be expressed more abundantly. The E2 expression has been clearly identified in bovine warts caused by BPV, but its identification in human lesion is currently less conclusive. The detection of E5 by immunostaining is compromised by the fact that the protein is predominantly membrane bound, therefore limiting epitope availability; as with E2, there are only a limited number of studies that have investigated E5 distribution in vivo, and it is difficult to be certain as to its precise expression pattern (Middleton et al. 2003; Doorbar 2007; Doorbar et al. 2012).

The detection of the High-risk α-HPV life cycle markers in cervical cancer is related to the different grades of the disease (summarized in Figure 6). During productive infection as it occurs in CIN1 lesions, cell cycle markers such as MCM are expressed preferentially in the basal and parabasal layers, with their presence being a direct consequence of E7 activity; these E7 surrogate markers localize to the nucleus, while for E7 both cytoplasmic and nuclear localization have been reported. In the mid layers, proteins necessary for genome amplification become elevated in these cells, allowing genome amplification to occur, also supporting the expression of the viral E4 protein.

E4 appears in the cytoplasm as MCM signal begins to decline in the mid-upper epithelial layers of the lesion and is generally a marker of low-grade disease; E4-positive cells are typically in S-phase or G2-phase and usually display detectable viral DNA. Cells supporting genome amplification present a stain positive with only a narrow overlapping for E4 and E7 / E7 surrogate markers, probably due to the down-regulation of E7 expression by increased E2 levels thought to accompany E4. The final stage in the virus life cycle involves the packaging of viral particles starts only in cells that have already undergone genome amplification follows genome amplification, and is marked eventually by the appearance of the capsid proteins (L1 and L2) in the nuclei of a subset of E4-positive cells in the upper epithelial layers.

Low-risk α-HPV life cycle markers in associated lesions show expression patterns similar to those found in early productive high-risk α-HPV infections; MCM is apparent only in the upper epithelial layers where E4 also becomes abundant.

E4 expression is gradually lost during cervical neoplastic progression, remaining restricted to isolated pockets close to the upper epithelial layers; the extent of E4 expression is progressively decreased and correlates inversely with disease severity, suggesting that the assessment of its distribution in the context of the lesions could be exploited as a useful tool for disease staging. Consistently with the reduction in E4 expression, viral DNA is usually not detected in HSIL, and cervical cancer as viral replication is increasingly driven down.

E7 expression becomes deregulated during disease progression and unlike E4 is more easily detectable in high-grade neoplasia than in productive lesions. E7 / E7 surrogate markers extend to the epithelial surface (e.g. MCM is elevated to different extents in neoplasia); the thickness of the E7 / E7 surrogate marker-expressing layer increases markedly with increasing disease severity from CIN1 to cervical cancer.

In high-risk α-HPV infections, p16ink4a is unable to exert a regulatory effect as S-phase entry is stimulated by E7 rather than cyclinD/Cdk; the absence of effective inhibition of cell cycle progression by p16ink4a can lead to its accumulation in the cell and to an elevation of E7 surrogate markers throughout the infected epithelial layers. Detectable p16ink4A is also regarded as a surrogate marker of elevated E7 expression, and can be seen in some CIN1 lesions as well as in CIN2 and CIN3; p16ink4a expression is used for routine diagnostic purposes as marker of high-risk α-HPV infection in cervical or oropharyngeal cancers.

As HSIL and cervical cancer are characterized by abortive infections, expression of capsid proteins L1 and L2 occurs rarely because viral particles are not produced and cannot be detected by immunostaining (Doorbar 2007; Doorbar et al. 2012; Griffin et al. 2012).

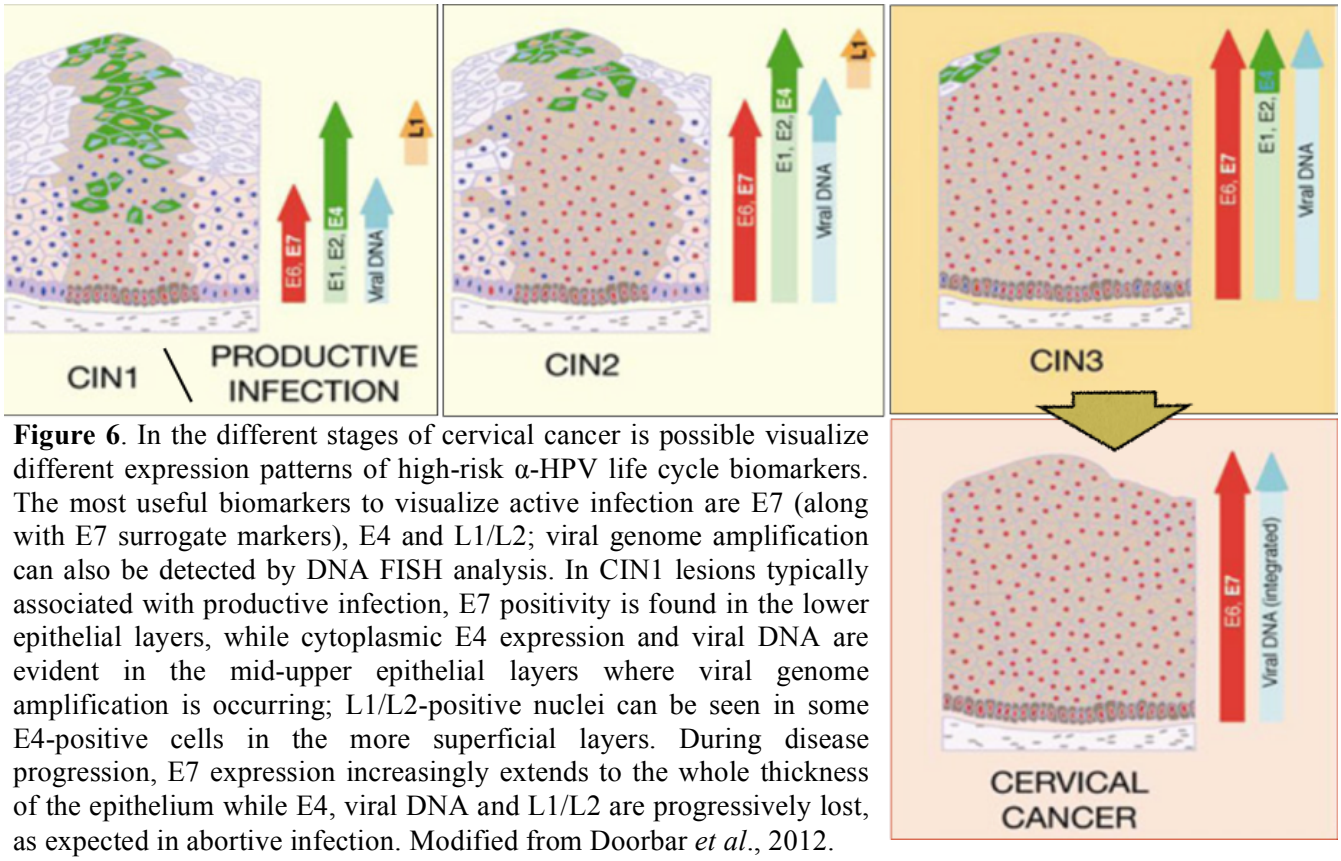

Figure 6. In the different stages of cervical cancer is possible visualize different expression patterns of high-risk α-HPV life cycle biomarkers. The most useful biomarkers to visualize active infection are E7 (along with E7 surrogate markers), E4 and L1/L2; viral genome amplification can also be detected by DNA FISH analysis. In CIN1 lesions typically associated with productive infection, E7 positivity is found in the lower epithelial layers, while cytoplasmic E4 expression and viral DNA are evident in the mid-upper epithelial layers where viral genome amplification is occurring; L1/L2-positive nuclei can be seen in some E4-positive cells in the more superficial layers. During disease progression, E7 expression increasingly extends to the whole thickness of the epithelium while E4, viral DNA and L1/L2 are progressively lost, as expected in abortive infection. Modified from Doorbar *et al*., 2012.

In a recent study has characterized for the first time β-HPV life cycle events in skin tumors from EV patients – the human model of β-HPV-induced skin cancer – showing how changes in E4 expression patterns relate to disease severity.

Abundant cytoplasmic E4 expression and viral DNA have been detected in differentiating cells undergoing viral genome amplification, especially in lower-grade lesions. Interestingly, E4 expression has also been seen in PCNA-positive basal cells in some more dysplastic EV skin tumors; in these lesions, E4 has been found to extend throughout the full thickness of the epithelium and to be apparent in the markedly atypical cells. The loss of such staining at the tumor border suggests a distinct type of E4 deregulation that may be exploited as a marker of viral expression during β-HPV-associated skin cancer progression (Borgogna et al. 2012).

Influence of HPV gene expression on keratinocytes differentiation in *in-vivo* model.

Different transgenic mice models have been developed so far for investigate the role of HPVs in skin cancer. The first transgenic mouse model for skin-associated HPVs was generated in 1992 at the University of Birmingham Medical School, by Searle's group. In this model the HPV-1 early region was placed under the control of epidermis specific promoter from keratin genes. Morphological alterations of the normal epithelial differentiation have been observed, the effect was greatest on the tail, where the epithelium became hyperproliferative in appearance, with several layers of irregular or cuboidal cells above the basal layer, an increase in the total number of cell layers, and abnormal cornification. A similar transient flaky appearance also associated with epidermal hyperplasia was observed on other regions of the skin around 7 days of age. It is worth mention that this transgenic mice didn't able to develop any spontaneous skin lesion.

Pfister and co-worker, to further evaluate the possible contribution of β-HPV in a proliferative tissue compartment during carcinogenesis, established a transgenic mouse model where the complete early region of HPV8 are expressed in the undifferentiated basal layer of epithelia under the control of the Keratin 14 promoter. In this transgenic mice the first phenotypic alterations detectable started around 14 weeks of age showing the presence of tumor like growth on the back with hair loss, hyperkeratosis and ulceration.

Almost all of these HPV8 trangenic mice (91%) developed without any treatment with physical or chemical carcinogen, single or multifocal benign tumors, characterized by papillomatosis, acanthosis, hyperkeratosis, and varying degrees of epidermal dysplasia; furthermore 6% of this transgenic mice developed SCC. The rapidity of skin tumor development in the mice is certainly related to the permanent expression of the HPV8 early genes in all proliferation competent keratinocytes driven by the K14 promoter. With this model could thus be shown for the first time that expression of a β-HPV leads to skin cancer development without exposure to any further physical or chemical carcinogens. Using transgenic technology, several others mouse lines have been developed expressing HPV8 E2 protein or also E6/E7 from other β-genotypes, such as HPV38 and HPV20 but HPV8 has thus far been the only cutaneous HPV type found to induce skin cancer completely on its own in the absence of any additional co-carcinogenic treatment, making it a good candidate to investigate how β-HPV gene expression can influence the keratinocytes differentiation (Tinsley, Fisher, and Searle 1992; Schaper et al. 2005; Akgül, Cooke, and Storey 2006; Viarisio et al. 2011; De Andrea et al. 2010).

MATERIALS & METHODS

β-HPV detection in a cohort of Organ Transplant Recipients

Collection of skin lesion specimens

All formalin-fixed and paraffin-embedded (FFPE) blocks analyzed in this study were retrieved from the medical material archives of the University-Hospital in Novara.

From the cohort of kidney transplant recipients (KTRs) a total amount of 111 FFPE blocks were obtained from 79 skin lesions. As the lesions were of different sizes, the samples were either split into more section or kept intact. A single block was available for 51 lesions, denominated "whole lesion". The remaining 28 lesions were sectioned in two or more blocks, and corresponded to the core and edges of the lesion. All lesions were excised from 17 KTRs (16 male and 1 female), receiving transplants between 1998 and 2009. However, one patient among them also had heart transplant twice.

Data on skin lesion development and characteristics were retrieved from pathology archives and clinical records. The mean length of patient follow-up following kidney transplantation was 15 years and 4 months±7 years and 8 months.

In the present study a single FFPE block from a wart-like lesion of a patient with epidermodysplasia verruciformis (EV) was used as a positive control.

Written informed consent was obtained by all subjects according to the Declaration of Helsinki, and approval was obtained from local ethic committee.

β-HPV DNA detection and genotyping

DNA extraction has been performed from three consecutive 10 μm-thick sections which were retrieved from FFPE blocks, purified DNA was obtained by means of the QIAamp Tissue Kit (Qiagen) according to the manufacturer's instructions. To prevent cross-contamination, the microtome was thoroughly cleaned following the sectioning of each block and a new blade installed for the next one.

β-HPV DNA detection and genotyping was carried out using the PM-PCR reverse hybridization assay (Skin β-HPV prototype research assay; Diassay BV, Rijswijk, The Netherlands), as

previously described (De Koning et al 2006). The method was designed to identify the 25 established β-HPV types (i.e., HPV 5, 8, 9, 12, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25, 36, 37, 38, 47, 49, 75, 76, 80, 92, 93, 96). PM-PCR amplification, generating a biotinylated amplimer of 117 bp from the E1 region, was carried out following all the precautions indicated by the manufacturer to avoid recurrence of cross-contamination.

Briefly, PM-PCR reaction was performed in a final reaction volume of 50µL, containing 10µL of the isolated DNA, 2.5mM $MgCl_2$, 1x GeneAmp PCR buffer II, 0.2mM deoxynucleoside triphosphates, 1.5 U Amplitaq Gold DNA polymerase and 10µL of the PM primer mix. The PCR reaction was performed as follows:

- 9' pre-heating step at 94°C
- 35 amplification cycles:
    - 30" at 94°C,
    - 45" at 52°C
    - 45" at 72°C
- 5' final elongation step at 72°C.

As a positive PCR control, a β-HPV plasmid clone was included. Identification of the amplified β-HPV types was performed by reverse hybridization analysis of the amplimers on genotyping strips provided by the manufacturer. In each strip a probe line contains a mix of "universal" β-HPV probes: in some cases these probes are positive without a specific β-HPV probe being positive, indicating the presence of an unspecified β-HPV genotype.

Antibodies

To generate polyclonal antibodies against the E4 proteins of the β-HPV genotypes, DNA comprising the full-length spliced E1^E4 gene (Doorbar, 2013) was amplified by PCR from an HPV5 genomic clone isolated from an EV patient. For PCR amplification of the E1^E4 coding region, the following primers were used:

- E4HPV5 forward: 5'GGAATTCAGAATGACGGATCCTAATTCTAAAGCTCCACGCCTCCAGGGTCGCCAGGAG-3′
- E4HPV5 reverse: 5′GCTCTAGAGTCGACCTATTACTGGGGGGTCGCGAGCTTCTTCCA-3′

The forward primer included an EcoRI site (underlined) upstream of the ATG initiation codon, while the reverse primers contained a SalI sequence (underlined).

To generate polyclonal antibodies that can broadly identify the L1 proteins of the β-HPV genotypes, sequence alignments were first carried out and the highly conserved region between aminoacid 200 and 300 was chosen. The corresponding coding region within the full length L1 gene was amplified by PCR from the same HPV5 genomic clone used for the generation of anti-HPV5 E1^E4 antobodies. For PCR amplification, primers with the following sequences were used:

- L1 forward: 5′-CGCGGATCCCGTCTGTATCCAAAACCCTTG-3′
- L1 reverse: 5′-CCGCTCGAGTTATCCTATAGTCTTTTGAGCTTG-3′

The forward primer included a BamHI site (underlined) upstream of the ATG initiation codon, while the reverse primers contained a XhoI sequence (underlined).

In both cases, amplimers were cloned into the pGEX-4T-2 vector (GE Healthcare) using standard methods, and then expressed in the *Escherichia coli* host BL21. GST-HPV5 L1 and GST-HPV5 E1^E4 fusion proteins were dialyzed overnight at 4°C in 50mM Tris-HCl pH 8.5, 0.5% β-mercaptoethanol and 3M urea, and then re-dialyzed for 12h at 4°C in 50mM Tris-HCl pH 8.5 and protease inhibitors (Sigma). Anti-sera were raised by injecting rabbits with the dialyzed GST-HPV5 L1 and GST-HPV5 E1^E4 fusion proteins. Rabbits were immunized at multiple subcutaneous sites with 250 μg of protein in Freund's complete adjuvant, followed by booster immunizations at 14-day intervals with the 250 μg antigen in Freund's incomplete adjuvant. Animals were bled 1 week after the fourth immunization and serum immunoglobulins obtained by precipitation with 45% saturated ammonium sulfate. The precipitate was then resuspended in PBS and purified on a protein A affinity column (GE Healthcare) according to the supplier's specifications. Antibody titres were evaluated by ELISA. Preimmune serum was collected prior to the initiation of the immunization protocol and was used as a control in the immunostaining experiments.

To verify the reactivity spectrum of anti-HPV5 E1^E4 antibodies amongst different β-HPV genotypes, Western blot analysis was performed for different β-HPV E1^E4 recombinant proteins. The antibodies were able to cross-react with many genotypes belonging to β1 species including HPV 8, 14, 20, 24, 25, 36.

Antibodies for the proliferation marker minichromosome maintenance protein 7 (MCM7), a commercial monoclonal antibody was used according to the manufacturer's instructions. (Neomarkers, Fremont, CA, USA).

Immunofluorescent detection

Consecutive 5-µm thick tissue sections were cut from FFPE blocks onto Superfrost™ Ultra Plus Adhesion Slides (Thermo Scientic), incubated at 56°C for 50' to increase adhesion on slide surface, dewaxed and rehydrated as follows: twice in xylene for 15' each, twice in 100% ethanol for 10 min each, followed by a series of graded ethanol (95%, 90%, 75%) for 5' each and finally 1X PBS for 5'.

To quench endogenous peroxidase, tissue sections were incubated with 3% hydrogen peroxide diluted in 1X PBS for 15' at room temperature with shaking and then rinsed in 1X PBS for 5'. For antigen unmasking, slides were placed in a glass slide holder and pre-soaked for 5 min in 10mM citrate buffer at pH 6.0 (Vector Laboratories) diluted in bidistilled water, then heated for three times in microwave oven at 750W for 4'30" each with 1' intervals inside a pressure cooker and finally cooled for 20' and washed for 5' in 1X PBS.

All the subsequent incubation steps performed in the staining procedures described below were carried out putting the slides inside a dark humidified box.

For immunofluorescent detection of β-HPV E4 coupled to DNA-FISH, DNA probes consisting of complete cloned β-HPV genomes were biotin-labelled using the Biotin-Nick Translation Mix (Roche) according to the manufacturer's instructions. Biotinylated probes were then 1:25 diluted in hybridization buffer (50% formamide, 5X SSC, 0.1% Tween-20, 0.2 ng/µl yeast tRNA) and the probe mix was added to tissue sections previously allowed to air dry after antigen unmasking. After was lowered a cover-slip onto tissue sections, and sealed with Rubber Gum (Rubbercement) to avoid evaporation. The slides were heated on a hot block for 5' at 95°C for DNA denaturation and then cooled on ice and finally incubated overnight at 37°C for probe annealing.

The following day, stringent washes with pre-warmed SSC solutions at decreasing concentrations were performed for 10' each as follows: 2X SSC (allowing also the peeling off of rubber gum and loosening of cover-slips), formamide wash buffer (50% formamide, 2X SSC, 0.05% Tween-20), 1X SSC, 0.5X SSC. Tissue sections were subsequently blocked with 10% NGS / 1X PBS for 1 h at room temperature. After blocking, primary rabbit polyclonal anti-HPV5 E1^E4 antibody was 1:1000 diluted in 5% NGS / 1X PBS and added onto tissue sections overnight at 4˚C. The following day, slides were then washed in 1X PBS / 0.05% Tween-20 for 5' at room temperature with shaking. Next, a mixture containing 1:100 diluted streptavidin-HRP (TSA^{TM} Tetramethylrhodamine System, PerkinElmer), 1:200 diluted FITC-labelled secondary anti-rabbit antibody (Invitrogen), and DAPI as nuclear counterstaining diluted in 1X PBS was added onto tissue sections and slides were incubated for 1 h at room temperature. After a 5' wash in 1X PBS, TSA fluorescent substrate

(TSA™ Tetramethylrhodamine System, PerkinElmer) prepared according to the supplier's specifications was added for 10' at room temperature, followed by a 5' wash in 1X PBS. After final wash twice 5' in 1X PBS, slides were mounted with ProLong® Gold Antifade Mountant (Life Technologies).

For immunofluorescent detection of β-HPV E4 coupled to MCM7, the tissue, sections after antigen retrieval, were blocked with 10% NGS / 1X PBS for 1 h at room temperature. After blocking, primary mouse monoclonal anti-MCM7 antibody was 1:200 diluted in 5% NGS / 1X PBS and incubated onto tissue sections overnight at 4°C.

The following day, slides were washed in 1X PBS / 0.05% Tween-20 for 5' at room temperature with shaking and tissue sections incubated with secondary biotinylated universal antibody (Vector Laboratories) for 30' at room temperature. For amplified the signal, the slides were washed 5' in 1X PBS, and then incubated with the Avidin/Biotin Blocking Kit (Vector Laboratories; 3.6 µl Avidin Solution + 3.6 µl Biotin Solution in 400 µl 50 mM Tris-HCl pH 7.5) for 30' at room temperature. After a 5' wash in 1X PBS, streptavidin-HRP (TSA™ Tetramethylrhodamine System, PerkinElmer) 1:100 diluted in 1X PBS was added onto tissue sections for 1 h at room temperature. Proceeding with 5' wash in 1X PBS, TSA fluorescent substrate (TSA™ Tetramethylrhodamine System, PerkinElmer) prepared according to the supplier's specifications was added for 10' at room temperature, followed by a further 5' wash in 1X PBS. Tissue sections were subsequently incubated overnight at 4°C with primary rabbit polyclonal anti-HPV5 E1^E4 antibody 1:1000 diluted in 5% NGS / 1X PBS.

On the third day, after a 5' wash in 1X PBS/ 0.05% Tween-20 at room temperature with shaking, FITC-labelled secondary anti-rabbit antibody (Invitrogen) 1:200, together with nuclei counterstaining DAPI diluited in 1X PBS was added onto tissue sections and slides were incubated for 1 h at room temperature. After wash twice in 1X PBS for 5', tissue sections were finally mounted with ProLong® Gold Antifade Mountant (Life Technologies).

For immunofluorescent detection of β-HPV L1, tissue sections after antigen retrieval were blocked with 10% NGS / 1X PBS for 1 h at room temperature. After blocking, tissue sections were incubated overnight at 4°C with primary rabbit polyclonal anti-HPV5 L1 antibody 1:1000 diluted in 5% NGS / 1X PBS.

The following day, after a 5' wash in 1X PBS / 0.05% Tween-20 at room temperature with shaking, TexasRed-labelled secondary anti-rabbit antibody (Invitrogen) 1:200 together with nuclei counterstaining DAPI diluted in 1X PBS was added onto tissue sections and slides were incubated

for 1 h at room temperature. After twice wash 5' in 1X PBS, tissue sections were finally mounted with ProLong® Gold Antifade Mountant (Life Technologies).

For assessment of histological features, slides analyzed by anti-HPV5 E1^E4 / anti-MCM7 antibodies were disassembled and stained with hematoxylin and eosin (H&E) as follows: hematoxylin 10', wash in tap water for 5-10', eosin for 5' and a final quick wash in tap water. Tissue sections were then dehydrated in a series of graded ethanol (75% for 20", 95% for 20", 100% for 2"), diaphanized twice in xylene for 2" each and finally mounted with VectaMount Permanent Mounting Medium (VECTOR Laboratories).

Images were acquired using a digital scanner (Pannoramic MIDI, 3D Histech Kft) allowing overlay of immunofluorescent and H&E staining patterns.

β-HPV influence on keratinocyte differentiation in HPV8 transgenic mice

Genotyping of progeny

To identified HPV8 transgenic from wild type littermates, genomic DNA was isolated from tail biopsies of 7-day-old mice using the QIAamp Tissue kit (Qiagen) or from 0.5% dispase II (Roche)–and trypsin (Sigma)–treated full-thickness skin. Samples of genomic mouse DNA were analyzed for presence of the transgene by PCR, using primers that bind outside the early genes E6 and E7 (5V-CAATTTTCCTAAGCAAATGGAC and 3V- CACTACATTCAGCTTCCAAAATACA), and ~0.3 μg of DNA was analyzed in a final volume of 25 μL using REDTaq ReadyMix PCR Reaction Mix (Sigma). PCRs were performed on a MJ PTC-200 thermal cycler (Bio-Rad) using the following protocol:

- 2' denaturation step at 94°C
- 35 amplification cycles:
    - 45" at 94°C
    - 1' at 50°C
    - 1'30" at 72°C
- 72°C for 7' for a final extension

PCR products were separated by 2% agarose–TAE gel electrophoresis, and ethidium bromide staining was used to detect the separated bands. Mice were kindly provide by Herber Pfister's group and housed in accordance with The Guide for the Care and Use of Laboratory Animals, in the animal facility at the University of "Piemonte Orientale", Novara.

Skin Samples

Skin samples were collected from 5 HPV8 transgenic mice and 5 wild type littermates. For immunofluorescent experiments, skin samples were formalin-fixed in 10% neutral-buffered formalin (Sigma-Aldrich) over night at 4˚C. After a 5' wash in 1X PBS, the samples were dehydrated by a series of grated ethanol (75%, 90%, 95) for 1 h each and then twice in 100% ethanol for 1 h each. Finally tissue samples were diaphanized twice in xylene for 1h 30' and then paraffin embedded.

Consecutive 5-µm thick tissue sections were cut from FFPE blocks onto Superfrost™ Ultra Plus Adhesion Slides (Thermo Scientific), incubated at 56°C for 50' to increase adhesion on slide surface, dewaxed and rehydrated as follows: twice in xylene for 15' each, twice in 100% ethanol for 10 min each, followed by a series of graded ethanol (95%, 90%, 75%) for 5' each and finally 1X PBS for 5'. Finally the sections were stained with hematoxylin and eosin (H&E) as follows: hematoxylin 10', wash in tap water for 5-10', eosin for 5' and a final quick wash in tap water. Tissue sections were then dehydrated in a series of graded ethanol (75% for 20", 95% for 20", 100% for 2"), diaphanized twice in xylene for 2" each and finally mounted with VectaMount Permanent Mounting Medium (VECTOR Laboratories).

Whole mount protocol

Whole mount is a well-established technique applied to characterize normal and genetically modified mice with skin defects. It has previously been determined that mouse tail epidermis is very informative, as robust whole mounts can be generated from any stage of the hair cycle. In addition, tail skin contains large hair follicles with clear anatomical features, which are ideal for microscopic visualization. In contrast however, the consistent generation of high-quality epidermal sheet for the dorsal epidermis is prevent by the fact that it is more fragile and contains a much higher density of hair follicles. Hair follicles in the tail grow as triplets and undergo cycles of growth with similar timing to dorsal follicles, although the degree of synchronization is less tight.

The epidermal whole mount protocol was established while working in the laboratory of Dr G. Patel (European Cancer Stem Cell Research Institute, Cardiff University, UK) as a visiting scientist, over a period of 7 months as part of the Ph.D program.

Transgenic mice and wild type littermates, 5 each, were killed using an approved technique and the tails were removed by slicing with a scalpel at the point at the attachment to the body. Then the tail was slit with a sterile scalpel and using a pair of forceps the skin was peeled off from the underlying connective tissue.

Using a petri dish as support, the skin was cut in different pieces of 5x5 mm^2, almost 12 pieces were collected from a single tail, and incubated overnight with 2.5 ml of Neutral protease (Dispase®, Worthington Biochemical Corporation) 25U/ml in a 6-well plate; every well contained not more than 3 pieces.

The following day the pieces of skin were placed on a sterile petri dish, and the intact epidermis sheet was gently removed from the dermis using two pairs of forceps. After that, the epidermal whole mounts were formalin-fixed in 10% neutral-buffered 2 h at room temperature. The samples were then stored for following immunofluorescent experiments in 1x PBS with 0.2% sodium azide at 4˚C.

Immunofluorecent staining on epidermal whole mount samples

This is the general protocol used to investigate different markers of stem cell subpopulations in the hair follicle of both transgenic and wild time mice. Stem cell markers were coupled with the proliferation marker Ki67.

The complete list of primary and secondary antibodies used on epidermal whole mount samples is described Table 1.

Each epidermal sheet was placed in an individual well of a 48-well tissue culture plate, and blocked with 100 µl of Blocking Buffer, (0.5% skim milk powder, 0.25% fish skin gelatin) in 1X Sodium Buffer (0.9% NaCl, 20 mM HEPES, pH 7.2), 30’ at room temperature. After the aspiration of the Blocking Buffer the epidermal sheets were incubated with the primary antibody diluted in Blocking Buffer, in gentle agitation overnight at room temperature.

The second day the samples were carefully moved in a new well and washed 4 times for 45’ each in 0.2% Tween 20 in 1X PBS. After the last washing the epidermal sheets were incubated with spieces-specific secondary antibodies diluted with also DAPI at a final concentration of 1 µg/ml, in Blocking Buffer. Tissue culture plates were covered with aluminum foil for the remainder of the protocol as the dyes are light-sensitive; secondary fluorescent antibodies were incubate over night with gentle agitation at room temperature.

The third day the secondary antibodies were aspirated and the epidermal sheet were washed 4 times for 45' each in 0.2% Tween 20 in 1X PBS. After the samples were mounted on a microscope slide with the basal surface facing the coverslip using the ProLong® Gold Antifade Mountant (Life Technologies).

Antibodies for keratinocyte stem cell characterization in hair follicle

In the hair follicle there are at least 4 keratinocyte stem cell populations in the hair follicle that can recreate the entire interfollicular epidermis and hair follicle, but in homeostasis each one are responsible for the turnover of a area in the hair follicle and the adjacent overlying epidermis.

Table 1. Complete list of stem cell markers used in the whole mount protocol.

Name of marker /stem cell population	Diluition	Company	Location in skin	Stem cell properties	References
CD34	1:200	553731, BD Pharmingen	Bulge region of mouse, external root sheath in human	Used for enrichment of HF bulge keratinocytes/ able to reconstitute epidermis	(Blanpain et al. 2004; Trempus et al. 2003)
Lgr5+ cells	1:500	NLS1236, Novus Biologicals	Bulb region	Contribute to all hair lineages but not to SG and IFE	(Jaks et al. 2008)
Lgr6+ cells	1:50	ab126747, Abcam	Isthimus	Prenatal Lgr6+ cells can form HF, SG and IFE, they are not the LRCs	(Snippert et al. 2010)
Lrig 1	1:100	AF3688, R&D Systems	IFE, Isthimus.	express Lgr6 but not the Lgr5 and CD34, and are able to reconstitute the IFE and SC	(Jensen et al. 2009)
K15	1:50	MA1-90929, Thermo Scientific	Bulge region of HF of mice and human	Capable of generating epidermis, HF and SG	(Morris et al. 2004)

Immunofluorescent stainings of all the keratinocyte stem cell markers were performed in coupled with the proliferation marker Ki67, a commercial monoclonal antibody used according to the manufacturer's instructions (ab16667, Abcam).

Images were acquired using The Leica DMI6000 B inverted microscope (Leica Microsystem) and analyzed using ImageJ, a public domain, Java-based image processing program developed by Wayne Rasband at the National Institutes of Health.

RESULTS

β-HPV detection in Kidney transplant recipients (KTRs)

Characteristics of the study cohort.

The cohort of KTRs in this study is composed of 17 patients (16 males and 1 female) and is described in Table 1. Patients were numbered in ascending order based on the number of tumors each developed. A total of 79 skin lesions were recorded in this study. The median time taken for of skin lesions to develop was 7 ± 5 years after transplantation. Within 10 years from transplantation, 5 patients developed more than 5 lesions (patients 13–17), mostly in sun-exposed sites, confirming sun exposure to be a major contributor to epithelial transformation. Multiple skin lesions (range 2-14) arose in a total of 10 patients. The proportion of lesions affecting sun-exposed skin areas (head, hands and forearms) was 59 out of 79 lesions (75%).

Histologically, the cutaneous lesions included:

- High-grade tumors
    - basal cell carcinoma (BCC) → n = 31
    - squamous cell carcinoma (SCC) → n = 14
- Precancerous lesions
    - actinic keratosis (AK) → n = 19
    - keratoacanthoma (KA) → n = 7
    - Bowen's disease (BD) → n=1
- Benign lesions
    - seborrheic keratosis (SK) → n=7

Table 1. Baseline characteristics of the study cohort of kidney transplant recipients.

Patients	Birth date	Tx date	Years after transplantation																								Follow Up (years)	Total n. of Tumours
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	32		
1 M	1961	2005 K				BCC*																							8	1
2 F	1946	1995 K												SCC															18	1
3 M	1952	1996 K					BCC																						17	1
4 M	1939	1994 K											BCC*																19	1
5 M	*1952*	*2002 K*					*BCC**																						11	1
6 M	1940	1988 K																		SK*									25	1
7 M	1943	2009 K		AK*																									4	1
8 M	*1938*	*2005 K*						*AK** SCC*																					8	2
9 M	1940	2004 K	AK*					AK*																					9	2
10 M	1941	1988 H 1996 H 2006 K																		AK* SCC*									25	2
11 M	1932 2008†	1992 K				SCC*										BD* BCC*		BCC											16	4
12 M	1969	1992 K										2BCC* BCC*					SK												21	4
13 M	1934	1981 K												SK BCC					KA	KA*	SCC*		2KA* KA						32	8
14 M	1940	2000 K						AK* SCC*	3BCC* 2BCC*			BCC	SK* BCC	BCC															13	10
15 M	1946	2005 K	BCC	BCC		BCC*		SK 2BCC 2SCC*	AK* 2BCC* BCC																				8	12
16 M	*1948 2005†*	*1993 K*			AK*			*2AK** *2AK** 3SCC*		*AK** KA*	2AK*		AK* KA*																12	14
17 M	*1940*	*1999 K*	BCC*	SCC*			1SK* *SCC** 2SCC*	SK* 3AK*				2BCC* BCC*	BCC*	BCC*															14	14

SK: Seborrheic keratosis; KA: Keratoacanthoma; BD: Bowen's disease; BCC: Basal cell carcinoma; AK: Actinic keratosis ; SCC: Squamous cell carcinoma

*lesion in sunlight-exposed body sites.

β-HPV DNA detection and genotyping in skin lesions

The results of β-HPV testing of DNA extracted from the 79 skin lesions are shown in Table 2. As already mentioned, in 28 cases multiple biopsy blocks were available corresponding to either the core or edges of the lesions. Out of the total 111 formalin fixed paraffin embedded (FFPE) blocks analyzed, 94 (85%) were positive for β-HPV DNA. Taking into account the original lesions from which these FFPE blocks were split, overall 69 out of 79 lesions (87%) contained β-HPV DNA.

Where multiple FFPE blocks were derived from a single lesion, the genotypes found in the core and edges of the lesions were the same. The most frequently observed genotypes were HPV 5 and 8 (both belonging to β1 species), which were found in 51 (65%) and 20 (25%) lesions respectively. HPV5 was detected as a single infection in 18 out of 79 lesions (23%) and HPV8 was detected as a single infection in two lesions (3%). Infections with two genotypes were found in 13 lesions (16%), while more than two genotypes were found in 28 lesions (35%). In the 5 patients who exhibited more than 5 lesions, HPV 5 alone or in multiple infections was found in 38 (66%) of the 58 lesions developed by these patients. β1 species, including HPV 5, 8, 14, 24 and 36, accounted for 61 lesions (77%) which were positive for at least one genotype. Among the genotypes detected from β2 species, the most frequently observed were HPV 80 and 38. In patients with multiple lesions, a good intra-patient concordance for the genotypes detected among the different lesions developed over time was generally observed.

Table 2. β-HPV DNA detection in the different types of skin lesions and in the FFPE blocks in which these lesion were splitted.

Skin lesions	β-HPV DNA-positive/ total		FFPE blocks (β-HPV DNA-positive/total)		
	Lesion	FFPE Blocks	WL	C	E
Total	70/79	94/111	43/51	24/28	27/32
BCC	25/31	33/44	17/21	8/10	8/13
SCC	14/14	19/20	8/9	5/5	6/6
AK	17/19	22/25	11/13	5/6	6/6
KA	7/7	12/13	1/1	5/6	6/6
BD	1/1	1/1	1/1	0/0	0/0
SK	6/7	7/8	5/6	1/1	1/1

WL, whole lesion; C, core; E, edge

Visualization of viral proteins in skin lesions

The selection of biomarkers used as useful tool to visualize the presence of active infections in lesional areas has been deeply investigated for α-HPV types associated with lesions from previous studies. The viral E4 and L1 proteins and cellular proliferation marker MCM7 resulted useful biomarkers for the visualization of viral life cycle events in α-HPV-induced lesions, including cervical tumors associated with high-risk α-HPV infection (Middleton et al. 2003; Borgogna et al. 2012; Doorbar 2007; Doorbar et al. 2012). In addition it has been reported that detection of E4 is helpful for the visualization of active β-HPV infection in skin tumors from epidermodysplasia verruciformis (EV) patients, especially in regions where some morphological differentiation of the epithelium is still present (Borgogna et al. 2012).

β-HPVs are known to be transcriptionally active in the skin tumors of patients presenting with Epidermodysplasia verruciformis, therefore tissue sections from a benign hyperplastic intraepidermal wart-like lesion from an EV patient were used as a positive control for immunostaining procedures. In almost all EV lesions it is possible to visualise viral life cycle events - already validated for α-HPVs - based on detection of viral protein markers (β-HPV E4 and L1), viral genome amplification and cellular proliferation marker MCM7 (Figure 1). The lesion in Figure 1 shows representative images of tissue sections from a benign wart-like lesion from an EV patient displaying unequivocal histological features associated with the infection, including acanthosis and the disorganization of the granular layers defined by an abrupt variation in keratoyaline granules. The core region of the lesion exhibited typical HPV-induced cytopathic effects in the spinous and granular layers, characterized by swollen and irregularly shaped keratinocytes containing enlarged hyperchromatic nuclei and pale, granular and often vacuolated cytoplasm with occasional perinuclear halos (Cubie 2013; Elder et al. 2005). Cells in this region displayed typical cytoplasmic E4 staining. The proliferation marker MCM7, whose expression is normally restricted to the basal layer, was also strongly increased compared with the adjacent normal epithelium, being most apparent in the basal and upper layers.

The abnormal expression of MCM7 shows that in order to support viral replication, E4-positive cells in the upper layers were driven to re-enter the cell cycle. The lack of E4 positivity in adjacent normal epithelium overlapped with a reduction in MCM7 expression, being lowered to the basal layer. The positivity for HPV DNA in FISH analysis overlapping the E4 staining confirmed that these cells were supporting viral genome amplification. As expected, L1-positive nuclei were detected in the more superficial layers of the E4-positive area, indicating completion of the productive viral lifecycle with the assembly and release of new infectious particles (Doorbar 2007; Doorbar et al. 2012).

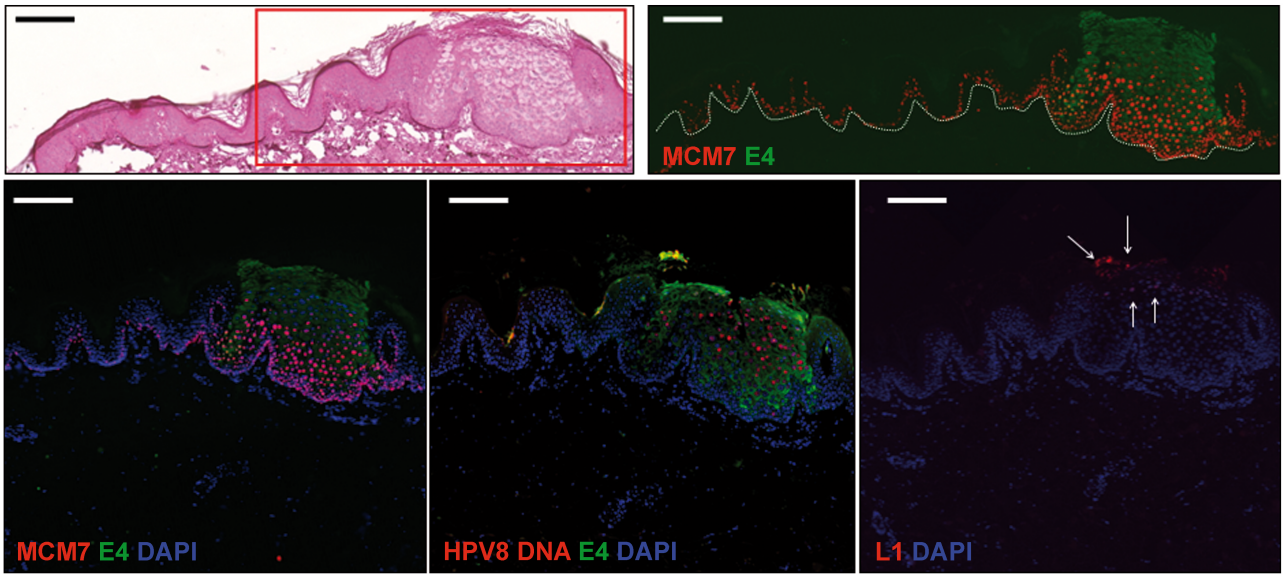

Figure 1. Distribution of the viral and cellular markers E4, L1, HPV8 DNA and MCM7 in a wart-like lesion from an EV patient (elbow). The top panels show (i) an H&E-stained section with the area of interest boxed in red (left) and (ii) immunofluorescent staining of the same section. MCM7 is labelled in red and E4 in green (right; the white dotted line indicates the basal layer). The region shown in the lower panels corresponds to the red square highlighted in the left-hand top panel. MCM7/E4 co-staining is reproduced (left) and serial sections were double stained to demonstrate viral genome amplification by HPV8 DNA-FISH (red) and for E4 expression (green) (middle).The right-hand section was stained with antibodies to L1 (red) (right; the white arrows indicate nuclear L1 staining). All sections were counterstained with DAPI (blue) to visualize cell nuclei. Scale bars: 100 µm.

To investigate whether similar patterns of productive infection seen in EV patients could be visualized in the lesions arose in a KTR, all the skin lesions of our cohort were analyzed with the viral protein markers. Tissue sections from all 111 FFPE blocks were co-stained for β-HPV E4 and L1 to detect viral antigen expression and for MCM7 as a cellular proliferation marker. As the quality of DNA extracted from FFPE material is generally poor, affecting the sensitivity and reliability of PCR-based assays, immunofluorescent screening was performed on all tissue specimens regardless of the results of β-HPV DNA detection.

Out of the 111 FFPE blocks analyzed, E4-positive cells were found in six blocks (for brevity, referred to as IF-positive), as follows:

- 4 AK (1 from Patient 8 and 3 from Patient 16)
- adjacent pathological epithelium of 1 SCC (Patient 17)
- adjacent pathological epithelium of 1 BCC (Patient 15)

For the first time active infection of β-HPV was found in a skin lesion developed by an organ transplant recipient.

Patients 5 and 8 received transplants in 2002 and 2005 respectively; Patient 5 developed a single lesion (1 BCC, IF-positive) while Patient 8 had 1 SCC prior to the IF-positive AK .
Patients 16 and 17 received transplants in 1993 and 1999 respectively and developed 14 skin lesions each in their post-transplant period.
Details of the IF-positive patients along with their skin lesions are summarized in Table 3, reporting the specific FFPE blocks (edge, core or whole lesion) where positivity was found and the genotyping results relative to these specimens. As expected from the spectrum of the different genotypes detected by the polyclonal anti-E4 antibody, all the IF-positive sections harbored genotypes belonging to species 1.

Table 3. Characteristics of the IF-positive patients from the KTR cohort and their skin lesions.

Patients	**Birth date**	**Tx date**	**Total skin lesions**	**IF-positive skin lesions (FFPE block) years post-tx (y/p-t) β-HPV genotypes**		
5 (M)	1952	2002	1 1 BCC	BCC (WL) 5 y/p-t HPV 8		
8 (M)	1938	2005	2 1 AK 1 SCC	AK (WL) 6 y/p-t HPV 8, 12, 36		
16 (M)	1948 (2005†)	1993	14 9 AK 2 KA 3 SCC	AK (WL) 6 y/p-t HPV 25	AK (E) 6 y/p-t HPV 36, 75	AK (C) 8 y/p-t HPV 8, 14, 19, 38, 75
17 (M)	1940	1999	14 2 SK 3 AK 4 SCC 5 BCC	SCC (E) 5 y/p-t HPV 24		

Tx, transplantation; WL, whole lesion; C, core; E, edge; BCC, basal cell carcinoma; AK, actinic keratosis; KA, keratoacanthoma; SCC, squamous cell carcinoma; SK, seborrheic keratosis

Having established the EV staining pattern, all the IF-positive areas identified in the kidney transplant recipient's lesions were analyzed with respect to their histological characteristics and staining patterns.
Representative images are presented below.

Figure 2 shows one of the two IF-positive areas detected in the adjacent pathological epithelium of a BCC from Patient 15 (neck). The epithelial lesion displayed acanthosis and E4/MCM7 and L1 staining patterns strongly resembled those observed in EV wart-like lesions (Figure 1), with typical features of β-HPV productive infection.

Figure 3 summarizes immunostaining in Patient 8, focusing on the IF-positive area found in the bowenoid AK located next to the SCC on the neck. This area shows clear signs of β-HPV-related cytopathic effects, enlarge keratinocytes with pail cytoplasm, with some cells displaying pleomorphism and nuclear atypia. Both early and late viral markers of β-HPV infection are present, suggesting the presence of β-HPV productive infection.

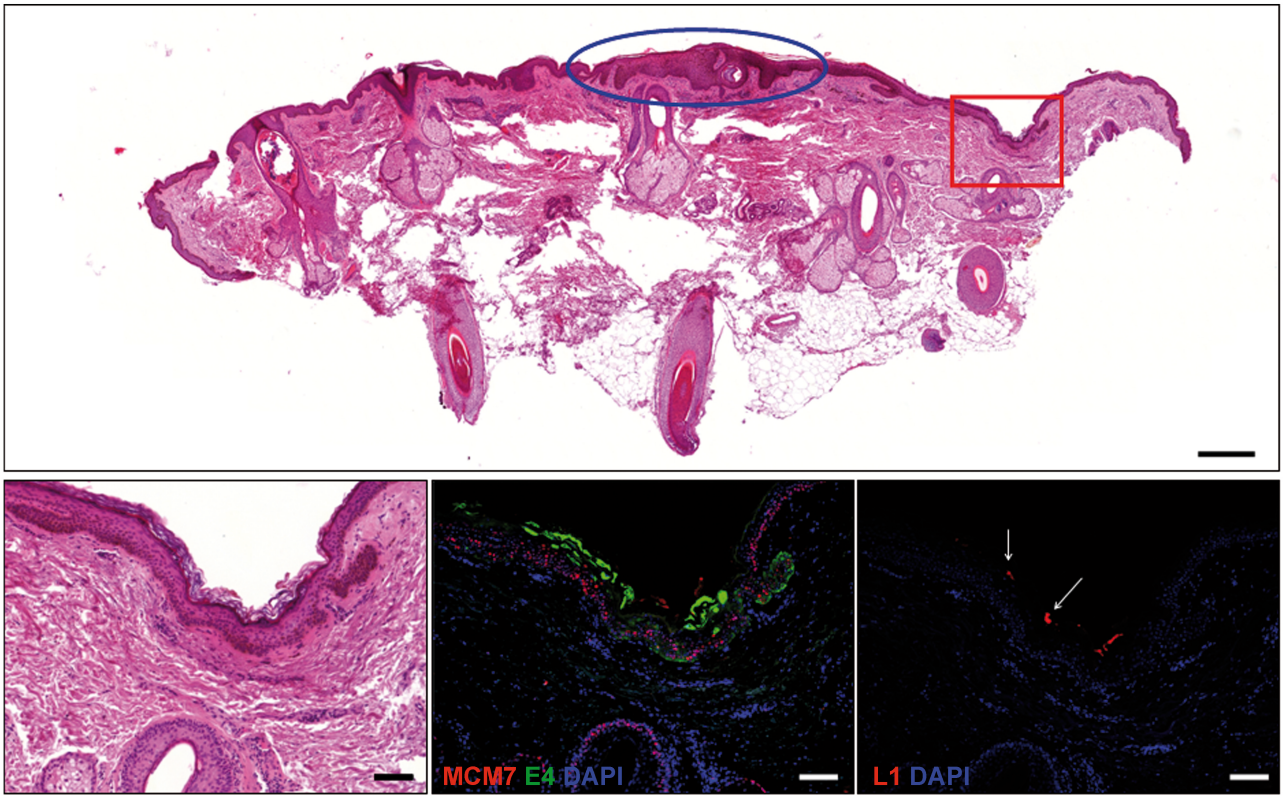

Figure 2. Distribution of the viral and cellular markers E4, L1 and MCM7 in a basal cell carcinoma from a kidney transplant recipient (Patient 5, neck). The top panel shows the scanned image of H&E stained section (scale bar: 1000 μm) where the blue circle indicates the BCC core region and the red square region of interest in the adjacent pathological epithelium. The area shown in the lower panels (scale bars: 100 μm) corresponds to the red square highlighted in the top panel. The same section was double stained using antibodies to E4 (green) and MCM7 (red) (middle image) while an adjacent section was stained with antibodies to L1 (red) (right image; the white arrows indicate nuclear L1 staining). All sections were counterstained with DAPI (blue) to visualize cell nuclei.

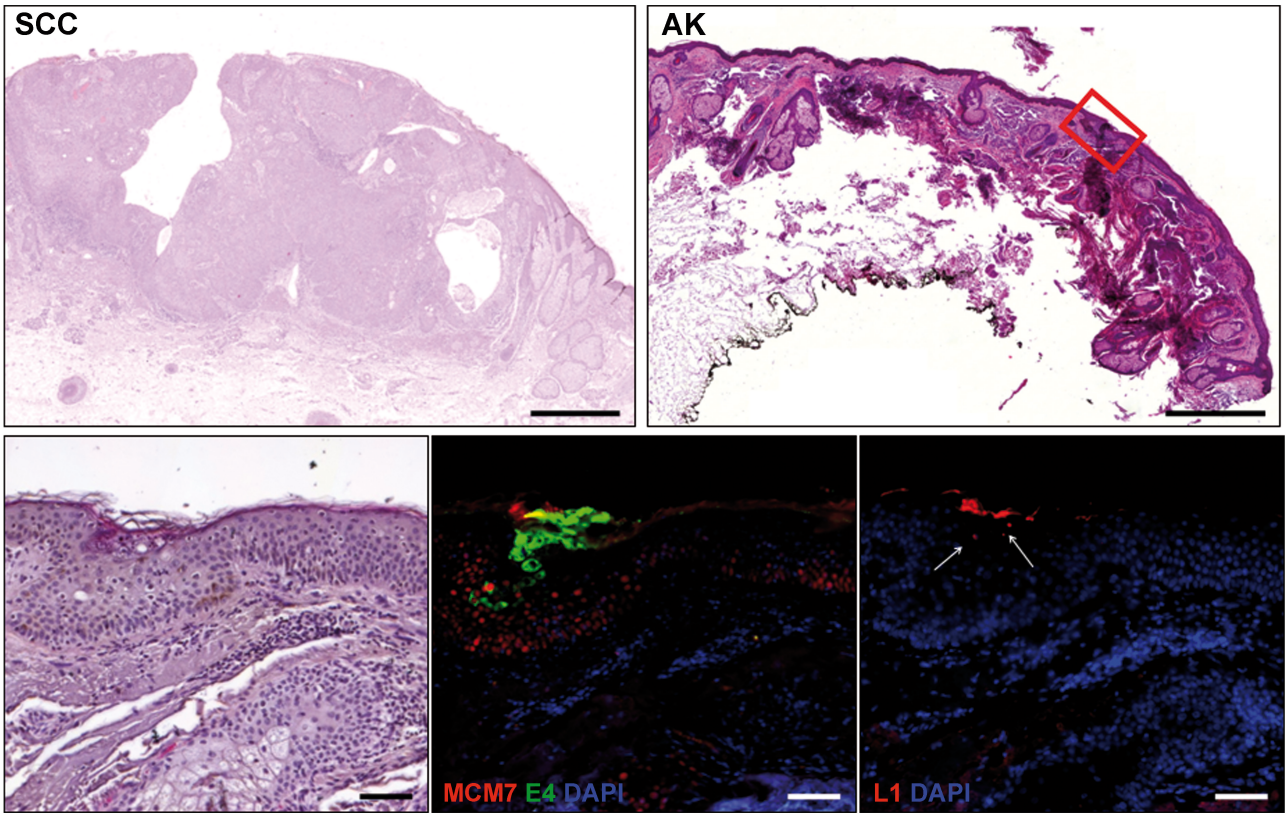

Figure 3. Distribution of the viral and cellular markers E4, L1 and MCM7 in a bowenoid actinic keratosis from a kidney transplant recipient (Patient 8, neck). The top two images (scale bars: 1000 µm) show H&E stained sections; the right-hand panel demostrating the AK with the area of interest boxed in red, while the left-hand panel illustrates a nearby SCC. The region shown in the lower panels (scale bars: 50 µm) corresponds to the red square highlighted in the right-hand top panel. The same section was double stained using antibodies to E4 (green) and MCM7 (red) (middle picture) and a serial section was stained with antibodies to L1 (red) (right; the white arrows indicate nuclear L1 staining). All sections were counterstained with DAPI (blue) to visualize cell nuclei.

A total of 3 hypertrophic AK developed from the same patient (Patient 16) are shown in Figures 4-6.

Figure 4 shows an AK (hand) with an unambiguous area of positivity found in an epithelial crevice, where disruption of the granular layer was clearly evident alongside parakeratosis, a histological feature indicative of HPV infection (Elder et al. 2005). In this case, it was possible to perform FISH analysis for the single genotype found, specifically HPV 25. FISH-positive nuclei neatly overlapped with some E4-positive cells in the cytoplasm, while L1 positivity was confined to the upper epithelial layers and some parakeratotic nuclei.

The IF-positive region reported in Figure 5 was found in the pathological edge of an AK (face) and showed the typical staining pattern and cytopathic effects of productive β-HPV infection. The AK of Figure 6 (hand) showed an IF-positive area corresponding to a highly parakeratotic epithelium that abruptly changed to normal orthokeratosis on both sides; as expected from the high grade of

parakeratosis, many L1-positive nuclei were found above the E4-positive cells. Consistent with the typical staining pattern of EV wart-like lesions shown in figure 1, MCM7 expression was increased in all the IF-positive areas being present in the basal layer and above. This pattern of staining decreased with the onset of E4 expression, although an overlap region was well apparent where E4/MCM7 double positive cells were found (see especially Figure 5).

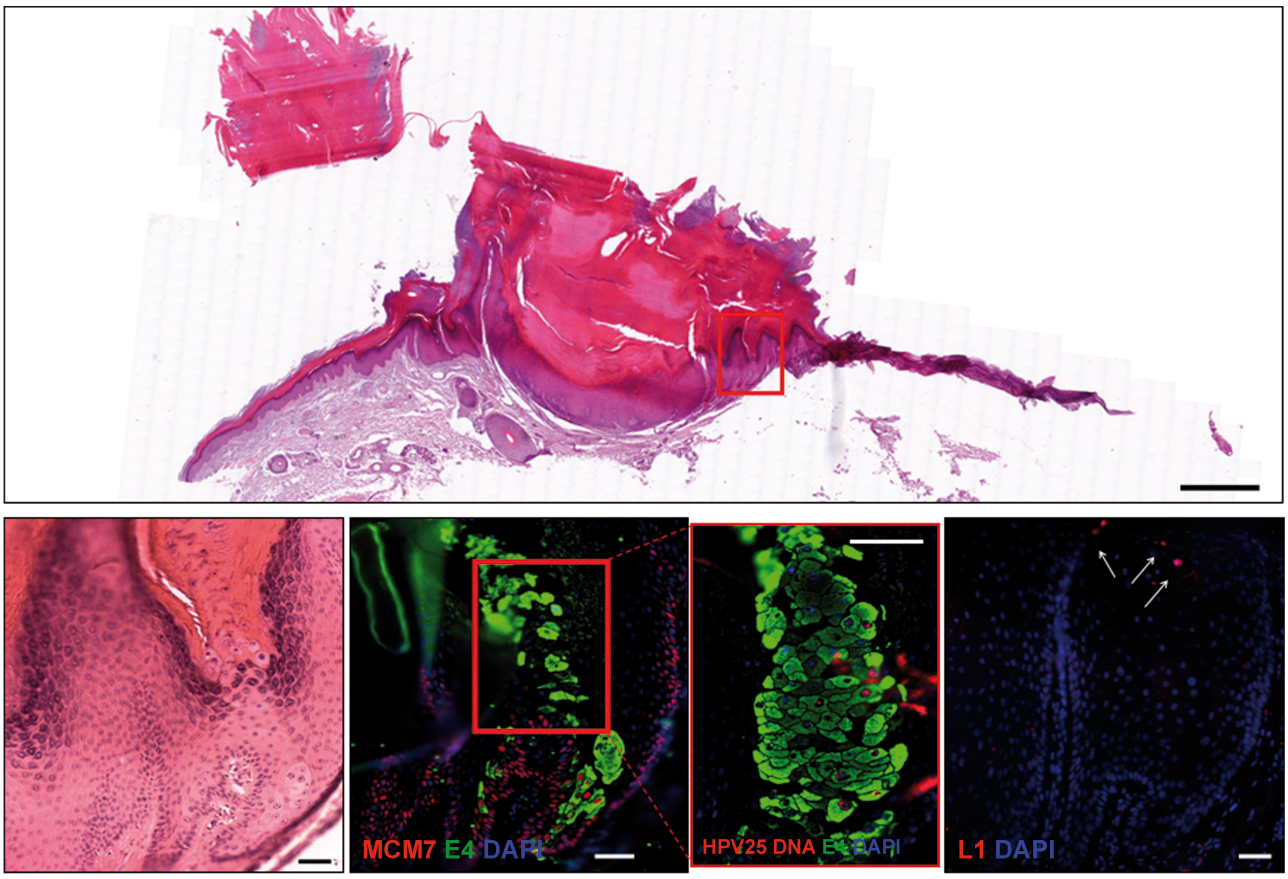

Figure 4. Distribution of the viral and cellular markers E4, L1, HPV25 DNA and MCM7 in a hypertrophic actinic keratosis from a kidney transplant recipient (Patient 16, hand). The top panel (scale bar: 1000 µm) shows an H&E stained section with the area of interest boxed in red. The region shown in the lower panel (scale bars: 50 µm) corresponds to the red square highlighted in the top panel. Serial sections were double stained using antibodies to E4 (green) and MCM (red; second image). Sections were also double stained to show viral genome amplification HPV25 DNA-FISH (red) with E4 expression (green; third image); and also stained with antibodies to L1 (red) (fourth panel; the white arrows indicate nuclear L1 staining). All sections were counterstained with DAPI (blue) to visualize cell nuclei.

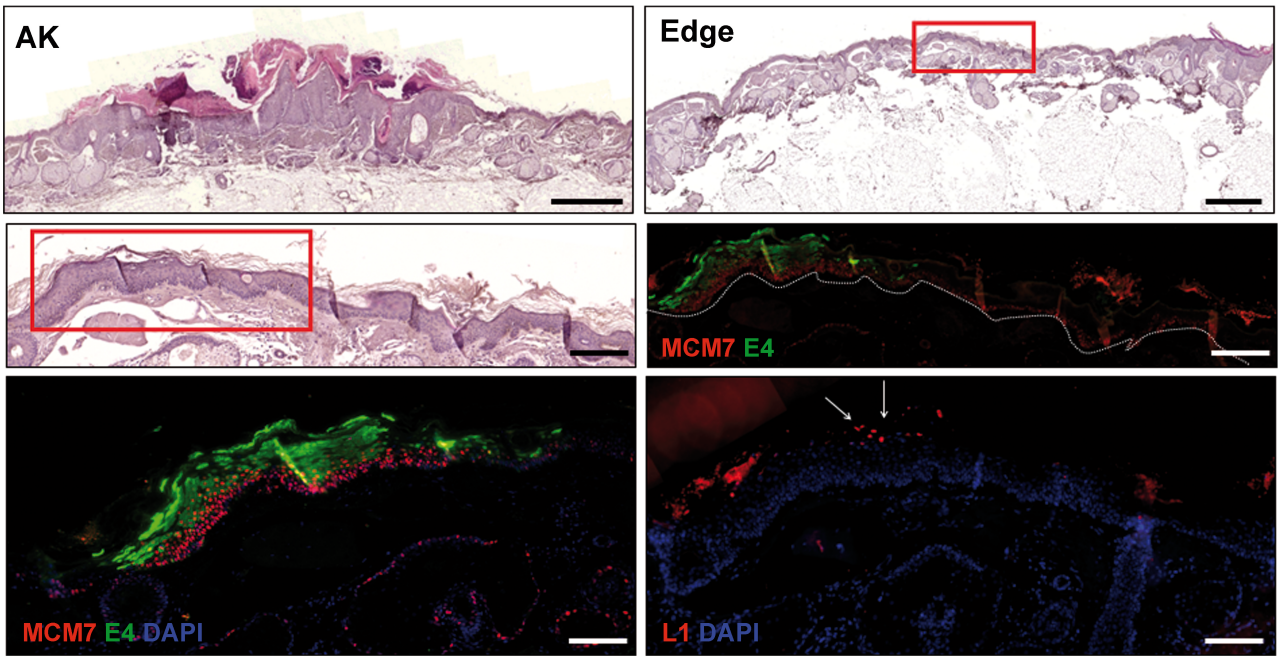

Figure 5. Distribution of the viral and cellular markers E4, L1, HPV25 DNA and MCM7 in a hypertrophic actinic keratosis from a kidney transplant recipient (Patient 16, face). The top two micrographs (scale bars: 1000 µm) show H&E stained sections of the core region of the lesion (left) and its edge (right) with the area of interest boxed in red. The middle panel (scale bars: 200 µm) shows the histology (H&E staining) of the area from the edge where viral markers were expressed, highlighted in the red square (left), and an overall picture of immunofluorescent staining (E4 in green; MCM7 in red) of the same tissue section (right; the white dotted line indicates the basal layer). The lower panel (scale bars: 100 µm), shows a magnified image of MCM7/E4 co-staining (left). Serial sections were stained with antibodies to L1 (red) (right; the white arrows indicate nuclear L1 staining). All sections were counterstained with DAPI (blue) to visualize cell nuclei.

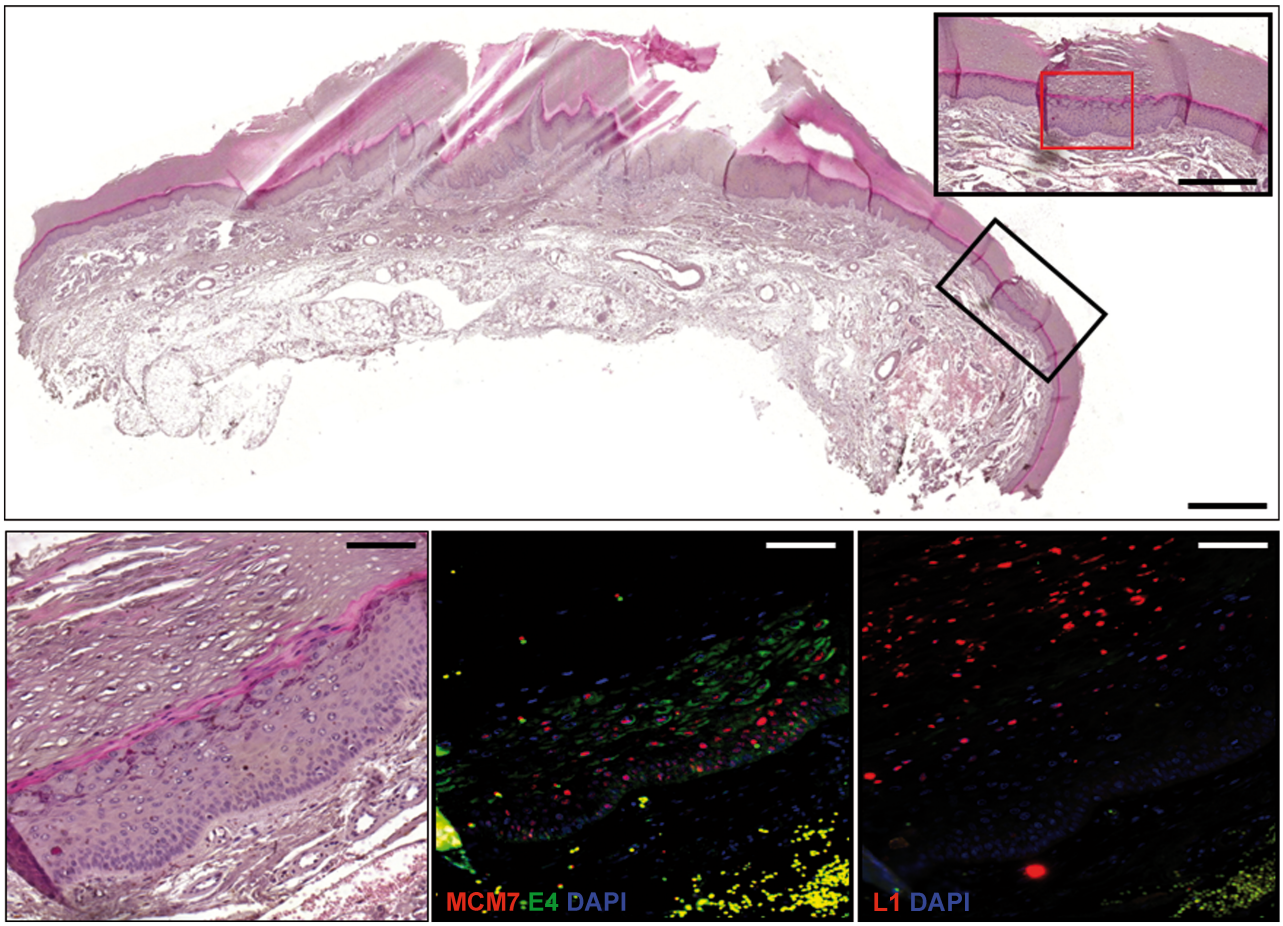

Figure 6. Distribution of the viral and cellular markers E4, L1, HPV25 DNA and MCM7 in a hypertrophic actinic keratosis from a kidney transplant recipient (Patient 16, hand). The top panel (scale bar: 100 µm) shows an H&E stained section, with a magnified insert (scale bar: 400 µm) illustrating a region of positive parakeratotic epithelium that abruptly changes to normal orthokeratosis on both sides. The region shown in the lower panel (scale bars: 50 µm) corresponds to the red square highlighted in the insert in the top panel. The first image (left) shows H&E staining of this area. Adjacent serial sections were immunostained using antibodies to E4 (green) and MCM7 (red; middle image) and L1 (red; right hand image). All sections were counterstained with DAPI (blue) to visualize cell nuclei.

Adjacent to the pathological epithelium of a SCC from Patient 17 (ear), a clear positive area was observed between two hair follicles (red box top right panel, Figure 7). Here, the cytopathic effect was remarkable consistent to that generally found in EV wart-like lesions; many E4-positive cells were present, as well as superficial L1-positive nuclei.

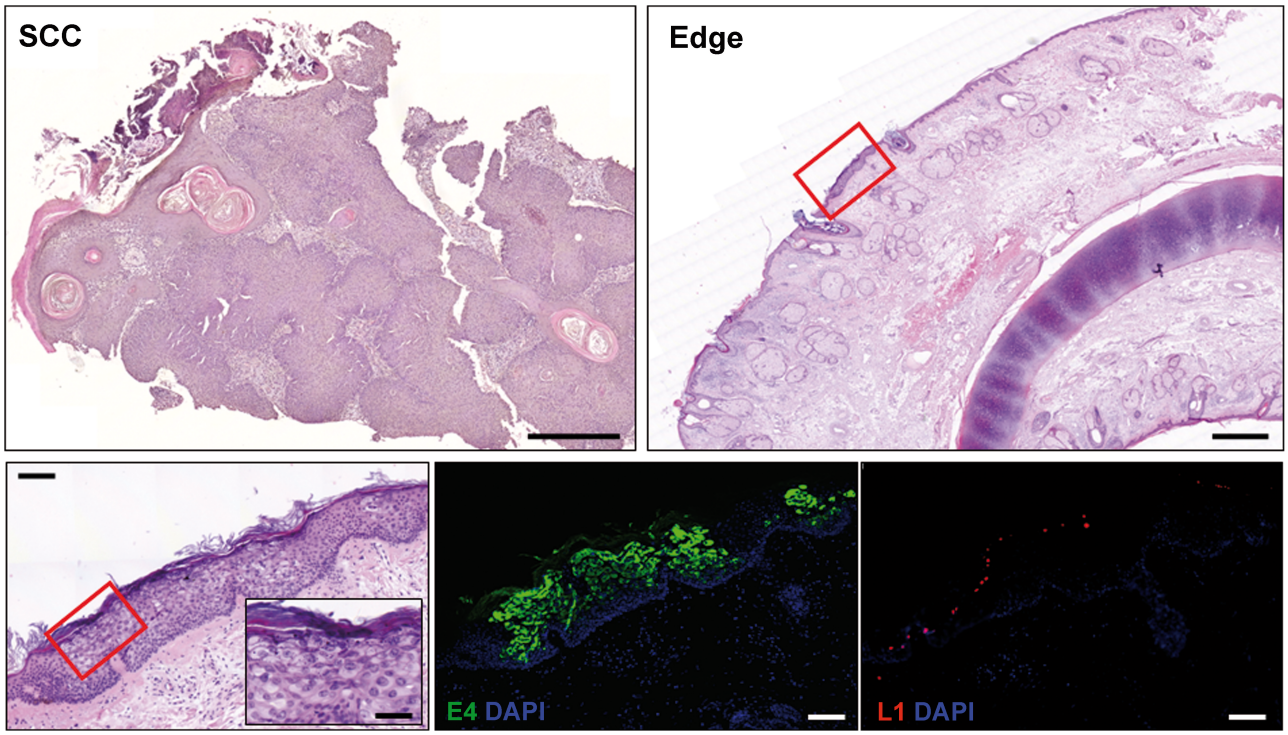

Figure 7. Distribution of the viral and cellular markers E4, L1 and MCM7 in the adjacent pathological epithelium of a squamous cell carcinoma from a kidney transplant recipient (patient 17, ear). The top panel shows H&E stained images of the SCC core region (left; scale bar: 500 µm) and its edge (right) with the area of interest boxed in red (scale bar: 1000 µm). The region shown in the lower panel (scale bars: 50 µm) corresponds to the red square in the top right-hand micrograph. The left hand image shows an H&E stained section with a magnified insert (scale bar: 20 µm) showing the cytopathic effect. Serial sections were double immunostained using antibodies to E4 (green; middle image) and to L1 (red; right hand image). All sections were counterstained with DAPI (blue) to visualize cell nuclei.

Overall, the cytological detail of histologically stained sections and immunostaining patterns for viral E4 and L1 proteins and cellular proliferation marker MCM7 found in the IF-positive KTR skin lesions are strikingly similar to those typically observed in EV wart-like lesions, exemplified in the representative images shown in Figure 1, associated with β-HPV productive infection.

H&E stained sections of all the tissue sections, which did not demostrate viral marker expression, were carefully screened for the presence of any HPV-related cytophatic effects. Consistently with the absence of detectable viral activity, no such features were found in any of these negative specimens.

HPV8 Transgenic mice

Phenotype of mice expressing HPV8 early genes

To define the influence of βHPV gene expression on keratinocytes differentiation, the skin of βHPV8 transgenic mice was investigated. This *in-vivo* model expresses the complete early region genes under the control of the epithelia specific K14 promoter. Previous studies have shown that in this model, 91% of HPV8-transgenic mice developed single or multifocal benign tumours such as papillomatosis, acanthosis, hyperkeratosis and epidermal dysplasia; 6% of them also developed a squamous cell carcinomas (Schaper et al. 2005).

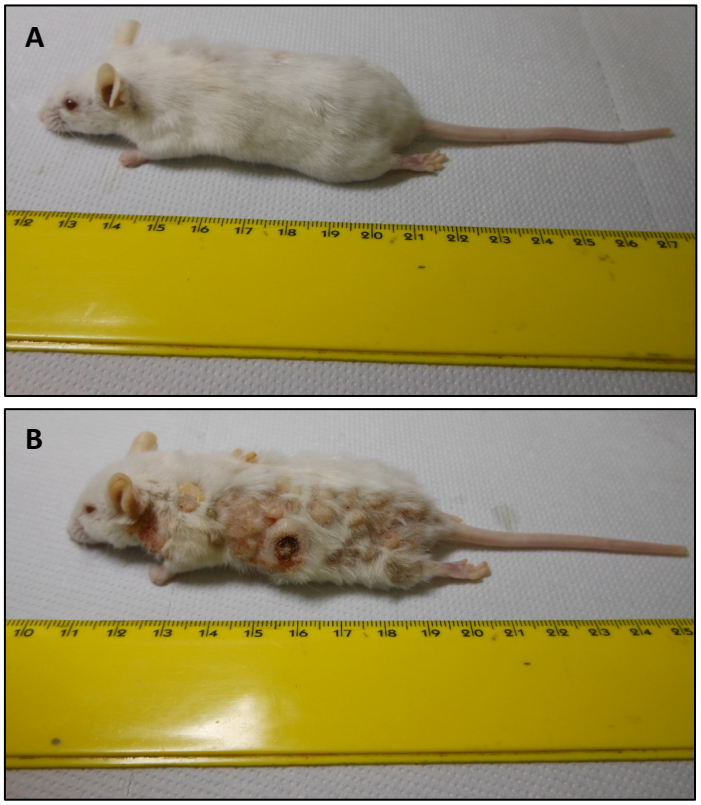

Figure 8. Comparison between 28 weeks old wild type (A) and HP8 transgenic mice (B). In transgenic mice tumours spontaneously developed during 5 weeks are clearly visible and diffusely spread

Morphogical and histological characteristics of 5 wild type mice and 5 HPV8 transgenic mice were analysed revealing that the skin of βHPV8 transgenic mice was thicker than wild type littermates with mean thicknesses of ears (0.67 vs 0.42 mm) and tails (3.51 vs 3.39 mm). Consistent with the observation of thicker skin in our transgenic model. H&E staining of sections from transgenic mice showed taller keratinocyte columns in adult βHPV8 transgenic mice hair follicle and adjoining interfollicular epidermis, 4.20 ± 0.47 vs 2.00 ± 0.00 cells and 3.83 ± 0.49 vs 1.5 ± 0.43 cells respectively (n=5). However, the weight and tail length were not significantly different between transgenic mice and wild type controls.

Visualization of keratinocyte stem cell marker phenotypes in transgenic and wild type mice

The epidermal whole mount protocol was established while working in the laboratory of Dr G. Patel (European Cancer Stem Cell Research Institute, Cardiff University, UK) as a visiting scientist, over a period of 7 months as part of the Ph.D program.

Figure 9 shows representative epidermal whole mount samples of wild type and HPV8 Transgenic mice stained with toluidine blue. As illustrated in these images, the epidermal whole mount protocol led to a complete visualization of all the keratinocyte populations all along the hair follicle.

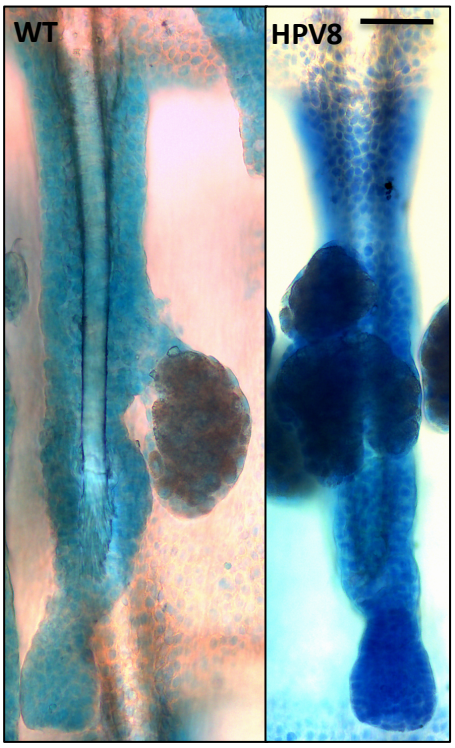

Figure 9. Epidermal whole mount samples of wild type and HPV8 Transgenic mice stained with toluidine blue. (scale bar: 100 μm)

As already mentioned, there are at least 4 keratinocyte stem cell populations in the hair follicle that can recreate the entire interfollicular epidermis and hair follicle. However, in homeostasis, the different populations display different functions. LGR5+ cells in the hair bulb and lower portion of the bulge support hair shaft formation. CD34 and K15 positive cells of the bulge are involved in hair cycling, supporting regrowth of the hair follicle during the anagen phase. LGR6 positive cells, at the entry point of the sebaceous gland and within the sebaceous gland maintain sebaceous gland turnover. Lrig 1 positive cells at the junctional zone support turnover of the infundibulum and adjacent overlying epidermis.

To determine which population of hair follicle keratinocyte stem cells was expanded in βHPV8 transgenic mice compared to wild type littermates, epidermal whole mount samples were co-stained with the KSC markers CD34, K15, LGR6, LGR5 and Lrig1 and with the proliferation marker Ki67 (Figure 10). So far unfortunately it wasn't possible to obtain a reliable LGR5 labelling.

Hair follicle size, and the positive areas of all the KSC markers were analysed in both wild type and transgenic mice using ImageJ software (Figure 10). No difference in hair follicle length was observed, however the width and therefore overall area of the hair follicles was greater in the transgenic mice correlating with expansion of the Lrig1+ area. The mean area of the hair follicle Lrig1+ population was markedly increased in the βHPV8 transgenic mice compared to wild type mice (23,845+/-13,480 vs 14,907+/- 3,793 μm^2, $p<0.001$), compared to the CD34+ (8,356+/-1,465 vs 8,077+/- 1,510 μm^2, p=NS), and LGR6+ population (60,250 +/- 9,972 vs 49,216 +/- 13,540 μm2, p=0.01). In line with these findings the hair follicle keratinocyte stem cell proliferation, validated with the marker of proliferation Ki67, was higher within the Lrig1+ (69 vs 55%, $p<0.001$),

compared to the CD34+ (1 vs 1%), and LGR6 (29 vs 40%) populations, of βHPV8 transgenic compared to wild type mice.

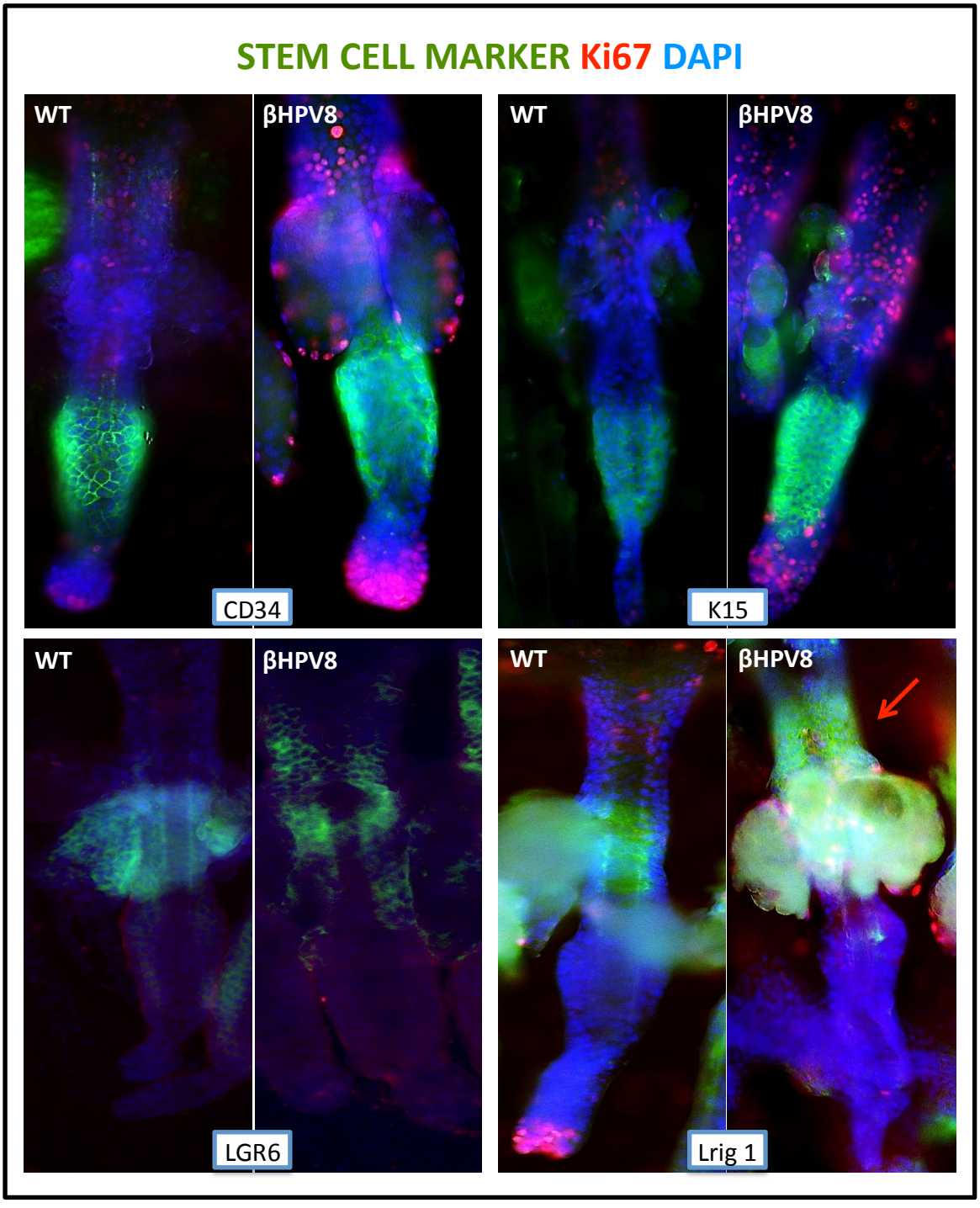

Figure 10. Distribution of keratinocyte stem cell markers CD34, K15 LGR6 and Lrig1 (green) co-stained with the marker of proliferation Ki67 (red), in whole mount analysis comparing wild type and transgenic mice. In all the sections of transgenic mice is possible to visualize an increase in proliferation overlapping the Lrig1 positive area. The red arrow in the lower right hand panel shows the increased Lrig 1 positive area in transgenic mice. All sections were counterstained with DAPI (blue) to visualise cell nuclei.

Conclusions

Collectively, the results obtained in the KTR study cohort indicate that detection of β-HPV infection at the DNA level does not prove any viral activity in the context of the lesional epithelium. Immunohistochemical screening for viral markers revealed active β-HPV infection in precancerous lesions (4 AK) and the adjacent pathological epithelium of high-grade lesions (1 BCC and 1 SCC) from the KTR cohort, suggesting that these viruses are actively implicated in the pathogenic mechanisms underlying skin carcinogenesis in the immunosuppressed setting of organ transplant recipients. Furthermore, results obtained using an *in-vivo* model of HPV8 transgenic mice suggest an interaction between β-HPV gene expression and a specific keratinocyte stem cell population. In accordance with a previously published report (Schaper et al. 2005), an increase in keratinocyte proliferation was demonstrated in the HPV8 model. Despite the expression of the transgene throughout the basal layer of skin and appendages, the whole mount protocol revealed that keratinocyte proliferation in βHPV8 transgenic mice, when compared to that in wild type mice, was localized only around the hair follicle isthmus, infundibulum and adjacent epidermis and overlapping the same area with the increased expression of the stem cell marker Lrig 1.

DISCUSSION

To our knowledge, this is the first study in which the association between skin lesions and β-HPV infection has been assessed at both the DNA level by PCR analysis and the protein level by immunofluorescence analysis. The first aim of the study was to determine whether detection of transcriptionally active β-HPV infection and active β-HPV replication could also be observed in cutaneous lesions from kidney transplant recipients (KTRs), as already defined in skin tumors from epidermodysplasia verruciformis (EV) patients, who display a high susceptibility of β-HPV infection associated with the development of disseminated wart-like lesions with a high propensity for malignant progression (Borgogna et al. 2012). The second aim was addressed to investigate the influence of HPV gene expression on keratinocytes differentiation in *in-vivo* model, using the β-HPV transgenic mice, expressing the complete early region of HPV8; described to develops spontaneously skin cancer in the absence of any additional co-carcinogenic treatments (Schaper et al. 2005; Akgül et al. 2006; Pfister 2003).

KTRs, affected by an acquired immunodeficiency status, were compared with the primary immunodeficiency setting of EV patients because they share the following two main features:

- A status of chronic immunosuppression, which may favor persistence or reactivation of latent skin-tropic viruses including β-HPV;
- The high risk of developing multiple skin tumors (Euvrard et al. 2003; Wisgerhof et al. 2010).

These commonalities are also valid for patients affected by primary immunodeficiencies in general (Leiding & Holland 2012).

In addition, in this study the EV model served as an important tool for optimizing the staining procedures for β-HPV E4 and L1 proteins and was used as a positive control when examining KTR tissue sections. A polyclonal antibody recognizing the E4 protein from β1 species was used alongside a polyclonal antibody raised against a highly conserved region of the β-HPV L1 protein.

From the cohort of KTRs a total amount of 111 FFPE blocks were obtained, previously collected from 79 skin lesions. Due to the different size of the lesions the samples were either split in more sections or maintained in a single intact. For 51 lesions a single block was available, denominated "whole lesion"; 28 lesions were sectioned in two or more blocks, corresponding to the core and

edges of the lesion. All lesions were excised from 17 KTRs (16 male and 1 female), receiving transplants between 1998 and 2009.

The initial PCR analysis demonstrated that β-HPV DNA was highly present in these specimens, with 94 FFPE blocks (85%) and 69 original lesions (87%) resulting positive. Multiple infections were observed in 41 lesions (52%), and at least one genotype belonging to the b1 species was found in 79 of the 111 blocks analyzed by PCR (71%).

Immunofluorescence analysis revealed positivity for viral markers in 6 FFPE blocks, corresponding to 4 actinic keratoses (AK) and the adjacent pathological epithelium of 1 basal cell carcinoma (BCC) and 1 squamous cell carcinoma (SCC). Positivity for β-HPV E4 was found in the context of the disorganized epithelium in the AK lesions, while in the 2 high-grade tumors they were localized to the lesional area surrounding the core region of the lesions. In all these specimens, the β-HPV E4 protein displayed the expected cytoplasmic localization in the mid-superficial layers of the epithelium accompanied by an elevation of the cellular proliferation marker MCM7 in the basal and suprabasal layers; the disappearance of E4-positivity in the adjacent normal epithelium constantly overlapped with a reduction in MCM7 expression, which was restricted to the basal layer. These findings support the hypothesis that β-HPV replication drives the cells above the basal layer to re-enter the cell-cycle in order to support viral genomic amplification; consistently with this observation, FISH-positive nuclei were found in some of the E4-positive cells in one AK lesion. As further proof that the productive viral lifecycle was being completed, all the E4-positive areas showed expression of the major coat protein L1 which also occurred in a subset of E4-positive cells in the upper layers. This is fully consistent with what has been seen in the case of other PV types including α-HPVs, where the onset of viral genomic amplification coincides closely with the expression of cytoplasmic E4 and nuclear L1 in some very superficial cells during productive infection (Doorbar 2007; Doorbar et al. 2012; Middleton et al. 2003). All the positive lesions displayed histological signs associated with HPV infection, including the typical cytopathic effect and parakeratosis (Cubie 2013; Elder et al. 2005); in one highly differentiated AK lesion, many L1 positive nuclei were detected and most of them localized to the thick upper parakeratotic layers.

A common trait of cancers with a well-documented viral etiology is their high incidence in immunosuppressed individuals (Piselli et al. 2009; Schulz 2009; Grulich et al. 2007). Based on the relevance that non-melanoma skin cancer (NMSC) is the most prevalent type of malignancy in the immunosuppressed setting of organ transplant recipients (OTRs) undergoing immunosuppressive therapy, one would expect that viruses may contribute to this widely reported increased susceptibility and incidence (Aldabagh et al. 2013). The detection of active β-HPV infection in EV

patients provided an important new insight into the viral oncogenesis of NMSC which might be extendable to further subsets of patients with impaired immune function including individuals with other primary immunodeficiencies and particularly long-lasting iatrogenically immunosuppressed subjects, such as OTRs. Despite many efforts, direct evidence of active β-HPV infection in skin lesions from non-EV patients were still missing and the major criticism against a direct involvement of these viruses in the pathogenesis of NMSC is that they do not seem to be maintained in high-grade tumors, such as SCC.

To date, the evidence suggesting a causal role of β-HPVs in skin carcinogenesis has been tainted by the main following reasons:

- It is possible to detect viral DNA in normal skin in both healthy and immunosuppressed individuals;
- Viral genome copy number is present in much less than one genome copy per tumor cell in non-EV skin lesions, and always in episomal form;
- Viral transcripts have not been identified by high-throughput mRNA sequencing in non-EV SCC;
- β-HPV E6/E7 oncoproteins exhibit different properties and a lower transforming potential compared to high-risk α-HPV oncoproteins (Akgül et al. 2006; Pfister 2003; Feltkamp et al. 2008; Sarah T Arron et al. 2011; Aldabagh et al. 2013).

Alongside the fact that ubiquitous β-HPV DNA carriage does not necessarily indicate active infection, these issues have created and so far maintained a state of uncertainty about the causative role of β-HPVs in non-EV skin cancer. Although the present study does not directly demonstrate a causal role of these viruses, the detection of β-HPV E4 and L1 expression in 4 AK, widely regarded to be SCC precursors or in situ carcinoma, and the adjacent pathological epithelium of 1 SCC and 1 BCC, clearly shows that β-HPVs do actively replicate in OTR lesional skin.

The findings obtained in this study for KTR skin lesions, showing active β-HPV infection in precancerous lesions and in the vicinity of malignant tumors, are fully compatible with a role of these viruses in the initial phases of skin carcinogenesis at least in the OTR immunosuppressed setting. These data are consistent with previous findings showing that the highest β-HPV loads are present in AK rather than in SCC, suggesting that their persistence may not be necessary for the maintenance of the transformed phenotype (Weissenborn et al. 2005). All these evidences are consistent with the "hit and run" model of β-HPV-induced skin carcinogenesis; according to this model, the viruses play a transient role in the early steps of the process being important for tumor initiation and progression, probably acting as co-factors enhancing the carcinogenic potential of UV damage, but they are likely not to be necessary for tumor maintenance. Consistent with a causal role

of β-HPVs in the initial stages of the disease, E4-positive areas were clearly visualized in 1 AK located adjacent to 1 SCC in the neck region of a patient. Furthermore the observed stimulation of basal and suprabasal cell proliferation shown by MCM7 expression in the upper epithelial layers of all the E4–positive areas may contribute, in association with other transforming agents such as UV irradiation, to the transformation process without necessarily being maintained in the more advanced disease (Peh & Doorbar 2005; Doorbar 2005; Doorbar et al. 2012; Akgül et al. 2006; Feltkamp et al. 2008). In this study was confirmed the crucial role of UV light in skin carcinogenesis, as a substantial proportion of the lesions developed by KTR cohorts were located in sun-exposed body sites.

Assuming that viral replication is more active at very early phases of carcinogenesis and perhaps when lesions are not yet even clinically evident, it is reasonable to propose that when lesions are removed, especially in more advanced stages, only some residual areas are detectable that exhibit viral replication. It is also worth mentioning that the areas of positivity were usually found in lesions which were originally split in more than one FFPE block; this procedure is currently applied when the surgical specimen is quite big and the different areas of the lesion are macroscopically evident, as it occurs when the erythematous area surrounding the tumor is also surgically removed. This context is defined as field cancerization. These assumptions might explain the low percentage of positivity found in this kind of study and the need of more thorough investigation of the perilesional skin where the likelihood of finding active β–HPV infection should be higher.

Furthermore the findings achieved in this study shows how the mere presence of viral DNA in a lesion does not necessarily indicate that the virus is driving or contributing to tumor development or progression. Detection methods allowing direct visualization of viral replication and transcriptional activity, like FISH analysis for viral genomic amplification and staining for β-HPV E4 and L1 proteins as done in this study, are fundamental to demonstrate biologically significant viruses in the context of lesional tissues. As these methods are getting compelling evidence that reactivation of ubiquitous viruses, such as β-HPVs in the OTR setting, may be actively involved in NMSC initiation and progression, more efforts should be addressed to visualize active viral replication and protein expression rather than limiting the analysis at the DNA level.

To date are present a reasonable amount of studies aimed to clarify the mechanism that lead HPVs to a persistent infection and lesion formation.

The general hypothesis is that lesion formation begins with the infection of a basal stem cell (rather than a basal transiently amplifying cell) and that the longevity of the stem cells is a key factor in the formation of a persistent lesion, with these cells being a reservoir for the infection. Cancer

development requires the virus to escape the host immune response over an extended period to lead the accumulation of genomic and genetic abnormalities. High-risk α-HPV chronic infections are characterized by long persistence in the slow-cycling epithelial stem cells, and this is a condition sine qua non for the development of HPV-associated malignancy.

Several studies have reported the ubiquitously presence of these β-HPV types on the body surface in immunosuppressed individual and in general population, with greater carriage in sun-exposed sites (e.g. forehead) that in sun-protected skin when their presence were detected in plucked hairs and skin swabs. They appear to establish a reservoir of infection in the follicular stem cells of hair bulbs, which are thought to be an immune privileged site protecting them from immune clearance and therefore enabling the persistence of multiple β-HPV types.

The capability to establish a reservoir of infection in the keratinocyte stem cell and to drive the cells to re-enter the cell-cycle in order to support viral genome amplification, represent the assumption upon which is based the second aim of this study. This aim was addressed to investigate the influence of βHPV gene expression on keratinocytes differentiation, and to pursuit this purpose it has been chosen the transgenic mice model expressing the entire early region of HPV8; so far the first model capable to develops spontaneously skin cancer in the absence of any additional co-carcinogenic treatments (Schaper et al. 2005; Akgül et al. 2006; Pfister 2003). To investigate the keratinocyte stem cells (KTCs) in the hair follicle were selected 4 different stem cell populations; which can recreate the entire hair follicle but in homeostasis support the turnover of different areas along all the hair follicle (Ghadially 2012; Cotsarelis 2006; Schepeler et al. 2014; Hsu et al. 2011; Snippert et al. 2010; Page et al. 2013). To distinguish the different population immunofluorescent staining for the KSC markers CD34, K15, LGR6, LGR5 and Lrig1 in correlation with the proliferation marker Ki67 have been established. So far unfortunately it wasn't possible to obtain a reliable LGR5 labelling.

The analysis of epidermal whole mount samples from both HPV8 transgenic mice and wild type littermates shows no difference in hair follicle length, however the width and so overall area of the hair follicles was greater in the transgenic mice correlating with expansion of the Lrig1+ area. The mean area of the Lrig1+ population was markedly increased in the βHPV8 transgenic compared to wild type mice (23,845 vs 14,907 μm^2), compared to the CD34+ (8,356 vs 8,077 μm^2), LGR6+ (60,250 vs 49,216 μm^2). In line with these findings the hair follicle keratinocyte stem cell proliferation was greater within the Lrig1+ (69 vs 55%), compared to the CD34+ (1 vs 1%), and LGR6 (29 vs 40%) populations, of βHPV8 transgenic compared to wild type mice.

These data suggesting that the early region genes of βHPV8 specifically target Lrig1+ cells, forcing the keratinocyte stem cells to proliferate and leading to an expansion of the keratinocytes

both in the hair follicle infundibulum and in the overlying interfollicular epidermis. The continuous proliferation of the Lrig1 population would compete with the normal epidermal keratinocytes and may result in its persistence over the entire epidermis, leading to the development of the field cancerization.

In summary, this work shows that careful detection of β-HPV activity markers (gene products and viral genomic amplification) at the single cell level allows to visualize their sites of replication, especially in intraepidermal precursor lesions and the marginal zones of more advanced disease. Also reveals the possible influence of the viral gene expression on the normal keratinocyte differentiation; identifying as target the specific KSC population expressing the Lrig1 marker in the β-HPV8 in-vivo model. Next step will be to validate these findings in human, looking for an Lrig1 or equivalent stem cell population expansion.

Further research are required to improve our knowledge of natural β-HPV infection and reactivation which will help understand how it is influenced by the epithelial site, UVB exposure and the immune system.

The viral lifecycle biomarkers analyzed could potentially be validated as diagnostic and prognostic tools for the assessment of the risk of keratinocyte carcinoma development in immunosuppressed patients like OTRs based on the activity status of these viruses. That would be a good starting point for the planning of adequate preventive strategies.

REFERENCES

Akgül, Baki, James C Cooke, and Alan Storey. 2006. "HPV-Associated Skin Disease." *The Journal of Pathology* 208 (2): 165–75. doi:10.1002/path.1893. http://www.ncbi.nlm.nih.gov/pubmed/16362995.

Akgül, Baki, Lucy Ghali, Derek Davies, Herbert Pfister, Irene M Leigh, and Alan Storey. 2007. "HPV8 Early Genes Modulate Differentiation and Cell Cycle of Primary Human Adult Keratinocytes." *Experimental Dermatology* 16 (7): 590–99. doi:10.1111/j.1600-0625.2007.00569.x. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2423465&tool=pmcentrez&rendertype=abstract.

Aldabagh, Bishr, Jorge Gil C. Angeles, Adela R. Cardones, and Sarah T. Arron. 2013. "Cutaneous Squamous Cell Carcinoma and Human Papillomavirus: Is There An Association?" *Dermatologic Surgery : Official Publication for American Society for Dermatologic Surgery [et Al.]* 39: 1–23. doi:10.1111/j.1524-4725.2012.02558.x.Cutaneous.

Arron, S Tuttleton, L Jennings, I Nindl, F Rosl, J N Bouwes Bavinck, D Seçkin, M Trakatelli, and G M Murphy. 2011. "Viral Oncogenesis and Its Role in Nonmelanoma Skin Cancer." *The British Journal of Dermatology* 164 (6): 1201–13. doi:10.1111/j.1365-2133.2011.10322.x. http://www.ncbi.nlm.nih.gov/pubmed/21418174.

Arron, Sarah T, J Graham Ruby, Eric Dybbro, Don Ganem, and Joseph L Derisi. 2011. "Transcriptome Sequencing Demonstrates That Human Papillomavirus Is Not Active in Cutaneous Squamous Cell Carcinoma." *Journal of Investigative Dermatology* 131 (8). Nature Publishing Group: 1745–53. doi:10.1038/jid.2011.91. http://dx.doi.org/10.1038/jid.2011.91.

Bachmann, Martin F, Urs Hoffmann Rohrer, Thomas M Kundig, Kurt Burki, Hans Hengartner, and Rolf M Zinkernagelt. 1993. "The Influence of Antigen Organization B Cell Responsiveness." *Science (New York, N.Y.)* 262 (November): 1–4.

Bauer, Birke, Paola Zigrino, Alan Storey, Cornelia Mauch, and Herbert Pfister. 2011. "Upregulation of Lipocalin-2 in Human Papillomavirus-Positive Keratinocytes and Cutaneous Squamous Cell Carcinomas." *Journal of General Virology*, 395–401. doi:10.1099/vir.0.025064-0.

Bergvall, Monika, Thomas Melendy, and Jacques Archambault. 2013. "The E1 Proteins." *Virology* 445 (1-2): 35–56. doi:10.1016/j.virol.2013.07.020. http://www.sciencedirect.com/science/article/pii/S0042682213004406.

Bernard, Hans-Ulrich. 2013. "Taxonomy and Phylogeny of Papillomaviruses: An Overview and Recent Developments." *Infection, Genetics and Evolution : Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases* 18 (August): 357–61. doi:10.1016/j.meegid.2013.03.011. http://www.ncbi.nlm.nih.gov/pubmed/23523816.

Blanpain, Cedric, William E Lowry, Andrea Geoghegan, Lisa Polak, and Elaine Fuchs. 2004. "Existence of Two Cell Populations within an Epithelial Stem Cell Niche" 118: 635–48.

Borgogna, Cinzia, Elisa Zavattaro, Marco De Andrea, Heather M Griffin, Valentina Dell'Oste, Barbara Azzimonti, Manuela M Landini, et al. 2012. "Characterization of Beta Papillomavirus E4 Expression in Tumours from Epidermodysplasia Verruciformis Patients and in Experimental Models." *Virology* 423 (2): 195–204. doi:10.1016/j.virol.2011.11.029. http://www.ncbi.nlm.nih.gov/pubmed/22217391.

Boulenouar, Selma, Christine Weyn, Melody Van Noppen, Mohamed Moussa Ali, Michel Favre, Philippe O Delvenne, Françoise Bex, Agnès Noël, Yvon Englert, and Véronique Fontaine. 2010. "Effects of HPV-16 E5, E6 and E7 Proteins on Survival, Adhesion, Migration and Invasion of Trophoblastic Cells." *Carcinogenesis* 31 (3): 473–80. doi:10.1093/carcin/bgp281. http://www.ncbi.nlm.nih.gov/pubmed/19917629.

Bouwes Bavinck, Jan Nico, Elsemieke I Plasmeijer, and Mariet C W Feltkamp. 2008. "Beta-Papillomavirus Infection and Skin Cancer." *The Journal of Investigative Dermatology* 128 (6): 1355–58. doi:10.1038/jid.2008.123. http://www.ncbi.nlm.nih.gov/pubmed/18478011.

Buck, Christopher B, Patricia M Day, and Benes L Trus. 2013. "The Papillomavirus Major Capsid Protein L1." *Virology* 445 (1-2): 169–74. doi:10.1016/j.virol.2013.05.038. http://www.sciencedirect.com/science/article/pii/S0042682213003322.

Burk, Robert D. 2014. "Human Papillomavirus Genome Variants." *Virology* 445 (0): 232–43. doi:10.1016/j.virol.2013.07.018.Human.

Campo, M S, S V Graham, M S Cortese, G H Ashrafi, E H Araibi, E S Dornan, K Miners, C Nunes, and S Man. 2010. "HPV-16 E5 down-Regulates Expression of Surface HLA Class I and Reduces Recognition by CD8 T Cells." *Virology* 407 (1): 137–42. doi:10.1016/j.virol.2010.07.044. http://www.ncbi.nlm.nih.gov/pubmed/20813390.

Cid Arregui, A., P. Gariglio, T. Kanda, and John Doorbar. 2012. "High-Risk Human Papillomaviruses : Towards a Better Understanding of the Mechanisms of Viral." *The Open Virology Journal*, 160–62.

Ciesielska, Urszula, Katarzyna Nowińska, Marzena Podhorska-okołów, and Piotr Dzięgiel. 2012. "The Role of Human Papillomavirus of Cervix Epithelial Cells and the Importance." *Adv Clin Exp Med* 21 (12): 235–44.

Connolly, Kate, Pete Manders, Peter Earls, and Richard J Epstein. 2014. "Papillomavirus-Associated Squamous Skin Cancers Following Transplant Immunosuppression: One Notch Closer to Control." *Cancer Treatment Reviews* 40 (2). Elsevier Ltd: 205–14. doi:10.1016/j.ctrv.2013.08.005. http://www.ncbi.nlm.nih.gov/pubmed/24051018.

Cotsarelis, George. 2006. “Epithelial Stem Cells: A Folliculocentric View.” *The Journal of Investigative Dermatology* 126 (7): 1459–68. doi:10.1038/sj.jid.5700376. http://www.ncbi.nlm.nih.gov/pubmed/16778814.

Crequer, Amandine, Capucine Picard, Etienne Patin, Aurelia D’Amico, Avinash Abhyankar, Martine Munzer, Marianne Debré, et al. 2012. “Inherited MST1 Deficiency Underlies Susceptibility to EV-HPV Infections.” *PloS One* 7 (8): e44010. doi:10.1371/journal.pone.0044010. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3428299&tool=pmcentrez&rendertype=abstract.

Crequer, Amandine, Anja Troeger, and Etienne Patin. 2012. “Human RHOH Deficiency Causes T Cell Defects and Susceptibility to EV-HPV Infections.” *The Journal of ...* 122 (9): 3239–47. doi:10.1172/JCI62949DS1. http://www.jci.org/articles/view/62949?key=e8f5d52df7d9367c509f.

Crosbie, Emma J, Mark H Einstein, Silvia Franceschi, and Henry C Kitchener. 2013. “Human Papillomavirus and Cervical Cancer.” *Lancet* 382 (9895). Elsevier Ltd: 889–99. doi:10.1016/S0140-6736(13)60022-7. http://www.ncbi.nlm.nih.gov/pubmed/23618600.

Cubie, Heather A. 2013. “Diseases Associated with Human Papillomavirus Infection.” *Virology* 445 (1-2): 21–34. doi:10.1016/j.virol.2013.06.007. http://www.sciencedirect.com/science/article/pii/S0042682213003565.

De Andrea, Marco, Massimo Rittà, Manuela M Landini, Cinzia Borgogna, Michele Mondini, Florian Kern, Karin Ehrenreiter, et al. 2010. “Keratinocyte-Specific stat3 Heterozygosity Impairs Development of Skin Tumors in Human Papillomavirus 8 Transgenic Mice.” *Cancer Research* 70 (20): 7938–48. doi:10.1158/0008-5472.CAN-10-1128. http://www.ncbi.nlm.nih.gov/pubmed/20876801.

De Villiers, E-m. 1998. “Dossier “ Virus and Cancer Human Papillomavirus Infections in Skin Cancers.” *Biomed & Pharmacother* 52: 26–33.

Doorbar, John. 2005. “The Papillomavirus Life Cycle.” *Journal of Clinical Virology : The Official Publication of the Pan American Society for Clinical Virology* 32 Suppl 1 (March): S7–15. doi:10.1016/j.jcv.2004.12.006. http://www.ncbi.nlm.nih.gov/pubmed/15753007.

———. 2007. “Papillomavirus Life Cycle Organization and Biomarker Selection” 23: 297–313.

———. 2013. “The E4 Protein; Structure, Function and Patterns of Expression.” *Virology* null (null). doi:10.1016/j.virol.2013.07.008. http://dx.doi.org/10.1016/j.virol.2013.07.008.

Doorbar, John, Wim Quint, Lawrence Banks, Ignacio G Bravo, Mark Stoler, Tom R Broker, and Margaret A Stanley. 2012. “The Biology and Life-Cycle of Human Papillomaviruses.” *Vaccine* 30 Suppl 5 (November): F55–70. doi:10.1016/j.vaccine.2012.06.083. http://www.ncbi.nlm.nih.gov/pubmed/23199966.

Dubina, Meghan, and Gary Goldenberg. 2009. “Viral-Associated Nonmelanoma Skin Cancers: A Review.” *The American Journal of Dermatopathology* 31 (6): 561–73. doi:10.1097/DAD.0b013e3181a58234. http://www.ncbi.nlm.nih.gov/pubmed/19590418.

Elder, Editors, E David, L Bernett, F George, and David E Elder Mb. 2005. *Lever's Histopathology of the Skin, 9th Edition*.

Euvrard, Sylvie, Jean Kanitakis, and Alain Claudy. 2003. "Skin Cancers after Organ Transplantation." *The New England Journal of Medicine* 348 (17): 1681–91. doi:10.1056/NEJMra022137. http://www.ncbi.nlm.nih.gov/pubmed/12711744.

Feltkamp, Mariet C W, Maurits N C de Koning, Jan Nico Bouwes Bavinck, and Jan Ter Schegget. 2008. "Betapapillomaviruses: Innocent Bystanders or Causes of Skin Cancer." *Journal of Clinical Virology : The Official Publication of the Pan American Society for Clinical Virology* 43 (4): 353–60. doi:10.1016/j.jcv.2008.09.009. http://www.ncbi.nlm.nih.gov/pubmed/18986829.

Ghadially, Ruby. 2012. "25 Years of Epidermal Stem Cell Research." *The Journal of Investigative Dermatology* 132 (3 Pt 2). Nature Publishing Group: 797–810. doi:10.1038/jid.2011.434. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3998762&tool=pmcentrez&rendertype=abstract.

Griffin, Heather, Zhonglin Wu, Rebecca Marnane, Vincent Dewar, Anco Molijn, Wim Quint, Christine Van Hoof, et al. 2012. "E4 Antibodies Facilitate Detection and Type-Assignment of Active HPV Infection in Cervical Disease." *PloS One* 7 (12): e49974. doi:10.1371/journal.pone.0049974. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3513315&tool=pmcentrez&rendertype=abstract.

Grulich, Andrew E, Marina T van Leeuwen, Michael O Falster, and Claire M Vajdic. 2007. "Incidence of Cancers in People with HIV/AIDS Compared with Immunosuppressed Transplant Recipients: A Meta-Analysis." *Lancet* 370 (9581): 59–67. doi:10.1016/S0140-6736(07)61050-2. http://www.ncbi.nlm.nih.gov/pubmed/17617273.

Harwood, Catherine A, and Charlotte M Proby. 2002. "Human Papillomaviruses and Non-Melanoma Skin Cancer." *Current Opinion in Infection Disease* 15: 101–14.

Hofbauer, Günther F L, Jan Nico Bouwes Bavinck, and Sylvie Euvrard. 2010. "Organ Transplantation and Skin Cancer: Basic Problems and New Perspectives." *Experimental Dermatology* 19 (6): 473–82. doi:10.1111/j.1600-0625.2010.01086.x. http://www.ncbi.nlm.nih.gov/pubmed/20482618.

Hsu, Ya-Chieh, Amalia Pasolli, and Elaine Fuchs. 2011. "Dynamics Between Stem Cells, Niche and Progeny in the Hair Follicle." *Cell* 144 (1): 92–105. doi:10.1016/j.cell.2010.11.049.Dynamics.

Hu, Lulin, Kendra Plafker, Valeriya Vorozhko, Rosemary E Zuna, Marie H Hanigan, Gary J Gorbsky, Scott M Plafker, Peter C Angeletti, and Brian P Ceresa. 2009. "Human Papillomavirus 16 E5 Induces Bi-Nucleated Cell Formation by Cell-Cell Fusion." *Virology* 384 (1). Elsevier Inc.: 125–34. doi:10.1016/j.virol.2008.10.011. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2658674&tool=pmcentrez&rendertype=abstract.

Hufbauer, Martin, Daliborka Lazić, Markus Reinartz, Baki Akgül, Herbert Pfister, and Sönke Jan Weissenborn. 2011. "Skin Tumor Formation in Human Papillomavirus 8 Transgenic Mice Is

Associated with a Deregulation of Oncogenic miRNAs and Their Tumor Suppressive Targets." *Journal of Dermatological Science* 64 (1): 7–15. doi:10.1016/j.jdermsci.2011.06.008. http://www.ncbi.nlm.nih.gov/pubmed/21763111.

Jaks, Viljar, Nick Barker, Maria Kasper, Johan H van Es, Hugo J Snippert, Hans Clevers, and Rune Toftgård. 2008. "Lgr5 Marks Cycling, yet Long-Lived, Hair Follicle Stem Cells." *Nature Genetics* 40 (11): 1291–99. doi:10.1038/ng.239. http://www.ncbi.nlm.nih.gov/pubmed/18849992.

Jensen, Kim B, Charlotte a Collins, Elisabete Nascimento, David W Tan, Michaela Frye, Satoshi Itami, and Fiona M Watt. 2009. "Lrig1 Expression Defines a Distinct Multipotent Stem Cell Population in Mammalian Epidermis." *Cell Stem Cell* 4 (5). Elsevier Ltd: 427–39. doi:10.1016/j.stem.2009.04.014. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2698066&tool=pmcentrez&rendertype=abstract.

Knipe, David, and Peter M Howley. 2007. *Fields Virology 5th Edition*.

Lazarczyk, Maciej, Patricia Cassonnet, Christian Pons, Yves Jacob, and Michel Favre. 2009. "The EVER Proteins as a Natural Barrier against Papillomaviruses: A New Insight into the Pathogenesis of Human Papillomavirus Infections." *Microbiology and Molecular Biology Reviews : MMBR* 73 (2): 348–70. doi:10.1128/MMBR.00033-08. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2698414&tool=pmcentrez&rendertype=abstract.

Leiding, Jennifer W, and Steven M Holland. 2012. "Warts and All: Human Papillomavirus in Primary Immunodeficiencies." *The Journal of Allergy and Clinical Immunology* 130 (5). Elsevier Ltd: 1030–48. doi:10.1016/j.jaci.2012.07.049. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3517887&tool=pmcentrez&rendertype=abstract.

Mahy, Brian W. J., and Marc H. V. Van Regenmortel. 2008. *Encyclopedia of Virology Third Edition*. Vol. 40. doi:10.1002/1521-3773(20010316)40:6<9823::AID-ANIE9823>3.3.CO;2-C.

McBride, Alison A. 2013. "The Papillomavirus E2 Proteins." *Virology* 445 (1-2): 57–79. doi:10.1016/j.virol.2013.06.006. http://www.sciencedirect.com/science/article/pii/S0042682213003553.

McLaughlin-Drubin, Margaret E, Jordan Meyers, and Karl Munger. 2012. "Cancer Associated Human Papillomaviruses." *Current Opinion in Virology* 2 (4). Elsevier B.V.: 459–66. doi:10.1016/j.coviro.2012.05.004. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3422426&tool=pmcentrez&rendertype=abstract.

Meyer, Thomas, and Enno Christophers. 2000. "Frequency and Spectrum of HPV Types Detected in Cutaneous Squamous-Cell Carcinomas Depend on the HPV Detection System : A Comparison of Four PCR Assays." *Dermatology* 201: 204–11.

Middleton, K., W. Peh, S. Southern, H. Griffin, K. Sotlar, T. Nakahara, a. El-Sherif, et al. 2003. "Organization of Human Papillomavirus Productive Cycle during Neoplastic Progression Provides a Basis for Selection of Diagnostic Markers." *Journal of Virology* 77 (19): 10186–

201. doi:10.1128/JVI.77.19.10186-10201.2003. http://jvi.asm.org/cgi/doi/10.1128/JVI.77.19.10186-10201.2003.

Moody, Cary a, and Laimonis a Laimins. 2010. "Human Papillomavirus Oncoproteins: Pathways to Transformation." *Nature Reviews. Cancer* 10 (8). Nature Publishing Group: 550–60. doi:10.1038/nrc2886. http://www.ncbi.nlm.nih.gov/pubmed/20592731.

Morris, Rebecca J, Yaping Liu, Lee Marles, Zaixin Yang, Carol Trempus, Shulan Li, Jamie S Lin, Janet a Sawicki, and George Cotsarelis. 2004. "Capturing and Profiling Adult Hair Follicle Stem Cells." *Nature Biotechnology* 22 (4): 411–17. doi:10.1038/nbt950. http://www.ncbi.nlm.nih.gov/pubmed/15024388.

Nindl, I, and F Rösl. 2008. "Molecular Concepts of Virus Infections Causing Skin Cancer in Organ Transplant Recipients." *American Journal of Transplantation : Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 8 (11): 2199–2204. doi:10.1111/j.1600-6143.2008.02392.x. http://www.ncbi.nlm.nih.gov/pubmed/18785959.

Nindl, Ingo, Marc Gottschling, and Eggert Stockfleth. 2007. "Human Papillomaviruses and Non-Melanoma Skin Cancer: Basic Virology and Clinical Manifestations." *Disease Markers* 23 (4): 247–59. http://www.ncbi.nlm.nih.gov/pubmed/17627060.

Oberyszyn, Tatiana M. 2008. "Non-Melanoma Skin Cancer: Importance of Gender, Immunosuppressive Status and Vitamin D." *Cancer Letters* 261 (2): 127–36. doi:10.1016/j.canlet.2008.01.009. http://www.ncbi.nlm.nih.gov/pubmed/18267352.

Orth, Gérard. 2006. "Genetics of Epidermodysplasia Verruciformis: Insights into Host Defense against Papillomaviruses." *Seminars in Immunology* 18 (6): 362–74. doi:10.1016/j.smim.2006.07.008. http://www.ncbi.nlm.nih.gov/pubmed/17011789.

Page, Mahalia E, Patrick Lombard, Felicia Ng, Berthold Göttgens, and Kim B Jensen. 2013. "The Epidermis Comprises Autonomous Compartments Maintained by Distinct Stem Cell Populations." *Cell Stem Cell* 13 (4): 471–82. doi:10.1016/j.stem.2013.07.010. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3793873&tool=pmcentrez&rendertype=abstract.

Peh, Woei Ling, and John Doorbar. 2005. "Detection of Papillomavirus Proteins and DNA in Paraffin-Embedded Tissue Sections." *Methods in Molecular Medicine* 119 (January): 49–59. doi:10.1385/1-59259-982-6:049. http://www.ncbi.nlm.nih.gov/pubmed/16350396.

Pfefferle, Regina, Gian Paolo Marcuzzi, Baki Akgül, Hans Udo Kasper, Falko Schulze, Ingo Haase, Claudia Wickenhauser, and Herbert Pfister. 2008. "The Human Papillomavirus Type 8 E2 Protein Induces Skin Tumors in Transgenic Mice." *The Journal of Investigative Dermatology* 128 (9): 2310–15. doi:10.1038/jid.2008.73. http://www.ncbi.nlm.nih.gov/pubmed/18401427.

Pfister, Herbert. 2003. "Chapter 8: Human Papillomavirus and Skin Cancer." *Journal of the National Cancer Institute. Monographs*, no. 31 (January): 52–56. http://www.ncbi.nlm.nih.gov/pubmed/12807946.

Pfister, Herbert, Pawel G Fuchs, Slawomir Majewski, Stefania Jablonska, Iwona Pniewska, and Magdalena Malejczyk. 2003. "High Prevalence of Epidermodysplasia Verruciformis-

Associated Human Papillomavirus DNA in Actinic Keratoses of the Immunocompetent Population." *Archives of Dermatological Research* 295 (7): 273–79. doi:10.1007/s00403-003-0435-2. http://www.ncbi.nlm.nih.gov/pubmed/14618345.

Piselli, P, G Busnach, F Citterio, M Frigerio, E Arbustini, P Burra, A D Pinna, et al. 2009. "Risk of Kaposi Sarcoma after Solid-Organ Transplantation: Multicenter Study in 4,767 Recipients in Italy, 1970-2006." *Transplantation Proceedings* 41 (4): 1227–30. doi:10.1016/j.transproceed.2009.03.009. http://www.ncbi.nlm.nih.gov/pubmed/19460525.

Roden, Richard B S, Douglas R Lowy, and John T Schiller. 1997. "Papillomavirus Is Resistant to Desiccation." *The Journal of Infectious Diseases*, 1076–79.

Roman, Ann, and Karl Munger. 2013. "The Papillomavirus E7 Proteins." *Virology* 445 (1-2): 138–68. doi:10.1016/j.virol.2013.04.013. http://www.sciencedirect.com/science/article/pii/S0042682213002225.

Schaper, Inke Diana, Gian Paolo Marcuzzi, Sönke Jan Weissenborn, Jan Weissenborn, and Hans Udo Kasper. 2005. "Development of Skin Tumors in Mice Transgenic for Early Genes of Human Papillomavirus Type 8." *Cancer Res.*, 1394–1400.

Schepeler, Troels, Mahalia E Page, and Kim B Jensen. 2014. "Heterogeneity and Plasticity of Epidermal Stem Cells." *Development (Cambridge, England)* 141 (13): 2559–67. doi:10.1242/dev.104588. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4067958&tool=pmcentrez&rendertype=abstract.

Schiller, John T, and Christopher B Buck. 2011. "Cutaneous Squamous Cell Carcinoma: A Smoking Gun but Still No Suspects." *The Journal of Investigative Dermatology* 131 (8). Nature Publishing Group: 1595–96. doi:10.1038/jid.2011.151. http://www.ncbi.nlm.nih.gov/pubmed/21753763.

Schiller, John T, and Douglas R Lowy. 2012. "Understanding and Learning from the Success of Prophylactic Human Papillomavirus Vaccines." *Nature Reviews. Microbiology* 10 (10): 681–92. doi:10.1038/nrmicro2872. http://www.ncbi.nlm.nih.gov/pubmed/22961341.

Schulz, Thomas F. 2009. "Cancer and Viral Infections in Immunocompromised Individuals." *International Journal of Cancer. Journal International Du Cancer* 125 (8): 1755–63. doi:10.1002/ijc.24741. http://www.ncbi.nlm.nih.gov/pubmed/19588503.

Snippert, Hugo J, Andrea Haegebarth, Maria Kasper, Viljar Jaks, Johan H van Es, Nick Barker, Marc van de Wetering, et al. 2010. "Lgr6 Marks Stem Cells in the Hair Follicle That Generate All Cell Lineages of the Skin." *Science (New York, N.Y.)* 327 (5971): 1385–89. doi:10.1126/science.1184733. http://www.ncbi.nlm.nih.gov/pubmed/20223988.

Tinsley, J. M., C. Fisher, and P. F. Searle. 1992. "Abnormalities of Epidermal Differentiation Associated with Expression of the Human Papillomavirus Type 1 Early Region in Transgenic Mice." *Journal of General Virology* 73 (5): 1251–60. doi:10.1099/0022-1317-73-5-1251. http://vir.sgmjournals.org/cgi/doi/10.1099/0022-1317-73-5-1251.

Tommasino, Massimo. 2014. "The Human Papillomavirus Family and Its Role in Carcinogenesis." *Seminars in Cancer Biology* 26 (June). Elsevier Ltd: 13–21. doi:10.1016/j.semcancer.2013.11.002. http://www.ncbi.nlm.nih.gov/pubmed/24316445.

Trempus, Carol S, Rebecca J Morris, Carl D Bortner, George Cotsarelis, Randall S Faircloth, Jeffrey M Reece, and Raymond W Tennant. 2003. "Enrichment for Living Murine Keratinocytes from the Hair Follicle Bulge with the Cell Surface Marker CD34." *The Journal of Investigative Dermatology* 120 (4): 501–11. doi:10.1046/j.1523-1747.2003.12088.x. http://www.ncbi.nlm.nih.gov/pubmed/12648211.

Van Doorslaer, Koenraad. 2013. "Evolution of the Papillomaviridae." *Virology* 445 (1-2): 11–20. doi:10.1016/j.virol.2013.05.012. http://www.sciencedirect.com/science/article/pii/S0042682213002924.

Vande Pol, Scott B, and Aloysius J Klingelhutz. 2013. "Papillomavirus E6 Oncoproteins." *Virology* 445 (1-2): 115–37. doi:10.1016/j.virol.2013.04.026. http://www.sciencedirect.com/science/article/pii/S0042682213002481.

Vandermark, Erik R., Krysta A. Deluca, Courtney R. Gardner, Daniel F. Marker, Cynthia N. Schreiner, David A. Strickland, Katelynn M. Wilton, Sumona Mondal, and Craig D. Woodworth. 2012. "Human Papillomavirus Type 16 E6 and E 7 Proteins Alter NF-kB in Cultured Cervical Epithelial Cells and Inhibition of NF-kB Promotes Cell Growth and Immortalization." *Virology* 425 (1): 53–60. doi:10.1016/j.virol.2011.12.023.Human.

Venuti, Aldo, Francesca Paolini, Lubna Nasir, Annunziata Corteggio, Sante Roperto, Maria S Campo, and Giuseppe Borzacchiello. 2011. "Papillomavirus E5: The Smallest Oncoprotein with Many Functions." *Molecular Cancer* 10 (1). BioMed Central Ltd: 140. doi:10.1186/1476-4598-10-140. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3248866&tool=pmcentrez&rendertype=abstract.

Viarisio, Daniele, Karin Mueller-decker, Ulrich Kloz, Birgit Aengeneyndt, Annette Kopp-schneider, Tarik Gheit, Christa Flechtenmacher, Lutz Gissmann, and Massimo Tommasino. 2011. "E6 and E7 from Beta Hpv38 Cooperate with Ultraviolet Light in the Development of Actinic Keratosis-Like Lesions and Squamous Cell Carcinoma in Mice." *PLoS Pathogens* 7 (7). doi:10.1371/journal.ppat.1002125.

Villiers, Ethel-michele De, Claude Fauquet, Thomas R Broker, and Hans-ulrich Bernard. 2004. "Classification of Papillomaviruses." *Molecular Biology* 324: 17–27. doi:10.1016/j.virol.2004.03.033.

Wallace, Nicholas A, Kristin Robinson, Heather L Howie, and Denise A Galloway. 2012. "HPV 5 and 8 E6 Abrogate ATR Activity Resulting in Increased Persistence of UVB Induced DNA Damage." *PLoS Pathogens* 8 (7): e1002807. doi:10.1371/journal.ppat.1002807. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3395675&tool=pmcentrez&rendertype=abstract.

Wang, Joshua W, and Richard B S Roden. 2013. "L2, the Minor Capsid Protein of Papillomavirus." *Virology* 445 (1-2): 175–86. doi:10.1016/j.virol.2013.04.017. http://www.sciencedirect.com/science/article/pii/S0042682213002262.

Weissenborn, Soenke Jan, Ingo Nindl, Karin Purdie, Catherine Harwood, Charlotte Proby, Judy Breuer, Slawomir Majewski, Herbert Pfister, and Ulrike Wieland. 2005. "Human Papillomavirus-DNA Loads in Actinic Keratoses Exceed Those in Non-Melanoma Skin Cancers." *The Journal of Investigative Dermatology* 125 (1): 93–97. doi:10.1111/j.0022-202X.2005.23733.x. http://www.ncbi.nlm.nih.gov/pubmed/15982308.

Wentzensen, Nicolas, Svetlana Vinokurova, and Magnus Von Knebel Doeberitz. 2004. "Systematic Review of Genomic Integration Sites of Human Papillomavirus Genomes in Epithelial Dysplasia and Invasive Cancer of the Female Lower Genital Tract." *Cancer Research* 64: 3878–84.

Wisgerhof, Hermina C, Jeroen R J Edelbroek, Johan W de Fijter, Geert W Haasnoot, Frans H J Claas, Rein Willemze, and Jan N Bouwes Bavinck. 2010. "Subsequent Squamous- and Basal-Cell Carcinomas in Kidney-Transplant Recipients after the First Skin Cancer: Cumulative Incidence and Risk Factors." *Transplantation* 89 (10): 1231–38. doi:10.1097/TP.0b013e3181d84cdc. http://www.ncbi.nlm.nih.gov/pubmed/20410852.

Yang, Binh H, Freddie I Bray, D Maxwell Parkin, John W Sellors, and Zuo-Feng Zhang. 2004. "Cervical Cancer as a Priority for Prevention in Different World Regions: An Evaluation Using Years of Life Lost." *International Journal of Cancer. Journal International Du Cancer* 109 (3): 418–24. doi:10.1002/ijc.11719. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4167424&tool=pmcentrez&rendertype=abstract.