UNIVERSITA' DEGLI STUDI DI NAPOLI FEDERICO II

DIPARTIMENTO DI MEDICINA VETERINARIA E PRODUZIONI ANIMALI

DOTTORATO DI RICERCA IN BIOLOGIA, PATOLOGIA ED IGIENE AMBIENTALE IN MEDICINA VETERINARIA

XXVII CICLO

Coordinatore: Chiar.mo Prof. G. Cringoli

TESI DI DOTTORATO

"Felis catus papillomavirus type 2 E6 and E7 oncogenes are actively transcribed in feline skin cancer *in vivo* and display transforming properties *in vitro*"

Tutor:

Chiar.mo Prof. G. Borzacchiello

Candidato:

Dr. Gennaro Altamura

ANNO ACCADEMICO 2014-2015

INDEX

1. INTRODUCTION

1.1 Papillomaviruses: phylogenesis and taxonomy

Papillomaviruses (PVs) are DNA viruses infecting a large number of mammals including humans and considered to be oncogenic; indeed, they were firstly detected in rabbits and cattle and in human skin warts (Shope and Hurst, 1933; Rowson and Mahy, 1967; Olson et al., 1951; zur Hausen, 2009). Their tropism is restricted to skin, squamous mucosal epithelia of mouth, anus and genital tract and are usually considered to be strictly specie-specific, although some rare cases of cross infection are known (Campo, 2002). Phylogenetic studies strongly suggest linked evolution between PVs and their host specie as a possible mechanism defining the evolutionary history of the virus, meaning that major part of evolutionary diversification of PVs must have proceeded thrugh molecularly unknown mechanisms that restrict PVs to their host species (Bernard, 2013).

PVs were originally grouped toghether with polyomaviruses in the family of "Papovaviridae", based on shared rare features: both virus groups harbour a double stranded circular DNA genome with a size of 5kb for polyomaviruses and 8kb for PVs and have a non-enveloped icosahedral capside (Bernard, 2013). However, the different structural and functional genomic organization of PVs and the unique combination of biological properties led the International Committee on Taxonomy of Viruses (ICTV) to classify this group as a separate "family", named Papillomaviridae (De Villiers et al., 2004).

Many of the known PVs were originally defined using the abbreviation of the name referred to the host species followed by a number indicating the "type" (e.g human PV type

16 is termed HPV16); however, today the scientific name of the host species is preferred to the informal and common one when a new PV is identified (Bernard, 2010).

The phylogenetic tree of Papillomaviridae family includes 189 "types" forming 70 species (corresponding to the minor branches) grouped in 30 genera (represented by the major branches) (**Figure 1**) (Bernard, 2010).

According to the last official classification, 120 PVs types are isolated from humans, 64 from mammals, 3 from birds and 2 from reptiles (Bernard, 2010). The classification of PVs was originally based on the biological and clinical properties, but taxonomic decisions were ultimately based on the phylogenetic tree topology. This new way of classification is based on the sequence of L1 gene: a PV is defined as a new "type" when a difference of at least 10% is detectable with all the known types, whilst isolates with 2-10% diversity are termed as "subtypes" (Bernard, 2013). Several studies suggest that genomic variation at this level are extremely rare and probably of little biological and clinical significance, in contrast to genomic variation up to 2%, termed as "variants" (Calleja-Macias et al., 2005). The 30 genera are indicated by Greek letters (*alpha, beta, gamma, delta, epsilon* and so on) and the species by addition of a number. For instance, the HPV16, the causative agent of most women cervical cancers, indicates the HPV type 16, belonging (toghether with other types) to the *alpha* papillomavirus genus, species *alpha* 9 (Bernard, 2013).

However, the great diversity among PVs and the putative differences in the pathogenetic mechanisms are still object of phylogenetic and biology studies leading to the discovery of new animal and human PVs types, so that the phlylogenetic tree of Papillomaviridae has to be continuously updated.

Figure 1

Philogenetic tree of the 189 papillomavirus types based on the nucleotide sequence of the L1 gene (modified from Bernard et al., 2010). The sequence identities decrease from close to 90% to about 45% in intraspecies, interspecies and intergeneric comparison. *Alpha* and *beta* genera (red squares) contains most of oncogenic HPVs

1.2 Genomic Organization of PVs

PVs are generally characterized by a proteic icosahedric capside with a 55 nm diameter, made of 72 capsomeres, each resulting from the assembling of 5 monomers of L1 structural protein. The capside encloses a double-stranded circular DNA genome of approximatively 8kb, whose structural and functional organization is highly conserved among the different PVs. It contains 8 open reading frames (ORFs), all being transcribed from one DNA strand only and encoding for a specific mRNA and its relative protein (Fehrmann and Laimins, 2003). Each of these proteins display a specific function in the life cycle of the virus. The HPV16 genome can be shown as the prototype of most of PVs genomes (**Figure 2**), which is organized in 3 functionally different regions:

1) E (early) region, showing different ORFs which encode for early proteins E1, E2, E4, E5, E6, E7, E8. Taken togheter, they consitute the 45% of the whole viral genome. These proteins are basically expressed in the early steps of viral cycle, being involved in viral replication and gene transcription. They also display transforming properties through molecular mechanisms leading to inhibition of cell death and triggering unscheduled cell proliferation.
2) L (late) region, it accounts for 40% of viral genome and is highly conserved. It is constituted by 2 ORFs encoding for major and minor capsidic structural proteins, L1 and L2, respectively. These proteins are responsible for the evocation of host immune response.
3) LCR (Long Control Region) also known as NCR (Non Coding Region), representing 7-10% of viral genome, it does not show any transcriptional activity, but containing the replication origins as well as transcriptional enhancer and promoter sequences playing a

role in the control of viral replication and transcription. Generally, it is positioned between the end of L1 gene and the beginning of E6 ORF.

Despite a high degree of conservation in the genome layout, a number of reported PVs show a deviating genomic organization, but the core ORFs E1, E2, L1 and L2 are present in all papillomaviruses known so far (Bernard 2013).

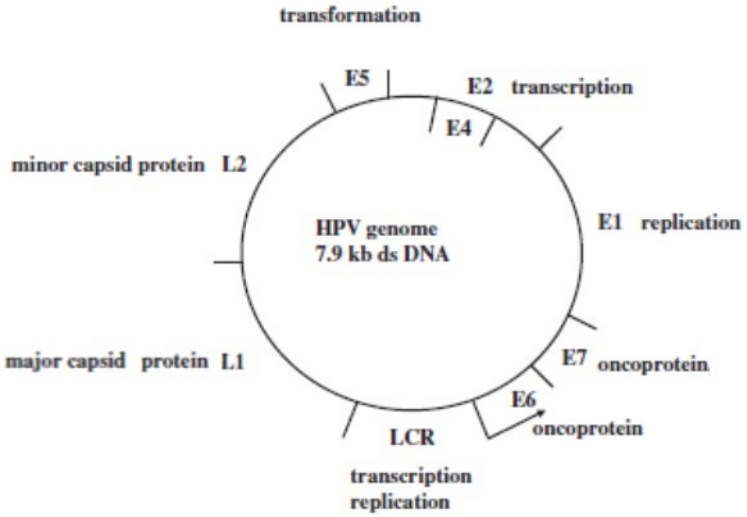

Figure 2

The genome of HPV16 has a size and gene arrangement typical for most of papillomaviruses. Some few types have rearrangement or replacement of E6 and E7 or may lack E5 (modified from Bernard, 2013).

1.3 PVs life cycle

Several studies have shown that DNA of the most prevalent HPVs can be readily detected on the healthy skin with no signs of apparent infection (Doorbar, 2005, Boxman et al., 2001). Similar findings have been exstensively reported also for animals, suggesting that PVs may be ubiquitarious inert hosts of human and animal skin (Antonsson et al., 2000; Antonsson et al., 2002). To actively undergo the infectious cycle, PVs particles must have access to cells in the basal layer. In this regard, the hair follicles may represent an important site of entry (Boxman et al., 2001). It is thought that the initial infection requires a break in the stratified epithelium. Such breaks may not be readily apparent, and may occur under conditions where the skin is subjected to environments where micro traumas may develop (Doorbar, 2005). Indeed, infectious cycle of PVs begins with an injury to the cutaneous or mucosal squamous epithelium, exposing the basement membrane and basal cells layer to the virus (Doorbar, 2005). The exact mechanism of virust entry in the cells is not fully understood, as well as the cell surface receptor allowing its attachment to the cell membrane; a possible mechanism proposed is the internalization via endocytosis of clathrin coated vescicles, a process requiring several hours (Giroglou et al., 2001; Culp and Christensen, 2004; Day et al., 2003, Selinka et al., 2002). The viral uncoating is facilitated by the disruption of intracapsomeric disulphide bonds in the reducing enviroment of the cells, allowing viral DNA to be transported into the nucleus (Li et al., 1998)

. At the initial stage, the viral genome replicates as an episomal plasmid to low copy numbers in proliferative basal epithelial cells. Expression of E1 and E2 genes is essential to mantain the viral genome as episome and to facilitate the correct segregation of genomes during cell division (Wilson et al. 2002; You et al., 2004). In uninfected epithelia, upon cell

division, daughter cells move up to the spinous layer and initiates differentiation. During PVs infection, the expression of early genes like E5, E6 and E7, forces a subset of infected cells in the spinous layer to aberrantly re-enter the S-phase of cell cycle to enhance viral DNA replication, so that the restraint on cell cycle progression is abolished and normal terminal differentiation is delayed (Sherman et al., 1997). Indeed, E5, E6 and E7 genes products are considered as oncoproteins, since they stimulate cell proliferation and cell survival (Doorbar, 2005). As cells with amplified viral DNA move to upper epithelial layers, Late genes L1 and L2 are expressed immediately after E4, to encode the proteins aimed to encapsidate viral DNA (Doorbar et al., 1997). The major capsid protein L1 is expressed after L2; they both allow the assembly of infectious particles (Florin et al., 2002). Subsequently, infectious viral particles are released from the surface of stratified epithelium within desquamated cells, allowing the viral spreading mostly through direct contact (**Figure 3**).

Although genome amplification and packaging is necessary for the formation of new replicative virions, infection can have different outcomes. Usually, the viral replication is slow and PVs infections remain asympthomatic, due to an efficient immune response by the host, thus slow subclinical infections appear to be the most common exitus. However, the onset of this response may be variable depending on the conditions of the immune system of the infected animal: a delay in the development of a response may trigger rapid replication of PVs, which in turn increases basal cell replication and terminal differentiation, resulting in the development of an exophytic mass known as papilloma or wart (Doorbar, 2005). Usually they persist for a relatively short period in terms of weeks (generally in all the species including humans), until they regress upon the rescue of immune system functionality, mostly driven by lymphocyte infiltration (Nicholls et al., 2001). Following regression, PV DNA is thought to

remain in the basal epithelial cells establishing a latent infection, prone to be reactivated when levels of immunosurveillance decline (Doorbar, 2005). Whether the immune system remains unable to develop an efficient cell mediated response for a prolonged time, a florid infection followed by lesion persistence or neoplastic transformation can occurr (Campo, 2002).

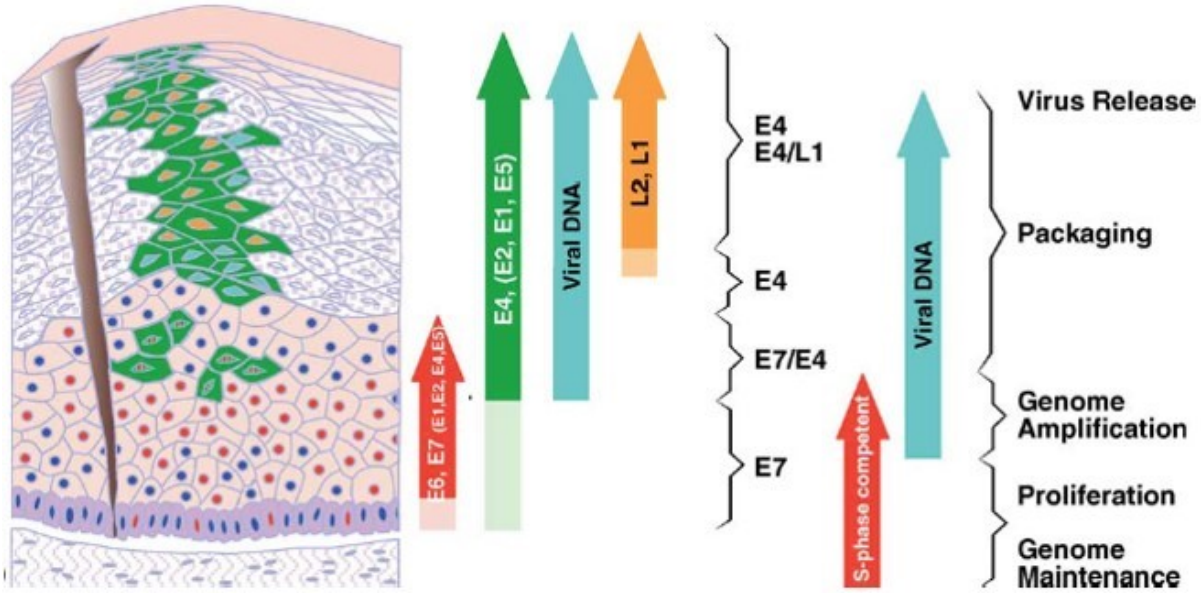

Figure 3

Schematic representation of papillomavirus life cycle (modified from Doorbar, 2005). Viral genes expression profiles are show for each phase of the infectious cycle.

1.4 Papillomaviruses and cancer

PVs are considered to be oncogenic: the first evidences of their carcinogenic potential came from experimental observations during the early decades of '900 on human and animal tumours. However, it was since 1960s that, with the evolution of molecular techniques, there was a great acceleration in the discovering of causal association of PVs with different forms of cancer so that, today, the carcinogenic role of several PVs is well established in humans and animals (see zur Hausen 2009).

Most of HPVs known so far are classified into two genera: *alpha* and *beta*. Among the genus *alpha* HPVs there are cutaneous types inducing benign, non-genital skin warts, and mucosal types, divided in two groups: the low-risk (LR) types inducing genital warts and laryngeal papillomas and the high-risk (HR) HPVs which are causative agents of cervical, anogenital and oropharyngeal cancers (reviewed in Howley, 2013). HPV16 and HPV18 are the most frequently types detected in cervical cancers worldwide, thus the majority of the biological studies have been focused on these two types. The oncogenic potential of HR HPVs is due to the transforming properties of E6 and E7 viral oncogenes, which are invariably highly expressed throughout the development of cervical cancer (see below).

The *beta* HPV types were firstly isolated in skin cancer-prone patients suffering from an immunity disorder known as Epidermodysplasia verruciformis (EV). These patients suffer from persistent HPV infection of the skin causing extensive flat warts, mostly progressing to squamous cell carcinoma (SCC) in sun-exposed regions. Immunosuppressed organ transplant recipients are also predisposed to *beta* HPVs-induced skin cancer; additionally, *beta* HPVs types infection is very common in the skin of healthy individuals, suggesting that immune system decline and other co-factors such as UV exposure are necessary for development of

HPV associated skin cancer. The precise role of *beta* HPVs in skin cancer is not fully understood so far, especially in non EV patients, however a number of studies on the transforming properties of *beta* types E6 and E7 oncogenes confirmed their carcinogenic potential (reviewed in Howley and Pfister, 2015).

Among animal PVs, 13 bovine papillomavirus (BPVs) types have been identified and associated to different skin and mucosal cancers in bovids and other species. The most studied BPVs types are BPV1 and BPV2 (genus *delta*) which cause cutaneous fibropapillomas and urinary bladder cancer and BPV4 (genus *Xi*) that induces esophageal cancer in cattle. As for the human counterpart, additional co-factor are required for carcinogenesis: the immunosuppressive and carcinogenic chemicals contained in the bracken fern are known to play a key role in BPVs associated bovine cancer. Although PVs are generally species specific, BPV1, 2 and recently 5 and 13 represent an exception, being able to infect buffaloes, equines, yaks, tapirs, giraffes, donkeys, bisons and zebras (for review see Bocaneti et al., 2014). In equids, BPV1 and 2 are known to contribute to the development of a fibroblastic dermal tumour termed sarcoid, the most common skin neoplasia in horses worldwide (reviewed in Nasir and Brandt, 2013). Differently from HPVs, the main oncogene of BPVs is E5 (see below) with a minor role played by E6 and E7 (see Corteggio et al., 2013 for review).

Fifteen canine PVs (CPVs) have been recognized so far, belonging to different genera. Although less clearly established and not extensively deepened through molecular studies, a role for CPVs infection has been hypothesized also for oral and skin epithelial benign lesions, possibly progressing to malignant cancer in dogs (for review see Rector and Van Ranst 2013 and Munday and Kiupel 2009).

The first evidences of a role for cottontail rabbit PV (CRPV) in the early ‘900 have been strenghtened by dozens of studies throughout the decades. CRPV induces cutaneous papillomas which in most of the cases progress to invasive SCC without any other known co-factors. Both E6 and E7 encoded by CRPV display transforming properties (Ganzenmueller et al., 2008).

1.5 Viral proteins

As stated before, PVs genome encodes for early and late proteins, differently involved the PVs lyfe cycle. Indeed, whilst L1 and L2 are basically structural proteins forming the viral capside, early proteins show a wide range of molecular function during the PVs infection.

1.5.1 Early proteins

Here, a comprehensive overview of early genes functions is reported. As thoroughly described below, the E1, E2 and E4 proteins display regulative functions in specific stages of the viral lyfe cycle, such as viral transcription and replication or viral particles release, while E5, E6 and E7 are considered to be oncogenes for their tranforming activities including the impairing of apoptosis and the increasing of cellular proliferation whithin the infected epithelia.

E1 proteins

The E1 has a molecular weight of 68 KDa and is expressed at low levels in PVs infected cells. Its main function is to bind the replication origin contained in the LCR and is characterized by an ATPase and 3'-5' helicase activity (Hughes and Romanos, 1993; Seo et al., 1993; Yang et al., 1993).

E1 recognizes and weakly binds to AT rich regions within the replication origins of PVs (Frattini and Laimins, 1994; Muller et al., 1997). This binding is stabilized through forming a complex with E2 protein (Frattini and Laimins, 1994; Dixon et al., 2000; Lu et al., 1993). The E1 binding sites (E1BS) are flanked by two E2 binding sites (E2BS) and E2

allows the correct positioning on the origin of replication by E1, which in turns forms hexamers with high affinity for DNA (Sedman and Stenlund, 1998).

Additionally, E1 proteins are able to bind the DNA polimerase α of the host cell and to trigger the recruitment of protein complexes involved in DNA replication to the viral replication origin (Conger et al., 1999; Masterson et al., 1998).

E4 proteins

Generally, the papillomavirus E4 ORF is contained within the E2 ORF, with the primary E4 gene-product being translated from a spliced mRNA that includes the E1 initiation codon and adjacent sequences; indeed, the E4 is located centrally within the E2 gene (Wang et al., 2011). Although a number of minor E4 transcripts have been reported, the product of the most abundant full-lenght mRNA has been most extensively analysed (Chow et al., 1987; Doorbar et al., 1990; Wang et al., 2011). During the papillomavirus life cycle, the E4 protein is expressed at the onset of vegetative viral genome amplification as the late stages of infection begin. E4 contributes to genome amplification success and virus synthesis, with its high level of expression suggesting additional roles in virus release and/or transmission. Thus, the E4 protein can serve as a biomarker of active virus infection (Middleton, 2003; Borgogna et al., 2012). In some cutaneous lesions, E4 is expressed at higher levels and invariably before the virion coat proteins L1 and L2. The E4 proteins of the *beta*, *gamma* and *mu* HPVs assemble into distinctive cytoplasmic, and sometimes nuclear, inclusion granules (Laurent et al., 1982; Egawa, 1994). The E4 structure and function is affected by cellular kinases (protein kinase A, Cyclin-dependent kinase, members of the MAP Kinase family and protein kinase C) as the infected cell progresses through the S and G2 cell cycle phases, but also by

proteases as the cells undergo through terminal differentiation (Doorbar, 2013). For HPV16, these kinases regulate the E4 main function, that is the association with the cellular keratin network, and eventually also its cleavage by the protease calpain which allows assembly into amyloid-like fibres (Doorbar, 2013; McIntosh et al., 2008); this reorganisation of the keratin network makes it mechanically unstable and thus facilitates the release of viral particles (Doorbar et al., 1991).

Although different in the primary amino acidic sequences, the E4 proteins share a recognisable modular organisation and pattern of expression, which may underlie conserved functions and regulation. Assembly into higher-order multimers and suppression of cell proliferation are common to all E4 proteins known so far. Although not yet formally demonstrated, a role in virus release and transmission remains a likely function for E4 (Brown and Bryan, 2000; Brown et al., 2006; Bryan and Brown, 2001).

E2 proteins

The full-lenght E2 protein is an essential regulatory protein encoded by all PVs Additionally, all the viruses potentially encode for shorter E2 species (McBride, 2013). E2 are DNA binding proteins which specifically recognize and bind to a 12 base pairs (bp) motifs located within the LCR. These proteins display multiple functions, mostly associated with transcription and replication of viral genome and are mostly expressed during the early and intermediate stages of the viral lyfe cycle (McBride, 2013). The E2 proteins are the main transcriptional regulators of the PVs, acting by recruiting cellular factors to the viral genome; thus E2 may activate or repress transcription depending on the context of these binding partners (Choe et al., 1989; McBride, 2013). On the contrary, the shorter E2 isoforms deriving

from alternative splicing of the mRNA, invariably act as transcriptional repressors by different mechanisms (i.e. dimerization with full-lenght E2 or competition for binding sites) (Barsoum et al., 1992; Lim et al., 1998). The fine regulatory function of E2 can be disrupted by mutation or integration of the viral genome, leading to alleviation of E2-mediated repression and increased expression of E6 and E7 transforming genes of HR PVs (Bernard et al., 1989). Low levels of E6 and E7 upon E2 transcriptional repression are essential for keeping a non-transformed phenotype in non-productive stages of PVs infection, thus lost of E2 by viral genome integration strongly contributes to malignant progression in cervical cancer in women (McBride AA, 2013).

Nevertheless, the E2 proteins play a key role in the intiation of viral replication by loading the E1 helicase onto the replication origin, which contains an E1BS and at least one E2 binding sequence (Sanders and Stenlund, 2000; Ustav et al., 1993). E2 is essential for viral replication also through displacing nucleosomes from the origin to alleviate transcriptional repression (Li and Botchan, 1994). Additionally, viral replication occurr in nuclear foci, and the formation of these foci is dependent upon E2 expression (Fradett-Turkotte et al., 2011). On the contrary, several alternative shorter E2 isoforms act as inhibitors of viral DNA replication as well as transcriptional repressors: this is believed to be important in the manteinance phase of replication or for episomal manteinance (Lace et al., 2008). In this regard, another important function of E2 is to tether viral genome (through binding E2BS of viral DNA) to mitotic chromosomes through protein-protein interaction (through different E2 protein motifs) in order to facilitate retention, manteinance and partitioning of the virale genome (Bastien and McBride., 2000; Kurg et al., 2010).

Several studies suggest additional functions for E2, among these a role in the packaging of viral DNA, post-transcriptional RNA processing, regulation of cellular gene expression or growth inhibition and apoptosis both in PVs positive and negative cells through different mechanisms (Zhao et al, 2000; Johansson et al., 2012; McBride, 2013; Johung et al., 2007).

In summary, expression and functional activity of E2 indicates that a PV is biologically active within an infected epithelium.

E5 proteins

Many but not all the PVs encode E5 proteins, short membrane-associated proteins with potent transforming activity but poorly defined roles in productive infection. Deriving from a spliced mRNA initiating upstream of the E2 gene, this protein varies from a size of approximatively 40 to 85 amino acids being predominantly hydrophobic, clusterd in one or more putative transmembrane domains. Despite the globular structure conferred by the aminoacidic composition and the very small size, E5 show an energetically favorable shape which favours the disposition of side-chain outside the core of the protein, facilitating the interaction with a great numbers of cellular targets (DiMaio and Petti, 2013).

The most studied E5 are those from BPV1 and HPV16: despite they show minimal sequences similarity with each other, they display overlapping transforming activities and share molecular targets (DiMaio and Petti, 2013). BPV1 E5 is the prototype of E5 protein encoded by the fibropapillomaviruses belonging to the genus *delta* (van Doorslaer, 2013). It is expressed in basal keratinocytes and superficial differentiated keratinocytes of the highly proliferative epithelia within bovine skin warts, as well as in the fibroblasts of dermal

prominent component of these tumuors (Burnett et al., 1992). Here, as in bovine urinary bladder tumours and BPV1 induced equine sarcoid, the main tranforming activity of this E5 is linked and dependent by its ability to bind to and activate the plateled-derived growth factor beta receptor (PDGFβR); this highly specific interaction takes place in the inner intermembranes space, as E5 forms homodimers which can recruit two monomers of PDGFβR that in turn may auto-transphosphorylate thus activating a signaling cascade resulting in recruitment and activation of cellular singalling proteins and finally mitogenesis (for review see Venuti et al., 2011 and Corteggio et al., 2013). Another transforming activity of BPV1 E5 is linked to its localization in the membrane of Golgi apparatus, where it binds to the subunit c of the vacuolar ATPase, causing the alkalinization and swelling of this and other vescicular structures (Goldstain et al., 1992; Tsirimonaki et al., 2006)

Additionally, BPV1 E5 contributes to the evasion of immune response by infected cells through inhibition of cell surface expression of major histocompatibility complex class I (MHCI) antigens, by its transcriptional repression and impairment of intracellular trafficking (Ashrafi et al., 2006; Marchetti et al., 2002).

HPVs E5 is highly hydrophobic as BPV1 E5, although no sequence homology is shared. Not all the HPVs encode E5 and E5 proteins from different HPV types display different biological and transforming properties (Bravo and Alonso, 2004). The most potent E5 among HPVs is HPV16 E5, which localizes mostly in the endoplasmic reticulum (ER), Golgi apparatus (GA), plasma membrane and nuclear envelope (DiMaio and Petti, 2013). However, this E5 display a weak transforming activity when compared to BPV1 E5, since it is more likely to cooperate with other oncogenes such as E6 and E7 in malignant transformation (DiMaio and Petti, 2013); its main oncogenic property is to sustain the

activation of epidermal growth factor receptor (EGFR) pathway through different mechanism, for instance by increasing its expression on cell surface or reducing its degradation, thus sensitising cells to mitogenic signaling by EGF or stimulating the expression or activation of signaling molecules involved in the downstream pathway (Tomakidi et al., 2000; Zhang et al., 2005). Additionally, as BPV1 E5, HPV6 and HPV16 E5 are able to bind the subunit c of the vacuolar ATPase, but the role of this interaction in oncogenesis is not yet fully understood (DiMaio and Petti, 2013). Another function shared with BPV1 E5 is the impairing of MHC antigens expression on cell surface, acting at different levels of their cellular trafficking, mainly through physical binding to MHC molecules and cellular chaperones (Ashrafi et al., 2006; DiMaio and Petti, 2013). HPV16 E5 is also able to counteract apoptosis through multiple mechanisms involving EGFR pathway, pro-apoptotic Bax and Fas proteins and COX-2-PGE-protein kinase A pathway (DiMaio and Petti, 2013).

Whilst the transforming activity of HPVs E5 has been clarified, the role of this oncogene in viral cycle is not fully clear. E5 mRNA is expressed in the suprabasal differentiating cells of HPV-infected epithelia, where it is suggested to contribute to enabling these cells to retain their proliferative capacity and perturb keratinocytes differentiation thus favouring viral DNA replication (Longworth and Laimins, 2004).

E6 proteins

All papillomaviruses encode an E6 ORF, positioned immediately downstream of the LCR. E6 proteins are approximatively 150 amino acids long and show a conserved structure: there are two CX_2C-X_{29}-CX_2C zinc-like fingers linked by a sequence of 36 aminoacids and flanked by short C-terminal and N terminal domains of variable length (Cole and Danos, 1987). E6 is expressed at low levels and is located in the nucleus (Lowy and Howley, 2001; Lipari et al., 2001). No enzymatic activity has been identified for E6 and most of E6 functions are thought to be mediated by protein-protein interactions (Howie et al., 2009) (**Figure 4**). The first partner identified to interact with E6 was the E6 associated protein (E6AP), an E3 ubiquitin ligase, involved in the pathway targeting the proteins to proteasomal degradation (Scheffner et al., 1993). The E6AP binds both to E6 and E6 bound proteins, thus targeting the partners in the complex to proteasome mediated degradation (Scheffner et al., 1993). Despite a large number of studies, the exact residues of E6 binding to E6AP have not yet fully identified, while the motif on E6AP that interacts with E6 has been characterized and is referred to as an LXXLL motif (Nomine et al., 2006); this motif is present also in other proteins able to bind E6, among these paxillin. The ability of E6 to bind this motif is conserved among a large number of PVs, including animal PVs such as BPV1 (Tong and Howley, 1997).

HR HPVs E6 is able to bind to another class of cellular partners known as PDZ proteins: the name of these protein comes from the PDZ domain, a 90 amino acids stretch found in a variety of proteins sharing this domain, among these hDlg proteins, MAG 1-3, MUPP1, PTPN3 (Kiyono et al., 1997; Thomas et al, 2001; Lee et al., 2000; Spanos et al., 2008). This binding is mediated by a motif designated as S/TXV domain on the E6 C-termini.

The PDZ proteins are targeted to proteasomal degradation in case of concurrent binding of E6 to E6AP, although some other ubiquitin ligases have been implicated (Sterlinko et al., 2004).

Other proteins such as p53 and p300 have been reported to bind E6 despite lacking an LXXLL or a PDZ domain. The may bind E6 through motifs not yet identified or indirectly through binding to E6AP or other E6 associated proteins (Howie et al., 2009).

The most studied interacting protein of E6 is the tumor suppressor p53, a transcription factor coordinating the cellular response to genotoxic or citotoxic stress. Normally present at low levels and functionally inactive, cellular damage triggers an increase in p53 protein level and an activation via post-translational modifications. Once activated, p53 can direct the cellular response towards DNA repair, cell cycle arrest and/or programmed cell death (apoptosis), depending on the type and the extent of the damage (Murray-Zmijevski et al., 2008). PVs have evolved a number of mechanisms to block p53 function in infected cells, mainly through E6 oncoproteins (Howie et al., 2009). Indeed, the principle mechanism of p53 inactivation, typical of HR HPVs, is the binding to E6AP through the mediation of E6, which leads p53 to proteasomal degradation (Scheffner et al., 1990); normally, E6AP does not bind to p53, but when E6 binds to E6AP, this becomes able to bind and ubiquitinate p53 (Huibregtse et al., 1993). Both HR and LR HPVs E6 binds to p53; however, while both HR and LR bind to p53 C-termini, only HR E6 bind to the core region, and this is known to be necessary for p53 protein degradation (Crook et al., 1991; Li and Coffino, 1996). Thus, LR HPVs E6 have evolved different strategies to inactivate p53, including inhibition of its binding to DNA, aberrant localization and post-translational modifications. Indeed, the binding of these E6 to p53 may induce p53 conformational changes, disrupting the interaction with sequence-specific DNA motifs, or mask the nuclear localization sequences (NLS) at the

C-termini leading to cytoplasmic accumulation of p53 (Howie et al., 2009). Another known mechanism involves the binding of E6 to p300: normally, p53 is acetylated by p300 following to DNA damage, thus enhancing its transcriptional activity. When bound to E6, p300 is not able to acetylate and activate p53, resulting in lower p53 function (Zimmermann et al., 2000). Several other studies report a role for E6 in interacting, degrading and abrogating the function of histone acetyltranferase hADA3, which leads to the loss of p53 transactivation of downstream genes (Kumar et al., 2002).

One of the main consequences of E6 degrading or blocking p53 function is to inhibit apoptotic signaling that would otherwise eliminate the infected cells, as several pro-apoptotic genes are among the p53 transcriptional targets. Interstingly, E6 is known to interfere with both intrinsic and extrinsic pathways of apoptosis (Howie et al., 2009). E6 has been shown to inhibit the extrinsic pathway acting at each level of the early stages, for instance by binding and triggering degradation of death receptors, adaptor proteins or to Caspase 8 (Filippova et al., 2002; Filippova et al., 2004; Garnet et al., 2006).

Other cellular stresses such as DNA damage or oxidative stress activate a number of pathways that converge on the mithocondria, which act as a hub to sense the balance of pro- and anti-apoptotic signals. When the cells senses intrinsic stresses, the balance is upset in favour of pro-apoptotic proteins, allowing the formation of pores in the mithocondrial membranes comprised of Bax and Bak proteins, and the subsequent release of proteins including the cytochrome c. These proteins form the apoptosome, a signaling complex that results in cleavage of caspases, and ultimately cell death (Howie et al., 2009). Other than inhibiting the expression of pro-apoptotic p53-dependent genes including Bax through impairing p53 protein, the E6 from HPVs have been found to interact with Bak and induce its

proteasomal dependent degradation, thus blocking the intrinsic apoptotic pathway (Thomas and Banks, 1998; Underbrink et al., 2008).

Another important oncogenic function of E6 is the disruption of cell adhesion, polarity and epithelial differentiation, in order to allow proliferation of differentiated cells and inhibition of terminal differentiation to support viral replication. For instance, E6 from different human and animal PVs, binds to paxillin, a focal adhesion molecule involved in tethering the cellular cytoskeleton to the ECM and transmit signals along the actin network from ECM to the nucleus (Degenhardt and Silverstein, 2001; Tong and Howley, 1997). This binding results in the disruption of actin fibers and failure in mantaining the cell structure. Additionally, E6 binds to and inhibits proteins involved in epithelial tight junctions and may regulate cell proliferation, such as hDlg, MAG 1-3, MUPP1, PTPN3, thus impairing their function as tumor suppressors (Howie et al., 2009).

Other functions by E6 are known to be very important in the transforming processes leading to malignant transformation induced by PVs, among these the induction of the catalytic subunit of human telomerase hTERT, modulation of G-protein signaling and immune recognition, interaction with proteins involved in mantaining chromosomal stability, thus leading to accelerated progression to carcinogenesis (Klingelhutz et al., 1996; Howie et al., 2009).

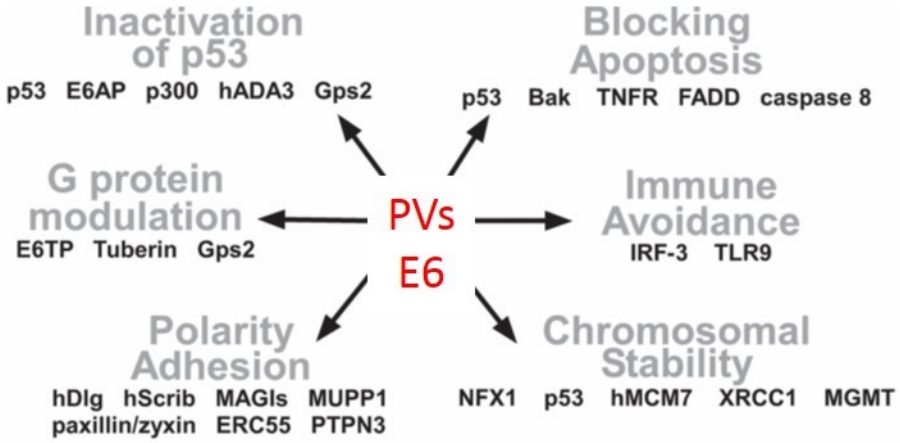

Figure 4

Binding partners of E6. E6 alters numerous cellular pathways mainly through protein-protein interactions (modified from Howie et al., 2009).

E7 proteins

The E7 proteins are small polypeptides of approximatively 100 amino acids. The amino-terminus of PVs E7 shares high homology with conserved region 1 and 2 (CR1 and CR2) of adenovirus EA1 and with the large tumor antigen of simian virus SV40 and these regions are necessary for the transforming properties of the three viruses (Figge et al, 1998; Phelps et al., 1988; Vousden et al., 1989). A conserved Leu-X-Cys-X-Glu (LXCXE) motif in the CR2 domain is necessary and sufficient for the binding to cellular retinoblastoma tumour suppressor protein (pRb) (Munger et al., 1989). Additionally, the C-termini of E7 contains a zinc-binding domain that is composed of two Cys-X-X-Cys motifs and function as a dimerization domain, although there are no definitive evidence for E7 acting as a dimer in vivo (Ohlenschlager et al., 2006). HPV16 E7 is located in the cytoplasm and, to a lesser extent, in the nucleus, despite no NLS is present in the amino acidic sequence (Ressler et al., 2007; Smith-McCune et al., 1999). Recent studies suggest that E7 can shuttle between cytoplasm and nucleus due to a nuclear export signal (Knapp et al., 2008). As for E6, E7 lacks intrinsic enzymatic activity and is widely accepted that its functions are linked to the ability to associate with and subvert the normal activity of cellular proteins (**Figure 5**) (McLaughlin and Munger, 2009).

HPVs E7 proteins associate with pRb and the related pocket proteins p107 and p130 through the LXCXE motif (Dyson et al., 1992). These proteins are involved in the regulation of the G1/S entry and progression by modulating the transcriptional activities of E2F transcription factors. Normally, pRb is complexed with E2F forming a transcriptional repressor acting specifically in G1. In late G1, cyclin dependent kinases (cdks) phosphorylate pRb leading to the disruption of pRb/E2F complex. Dissociated E2F acts as a transcriptional

activator of several genes necessary for entry and progression in S-phase (Frolov et al., 2004). The E7 binds to pRb preferentially in the G1 specific pRb/E2F repressor complexes, causing its disruption and uncontrolled G1 exit and S-phase entry (Dyson et al., 1992). The destabilization of pRb is mediated through inducing proteasomal degradation via association with cullin 2 ubiquitin ligase complex by E7 (Huh et al., 2007). The LR HPVs E7 binds to pRb with lower efficiency due to a single aminoacidic substitution (Gly22 vs Asp21) (Gage et al., 1990). The transforming ability of E7 correlates directly with pRb binding efficiency and thus with the specific aminoacid in that position (Heck et al., 1992; Sang et al., 1992). However, *beta* cutaneous HPVs conserve Asp21 and efficiently bind to pRb, but this does not associate with their classification as HR in vivo and does not necessary predict transforming potential in vitro (Ciccolini et al., 1994).

As a consequence of the enhanced E2F activity, its downstream genes involved in S-phase entry and progression are upregulated in E7 expressing cells, among these Cycline A and E which are the catalytic subunit of cdk2 (Zerfass et al., 1995). E7 dysregulates G1/S cell checkpoint by additional mechanisms. HPV16 E7 interacts with and abrogate the growth-inhibitory activities of p21 and p27 which are normally induced by antiproliferative signals and directly binds to cdk2/cyclins complexes resulting in enhanced cdk2 activity (Funk et al., 1997; Zerfass-Thome et al., 1996).

Additionally, E7 is known to impair anoikisis, a form of apoptosis triggered when cells enter the S-phase despite not attached to the ECM in the basal layer, the only site within epithelium where cells are committed to proliferate (Frisch et al., 2001). The resistance to anoikisis is a hallmark of cancer and is tightly mediated also by the Rb associated protein p600. HR and LR HPVs, as well as BPV1 E7, bind to p600, thus dysregulating anoikisis and

contributing to viral transformation (DeMasi et al., 2007; Huh et al., 2005; Corteggio et al., 2011).

Further additional mechanism of HPVs E7 mediated transformation include its ability to perturb the epigenetic program of host cells trough interaction with members of the polycomb group transcription factor complexes, to impair the TGFβ mediated growth inhibition, to interfere with interferon and insulin-like growth factor signaling and favour the chromosomal instability (McLaughlin and Munger, 2009).

Among animal PVs, BPV1 E7 is considered as a weak transforming gene, as it synergies with E5 in cell transformation during cancer development. E7 expression correlates *in vivo* with the over-expression of β1-integrin, which plays a role in the regulation of keratinocytes proliferation and differentiation; additionally, BPV1 E7 is involved in cell-mediated immune responses leading to tumour rejection and invasion process by upregulation of Matrix metalloproteinase1 (MMP-1) expression (for review of BPVs E7 see Corteggio et al., 2013). BPV4 E7, unlike BPV1 E7, possesses the canonical Cys-X-X-Cys motif and the pRb interaction domain, that is crucial for its transforming ability (Pennie et al., 1993). Cotton tail rabbit PV (CRPV) E7 binds and degrade pRb and show high transforming activity, since it is able to immortilize rabbit primary cells when expressed individually (Ganzenmueller et al., 2008).

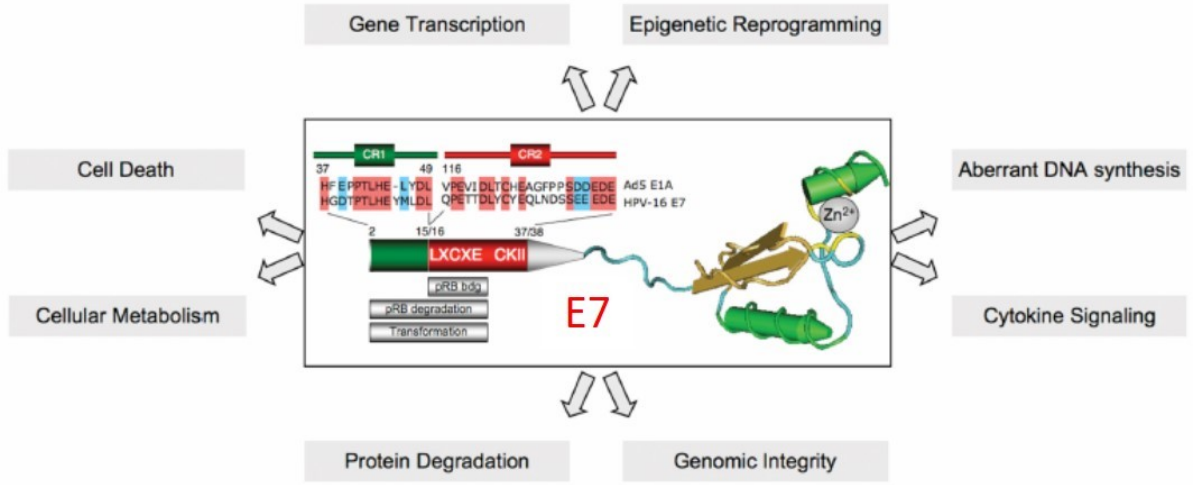

Figure 5

Schematic representation of HPV E7 and affected cellular processes (modified from McLaughlin and Murgen, 2009).

1.5.2 Late proteins

PVs Late genes encodes for the capsidic proteins L1 and L2. L1 is the major capsidic protein with a molecular weight of 55-60 KDa, whlist L2 is the minor protein with a size of 70 KDa. The viral capside is composed of 72 capsomeres. Each capsomer is a pentamer composed of 360 molecules of L1 proteins (**Figure 6**) and 12 up to 36 molecolules of L2 protein (Modis et al., 2002). Late genes transcription is strictly dependent upon the stage of keratinocytes differentiation (Doorbar, 2005). L1 is characterised also by some post-translational modifications such as phosphorylation and glycosilation. L1 is necessary for the virions assembly and production of complete infectious particles and is highly antigenic, whilst L2 plays both structural and regulatory functions: it accumulates nuclear structures known as PML bodies during virus assembly and recruits L1 to these domains (Becker et al., 2004). It has been suggested that PML bodies may be the sites of papillomavirus DNA replication and that capsid proteins accumulate at these sites to facilitate packaging (Swindle et al., 1999)

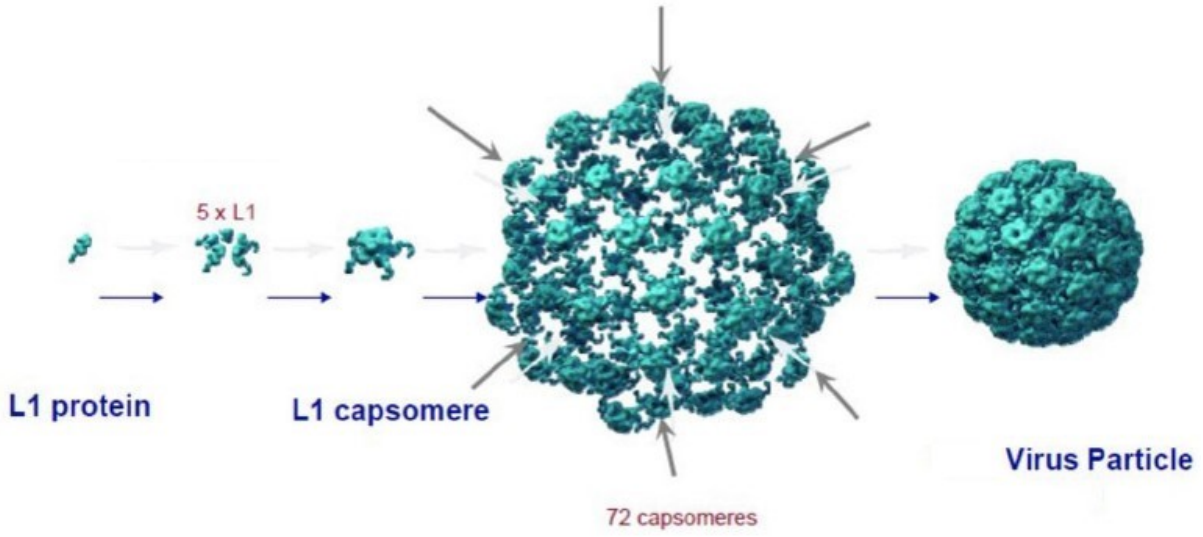

Figure 6

L1 is the major structural proteins involved in the assembly of PVs ichosaedric capside

1.6 Papillomaviruses in felids

It is worthwhile noting that PV-induced diseases in felids have been much less studied when compared to other species. Indeed, only in recent years several studies have been conducted to detect, classify and find possible associations of PVs infection with epithelial cancer in domestic as well as in exotic felids.

Among the PVs identified in felids, major efforts have been conducted to study the PVs of the domestic cat (Felis catus). Indeed, several PV genomes have been isolated from different species of the family Felidae, among these Lynx rufus (LrPV1), Pantera leo (PlPV1), Puma concolor (PcPV) and Uncia uncia (UuPV1) (Sundberg et al., 2000;), but the entire genomic DNA sequence has thus far been determined only for Felis catus Papillomavirus type 1, 2 (FcaPV1/2), and very recently for types 3 and 4 (FcaPV3/4) (Tachezy et al., 2002; Lange et al., 2009; Munday et al., 2013; Dunowska et al., 2014).

Felis catus papillomavirus type 1 (FcaPV1)

The FcaPV1 was originally isolated by from cutaneous hyperkeratotic lesions from an old domestic short-haired Persian cat, affected with an inherited form of immunodeficiency (Chediak–Higashi syndrome) (Tachezy et al., 2002). The cat had received longterm corticosteroid therapy, which might have contributed to the immune suppression (Carney et al., 1990), strenghtening the hypothesis of impaired immune system as a co-factor also in feline PVs associated skin lesions.

The complete genome contains 8300 bp (substantially larger than that of most of PVs) and has a GC-content of 46%. FcaPV1 contains seven major ORFs coding for five early proteins E1, E2, E4, E6, and E7 and two late proteins L1 and L2 (**Figure 7**).

The E6 contains four C-X-X-C motifs, whereas the E7 contains two such motifs. The E7 also contains the conserved pRb binding domain (DLRCYEQMPGEEE). The ATP-binding site of the ATP-dependent helicases (GPPNTGKS) is conserved in the carboxy-terminal part of E1. The E2 contains a leucine zipper domain (L-X_6-L-X_6-L-X_6-L). The E4 ORF is completely contained within the E2 gene. Typically, it does not contain a start codon. The peculiar high proline content is conserved in E4 ORF of FcaPV1.

Both L1 and L2 have a nuclear localization signal at the 3' ends. In FcaPV1, the LCR (or upstream regulative region URR) between the stop codon of L1 and the first ATG in E6 is only 384 bp long. PVs usually contain an E1-recognition site situated in the middle of two E2BS. The E1-recognition site (TGATTGTTGTTAACAAC) is present and three typical palindromic E2BS with the sequence ACCG-N4-CGGT, plus an additional degenerate one (believed to be functionally important) were identified. The LCR also contains at its 5' end the cis elements necessary for the processing of the L1 and L2 capsid mRNA transcripts. The

FcaPV1 genome contains a noncoding region (NCR2) of 1.3 kb between the end of E2 and the beginning of L2. No E5 or E5-like protein could be identified in this region, and, in contrast to the noncoding region between L1 and E6, there are no E2BS and no clusters of regulatory or promotor elements are identifiable. It is possible that this region comes from an early integration event with a piece of DNA of unknown function and origin.

FcaPV1 shows the highest percentage of similarity to Canine PV1 and is clusterd in *lambda* genus. Despite this virus has been firstly reported on skin lesions, it has rarely been detected subsequently and recent work suggest FcaPV1 as possibly involved in the pathogenesis of feline oral papillomas (Munday and French, 2015); thus, this virus appears to be an uncommon cause of skin disease in cats.

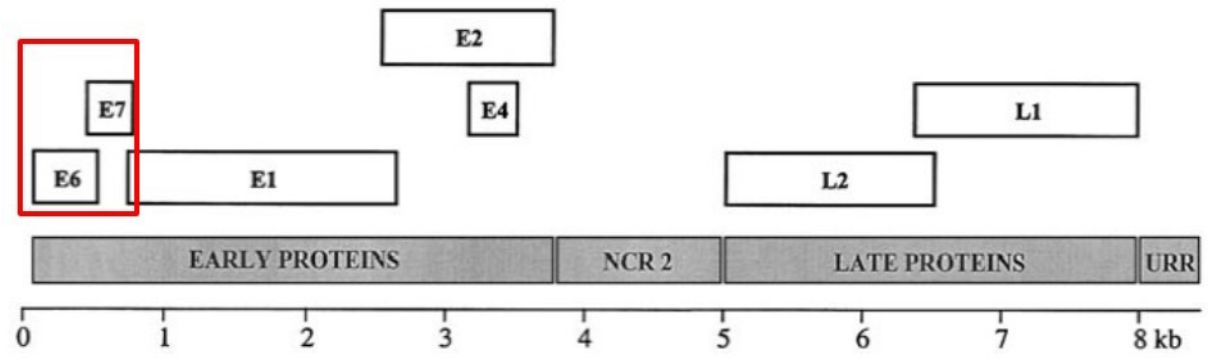

Figure 7

Linear representation of the ORFs of FcaPV1 genome. E6 and E7 putative transforming genes highlighted in red (modified from Tachezy et al., 2002)

Fcatus papillomavirus type 2 (FcaPV2)

FcaPV2 was identified by Lange et al. in 2009 and initially named as Felis domesticus papillomavirus type 2 (FdPV2). The entire viral genome was amplified from 3 Bowenoid in situ carcinomas (BISCs) of 3 different cats by rolling circle amplification (RCA) and subsequently sequenced and cloned (Lange et al., 2009).

FcaPV2 genome consists of 7899 bp with a GC content of 53%. Open reading frame analysis reveals the presence of six ORFs encoding the E1, E2, L2, L1, E6 and E7 proteins, (**Figure 8**). The analysis of their putative amino acids sequences reveals the typical features of PVs proteins: an ATP dependent helicase motif (GPPNTGKS) is identifiable in E1, two putative metal-binding motifs ($CX_2CX_{29}CX_2C$) in E6, one such motif and one pRb-binding domain (LXCXE) in E7 (Munger et al., 2004; Wilson et al., 2002). The deduced amino acid sequences of both structural proteins L1 and L2 were predicted to harbour a basic tail at their C-termini. Furthermore, a 615 bp long LCR between the stop-codon of the L1 gene and the start-codon of the E6 gene is present. Eight consensus sequences for E2 binding (ACCN6GGT) (Androphy et al., 1987) were identified, five of which located in the LCR and three within the putative E1 ORF. A putative origin of DNA replication, consisting of two E2-binding regions flanking an A/T-rich region, is also identifiable within the LCR, as well as a polyadenylation consensus sequence (AATAAA) at the 5' end of the L2 ORF for processing the early protein pre-mRNA. Thus, all important elements of a typical papillomavirus genome are detectable.

Phylogenetic analysis revealed that less than 60% of sequence homology is shared with the closest related PVs, suggesting that this viruses is the prototype of a new genus.

Indeed, in the last revision of PVs taxonomy it was designed as the first member of the new genus of *dyotheta*-PVs (Bernardt et al., 2010).

FcaPV2 is the PV most frequently associated with feline non-melanoma skin cancer. Its frequent detection in feline viral plaques, BISCs and SCCs allowed to hypothesize a role for this virus in the pathogenesis of these tumours. However, the virus is widespread and often amplified also from clinically healhy skin, suggesting that other co-factors are necessary for neoplastic transformation of the infected epithelia (Thomson et al., 2015). Most importantly, in all the studies published so far, only a frequent and specific association of viral DNA with skin lesions is reported. As for other animal and human PVs, the presence of a PV DNA does not prove its involvment in the carcinogenesis, since the virus may be considered a simple bystander or displaying a latent infection (Antonsson et al., 2000; Antonsson et al., 2002). Thus, a causative role for FcaPV2 in skin carcinogenesis is not yet proven. Different approaches should be needed to demonstrate this pathogenetic hypothesis. The first evidence may come from *in vivo* studies demonstrating the biological activity of the virus within the lesions. As stated before, only an association of viral DNA with the lesions has been reported, but no viral genes expression has been never demonstrated. Furthermore, while hundreds of in vitro studies have extensively demonstrated the transforming activity of BPVs and HPVs, functional studies focussed on the putative oncogenic properties of FcaPV2 are completely lacking. Hence the necessity of these new approaches to demonstrate a causal role of this virus in the pathogenesis of feline non-melanoma skin cancer, which is the main aim of this experimental work.

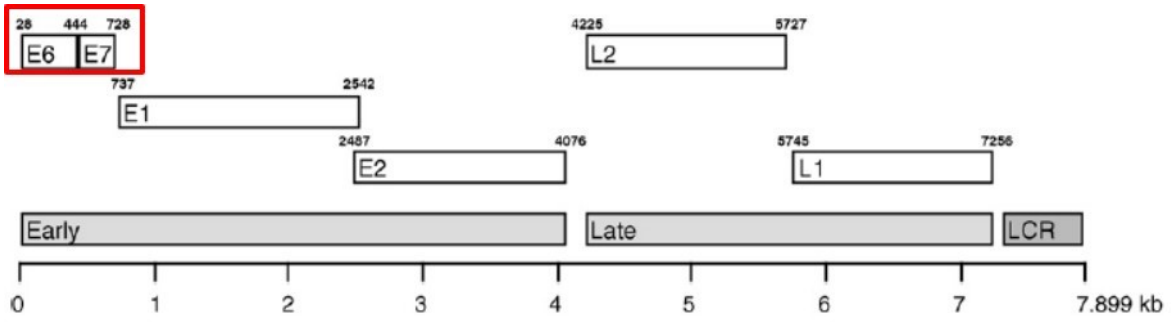

Figure 8

Schematic representation of the FcaPV2 genome and ORFs. Putative oncogenes E6E7 are highlighted in red (modified from Lange et al., 2009).

Felis catus papillomavirus type 3 (FcaPV3)

Several works report an association of BISCs with a short sequence of PV DNA not overlapping with FcaPV2 designated as FdPV-MY2 (Munday et al., 2011; O'Neill et al., 2011). Recently, the complete genome of this new PVcontaining the FdPV-MY2 sequence was amplified from a cat with multiple BISCs and it was designated as FcaPV3 (Munday et al., 2013).

The complete genome counts 7583 bp with a GC content of 46.7%. Differently from FcaPV1 and 2, FcaPV3 genome was predicted to encode for six early proteins E1, E2, E4, E5, E6, and E7 in addition to the two late proteins L1 and L2 (**Figure 9**). The E6 protein of FcaPV3 contains two conserved zinc-binding domains (CXXC-X_{29}-CXXC), which are separated by 36 amino acids. The E7 protein show a single zinc-binding domain but, notably, it lacks the pRb binding site (LXCXE). The predicted E1 protein of FcaPV3 contains two helicase domains. The C-terminal helicase domain contains the conserved ATP-binding site GPPDTGKS and the origin of replication binding domain. The FcaPV3 E1 also contains the Cyclin A interaction motifs (RXL), whose function in binding to cyclin/cyclin-dependent kinase complexes has been shown to be important for initiation of other papillomaviruses replication (Ma et al., 1999). A viral DNA binding domain and the N-terminal transactivation domains were identified within E2 gene. The FcaPV3 genome was predicted to encode a 218 aa protein downstream of E2 that partially overlaps L2. Based on the position of this predicted ORF within the E2-L2 region, it was annotated as ORF E5 (Bravo and Alonso, 2004). The putative ORF E5 of FcaPV3 shared 51.3% and 49.2% identity at the nucleotide level with the E5 of rabbit oral papillomavirus and Canine PV2. The E4 ORF was identified within the ORF E2, but in a different translation frame. It has high proline content (15.2%) as reported for

other PVs (Van Doorslaer et al., 2007). The LCR comprises 408 bp between L1 and E6. As expected, it contains regulatory sequences, including three polyadenylation sites and other motifs that have been shown to be necessary for the processing of the L1 and L2 mRNA (Birnstiel et al., 1985). An E1BS was present, althought slightly different from the one described for other papillomaviruses (Chen and Stenlund, 1998; Holt and Wilson, 1995). Three putative E2BS with a consensus sequence ACCN6GGT (Androphy et al., 1987) were identified flanking on either side the E1BS, as typical for the papillomavirus origin of replication (Stenlund, 2007). Unexpectedly, an additional ORF (nt 7244–7492) was predicted within the LCR region of FcaPV3. No ORF has previously been described in this position of a PV genome and it is unknown whether or not this ORF is expressed during the life cycle of FcaPV3.

FcaPV3 forms a well-supported clade with CPV-2 and CPV-7, and is classified as a *tau*-papillomavirus (Munday et al., 2013).

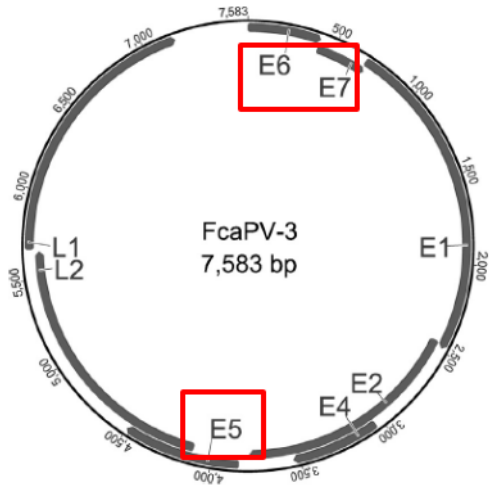

Figure 9

Schematic representation of the FcaPV3 genome. E5, E6 and E7 (highlighted in red) are putative transforming proteins (modified from Munday et al., 2013)

Felis catus papillomavirus type 4 (FcaPV4)

The complete genome of FcaPV4 comprised 7,616 bp with a GC content of 48.8 %. As FcaPV3, FcaPV4 genome was predicted to contain eight ORFs coding for E1, E2, E4, E5, E6, E7, L1 and L2 (**Figure 10**) (Munday et al., 2014). The putative E6 protein of FcaPV4 was predicted to contain two conserved zinc-binding domains (CXXC-X_{29}-CXXC), but lacked a PDZ-binding motif (ETQL) in its C-terminus. Similarly to FcaPV3, FcaPV4 E7 protein contains a single zinc-binding domain, but not the pRb protein-binding site (LXCXE). The predicted E1 contained two helicase domains and the conserved ATP-binding site GPPDTGKS and the origin of replication-binding domain were identified within the C-terminal one. The FcaPV4 E1 was also predicted to contain four Cyclin A interaction motifs (RXL) at positions 124, 194, 287 and 592. The conserved viral DNA-binding domain and the N-terminal transactivation domain were also identified within the E2 included. The E4 ORF was identified in a different translation frame within the E2 ORF. It had high proline content (16.5%) as reported for other PVs (Van Doorslaer., 2007) and a predicted signal peptide in the N-terminus. The LCR comprised 429 bp between L1 and E6. The FcaPV4 LCR was predicted to contain all the expected regulatory sequences, considered as important for the processing of eukaryotic mRNAs, although the distances between these elements in FcaPV4 genome are longer than those described for other eukaryotic genes (Birnstiel et al., 1985). A palindromic (50-ATTGTTXXXAACAAT-30) E1BS (Chen and Stenlund., 1997) and four putative E2BS with a consensus sequence ACCN6GGT (Androphy et al., 1987) were predicted in the FcaPV4 genome at the relative positions of E1BS and E2BS, with E1BS flanked on either side by the E2BS, typical for the papillomavirus origin of replication (Stenlund et al., 2007). Among the PVs known so far, FcaPV4 was most similar to FcaPV3; based on the actual

classification criteria, FcaPV3 and FcaPV4 should be classified as two species within *tau*-PVs (Munday et al., 2014).

While short sections of this virus have previously been identified in cutaneous SCCs (Feline papillomavirus MY03 sequence, GenBank: HM802139.1, Munday JS, unpublished data), the virus was cloned and sequenced from the oral cavity of a cat with ulcerative gingivitis (Munday et al., 2014).

Thus, the role of this virus in the development of diseases in domestic cats remains uncertain.

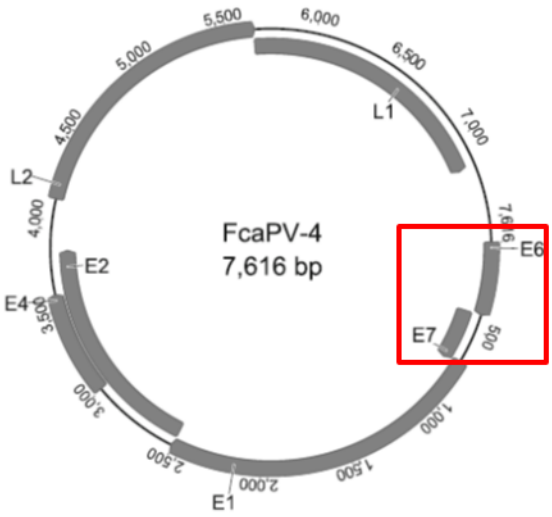

Figure 10

Schematic genomic organization of FcaPV4. E6 and E7 ORFs encoding for putative oncogenes are highlighted in red (modified from Dunowska and Munday, 2014)

1.6.1 Cutaneous lesions associated with papillomaviruses in the domestic cat (Felis catus)

Cutaneous Papillomas

Differently from other domestic species, PVs associated cutaneous papillomas are rare in the domestic cat. Only two cases have been reported in the literature, one arising on the eyelid on the site of a previous surgery, the other one on the nasal planum (Carpenter et al., 1992; Munday et al., 2007). Microscopically, both papillomas consisted of thickened, folded epidermis that contained prominent viral cytopathology. PVs infection was detected by non type-specific immunohistochemistry (IHC) in the former case, by PCR in the latter one, where HPV9 DNA was detected (Carpenter et al., 1992; Munday et al., 2007). Since PVs are usually specie specific and the presence of viral DNA does not necessary mean that the virus is biologically active, this apparent cross-infection cannot be considered as a causal factor in the development of the lesion and a contamination by contact with human skin cannot be ruled out.

Viral plaques

Viral plaques are considered to be uncommon in cats, although lesions that remain small might be unnoticed. Plaques typically present as multiple, non-painful, non-pruritic, ovoid, slightlyraised, <8 mm diameter lesions. They can be pigmented or non-pigmented and are often covered with a keratin crust (**Figure 11**). No area of the body appears to be predisposed to development of these plaques (Wilhelm et al., 2006). Microscopically, they show a sharply demarcated focus of mildly thickened epidermis containing prominent viral cytopathology, but they do not exhibit significant cell dysplasia. The epidemiology and pathogenesis of viral plaques in cats is unclear. PVs infection has been detected using different approaches in a series of studies (Wilhelm et al., 2006). FcaPV2 is the most common virus associated with viral plaques, whilst FcaPV1 has been very rarely detected, suggesting that the former PV might be the main cause of these lesions in domestic cats (Tachezy et al., 2002; Terai and Burk, 2002; Munday and Peters-Kennedy, 2010). FcaPV2 is highly diffused and viral DNA is often amplified from the skin of clinically normal domestic cats, suggesting that subclinical infection is common in cats (Munday and Witham, 2010). Therefore, since FcaPV2 infection is common but viral plaques are rare, the disease is likely to be multifactorial. Viral plaques were initially reported in immunosuppressed cats (Carney et al.,1990; Egberink et al., 1992) and, although viral plaques have subsequently been reported in seemingly immunocompetent cats (Wilhelm et al., 2006), a delayed or impaired cell-mediated immune response to FcaPV2 could play a role in disease development. The biological behaviour of viral plaques is variable, with some plaques remaining small before resolving spontaneously, while others progress to BISCs (Wilhelm et al., 2006).

Figure 11

Feline viral plaque. European cat affected with a viral plaque appearing as a small crusted lesion on the left eyelid (black arrow), Conceicao et al., 2007, available at: <http://www.scielo.br/scielo.php?script=sci_arttext&pid=S010209352007000300039&lng=en&nrm=iso>.ISSN 01020935. http://dx.doi.org/10.1590/S0102-093520070003000).

Bowenoid in situ squamous cell carcinomas (BISCs)

BISCs are rare lesions that tend to affect middle-aged to older cats (Miller et al., 1992; Baer and Helton, 1993). They usually present as multiple crusting or ulcerated plaques, although they may rarely appear as a single lesion. Several authors observed that hairless breeds are predisposed to BISCs and lesions are more severe in these breeds (**Figure 12**). Despite BISCs can develop also in haired and heavily pigmented skin (Baer and Helton, 1993), a role for ultraviolet (UV) light can not be ruled out, since some UV exposed sites suche as face, neck and limbs are possibly predisposed (Wilhelm et al., 2006). BISCs are often larger than viral plaques, and histopathological analysis reveals well-demarcated foci containing epidermal thickening and dysplasia within all levels of the epidermis and extending into hair follicles, but remaining confined by the basement membrane. The dysplastic cells can have elongated nuclei that tend to be orientated in one direction (the so called 'windblown' appearance; Gross et al., 2005). PVs infection in BISCs was initially highlighted through different techniques such as cytopathology or IHC with variable success (LeClerc and Haines, 1997; Gill et al., 2008). However, the improving of high specific molecular assays led to a marked increasing of type-specific PV DNA detection in feline BISCs (Nespeca et al., 2006; Munday et al., 2007; Munday et al., 2008; Munday et al., 2011). From this studies, a frequent and clearly specific association of FcaPV2 DNA is evident; as a matter of fact, in 2009, DNA extracted from a BISC was used to determine the complete genome of FcaPV2 (Lange et al., 2009). Despite FcaPV2 is the PV that is most frequently detected in feline BISCs, some feline BISCs contain other PV types, including human PVs (Munday et al., 2007; O'Neill et al., 2011) and the novel PV designated as FcaPV3 (Munday et al., 2013). Thus, viral plaques and BISCs share some histological features and both are

likely to be caused predominantly by FcaPV2. However, it is currently unknown whether all BISCs develop as progressions from viral plaques or whether BISCs can develop de novo. The clinical behaviour of BISCs is highly variable, but in many cases a rapid progression with the development of numerous large additional lesions can be observed. Additionally, BISCs also develop an invasive phenotype and progress to SCC (Baer and Helton, 1993) and a prompt removal of any lesions that show rapid progression or the development of an invasive phenotype may be necessary (Scott et al., 2001). Surgical excision in not curative, since additional BISC lesions often develop in the same cat (Baer and Helton, 1993). Several therapies have been proposed for BISCs, such as the treatment with imiquimod cream in one published case series, however this therapy was not succesfull (Gill et al., 2008). Although several studies report that suppression of cats suffering from BISCs is uncommon (Baer and Helton, 1993; Scott et al., 2001; Gill et al., 2008), BISCs can lead to the consideration of euthanasia if extensive areas of the skin are affected.

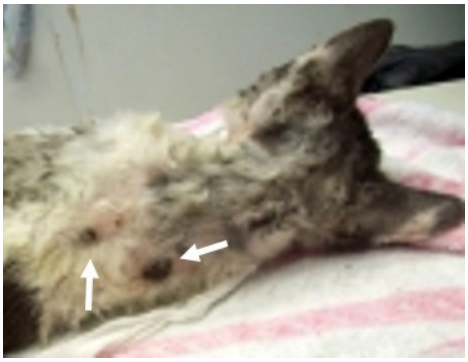

Figure 12

European cat affected with multifocal BISC (white arrows)

(https://www.vetstream.com/felis/Content/Disease/dis60148.asp).

Feline cutaneous squamous cell carcinomas (SCCs)

Cutaneous SCCs are very frequent in cats, most commonly developing on the nasal planum, pinnae and eyelids of cats. Based on these anatomical sites, a role for chronic solar exposure has been hypothesized; additionally, white-haired cats have 13.4 times risk of developing SCC than other colored cats and generally they arise in non-haired, non-pigmented areas of the body, strenghtening the hypothesis of a key role for UV light (Thomson, 2007). Usually, SCCs affect old animals, with a mean age of 12 years (Vail et al., 1998; Ruslander et al., 1997; Dorn et al., 1971). These tumors are generally locally invasive resulting in destruction of underlying tissue, over a long period of time from months to years; although slow to metastasize, several cases of more aggressive lesions with highly and rapid metastasizing phenotype have been reported (Ravens et al., 2013; Thomson, 2007). Metastases to local lymph nodes and lungs can occur in advanced or poorly differentiated lesions (Thomson, 2007).

Macroscopically, feline SCCs appear as flat, ulcerated masses (**Figure 13**), whilst at microscopy analysis they show classical PVs associated cytopathology including cells with expanded greyish blue, slightly granular cytoplasm, cells with dark shrunken nuclei surrounded by a clear cytoplasmic halo (koilocytes) and clumping of keratohyaline granules (Munday, 2013). This histopathological pattern and the observation that some feline cutaneous SCCs evolve from PV-induced BISCs strongly suggest that PVs infection may have a causal role in the development of these tumours. Most importantly, PVs DNA has been detected in SCCs (Nespeca et al., 2006) and later, FcaPV2 has been frequently and specifically amplified from cutaneous SCCs (Munday et al., 2008). While this shows that FcaPV2 was the virus most frequently associated with SCCs, the nature of the association is

still unclear, since cats are often subclinically infected with FcaPV2 (Munday and Witham, 2010). A possible hypothesis may be that other co-factors are needed for the development of feline skin cancer, as already known for human counterpart and in other species, among these, immunosuppression and UV light. Surprisingly, a study that compared SCCs from UV-protected skin (thus less likely to be caused by UV exposure) to SCCs from UV-exposed skin revealed that PV DNA was present significantly more frequently in the UV-protected SCCs. Despite the appearent paradox, the detection of significantly different frequencies of PV DNA within the two groups of SCCs suggests the virus was not inert and could have contributed to the development of neoplasia (Munday et al., 2011). Indeed, as for the human counterpart, the "hit and run" hypothesis is considered to be the most likely: in other words, the virus is necessary in the first stage of cell tranformation, acting sinergistically with the UV light, but the expression of viral genes is not required to sustain malignant progression and virus is no longer detectable at later stages of the lesions. In the end, a possible cooperation of PV infection with UV light in the development of feline SCCs has to be taken into account (Munday et al., 2009, Munday et al., 2011; O'Neill et al., 2011). However, although the high frequency of FcaPV2 DNA detection in feline SCCs specimens suggests a possible causal association, further evidence that the PV causes neoplasia is required, since alle the studies available so far are based only on the association of the viral DNA with skin lesions. Additionally, since many cats are infected by FcaPV2, but comparatively few develop SCCs, it appears likely that additional cofactors are required for development of neoplasia (Munday et al., 2013).

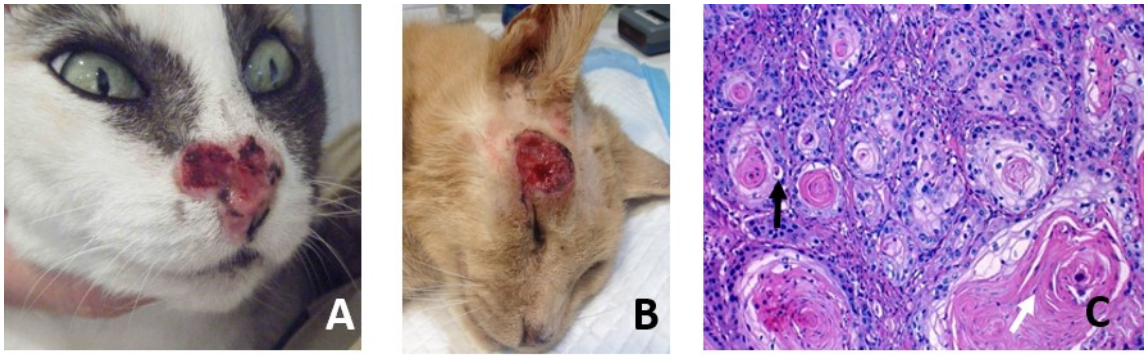

Figure 13

Feline cutaneous SCCs. (A) Cutaneous SCC on the nasal planum of an 21-year-old cat. **(B)** SCC on the right lateral auricular region in a 15-year-old cat (Photos by Dr J. Euclid, University of Sidney, available at vetbook.org/wiki/cat/.../Squamous_cell_carcinoma). **(C)** Photomicrograph of a SCC from the pinna of a cat showing the presence of viral cytopathology (koilocytes, black arrows) and corneal pearls (arrowheads) (available at http://www.vet.uga.edu/ivcvm/courses/VPAT5200/04_growth/05_carcinogenesis/carcino02.htm

Feline sarcoids

Feline sarcoids are rare neoplasms of domestic cats resembling the equine sarcoids, the most common skin neoplasm of horses (Nasir and Campo, 2008). They typically develop on the nose, upper lips and digits of young to middle-aged cats from rural areas (Schulman et al., 2001). Sarcoids appear as a single, firm and exophytic mass, they may become ulcerated and are rarely multiple (**Figure 14**). Microscopically they show the typical features of equine sarcoids, appearing as an expansion of the dermis made by hyperproliferative spindle-shaped mesenchymal cells, covered by hyperplastic epidermis (Gross et al., 2005). Feline sarcoids have not been reported to metastasise and surgical excision should be curative, despite local recurrence can be observed (Schulman et al., 2001). There are no evidences that sarcoids permit viral replication. However, PV DNA has been amplified in most of feline sarcoids using molecular methods (Schulman et al., 2001; Teifke et al., 2003). Since the PV DNA sequence that has been consistently amplified from feline sarcoids (FeSarPV) has never been detected in any non-sarcoid feline samples, cats do not appear to be the natural host of this PV (Munday et al., 2010). Consistently, FeSarPV is similar to sequences of ruminant PVs and has been found in bovine papillomas, as well as in normal bovine skin (Munday and Knight, 2010). These results suggest that FeSarPV is likely to be a bovine PV and that, similarly to equine sarcoids which are caused by cross-species infection with BPV1 or BPV2, feline sarcoids are the result of cross-species infection. Interestingly, neither BPV1 nor BPV2 has been detected in feline sarcoids and FeSarPV has never been detected in equine sarcoids.

Figure 14

Feline sarcoid. Exophytic mass present on the nasal philtrum of this cat diagnosed as feline sarcoid (https://www.vetstream.com/felis/Content/Disease/dis06228.asp).

1.6.2 Oral lesions associated with papillomaviruses in domestic cats

Oral papillomas

Very few cases of feline oral papillomas have been reported in literature. They are characterized by well-demarcated sessile or papillomatous proliferations of hyperplastic keratinocytes and have been shown to be positive for PVs immunostaining (Sundberg et al., 2000). Recently, feline oral papillomas showing the typical cytopathology of PVs induced lesions have been found to be specifically associated with FcaPV1 (Munday and French, 2015). Additionally, the recently identified FcaPV4 has been isolated from the salivary duct and gingival epithelium showing viral cytopathology (Dunowska et al., 2014).

Oral squamous cell carcinomas

Oral SCCs are the fourth most common feline neoplasm and are often rapidly metastasizing and thus fatal (Munday, 2013). The most affected sites are tongue, mandible and the maxilla (Withrow, 2001). In human beings, PVs cause up to 25% of oral SCCs (Gillison et al., 2000). On the contrary, PVs DNA has been very rarely detected in feline oral SCCs and did never correspond to any FcaPV known so far (Munday et al., 2009; Anis et al., 2010; O'Neill et al., 2011; Munday et al., 2011c). Currently there is little evidence that PVs cause feline oral SCCs, thus further studied are needed to clarify this issue.

1.6.3 Papillomaviral diseases in exotic felids

PV-induced disease is of potential importance in some highly endangered exotic felids and, since some exotic felids are becoming increasingly inbred, they may become more susceptible to PV-infections and subsequent epithelial lesions (Rector et al., 2007).

Cutaneous lesions of exotic cats

PV-induced cutaneous plaques and SCCs have only been reported in snow leopards (Uncia uncia); the specific PV has been designated as UuPV1 (Rector et al., 2007). Previous works reported PVs infection in viral plaques, mainly on the face and forelimbs, possibly evolving to SCCs. The PV type associated to the viral plaques was not determined, but appears to be different to UuPV1 (Sundberg et al., 2000; Joslin et al., 2000).

Sarcoids have been reported in captive African lions (Panthera leo), most frequently around the mouth and front limbs; FeSarPV DNA was amplified from these lesions and, since the lions were primarily fed un-skinned cattle carcasses, infection from cattle was suspected (Orbell et al., 2011). One case of sarcoid has been described also in captive mountain lion (Felis concolor), containing PV DNA with 96% similarity to FeSarPV. Despite rare recurrance, surgical excision seems to be curative for exotic feline sarcoid (Schulman et al., 2003).

Oral lesions of exotic cats

PV-induced oral papillomas have been reported in six species of exotic cats. PVs were sequenced from lesions of the tongue in Asian lions (Panthera leo persica: PlpPV1) (Sundberg et al., 1996); papillomas were more common in lions infected by a lion-specific strain of feline immunodeficiency virus, but whether oral papillomas in lions result in significant disease is uncertain (Roelke et al., 2009).

Oral papillomas are associated with UuPV1 in snow leopards and appear as multiple small pale nodules on the ventral surface of the tongue (Sundberg et al., 2000; Rector et al., 2007; Mitsouras et al., 2011). Snow leopards frequently develop oral SCCs and it is possible that UuPV1 could cause both papillomas and SCCs in this species. However, UuPV1 DNA can be amplified also from oral swabs of animals with no detectable disease (Mitsouras et al., 2011), so the relationship between this PV and oral neoplasia is still to be proven.

A PV aetiology was confirmed by IHC in papillomas from a clouded leopard (Neofelis nebulosa) and an Asian desert cat (Felis bieti; Sundberg et al., 1996; Sundberg et al., 2000). PVs from oral lesions of bobcats (Lynx rufus) and Florida panthers (Puma concolor; Rector et al., 2007) were also sequenced.

All the PVs sequenced from oral papillomas of exotic felids belong to *lambda* genus and are genetically similar to each other and FcaPV1, suggesting co-speciation of each PV and its host (Rector et al., 2007).

2. AIM OF THE STUDY

A consistent number of studies simply suggest an association of FcaPV2 DNA with feline skin SCC and precursors; however, expression of FcaPV2 genes in feline skin tumours has never been proven. Nevertheless, a consistent detection of the virus in clinically healthy skin samples has been reported in domestic cat (Munday, 2013). In general, the presence of PVs DNA in samples does not necessary indicate that an active infection is ongoing and that it has a role in the skin carcinogenesis.

Additionally, differently from other PVs proven to be oncogenic, functional in *vitro* models of PVs induced feline skin cancer are completely lacking. Thus, the causative role of FcaPV2 in the development of feline SCC and precursors is far from being demonstrated.

The first aim of this work was to study the expression of viral genes E2, E6 and E7 in a subset of cutaneous skin tumours and normal skin samples.

The second part of this study was focused on the establishment of a new *in vitro* model to investigate the possible transforming activity of FcaPV2, in order to understand possible oncogenic properties of viral genes E6 and E7. The molecular pathway of tumour suppressors p53 and pRb were investigated, as well as the ability of FcaPV to impair UVB induced apoptosis, a well known mechanism triggering PVs induced skin cancer.

The final results of this study will allow to establish whether FcaPV2 is a causative agent or an innocent bystander in feline skin cancer, making a contribution to a poorly investigated field in veterinary oncology, and if this may be considered as a general model of spontaneous PV-induced cancer, useful for sfurther studies in the field of comparative pathology.

3. MATERIALS AND METHODS

Samples collection

Ten (10) normal skin samples (N1-N10) were collected during routine sterilization surgeries at The Veterinary Hospital of Naples Frullone. One oral SCC (T1), eight (8) SCCs (T2-T9) and 2 non SCCs skin diseases were also collected (T10 and T11); most of these biopsies were taken from hospitalized cats during normal clinical activities at the Department of Veterinary medicine and animal Productions of University of Naples Federico II or with the help of practitioners from private clinics of differents regions of Southern Italy; other samples were sent from University of Veterinary Medicine of Vienna, Austria and from University of Agriculture Sciences and Veterinary Medicine, Ion Ionescu de la Brad, Iasi, Romania. All the samples were from cats of european short-hair breed, except for T9 that was collected from a Siamese cat. The anatomical site of the lesions was not known for most of the samples; T1 was an oral SCC of the mandible, T7 SCC was removed from the right ear, T9 from the left side of the face, T11 from the right forepaw. All the biopsies were collected under local or total anaesthesia without causing pain or any kind of suffering to the animals; sample T1 was removed from an euthanized cat suffering from a devasting oral SCC. After surgical excision, the samples were immediatey submerged in RNA*later*® RNA stabilization solution (Life Technologies) in 1,5 mL tubes and stored at -80 °C in order to preserve the integrity of both DNA and RNA.

DNA and RNA extraction

Samples were removed from RNA*later*® and approximatively 20 mg of tissue were cut with sterile disposable scalpels. Total DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen #69504) according to the manufacturer's protocol.

Cellular RNA was obtained using RNeasy Mini Kit (Qiagen #74104) following the brand recommendations. Sample T8 and T9 were not available for RNA extraction.

The DNA and RNA concentrations of each sample were measured using a spectrophotometer.

Polymerase chain reaction (PCR) and Reverse Transcription (RT)-PCR

DNA samples were subjected to PCR amplifying a specific fragment of FcaPV2 L1 gene. FcaPV2 genome cloned into pBluescript II-KS^{+} vector was kindly provided by Prof. C.E. Lange (Dermatology Unit, Clinic for Small Animal Internal Medicine, Vetsuisse Faculty, Zurich, Switzerland) and was used as positive control of the reaction. The primers sequences, the size of amplified fragment, and the annealing temperatures are summarized in **Table 1.** PCR conditions were as follows: initial denaturation at 95 °C for 5', followed by 36 cycles at 95 °C for 30" (denaturation), Tm °C for 30" (annealing), and 72 °C for 30" (extension), with a final extension at 72 °C for 5'. PCR products were separated by electrophoresis in 1,5 % agarose gels with Tris borate ethylene diamine tetra acetic acid (EDTA) buffer (TBE; 89 mM Tris base, 89 mM Boric acid, and 2 mM EDTA), stained with ethidium bromide, and visualised under ultraviolet light. Amplicons were purified using the MiniElute Gel Extraction Kit (Qiagen #28606) following manufacturer's instructions and sent to "Research Center CEINGE Advanced Biotechnologies" for sequencing. The sequences

were aligned to FcaPV2 L1 gene sequence using Basic Local Alignment Search Tool (NCBI/BLAST).

Reverse transcription (RT) to cDNA was performed on 500 ng of each RNA sample using iScript™ cDNA Synthesis Kit (Bio-Rad #170-8891) according to manufacturer's protocol. PCR was carried out to amplify specific fragments of E2, E6 and E7 FcaPV2 genes and feline GAPDH in 25 µl of reaction mixture, containing 50 ng of cDNA from each sample, 2 mM $MgCl_2$, 200 µM each dNTP, 480 nM of each primer and 2.5 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems). The PCR protocol was the following: initial denaturation step of 5' at 95 °C, then 36 cycles of amplification with a denaturation step at 95 °C for 30", an annealing step at specific Tm °C for 30" and an extension step at 72 °C for 30". A final extension step at 72 °C for 5' was performed in each PCR assay. For detection of E7, a second PCR using 1 uL of the first PCR product as stamp was necessary for samples T1, T3, T4 and T5. The same reactions were performed in parallel on FcaPV2 genome cloned in pBluescript II-KS^+ as positive control of the amplifications (not shown). The primers sets, the size of amplified fragments and the annealing temperatures are summarized in **Table 1**. Detection of the amplified products was carried out by electrophoresis on 1,5 % ethidium bromide-stained agarose gel. Amplicons were purified using the MiniElute Gel Extraction Kit (Qiagen #28606) following manufacturer's instructions and sent to "Research Center CEINGE Advanced Biotechnologies" for sequencing. Alignment of the sequences with FcaPV2 E2, E6 and E7 genes was performed using NCBI/BLAST.

Cells and cells culture

Crandel-Rees feline kidney (CRFK) cells were purchased from ATCC® cell bank. NIH3T3 mouse fibroblasts cell line and HaCat immortalized human keratinocytes was available in the laboratory of Infections and Cancer Biology Group (ICB) at International Agency for Research on Cancer (IARC), Lyon, France, where most of this work was developed. Phoenix cell line, developed from the 293T cell line transformed with adenovirus E1a and carrying a temperature sensitive T antigen (Nolan laboratory, Stanford University) was already available at IARC as well. This cell line can be used as a packaging line since it carries a construct capable of producing *gag*, *pol* and *env* proteins for ecotropic and amphotropic viruses to act in *trans*.

CRFK, NIH3T3, Phoenix and HaCat cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose, supplemented with 10% fetal bovine serum (FBS) (PAA), 100 U/mL penicillin and 100 µg/mL streptomycin (Pen Strep, Gibco, Invitrogen), 2 mM L-glutamine (PAA) and 10 µg/mL ciprofloxacin hydrochloride (EUROMEDEX).

Human primary keratinocytes (HPK) from 2 different donors were obtained by the "Laboratoire des Substituts Cutanes", Hopital E. Herriot (Lyon, France). Primary cells were cultured in FAD medium, composed with: 25% DMEM (PAA), 75% Ham's F12 (PAA), 5% FBS (PAA), 100 U/mL penicillin and 100 µg/mL streptomycin (Pen Strep, Gibco, Invitrogen), 10 µg/mL Cyprofloxacine (EUROMEDEX), 10 ng/mL epidermal growth factor (EGF, R&D), 10 mM Cholera toxin (List Biological Laboratories INC.), 400 ng/mL hydrocortisone (Sigma), 24 ug/mL Adenine (Sigma), 5 µg/mL human Insulin (Sigma).

All cell lines were grown at 37 °C in humified atmosphere at 5% CO_2. In order to form colonies, the keratinocytes must be suitably supported by fibroblastic cells, in particularly by

soluble products elaborated by the fibroblasts (Green et al., 1977). Thus, HPK were co-cultured with a feeder layer of NIH3T3 previously treated with Mytomicin (Sigma) for 2 h to arrest cell division in order to avoid overgrowing in co-culture flasks. The feeder layer was changed every 2 days and at each passage of HPK.

Cloning strategies and Constructs

The whole operon E6E7 and the E6 and E7 genes individually, were cloned into pCEFL-HA using standard procedures for molecular cloning. Firstly, the different genes were subcloncloned by PCR from FcaPV2 genome cloned into pBluescript II-KS^{++}, using specific primers containing sites for EcoRI and XbaI restriction enzymes (RE). The PCR reactions were carried out using the reagents and protocols assessed above; primers details are summarized in **Table 1**. The PCR products corresponding to E6E7, E6 and E7 genes and th pCEFL-HA vector were double-digested with EcoRI and XbaI RE (New England Biolabs, NEB) following manufacturer's instructions, at 37 °C for 2h. After purification of the digestion products using the QIAquick® PCR Purification Kit (Qiagen #28106), digested pCEFL-HA was dephosphorylated using Rapid DNA Dephos&Ligation kit (Roche #04898117001) to avoid self-ligation. Following another step of purification of the digested and dephosphorylated pCEFL-HA with the same aforementioned kit, the final products wer subjected to ligation reaction using DNA Ligation Kit (TAKARA BIO INC. #6021), according to manufacturer's recommendations. Ligation products were transformed by heat shock (30' on ice, 45" at 42 °C, 2' on ice) in Subcloning Efficiency™DH5α™Competent Cells (Invitrogen) and the final constructs consisting in pCEFLE6E7, pCEFLE6 and pCEFLE7 were purified by maxiprep using Plasmid Maxi Kit (Qiagen #12263).

The pLXSN vector was available at ICB laboratory at IARC. The whole operon E6E7, E6 and E7 ORFs were subcloncloned by PCR using specific primers containing sites for EcoRI and BamHI RE. The PCR reactions were carried out using the reagents and protocols assessed above; primers details are summarized in **Table 1**. The PCR products corresponding to E6E7, E6 and E7 genes and the empty pLXSN vector were double-digested with EcoRI and BamHI RE (Thermo Scientific) following manufacturer's instructions, at 37 °C for 2h. The digestion products were run in agarose gel and then purified by using the MiniElute Gel Extraction Kit (Qiagen #28606). The digested pLXSN was dephosphorylated using Shrimp Alkaline Phosphatase (SAP) kit (USB Corporation #70092Z) to avoid self-ligation. Following another step of purification by gel extraction of the digested and dephosphorylated pLXSN using the same aforementioned kit, the final products wer subjected to ligation reaction using the Ligase T4 ligation kit (Roche #10481220001) according to manufacturer's recommendations. Ligation products were transformed by heat shock (30' on ice, 45" at 42 °C, 2' on ice) in Subcloning Efficiency™DH5α™Competent Cells (Invitrogen) and the final constructs consisting in pLXSNE6E7, pLXSNE6 and pLXSNE7 were purified by midiprep using PureLink® HiPure Plasmid Midiprep Kit (Invitrogen).

To generate recombinant bacterial fusion proteins, FcaPV2 E6 and E7 genes were cloned in the glutathione-S-transferase (GST) fusion vector pGEX4T1. HPV16 E6 and E7 cloned respectively in pGEX4T1 and pGEX2T1 vectors were already available at ICB laboratories. FcaPV2 E6 and E7 were subcloned by PCR using specific primers containing sites for EcoRI and NotI RE. The PCR reactions performed using the reagents and protocols assessed above; primers details are summarized in **Table 1**. The PCR products corresponding to E6 and E7 genes and the empty pGEX4T1 vector were double-digested with EcoRI and

NotI RE (Thermo Scientific) following manufacturer's instructions, at 37 °C for 2h. The digestion products were run on agarose gel and then purified by using the MiniElute Gel Extraction Kit (Qiagen #28606). The digested pGEX4T1 was dephosphorylated using Shrimp Alkaline Phosphatase (SAP) kit (USB Corporation #70092Z) to avoid self-ligation. Following another step of purification by gel extraction of the digested and dephosphorylated pGEX4T1 using the same aforementioned kit, the final products wer subjected to ligation reaction using the Ligase T4 ligation kit (Roche #10481220001) according to manufacturer's recommendations. Ligation products were transformed by heat shock (30' on ice, 45" at 42 °C, 2' on ice) in One Shot® MAX Efficiency® DH5α™-T1^{R} Competent Cells (Invitrogen) and the final constructs consisting in pGEX4T1FcaPV2 E6 and pGEX4T1FcaPV2 E7 were purified by maxiprep using PureLink® HiPure Plasmid Maxiprep Kit (Invitrogen).

Transfection and retroviral transduction procedures

CRFK cells were plated at 4x10^{6} in 100mm petri dishes. After 24 h, cells were transfected with empty pCEFL-HA vector, pCEFLE6E7, pCEFLE6 and pCEFLE7 using Lipofectamine®2000 (Life Technologies) following the instructions provided by the manufacturer. Cells were splitted 48 h after transfections and subjected to selection by adding neomicin 500 μg/mL to the growth medium. Cells selected by resistance to the antibiotic conferred by the vector were pooled after 2 weeks and tested for the expression of the transfected genes by RT-PCR as described above using the same primers designed for cloning.

Phoenix cells were plated in 100mm petri dishes. After 24 h, when cells reached a 50/70% confluence, they were transfected with empty pLXSN, pLXSNE6E7, pLXSNE6 and

pLXSNE7 using CalPhos Mammalian transfection kit (K2051-1 BD-Biosciences). Briefly, samples were prepared as follows: 10 µg of the DNA corresponding to each construct were mixed with 440 µl of H_2O and 62 µl of $CaCl_2$ (stock 2M) in a 15 ml tube and then 500 µl of HBS (2X) were added drop by drop to obtain 1 mL of transfection solution. Growth medium was replaced by 5mL of fresh one added with Cloraquine at 25µM final concentration and the transfection solution was added drop by drop. After 6 h the medium was changed with fresh complete DMEM and 24 h after transfection it was changed again. Further 24 h later the medium of transfected cells containing the retroviral particles was collected and passed twice through 0.22 µm filters. The retroviral suspension was added with 5uL of Polybren (from a stock 5mg/mL) and applied to HPK monolayer. After 6 h it was removed and replaced by complete fresh FAD medium. Cells were splitted 1:1 24 h after the infection and neomycin (0,1mg/mL final concentration) was added to the culture medium to start selection of transduced cells to be ended after 5 days.

UVB irradiation

For UVB irradiation, 3,5 x 10^6 CRFK cells were plated in 6 well plates. After 24 h, when cells were at 60-70% confluence, growth medium was removed and 400 uL of PBS were added to each well to avoid the cell monolayer to get dry during irradiation. Thus cells were irradiated at 25 mJ/cm^2 (0,025 J/cm^2) using BIO-SUN irradiation system 365/312nm (Vilbert Lourmat, France) and PBS was replaced by fresh medium. For not irradiated cells used as control, medium was simply replaced by fresh one after a washing in 400 uL of PBS. All the cells were harvested after 8 h to be analyzed by Western blotting or qPCR.

Western blotting procedures and antibodies

To prepare whole cell protein extracts for Western blotting analysis, cells were lysed using RIPA buffer (50mM Tris, 150mM NaCl, 1% Triton X-100, 1mM EDTA, 0,25 % Deoxicholate) added with protease and tyrosin and serine-threonine phosphatase inhibitors cocktails (10 uL for each mL, Sigma), for 20' on ice. Cell lysates were clarified by centrifugation at 13000 rpm for 10' at 4 °C. Protein concentration was measured by BCA protein assay performed according to manufacturer protocol. Equal amounts of each protein sample were mixed to Laemmli sample buffer (sodiumdodecyl sulphate (SDS), Tris-HCl pH 6,8%, glycerol, bromophenol blue, and β-mercaptoethanol), boiled and loaded onto polyacrylammide gels to be subjected to separation by electrophoresis. Proteins were transferred onto PVDF membranes using IBlot II apparatus (Life Technologies). Membranes were incubated with 5% Non Fat Dry Milk (NFDM) in Phosphate Buffered Saline (PBS) 0,1% Tween-20 (PBST) for 1 h at room temperature (rt), to block aspecific bindings, and then the primary antibodies diluted in PBST 5% NFDM were applied over night (O/N) at 4 °C with gentle agitation. After washing steps in PBST, membranes were incubated with appropriate secondary antibodies conjugated with Horseradish Peroxidase (HRP) (mouse or rabbit HRP linked immunoglobulins, depending on the species of the primary antibody, Promega) for 1h at rt. Following further washings in PBST, bound antibodies were visualized by enhanced chemiluminescence (ECL, Clarity™ ECL Western Blotting Substrate, Bio-Rad). Protein levels were quantitatively estimated by densitometric analysis using ChemiDoc gel scanner (Bio-Rad) equipped with a densitometric workstation (Image Lab software, Bio-Rad).

For the analysis of p53 pathway, rabbit anti-human p53 (Santa Cruz, sc-6243) and rabbit anti-human p21 (Santa Cruz, sc-397) were used at 1:1000 dilution.

For the biochemical detection of apoptosis, rabbit anti-human Caspase3 (Cell Signaling #9662), rabbit anti-human Bax (Santa Cruz, sc-493) and Bak (Santa Cruz, sc-7873) antibodies were applied, at 1:1000 dilution for the two formers, 1:500 for the latter.

The proteins of the pRb pathway were detected using mouse anti-human pRb (BD Pharmingen #554136), rabbit anti-human Cycline A (Santa Cruz, sc-751) and mouse anti-human Cdc2 (Santa Cruz, sc-54) at 1:1000 dilution.

All the blots were stripped and reprobed with mouse anti-human β-actin antibody (Calbiochem) to allow normalization.

RNA extraction and Real-time quantitative PCR (Real-time qPCR)

Total RNA was extracted from cells using the Absolutely RNA Miniprep Kit (Agilent, #400800) following the manufacturer recommendations. Sample concentrations were calculated by a spectrophotomer and RT was performed on 500 ng of total RNA using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific #K1632); 50 ng of cDNA were used to prepare serial dilutions, which were subjected to qPCR for feline p53, Bcl-Xl, Cdc2, cMyc using Mesa Green qPCR Mastermix Plus (RT-SY2X-06+WOULR, Eurogentec) according to the brand instructions and run in Mx3005P qPCR System (Agilent Technologies) with the following protocol: 95 °C 10', followed by 95 °C 15'', Tm °C 45 '', 72 °C 45" (45 cycles), 95 °C 1', 55 °C 30", 95 °C 30". Amplification of feline GAPDH was performed in parallel to allow normalization of the results.The primers sequences and the specific Tm °C are summarized in **Table 1.** The results were analyzed using the software provided by the machinery.

GST pull-down assay

Recombinant bacterial fusion proteins were generated cloning the appropriate PVs E6 and E7 genes in the glutathione-S-transferase (GST) fusion vector pGEX4T1 and pGEX2T1 as described above.

The empty vector pGEX4T1, FcaPV2 E6, FcaPV2 E7 and HPV16 E6 cloned in pGEX4T1 and HPV16 E7 cloned in pGEX2T1 were transformed (~3 ug) by thermal shock (30' on ice, 45" at 42°, 2' on ice) in Rosetta™ competent bacterial strain (Life Technologies). Cells were resuspended in 250 uL of Sock medium and incubated at 37° in agitation for 45'. Fifty uL of each cell suspension were plated on LB-Agar plates added with Ampicillin 100ug/mL (LB Amp+) and let grow O/N.

Grown colonies for each construct were picked and grown O/N in snapcap tubes in 5 mL of LB Amp+. Of each cell suspension, 1 mL was added to 100 mL of LB Amp+ and grown overday monitoring the OD_{600nm}. When bacterial suspensions reached an OD_{600nm} of 0,4, Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the bacterial suspension at 0,1 mM final concentration to stimulate fusion proteins sinthesys. Each culture was grown for further 4 hours at 30 °C and the bacteria were collected by centrifugation. For cell lysis and protein purification, the bacterial pellets were resuspended in 2 mL of NETN buffer (20mM Tris-HCl pH8, 10mM NaCl, 0,5 % Nonidet p40, 1mM EDTA, 1mM NaF) added with protease and phosphatases inhibitors and subjected to sonication on ice in a Branson Sonifier 250 (4 cycles of 20" with an interval of 1' on ice between cycles). The insoluble bacterial debris was removed by centrifugation at 10000 rpm for 15' and the supernatant was filtered through steyle 0,45 μm filter to obtain a clear bacterial extract.

The fusion proteins were purified by incubating the bacterial lysates with 75 uL of Glutathione Sepharose-4B (GelHealthcare) previously washed in 0,5% NFDM NETN buffer, at 4 °C for 1h on rotating wheel. The beads were finally recovered by centrifugation (1' at 6000 rpm at 4 °C) and washed 5 times with NETN buffer to grant purity of the preparation.

Expression and purity of the recombinant proteins was verified by SDS polyacrylammide gel electrophoresis followed by Comassie Blue staining: 10 uL of each beads-GST-fused proteins were added with 10 uL of Laemmly sample buffer 5X, boiled and cenrifugated, and then the supernatant was loaded onto 12% poliacrylammide gel. The gel staining allowed an approximative quantization and to check the purity of the obtained fusion proteins.

HaCat and CRFK cells were lysed in IP buffer (Tris pH 7.5 20mM, NaCl 200mM, EDTA 1mM, NP-40 0,5%) added with protease and phosphatase inhibitors as described above; 0,6 mg of whole cell lysates were incubated first with 5 uL of GST-Sepharose beads for 30' at 4 °C in rotation to allow a preclearing of non specific bindings to GST, then with equal amount of GST and GST fusion proteins-beads complexes for 1h at 4 °C. The beads were collected by centrifugation and washed 5 times with NETN buffer. They were resuspended in 10 uL of Laemmli sample buffer 5X, boiled, centrifugated and the supernatant was loaded onto appropriate SDS polyacrylamide gels. The samples were subjected to Western blotting for p53 and pRb detection as described above.

HPK immortalization assay

To check the immortalization or life-span prolonging ability of FcaPV2 putative oncogenes, HPK were transduced with empty pLXSN vector, pLXSNE6E7, pLXSNE6 and pLXSNE7. After selection, transduced cells were cultured for several passages. Simply, cells obtained after selection were assumed to be at population doubling (PD) 0. When reaching a confluence of~80%, cells were splitted 1:2 and they were considered to be at PD 1; at the second splitting 1:2, the PD 2 was counted, and so on. Since no immortalization phenomenon could be observed, cells were kept in culture until they underwent senescence and cell death and the PD number of oncogenes tranduced cell lines were compared to cell selected for empty pLXSN and to each other.

Statistical analysis

For statistical analysis, independent sample t-test ($p<0.05$ and/or $p<0,01$) was performed using SPSS 13 software.

Application	Genes	Primers sequences	Tm °C	Fragments size
PCR	L1	Fw: 5'-GACACTACACGCGGTACCAA-3' Rev: 5'-GGTAAGAGTGACCACGCACA-3'	60 °C	150 bp
RT-PCR	E2	Fw: 5'-CTGGTTTGGCCGGACACTAT-3' Rev: 5'-ATCCGTCCGGAACTTCACAC-3'	60 °C	126 bp
	E6	Fw: 5'-TACGAGCGCGAGCATTTTTG-3' Rev: 5'-TGCAGCTTTTCTCCTGGTGT-3'	59,9 °C	134 bp
	E7	Fw: 5'-TTGGCACGACACCTACCATT-3' Rev: 5'-ACTGCACACTCCACAGGGTA-3'	59,6°C	167 bp
	GAPDH	Fw: 5'-CCATGTTTGTGATGGGCGTG-3' Rev: 5'-TGATGGCATGGACTGTGGTC-3'	60 °C	159 bp
Cloning in pCEFL-HA RT-PCR	E6E7	Fw: 5'-AATTGAATTCGAGCGTCCTCGCACGGCT-3' Rev: 5'-AATTTCTAGACCCGTCCAGGTCACTACACT-3'	60 °C	784 bp
	E6	Fw: 5'-AATTGAATTCGAGCGTCCTCGCACGGCT-3' Rev: 5'-AATTTCTAGAGGTGCCTCCAGCAGTACAATG-3'	61,3 °C	463 bp
	E7	Fw: 5'-AATTGAATTCATTGGCACGACACCTACCAT-3' Rev: 5'-AATTTCTAGACCCGTCCAGGTCACTACACT-3'	59 °C	368 bp
Cloning in pLXSN	E6E7	Fw: 5'-AAAAGAATTCATGGAGCGTCCTCGCACG-3' Rev: 5'-AAAAGGATCCCTAGATGTAGCGTCTACT-3'	60 °C	723 bp
	E6	Fw: 5'-AAAAGAATTCATGGAGCGTCCTCGCACG-3' Rev: 5'-AAAAGGATCCTTATTGCAGGTACCTGCACAG-3'	62 °C	437 bp
	E7	Fw: 5'-AAAAGAATTCATGATTGGCACGACACCT-3' Rev: 5'-AAAAGGATCCCTAGATGTAGCGTCTACT-3'	50 °C	308 bp
Cloning in pGEX4T1	E6	Fw: 5'-AAAAGAATTCATGGAGCGTCCTCGCACG-3' Rev: 5'-AAAAGCGGCCGCTTATTGCAGGTACCTGCACAG-3'	62 °C	439 bp
	E7	Fw: 5'-AAAAGAATTCATGATTGGCACGACACCT-3' Rev: 5'-AAAAGCGGCCGCCTAGATGTAGCGTCTAGT-3'	50 °C	310 bp
Real-time qPCR	p53 *	Fw: 5'-GCGCCTATGGTTTCCATTTA-3' Rev: 5'-GGCAAAACAGCTTGTTGAGG-3'	60 °C	237 bp
	Bcl-Xl	Fw: 5'-GGAGCTGGTGGTTGACTTTC-3' Rev: 5'–AGGATGGGTTGCCATTGAT-3'	60 °C	237 bp
	Cdc2	Fw: 5'- CCCCCAATAATGAAGTGTGG-3' Rev: 5'-CCATTTTGCCAGAAATTCGT-3'	60 °C	176 bp
	cMyc	Fw: 5'-CAAAAGGTCGGAATCGGGGT-3' Rev: 5'-CGTGGCATCTCTTAAGGACCA-3'	60 °C	83 bp
	GAPDH	Fw: 5'-CCATGTTTGTGATGGGCGTG-3' Rev: 5'-TGATGGCATGGACTGTGGTC-3'	60 °C	159 bp

Table 1. Details of PCR primers used throughout the work for each application and each gene. The nucleotide sequences, the annealing temperatures (Tm °C) and the size of amplified fragments are indicated (bp:base pairs) (* from Mochizuchi et al., 2012)

4. RESULTS

4.1 FcaPV2 E2, E6 and E7 genes are expressed in feline SCC *in vivo*

To assess whether FcaPV2 was biologically active in feline skin cancer and to hypothesize a role in the pathogenesis of these tumours, we aimed to demonstrate putative viral oncogenes expression in a series of feline SCCs, non SCC skin lesions and normal skin biopsies.

Firstly, 10 normal skin samples (N1-N10), 1 oral SCC sample (T1), 8 cutaneous SCCs (T2-T9), 1 epithelial dysplasia of unknown origin (T10) and 1 sarcoma sample (T11) were analyzed by PCR amplifying a specific fragment of FcaPV2 L1 gene to check for the presence of viral DNA. A band of the expected molecular size was amplified from 4 out of 10 (40%) normal skins (N1, N2, N5, N9), from the oral SCC (T1) and from 8 out of 8 (100%) cutaneous SCCs (T2-T9); the epithelial dysplasia (T10) and the sarcoma sample (T11) were negative for viral DNA (data not shown). The amplicons from samples T1 and N5 were sequenced, revealing a 92% and 95% of sequence homology with the specific fragment of FcaPV2 L1.

RNA could be extracted from 4/4 DNA positive normal skins, from the oral SCC and from 7/8 DNA positive cutaneous SCCs and analyzed by RT-PCR to check for E2, E6 and E7 genes expression. The fragments of E2 and E6 genes were successfully amplified from samples T1, T3, T5, T6 and T7 (**Figure 15**A). The E7 amplicons were detected in samples T6 and T7, while a second amplification reaction on the first PCR products was necessary to

detect E7 amplicons in samples T1, T3, T4 and T5 **(Figure 15B).** Among the 4 normal skins analyzed, N1 was potitive for E2, E6 and E7 expression, whilst only E6 could be amplified from N2. GAPDH was amplified to chek the quality of the RNA and it was detected in all the samples (**Figure 15A**). E2, E6 and E7 amplicons from T6 and T7 were sequenced revealing 96% sequence homology with the FcaPV2 E2 for both, 94% and 97% with E6, and 100% and 94% with E7, respectively. E2 fragment amplified from N1 was 99% identical to the E2 sequence, whilst the E6 fragment sequenced from N2 showed 93% homology with the corresponding FcaPV2 gene.

In summary, 100% of SCCs (including 1 oral and 7 cutaneous samples), none of the non SCC skin diseases and 40% of normal skins were positive for viral DNA. Most importantly, the virus was found to be transcriptionally active in the 89% of the analyzed DNA positive feline SCC samples versus the 50% of DNA positive normal skins. E2 protein is involved in viral transcription and viral genome replication, whilst E6 and E7 are considered as putative oncogenes for analogy with other oncogenic PVs known so far. Thus, taken toghether, these findings demonstrate that FcaPV2 in biologically active *in vivo* and plays a causative role in the development of feline oral and skin cancer.

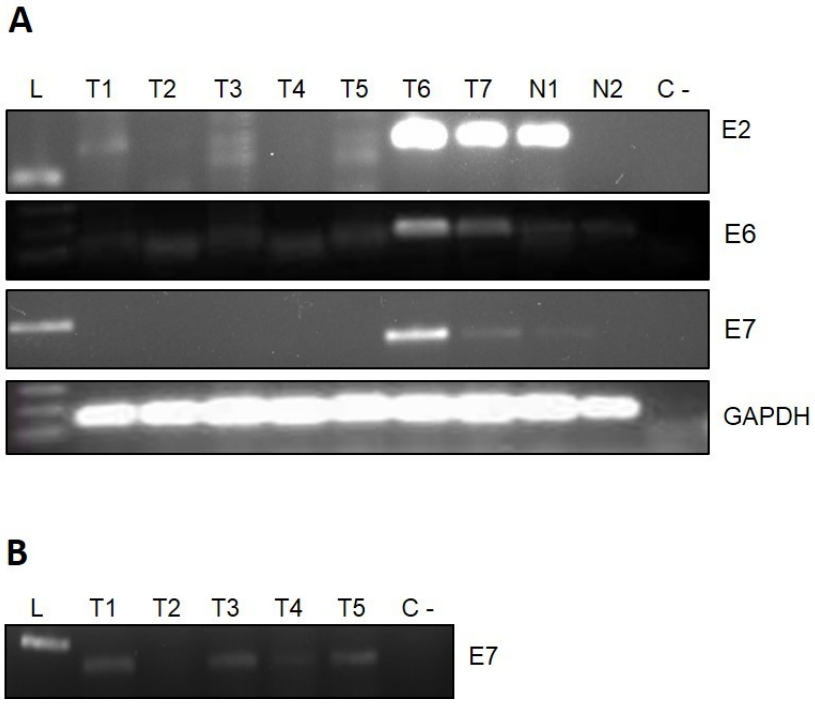

Figure 15

FcaPV2 is transcriptionally active in feline SCC *in vivo*. (A) E2 and E6 mRNA were detected in 5/7 tumor samples and in 1/2 normal skins; E7 was readily found to be expressed in 2 SCC and in 1 normal skin. GAPDH was successfully amplified from all the samples ensuring adequate RNA quality. **(B)** When performing a second amplification on the first PCR products, E7 expression was detected also in 4/5 of the additional samples analyzed. Representative panels from RT-PCR are shown.

4.2 Expression of FcaPV2 E6 and E7 oncogenes in feline epithelial cells CRFK

To establish an in vitro model of FcaPV2 associated feline skin cancer, feline epithelial cells CRFK were transfected with pCEFLE6E7 (CRFKE6E7). To further understand which among E6 and E7 genes was responsible for the biological effects to be investigated, pCEFLE6 and pCEFLE7 were also transfected individually (CRFKE6, CRFKE7). Once subjected to selection by the antibiotic resistance conferred by the vector, transfected cells pools were obtained and analyzed by RT-PCR for the expression of the transfected genes. As expected both E6 and E7 were succesfully amplified from CRFKE6E7, while only E6 and only E7 were expressed in CRFKE6 and CRFKE7, respectively; the specificity of PCR bands was ensured by using FcaPV2 genome as positive control and GAPDH cDNA, amplified to check the quality of the starting RNA, was detected in all the cell lines. (**Figure 16**). The amplicons corresponding to E6 and E7 genes were sequenced, revealing a 100% homology with FcaPV2 E6 and E7 sequences.

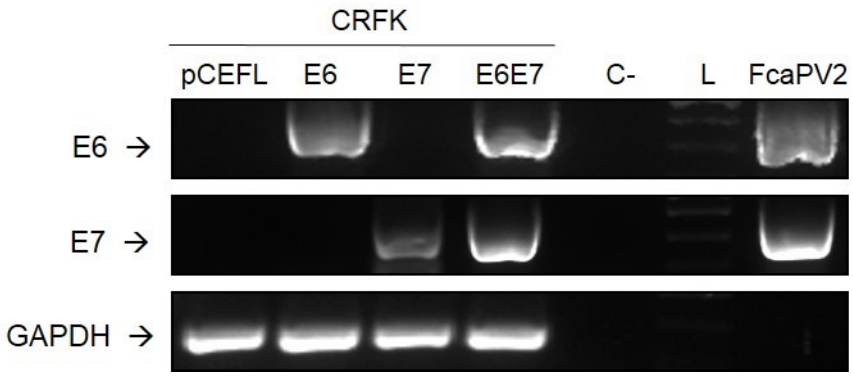

Figure 16

Expression of FcaPV2 oncogenes in feline epithelial cell lines CRFK. A representative RT-PCR gel showing mRNA expression of the indicated FcaPV2 putative oncogenes in transfected CRFK cells.

4.3 FcaPV2 impairs p53 at protein level upon E6 expression, while E7 downregulates p53 mRNA

Since tumour suppressor p53 is the cellular target most frequently impaired in PVs induced cancers, we firstly aimed at assessing the p53 protein levels in CRFKE6E7, CRFKE6 CRFKE7 by Western blotting analysis on whole cell lysates: **Figure 17A** shows that p53 protein is significantly downregulated in CRFKE6E7, CRFKE6 and CRFKE7 when compared to control cells (CRFKpCEFL), as confirmed by densitometric analysis normalized for β-actin on three independent experiments (**Figure 17B**).

While E6 from HR mucosal HPVs and from several cutaneous HPVs is known to degrade p53 protein, no mechanisms by E7 are known to impair p53 protein. To further investigate the mechanisms of p53 downregulation by E7, Real-time qPCR was performed on cDNA retrotranscribed from total RNA extracted from all the cell lines, twice in technical duplicate. As shown in **Figure 17C,** p53 was found to be downregulated at mRNA level specifically upon E7 expression, suggesting different and unreported mechanisms by this oncogene aimed at affecting p53 expression.

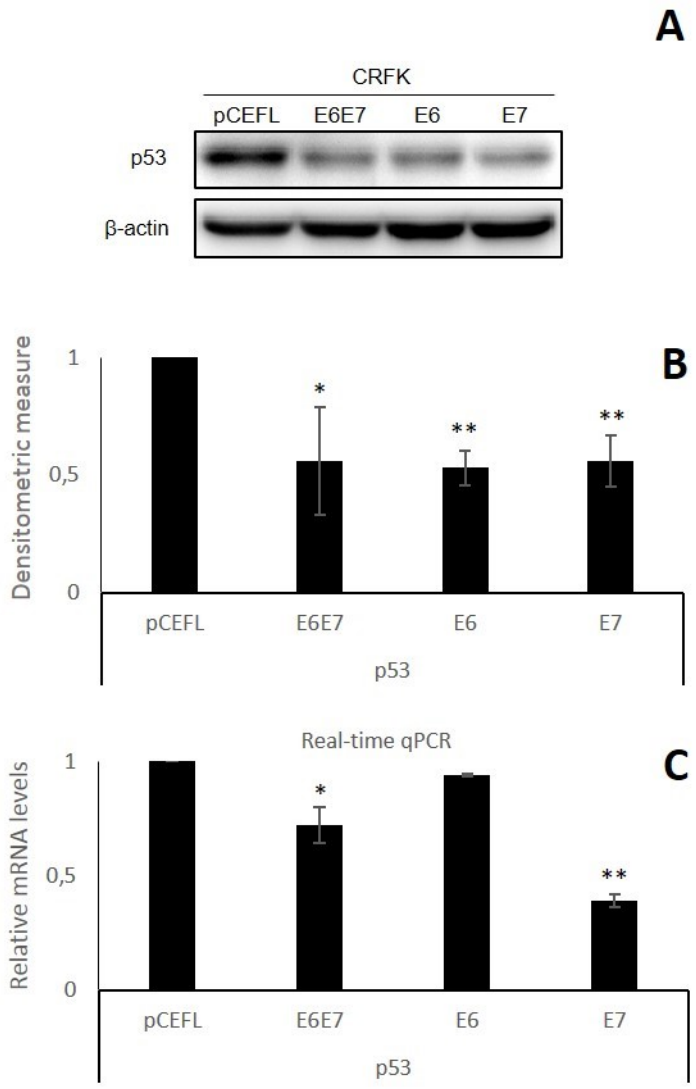

Figure 17

FcaPV2 oncogenes impair p53 levels. (A) A representative Western blotting showing dowregulation of p53 in cells expressing E6E7, E6 and E7. **(B)** Mean densitometric values normalized to β-actin and SD from 3 independent experiments. **(C)** FcaPV2 downregulates p53 mRNA level upon E7 expression; Real-time qPCR data from independent experiments normalized for GAPDH are shown. (*p<0,05; **p<0,01).

4.4 FcaPV2 oncogenes impair p53/p21 UVB responsive pathway

Several skin PVs are known to inhibit UVB induced cell cycle arrest through impairing p53/p21 pathway, mainly reducing p53 protein levels or its function through post-translational modifications, constituting therefore a possible mechanisms triggering skin carcinogenesis. To assess whether FcaPV2 is able to counteract p53/p21 responsiveness to UVB in our feline model, cells were irradiated with UVB light at 25 mJ/cm^2 and subjected to Western blotting for p53 and p21 after 8 h (**Figure 18A**). Importantly, the results confirmed the lower p53 protein levels in all the cell lines expressing the oncogens compared to control cells. As expected, p53 accumulation could be observed in CRFKpCEFL after UVB treatment, whilst almost undetectable protein augmentation was detected in CRFKE6E7, CRFKE6 and CRFKE7. Data from 3 independent experiments were normalized for β-actin levels and are presented as fold increase compared to the internal not irradiated control for each cell line put as 1 (**Figure 18B**). Consistently, p21 was accumulated in CRFKpCEFL treated with UVB, but not in other cell lines. Densitometric analysis normalized for β-actin from 3 independent experiments yielding comparable results is shown (**Figure 18C**). These results indicate that p53 downregulation by FcaPV2 oncogenes leads to its non-responsiveness to UVB irradiation. As a consequence, cells do not undergo p21-induced cell cycle arrest, suggesting that this common mechanism at the basis of PVs induced skin cancer is shared also by FcaPV2.

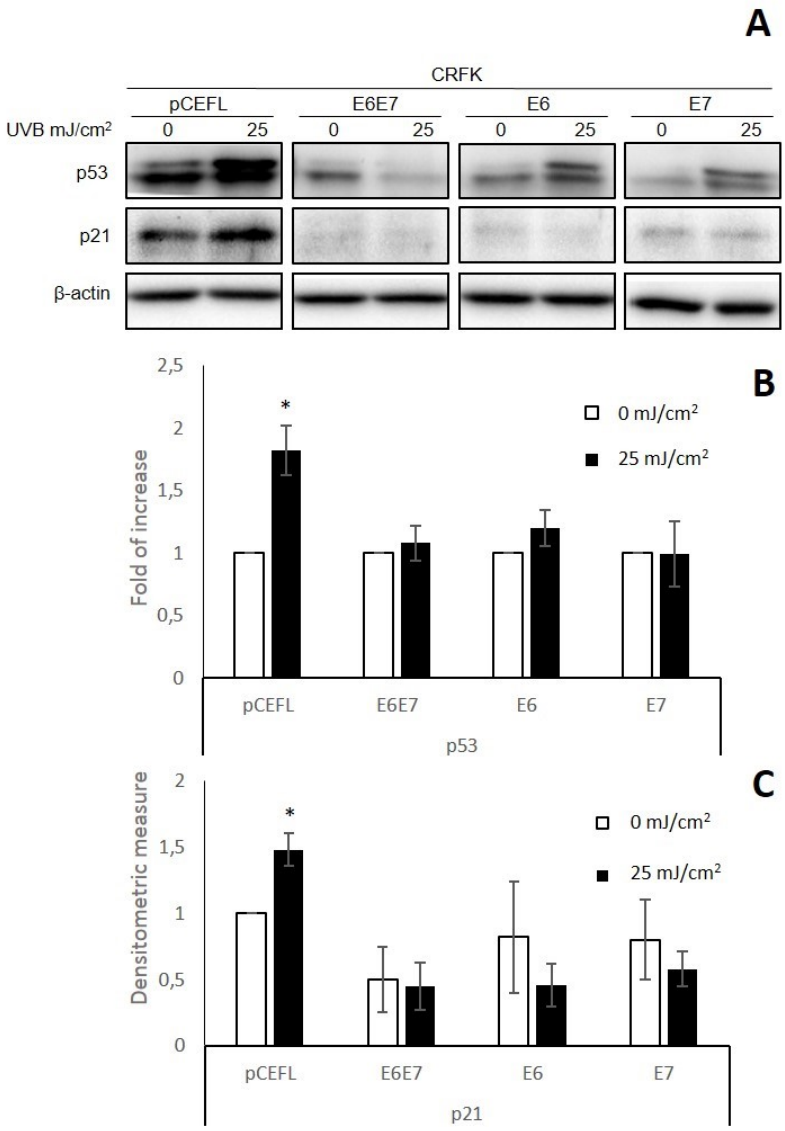

Figure 18

FcaPV2 oncogenes impair p53/p21 UVB responsive pathway. **(A)** p53 and p21 are upregulated after UVB irradiation (25 mJ/cm^2) in control cells CRFKpCEFL but not in cells expressing E6E7, E6 and E7; representative Western blotting are shown. **(B)** Mean densitometric values normalized to β-actin and SD from 3 independent experiments for p53 are presented as fold increase relative to not irradiated control for each cell line. **(C)** Mean densitometric values normalized to β-actin and SD from 3 independent experiments for p21. (*p<0,05).

4.5 FcaPV2 counteracts UVB induced apoptosis

Several cutaneous HPVs belonging to genus *beta* are know to impair UVB induced apoptosis, in order to counteract cell death and keep an high proliferative epithelium which may favour viral replication. Here, we show that FcaPV2 is able to counteract UVB induced apoptosis in feline epithelial cells. Western blotting analysis revealed that Caspase3 was highly cleaved in CRFKpCEFL after UVB exposure, while almost no cleavage could be detected in all the cell lines expressing the different oncogenes (**Figure 19A**). Consistently, a signficant accumulation of pro-apoptotic proteins Bax and Bak could be observed in UVB exposed CRFKpCEFL but not in CRFKE6E7, CRFKE6 and CRFKE7 (**Figure 19A**); densitometric data normalized for β-actin from at least two independent experiments are shown (**Figure 19B, C, D**). Bcl-Xl is a pro-survival protein which inhibits Bak pro-apoptotic function and is downregulated in apoptotic cells. As expected, Real-time qPCR showed that Bcl-Xl gene expression is downregulated in irradiated control cells, while its mRNA levels remain stable in cells expressing E6E7 and E6 when treated with UVB. Despite less potently, E7 inhibits Bcl-Xl downregulation as well. **Figure 19E** shows mean values and standard deviations (SD) from three independent experiments performed in technical duplicate, normalized for GAPDH. These results strongly suggest that FcaPV2 impairs UVB induced apoptosis, as already known for cutaneous HPVs. This effect is likely to be, at least in part, linked to p53 downregulation and its lower responsiveness to UVB assessed above.

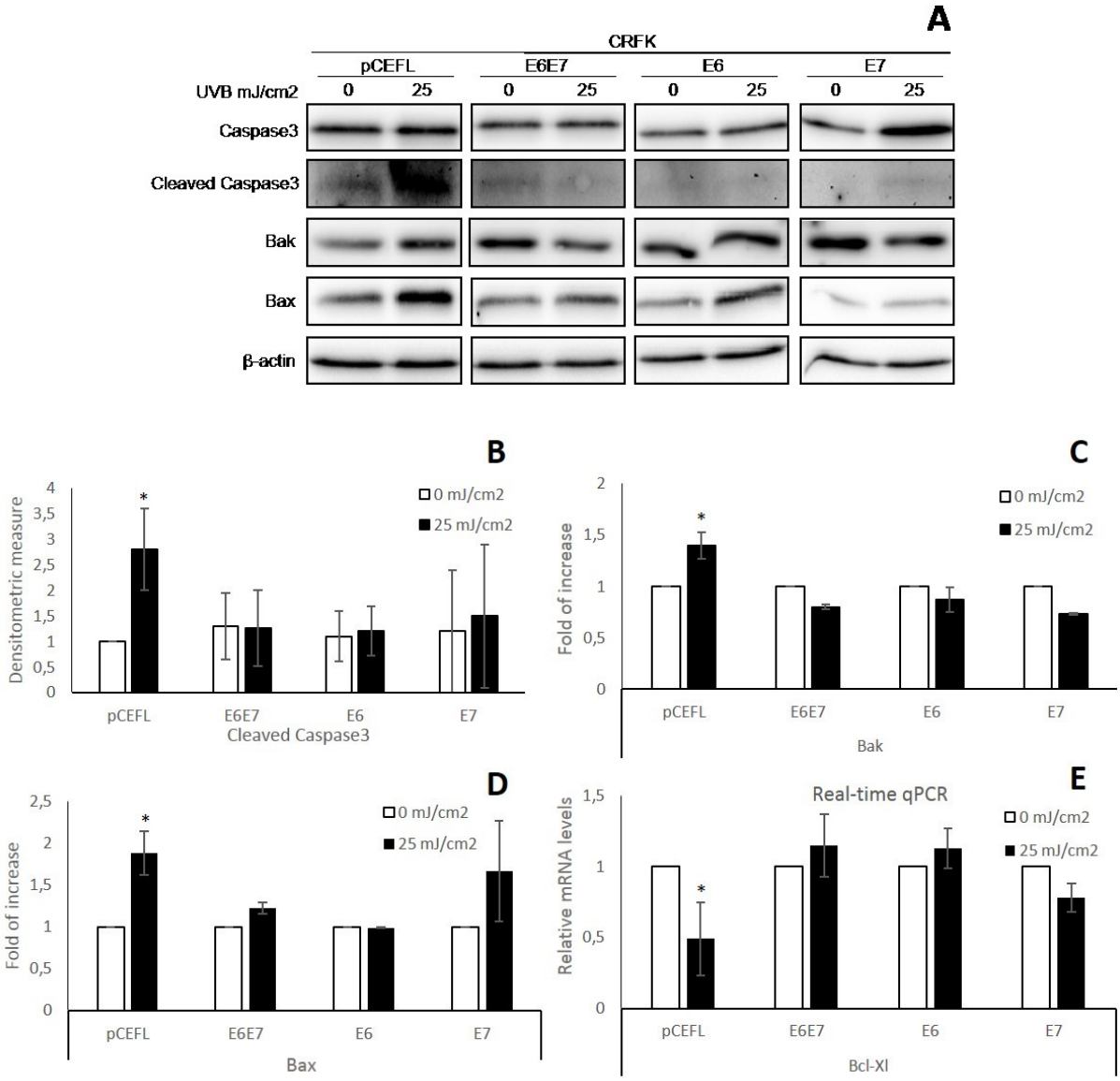

Figure 19

(continued on following page)

FcaPV2 oncogenes counteracts UVB induced apoptosis. (A) Caspase3 is cleaved after UVB irradiation (25 mJ/cm^2) in control cells CRFKpCEFL but not in cells expressing E6E7, E6 and E7; consistently, a signficant accumulation of pro-apoptotic proteins Bax and Bak could be observed in UVB exposed CRFKpCEFL but not in CRFKE6E7, CRFKE6 and CRFKE7; representative Western blotting are shown. **(B)** Mean densitometric values normalized to β-actin and SD from independent experiments for Caspase3 are shown. **(C)**, **(D)** Mean densitometric values normalized to β-actin and standard deviations SD from independent experiments for Bak and Bax are presented as fold increase relative to not irradiated control for each cell line. **(E)** Anti-apoptotic Bcl-Xl gene expression is downregulated in irradiated CRFKpCEFL cells, while its mRNA levels remain stable in cells expressing E6E7 and E6 when treated with UVB. Despite less potently, E7 inhibits Bcl-Xl downregulation as well. Mean values and SD from independent Real-time qPCR experiments normalized for GAPDH are shown (*p<0,05).

4.6 pRb pathway is impaired in cells expressing FcaPV2 oncogenes

The E7 from HR HPVs and some *beta* cutaneous HPVs inhibits pRb function thorugh different mechanisms, among these the direct binding to pRb and the induction of proteasomal degradation which in turn leads to expression of downstream genes involved in cell proliferation. Here we show by Western blotting that pRb is downregulated in CRFKE6E7, and both E7 and E6 are responsible of such effect when expressed individually (**Figure 20A**). Consistently, Cyclin A and Cdc2 are upregulated in CRFKE6E7 and CRFKE7, while in CRFKE6 Cdc2 but not Cyclin A is overexpressed, suggesting different mechanisms of action by E6 and E7 on pRb pathway (**Figure 20A**). The densitometric mean values normalized for β-actin levels from three independent experiments are shown (**Fig 20B, C, D**). Additionally, Real-time qPCR amplifying Cdc2 and cMyc was performed three times in technical duplicate, in parallel with GAPDH to allow normalization. As expected, Cdc2 was found to be upregulated also at mRNA level, thus strenghtening the biochemical findings (**Figure 20E**). The cell cycle promoter cMyc was found overexpressed in presence of the whole operon E6E7 in repeated experiments, but the specific contribution to its upregulation by E6 and E7 individually could not be fully clarified, since the results were not perfectly reproducible (data not shown).

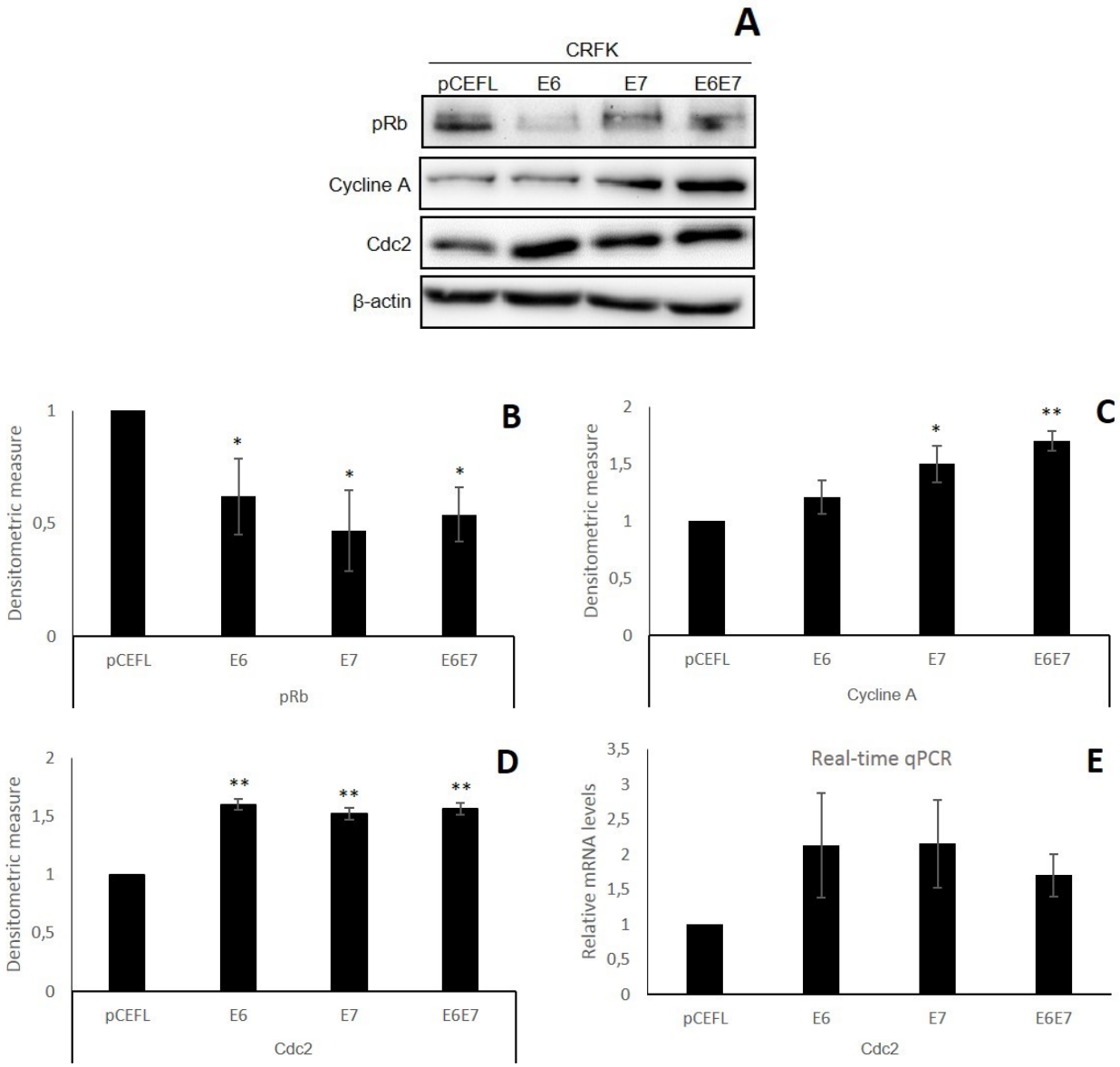

Figure 20

FcaPV2 oncogenes impair the pRb pathway. (A) Representative Western blotting showing dowregulation of pRb and upregulation of its downstream gene Cdc2 in cells expressing E6, E7 and E6E7; Cycline A is upregulated upon E7 expression but not in E6 expressing cells. **(B), (C), (D)** Mean densitometric values normalized to β-actin and SD from three independent experiments for pRb, Cycline A and Cdc2. **(E)** Real-time qPCR data from three independent experiments showing Cdc2 mRNA upregulation in CRFKE6, CRFKE7 and CRFKE6E7 compared to CRFKpCEFL. (*p<0,05, **p<0,01).

4.7 FcaPV2 E6 and E7 bind to feline p53

E6 proteins from *alpha* HR and several *beta* cutaneous HPVs are able to bind p53 protein directing it to proteasome and triggering its degradation. To check whether FcaPV2 E6 and E7 (since E7 expression was shown to downregulate p53 as well) were able to bind p53, GST-FcaPV2 E6 and GST-FcaPV2 E7 fusion proteins were generated and a GST pull-down assay was performed. **Figure 21A** shows that FcaPV2 E6 binds to feline p53 when incubated with CRFK cell lysate, as well as HPV16 E6. Since the binding HPV16 E6 with human p53 is widely known, the same experiment was performed incubating the fusion proteins with human cells lysate (HaCat) as positive control: the obtained results confirmed this interaction and showed that FcaPV2 E6 is able to bind also human p53 (**Figure 21B**). Surprisingly, HPV16 E7 and FcaPV2 E7 were shown to bind both human and feline p53 (**Figure 21C, D**). The ability of HPV16 E7 to bind p53 has never been reported and encourages new studies to unravel unexplored mechanism by which HPV16 may impair p53 tumor suppressor pathway. Despite strongly suggested by these new unreported results, whether the binding by FcaPV2 E7 is directly involved in p53 downregulation remains to be established in future studies.

4.8 FcaPV2 E7 binds to feline pRb

E7 proteins from HR *alpha* and several *beta* cutaneous HPVs bind pRb protein directing it to proteasome and triggering its degradation. To check whether FcaPV2 E7 and E6 (since pRb was downregulated upon E6 expression as well) were able to bind pRb, a GST pull-down assay was performed. **Figure 21C** shows that FcaPV2 E7 binds to feline pRb in CRFK cell lysate, as well as HPV16 E7. The binding of HPV16 E7 to pRb was also performed incubating the fusion proteins with human cells lysate (HaCat) as positive control (**Figure 21D**). However, FcaPV2 E6 and, as expected, HPV16 E6 were not able to bind feline nor human pRb, suggesting different mechanisms leading to pRb downregulation by FcaPV2 E6 and E7 (**Figure 21A, B**). Further studies will clarify this point.

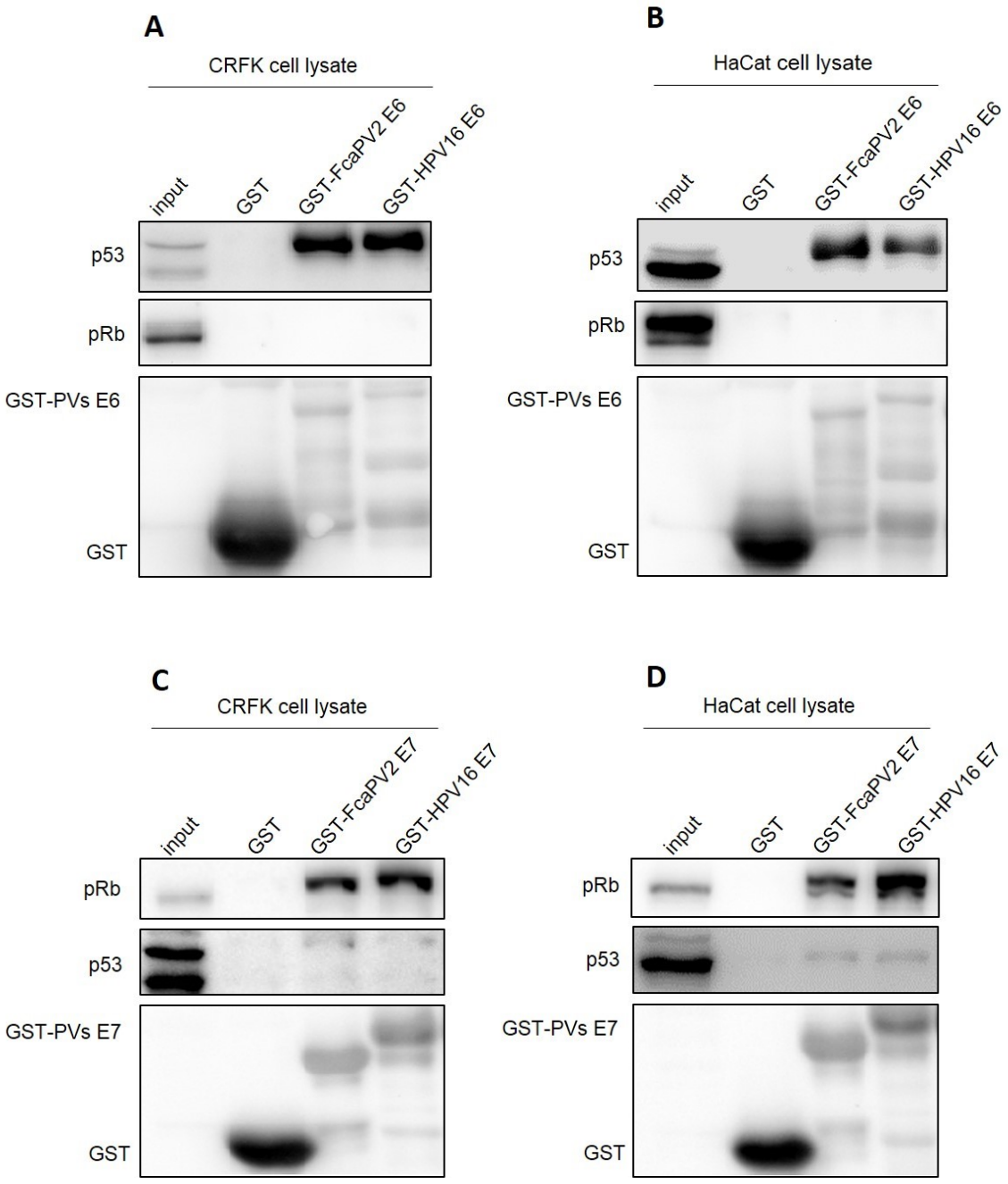

Figure 21

(continued on the following page)

FcaPV2 E6 binds to feline and human p53 but not to pRb, while FcaPV2 E7 binds to feline and human pRb and p53. (A) GST pull-down assay showing the binding of FcaPV2 and HPV16 GST-E6 fusion proteins to feline p53 but not pRb in CRFK cells. One-tenth of the total cellular extract (60 g) used in the GST pulldown assay was loaded on the gel as a control (input). **(B)** The same experiment on HaCat human cell line performed as positive control confirmed the interaction of HPV16 E6 with human p53 and showed the same ability by FcaPV2E6. One-tenth of HaCat cellular extract used in the GST pull-down assay was loaded as input. (**C)** GST pull-down assay showing the binding of FcaPV2 and HPV16 GST-E7 fusion proteins to feline pRb and p53 in CRFK cells. One-tenth of the total cellular extract (60 g) used in the GST pulldown assay was loaded on the gel input. (**D)** The same experiment on HaCat human cell line performed as positive control confirmed the interaction of HPV16 E7 with human pRb and showed the same ability by FcaPV2 E7; interactions of HPV16 E7 and FcaPV2 E7 with human p53 were detected. One-tenth of HaCat cellular extract used in the GST pull-down assay was loaded as input.

4.9 FcaPV2 prolongs the life-span of HPK

As in healthy epithelia, where they are committed to terminal differentiation after a proliferative stage, cultured human primary keratinocytes (HPK) are intended to senescence and cell death after several PD. Whether a PV is oncogenic, it is able to immortalize or prolong the life-span of HPKs as observed for human and animal oncogenic PVs. Here we show that the life-span of primary cells transduced with FcaPV2 E6E7 (pLXSNE6E7) is much longer when compared to control cells (pLXSN trandusced cells), with 11 PD versus 1 PD, respectively (**Table 2**). When looking at the effect of the single oncogenes, we found that HPKs expressing E7 alone (pLXSNE7) were able to undergo 6 PD, while the cells transduced with E6 (pLXSNE6) did not survive the selection. To further investigate E6 transforming potential, it was tranduced again in HPKs from a different donor, since cells from the previous one were no longer available: these primary cells (pLXSNE6) were succesfully selected and showed a delayed senescence phase with respect to control cells and grew until reaching 50% of confluence, but they were not able to further replicate (**Table 2**)**.** The growth curve showing the different growth profiles and the morphology of each cell line is shown in **Figure 22.** These data indicate that FcaPV2 display transforming properties prolonging the life-span of primary cells. Additionally, E7 but not E6 seems to be able to increase the proliferative ability of HPK, despite less potently than the whole natural operon E6E7.

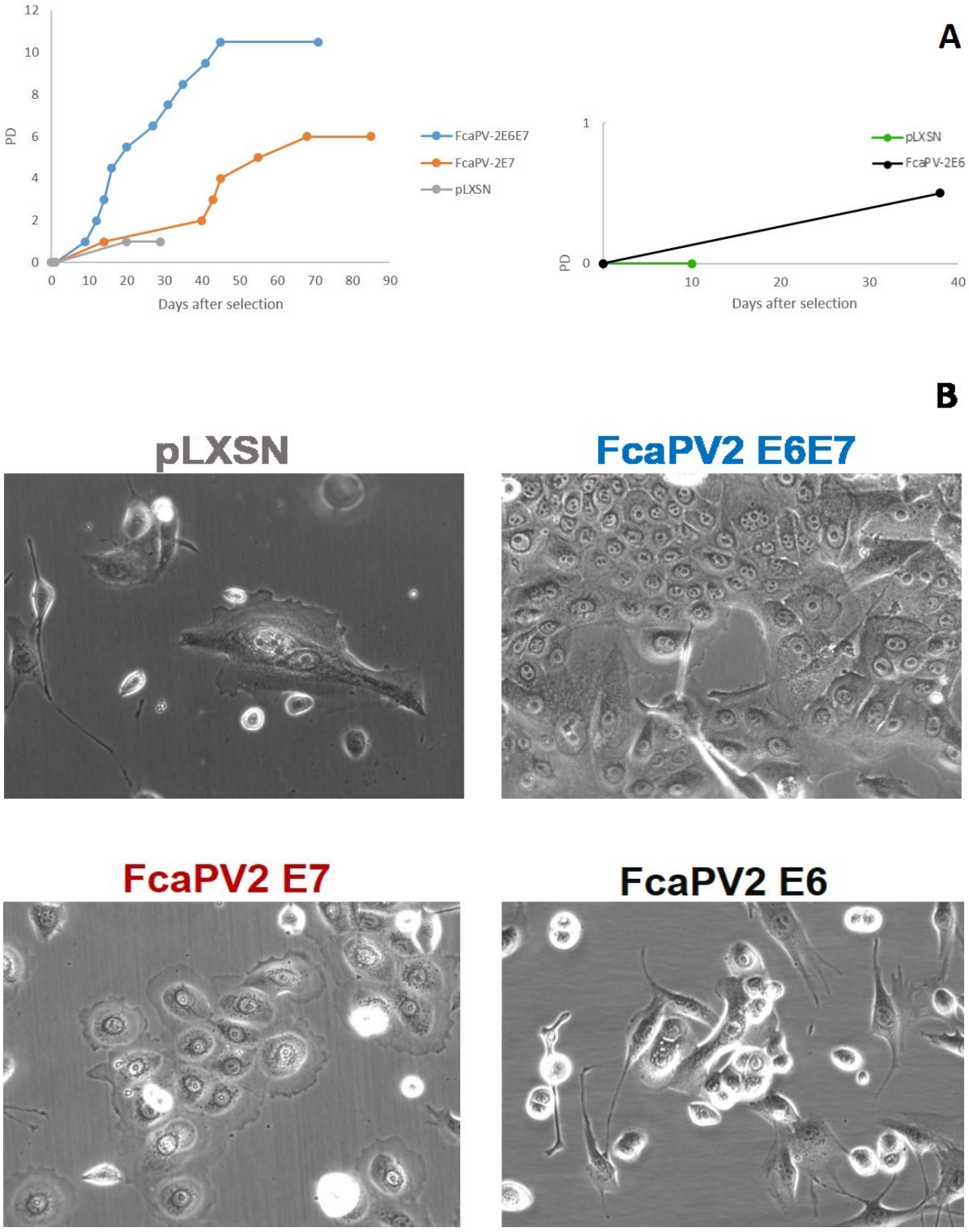

Figure 22

(continued on following page)

FcaPV2 prolongs the life-span of human primary keratinocytes (HPK). (A) Growth curves of HPK transduced with the indicated recombinant retroviruses showing the prolonged life-span and the enhanced proliferative ability conferred by FcaPV2 E6E7 and FcaPV2 E7 compared to control cells (pLXSN) (left panel); FcaPV2 E6 prolonged the senescence phase but did not affect proliferation rate compared to control cells (pLXSN) (right panel). (**B)** Morphology of HPK transduced with the indicated recombinant retroviruses 20 days postransduction. Note the features of cellular senescence in pLXSN and FcaPV2 E6 transduced cells with enlarged cytoplasms and intercellular bridges.

Retrovirus	Number of PD before senescence from donor:	
	1	2
None (empty pLXSN)	1	0
pLXSNE6E7	11	NA
pLXSNE6	-	0,5
pLXSNE7	6	NA

Table 2. Life-span of human primary keratinocytes transduced with empty vector pLXSN or with recombinant retroviruses pLXSNE6E7, pLXSNE6 and pLXSNE7 and cultured for several population dublings (PD) (-: not succesfully transduced; NA: not assessed).

5. DISCUSSION

PVs are double stranded DNA viruses infecting skin and squamous mucosal epithelia of mouth and ano-genital tract, where they are able to cause benign hyperproliferative lesions as well as malignant cancer in human and animal species (Campo, 2002). Different cutaneous lesions are known to occur in the domestic cat, among these SCCs and precursors such as viral plaques and BISCs, mostly affecting old, white coated animals in UV-exposed sites (nasal planum, ears, eyelids) (Thomson, 2007). This may suggest a role for chronic sun exposure, which is known to be a co-factor in the development of *beta* HPVs-associated cutaneous SCC in humans (Howley and Pfister, 2015). SCCs are the most common cutaneous and oral malignant cancer in cats; they are locally invasive, may metastasize and are not responsive to the current therapies and surgical excision, thus being often fatal or forcing vets to carry out euthanasia (Gross et al., 2005). Molecular investigations throughout the last years revealed a frequent and specific association of FcaPV2 DNA with cutaneous SCCs and precursors, suggesting that PV infection is involved in the pathogenesis of skin cancer also in domestic cat (reviewed in Munday, 2013). As many PVs, FcaPV2 potentially encodes for E6 and E7, which are considered to be oncogenes for analogy with other skin and mucosal PVs. FcaPV2 contains also an ORFs for E2 gene, usually involved in viral genes transcriptional regulation and viral replication (Lange et al., 2009; McBride, 2013). However, viral gene expression has never been demonstrated within feline skin cancer so far, thus a causative role cannot be yet proven. In this work we showed by type-specific PCR that 100% of feline SCCs (including one oral sample) were positive for FcaPV2 DNA versus 40% of normal skins and 0/2 non SCC skin lesions, in accordance with previous studies showing similarly higher rate of association in SCCs. Additionally, this widely reported FcaPV2 inert infection in clinically

healthy animals is very common also for human and other animal PVs: indeed, PVs may undergo latent infection without clinical manifestations until other co-factors, such as the aforementioned UV-exposure, interplay with the virus to trigger lesions development and/or malignant transformation (Thomson et al., 2015; Munday, 2013; Doorbar, 2005; Antonsson et al., 2000; Antonsson et al., 2002). Most importantly, FcaPV2 was found to be transcriptionally active in 89% of our SCCs samples but only in 20% of normal skin, thus corroborating the hypothesis of a carcinogenic role of the virus. Indeed, E2 expression suggest transcriptional and replicative activity by FcaPV2 *in vivo*. Furthermore, the expression of the putative oncogenes E6 and E7 strongly suggest that the virus may display transforming activity in the infected epithelia contributing to cancer development (Stoler et al., 1992). Unfortunately, we could not assess histopathological diagnosis of normal skin samples, thus the hypothesis that those found positive for FcaPV2 mRNA may be dysplastic or not fully healthy cannot be ruled out. Another possible explanation may be that the virus could have infected a subset of cells sufficient in supporting viral cycle but not in triggering development of macro/microscopically detectable neoplastic lesion (Doorbar et al., 2005).

A significant subset (about 20%) of oral SCC in humans is caused by HR *alpha* HPVs (reviewed in Howley, 2013). In domestic cat, FcaPV1 DNA has been found to be associated with oral papillomas and FcaPV4 DNA with an inflammatory disease of the oral cavity, but not with oral SCCs (Dunowska et al., 2014; Munday and French, 2015). Although referred to only one sample, FcaPV2 gene expression in the oral SCC analyzed in this study may suggest a mixed tropism of this virus, both for skin and oral squamous epithelia, and a possible role in oral SCC development.

Once the expression of viral putative oncogenes was demonstrated in skin tumours *in vivo*, we aimed at establishing an *in vitro* model, in order to molecularly characterize the possible transforming properties of FcaPV2, and the tumor suppressors p53 and pRb pathways were investigated, since they are frequently impaired in PVs induced cancer (for review see Nguyen et al., 2014).

Normally present at low levels in cells, p53 tumour suppressor is a transcription factor coordinating the cellular response to genotoxic or citotoxic stress such as UV-induced DNA damage. UV irradiation leads to an increasing in p53 protein levels and activation via post-translational modifications, resulting in increased expression of cell cycle inhibitors such as p21 and/or pro-apoptotic genes including Bax, so that cells are committed to DNA repair or apoptosis in order to avoid transmission of oncogenic mutations to doughter cells (Murray-Zmijevski et al., 2008). The E6 oncoproteins have been implicated in the potential carcinogenic activities of *beta* cutaneous HPVs, mostly through interfering with those processes, thus allowing cells with UV-triggered mutations to survive and progress to cancer. Differently from E6 of *alpha* HR HPVs that binds to p53 leading it to proteasomal degradation and loss of tumour suppressor activity (Scheffner et al., 1990), most of E6 proteins from *beta* cutaneous HPVs are known to impair p53 function through different mechanisms (reviewed in Howley et al., 2015). The only exception known so far is the *beta 3* type HPV49, whose E6 binds to and degrades p53, and HPV76 E6, which is associated with lower p53 protein levels through unknown mechanisms (Cornet et al., 2012; White et al., 2014). Here, we show by GST pull-down assay that FcaPV2 E6 binds to feline p53 protein which results to be downregulated, suggesting that this oncogene behaves similarly to those from *alpha* HR HPVs and *beta* HPV49 and 76. Surprisingly, p53 was found to be

downregulated also upon E7 expression (CRFKE6E7 and CRFKE7) and to bind E7 as well. Whether the physical interaction is involved in p53 downregulation by E7 has to be investigated in further studies. Additionally, when investigating p53 gene expression through qPCR, we found lower p53 mRNA levels in E7 expressing cells. To the best of our knowledge, no PVs E7 has been reported to downregulate p53 gene expression so far. Several works show that suppression of HPV16 E7 expression leads to restoring of p53 protein but not at mRNA level and E7 from *beta* cutaneous HPV38 is known to selectively inhibit expression of p53 downstream genes (Guo et al., 2011; Lee et al., 2011; Kim et al., 2012; Kim et al., 2013., Saidj et al., 2013); all these findings suggest that human and animal PVs may have evolved different mechanisms to reduntantly impair p53 pathway by E7 other than by E6. Consistently with low p53 basal levels, when subjected to Western blotting after UVB irradiation, feline epithelial cells expressing the FcaPV2 oncogenes did not show significant p53 accumulation and, as a consequence, the cell cycle inhibitor p21 was not upregulated. The *alpha* HR HPV16 and 18 E6 have been shown to inhibit p53 dependent-p21 accumulation and p21 was not responsive to UVB irradiation in cells expressing *beta* types HPV38, 49 and 76 due to p53 inactivation, suggesting that FcaPV2 E6 shares common mechanisms with mucosal and cutaneous HPVs to impair p21-dependent cell cycle arrest (Cornet et al., 2012; White et al., 2014). Additionally, FcaPV2 oncogenes were found to be able to counteract UVB induced apoptosis at the same experimental conditions by Western blotting for different apoptotic markers: indeed, Caspase3, a downstream effector of intrinsic apoptosis activated by proteolytic cleavage (for review see Shalini et al., 2015), was found to be cleaved only in control cells but not in cells expressing E6E7, E6 and E7 after UVB treatment; consistently Bax, a pro-apoptotic protein involved in the formation of the mythocondrial pores which

allows the formation of the apoptosome (Narita et al., 1998), was not accumulated after UVB irradiation in cells expressing FcaPV2 oncogenes, particularly upon E6 expression. Since Bax gene is under the transcriptional control of p53 and is upstream to Caspase3 in the apoptotic intrinsic pathway (Miyashita et al., 1994), the inhibition of apoptosis by FcaPV2 may be, at least in part, dependent on the impairment of p53 pathway by E6 and E7. However, suppression of E6 and E7 expression increases Caspase3 cleavage with a p53 independent manner in chemically-induced apoptosis in cells expressing HPV16 (Kim et al., 2013; Magal et al., 1998). Furthermore, anti-apoptotic activity of E6 from several cutaneous *beta* types has been shown to be uncoupled from p53 transcriptional activity and to be exerted also in p53 null cell lines after UVB exposure, thus further investigations are needed to clarify whether inhibition of apoptosis by FcaPV2 is mechanistically p53-dependent (Jackson and Storey, 2000; Giampieri et al., 2004). Another mechanism by which E6 proteins from a series of cutaneous HPVs such as HPV5, 8, 20, 22, 38, 76, 92 and 96 may counteract UVB-induced apoptosis is the degradation of pro-apoptotic protein Bak which, synergistically with Bax, normally contributes to the release of mythocondrial pro-apoptotic factors (Jackson et al., 2000; Jackson and Storey, 2000; Underbrink et al., 2008); several studies on different models show that, regardless of whether Bak degradation is constitutive or induced by UVB, it never accumulates in cells expressing cutaneous and mucosal HPVs E6, depending on proteasomal degradation (reviewed in Howley and Pfister, 2015). Additionally, E6 and E7 from *alpha* HR HPVs have been found to degrade Bak and impair apoptosis induced by different cellular stresses including UV (Liu et al., 2008; Du et al., 2004). Here, we showed that basal levels of Bak are variable among the different cell lines, but it does not accumulate in CRFK cells expressing FcaPV2 oncogenes after UVB treatment, suggesting that FcaPV2 shares similar

biological properties with oncogenic HPVs in impairing Bak, thus preventing UVB-induced apoptosis. Much more controversies exist about the regulation of anti-apoptotic members of Bcl-2 protein family by PVs oncogenes, among these Bcl-Xl. In non apoptotic cells, Bcl-Xl binds to Bak and blocks its heterodimerization with Bax (Sattler et al., 1997), thus inhibiting the mythocondrial apoptotic cascade; normally, following apoptotic stimuli such as UVB irradiation, Bcl-Xl is downregulated (Michels et al., 2013; Galluzzi et al., 2011). Whilst Bcl-Xl responsiveness to UVB irradiation has never been found to be affected, at least at protein level, by expression of *beta* HPVs E6 proteins, several studies indicate that suppression of HPV18 E6 and, less commonly, E7 expression leads to Bcl-Xl decreasing thus triggering apoptosis (Strujiki et al., 2008; Li et al., 2015; Ham et al., 2014; Underbrink et al., 2008). Our findings demonstrate by qPCR that Bcl-Xl gene is downregulated in irradiated control cells but, interestingly, this phenomenon is inhibited in presence of FcaPV2 oncogenes, with E6 being the major responsible of such biological effect, indicating that the virus counteracts also Bcl-Xl pathway to inhibit cell death as HPV18. Bcl-Xl is, at least in part, under transcriptional control of p53, thus UVB-induced Bcl-Xl downregulation may be inhibited along with the impairment of p53 pathway upon FcaPv2 oncogenes expression (Galluzzi et al., 2011). Taken together, all these findings strongly suggest that FcaPV2 acts synergistically with UV light in the development of feline SCC, as already known for human counterpart.

Along with p53, the pRb pathway is commonly impaired in PVs induced cancer. The tumour suppressor pRb is a transcriptional repressor that binds to and block E2F transcription factor, inhibiting the activation of genes involved in cell cycle progression and cell proliferation (for review see Frolov et al., 2004). E7 from HR HPVs binds to pRb protein through the pRb binding domain LXCXE directing the cellular tumour suppressor through

proteasomal degradation, whilst cutaneous *beta* HPVs E7 proteins enhance the phosphorylation of pRb: both mechanisms lead to the release of E2F, which in turn activates the expression of pro-proliferative genes Ciclyne A, Cdc2 and cMyc (Dyson et al., 1992; Huh et al., 2007; Zerfass et al., 1995; Cornet et al., 2012). In our model, Western blotting analysis showed lower pRb levels in cells expressing E6E7 and E7, but surprisingly we found pRb downregulation also when E6 was expressed individually. Consistently, both Western blotting and qPCR showed higher Cdc2 expression in CRFKE6E7, CRFKE7 and CRFKE6 compared to control cells. However, Cycline A was upregulated in E7 but not E6 expressing cells. Furthermore FcaPV2 E7 contains the canonical pRb binding domain LXCXE (Lange et al., 2009) and GST pull-down assay demonstrated that, as expected, E7 but not E6 was able to bind feline pRb, suggesting that E6 may act on pRb pathway with differents mechanisms with respect to E7, possibly affecting Cdc2 but not Cyclin A expression. Additionally, studies conducted on cutaneous HPV14 and 22, indicate that lower pRb levels does not necessary result in genes upregulation, indicating that additional events may be required to deregulate pRb downstream genes expression (Cornet et al., 2012). Similarly, qPCR showed higher levels of cMyc mRNA in presence of FcaPV2 E6E7 operon, but repeated experiments did not yield clearly reproducible results on cells expressing E6 and E7 individually, thus the exact contribution of each of the two oncogenes should be investigated by further studies. Previous studies reported pRb downregulation and Cyclin A overexpression in feline SCC *in vivo* (Munday and Aberdein, 2012; Murakami et al., 2000), thus the results obtained in our functional model strongly suggest a role of FcaPV2 in corrupting pRb pathway and contributing to SCC development; however, whilst FcaPVE7 seems to behave like HR HPVs

E7, further studies are required to understand the molecular mechanism by which E6 may differently impair pRb pathway.

To further characterize the transforming properties of FcaPV2 oncogenes, an *in vitro* immortalization assay was performed. Oncogenic HPVs are able to immortalize or prolong the life-span of HPK (Cornet et al., 2012). We observed that HPK transduced with recombinant retroviruses expressing E6E7 showed a longer life-span, being able to undergo more PD and to survive in culture for many additional days when compared to control cells, confirming the transforming potential of FcaPV2. When expressed individually, E6 was found only to be able to prolong the senescence phase without affecting the proliferation ability of HPK, whilst E7 enhanced the number of PD but less efficiently than E6E7, suggesting that they make different contributions to the transforming process and must act synergistically to display the full carcinogenic potential of FcaPV2. In veterinary oncology, similar findings were reported for the CRPV, where E7 was shown to prolong alone and in combination with E6 the life-span of HPK, suggesting that this is a reliable assay to analyze the transforming properties of E6 and E7 oncogenes of animal PVs (Ganzenmueller et al., 2008).

In summary, our study demonstrates for the first time a possible causative role of FcaPV2 *in vitro*. We showed viral gene expression *in vivo* in tumour samples and we established the first in vitro model in order to characterize the transforming properties of FcaPV2 E6 and E7 oncogenes. The p53 and pRb pathways classically corrupted in PV-induced cancer were found to be impaired by E6 and E7, respectively, and new possible cross-acting mechanisms on the reciprocal pathway were identified. Furthermore, the two oncogenes were able to impair UVB-induced cell cycle arrest and apoptosis, strengthening the

hypothesis of sun exposure as a co-factor in viral-triggered skin carcinogenesis in the domestic cat, and to prolong the life-span of primary cells, confirming definitively the transforming properties of FcaPV2.

6. FUTURE PROSPECTS

Skin tumours of domestic cat are of considerable interest in veterinary medicine, since they are not responsive to current therapies and are often lethal. Unraveling molecular pathways whose alterations are at the basis of feline skin cancer may open a new field of inquiry addressed to the formulation of vaccination principals for the prevention of these severe tumors of cats, as it has recently happened with HPV induced cervical cancer in women, and/or to the identification of molecular targets for a possible therapeutical approach. Additionally, they show high similarity with the human counterpart. Thus, feline SCC is considered a new model for the study of spontaneous SCC human as well.

7. ACKNOWLEDGMENTS

The author wishes to express his gratitude to: Prof. C.E. Lange for the kind gift of cloned FcaPV2 genome, to Dr. F. Albanese, Dr. F. Bocaneti, Prof. S. Brandt, Dr. E. Costa, Dr. E. Lanaro, Dr. M.R. Lorenzo, Dr F. Valentini, Prof. L. Navas, for having provided the samples for *in vivo* studies.

8. BIBLIOGRAPHY

Androphy EJ, Lowy DR, Schiller JT, Bovine papillomavirus E2 transactivating gene product binds to specific sites in papillomavirus DNA. Nature.1987 Jan 1-7;325(6099):70-3.

Anis EA, O'Neill SH, Newkirk KM, Brahmbhatt RA, Abd-Eldaim M, Frank LA, Kania SA, Molecular characterization of the L1 gene of papillomaviruses in epithelial lesions of cats and comparative analysis with corresponding gene sequences of human and feline papillomaviruses. Am J Vet Res. 2010 Dec;71(12):1457-61. doi: 10.2460/ajvr.71.12.1457.

Antonsson A, Hansson BG, Healthy skin of many animal species harbors papillomaviruses which are closely related to their human counterparts. J Virol. 2002 Dec;76(24):12537-42.

Antonsson A, Forslund O, Ekberg H, Sterner G, Hansson B, The ubiquity and impressive genomic diversity of human skin papillomaviruses suggest a commensalic nature of these viruses. J Virol. 2000 Dec;74(24):11636-41.

Ashrafi GH, Brown DR, Fife KH, Campo MS, Down-regulation of MHC class I is a property common to papillomavirus E5 proteins. Virus Res. 2006 Sep;120(1-2):208-11. Epub 2006 Jun 15.

Baer KE, Helton K, Multicentric squamous cell carcinoma in situ resembling Bowen's disease in cats. Veterinary Pathology 1993, 30, 535–543.

Barsoum J, Prakash SS, Han P, Androphy EJ, Mechanism of action of the papillomavirus E2 repressor: repression in the absence of DNA binding. J Virol. 1992 Jun;66(6):3941-5.

Bastien N, McBride AA, Interaction of the papillomavirus E2 protein with mitotic chromosomes, Virology. 2000 Apr 25;270(1):124-34.

Becker KA, Florin L, Sapp C, Maul GG, Sapp M, Nuclear localization but not PML protein is required for incorporation of the papillomavirus minor capsid protein L2 into virus-like particles. J Virol. 2004 Feb;78(3):1121-8.

Bernard BA, Bailly C, Lenoir MC, Darmon M, Thierry F, Yaniv M, The human papillomavirus type 18 (HPV18) E2 gene product is a repressor of the HPV18 regulatory region in human keratinocytes. J Virol. 1989 Oct;63(10):4317-24.

Bernard HU, Burk RD, Chen Z, van Doorslaer K, zur Hausen H, de Villiers EM, Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments.Virology. 2010 May 25;401(1):70-9. doi: 10.1016/j.virol.2010.02.002. Epub 2010 Mar 5.

Bernard HU, Taxonomy and phylogeny of papillomaviruses: an overview and recent developments.Infect Genet Evol. 2013 Aug;18:357-61. doi: 10.1016/j.meegid.2013.03.011. Epub 2013 Mar 20. Review.

Birnstiel ML, Busslinger M, Strub K, Transcription termination and 3' processing: the end is in site! Cell. 1985 Jun;41(2):349-59.

Bocaneti F, Altamura G, Corteggio A, Velescu E, Roperto F, Borzacchiello G, Bovine Papillomavirus: New Insights into an Old Disease. Transbound Emerg Dis. 2014 Mar 24. doi: 10.1111/tbed.12222. [Epub ahead of print]

Borgogna C, Zavattaro E, De Andrea M, Griffin HM, Dell'Oste V, Azzimonti B, Landini MM, Peh WL, Pfister H, Doorbar J, Landolfo S, Gariglio M, Characterization of beta papillomavirus E4 expression in tumours from Epidermodysplasia V erruciformis patients and in experimental models.Virology. 2012 Feb 20;423(2):195-204. doi: 10.1016/j.virol.2011.11.029. Epub 2012 Jan 2.

Boxman IL, Russell A, Mulder LH, Bavinck JN, ter Schegget J, Green A, Association between epidermodysplasia verruciformis-associated human papillomavirus DNA in plucked eyebrow hair and solar keratoses. J Invest Dermatol 2001;117:1108–12.

Bravo IG, Alonso A, Mucosal human papillomaviruses encode four different E5 proteins whose chemistry and phylogeny correlate with malignant or benign growth. J Virol. 2004 Dec;78(24):13613-26.

Brown DR, Bryan Brown JT, Abnormalities of cornified cell envelopes isolated from human papillomavirus type11-infected genital epithelium. Virology. 2000 May 25;271(1):65-70.

Brown DR, Kitchin D, Qadadri B, Neptune N, Batteiger T, Ermel A, The human papillomavirus type 11 E1∧E4 protein is a transglutaminase 3 substrate and induces abnormalities of the cornified cell envelope.Virology. 2006 Feb 5;345(1):290-8. Epub 2005 Oct 28.

BryanJT, BrownDR,Transmission of human papillomavirus type 11 infection by desquamated cornified cells.Virology. 2001 Mar 1;281(1):35-42.

Burnett S, Jareborg N, DiMaio D, Localization of bovine papillomavirus type 1 E5 protein to transformed basal keratinocytes and permissive differentiated cells in fibropapilloma tissue. Proc Natl Acad Sci U S A. 1992 Jun 15;89(12):5665-9.

Calleja-Macias IE, Kalantari M, Allan B, Williamson AL, Chung LP, Collins RJ, Zuna RE, Dunn ST, Ortiz-Lopez R, Barrera-Saldaña HA, Cubie HA, Cuschieri K, Villa LL, Bernard HU, Papillomavirus subtypes are natural and old taxa: phylogeny of human papillomavirus types 44 and 55 and 68a and -b.J Virol. 2005 May;79(10):6565-9.

Campo MS, Animal models of papillomavirus pathogenesis. Virus Res. 2002 Nov;89(2):249-61. Review.

Carney HC, England JJ, Hodgin EC, Whiteley HE, Adkison DL, Sundberg JP, Papillomavirus infection of aged Persian cats. J Vet Diagn Invest. 1990 Oct;2(4):294-9.

Carpenter JL, Kreider JW, Alroy J, Schmidt, GM, Cutaneous xanthogranuloma and viral papilloma on an eyelid of a cat. 1992 Veterinary Dermatology 3, 187–190.

Chen G, Stenlund A, Characterization of the DNA-binding domain of the bovine papillomavirus replication initiator E1. J Virol. 1998 Apr;72(4):2567-76.

Choe J, Vaillancourt P, Stenlund A, Botchan M, Bovine papillomavirus type 1 encodes two forms of a transcriptional repressor: structural and functional analysis of new viral cDNAs. J Virol. 1989 Apr;63(4):1743-55.

Chow LT, Reilly SS, Broker TR, Taichman LB, Identification and mapping of human papillomavirus type 1 RNA transcripts recovered from p lantarwarts and infected epithelial cell cultures. J Virol. 1987 Jun;61(6):1913-8.

Ciccolini F, Di Pasquale G, Carlotti F, Crawford L, Tommasino M, Functional studies of E7 proteins from different HPV types. Oncogene. 1994 Sep;9(9):2633-8.

Cole ST, Danos O, Nucleotide sequence and comparative analysis of the human papillomavirus type 18 genome. Phylogeny of papillomaviruses and repeated structure of the E6 and E7 gene products. J Mol Biol. 1987 Feb 20;193(4):599-608.

Conger K.L., Liu J.S., Kuo S.R., Chow L.T., Wang T.S., Human papillomavirus DNA replication: interactions between the viral E1 protein and two subunits of human DNA polymerase alpha/primase. J Biol Chem 274(5):2696-705, 1999.

Cornet I, Bouvard V, Campo MS, Thomas M, Banks L, Gissmann L, Lamartine J, Sylla BS, Accardi R, Tommasino M, Comparative analysis of transforming properties of E6 and E7 from different beta human papillomavirus types. J Virol. 2012 Feb;86(4):2366-70. doi: 10.1128/JVI.06579-11. Epub 2011 Dec 14.

Corteggio A, Altamura G, Roperto F, Borzacchiello G, Bovine papillomavirus E5 and E7 oncoproteins in naturally occurring tumors: are two better than one? Infect Agent Cancer. 2013 Jan 9;8(1):1. doi: 10.1186/1750-9378-8-1.

Corteggio A, Di Geronimo O, Roperto S, Roperto F, Borzacchiello G, Bovine papillomavirus E7 oncoprotein binds to p600 in naturally occurring equine sarcoids.J Gen Virol. 2011 Feb;92(Pt 2):378-82. doi: 10.1099/vir.0.025866-0. Epub 2010 Oct 21.

Crook T, Tidy JA, Vousden KH, Degradation of p53 can be targeted by HPV E6 sequences distinct from those required for p53 binding and trans-activation. Cell. 1991 Nov 1;67(3):547-56.

Culp TD, Christensen ND, Kinetics of in vitro adsorption and entry of papillomavirus virions. Virology. 2004 Feb 5;319(1):152-61.

Day PM, Lowy DR, Schiller JT, Papillomaviruses infect cells via a clathrin-dependent pathway. Virology. 2003 Mar 1;307(1):1-11.

de Villiers EM, Fauquet C, Broker TR, Bernard HU, zur Hausen H. Virology Classification of papillomaviruses. 2004 Jun 20;324(1):17-27. Review.

Degenhardt YY, Silverstein S, Interaction of zyxin, a focal adhesion protein, with the e6 protein from human papillomavirus type 6 results in its nuclear translocation. J Virol. 2001 Dec;75(23):11791-802.

DeMasi J, Chao MC, Kumar AS, Howley PM, Bovine papillomavirus E7 oncoprotein inhibits anoikis. J Virol. 2007 Sep;81(17):9419-25. Epub 2007 Jun 27.

DiMaio D, Petti LM, The E5 proteins.Virology. 2013 Oct;445(1-2):99-114. doi: 10.1016/j.virol.2013.05.006. Epub 2013 May 31. Review.

Dixon EP, Pahel GL, Rocque WJ, Barnes JA, Lobe DC, Hanlon MH, Alexander K.A., Chao S.F., Lindley K., Phelps W.C., The E1 helicase of human papillomavirus type 11 binds to the origin of replication with low sequence specificity. Virology 2000, 270(2): 345-57,

Doorbar J, Parton A, Hartley K, Banks L, Crook T, Stanley M, Crawford L, Detection of novel splicing patterns in a HPV16-containing keratinocyte cell line. Virology. 1990 Sep;178(1):254-62.

Doorbar J, Ely S, Sterling J, McLean C, Crawford L, Specific interaction between HPV-16 E1-E4 and cytokeratins results in collapse of the epithelial cell intermediatefilament network. Nature. 1991 352(6338): 824-7,

Doorbar J., The E4 protein; structure, function and patterns of expression. Virology. 2013 Oct;445(1-2):80-98. doi: 10.1016/j.virol.2013.07.008. Epub 2013 Sep 7.

Doorbar J, Foo C, Coleman N, Medcalf L, Hartley O, Prospero T, Napthine S, Sterling J, Winter G, Griffin H, Characterization of events during the late stages of HPV16 infection in vivo using high-affinity synthetic Fabs to E4. Virology. 1997 Nov 10;238(1):40-52.

Doorbar J, The papillomavirus life cycle. J Clin Virol. 2005 Mar;32 Suppl 1:S7-15. Review.

Dorn CR, Taylor DO, Schneider R, Sunlight exposure and risk of developing cutaneous and oral squamous cell carcinomas in white cats. J Natl Cancer Inst 46:1073-1078, 1971.

Du J, Chen GG, Vlantis AC, Chan PK, Tsang RK, van Hasselt CA, Resistance to apoptosis of HPV 16-infected laryngeal cancer cells is associated with decreased Bak and increased Bcl-2 expression. Cancer Lett. 2004 Mar 8;205(1):81-8.

Dunowska M, Munday JS, Laurie RE, Hills SF, Genomic characterisation of Felis catus papillomavirus 4, a novel papillomavirus detected in the oral cavity of a domestic cat. Virus Genes. 2014 Feb;48(1):111-9. doi: 10.1007/s11262-013-1002-3. Epub 2013 Nov 16.

Dyson N, Guida P, Münger K, Harlow E, Homologous sequences in adenovirus E1A and human papillomavirus E7 proteins mediate interaction with the same set of cellular proteins. J Virol. 1992 Dec;66(12):6893-902.

Egawa K, New types of human papillomaviruses and intracytoplasmic inclusion bodies: a classification of inclusion wartsaccording to clinical features, histology and associated HPV types. Br J Dermatol. 1994 Feb;130(2):158-66.

Egberink HF, Berrocal A, Bax HA, van den Ingh TS, Walter JH, Horzinek MC, Papillomavirus associated skin lesions in a cat seropositive for feline immunodeficiency virus.Vet Microbiol. 1992 Jun 1;31(2-3):117-25.

Fehrmann F, Laimins LA, Human papillomaviruses: targeting differentiating epithelial cells for malignant transformation. Oncogene. 2003 Aug 11;22(33):5201-7. Review.

Figge J, Webster T, Smith TF, Paucha E, Prediction of similar transforming regions in simian virus 40 large T, adenovirus E1A, and myc oncoproteins. J Virol. 1988 May;62(5):1814-8.

Filippova M, Parkhurst L, Duerksen-Hughes PJ, The human papillomavirus 16 E6 protein binds to Fas-associated death domain and protects cells from Fas-triggered apoptosis. J Biol Chem. 2004 Jun 11;279(24):25729-44. Epub 2004 Apr 8.

Filippova M, Song H, Connolly JL, Dermody TS, Duerksen-Hughes PJ, The human papillomavirus 16 E6 protein binds to tumor necrosis factor (TNF) R1 and protects cells from TNF-induced apoptosis. J Biol Chem. 2002 Jun 14;277(24):21730-9. Epub 2002 Apr 4.

Florin L, Sapp C, Streeck RE, Sapp M, Assembly and translocation of papillomavirus capsid proteins. J Virol. 2002 Oct;76(19):10009-14.

Fradet-Turcotte A, Bergeron-Labrecque F, Moody CA, Lehoux M, Laimins LA, Archambault J, Nuclear accumulation of the papillomavirus E1 helicase blocks S-phase progression and triggers an ATM-dependent DNA damage response. J Virol. 2011 Sep;85(17):8996-9012. doi: 10.1128/JVI.00542-11. Epub 2011 Jul 6.

Frattini MG and Laimins L.A., The role of the E1 and E2 proteins in the replication of human papillomavirus type 31b. Virology. 1994 Nov 1; 204(2):799-804.

Frisch SM, Screaton RA, Anoikis mechanisms. Curr Opin Cell Biol. 2001 Oct;13(5):555-62. Review.

Frolov MV, Dyson NJ, Molecular mechanisms of E2F-dependent activation and pRB-mediated repression. J Cell Sci. 2004 May 1;117(Pt 11):2173-81.

Funk JO, Waga S, Harry JB, Espling E, Stillman B, Galloway DA, Inhibition of CDK activity and PCNA-dependent DNA replication by p21 is blocked by interaction with the HPV-16E7 oncoprotein. Genes Dev. 1997 Aug 15;11(16):2090-100.

Gage JR1, Meyers C, Wettstein FO, The E7 proteins of the nononcogenic human papillomavirus type 6b (HPV-6b) and of the oncogenic HPV-16 differ in retinoblastoma protein binding and other properties. J Virol. 1990 Feb;64(2):723-30.

Galluzzi L, Morselli E, Kepp O, Vitale I, Pinti M, Kroemer G, Mitochondrial liaisons of p53 Antioxid Redox Signal. 2011 Sep 15;15(6):1691-714. doi: 10.1089/ars.2010.3504. Epub 2011 Jan 7. Review.

Ganzenmueller T, Matthaei M, Muench P, Scheible M, Iftner A, Hiller T, Leiprecht N, Probst S, Stubenrauch F, Iftner T, The E7 protein of the cottontail rabbit papillomavirus immortalizes normal rabbit keratinocytes and reduces pRb levels, while E6 cooperates in immortalization but neither degrades p53 nor binds E6AP. Virology. 2008 Mar 15;372(2):313-24. Epub 2007 Dec 11.

Garnett TO, Filippova M, Duerksen-Hughes PJ, Accelerated degradation of FADD and procaspase 8 in cells expressing human papilloma virus 16E6 impairs TRAIL-mediated apoptosis.Cell Death Differ. 2006 Nov;13(11):1915-26. Epub 2006 Mar 10.

Giampieri S, García-Escudero R, Green J, Storey A, Human papillomavirus type 77 E6 protein selectively inhibits p53-dependent transcription of proapoptotic genes following UV-B irradiation.Oncogene. 2004 Jul 29;23(34):5864-70.

Gill VL, Bergman PJ, Baer KE, Craft D, Leung C, Use of imiquimod 5% cream (Aldara) in cats with multicentric squamous cell carcinoma in situ: 12 cases (2002-2005). Vet Comp Oncol. 2008 Mar;6(1):55-64. doi: 10.1111/j.1476-5829.2007.00144.x.

Gillison ML, Koch WM, Capone RB, Spafford M, Westra WH, Wu L, Zahurak ML, Daniel RW, Viglione M, Symer DE, Shah KV, Sidransky D, Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J Natl Cancer Inst. 2000 May 3;92(9):709-20.

Giroglou T, Florin L, Schäfer F, Streeck RE, Sapp M, Human papillomavirus infection requires cell surface heparan sulfate. J Virol. 2001 Feb;75(3):1565-70.

Goldstein DJ, Toyama R, Dhar R, Schlegel R, The BPV-1 E5 oncoprotein expressed in Schizosaccharomyces pombe exhibits normal biochemical properties and binds to the endogenous 16-kDa component of the vacuolar proton-ATPase. Virology. 1992 Oct;190(2):889-93.

Green H, Rheinwald JG, Sun TT, Properties of an epithelial cell type in culture: the epidermal keratinocyte and its dependence on products of the fibroblast. Prog Clin Biol Res 1977;17:493-500.

Gross TL, Ihrke PJ, Walder EJ, Affolter VK, Skin Diseases of the Dog and Cat: Clinical and Histopathologic Diagnosis, 2005, Second Ed. Blackwell Science, Oxford, UK, 932 pp.

Guo C, Liu K, Zheng Y, Luo H, Chen H, Huang L, Apoptosis induced by an antagonist peptide against HPV16 E7 in vitro and in vivo via restoration ofp53.Apoptosis. 2011 Jun;16(6):606-18. doi: 10.1007/s10495-011-0594-0.

Ham S, Kim KH, Kwon TH, Bak Y, Lee DH, Song YS, Park SH, Park YS, Kim MS, Kang JW, Hong JT, Yoon DY, Luteolin induces intrinsic apoptosis via inhibition of E6/E7 oncogenes and activation of extrinsic and intrinsic signaling pathways in HPV-18-associated cells.Oncol Rep. 2014 Jun;31(6):2683-91. doi: 10.3892/or.2014.3157. Epub 2014 Apr 25.

Heck DV, Yee CL, Howley PM, Münger K, Efficiency of binding the retinoblastoma protein correlates with the transforming capacity of the E7 oncoproteins of the human papillomaviruses. Proc Natl Acad Sci U S A. 1992 May 15;89(10):4442-6.

Holt SE, Wilson VG, Mutational analysis of the 18-base-pair inverted repeat element at the bovine papillomavirus origin of replication:identification of critical sequences for E1 bindi ng and in vivo replication. J Virol. 1995 Oct;69(10):6525-32.

Howie HL, Katzenellenbogen RA, Galloway DA, Papillomavirus E6 proteins.Virology. 2009 Feb 20;384(2):324-34. doi: 10.1016/j.virol.2008.11.017. Epub 2008 Dec 10.

Howley PM, Schiller JY, Lowy DR, Papillomaviruses, In: Kinpe DM, Howley, PM (Eds), Fields Virology, 6th ed. Lippincott, Williams and Wilkins, Philadelphia. 2013 pp. 1662-1703.

Howley PM, Pfister HJ. Beta genus papillomaviruses and skin cancer. Virology. 2015 Feb 24. pii: S0042-6822(15)00047-1. doi: 10.1016/j.virol.2015.02.004. [Epub ahead of print].

Hughes FJ, Romanos MA, E1 protein of human papillomavirus is a DNA helicase/ATPase. Nucleic Acids Res. 1993 Dec 25;21(25):5817-23.

Huh K, Zhou X, Hayakawa H, Cho JY, Libermann TA, Jin J, Harper JW, Munger K, Human papillomavirus type 16 E7 oncoprotein associates with the cullin 2 ubiquitin ligase complex, which contributes to degradation of the retinoblastoma tumor suppressor. J Virol. 2007 Sep;81(18):9737-47. Epub 2007 Jul 3.

Huh KW, DeMasi J, Ogawa H, Nakatani Y, Howley PM, Münger K, Association of the human papillomavirus type 16 E7 oncoprotein with the 600-kDa retinoblastoma protein-associated factor, p600. Proc Natl Acad Sci U S A. 2005 Aug 9;102(32):11492-7. Epub 2005 Aug 1.

Huibregtse JM, Scheffner M, Howley PM, Cloning and expression of the cDNA for E6-AP, a protein that mediates the interaction of the human papillomavirus E6 oncoprotein with p53. Mol Cell Biol. 1993 Feb;13(2):775-84.

Jackson S, Harwood C, Thomas M, Banks L, Storey A, Role of Bak in UV-induced apoptosis in skin cancer and abrogation by HPV E6 proteinsGenes Dev. 2000 Dec 1;14(23):3065-73.

Jackson S, Storey A, E6 proteins from diverse cutaneous HPV types inhibit apoptosis in response to UV damage. Oncogene. 2000 Jan 27;19(4):592-8.

Johansson C, Somberg M, Li X, Backström Winquist E, Fay J, Ryan F, Pim D, Banks L, Schwartz S.HPV-16 E2 contributes to induction of HPV-16 late gene expression by inhibiting early polyadenylation. EMBO J. 2012 May 22;31(14):3212-27. doi: 10.1038/emboj.2012.147.

Johung K, Goodwin EC, DiMaio D, Human papillomavirus E7 repression in cervical carcinoma cells initiates a transcriptional cascade driven by the retinoblastoma family, resulting in senescence. J Virol. 2007 Mar;81(5):2102-16. Epub 2006 Dec 20.

Joslin J, Garner M, Collins D, Kamaka E, Sinabaldi K, Meleo K, Montali RJ, Sundberg, JP, Jenson AB, Ghim S, et al.,Viral papilloma and squamous cell carcinomas in snow leopards (Uncia uncia). In: Proceedings of the American Association of Zoo Veterinarians and International Association for Aquatic Animal Medicine Joint Conference, New Orleans, Louisiana, USA, 17–21September 2000, pp. 155–158.

Kim MS, Bak Y, Park YS, Lee DH, Kim JH, Kang JW, Song HH, Oh SR, Yoon do Y, Wogonin induces apoptosis by suppressing E6 and E7 expressions and activating intrinsic signaling pathways in HPV-16 cervical cancer cells. Cell Biol Toxicol. 2013 Aug;29(4):259-72. doi: 10.1007/s10565-013-9251-4. Epub 2013 Aug 18.

Kim MS, Kim JH, Bak Y, Park YS, Lee DH, Kang JW, Shim JH, Jeong HS, Hong JT, Yoon do Y, 2,4-bis (p-hydroxyphenyl)-2-butenal (HPB242) induces apoptosis via modulating E7 expression and inhibition of PI3K/Akt pathway in SiHa human cervical cancer cells.Nutr Cancer. 2012;64(8):1236-44. doi: 10.1080/01635581.2012.718405.

Kiyono T, Hiraiwa A, Fujita M, Hayashi Y, Akiyama T, Ishibashi M, Binding of high-risk human papillomavirus E6 oncoproteins to the human homologue of the Drosophila discs large tumor suppressor protein. Proc Natl Acad Sci U S A. 1997 Oct 14;94(21):11612-6.

Klingelhutz AJ, Foster SA, McDougall JK, Telomerase activation by the E6 gene product of human papillomavirus type 16. Nature. 1996 Mar 7;380(6569):79-82.

Knapp AA, McManus PM, Bockstall K, Moroianu J, Identification of the nuclear localization and export signals of high risk HPV16 E7 oncoprotein. Virology. 2009 Jan 5;383(1):60-8. doi: 10.1016/j.virol.2008.09.037. Epub 2008 Nov 8.

Kumar A, Zhao Y, Meng G, Zeng M, Srinivasan S, Delmolino LM, Gao Q, Dimri G, Weber GF, Wazer DE, Band H, Band V, Human papillomavirus oncoprotein E6 inactivates the transcriptional coactivator human ADA3. Mol Cell Biol. 2002 Aug;22(16):5801-12.

Kurg R, Uusen P, Võsa L, Ustav M, Human papillomavirus E2 protein with single activation domain initiates HPV18 genome replication, but is not sufficient for long-term maintenance of virus genome. Virology. 2010 Dec 20;408(2):159-66. doi: 10.1016/j.virol.2010.09.010. Epub 2010 Oct 12.

Lace MJ, Anson JR, Thomas GS, Turek LP, Haugen TH, The E8--E2 gene product of human papillomavirus type 16 represses early transcription and replication but is dispensable for viral plasmid persistence in keratinocytes. J Virol. 2008 Nov;82(21):10841-53. doi: 10.1128/JVI.01481-08. Epub 2008 Aug 27.

Lange CE, Tobler K, Markau T, Alhaidari Z, Bornand V, Stöckli R, Trüssel M, Ackermann M, Favrot C, Sequence and classification of FdPV2, a papillomavirus isolated from feline Bowenoid in situ carcinomas.Vet Microbiol. 2009 May 28;137(1-2):60-5. doi: 10.1016/j.vetmic.2009.01.002. Epub 2009 Jan 6.

Laurent R, Kienzler JL, Croissant O, Orth G, Two anatomoclinical types of warts with plantar localization: specific cytopathogenic effects of papillomavirus. Type I (HPV-1) and type 2 (HPV-2). Arch Dermatol Res. 1982;274(1-2):101-11.

LeClerc, SMC, Haines, EG, Papillomavirus infection in association with feline cutaneous squamous cell carcinoma in situ. In: Proceedings of the 40th Meeting of the American Association of Veterinary Diagnosticians and the Meeting of the American College of Veterinary Dermatologists, Louisville, Kentucky, USA, 17–24 October 1997, pp. 125–126.

Lee SS, Glaunsinger B, Mantovani F, Banks L, Javier RT, Multi-PDZ domain protein MUPP1 is a cellular target for both adenovirus E4-ORF1 and high-risk papillomavirus type 18 E6 oncoproteins. J Virol. 2000 Oct;74(20):9680-93.

Lee S, Kim H, Kang JW, Kim JH, Lee DH, Kim MS, Yang Y, Woo ER, Kim YM, Hong J, Yoon DY, The biflavonoid amentoflavone induces apoptosis via suppressing E7 expression, cell cycle arrest at sub-G_1 phase, and mitochondria-emanated intrinsic pathways in human cervical cancer cells.J Med Food. 2011 Jul-Aug;14(7-8):808-16. doi: 10.1089/jmf.2010.1428. Epub 2011 Jun 11.

Li M, Beard P, Estes PA, Lyon MK, Garcea RL, Intercapsomeric disulfide bonds in papillomavirus assembly and disassembly. J Virol. 1998 Mar;72(3):2160-7.

Li R, Botchan MR, Acidic transcription factors alleviate nucleosome-mediated repression of DNA replication of bovine papillomavirus type 1. Proc Natl Acad Sci U S A. 1994 Jul 19;91(15):7051-5.

Li X, Coffino P, High-risk human papillomavirus E6 protein has two distinct binding sites within p53, of which only one determines degradation. J Virol. 1996 Jul;70(7):4509-16.

Li Y, Qi H, Li X, Hou X, Lu X, Xiao X, A novel dithiocarbamate derivative induces cell apoptosis through p53-dependent intrinsic pathway and suppresses the expression of the E6 oncogene of human papillomavirus 18 in HeLa cells. Apoptosis. 2015 Mar 13. [Epub ahead of print]

Lim DA, Gossen M, Lehman CW, Botchan MR, Competition for DNA binding sites between the short and long forms of E2 dimers underlies repression in bovine papillomavirus type 1 DNA replication control. J Virol. 1998 Mar;72(3):1931-40.

Lipari F, McGibbon GA, Wardrop E, Cordingley MG, Purification and biophysical characterization of a minimal functional domain and of an N-terminal Zn2+-binding fragment from the human papillomavirus type 16 E6 protein. Biochemistry. 2001 Feb 6;40(5):1196-204.

Liu HC, Chen GG, Vlantis AC, Tse GM, Chan AT, van Hasselt CA, Inhibition of apoptosis in human laryngeal cancer cells by E6 and E7 oncoproteins of human papillomavirus 16.J Cell Biochem. 2008 Mar 1;103(4):1125-43.

Longworth MS, Laimins LA, Pathogenesis of human papillomaviruses in differentiating epithelia. Microbiol Mol Biol Rev. 2004 Jun;68(2):362-72.

Lowy DR, Howley PM, Papillomaviruses, In: Knipe, DM, Howley, PM (Eds), Fields Virology. Fourth ed. Lippincott Williams and Wilkins. 2011 pp. 2231-2264

Lu J.Z., Sun Y.N., Rose R.C., Bonnez W., McCance D.J., Two E2 binding sites (E2BS) alone or one E2BS plus an A/T-rich region are minimal requirements for the replication of the human papillomavirus type 11 origin. J Virol 1993 Dec;67(12):7131-9 .

Ma T, Zou N, Lin BY, Chow LT, Harper JW, Interaction between cyclin-dependent kinases and human papillomavirus replication_initiation protein E1 isrequired for e fficient viral replication. Proc Natl Acad Sci U S A. 1999 Jan 19;96(2):382-7.

Magal SS, Jackman A, Pei XF, Schlegel R, Sherman L, Induction of apoptosis in human keratinocytes containing mutated p53 alleles and its inhibition by both the E6 and E7 oncoproteins. Int J Cancer. 1998 Jan 5;75(1):96-104.

Marchetti B, Ashrafi GH, Tsirimonaki E, O'Brien PM, Campo MS, The bovine papillomavirus oncoprotein E5 retains MHC class I molecules in the Golgi apparatus and prevents their transport to the cell surface.Oncogene. 2002 Nov 7;21(51):7808-16.

Masterson PJ, Stanley MA, Lewis AP, Romanos MA, A C-terminal helicasedomain of the human papillomavirus E1 protein binds E2 and the DNA polymerase alpha-primase p68 subunit. J Virol. 1998 Sep;72(9):7407-19.

McBride AA, The papillomavirus E2 proteins. Virology. 2013 Oct;445(1-2):57-79. doi: 10.1016/j.virol.2013.06.006. Epub 2013 Jul 10. Review.

McIntosh PB, Martin SR, Jackson DJ, Khan J, Isaacson ER, Calder L, Raj K, Griffin HM, Wang Q, Laskey P, Eccleston JF, Doorbar J, Structural analysis reveals an amyloid form of the human papillomavirus type 16 E1--E4 protein and provides amolecular basis for its accumulation. J Virol. 2008 Aug;82(16):8196-203. doi: 10.1128/JVI.00509-08. Epub 2008 Jun 18.

McLaughlin-Drubin ME, Münger K. The human papillomavirus E7 oncoprotein. Virology. 2009 Feb 20;384(2):335-44. doi: 10.1016/j.virol.2008.10.006. Epub 2008 Nov 12. Review.

Michels J, Kepp O, Senovilla L, Lissa D, Castedo M, Kroemer G, Galluzzi L. Functions of BCL-XL at the Interface between Cell Death and Metabolism. Int J Cell Biol. 2013;2013:705294. doi: 10.1155/2013/705294. Epub 2013 Feb 28.

Middleton K, Peh W, Southern S, Griffin H, Sotlar K, Nakahara T, El-Sherif A, Morris L, Seth R, Hibma M, Jenkins D, Lambert P, Coleman N, Doorbar J, Organization of human papillomavirus productive cycle during neoplastic progression provide s a basis forselection of diagnostic markers. J Virol. 2003 Oct;77(19):10186-201.

Miller WH, Affolter V, Scott, DW, Suter MM, Multicentric squamous cell carcinomas in situ resembling Bowen's disease in 5 cats. Veterinary Dermatology. 1992; 3, 177–182.

Mitsouras K, Faulhaber EA, Hui G, Joslin JO, Eng C, Barr MC, Irizarry KJ, Development of a PCR assay to detect papillomavirus infection in the snow leopard.BMC Vet Res. 2011 Jul 18;7:1-11. doi: 10.1186/1746-6148-7-38.

Miyashita T, Krajewski S, Krajewska M, Wang HG, Lin HK, Liebermann DA, Hoffman B, Reed JC, Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. Oncogene. 1994 Jun;9(6):1799-805.

Mochizuki H, Goto-Koshino Y, Sato M, Fujino Y, Ohno K, Tsujimoto H. Comparison of the antitumor effects of an MDM2 inhibitor, nutlin-3, in feline lymphoma cell lines with or without p53 mutation. Vet Immunol Immunopathol. 2012 Jun 30;147(3-4):187-94. doi: 10.1016/j.vetimm.2012.04.017. Epub 2012 Apr 20.

Modis Y, Trus BL, Harrison SC, Atomic model of the papillomavirus capsid. EMBO J. 2002 Sep 16;21(18):4754-62.

Muller F., Giroglou T., Sapp. M., Characterization of the DNA-binding activity of the E1 and E2 proteins and the E1/E2 complex of human papillomavirus type 33. J Gen Virol. 1997 Apr;78 (Pt 4):911-5.

Munday JS, Papillomaviruses in felids. Vet J. 2014 Mar;199(3):340-7. doi: 10.1016/j.tvjl.2013.11.025. Epub 2013 Dec 2.

Munday JS, Aberdein D. Loss of retinoblastoma protein, but not p53, is associated with the presence of papillomaviral DNA in feline viral plaques, Bowenoid in situ carcinomas, and squamous cell carcinomas.Vet Pathol. 2012 May;49(3):538-45. doi: 10.1177/0300985811419534. Epub 2011 Sep 19.

Munday JS, Dunowska M, De Grey S, Detection of two different papillomaviruses within a feline cutaneous squamous cell carcinoma: case report and review of the literature. N Z Vet J. 2009 Aug;57(4):248-51. doi: 10.1080/00480169.2009.36911. Review.

Munday JS, Dunowska M, Hills SF, Laurie RE, Genomic characterization of Felis catus papillomavirus-3: a novel papillomavirus detected in a feline Bowenoid in situ carcinoma. Vet Microbiol. 2013 Aug 30;165(3-4):319-25. doi: 10.1016/j.vetmic.2013.04.006. Epub 2013 Apr 13.

Munday JS, French AF, Felis catus papillomavirus types 1 and 4 are rarely present in neoplastic and inflammatory oral lesions of cats. Res Vet Sci. 2015 Mar 6. pii: S0034-5288(15)00056-9. doi: 10.1016/j.rvsc.2015.03.002. [Epub ahead of print]

Munday JS, French AF, Peters-Kennedy J, Orbell GM, Gwynne K, Increased p16CDKN2A protein within feline cutaneous viral plaques, bowenoid in situ carcinomas, and a subset of invasive squamous cell carcinomas.Vet Pathol. 2011 Mar;48(2):460-5. doi: 10.1177/0300985810374844. Epub 2010 Jun 29.

Munday JS, Gibson I, French AF, Papillomaviral DNA and increased p16CDKN2A protein are frequently present within feline cutaneous squamous cell carcinomas in ultraviolet-protected skin.Vet Dermatol. 2011 Aug;22(4):360-6. doi: 10.1111/j.1365-3164.2011.00958.x. Epub 2011 Mar 10.

Munday JS, Hanlon EM, Howe L, Squires RA, French AF, Feline cutaneous viral papilloma associated with human papillomavirus type 9. Vet Pathol. 2007 Nov;44(6):924-7.

Munday JS, Howe L, French A, Squires RA, Sugiarto H. Detection of papillomaviral DNA sequences in a feline oral squamous cell carcinoma. Res Vet Sci. 2009 Apr;86(2):359-61. doi: 10.1016/j.rvsc.2008.07.005. Epub 2008 Aug 19.

Munday JS, Kiupel M, Papillomavirus-associated cutaneous neoplasia in mammals.Vet Pathol. 2010 Mar;47(2):254-64. doi: 10.1177/0300985809358604. Epub 2009 Dec 31.

Munday JS, Kiupel M, French AF, Howe L, Amplification of papillomaviral DNA sequences from a high proportion of feline cutaneous in situ and invasive squamous cell carcinomas using a nested polymerase chain reaction.Vet Dermatol. 2008 Oct;19(5):259-63. doi: 10.1111/j.1365-3164.2008.00685.x. Epub 2008 Aug 7.

Munday JS, Kiupel M, French AF, Howe L, Squires RA, Detection of papillomaviral sequences in feline Bowenoid in situ carcinoma using consensus primers.Vet Dermatol. 2007 Aug;18(4):241-5.

Munday JS, Knight CG, Amplification of feline sarcoid-associated papillomavirus DNA sequences from bovine skin.Vet Dermatol. 2010 Aug;21(4):341-4. doi: 10.1111/j.1365-3164.2010.00872.x. Epub 2010 Mar 29.

Munday JS, Knight CG, French AF, Evaluation of feline oral squamous cell carcinomas for p16CDKN2A protein immunoreactivity and the presence of papillomaviral DNA. Res Vet Sci. 2011 Apr;90(2):280-3. doi: 10.1016/j.rvsc.2010.06.014.

Munday JS, Knight CG, Howe L, The same papillomavirus is present in feline sarcoids from North America and New Zealand but not in any non-sarcoid feline samples. J Vet Diagn Invest. 2010 Jan;22(1):97-100.

Munday JS, Peters-Kennedy J, Consistent detection of Felis domesticus papillomavirus 2 DNA sequences within feline viral plaques. J Vet Diagn Invest. 2010 Nov;22(6):946-9.

Munday JS, Witham AI, Frequent detection of papillomavirus DNA in clinically normal skin of cats infected and noninfected with feline immunodeficiency virus. Vet Dermatol. 2010 Jun;21(3):307-10. doi: 10.1111/j.1365-3164.2009.00811.x. Epub 2009 Dec 23.

Münger K, Werness BA, Dyson N, Phelps WC, Harlow E, Howley PM, Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. EMBO J. 1989 Dec 20;8(13):4099-105.

Münger K, Baldwin A, Edwards KM, Hayakawa H, Nguyen CL, Owens M, Grace M, Huh K, Mechanisms of human papillomavirus-induced oncogenesis. J Virol. 2004 Nov;78(21):11451-60.

Murakami Y, Tateyama S, Rungsipipat A, Uchida K, Yamaguchi R, Immunohistochemical analysis of cyclin A, cyclin D1 and P53 in mammary tumors, squamous cell carcinomas and basal cell tumors of dogs and cats. J Vet Med Sci. 2000 Jul;62(7):743-50.

Murray-Zmijewski F, Slee EA, Lu X, A complex barcode underlies the heterogeneous response of p53 to stress. Nat Rev Mol Cell Biol. 2008 Sep;9(9):702-12. doi: 10.1038/nrm2451. Review.

Narita M, Shimizu S, Ito T, Chittenden T, Lutz RJ, Matsuda H, Tsujimoto Y, Bax interacts with the permeability transition pore to induce permeability transition and cytochrome c release in isolated mitochondria. Proc Natl Acad Sci U S A. 1998 Dec 8;95(25):14681-6.

Nasir L, Brandt S, Papillomavirus associated diseases of the horse.Vet Microbiol. 2013 Nov 29;167(1-2):159-67. doi: 10.1016/j.vetmic.2013.08.003. Epub 2013 Aug 18. Review.

Nasir L, Campo MS, Bovine papillomaviruses: their role in the aetiology of cutaneous tumours of bovids and equids.Vet Dermatol. 2008 Oct;19(5):243-54. Review.

Nespeca G, Grest P, Rosenkrantz WS, Ackermann M, Favrot C, Detection of novel papillomaviruslike sequences in paraffin-embedded specimens of invasive and in situ squamous cell carcinomas from cats. Am J Vet Res. 2006 Dec;67(12):2036-41.

Nguyen HP, Ramírez-Fort MK, Rady PL, The biology of human papillomaviruses.Curr Probl Dermatol. 2014;45:19-32. doi: 10.1159/000355959. Epub 2014 Mar 13. Review.

Nicholls PK, Moore PF, Anderson DM, Moore RA, Parry NR, Gough GW, Stanley MA, Regression of canine oral papillomas is associated with infiltration of CD4+ and CD8+ lymphocytes. Virology. 2001 Apr 25;283(1):31-9.

Nominé Y, Masson M, Charbonnier S, Zanier K, Ristriani T, Deryckère F, Sibler AP, Desplancq D, Atkinson RA, Weiss E, Orfanoudakis G, Kieffer B, Travé G, Structural and functional analysis of E6 oncoprotein: insights in the molecular pathways of human papillomavirus-mediated pathogenesis. Mol Cell. 2006 Mar 3;21(5):665-78.

Ohlenschläger O, Seiboth T, Zengerling H, Briese L, Marchanka A, Ramachandran R, Baum M, Korbas M, Meyer-Klaucke W, Dürst M, Görlach M, Solution structure of the partially folded high-risk human papilloma virus 45 oncoprotein E7. Oncogene. 2006 Sep 28;25(44):5953-9. Epub 2006 Apr 24.

Olson C Jr, Cook RH, Cutaneous sarcoma-like lesions of the horse caused by the agent of bovine papilloma. Proc Soc Exp Biol Med. 1951 Jun;77(2):281-4.

O'Neill SH, Newkirk KM, Anis EA, Brahmbhatt R, Frank LA, Kania SA, Detection of human papillomavirus DNA in feline premalignant and invasive squamous cell carcinoma. Vet Dermatol. 2011 Feb;22(1):68-74. doi: 10.1111/j.1365-3164.2010.00912.x.

Orbell GM, Young S, Munday JS. Cutaneous sarcoids in captive African lions associated with feline sarcoid-associated papillomavirus infection.Vet Pathol. 2011 Nov;48(6):1176-9. doi: 10.1177/0300985810391111. Epub 2010 Dec 17.

Pennie WD, Grindlay GJ, Cairney M, Campo MS, Analysis of the transforming functions of bovine papillomavirus type 4. Virology. 1993 Apr;193(2):614-20.

Phelps WC, Yee CL, Münger K, Howley PM,The human papillomavirus type 16 E7 gene encodes transactivation and transformation functions similar to those of adenovirus E1A. P Cell. 1988 May 20;53(4):539-47.

Ravens PA, Vogelnest LJ, Tong LJ, Demos LE, Bennett MD, Papillomavirus-associated multicentric squamous cell carcinoma in situ in a cat: an unusually extensive and progressive case with subsequent metastasis.Vet Dermatol. 2013 Dec;24(6):642-5, e161-2. doi: 10.1111/vde.12080. Epub 2013 Oct 11.

Rector A, Lemey P, Tachezy R, Mostmans S, Ghim SJ, Van Doorslaer K, Roelke M, Bush M, Montali RJ, Joslin J, Burk RD, Jenson AB, Sundberg JP, Shapiro B, Van Ranst M, Ancient papillomavirus-host co-speciation in Felidae. Genome Biol. 2007;8(4):R57.

Rector A, Van Ranst M, Animal papillomaviruses. Virology. 2013 Oct;445(1-2):213-23. doi: 10.1016/j.virol.2013.05.007. Epub 2013 May 25. Review.

Ressler S, Scheiden R, Dreier K, Laich A, Müller-Holzner E, Pircher H, Morandell D, Stein I, Viertler HP, Santer FR, Widschwendter A, Even J, Jansen-Dürr P,Capesius C, Zwerschke W, High-risk human papillomavirus E7 oncoprotein detection in cervical squamous cell carcinoma. Clin Cancer Res. 2007 Dec 1;13(23):7067-72.

Roelke ME, Brown MA, Troyer JL, Winterbach H, Winterbach C, Hemson G, Smith D, Johnson RC, Pecon-Slattery J, Roca AL, Alexander KA, Klein L, Martelli P, Krishnasamy K, O'Brien SJ. Pathological manifestations of feline immunodeficiency virus (FIV) infection in wild African lions.Virology. 2009 Jul 20;390(1):1-12. doi: 10.1016/j.virol.2009.04.011. Epub 2009 May 22.

Rowson KEK, Mahy BWJ, Human papova (wart) virus. Bacteriol Rev, 31 (1967), pp. 110–131

Ruslander D, Kaser-Hotz B, Sardinas JC, Cutaneous squamous cell carcinoma in cats. Comp Cont Ed 19:1119-1129, 1997.

Saidj D, Cros MP, Hernandez-Vargas H, Guarino F, Sylla BS, Tommasino M, Accardi R, Oncoprotein E7 from beta human papillomavirus 38 induces formation of an inhibitory complex for a subset of p53-regulated promoters.J Virol. 2013 Nov;87(22):12139-50. doi: 10.1128/JVI.01047-13. Epub 2013 Sep 4.

Sanders CM, Stenlund A. Transcription factor-dependent loading of the E1 initiator reveals modular assembly of the papillomavirus origin melting complex. J Biol Chem. 2000 Feb 4;275(5):3522-34.

Sang BC, Barbosa MS, Single amino acid substitutions in "low-risk" human papillomavirus (HPV) type 6 E7 protein enhance features characteristic of the "high-risk" HPV E7 oncoproteins. Proc Natl Acad Sci U S A. 1992 Sep 1;89(17):8063-7.

Sattler M, Liang H, Nettesheim D, Meadows RP, Harlan JE, Eberstadt M, Yoon HS, Shuker SB, Chang BS, Minn AJ, Thompson CB, Fesik SW, Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis. Science. 1997 Feb 14;275(5302):983-6.

Scheffner M, Huibregtse JM, Vierstra RD, Howley PM, The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell. 1993 Nov 5;75(3):495-505.

Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM, The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell. 1990 Dec 21;63(6):1129-36.

Schulman FY, Krafft AE, Janczewski T, Mikaelian I, Irwin J, Hassinger K, Cutaneous fibropapilloma in a mountain lion (Felis concolor). J Zoo Wildl Med. 2003 Jun;34(2):179-83.

Schulman FY, Krafft AE, Janczewski T, Feline cutaneous fibropapillomas: clinicopathologic findings and association with papillomavirus infection.Vet Pathol. 2001 May;38(3):291-6.

Scott DW, Miller WH, Griffin CE, 2001. Muller and Kirk's Small Animal Dermatology, Sixth Ed. W.B. Saunders, Philadelphia, Pennsylvania, USA, 1528 pp.

Sedman J, Stenlund A., The papillomavirus E1 protein forms a DNA-dependent hexameric complex with ATPase and DNA helicase activities.J Virol. 1998 Aug;72(8):6893-7.

Selinka HC, Giroglou T, Sapp M, Analysis of the infectious entry pathway of human papillomavirus type 33 pseudovirions. Virology. 2002 Aug 1;299(2):279-287.

Seo Y.S., Müller F., Lusky M., Hurwitz J, Bovine papillomavirus (BPV)-encoded E1 protein contains multiple activities required for BPV DNA replication. Proc NatlAcad Sci U S A. 1993 Jan 15;90(2):702-6.

Shalini S, Dorstyn L, Dawar S, Kumar S, Old, new and emerging functions of caspases.Cell Death Differ. 2015 Apr;22(4):526-539. doi: 10.1038/cdd.2014.216. Epub 2014 Dec 19. Review.

Sherman L, Jackman A, Itzhaki H, Stöppler MC, Koval D, Schlegel R, Inhibition of serum- and calcium-induced differentiation of human keratinocytes by HPV16 E6oncoprotein: role of p53 inactivation. Virology. 1997 Oct 27;237(2):296-306.

Shope RE, Hurst EW, INFECTIOUS PAPILLOMATOSIS OF RABBITS: WITH A NOTE ON THE HISTOPATHOLOGY. J Exp Med. 1933 Oct 31;58(5):607-24.

Smith-McCune K, Kalman D, Robbins C, Shivakumar S, Yuschenkoff L, Bishop JM, Intranuclear localization of human papillomavirus 16 E7 during transformation and preferential binding of E7 to the Rb family member p130. Proc Natl Acad Sci U S A. 1999 Jun 8;96(12):6999-7004.

Spanos WC, Geiger J, Anderson ME, Harris GF, Bossler AD, Smith RB, Klingelhutz AJ, Lee JH, Deletion of the PDZ motif of HPV16 E6 preventing immortalization and anchorage-independent growth in human tonsil epithelial cells. Head Neck. 2008 Feb;30(2):139-47.

Stenlund A, DNA replication of papillomaviruses. In: Garcea, RL, DiMaio, D (Eds.), The Papillomaviruses. Springer, New York. 2007 pp. 145–175.

Sterlinko Grm H, Weber M, Elston R, McIntosh P, Griffin H, Banks L, Doorbar J, Inhibition of E6-induced degradation of its cellular substrates by novel blocking peptides. J Mol Biol. 2004 Jan 23;335(4):971-85.

Stoler MH, Rhodes CR, Whitbeck A, Wolinsky SM, Chow LT, Broker TR, Human papillomavirus type 16 and 18 gene expression in cervical neoplasias.Hum Pathol. 1992 Feb;23(2):117-28.

Struijk L, van der Meijden E, Kazem S, ter Schegget J, de Gruijl FR, Steenbergen RD, Feltkamp MC, Specific betapapillomaviruses associated with squamous cell carcinoma of the skin inhibit UVB-induced apoptosis of primary human keratinocytes. J Gen Virol. 2008 Sep;89(Pt 9):2303-14. doi: 10.1099/vir.0.83317-0.

Sundberg JP, Montali RJ, Bush M, Phillips LG, O'Brien SJ, Jenson AB, Burk RD, Van Ranst, M, Papillomavirus-associated focal oral hyperplasia in wild and captive Asian lions (Panthera leo persica). Journal of Zoo and Wildlife Medicine. 1996 27, 61–70.

Sundberg JP, Van Ranst M, Montali R, Homer BL, Miller WH, Rowland PH, Scott DW, England JJ, Dunstan RW, Mikaelian I, Jenson AB, Feline papillomas and papillomaviruses.Vet Pathol. 2000 Jan;37(1):1-10.

Swindle CS, Zou N, Van Tine BA, Shaw GM, Engler JA, Chow LT, Human papillomavirus DNA replication compartments in a transient DNA replication system. J Virol. 1999 Feb;73(2):1001-9.

Tachezy R, Duson G, Rector A, Jenson AB, Sundberg JP, Van Ranst M, Cloning and genomic characterization of Felis domesticus papillomavirus type 1.Virology. 2002 Sep 30;301(2):313-21.

Teifke JP, Kidney BA, Löhr CV, Yager JA, Detection of papillomavirus-DNA in mesenchymal tumour cells and not in the hyperplastic epithelium of feline sarcoids.Vet Dermatol. 2003 Feb;14(1):47-56.

Terai M, Burk RD, Felis domesticus papillomavirus, isolated from a skin lesion, is related to canine oral papillomavirus andcontains a 1.3 kb non-coding region between the E2 and L2 open reading frames. J Gen Virol. 2002 Sep;83(Pt 9):2303-7.

Thomas M, Banks L, Inhibition of Bak-induced apoptosis by HPV-18 E6. Oncogene. 1998 Dec 10;17(23):2943-54.

Thomas M, Glaunsinger B, Pim D, Javier R, Banks L, HPV E6 and MAGUK protein interactions: determination of the molecular basis for specific protein recognition and degradation. Oncogene. 2001 Sep 6;20(39):5431-9.

Thomson M, Squamous cell carcinoma of the nasal planum in cats and dogs, Clin Tech Small Anim Pract. 2007 May;22(2):42-5.

Thomson NA, Dunowska M, Munday JS, The use of quantitative PCR to detect Felis catus papillomavirus type 2 DNA from a high proportion of queens and their kittens.Vet Microbiol. 2015 Feb 25;175(2-4):211-7. doi: 10.1016/j.vetmic.2014.11.028. Epub 2014 Dec 11.

Tomakidi P, Cheng H, Kohl A, Komposch G, Alonso A, Modulation of the epidermal growth factor receptor by the human papillomavirus type 16 E5 protein in raft cultures of human keratinocytes. Eur J Cell Biol. 2000 Jun;79(6):407-12.

Tong X, Howley PM, The bovine papillomavirus E6 oncoprotein interacts with paxillin and disrupts the actin cytoskeleton. Proc Natl Acad Sci U S A. 1997 Apr 29;94(9):4412-7.

Tsirimonaki E, Ullah R, Marchetti B, Ashrafi GH, McGarry L, Ozanne B, Campo MS, Similarities and differences between the E5 oncoproteins of bovine papillomaviruses type 1 and type 4: cytoskeleton, motility and invasiveness in E5-transformed bovine and mouse cells. Virus Res. 2006 Feb;115(2):158-68. Epub 2005 Sep 15.

Underbrink MP, Howie HL, Bedard KM, Koop JI, Galloway DA, E6 proteins from multiple human betapapillomavirus types degrade Bak and protect keratinocytes from apoptosis after UVB irradiation. J Virol. 2008 Nov;82(21):10408-17. doi: 10.1128/JVI.00902-08. Epub 2008 Aug 20.

Ustav E, Ustav M, Szymanski P, Stenlund A, The bovine papillomavirus origin of replication requires a binding site for the E2 transcriptional activator. Proc Natl Acad Sci U S A. 1993 Feb 1;90(3):898-902.

Vail DM, Withrow SJ, Tumours of the skin and subcutaneous tissues, in Withrow SJ, MacEwen EG (eds): Small Animal Clinical Oncology (ed 3). Philadelphia, PA, Saunders, 1998, pp 233-260.

Van Doorslaer K, Evolution of the papillomaviridae.Virology. 2013 Oct;445(1-2):11-20. doi: 10.1016/j.virol.2013.05.012. Epub 2013 Jun 14.

Van Doorslaer K, Rector A, Jenson AB, Sundberg JP, Van Ranst M, Ghim SJ, Complete genomic characterization of a murine papillomavirus isolated from papillomatous lesions of a European harvest mouse (Micromys minutus). J Gen Virol. 2007 May;88(Pt 5):1484-8.

Venuti A, Paolini F, Nasir L, Corteggio A, Roperto S, Campo MS, Borzacchiello G, Papillomavirus E5: the smallest oncoprotein with many functions. Mol Cancer. 2011 Nov 11;10:140. doi: 10.1186/1476-4598-10-140. Review.

Vousden KH, Jat PS, Functional similarity between HPV16E7, SV40 large T and adenovirus E1a proteins. Oncogene. 1989 Feb;4(2):153-8.

Wang X, Meyers C, Wang HK, Chow LT, Zheng ZM, Construction ofa full transcription map of human papillomavirus type 18 during productive vi ral infection. J Virol. 2011 Aug;85(16):8080-92. doi: 10.1128/JVI.00670-11. Epub 2011 Jun 15.

White EA, Walther J, Javanbakht H, Howley PM, Genus beta human papillomavirus E6 proteins vary in their effects on the transactivation of p53 target genes. J Virol. 2014 Aug;88(15):8201-12. doi: 10.1128/JVI.01197-14. Epub 2014 May 21.

Wilhelm S, Degorce-Rubiales F, Godson D, Favrot C, Clinical, histological and immunohistochemical study of feline viral plaques and bowenoid in situ carcinomas. Vet Dermatol. 2006 Dec;17(6):424-31.

Wilson VG, West M, Woytek K, Rangasamy D, Papillomavirus E1 proteins: form, function, and features. Virus Genes. 2002 Jun;24(3):275-90. Review.

Withrow SJ, Cancer of the gastrointestinal tract. In: Small Animal Clinical Oncology, 3rd edn, SJ Withrow and EG MacEwen, eds, Philadelphia, PA, WB Saunders Co, 2001: 304-353.

Yang L., Mohr I., Fouts E., Lim D.A., Nohaile M., Botchan M, The E1 protein of bovine papillomavirus 1 is an ATP-dependent DNA helicase. Proc Natl Acad Sci U S A. 1993 Jun 1;90(11):5086-90.

You J, Croyle JL, Nishimura A, Ozato K, Howley PM, Interaction of the bovine papillomavirus E2 protein with Brd4 tethers the viral DNA to host mitotic chromosomes. Cell. 2004 Apr 30;117(3):349-60.

Zerfass K, Schulze A, Spitkovsky D, Friedman V, Henglein B, Jansen-Dürr P, Sequential activation of cyclin E and cyclin A gene expression by human papillomavirus type 16 E7through sequences necessary for transformation. J Virol. 1995 Oct;69(10):6389-99.

Zerfass-Thome K, Zwerschke W, Mannhardt B, Tindle R, Botz JW, Jansen-Dürr P, Inactivation of the cdk inhibitor p27KIP1 by the human papillomavirus type 16 E7 oncoprotein. Oncogene. 1996 Dec 5;13(11):2323-30.

Zhang B, Srirangam A, Potter DA, Roman A, HPV16 E5 protein disrupts the c-Cbl-EGFR interaction and EGFR ubiquitination in human foreskin keratinocytes. Oncogene. 2005 Apr 7;24(15):2585-8.

Zhao KN, Hengst K, Liu WJ, Liu YH, Liu XS, McMillan NA, Frazer IH, BPV1 E2 protein enhances packaging of full-length plasmid DNA in BPV1 pseudovirions. Virology. 2000 Jul 5;272(2):382-93.

Zimmermann H, Koh CH, Degenkolbe R, O'Connor MJ, Müller A, Steger G, Chen JJ, Lui Y, Androphy E, Bernard HU, Interaction with CBP/p300 enables the bovine papillomavirus type 1 E6 oncoprotein to downregulate CBP/p300-mediated transactivation by p53. J Gen Virol. 2000 Nov;81(Pt 11):2617-23.

zur Hausen H, Papillomaviruses in the causation of human cancers - a brief historical account. Virology. 2009 Feb 20;384(2):260-5. doi: 10.1016/j.virol.2008.11.046. Epub 2009 Jan 8. Review.

.