Published in final edited form as:
Virology. 2009 February 20; 384(2): 335–344. doi:10.1016/j.virol.2008.10.006.

The Human Papillomavirus E7 Oncoprotein

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Abstract

The human papillomavirus (HPV) E7 oncoprotein shares functional similarities with such proteins as adenovirus E1A and SV40 large tumor antigen. As one of only two viral proteins always expressed in HPV-associated cancers, E7 plays a central role in both the viral life cycle and carcinogenic transformation. In the HPV viral life cycle, E7 disrupts the intimate association between cellular differentiation and proliferation in normal epithelium, allowing for viral replication in cells that would no longer be in the dividing population. This function is directly reflected in the transforming activities of E7, including tumor initiation and induction of genomic instability.

Keywords

Human papillomavirus; E7 oncoprotein; cellular transformation; retinoblastoma tumor suppressor; p53; genomic instability; centrosome duplication; ubiquitin ligase; cell death; cellular metabolism

Introduction

Human papillomaviruses (HPVs) are a DNA virus family of approximately 200 types that display a marked tropism for squamous epithelium. Despite a similar genomic make-up, different HPVs infect epithelia at distinct anatomic locations. Approximately 30 HPV types infect the anogenital and oral mucosa and can be further classified as “low-risk” and “high-risk” based on the clinical prognosis of their associated lesions. Low-risk HPVs cause benign epithelial hyperplasias (warts), while high-risk HPVs cause lesions that have a propensity for malignant progression. Virtually all cervical carcinoma cases are associated with high-risk HPV infection, and two viral proteins, E6 and E7, which are consistently expressed in the tumors, are required for both the induction and maintenance of the transformed phenotype (Munger et al., 2004).

Cellular transformation activities of HPV E7 proteins

In the mid 1980s it was recognized that high-risk HPV genomes encode cellular transformation activities when assayed in rodent fibroblast lines such as NIH3T3 cells (Yasumoto et al., 1986) or in the classical *ras* oncogene cooperation assay in baby rat kidney cells (Matlashewski et al., 1987). Subsequent mutational analyses revealed that the E7 protein scored as the major transforming activity of high-risk HPVs in these assay systems (Bedell et al., 1989; Kanda et

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al., 1988; Phelps et al., 1988; Tanaka et al., 1989; Vousden et al., 1988; Watanabe et al., 1988; Yutsudo et al., 1988).

Later studies revealed that expression of cloned high-risk HPV genomes in the natural host cell type of HPVs, primary human genital epithelial cells, causes life span extension and cellular immortalization and inhibits keratinocyte differentiation and facilitates immortalization (Schlegel et al., 1988; Woodworth et al., 1988; Woodworth et al., 1989). High-risk HPV genome expressing keratinocytes grown in organotypic raft cultures display cellular alterations and abnormalities in tissue architecture that resemble high-grade HPV associated clinical lesions (McCance et al., 1988; Woodworth et al., 1990). The resulting cell lines were non-tumorigenic in nude mice but tumorigenic clones were obtained when cultured over extended periods of time or when additional oncogenes such the *ras* or *fos* oncogenes were co-expressed (DiPaolo et al., 1989; Durst et al., 1989; Hurlin et al., 1991; Pei et al., 1993). Mutational analyses revealed that the E7 protein in cooperation with E6 is necessary for these transforming activities in human epithelial cells (Bedell et al., 1989; Hawley-Nelson et al., 1989; Hudson et al., 1990; Munger et al., 1989a). The transforming activities of HPV E7 proteins correlate with the low-risk/high-risk classification, as low-risk HPV E7 proteins, such as HPV6 and 11 E7, have greatly decreased transforming and immortalizing activities as compared to high-risk HPV E7 proteins (Barbosa et al., 1991; Halbert et al., 1992).

Biochemical characterization of E7 proteins

The E7 proteins are small, acidic polypeptides composed of approximately 100 amino acids (Figure 1). The amino terminus of E7 contains a region of sequence similarity to a portion of conserved region (CR) 1 and the entire CR2 of adenovirus (Ad) E1A and related sequences in simian vacuolating virus 40 (SV40) large tumor antigen (T Ag) (Figge et al., 1988;Phelps et al., 1988;Vousden et al., 1989). As with SV40 T Ag and Ad E1A, these two conserved regions significantly contribute to the transforming activities of high-risk HPV E7 oncoproteins (Edmonds et al., 1989;Jewers et al., 1992;Phelps et al., 1992;Storey et al., 1990;Watanabe et al., 1990). A conserved Leu-X-Cys-X-Glu (LXCXE) motif in the CR2 homology domain is necessary and sufficient for the association of E7 protein with the retinoblastoma tumor suppressor protein, pRB (Munger et al., 1989b). Some studies claim that the C-terminal E7 domain contains an independent, low-affinity pRB binding site (Liu et al., 2006;Patrick et al., 1994), but HPV16 E7 mutants with a deletion of the pRB core binding site in CR2 fail to associate with pRB family members as determined by Western blotting and extensive proteomic analyses of associated cellular protein complexes (K.W. Huh, M. Grace, C. L. Nguyen and K. Munger, unpublished data). Adjacent to the LXCXE motif is a consensus casein kinase II (CK II) phosphorylation site (Barbosa et al., 1990;Firzlaff et al., 1989). The carboxyl terminus is phosphorylated by an as yet unidentified kinase (Massimi et al., 2000) and importantly contributes the transforming activities of E7 (Helt et al., 2002;Helt et al., 2001). The E7 carboxyl terminus contains a zinc-binding domain that is composed of two Cys-X-X-Cys motifs (Barbosa et al., 1989) and functions as a dimerization domain (Clemens et al., 1995;Liu et al., 2006;McIntyre et al., 1993;Ohlenschlager et al., 2006), although there is no compelling evidence that E7 exists as a dimer *in vivo* and/or that dimerization is necessary for the biological activities of E7. Interestingly, HPV E6 proteins consist of two tandem copies of a Cys-X-X-Cys motif that shares sequence similarity to the E7 carboxyl terminus, indicating that E6 and E7 may have a common ancestral precursor (Cole et al., 1987). The 3-dimensional structure of HPV E7 proteins has been solved by NMR (Liu et al., 2006) and X-ray crystallography (Ohlenschlager et al., 2006). These studies have revealed that the amino terminal domain is unfolded whereas the C-terminal domain forms a unique, tightly packed zinc-binding fold (Liu et al., 2006;Ohlenschlager et al., 2006) (Figure 1).

HPV16 E7 migrates on SDS polyacrylamide gels with an apparent molecular size of 18 to 20 kDa, rather than according to its predicted molecular weight of ~11 kDa (Smotkin et al., 1986; Smotkin et al., 1987); this aberrant migration is mediated by the amino terminal CR1 homology domain (Heck et al., 1992; Münger et al., 1991) and may be caused by the high content of acidic residues (Armstrong et al., 1993). HPV16 E7 is located largely in the cytoplasm (Huh et al., 2005; Nguyen et al., 2007; Ressler et al., 2007; Smotkin et al., 1987) but also exists in nuclear pools (Greenfield et al., 1991; Sato et al., 1989; Smith-McCune et al., 1999). Although HPV16 E7 does not have a prototypical nuclear targeting sequence, it is actively transported into the nucleus through a novel Ran-dependent that does not involve Kap β import receptors (Angeline et al., 2003). More recent studies have shown that E7 also contains a nuclear export sequence and can shuttle between the cytoplasm and nucleus (Knapp et al., 2008). In addition to nuclear and cytoplasmic pools of E7, nucleolar HPV16 E7 localization has also been reported (Zatsepina et al., 1997), but in most immunofluorescence studies nuclear HPV16 E7 staining seems to specifically exclude nucleolar structures. The half-life of HPV16 E7 is less than 2 hours (Smotkin et al., 1987). E7 is degraded via a ubiquitin-mediated proteasomal mechanism that involves conjugation of ubiquitin to the amino terminus of E7 (Reinstein et al., 2000). A cullin 1 containing ubiquitin ligase complex has been implicated in HPV16 E7 degradation (Oh et al., 2004a) although association of HPV16 E7 with cullin 1 was not detected in other studies (Huh et al., 2007).

Glycerol gradient centrifugation experiments demonstrated that HPV16 E7 is a component of higher molecular weight complexes, indicating that it may associate with cellular protein complexes (Smotkin et al., 1987). E7 lacks intrinsic enzymatic activities and specific DNA binding activities and it is now widely accepted that its biological activities are linked to its ability to associate with and subvert the normal activities of cellular regulatory complexes (Figure 1).

HPV E7 and the viral life cycle

During carcinogenic progression the HPV genome frequently integrates into a host cell chromosome and, as a result, the viral oncoproteins, E6 and E7, are the only viral proteins that are consistently expressed in HPV positive cervical carcinomas. Viral genome integration is a terminal event and is thus not a manifestation of the normal viral life cycle. Hence, the oncogenic activities of high-risk HPV E7 proteins reflect their functions during the viral life cycle. The HPV life cycle is intimately associated with the differentiation process of the infected epithelial cell. The skin is the largest organ of the human body and is subject to continuous turnover. It contains a single layer of dividing cells, the basal cells. Basal cells undergo asymmetric mitosis; one daughter cell remains an undifferentiated, proliferating basal cell whereas the other daughter becomes a differentiating suprabasal cell. The suprabasal daughter cell withdraws from the cell division cycle and undergoes a program of terminal differentiation. This ensures the mechanical stability of the skin and provides protection for the dividing basal cells from direct exposure to environmental mutagens. In order to establish a persistent infection, papillomaviruses need to infect basal cells where the viral genomes are maintained at a low copy number. The production of infectious progeny virus, however, exclusively occurs in the terminally differentiated layers of the epithelium (reviewed in Lee et al., 2007).

With the exception of the E1/E2 complex, papillomaviruses lack essential enzymes for viral genome replication; the host cell's DNA synthesis machinery must be utilized for this purpose (reviewed in Stubenrauch et al., 1999). In normal epithelia, cellular DNA synthesis no longer occurs in differentiated epithelial cells, making them inherently incapable of supporting HPV replication. Consequently, papillomaviruses need to uncouple the processes of cellular differentiation and proliferation. The strategies that some HPVs have developed to achieve this

contribute to their transforming activities. Infected cells leaving the basal layer remain competent to support DNA synthesis largely due to the actions of the E7 protein (Cheng et al., 1995). There appear to be HPV type-specific differences with regard to the specific roles that E7 proteins play in the viral life cycle, as HPV11 and 31 E7 are where shown to be required for episomal maintenance of the viral genome (Oh et al., 2004b; Thomas et al., 1999), whereas HPV16 and 18 E7 are necessary for the productive stage of the viral life cycle (Flores et al., 2000; McLaughlin-Drubin et al., 2005). While many of the biological activities of E7 that are necessary for the viral life cycle remain to be elucidated, it has been shown that the ability of HPV16 E7 to allow suprabasal cells to support DNA synthesis is linked to E7's ability to bind pRB family members but not with its ability to induce their degradation (Collins et al., 2005). On the other hand, the capacity of HPV16 E7 to perturb differentiation correlates with both E7's binding to and degradation of pRB family members, suggesting that different key stages of the productive stage of the HPV16 life cycle rely on different functions of E7 (Collins et al., 2005).

Activation of E2F-dependent transcription through degradation of the retinoblastoma tumor suppressor protein, pRB

HPV E7 proteins associate with pRB and the related pocket proteins, p107 and p130, via the LXCXE motif in CR2 (Dyson et al., 1992; Munger et al., 1989b). The best-studied function of the pocket proteins is the ability to regulate G1/S entry and progression by modulating the transcriptional activities of E2F transcription factors (reviewed in Frolov et al., 2004). E2F transcription factors are heterodimers that contain an E2F (E2F1-8) and a DP (DP-1, DP-2) subunit and are critical regulators of G1 exit and S-phase progression. In addition, a number of other cellular processes, including cellular differentiation apoptosis, and genomic instability, are controlled by E2Fs (reviewed in Dyson, 1998). The G1 specific pRB/E2F complex acts a transcriptional repressor. In normal cells, disruption of the pRB/E2F repressor complex is triggered by cdk4/6 and cdk2 mediated pRB phosphorylation in late G1; the dissociated E2F acts as a transcriptional activator of genes necessary for S-phase entry and progression. Like SV40 T Ag, high-risk HPV E7 preferentially associates with G1-specific, E2F-bound pRB (Dyson et al., 1992). This causes disruption of pRB/E2F repressor complexes and, thus, uncontrolled G1 exit and S-phase entry. In addition to the LXCXE motif in CR2, carboxyl terminal E7 sequences are necessary for disruption of pRB/E2F complexes (Huang et al., 1993; Wu et al., 1993).

However, unlike SV40 T Ag and Ad E1A, which inactivate pRB/E2F complexes by stoichiometric pRB association, high-risk HPV E7 proteins destabilize pRB through proteasomal degradation (Boyer et al., 1996; Jones et al., 1997b) via a mechanism that involves association with and reprogramming of the cullin 2 ubiquitin ligase complex by HPV16 E7 (Huh et al., 2007). The induction of the aberrant pRB degradation by high-risk HPV E7 proteins and the resulting activation of E2F-mediated transcription represent important mechanisms by which these viruses achieve and maintain S-phase competence in differentiated epithelial cells. In addition to the core pRB binding site in CR2, sequences in the CR1 homology domain are necessary for pRB degradation and mutational studies suggest that the ability of high-risk HPV E7 proteins to destabilize pRB family members is necessary for cellular transformation (Jones et al., 1997b). Low-risk HPV E7 proteins bind pRB with lower efficiency (approximately 10-fold lower) than the high-risk HPV E7 proteins (Gage et al., 1990; Munger et al., 1989b). This difference in pRB binding efficiency maps to a single amino acid (Asp 21 in HPV16 E7 versus Gly 22 in HPV6 E7) and substitution experiments indicated that this single amino acid residue was the primary determinant for pRB-binding affinity and transformation capacity of the mucosal HPV E7 proteins (Heck et al., 1992; Sang et al., 1992). Interestingly, for cutaneous HPV E7 proteins, the presence of an aspartate residue and efficient pRB binding is not

associated with a high-risk clinical classification *in vivo* and does not predict transforming potential *in vitro* (Ciccolini et al., 1994).

HPV16 E7 can directly bind E2F1 and enhance E2F1-mediated transcription (Hwang et al., 2002). E2F1 plays a role in mediating the transcriptional control of the E2F6 gene, which is upregulated at the G1/S-phase transition to exert an opposing effect on the activities of E2F-responsive promoters, thereby directing appropriate cell cycle exit and differentiation (Lyons et al., 2006). Interestingly, HPV E7 associates with E2F6 and abrogates its ability to function as a transcriptional repressor (McLaughlin-Drubin et al., 2008), suggesting that the functional deregulation of E2F6 by HPV E7 is needed to counterbalance the up-regulation of E2F6 as a consequence of the activation of E2F1 by E7, thus ensuring that the cells remain in an S-phase-competent state that is necessary for the viral life cycle. Moreover, given E2F6′s possible role in maintaining quiescence (Ogawa et al., 2002), it is conceivable that the deregulation of E2F6 by HPV E7 aids in allowing HPVs to bypass negative growth signals, thus allowing cells to exit from G0.

Other mechanisms of G1/S cell checkpoint dysregulation

In addition to targeting pRB for proteasomal degradation, high-risk HPV E7 proteins also contribute to cell cycle dysregulation through several additional mechanisms.

Cyclin dependent kinases (cdks) are the motors that drive the cell division cycle. Multiple mechanisms, most importantly association with positive regulatory subunits, cyclins, and negative regulators, cyclin-dependent kinase inhibitors (CKIs), regulate their activity. Expression of cyclins E and A, the regulatory subunits of cdk2, which drive S-phase entry and progression, is under E2F control and they are both expressed at higher levels in E7 expressing cells (Zerfass et al., 1995).

HPV16 E7 has been shown to interact with and abrogate the growth-inhibitory activities of the CKIs $p21^{CIP1}$ (Funk et al., 1997; Jones et al., 1997a) and $p27^{KIP1}$ (Zerfass-Thome et al., 1996), which are induced by anti-proliferative signals, including growth factor withdrawal (Firpo et al., 1994), activation of p53 (el-Deiry et al., 1993), and loss of cellular adhesion (Assoian, 1997; Fang et al., 1996). Interestingly, $p21^{CIP1}$ has been implicated in coupling cell cycle arrest and differentiation in keratinocytes (Alani et al., 1998; Di Cunto et al., 1998; Missero et al., 1996). The steady state levels of $p21^{CIP1}$ increase during differentiation, subsequently inhibiting cdk2 activity and inducing a G1 growth arrest; interestingly, $p21^{CIP1}$ levels are increased further through a non-transcriptional mechanism in the presence of HPV E7. Although HPV16 E7 expression increases $p21^{CIP1}$ levels through protein stabilization (Jian et al., 1998; Jones et al., 1999; Noya et al., 2001), cdk2 remains active in HPV E7-expressing cells (Funk et al., 1997; Jones et al., 1997a; Ruesch et al., 1997). The ability of HPV E7 to abrogate CKIs, together with its ability to disrupt pRB/E2F complexes, which results in increased cyclin E and cyclin A levels, retains differentiating keratinocytes in a DNA synthesis competent state. HPV16 E7 can also directly associate with cdk2/cyclin A and cyclin E complexes (He et al., 2003; Nguyen et al., 2008b; Tommasino et al., 1993) resulting in increased cdk2 activity as measured on a pRB-derived substrate (He et al., 2003).

HPV16 E7 and epigenetic reprogramming

In addition to a DP1/2 containing repressive complex, E2F6 is also a component of polycomb group (PcG) transcription factor complexes. Mammalian PcG complexes act as transcriptional repressors, some of which contain histone methyltransferases (Attwooll et al., 2005; Ogawa et al., 2002; Trimarchi et al., 2001). Given that in addition to DP1 and DP2 we also identified the polycomb group proteins Ring1, Bmi1, Mel18, hpc2, and L3MBTL2 as HPV16 E7 associated proteins by tandem affinity purification (McLaughlin-Drubin et al., 2008), we presume that

HPV16 E7 associates with both E2F6/DP and E2F/PcG complexes. This is supported by the finding that some E2F6 specific cellular genes are upregulated, and that E2F6 containing polycomb bodies are detected at lower levels in E7 expressing cells (McLaughlin-Drubin et al., 2008).

In addition, E7 interacts with class I histone deacetylases (HDACs) (Brehm et al., 1999; Longworth et al., 2004), which function as transcriptional co repressors by inducing chromatin remodeling by reversing acetyl modifications of lysine residues on histones. The association between E7 and HDACs results in increased levels of E2F2-mediated transcription in differentiating cells (Longworth et al., 2005), possibly influencing S-phase progression. Moreover, E7 can also associate, directly or indirectly, with histone acetyl transferases (HATs) including p300, pCAF, and SRC1 (Avvakumov et al., 2003; Baldwin et al., 2006; Bernat et al., 2003; Huang et al., 2002) and has been shown to abrogate SRC1 associated HAT activity (Baldwin et al., 2006).

Subversion of p53 functions by HPV E7

The levels and half-life of p53 are increased in E7 expressing cells, indicating that E7 may perturb p53 degradation (Demers et al., 1994; Jones et al., 1997a; Jones et al., 1997b). Although the mechanism of p53 stabilization in E7 expressing cells is unclear, it is known that it is independent of $p14^{ARF}$, an inhibitor of mdm2-mediated p53 degradation (Bates et al., 1998; Zhang et al., 1998). Nevertheless, normal mdm2 mediated p53 turnover is defective in high-risk HPV E6/E7 expressing cervical carcinoma cells lines (Hengstermann et al., 2001), and the p53-specific ubiquitin ligase mdm2 is not as efficiently bound to p53 in E7 expressing cells as compared to normal cells (Seavey et al., 1999). This is intriguing, as mdm2 has been implicated in the degradation of pRB (Uchida et al., 2005), and there is a correlation between E7 mediated pRB degradation and p53 stabilization (Jones et al., 1997b). Despite the high levels of p53 in E7 expressing cells, the transcriptional activity of p53 is not activated (Jones et al., 1999), in fact, E7 has been shown to inhibit p53 transcriptional activity in reporter assays (Eichten et al., 2002).

Moreover, HPV16 E7 interferes with p53-mediated G1 growth arrest signaling in response to DNA damage (Demers et al., 1994; Hickman et al., 1994; Slebos et al., 1994; Vousden et al., 1993). The mechanistic details of this are unclear but may include aberrant expression of E2F transcriptional targets (Hickman et al., 1994; Katich et al., 2001), inactivation of the p53-responsive CKI $p21^{CIP1}$ (Funk et al., 1997; Jones et al., 1997a), and destabilization of pRB (Jones et al., 1997b).

HPV E7 and cell death signaling

Primary human fibroblasts expressing HPV16 E7 are predisposed to apoptosis, especially under conditions of growth factor deprivation or when the cells reach confluence. This has been observed with other viral and cellular oncogenes (Evan et al., 1992; White et al., 1991) and is sometimes referred to as the “trophic sentinel response”. The trophic sentinel response represents a cell intrinsic tumor suppressive pathway that is triggered when the oncogenic aberrant proliferative signal is in conflict with a growth inhibitory signal generated by a lack of mitogenic stimulation. Generation of this signal in E7 expressing fibroblasts requires integrity of the p53 tumor suppressor pathway, but does not involve increased expression of p53-responsive genes (Eichten et al., 2004). While it is accompanied by caspase activation, the HPV16 E7 induced trophic sentinel response still occurs when caspases are inhibited and hence represents a form of cell death that is distinct from apoptosis. Recent experiments with normal human keratinocytes have revealed that HPV16 E7 expression may cause metabolic stress that causes activation of an autophagy-like process (Zhou et al., 2008).

Anoikis, a form of apoptosis, is triggered when cells attempt to enter S-phase despite a lack of matrix attachment (Frisch et al., 2001). The resistance to anoikis is a hallmark of cellular transformation and is characterized by the ability of transformed cells to form foci and display anchorage independent growth. High- and low-risk, as well as BPV1 E7, associate with the 600 kD retinoblastoma protein-associated factor, p600 (DeMasi et al., 2005; Huh et al., 2005). Although the biological functions of p600 have not been fully elucidated in mammalian cells, reports on p600 homologs in Drosophila and Arabidopsis suggest that p600 may play a role in chromosome segregation (Sekelsky et al., 1999), synaptic transmission at the neuromuscular junction (Richards et al., 1996), calcium influx (Xu et al., 1998), and/or polar auxin transport (Gil et al., 2001). A recent study identified p600 as a novel microtubule-associated protein (MAP) that is developmentally regulated in neurons (Shim et al., 2008). The p600 protein contains a RING finger/UBR domain and functions as a ubiquitin ligase in the N-end rule pathway (Tasaki et al., 2005) that is involved in the ubiquitination of proteins through an interaction with their N-terminus (reviewed in Mogk et al., 2007). Experiments involving RNAi knockdown of p600 in mammalian cells have implicated p600 in anoikis (DeMasi et al., 2007; Huh et al., 2005). Therefore, the interaction between E7 and p600 may deregulate anoikis and protect detached cells from apoptosis, thereby contributing to viral transformation (DeMasi et al., 2007; Huh et al., 2005), Consistent with this idea, HPV16 E7 associates with p600 through the CR1 domain, which, is necessary for the transformation capability of HPV16 E7 (Edmonds et al., 1989; Gulliver et al., 1997; Phelps et al., 1992), as well as BPV1 E7 (DeMasi et al., 2005).

Modulation of cytostatic cytokine signaling by HPV E7

Transforming growth factor β (TGF-β) is potent inhibitor of epithelial cell growth, and acquisition of TGF-β resistance is a hallmark of epithelial tumors (reviewed in Polyak, 1996). Indeed, cervical carcinoma cell lines are TGF-β resistant (De Geest et al., 1994), and ectopic HPV16 E7 expression abrogates TGF-β mediated growth inhibition (Pietenpol et al., 1990). Both $p21^{CIP1}$ and $p27^{KIP1}$ have been implicated in TGF-β mediated growth inhibition (Datto et al., 1995; Elbendary et al., 1994; Polyak et al., 1994); and HPV16 E7's ability to inactivate these CKIs may contribute to its ability to abrogate TGF-β mediated growth inhibition. TGF-β also induces a cdk4/cdk6 specific CKI, $p15^{INKB}$ (Hannon et al., 1994) and $p15^{INKB}$-induced growth suppression may require functional pRB, which is targeted fro degradation by HPV16 E7.

However, acquisition of TGF-β resistance in HPV positive cell line is a multi-step process. TGF-β treatment leads to decreased expression of the HPV E6 and E7 oncoproteins; consequently, freshly derived HPV-containing epithelial cell lines remain sensitive to TGF-β (Creek et al., 1995; Woodworth et al., 1990). This effect is mediated by a mechanism that involves the ski oncogene and NFI sites in the viral upstream regulatory region (URR) and acquisition of TGF-β resistance involves this complex (Baldwin et al., 2004).

HPV positive cervical carcinoma cell lines also acquire resistance to tumor necrosis factor α (TNF) (Villa et al., 1992), an important immune response mediator that is produced by cytotoxic T cells in response to a viral infection. Normal keratinocytes undergo G1 growth arrest and cellular differentiation in response to TNF (Basile et al., 2001) through NF-kB-mediated induction of $p21^{CIP1}$ (Basile et al., 2003). TNF-related cytokines such as Fas ligand and TNF-related apoptosis inducing ligand (TRAIL) do not affect keratinocyte proliferation (Basile et al., 2001). In contrast, HPV16 E7-expressing keratinocytes continue to proliferate in the presence of TNF (Vieira et al., 1996; Villa et al., 1992).

TNF triggers apoptosis when co-administered with the protein synthesis inhibitor cycloheximide, and HPV E7 expressing keratinocytes exhibit increased apoptosis upon TNF/cycloheximide treatment (Basile et al., 2001; Stoppler et al., 1998).

HPV E7 also compromises interferon (IFN) signaling through association with and inhibition of IFN-α induced nuclear translocation of p48, the DNA binding component of ISGF-3 (Barnard et al., 1999; Barnard et al., 2000). Interferon expression is activated by interferon regulatory factors (IRFs) and HPV16 E7 can associate with IRF-1 and impair its transcriptional activity (Park et al., 2000; Perea et al., 2000). Moreover, IFN-γ has been shown to inhibit HPV16 E7 expression, and IFN-γ-induced suppressor of cytokine signaling-1 (SOCS-1)/JAB can associate with and induce ubiquitin-mediated degradation of E7 (Kamio et al., 2004).

HPV16 E7 may also interfere with insulin-like growth factor (IGF) signaling, which regulates cell survival. HPV16 E7 has been reported to associate with insulin like growth factor binding protein-3 (IGFBP-3) (Mannhardt et al., 2000) and accelerate its proteasome-mediated degradation (Santer et al., 2007). HPV16 E7 expressing cells express increased IGFBP-2 and IGFBP-5 levels through an NF-kB dependent mechanism, specifically under conditions of growth factor depletion (Eichten et al., 2004).

Effects of HPV E7 on cellular metabolism

The metabolism of cancer cells is based on energy generation through glycolytic processes rather than mitochondrial respiration (reviewed in Aisenberg, 1961; Warburg, 1936). HPV16 E7 can associate with and alter the activity of the metabolic enzyme pyruvate kinase (PK) (Zwerschke et al., 1999). Presumably as a consequence of this association, HPV16 E7 transformed cells contain high levels of a relatively inactive, dimeric form of PK and such cells derive their metabolic energy mostly from glycolytic processes rather than from oxidative phosphorylation (Mazurek et al., 2001).

HPV16 E7 has also been found to associate with and allosterically activate α-glucosidase (Zwerschke et al., 2000). This enzyme regulates glycogen catabolism and similar to many other tumor types, HPV-associated cervical cancers contain relatively low glycogen levels (Pedersen, 1975).

Transformed cells often have a higher pH and HPV E7 expression in NIH 3T3 cells causes intracellular alkalinization potentially due to increased activity of the Na^+/H^+ exchanger protein. Inhibition of this process decreased cell proliferation and inhibited anchorage independent growth of HPV16 E7 transformed NIH 3T3 cells (Reshkin et al., 2000).

HPV E7 and chromosomal instability

High-risk HPV oncogene expression in primary human keratinocytes causes cellular immortalization, and these cells exhibit many of the hallmarks of premalignant lesions. However, these cells are not fully transformed, as they do not form tumors when injected into nude mice. Malignant progression of these cells occurs after prolonged passaging in culture or when additional oncogenes, such as *ras* or *fos*, are expressed (DiPaolo et al., 1989; Durst et al., 1989; Hurlin et al., 1991; Pei et al., 1993). Similarly, the development of cervical cancer in HPV16 E6 and E7 transgenic mice is dependent on long-term estrogen exposure (Arbeit et al., 1996). This is comparable to the extended period of time between initial HPV infection and the development of invasive cervical carcinoma (reviewed in Schiffman et al., 2007).

Thus, while the expression of the HPV oncogenes is necessary and sufficient for the initiation of cervical carcinogenesis, additional host genome mutations are needed for malignant progression. Indeed, cervical cancer cells have accumulated a wide range of numerical and

structural chromosomal abnormalities (Mitelman et al., 2007) some of which likely contribute to malignant progression. Gains of chromosome 3q have been reported to correlate with progression of severe dysplasia to invasive carcinoma (Heselmeyer et al., 1997; Heselmeyer et al., 1996) and transfer of chromosome 11 suppresses the malignant phenotype of the HPV18-positive HeLa cervical carcinoma cell line (Rosl et al., 1991; Stanbridge et al., 1982). Development of aneuploidy is specifically associated with high-risk, but not low-risk, HPV infection in vivo (Rihet et al., 1996) and is detected in premalignant lesions (Bibbo et al., 1989; Steinbeck, 1997) prior to the integration of HPV genomes into host chromosomes (Bulten et al., 1998; Southern et al., 1997).

A variety of cytogenetic abnormalities have also been detected in HPV immortalized keratinocytes (Cottage et al., 2001; Smith et al., 1989) suggesting that HPV oncogene expression may somehow facilitate genomic destabilization. This concept was experimentally proven when it was shown that expression of high-risk HPV E6 and/or E7 in primary human cells increases genomic instability (Hashida et al., 1991; White et al., 1994).

Aneuploidy in HPV E6 and/or E7 expressing cells arises as a consequence of gains and/or losses of entire chromosomes during mitosis that can arise as a consequence of multiple types of mitotic abnormalities including lagging chromosomal material, anaphase bridges, and multipolar mitoses (Duensing et al., 2002).

Abnormal, multipolar mitoses are histopathological hallmarks of high-risk HPV-associated premalignant lesions and cancers (Winkler et al., 1984) and are induced by supernumerary centrosomes (Duensing et al., 2000). The synthesis of centrosomes is intimately linked to the cell division cycle. After cell division each daughter cell contains a single centrosomes, which consist of two centrioles. Upon S-phase entry the two centrioles separate and each centriole then serve as a template for the synthesis of one daughter centriole. After completion of S-phase, cells contain two centrosomes that form the poles of the bipolar mitotic spindle, thus ensuring equal and symmetric chromosome segregation during cell division (reviewed in Bettencourt-Dias et al., 2007).

High-risk, but not low-risk, HPV E6 and E7 proteins cooperate to generate mitotic defects and aneuploidy through induction of supernumerary centrosomes and multipolar mitoses in primary human epithelial cells (Duensing et al., 2000). Supernumerary centrosomes and associated multipolar mitoses have been detected in cells that express episomal HPV16 at low copy number (Duensing et al., 2001b) and their incidence increases in cells with integrated HPV (Pett et al., 2004; Skyldberg et al., 2001) presumably due to higher E6/E7 expression. In addition, supernumerary centrosomes have been documented in HPV16 E6 and/or E7 expressing transgenic mice that develop cervical (Balsitis et al., 2003; Riley et al., 2003) or skin lesions (Schaeffer et al., 2004). HPV16 E7 induces supernumerary centrosomes by uncoupling centriole synthesis from the cell division cycle causing aberrant centrioles synthesis (Duensing et al., 2001a; Guarguaglini et al., 2005) through concurrent formation of multiple daughter centrioles from a single maternal template (Duensing et al., 2007). Unlike normal centrosome duplication, the ability of HPV16 E7 to induce aberrant centriole synthesis is dependent on cdk2 activity. The inhibition of cdk2 interferes with E7 mediated induction of supernumerary centrosomes and decreases the incidence of centrosome abnormalities and aneuploidy in E7 expressing cells (Duensing et al., 2006; Duensing et al., 2004). As discussed in a previous section, HPV16 E7 expression causes aberrant cdk2 activation through multiple pathways. Since HPV16 E7 expression augments the incidence of supernumerary centrosomes in mouse embryo fibroblasts that lack pRB, p107 and p130, however, the ability of HPV16 E7 to induce supernumerary centrosomes is at least in part independent of the ability to target pRB family members (Duensing et al., 2003). A possible pRB/p107/p130 independent mechanism for the emergence of supernumerary centrosomes in HPV16 E7 expressing cells involves the

association of E7 with the centrosomal regulator γ-tubulin through sequences that overlap the pRB core-binding site and interfere with γ-tubulin recruitment to centrosomes. The association of HPV16 E7 with γ-tubulin was pRB/p107/p130 independent and binding correlated with the ability of HPV16 E7 to induce supernumerary centrosome abnormalities in pRB/p107/p130 deficient cells. Moreover, HPV16 E7 expression significantly inhibited γ-tubulin recruitment to the centrosome (Nguyen et al., 2007).

Supernumerary centrosomes do not necessarily cause multipolar mitoses due to a cellular defense mechanism, centrosome coalescence, which causes formation of a single mitotic spindle pole by multiple centrosomes. Such cells undergo pseudo-bipolar mitoses, although such mitotic events may lead to chromosome segregation errors. A recent study with high-risk HPV associated anal carcinomas has revealed that the vast majority of cells with supernumerary centrosomes appear to undergo pseudo-bipolar mitosis (Duensing et al., 2008). Nonetheless, tripolar mitoses are hallmarks of high-risk HPV associated lesions and cancers (Crum et al., 1984; Skyldberg et al., 2001) and a significant fraction of HPV16 E6/E7 expressing keratinocytes undergo multipolar mitoses (Duensing et al., 2000). In some cell types, most notably U2OS osteosarcoma cells, E7 expression is sufficient for the induction of multipolar mitoses, suggesting that HPV E7 contributes to subversion of centrosome coalescence.

A study from Bill Saunder's group has shown that the nuclear and mitotic apparatus protein-1 (NuMA) and the microtubule motor protein dynein play significant roles in regulating this process. Oral cancer cell lines with NuMA overexpression show delocalization of dynein from the mitotic spindles, which in turn was correlated with abrogation of centrosome coalescence (Quintyne et al., 2005). HPV16 E7 expression causes dynein delocalization from mitotic spindles (Nguyen et al., 2008a). This activity of E7 maps to a carboxyl terminal domain that is distinct from the LXCXE sequences that are necessary for the induction of supernumerary centrosomes. Interestingly, however, in the context of a normal cell, HPV16 E7 mediated dynein delocalization is not sufficient to induce induction of multipolar mitoses (Nguyen et al., 2008a). It will be interesting to determine whether the ability of HPV16 E7 to induce dynein delocalization is mediated through NuMA and whether it contributes to induction of multipolar mitoses in the context of HPV16 E6 expression which has been shown to greatly increase the incidence of multipolar mitoses in E7 expressing keratinocytes (Duensing et al., 2000).

HPV16 E7 expression also causes other types of mitotic abnormalities including lagging chromosomal material and anaphase bridges that may represent chromosomal fusions caused by double stand DNA breaks (Duensing et al., 2002). The presence of DNA repair foci seen in HPV16 E7 expressing cells indicates that E7 may induce double strand DNA breaks or interfere with break repair. This may facilitate viral genome integration. Consequently, E7 may be a driving force for integration of high-risk HPV genomes into host cellular chromosomes (Kessis et al., 1996), an event that frequently accompanies malignant progression of high-risk HPV associated lesions. Induction of genomic instability and HPV genome integration may also arise as a direct consequence of HPV genome replication, through “onion skin” DNA replication, where multiple replication initiation events occur on a single viral genome template so that replication is occurring at different stages simultaneously (Kadaja et al., 2007; Mannik et al., 2002). This may result in double stranded HPV DNA fragments breaking off of the circular genome and being integrated into the host genome via the endogenous DNA double strand break (DSB) repair machinery (Kadaja et al., 2007). If the upstream regulatory region is integrated into the host DNA, it may be the site of continued “onion skin” replication as long as the viral E1 and E2 replication proteins are expressed (Kadaja et al., 2007). This abnormal generation of multiple double stranded DNA fragments can also initiate DNA DSB repair, which may result in several possible outcomes: excision of HPV DNA, excision of host DNA, rearrangement of host or HPV DNA, repeated integration of HPV DNA, or repeated fragments of host DNA.

Fanconi anemia (FA) patients have an increased incidence of squamous cell carcinomas at sites that are infected by HPVs (reviewed in zur Hausen, 2002) and oral cancers arising in FA patients are HPV positive at a significantly higher rate than in the general population (Kutler et al., 2003). The FA pathway is activated by DNA crosslinking agents and stalled replication forks (reviewed in Kennedy et al., 2005), and it was recently demonstrated that the FA pathway is activated by HPV16 E7 expression and that the capacity of HPV16 E7 to induce DNA repair foci is enhanced in FA patient derived cell lines (Spardy et al., 2007).

Concluding Remarks

At the time this review was written it has been more that 20 years since the discovery of transforming activity of the HPV16 E7 protein. Thus one might question (and some grant reviewers do, indeed), whether or not continued studies on HPV oncoproteins can still yield conceptually novel insights. Does the fact that one of the authors of this article (KM) has been studying biological activities and cellular targets of the HPV E7 proteins for more than 20 years represent relentless scientific curiosity or is simply a reflection of intellectual lethargy? While readers of this article might disagree on the answer of the latter question, these authors would strongly argue that HPV16 E7 will continue to provide a powerful tool to discover novel cellular signaling pathways that importantly contribute to tumorigenesis. HPV associated cancers are unique amongst human solid tumors since they are uniformly caused by the same carcinogens, expression of high-risk HPV E6 and E7 oncoproteins. The findings that HPV16 E7 expression in a transgenic mouse model is sufficient for cervical cancer development (Riley et al., 2003) and that its continued expression remains necessary for the maintenance of the transformed state of cervical cancer derived cells (DeFilippis et al., 2003) clearly validate the premise that the HPV E7 oncoprotein importantly contributes to induction and progression of HPV associated cancers. Clearly, genome-wide RNA expression analyses as well as novel proteomic methodologies made it possible to identify novel and unexpected cellular pathways that HPV16 E7 targets. The finding that HPV16 E7 expression induces certain aspects of angiogenesis serves as a powerful illustration of this point (Chen et al., 2007; Toussaint-Smith et al., 2004).

With the availability of genome wide libraries of siRNA and cDNA expression clones, it is now possible to perform loss- and gain-of-function experiments in mammalian cells that for all practical purposes approach genetic screens in genetically tractable model organisms. Such studies, in combination with sophisticated analytical tools that are developed through the emerging field of network biology, will yield novel and unexpected insights into the molecular signaling networks that are targeted by HPV E7. We have recently performed a small-scale, proof-of-principle shRNA screen to investigate whether HPV16 E7 expression alters the kinase requirements of the RKO colon cancer cell line. These experiments revealed that E7 expression in these cells abrogates the requirement of RKO cells for CDK6, ERBB3, FYN, AAK1, and TSSK2. The reduced requirement for CDK6 in E7 expressing RKO cells is not surprising given that HPV16 E7 targets its major phosphorylation substrate, the retinoblastoma tumor suppressor pRB, for degradation. On the other hand, the biochemical mechanisms by which E7 expression abrogates the requirement for the four other kinases in RKO cells are unknown (Baldwin et al., 2008). Given that kinase signaling pathways are remarkably distinct in different cell types (Grueneberg et al., 2008), similar studies that are currently performed in a more appropriate “wild-type” cellular background promise to reveal exciting new avenues of research.

Acknowledgements

This article is dedicated to all Münger Lab members, past and present, whose hard and dedicated work has provided many critical insights into the biochemistry and biology of human papillomavirus oncoproteins. KM also acknowledges the spiritual support of M. An Hattens and Chung Hwa, and MEM-D acknowledges the support of P.

R. Cocet. Supported by NIH grants R01CA066980 and R01CA081135 (KM) and American Cancer Society Postdoctoral Fellowship PF-07-072-01-MBC (MEM-D).

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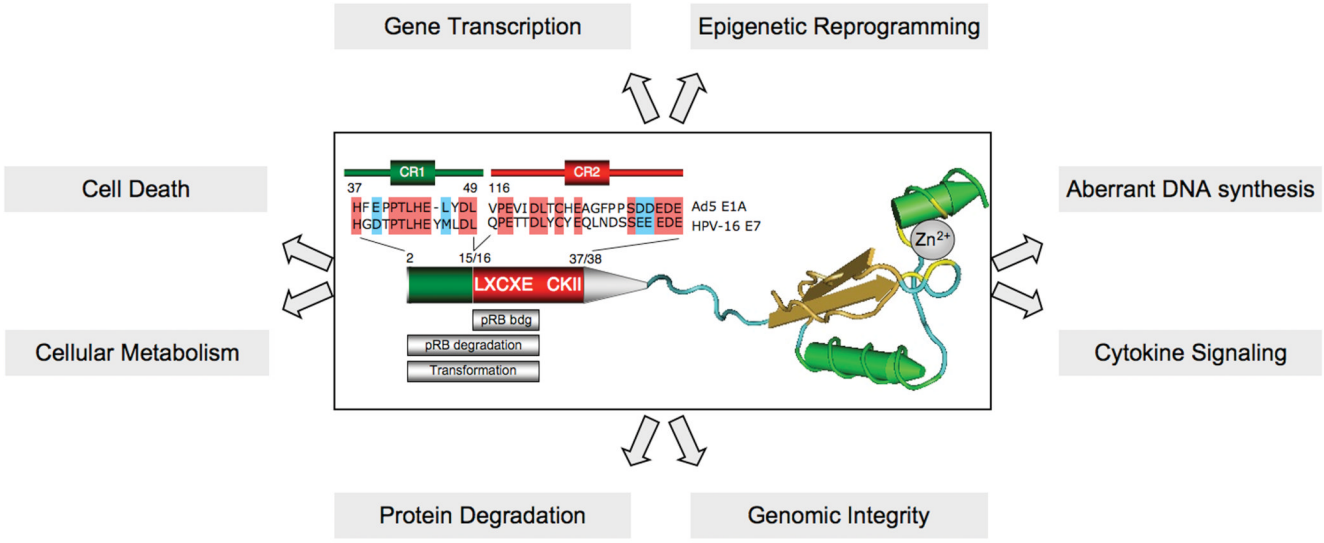

Figure 1. Schematic representation of the HPV E7 oncoprotein and affected cellular processes

The unstructured amino terminal 37 amino acid residues of HPV16 E7 have sequence similarity to a portion of CR1 (green) and the entire CR2 (red) of Ad E1A, with specific identical and chemically similar amino acid residues indicated by red and blue boxes, respectively. CR1 sequences are required for cellular transformation and pRB degradation but do not directly contribute to pRB binding. The core pRB binding site (LXCXE), also required for transformation, is located within CR2, adjacent to a casein kinase II consensus phosphorylation site (CKII). The carboxyl terminal E7 domain shown here is from HPV45 E7 as determined by X-ray crystallography (Ohlenschlager et al., 2006). This portion of the E7 sequence has a compact β1β2αIβ3α2 topology that represents a unique zinc-binding fold. The cysteine residues involved in zinc binding are indicated in yellow. They are arranged as two Cys-X-X-Cys motifs separated by 29 amino acid residues. Cellular processes affected by E7 and discussed in this chapter are indicated in the grey boxes. See text for details and references.