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Minireview

Cellular transformation by human papillomaviruses: Lessons learned by comparing high- and low-risk viruses

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ABSTRACT

The oncogenic potential of papillomaviruses (PVs) has been appreciated since the 1930s yet the mechanisms of virally-mediated cellular transformation are still being revealed. Reasons for this include: a) the oncoproteins are multifunctional, b) there is an ever-growing list of cellular interacting proteins, c) more than one cellular protein may bind to a given region of the oncoprotein, and d) there is only limited information on the proteins encoded by the corresponding non-oncogenic PVs. The perspective of this review will be to contrast the activities of the viral E6 and E7 proteins encoded by the oncogenic human PVs (termed high-risk HPVs) to those encoded by their non-oncogenic counterparts (termed low-risk HPVs) in an attempt to sort out viral life cycle-related functions from oncogenic functions. The review will emphasize lessons learned from the cell culture studies of the HPVs causing mucosal/genital tract cancers.

Introduction

Papillomaviruses cause benign hyperproliferative disease and malignances (zur Hausen, 2009). The purpose of this review is to discuss various aspects of cellular transformation mediated by human papillomaviruses (HPVs). In the case of polyomaviruses, e.g., SV40 and polyoma virus, the transforming functions of the viral proteins are only revealed when the replication functions of those proteins are limited, either by expressing the genome in a non-permissive cell where the viral genome cannot replicate (Murakami et al., 1986; Watkins and Dulbecco, 1967) or by expression of the genome in a permissive cell where the origin of replication has been mutated (Gluzman, 1981). In the case of the HPVs, transformation and replication are again mutually exclusive, but the infected host cell is the same so it is less easy to separate functions specific to the life cycle of the virus from those critical to cellular transformation.

All HPVs are presumed to infect the basal cells through a microabrasion. In the basal cells the viral genome is maintained extrachromosomally at 50–100 copies per cell and no viral structural proteins are produced; this phase is referred to as the non-productive phase. Both the low level expression and genome copy number are due, at least in part, to the presence in undifferentiated cells of cellular proteins which negatively regulate expression of genes from the viral genome and may compete for binding to the viral origin of replication (Ai et al., 2000; Ai et al., 1999; Narahari et al., 2006; O'Connor et al., 2000). Viral DNA amplification and virion production (the productive phase of the viral life cycle) occur in the differentiated compartment. These differentiated cells would normally have exited the cell cycle and would, therefore, not support the productive phase of the virus life cycle. Thus all HPVs have to uncouple the normal proliferation:differentiation switch to maintain or create an environment compatible with viral DNA amplification within the differentiated compartment. One could imagine that this uncoupling would give all the HPVs the potential to cause cancer yet only a subset of HPVs is oncogenic. The perspective of this review will be, wherever possible, to distinguish oncogenic functions from life cycle functions by comparing activities of proteins encoded by HPVs associated with cancer to activities of proteins encoded by HPVs associated only with benign disease.

There has been a flurry of excellent reviews recently of the human papillomaviruses and their gene products (Ghittoni et al., 2010; Howie et al., 2009; McLaughlin-Drubin and Munger, 2009; Moody and Laimins, 2010; Pim and Banks, 2010; Wise-Draper and Wells, 2008). This review will emphasize lessons learned from the cell culture studies of the human alpha-PV oncoproteins (E6, and E7) each in contrast to

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their non-oncogenic counterparts. There will also be a more limited discussion of the human beta-PV E6 and E7 proteins and of the HPV E5 protein. The human alpha-PVs are those infecting the mucosa/genital epithelium and causing lower genital tract and head and neck disease; the human beta-PVs infect cutaneous tissue and are associated with non-melanoma skin cancer (de Villiers et al., 2004). Topics will include regulation of cell cycle and differentiation; apoptosis, senescence and autophagy; genomic instability; and metabolism. Although this review will emphasize results of experiments using the reductionist approach, i.e. analyzing the activity of a single protein, it should be borne in mind that these viral proteins are acting in the context of each other. For example, the ability of high-risk HPV E7 to target Rb for degradation can trigger cell death but the ability of high-risk HPV E6 to target p53 for degradation abrogates this death.

HPV overview

Establishing the oncogenic potential of high-risk HPVs and limited oncogenic potential of low-risk HPVs

In the early 1980s several HPV genomes were isolated, some from benign lesions and others from cervical carcinomas. Subsequent epidemiological data established the grouping of HPV genomes into high-risk (e.g., HPV 16, 18, 31, 33, 45) and low-risk (e.g., HPV 6, 11) with respect to cancer (zur Hausen, 2002). Co-incident with the accumulation of epidemiological data, the oncogenic potential of HPVs was defined in the late 1980s and early 1990s through a variety of in vitro assays. Initial experiments established that high-risk HPVs could morphologically transform permanent rodent cells (Tsunokawa et al., 1986; Yasumoto et al., 1986). Further, high-risk HPVs could cooperate with activated *ras* to transform primary rodent cells (Matlashewski et al., 1987).

As early as 1987 a distinction in the in vitro activity of high-risk versus low-risk HPV genomes was reported. High-risk HPVs could cooperate with activated *ras* to transform primary rodent cells but low-risk HPVs could not (Storey et al., 1988). The high-risk HPV genome but not the low-risk HPV genome could cooperate with activated *ras*, in the presence of dexamethasone, to morphologically transform primary rodent cells (Pater et al., 1988). In contrast, others reported that low-risk HPVs could cooperate with *ras* to transform primary rodent cells although requiring more time in culture (Cerni et al., 1990; Chesters and McCance, 1989). Some differences in outcome from different laboratories probably relate to whether or not selection was used to isolate transformed colonies and the assays used to score transformation.

The ability to conduct experiments in the HPV host cell, human primary foreskin keratinocytes, led to exploration of HPV activity in these latter cells. It was quickly shown that DNA from high-risk HPVs could immortalize primary human keratinocytes derived from foreskin or cervical tissue but that such cells were not tumorigenic in nude mice (Kaur and McDougall, 1988; Pirisi et al., 1988; Pirisi et al., 1987; Woodworth et al., 1988). Such cells became tumorigenic if kept in culture for a period of time or if activated *ras* was introduced (DiPaolo et al., 1989; Hurlin et al., 1991; Pecoraro et al., 1991). Further, the high-risk HPV genome could alter differentiation of an organotypic raft culture (McCance et al., 1988). The ability of HPVs to immortalize human primary keratinocytes and inhibit differentiation was initially reported to be limited to high-risk HPVs (Pecoraro et al., 1989; Schlegel et al., 1988; Woodworth et al., 1989). However, later experimental protocols indicated that low-risk HPVs could also delay or alter differentiation (Thomas et al., 2001).

Defining the oncogenes

Analysis of cervical carcinomas indicated that the high-risk HPV genome is often integrated and E6 and E7 are the genes that are expressed (Schwarz et al., 1985; Yee et al., 1985). Later experiments showed that the integration enhanced their activity (Jeon et al., 1995). Almost simultaneously with the establishment that high-risk HPVs could transform permanent rodent cells and cooperate with activated *ras* to transform primary rodent cells, was the documentation that the critical genes for this transformation were E6 and E7 (Bedell et al., 1987; Vousden et al., 1988), with E7 having stronger transforming activity (Vousden and Jat, 1989). Early on there was some controversy over whether the E6 and E7 genes of low-risk HPVs had transforming activity in rodent cells (Chesters and McCance, 1989; Hiraiwa et al., 1993; Storey et al., 1990b). Some differences in outcome may have been related to the level of expression of proteins, either because of the promoter used to drive expression or because of the level of splicing achieved. Later experiments tended to use retroviral transduction, which also influences the extent of splicing but also allows delivery of the genome to a much higher percentage of cells compared to transfection.

In human keratinocytes, transfection of the E6 and E7 genes of high-risk HPVs is necessary and sufficient for immortalization and inhibition of keratinocyte differentiation but not for tumorigenicity (Barbosa and Schlegel, 1989; Hawley-Nelson et al., 1989; Hudson et al., 1990; Kaur et al., 1989; Munger et al., 1989a; Sedman et al., 1991). The relative contribution of E6 and E7 to immortalization of keratinocytes has also been examined using retroviral vectors. With that protocol, E7, but not E6, alone can immortalize cells; however, the efficiency of immortalization is much greater when E6 and E7 are expressed together (Halbert et al., 1991).

In contrast, low-risk HPV E6 and E7 have little or no immortalizing activity. When transfected into keratinocytes, neither can complement the corresponding gene from HPV 16 or HPV 18 (Barbosa et al., 1991). In addition, neither gene is able to immortalize keratinocytes when transduced into cells (Halbert et al., 1991). However, by transduction, weak immortalizing activity of HPV 6 E6 or HPV 6 E7 can be detected when HPV 16 E7 or HPV 16 E6, respectively, is present (Halbert et al., 1992).

Two issues arose during the course of these experiments. First, the transcription and translation of E6 and E7 are regulated differently between the high-risk and low-risk HPVs. While low-risk HPVs have a promoter upstream of the E6 gene and another upstream of the E7 gene, in high-risk HPVs there is a single promoter upstream of E6 and a polycistronic mRNA is produced (Smotkin et al., 1989). Further, in the high-risk HPVs, the relative levels of translation of E6 and E7 are determined by whether the splice site within E6 is used, which favors production of E7 (Sedman et al., 1991; Tang et al., 2006). Thus it was possible that in instances where E6 seemed less active, this is due to the level of expression of E6 versus E7 and similarly where low-risk proteins seem less active, it could be due to differences in expression levels compared to high-risk. However, when the early region of both high-risk HPV and low risk HPV is expressed from a strong promoter, only the high risk HPV early region can immortalize keratinocytes (Morgan et al., 1992). Similarly, expression of individual genes from the same promoter supports the conclusion that the low-risk HPV E6 and E7 genes encoded proteins lacking in oncogenic potential (Barbosa et al., 1991). Second, early data indicated that predominantly high-risk spliced mRNA was seen in patient biopsies and immortalized cell lines (Bohm et al., 1993; Doorbar et al., 1990; Sherman et al., 1992). In contrast, when cloned into a retrovirus, the predominant mRNA species is unspliced. This is dictated by the distance between the promoter and splice donor (Zheng et al., 2004). The potential differences in relative levels of E6 and E7 expression depending upon the model system used should be kept in mind.

Finally, transgenic mouse models have validated the oncogenic potential of high-risk alpha-HPV E6 and E7 in the skin, cervix, and head and neck and the beta-HPV E6 and E7 in the skin (Dong et al., 2005; Lambert et al., 1993; Riley et al., 2003; Strati et al., 2006). These models have also revealed that the relative impact of E6 and E7 in vivo is HPV type and tissue-specific. For example, alpha-HPV E7 is the more potent oncogene in the cervix and head and neck but alpha and beta-HPV E6s are more potent in the skin (Herber et al.,

1996; Marcuzzi et al., 2009; Riley et al., 2003; Song et al., 1999; Strati and Lambert, 2007). There are no published results on the activity of low-risk HPV E6 and/or E7 in the transgenic mouse model.

The HPV E6 proteins

E6 and transformation

While E6 and E7 are co-expressed in the course of HPV infection and in cancers, dissection of the individual contribution of each oncogene is important for determining their role in replication and transformation. While a number of E6 functions and interactions help to explain how high-risk HPV E6s cause transformation, surprisingly few functions have been attributed to E6s from low-risk types. Those that are shared between low- and high-risk HPV E6s might point to what functions are important for the virus life cycle. In the following sections, high and low risk E6s will be compared when the information is available. Of course, because of the association with development of cancer, most studies have focused on high-risk HPV E6s.

E6s from high-risk types such as HPV 16 and 18 have transforming potential in various assays. Early studies indicated that high-risk HPV E6s are not particularly effective in cooperating with activated *ras* to cause transformation of baby rat kidney (BRK) cells (Bedell et al., 1989; Phelps et al., 1988; Sedman et al., 1991; Storey et al., 1988). Taking the cue from SV40 T antigen and adenovirus E1B, researchers determined that high-risk HPV E6 binds to p53 (Werness et al., 1990). Later it was shown that E6 targets p53 for degradation (see below) (Scheffner et al., 1990). These findings spawned a great deal of interest in E6 and its involvement in transformation. While early studies suggested that p53 mutation and E6 expression were equivalent in the transformation of mouse cells in cooperation with mutant *ras* (Storey and Banks, 1993), the finding that certain E6 mutants are unable to target p53 for degradation but still have transforming properties indicated that E6 has other functions (Pim et al., 1994; Storey and Banks, 1993). E6 from high-risk types does not cause immortalization of human keratinocytes on its own but significantly increases the efficiency of immortalization of human keratinocytes by high-risk HPV E7 (Halbert et al., 1991, 1992; Howley and Lowy, 2007). In apparent contrast to these findings, high-risk HPV E6 alone can immortalize human mammary epithelial cells (Band et al., 1991; Dalal et al., 1996). However, it was later demonstrated that loss of expression of $p16^{Ink4a}$, a cyclin dependent kinase inhibitor, along with E6 expression is necessary for mammary epithelial cell immortalization, indicating a requirement for abrogation of the Rb pathway (Foster et al., 1998; Kiyono et al., 1998). Degradation of p53 is not essential for immortalization of human keratinocytes and mammary epithelial cells (Kiyono et al., 1998; Liu et al., 1999) and, in fact, immortalization of these cells is more associated with the ability of E6 to activate telomerase (discussed below) (Kiyono et al., 1998). Low-risk HPV E6s have little to no transforming or immortalizing potential in transformation assays (Band et al., 1993; Halbert et al., 1992).

Studies with transgenic animals have also provided insight into how high-risk HPV E6s cause transformation. In transgenic mice that express high-risk HPV E6 and E7 from a keratin 14 (K14) promoter, tumors develop in the skin and cervix, the latter in conjunction with chronic estrogen treatment (Riley et al., 2003; Song et al., 1999). These studies demonstrate that both oncogenes have transforming properties in vivo. While the E6 transgene plus estrogen can cause tumors, similar studies with the E7 transgene show that it is more efficient at tumor initiation. E6 may be more important for tumor progression (Riley et al., 2003). In these studies with transgenic animals, two functions of E6 emerged as being important for tumorigenicity, the ability to bind to alpha-helix containing proteins and the ability to bind to PDZ (PSD95/Dlg/Zo-1) containing proteins (Shai et al., 2007).

Overall, these findings indicate that high-risk HPV E6s cooperate with abrogation of the Rb pathway (either through expression of E7 or other mechanisms) to cause transformation. Furthermore, they demonstrate that high-risk HPV E6s have multiple functions, aside from targeting p53 for degradation, that are important for transformation. They do not, however, tell us much about the role of E6 in virus replication.

Many different functions have been attributed to E6 (Table 1) and it has been shown that E6 has numerous interactions within the cell including both DNA binding properties and the ability to bind to many cellular proteins (Table 2). For recent reviews, see Howie et al. (2009); Liu et al. (2009); Pim and Banks (2010); Wise-Draper and Wells (2008); and Yugawa and Kiyono (2009). In this part of the review, functions and transformation properties of E6 will be discussed. Where applicable, comparisons will be made between those functions and binding partners that are shared between low- and high-risk HPV E6s, with the thought that this will shed light on

Table 1
Functions of HPV E6.

Function	High-risk HPV E6 (HPV 16, 18, or 31)	Low-risk HPV E6 (HPV 6 or 11)	References
Inhibition of p53 transactivation	+	+	Crook et al. (1991); Lechner and Laimins (1994); Pim et al. (1994); Thomas et al. (1995)
Inhibition of p53 acetylation	+	+	Jha et al. (2010); Patel et al. (1999); Thomas and Chiang (2005)
Bypass of growth arrest upon DNA damage	+	−	Havre et al. (1995); Jones and Munger (1997); Kessis et al. (1993)
Induction of genetic instability	+	?	Duensing and Munger (2002); Liu et al. (2007)
Immortalization of human cells (with Rb inactivation)	+	−	Dalal et al. (1996); Halbert et al. (1991)
Telomerase activation	+	−	Gewin and Galloway (2001); Kiyono et al. (1998); Klingelhutz et al. (1996); Veldman et al. (2001)
c-myc activation	+	−	Veldman et al. (2003)
Inhibition of keratinocyte differentiation	+	?	Alfandari et al. (1999); Duffy et al. (2003); Nees et al. (2000); Sherman et al. (1997); Sherman and Schlegel (1996)
Inhibition of interferon response	+	+/−	Cordano et al. (2008); Li et al. (1999); Nees et al. (2001); Ronco et al. (1998)
NF-κB activation	+	?	An et al. (2008); James et al. (2006b); Nees et al. (2001); Yuan et al. (2005)
Akt activation	+	?	Pim et al. (2009)
Wnt activation	+	?	Lin et al. (2009)
mTORC1 activation	+	?	Spangle and Munger (2010)
miR-34a downregulation	+	?	Wang et al. (2009b)

Table 2
Binding partners of HPV E6.

Binding partner	High-risk (16, 18 or 31)	Low-risk (1, 6 or 11)	References
p53	+ (binding and degradation)	+/− (binding)	Crook et al. (1991); Foster et al. (1994); Lechner and Laimins (1994); Li and Coffino (1996); Pim and Banks (1999); Pim et al. (1997); Scheffner et al. (1990); Thomas et al. (1995); Werness et al. (1990)
E6-AP	+ (binding)	+ (11E6 binds)	Bedard et al. (2008); Brimer et al. (2007); Scheffner et al. (1993)
Bak	+ (binding and degradation)	+ (binding and degradation)	Thomas and Banks (1999); Underbrink et al. (2008)
Dlg	+ (binding and degradation)	+/− (degradation)	Brimer et al. (2007); Gardiol et al. (1999); Kiyono et al. (1997); Pim et al. (2009)
MAGI-1	+ (binding and degradation)	−	Pim et al. (2009); Thomas et al. (2002)
MAGI-2	+ (binding and degradation)	−	Pim et al. (2009)
MUPP1	+ (binding and degradation)	−	Lee et al. (2000)
Scribble	+ (binding and degradation)	−	Nakagawa and Huibregtse (2000); Pim et al. (2009)
CAL	+ (binding and degradation)	?	Jeong et al. (2007)
PTPN3/PTPN13	+ (binding and degradation)	−	Jing et al. (2007); Spanos et al. (2008b)
p300/CBP	+ (binding prevents acetylation of p53)	+/− (binding prevents acetylation of p53)	Patel et al. (1999); Thomas and Chiang (2005); Zimmermann et al. (1999)
Tip60	+ (destabilizes)	+ (destabilizes)	Jha et al. (2010)
PML	+ (interaction)	+ (interaction)	Guccione et al. (2004)
E6TP1	+ (binding and degradation)	−	Gao et al. (1999)
Paxillin	+ (binding)	−	Tong et al. (1997)
E6BP/ERC-55	+ (interaction)	?	Chen et al. (1995)
Mcm7	+ (binding and degradation)	−	Kuhne and Banks (1998); Kukimoto et al. (1998)
MGMT	+ (binding and degradation)	?	Srivenugopal and Ali-Osman (2002)
NFX1-91	+ (binding and degradation)	−	Gewin et al. (2004); Xu et al. (2008)
TERT	+ (binding)	?	Liu et al. (2009)
Ada3	+ (binding and degradation)	−	Kumar et al. (2002)
Tyk2	+	+/−	Li et al. (1999)
FADD	+ (binding and degradation)	?	Filippova et al. (2004)
TNFR1	+ (binding)	?	Filippova et al. (2002)
IRF-3	+ (16 only, inhibits transactivation)	−	Ronco et al. (1998)
Procaspase 8	+ (degradation)	−	Filippova et al. (2007); Tungteakkhun et al. (2010)
c-myc	+ (co-activation)	−	Veldman et al. (2003)
CYLD	+ (binding and degradation)	?	An et al. (2008)

what properties of E6 are important for replication. Current knowledge on the function of E6s from beta-PVs will be discussed in a section devoted to beta-PVs toward the end of the review.

Features of the E6 protein

The E6 protein is approximately 150 amino acids in length with a molecular weight of 18 kDa (Howley and Lowy, 2007). E6 proteins from different HPV types are characterized by four C-X-X-C motifs that are involved in the formation of two zinc binding sites (Fig. 1). These zinc binding sites are essential for many of the known functions of E6, although there are some exceptions. A region in the second zinc binding site of E6 is involved in binding to a number of alpha-helix containing cellular proteins that have an LXXLL motif, including the ubiquitin ligase, E6-AP (Chen et al., 1998). In addition to this domain, other regions of E6 are important for various E6 functions and for binding to cellular proteins such as p53, E6-AP, p300, and PDZ-containing proteins. These will be discussed in more detail below.

Determining the exact structure of E6 has been difficult. In one study, the N and C terminal halves were separated and folded independently such that a pseudodimeric scaffold was predicted for the whole E6 protein (Nomine et al., 2006). Both the N and C termini

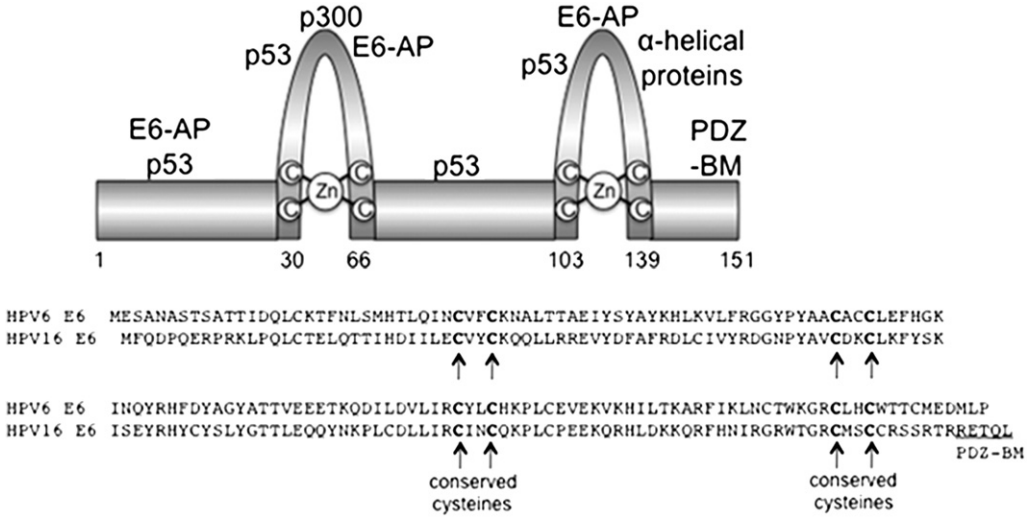

Fig. 1. Schematic of the HPV 16 E6 protein. The two zinc binding sites are conserved among the different HPV types. Protein binding regions are shown for HPV 16 E6. The text below the schematic displays the aligned sequences of high-risk HPV 6 E6 and low-risk HPV 16 E6.

are predicted to form a stably folded domain composed of a three-stranded beta sheet and two short helices. Antiparallel assembly of the N and C termini leads to a six-stranded beta sheet with the two H1 helices packing in a classical manner and the zinc binding sites located at opposite edges of the molecule. In this model, a number of conserved residues are exposed in all HPV species that could be involved in structure or generic function. Using a combination of software programs to predict ordered and disordered regions of proteins, high-risk HPV E6 is found to be more disordered (unfolded) than low-risk HPV E6 (Uversky et al., 2006), consistent with the finding that oncogenic proteins are more disordered.

E6 can be localized to both nuclear, cytoplasmic, and membrane fractions (Grossman et al., 1989; Guccione et al., 2002), a finding which supports its many proposed functions. There is evidence that E6 localization is different for low- and high-risk viruses and that this might partially explain differences in the way low and high-risk HPV E6 regulates cellular proteins such as p53 (Guccione et al., 2002; Pietsch and Murphy, 2008). For example, rather than targeting p53 for degradation, low-risk HPV E6s apparently sequester p53 in the cytoplasm to prevent it from transactivating p53 responsive genes in the nucleus (Pietsch and Murphy, 2008).

The understanding of E6 function is further complicated by the fact that certain high-risk HPV types generate one or more truncated E6 transcripts, called E6*. The proteins generated from these transcripts have their own inhibitory or activating functions (Filippova et al., 2007; Pim and Banks, 1999; Pim et al., 1997). For example, E6* inhibits E6-mediated degradation of p53 by binding the full-length E6 protein (Pim and Banks, 1999). E6* has also been shown to modulate how E6 affects apoptotic pathways (Filippova et al., 2009). Other functions of E6* have also been demonstrated as discussed below.

Interactions with p53 and E6-AP

Certainly, one of the best-known functions of E6 from high-risk HPV types is the binding and targeting of p53 for ubiquitin-mediated degradation (Scheffner et al., 1990). The ubiquitin ligase involved in this process is E6-AP (E6 associated protein) (Huibregtse et al., 1991; Scheffner et al., 1993). E6 domains necessary for binding and degradation of p53 are different, although the exact nature of these interactions has a history of controversy (Crook et al., 1991; Foster et al., 1994; Li and Coffino, 1996; Pim et al., 1994; Thomas et al., 1995). Both low- and high-risk HPV E6s can bind to p53 through E6's C-terminal half but this does not induce degradation (Lechner and Laimins, 1994). Binding to the N-terminal half of E6 is necessary for p53 degradation. Two different regions of p53 can be bound by E6, a region in the carboxy terminus that is bound by both low-and high-risk HPV E6's, and a region in the core region that correlates with p53 degradation (Li and Coffino, 1996). Binding to the core of p53 is necessary for degradation. The exact nature of E6's binding to E6-AP is also not entirely clear. However, it would appear that more than one region is necessary for effective binding (Huibregtse et al., 1993; Nomine et al., 2006; Pim and Banks, 2010). There is evidence that E6 from low-risk HPV 11 can also bind E6-AP (Brimer et al., 2007), indicating that E6-AP binding and p53 degradation are not always associated with one another and suggesting a potentially important role for binding of E6-AP by low and high risk E6s.

Other mechanisms of p53 pathway abrogation by low- and high-risk HPV E6s

Even though low-risk HPV E6s do not effectively target p53 for degradation they are capable of abrogating p53's function in other ways (Lechner and Laimins, 1994). For example, as mentioned above, low-risk HPV E6s may sequester p53 in the cytoplasm to affect function (Pietsch and Murphy, 2008). In addition, low-risk HPV E6s can also inhibit transcriptional activation by p53. Like high-risk HPV E6s, low-risk HPV E6s are capable of interacting with the p300/CBP complex, which is involved in p53 acetylation and this interaction does not involve E6-AP (Patel et al., 1999; Thomas and Chiang, 2005; Zimmermann et al., 1999). The binding is thought to prevent the acetylation of p53. Indeed, expression of E6 proteins represses the ability of p53 to activate p53 responsive promoters. While there have been conflicting reports over how low-risk HPV E6s affect p53 transactivation, some of the controversy is likely due to use of different p53 promoters (Pim and Banks, 2010). p53 is modified in a variety of ways (e.g. acetylation and phosphorylation) and many of these modifications affect the ability of p53 to differentially modulate the activity of different promoters. Other potential mechanisms have been proposed to explain how E6 affects acetylation of p53. For example, E6 can bind Ada3, another HAT, but this interaction has not been extensively studied for low-risk HPV E6 (Kumar et al., 2002).

Recently, a new interaction of both low- and high-risk HPV E6s with the nucleosomal acetyltransferase TIP60 has been demonstrated that also offers further insight into how E6 affects p53 function (Jha et al., 2010). TIP60 is involved in a number of processes including acetylation of histone H4, acetylation of p53 at K120, and, indirectly, in dephosphorylation of phosphoH2AX for termination of DNA damage response (Sun et al., 2010). E6's interaction with TIP60 destabilizes the TIP60 complex and causes disruption of TIP60-dependent transcriptional regulation and apoptosis. Interestingly, E6 affects the ability of TIP60 to mediate p53-associated upregulation of genes involved in inducing apoptosis, but it does not affect p53-associated upregulation of cell cycle arrest genes such as $p21^{WAF}$. In addition, TIP60 suppresses the HPV early promoter through binding to a Brd4 complex. E6's interaction with TIP60 ameliorated this effect. Another finding of this study is that E6's effect on TIP60 does not depend on binding to E6-AP or p53. In fact, E6*, which consists of only the first 43 amino acids of E6, modulates and causes destabilization of TIP60. Thus, these studies shed new light on how E6 from both low- and high-risk types regulate p53. Understanding E6's interaction with TIP60 might be a key to understanding how E6 is involved in the HPV life cycle, but further studies using HPV replication assays are warranted.

p53 and viral replication

The role of E6 in the HPV life cycle is not entirely clear, but studies using the entire HPV 11, 16 or 31 genomes have demonstrated that E6 is necessary for efficient replication in keratinocytes (Oh et al., 2004; Park and Androphy, 2002; Thomas et al., 1999). HPV 16 E6 mutants defective in p53 binding are loss of function for genome maintenance (Park and Androphy, 2002). For HPV 11, lack of E6 results in rapid loss of episomal forms of the viral genome and mutation of a p53 binding site in HPV 11 E6 results in inability to maintain episomes (Oh et al., 2004). Similar findings are observed for HPV 31 (Park and Androphy, 2002). A more recent study, using a raft culture system, indicated that lack of E6 results in p53 accumulation and a severe reduction in genome amplification during the productive stage of replication. In this setting, genome amplification is rescued by retroviral transduction of E6 (Wang et al., 2009a). Thus, it appears that E6 is essential for replication, most likely playing a significant role in the productive program, and that abrogation of p53, but not necessarily degradation, is important in this process.

Because both low- and high-risk HPV E6 inhibit the p53 pathway, it would certainly appear that this is an important concern for papillomaviruses, as it is for other viruses. The abrogation of key p53 functions is predicted to have significant consequences with regard to DNA damage response, apoptosis, and cellular transformation. The mechanism by which the different E6s inhibit p53 function could greatly affect the ability of p53 to confer either apoptosis or cycle arrest. E6s from high-risk virus types appear to have a much greater

capacity than E6s from low-risk virus types to bypass cell cycle arrest and prevent apoptosis and part of this is likely due to their more efficient ability to abrogate p53 function (Elbel et al., 1997; Foster et al., 1994). There is no definitive evidence that high-risk viruses replicate "better" than low-risk viruses, so the reason for the different mechanisms of p53 abrogation is unclear. One possibility is that E6 is necessary for counterbalancing the effects of E7, which by itself is known to upregulate p53 (Demers et al., 1994b). With this in mind, it is tempting to speculate that high-risk HPV E6s might have to be more effective at abrogating the p53 response because high-risk HPV E7 is more prone to activating it. However, there are also studies that indicate that high-risk HPV E7 stabilizes p53 without activating it and that high-risk HPV E7 might also inhibit p53 activity (Eichten et al., 2002; Jones et al., 1999). Thus, a simple explanation is not readily apparent.

E6 and Bak

In addition to E6's role in affecting the p53 pathway, E6 also directly affects apoptotic effector molecules and this appears to be conserved between low- and high-risk virus types. For example, both low- and high-risk HPV E6s bind to the pro-apoptotic protein, Bak, and cause its degradation (Thomas and Banks, 1999). This p53-independent interaction may affect responses to DNA damaging agents such as UV (Jackson and Storey, 2000). These findings suggest that E6's interaction with Bak is conserved across many HPV types and would indicate that it is important for the HPV life cycle.

E6 and PDZ containing proteins

Another important function of high-risk HPV E6s that has been given a lot of attention is its ability to bind to PDZ containing proteins through its C-terminal PDZ binding motif (Thomas et al., 2008). PDZ binding motifs are also found in many other viral proteins including HTLV-1 tax and adenovirus E4-ORF1 (Javier, 2008). This generality would suggest that high-risk papillomaviruses benefit from modulating these proteins for efficient replication. The PDZ binding motif of E6 is essential for transformation in vitro and in vivo, both in transgenic mouse models and in a xenograft model of HPV-associated oropharyngeal cancer (Simonson et al., 2005; Spanos et al., 2008b). The PDZ binding motif has also been implicated in causing an epithelial to mesenchymal transition in human epithelial cells (Watson et al., 2003), in activating NFκB (James et al., 2006b) and in increasing the efficiency of immortalization of human tonsillar epithelial cells (Spanos et al., 2008a). Numerous proteins contain PDZ domains and E6 binds to many of them to cause their degradation (see Table 2). Several PDZ containing proteins that bind to E6 belong to the MAGUK (Membrane Associated Guanylate Kinase) family of proteins (e.g. Dlg, Scribble, and MAGI-1, -2, and -3) which are generally believed to be associated with the cellular membrane and affect such processes as cell polarity, maintaining cell-to-cell interactions, and mediating signals from membrane (Gardiol et al., 1999; Jeong et al., 2007; Kiyono et al., 1997; Nakagawa and Huibregtse, 2000; Thomas et al., 2002). The E6 PDZ motif is also important for binding to several protein phosphatases (e.g. PTPN3 and PTPN13) that are believed to be involved in both inhibiting and activating a variety of cell signaling pathways (Jing et al., 2007; Spanos et al., 2008b). These phosphatases are also commonly mutated or downregulated in cancer and E6-mediated degradation of the phosphatases appears to be necessary for tumorigenic transformation by E6 (Spanos et al., 2008b). A recent study indicated that downregulation of PTPN13 by E6 is associated with activation of MAP kinase signaling (Hoover et al., 2009).

While the PDZ binding motif is conserved among high-risk virus types, the motif is generally not found in low-risk HPV E6's. Swapping the PDZ domain of high-risk HPV E6 onto low-risk HPV E6 results in degradation of PDZ containing proteins (Pim et al., 2002). The E6 protein of some so-called low-risk HPV types such as HPV-70 can bind to PDZ domain containing proteins (Muench et al., 2009). In addition, HPV-18 E6* (which does not contain a PDZ binding motif) causes the downregulation of many PDZ proteins such as Dlg, without a direct interaction between the two proteins, although the proteasome pathway is apparently involved (Pim et al., 2009). These studies indicate that inactivation of PDZ containing proteins is not exclusive to full-length high-risk HPV E6s and that the different viral types may have adapted different strategies to deal with these proteins. Like the interaction of E6 with p53, the different mechanisms by which the virus manipulates the PDZ containing proteins may determine whether the virus causes cancer or not. Further studies are warranted to determine whether inhibition of PDZ containing proteins is important for replication of high-risk viruses.

E6 and keratinocyte differentiation

Modulation of keratinocyte differentiation by E6 is a potential mechanism that HPVs might utilize to make the cellular environment more favorable for replication. E6s from high-risk types inhibit serum and calcium mediated differentiation (Alfandari et al., 1999; Sherman and Schlegel, 1996). Microarray analyses demonstrate that E6 expression causes downregulation of a large number of genes that are associated with keratinocyte differentiation (Duffy et al., 2003; Nees et al., 2000). Evidence suggests that E6 inhibits differentiation through downmodulation of the TGF-beta pathway (Nees et al., 2000). In addition, high-risk HPV E6s bind to cellular proteins such as ERC-55, a calcium binding protein, E6-TP1, a Rap1 GTPase-activating protein, and paxillin, a focal adhesion protein, all of which could play a role in altering the differentiation capacity of keratinocytes (Chen et al., 1995; Gao et al., 1999; Tong et al., 1997). More recently, high-risk HPV E6 was found to alter the Notch pathway, which is a well-studied effector of keratinocyte differentiation (Chakrabarti et al., 2004). One would expect that low-risk HPV types would also benefit from modulating cellular differentiation, but most of the interactions noted above are limited to E6s from high-risk types and studies implicating low-risk HPV E6s in modulating differentiation are insufficient for drawing conclusions.

E6 and NFκB activation

Another potentially important function of E6 in modulating the host cell environment is its ability to activate the NFκB pathways. Activation of NFκB has been demonstrated in most epithelial cancers where it is believed to play a role in a variety of cellular processes including inhibition of apoptosis, adaptation to hypoxia and epithelial to mesenchymal transition (EMT), among other possibilities (Prasad et al., 2010). Microarray analyses indicate that E6 activates a large number of NFκB responsive genes (Nees et al., 2001) and other studies demonstrate that E6 upregulates the NFκB responsive gene c-IAP2 to confer resistance to apoptosis (James et al., 2006b; Yuan et al., 2005). The mechanism by which E6 activates NFκB is not completely clear. One study indicated that the PDZ binding motif is involved (James et al., 2006b), whereas another study provided evidence that E6 inactivates a deubiquitinase called CYLD, which subsequently causes activation of the NFκB pathway during hypoxia (An et al., 2008). Further, E6 activates NFκB by interactions with the transcription factor NFX1-91 (Xu et al., 2010). Whether E6's from low-risk HPV types interact with either CYLD or NFX1-91 is currently unknown.

E6 and PMLs

It has been demonstrated that both low-risk and high-risk HPV E6 can abrogate PML (promyelocytic leukemia) induced cellular

senescence and high-risk HPV E6 can direct the proteolytic degradation of PML in primary baby rat kidney cells (Guccione et al., 2004). The interaction of E6 with PMLs is conserved across low- and high-risk viruses, indicating that it may be important for the viral life cycle. PML proteins are present in nuclear bodies referred to as PODs (PML oncogenic domains) or nuclear domain 10 (ND10) (Guccione et al., 2004). These subnuclear bodies are attached to the nuclear matrix and are believed to play a role in many cellular processes such as transcriptional regulation, protein modification, apoptosis, senescence, interferon response, autophagy, and chromatin modifications. Other nuclear proteins in PODs include Daxx, Sp100, and CBP. PODs have been implicated in cellular defense mechanisms against viral replication or, on the other hand, as possible sites for viral assembly for a variety of different viruses (Schiller et al., 2010). The HPV late protein L2 associates with PODs, suggesting that PODs may play a role in papillomavirus life cycle (Lin et al., 2009; Schiller et al., 2010). Another study suggests that PODs do not play a significant role in HPV replication (Nakahara and Lambert, 2007). Further research will be needed to resolve this controversy.

A new function for E6: activation of mTORC1

An additional high-risk HPV E6 function that has recently been demonstrated is its ability to regulate the mTORC1 pathway (Sabatini, 2006; Spangle and Munger, 2010). The mechanism by which this occurs is not entirely clear. Early studies indicated that the E6 protein caused degradation of the mTOR inhibitor TSC2 (tuberous sclerosis complex 2) (Lu et al., 2004; Zheng et al., 2008), but this finding was not repeated in more recent studies (Spangle and Munger, 2010). However, E6 increases protein synthesis by enhancing translation initiation complex assembly at the 5′ mRNA cap and increasing cap-dependent translation. This is likely due to mTORC1 activation; co-expression of E7 did not appear to affect this function. These findings suggest that E6 is involved in modulating cellular metabolism and could have significant implications for the potential role of E6 in promoting viral replication in differentiated cells, where nutrient supply would be low. Whether this function of E6 is conserved across different HPV types remains to be determined.

E6 and the cell cycle

Because HPVs replicate in terminally differentiating tissue, they have developed a number of strategies to push normally nondividing cells into states that allow DNA synthesis. As mentioned above, the abrogation of the p53 pathway by E6s allows bypass of p53-mediated checkpoints in the context of DNA damage or apoptotic signaling. As will be discussed, HPV E7 interferes with Rb signaling to mediate the transit of cells from G1 to S. There is also evidence that HPV E6 plays a direct role in this process. In human fibroblasts, expression of E6 causes phosphorylation of Rb along with concomitant upregulation of genes that are normally inhibited by Rb such as $p16^{INK4a}$, CDC2, E2F-1, and cyclin A (Malanchi et al., 2004). E6 expression is also associated with an increase in cyclin A/cyclin dependent kinase activity. In this context, p53-independent downregulation of the cyclin/cdk inhibitor $p21^{CIP1}$ is also observed. Interestingly, these activities were demonstrated for HPV 16 E6 and HPV 1 E6, indicating that both non-oncogenic and oncogenic E6s are involved in promoting G1 to S transit. Another study showed that in the transgenic mouse model high-risk HPV E6 causes upregulation of E2F responsive (e.g. S phase) genes such as Mcm7 and cyclin E (Shai et al., 2007). There is also evidence that high-risk HPV E6 can bind directly to Mcm7 (Kukimoto et al., 1998). Overall, these findings indicate that E6 plays a role in pushing cells into S phase and that this is not necessarily dependent on abrogation of the p53 pathway. Moving cells into S, particularly when DNA damage has occurred or when the cell is not ready to replicate its DNA would be expected to lead to chromosome instability. Indeed, high-risk HPV E6 and E7 can independently induce numerical and structural chromosome instability (Duensing and Munger, 2002) and E6 can cause polyploidy by a p53-independent mechanism (Heilman et al., 2009). The consequences of this instability could be an increased chance for development and progression of cancer.

E6 and interferon response

Most viruses have evolved mechanisms to avoid or repress the interferon response and papillomaviruses are no exception. Microarray studies indicate that expression of E6 causes downregulation of multiple IFN responsive genes including Stat-1 and 2′-5′ oligoadenylate synthetase and downregulation of IFN-α and -β (Nees et al., 2001). The E6 protein from both high- and low-risk HPV E6 binds to Tyk2 of the Jak–Stat pathway but only high-risk HPV E6 inhibits its function (Li et al., 1999). High-risk HPV E6 also binds interferon regulatory factor-3 (IRF-3) and inhibits its ability to activate transcription of IFN-β responsive genes (Ronco et al., 1998). Whether downregulation of the interferon response is an integral part of HPV infection and replication is not entirely clear. Both E6 and E7 and another viral protein, E1, are thought to play a role in suppressing the IFN response (Beglin et al., 2009) so the situation is complicated. The ability of E6 to mediate resistance to IFN induced growth arrest is dependent on inhibition of p53 acetylation by p300/CBP (Hebner et al., 2007). Since low-risk HPV E6s are capable of interacting with p300/CBP, it would seem likely that low-risk HPVs would be capable of suppressing the IFN response upon infection, but this remains to be determined.

E6 and telomerase

As mentioned above, exogenous expression of high-risk but not low-risk HPV E6s activates telomerase, the enzyme that adds telomere repeats to the ends of chromosomes (Klingelhutz et al., 1996). Telomerase activation has also been demonstrated in cells with replicating HPV genomes, indicating that this activation is not simply an artifact of overexpression of E6 through retroviruses (Sprague et al., 2002). The mechanism by which E6 activates telomerase is still not completely clear. Mutational analyses indicate that activation does not depend on p53 degradation or the PDZ binding motif (Kiyono et al., 1998; Klingelhutz et al., 1996). Expression of E6 causes transcriptional upregulation of TERT, the reverse transcriptase component of telomerase, and acetylation of histones at the TERT promoter is associated with this activation (Gewin and Galloway, 2001; Oh et al., 2001; Veldman et al., 2001). Initial studies indicated that E6 activation of telomerase relied on a proximal E-box in the TERT promoter. It was later demonstrated that E6 binds to c-myc and E6-AP in a complex and, although this does not lead to degradation of c-myc, the interaction might allow c-myc to be more active at the TERT promoter (Veldman et al., 2003). Other studies indicate that E6 can bind to and target for degradation a cellular protein called NFX1-91 that binds to mSin3a, a histone deacetylase that acts as a repressor of TERT transcription in the TERT promoter (Gewin et al., 2004; Xu et al., 2008). Splice variant NFX1-123 and cytoplasmic poly(A) binding proteins may also play a role in regulating TERT levels (Katzenellenbogen et al., 2009). While most mutational studies have implicated E6-AP binding as being necessary for telomerase activation by E6 (James et al., 2006a; Liu et al., 2005; Xu et al., 2008), a conflicting report indicates that binding of E6 to E6-AP is not involved in induction of telomerase (Sekaric et al., 2008). As discussed later in this review, expression of E7 can synergize with E6 to increase telomerase levels (Liu et al., 2008). A more recent study provides evidence that E6 can bind directly to the TERT protein, which could stabilize TERT and/or change its localization in the cell (Liu et al.,

2009). Mutational analysis indicates that the telomerase activation function of E6 from high-risk types is essential for immortalization of primary cells (Kiyono et al., 1998). Because E6s from low-risk HPV types do not activate telomerase, the role that E6's activation of TERT plays in the virus life cycle is unclear.

While TERT is involved in telomerase activation and telomere elongation, TERT also has telomere-independent functions (Bollmann, 2008), one of which is activation of the Wnt signaling pathway (Choi et al., 2008). It is interesting to note that E6 can activate the Wnt pathway, although the mechanism is unknown (Lichtig et al., 2010). Wnt signaling is involved in a variety of cellular processes and it is possible that activation of this pathway creates a cellular environment that is favorable for high-risk HPV replication.

Regulation of miRNAs by E6

The regulation of cellular miRNAs by viral proteins provides a potent means to affect a number of cellular processes. Not surprisingly, recent studies indicate that high-risk HPV E6 and E7 affect miRNAs that are known to be involved in growth regulation (Zheng and Wang, 2011). For example, high-risk HPV E6s downregulate miR-34a, an miRNA that targets numerous genes involved in cell cycle control (Wang et al., 2009b). High-risk HPV E6s also downregulate expression of miR-23b, which targets expression of the urokinase plasminogen activator gene, and subsequently may affect cell migration (Au Yeung et al., 2011). Much of high-risk HPV E6's ability to affect miRNA expression is apparently through its ability to target p53. A recent study, however, indicated that low-risk HPV-11 also modulates miRNA expression (Dreher et al., 2011). Thus, there is the possibility that both low and high-risk HPV E6 affect cell function, differentiation, and growth through modulation of miRNAs.

Summary of E6

The precise role of E6 in the HPV life cycle has been difficult to ascertain, partly because it is so multifunctional, but also because different HPV types have evolved into their own niches and have developed different strategies to efficiently replicate. Looking for common functions between E6s from different HPV types might provide some insight into what is necessary for replication but it should be kept in mind that each type has likely developed its own particular ways to overcome cellular defenses and create the right cellular environment for viral replication and maintenance. Even among the same HPV types there is considerable variation. For example, recent studies have demonstrated that a variant of HPV 16 with only minimal differences in E6 sequence from the prototype (Q14H/H78Y/L83V) is more efficient at immortalizing and transforming cells, even though p53 targeting and telomerase activation appear to be similar (Richard et al., 2010). This variant is found more frequently in cervical cancers, which may indicate that these amino acid alterations cause more efficient transformation, better replication, maintenance, or a combination of these factors. Certainly, improved model systems to study HPV replication as well as more rigorous comparisons between different HPV types and subtypes will be necessary to advance our understanding of the role of E6 in the HPV life cycle.

The HPV E7 proteins

E7 functions and posttranslational modifications recognized early

The E7 proteins range in size from 98 to 105 amino acids. The structure of two HPV E7s determined by NMR and by X-ray crystallography shows that the N-terminal half is disordered (extended) while the C-terminal half forms a well structured zinc binding site with a $\beta_1\beta2_\alpha1\beta_3\alpha_2$ topology (Liu et al., 2006; Ohlenschlager et al., 2006). The designation of a disordered structure in the N-terminus and an ordered structure in the C-terminus of E7 correlates with observations made on the disordered content of the E7 protein using a number of software programs (Uversky et al., 2006). Of note, compared to high-risk HPV E7s, the low-risk HPV E7s have a lower degree of disorder, perhaps indicative of a lower number of interactions with other proteins (see below) (Uversky et al., 2006). The N-terminus of

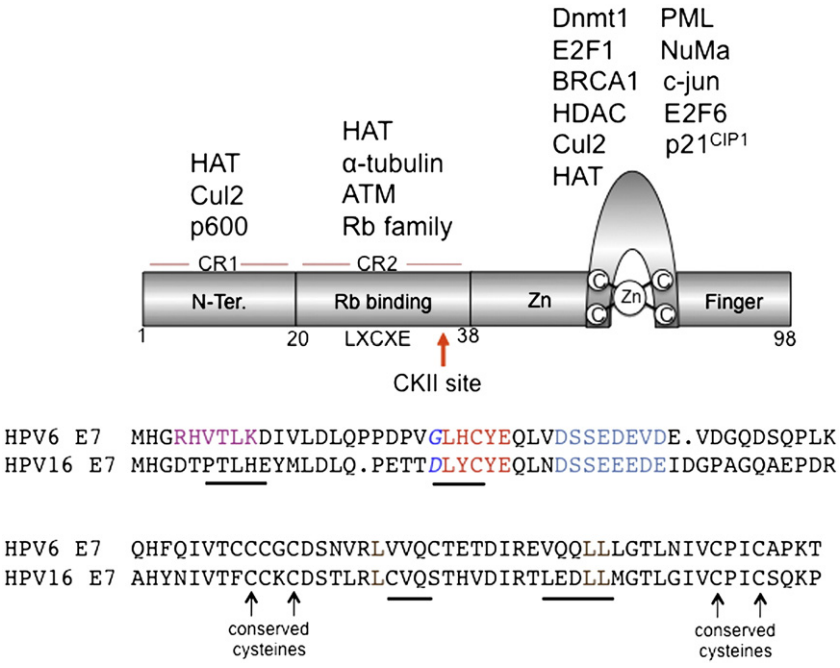

Fig. 2. Schematic of the HPV 16 E7 protein. The positions of CR1, CR2, and the zinc binding site, as well as the LXCXE motif and CKII site within CR2 are shown. The zinc binding site is conserved among the different HPV types. Protein binding regions are shown for HPV 16 E7. The text below the schematic displays the aligned sequences of high-risk HPV 6 E7 and HPV 16 E7. The PTLHE (6–10), DLYC (22–26) sequences referred to in the text are underlined; the amino acid conferring low or high Rb binding affinity is italicized; the PKC site in HPV 6 E7 is in purple; the LXCXE motif in red; and the CKII recognition sequence in blue. The proximity of the sequences required for abrogating $p21^{CIP1}$ activity (aa 68–70 and 79–83) and for binding HDAC is denoted by underlining and brown lettering, respectively.

Table 3
Binding partners of HPV E7.

Binding partner[a]	High-risk (HPV 16, 18, 31)	Low-risk (HPV 6 or 11)	References
Rb	+ (binding and degradation)	<[b](binding)	Dyson et al. (1989b); Munger et al. (1989); Firzlaff et al. (1991); Jones and Munger (1997); Berezutskaya et al. (1997); Helt and Galloway (2001)
p107	+ (binding and degradation)	< (binding)	Dyson et al. (1992); Helt and Galloway (2001); Gonzalez et al. (2001); Zhang et al. (2006)
p130	+ (binding and degradation)	< (binding and degradation)	Dyson et al. (1992); Helt and Galloway (2001); Zhang et al. (2006)
E2F1	+	<	Hwang et al. (2002)
E2F6	+	+	McLaughlin-Drubin et al. (2008)
Cyclin A	+		Dyson et al. (1992)
Casein kinase II	+	<	Barbosa et al. (1990); Firzlaff et al. (1989)
c-jun	+	?	Antinore et al. (1996)
MPP2	+	?	Luscher-Firzlaff et al. (1999)
SRC-1	+	+	Baldwin et al. (2006)
α-Tubulin	+	−	Nguyen et al. (2007)
NuMA	+	+	Nguyen and Munger (2009)
S4/26S	+	?	Berezutskaya and Bagchi (1997)
Cullin 2	+	−	Huh et al. (2007)
p600	+	+	Huh et al. (2005)
HDAC	+	?	Brehm et al. (1999)
HAT[c]	+	+	Bernat et al. (2003); Avvakumov et al. (2003); Huang and McCance (2002)
TBP	+	+	Massimi et al. (1996); Maldonado et al. (2002); Phillips and Vousden (1997)
PP2A	+	+	Pim et al. (2005)
PML	+	+	Bischof et al. (2005)
M2-PK	+	−	Zwerschke et al. (1999)
BRCA-1	+	?	Zhang et al. (2005b)
DNMT1	+	?	Burgers et al. (2007)

[a] Binding may be direct or indirect.
[b] < indicates that the E7 protein binds less well than the related HPV 16 E7 protein.
[c] Combined data for p300 and pCAF binding.

Table 4
Functions of HPV E7.

Function	High-risk HPV E7 (HPV 16, 18, or 31)	Low-risk HPV E7 (HPV 6 or 11)	References
Transformation of permanent rodent cells	+	−	Banks et al. (1990a); Barbosa et al. (1990); Edmonds and Vousden (1989); Watanabe et al. (1990); Watanabe et al. (1992)
Cooperation with ras	+	−	Banks et al. (1990a); Chesters et al. (1990); Firzlaff et al. (1991); Jewers et al. (1992); Phelps et al. (1992); Storey et al. (1990b)
Immortalization	+	−	Jewers et al. (1992); Melillo et al. (1994)
Transactivation of AdE2 promoter	+	+	Edmonds and Vousden (1989); Firzlaff et al. (1991); Phelps et al. (1992); Storey et al. (1990b); Watanabe et al. (1990)
Induction of DNA synthesis	+	+	Banks et al. (1990a); Morris et al. (1993); Watanabe et al. (1992)
Bypass of growth arrest	+	−	Demers et al. (1996); Helt and Galloway (2001)
Abrogation of $p21^{CIP1}$ inhibition	+	<	Funk et al. (1997); Helt et al. (2002); Jones et al. (1997a)
Abrogation of Rb-induced quiescence	+	?	Brehm et al. (1999); Helt and Galloway (2001)
Episomal maintenance	+	+	Flores et al. (2000); McLaughlin-Drubin et al. (2005);
	−		Oh et al. (2004); Thomas et al. (1999)
Amplification of viral genomes	+	?	Flores et al. (2000); McLaughlin-Drubin et al. (2005)
Genomic instability − centrosome duplications	+	−	Duensing et al. (2000); Duensing and Munger (2003)
Abrogation of senescence	+	+ (PML-induced senescence)	Bischof et al. (2005); DeFilippis et al. (2003); Psyrri et al. (2004); Wise-Draper et al. (2005)
Activation of the c-fos promoter	+	−	Morosov et al. (1994)
Activation of the p73 promoter	+	−	Brooks et al. (2002)
Activation of AKT	+	+	Menges et al. (2006); Pim et al. (2005)
Abrogation of BRCA1 repression of hTERT	+	?	Zhang et al. (2005b)
Induction of the telomerase promoter	+	?	Liu et al. (2008)
Induction of cell DNA synthesis in suprabasal cells	+	+	Cheng et al. (1995); Chien et al. (2000); Collins et al. (2005); Flores et al. (2000)
Delay of differentiation	+	+	Collins et al. (2005); Flores et al. (2000); Jones et al. (1997a); Zhang et al. (2006)
Downregulation of miR-203	+	?	Melar-New and Laimins (2010)
Upregulation of SIRT1	+	?	Allison et al. (2009)
Induction of EZH2	+	+	Holland et al. (2008)
Upregulation of Nucleophosmin	+	?	McCloskey et al. (2010)
Upregulation of KDM6A/B	+	?	McLaughlin-Drubin et al. (2011)
Prolonged G2	+	?	Banerjee et al. (2011)
Induction of mIR-16-1	+	?	Zheng and Wang (2011)
STAT-1 suppression	+	−	Hong et al. (2011); Thomas et al. (2001)

E7 shares homology with SV40 T antigen and adenovirus E1A, in two regions referred to as conserved region 1 (CR1) and conserved region 2 (CR2) (Phelps et al., 1992; Phelps et al., 1988) (Fig. 2). This conservation allowed the rapid recognition that high-risk HPV E7 binds the retinoblastoma tumor suppressor protein (Rb) (Fig. 2, Table 3) (Barbosa et al., 1990; Dyson et al., 1989). Further, adjacent to the Rb binding site is a casein kinase II recognition sequence, also shared by the three viruses (Barbosa et al., 1990; Firzlaff et al., 1989). In addition, E7 shares with adenovirus E1A the ability to transactivate the adenovirus E2 promoter (Phelps et al., 1988) and to induce cellular DNA synthesis (Sato et al., 1989) (Table 4). Two other post-translational modifications of E7 have been reported. An unknown kinase phosphorylates a site in the C terminus of high-risk HPV E7 during S phase (Massimi and Banks, 2000) and PKC, at least in vitro, can phosphorylate a site in CR1 of low-risk but not high-risk HPV E7 (Armstrong and Roman, 1995).

The intracellular localization of high-risk and low-risk HPV E7s and whether there are differences in location that might explain functional differences are not completely established. Using tagged HPV 16 E7 and immunofluorescence, the E7 protein was found mainly in the nucleus (Smith-McCune et al., 1999). By immunofluorescence, both untagged high-risk and low-risk HPV E7 proteins are mostly nuclear but only the low-risk HPV E7 co-localizes with PML in PML oncogenic domains (PODs) while the high-risk HPV E7 is distributed more diffusely (Guccione et al., 2002). In contrast, when analyzed by subcellular fractionation, high-risk HPV E7 is mostly cytoplasmic (Nguyen and Munger, 2009; Smotkin and Wettstein, 1987). Consistent with both nuclear and cytoplasmic localization, high-risk HPV E7 contains both nuclear localization and nuclear export signals (Knapp et al., 2009); HPV 6 E7 has a putative nuclear export signal (Barrow-Laing et al., 2010). Both high and low-risk HPV E7s can alter the intracellular location of cellular proteins, increasing their presence in the cytoplasm (Baldwin et al., 2006; Barrow-Laing et al., 2010; Westbrook et al., 2002).

With a few exceptions (see further binding partners in subsequent sections), the affinity of low-risk HPV E7 for a given target has often been found to be lower than that of the high-risk HPV E7. Both the affinity of binding of E7 to Rb and the efficiency of phosphorylation of E7 by casein kinase II are lower for low-risk HPVs than for high-risk HPVs (Barbosa et al., 1990; Gage et al., 1990; Munger et al., 1989b). Further, low-risk HPV E7 can stimulate DNA synthesis in rodent cells and in organotypic keratinocyte raft cultures and transactivate the Ad E2 promoter although perhaps to a lesser extent than high-risk HPV E7 (Cheng et al., 1995; Watanabe et al., 1992).

Information gained by the use of chimeras or amino acid swaps between high and low-risk HPV E7

In initial experiments designed to assign regions of E7 responsible for high-risk HPV E7 activities, chimeras were made of the N-terminal half of high-risk HPV 16 E7 and the C-terminal half of low-risk HPV 6 E7, and vice versa. The ability of these chimeric proteins to cooperate with *ras* to transform primary BRKs and to transactivate the AdE2 promoter was determined (Munger et al., 1991). The results suggested that the C-terminal halves of the proteins were comparable, that the N-terminal half contained the transforming activity, and that there was no significant difference in the ability of HPV 6 E7 and HPV 16 E7 or the chimeras to transactivate the AdE2 promoter (Heck et al., 1992; Munger et al., 1991; Takami et al., 1992). When the N-terminal half was subdivided into three parts, CR1, Rb binding site, and the CKII phosphorylation site, and all combinations of high-risk HPV E7 and low-risk HPV E7 were constructed, the Rb binding site fragment was the sole determinant of the affinity of E7 for Rb and of cooperation with *ras* to transform primary rodent cells. Amino acid swaps revealed that the amino acid N-terminal to the LXCXE Rb binding motif (aspartate in the case of HPV 16 compared to glycine in the case of HPV 6) dictated the affinity of E7 for Rb binding and also the efficiency of transformation of rodent cells (Heck et al., 1992; Sang and Barbosa, 1992). However, this single change did not result in gain of immortalization function by low-risk HPV E7 (Hu et al., 1995). These results are consistent with the interpretation that activities in CR1 are also necessary for immortalization. It is important to emphasize, however, that the N-terminal region swaps were all in the context of the HPV 16 E7 C-terminus. As will be described in the sections below, the C-terminal region is also pertinent to the oncogenic activity of high-risk HPV E7 and the activities of the C-termini of low-risk HPV E7 and high-risk HPV E7 are not equivalent.

Mutational analysis of E7 directed at dissecting its functions: transformation, Ad E2 transactivation, stimulation of DNA synthesis, and Rb binding

Extensive mutational analysis of the high-risk HPV E7s has allowed dissection of E7 activities. Experiments conducted with mutated E7 in rodent cells indicated that the induction of DNA synthesis and Rb binding can be distinguished from transactivation and from transformation in some cases but are overlapping in others (Banks et al., 1990b; Barbosa et al., 1990; Phelps et al., 1992; Rawls et al., 1990; Storey et al., 1990a; Watanabe et al., 1990). For example, the Rb binding motif in CR2 is required for stimulation of DNA synthesis, for transactivation and for the ability to cooperate with *ras* in transformation; amino acids 6–10 (PTLHE) in CR1 are not required for Rb binding or transactivation but are required for E7 to cooperate with *ras* in transformation; and another mutation in CR1 is proficient for inducing DNA synthesis but not for transformation. A high-risk HPV E7 mutated in the CKII site is fully competent to transactivate the AdE2 promoter (Firzlaff et al., 1991). However, the role of the CKII site in cooperation with activated *ras* to transform primary rodent cells is unclear: in one case, mutating the site results in loss of function; in another report, there is little effect (Firzlaff et al., 1991; Heck et al., 1992). In total, the results are consistent with the interpretation that stimulation of DNA synthesis and transactivation might be more relevant to E7 functions needed for completion of the virus life cycle while additional functions are required for the oncogenic activity of the high-risk HPV E7.

As noted earlier, the C-terminal half of E7 contains a zinc binding site, composed of two C-X-X-C motifs (Barbosa et al., 1989; McIntyre et al., 1993). Early experiments indicated that this region is important for protein stability (Watanabe et al., 1990). Thus, interpretation of mutations requires establishing either the stability of the protein or its ability to function in other assays. Originally the zinc binding site of high-risk HPV E7 appeared to be required for dimerization of E7 and for transformation of rodent cells as well as immortalization of keratinocytes (Jewers et al., 1992; McIntyre et al., 1993). However, recent mutational analysis has shown that dimerization is not required for E7 to cooperate with *ras* to transform rodent cells (Todorovic et al., 2011). The C-terminus in high-risk HPV E7 is also required to allow bypass of growth arrest (Helt et al., 2002), and to abrogate Rb-induced quiescence (Brehm et al., 1999; Helt et al., 2002).

E7 and the retinoblastoma tumor suppressor family, Rb, p107, p130 and other cell cycle regulators

Inducible expression of E7 in growth arrested cells indicates that E7 induces cellular DNA synthesis at the G1 to S transition (Banks et al., 1990a). The implication of E7's binding to the Rb protein became clear when it was shown that this releases the transcription factor E2F (later to become a family of transcription factors) (Chellappan et al., 1992). The ability to disrupt this complex requires sequences in the C-terminus and is greater for high-risk HPV E7 than for low-risk

HPV E7 (Helt and Galloway, 2001; Huang et al., 1993; Patrick et al., 1994; Wu et al., 1993). This ability, however, is not sufficient to maximally stimulate DNA synthesis (Morris et al., 1993). Further, in immortalization assays, addition of E2F1 is sufficient to complement an E7 mutant deficient in binding Rb, but is not sufficient to complement an E7 mutated in the N-terminus, indicating that E7-mediated immortalization requires more than release of E2F1 following binding and degradation of Rb (Melillo et al., 1994).

High-risk HPV E7 binds the other Rb family members, p107 and p130, and point mutations within the LXCXE motif show that high-risk HPV E7 is able to distinguish between Rb family members (Carlotti and Crawford, 1993; Ciccolini et al., 1994; Davies et al., 1993). Low-risk HPV E7 binds the Rb family members with lower affinity (Demers et al., 1996; Gage et al., 1990; Munger et al., 1989b). HPV 16 E7 targets all three Rb family members for degradation; HPV 6 E7 only targets p130 for degradation (Boyer et al., 1996; Demers et al., 1994a; Gonzalez et al., 2001; Helt and Galloway, 2001; Jones and Munger, 1997; Zhang et al., 2006). Degradation of Rb family members requires sequences in CR1 and CR3 in addition to the LXCXE motif (Giarre et al., 2001; Gonzalez et al., 2001; Helt and Galloway, 2001; Jones and Munger, 1997; Zhang et al., 2006). High-risk HPV 16 E7, but not HPV 18 E7, hijacks the cullin 2 E3 ubiquitin ligase complex to target Rb for degradation (Huh et al., 2007). The mechanism(s) of degradation of p130 by high-risk and low-risk HPV E7 appears to differ: higher levels of cytoplasmic p130 are seen in high-risk HPV E7 expressing cells while a shorter half life for p130 in the nucleus is seen when low-risk HPV E7 is expressed (Barrow-Laing et al., 2010). The ability of low-risk HPV E7 to target p130 for degradation is dependent upon CKII-phosphorylation of E7 (Genovese et al., 2008).

Differences in ability of high-risk and low-risk HPV E7 to transactivate E2F-responsive promoters may relate to which Rb family member(s) regulates that promoter. In particular, both high-risk HPV E7 and low-risk HPV E7 transactivate the AdE2 promoter to the same extent in a cervical carcinoma cell line which is HPV-negative and contains a mutated Rb (C33A cells), however, in primary human foreskin keratinocytes, expression of high-risk HPV E7 results in greater transactivation of the AdE2 promoter than low-risk HPV E7 (Armstrong and Roman, 1997). In these latter cells, both low and high-risk HPV E7 transactivate the B-myb promoter, regulated by p107/p130, equally (Armstrong and Roman, 1997). These data suggest that the ability of low-risk HPV E7 to disrupt the interaction of p107/p130 with E2F is equivalent to high-risk HPV E7, while low-risk HPV E7 is less efficient than high-risk HPV E7 in disrupting the interaction of Rb with E2F.

The outcome of disruption of Rb family-E2F complexes is the release of repression of E2F-responsive genes. An ever expanding number of such genes are recognized, including cyclin A and E, $p21^{CIP1}$, DNA polymerase α, PCNA, which serve to drive cells into S phase and induce cellular DNA synthesis as well as functioning to turn off the cycle. Interestingly, hTERT also contains an E2F site and high-risk HPV E7 can enhance expression of E6-mediated hTERT through this site (Liu et al., 2008). This activity is dependent upon an intact Rb binding site motif. The high-risk HPV E7s, but not low-risk E7, transactivate the p73 promoter in HFKs, an activity dependent upon the Rb binding site (see Table 3) and suggested to be dependent upon E2F1 (Brooks et al., 2002).

Further, the high-risk HPV E7s bind cyclin A and cdk2 (Dyson et al., 1992; Tommasino et al., 1993). Similar to binding to Rb family members, high-risk HPV E7s have a greater affinity for cyclin A than do low-risk HPV E7s (Ciccolini et al., 1994). High-risk but not low-risk HPV E7 binds the E2F-cyclin A complex; this binding requires the Rb binding site but not the CR1 PTLHE sequence (Arroyo et al., 1993). In a separate series of experiments, both low and high-risk HPV E7 were found to bind purified cdk2 and stimulate its kinase activity (He et al., 2003).

It is interesting to note that while high-risk HPV E7 can bypass growth arrest mediated by DNA damage or differentiation, low-risk HPV E7 cannot (Demers et al., 1996). Yet, as noted above, both high and low-risk HPV E7 can stimulate DNA synthesis although high-risk HPV E7 is more effective. This is consistent with later observations that bypass of growth arrest requires both degradation of Rb and abrogation of $p21^{CIP1}$, (Helt et al., 2002; Helt and Galloway, 2001). Abrogation of $p21^{CIP1}$ inhibition requires sequences in the C-terminus of E7: the zinc binding site mutants are proficient for degradation of all three Rb family members, yet unable to overcome growth arrest (Helt et al., 2002). Not only does low-risk HPV E7 not target Rb for degradation, it also is less efficient than high-risk HPV E7 in abrogating $p21^{CIP1}$ activity (Funk et al., 1997; Jones et al., 1997a).

HPV 1 E7: an informative anomaly

While it would be tempting to say that the affinity of E7 for Rb family members and cyclin A/cdk complexes dictates whether the protein can activate an E2F-regulated promoter and is oncogenic, HPV 1 E7 precludes reaching this conclusion. HPV 1 is a Mu-HPV that is associated with benign skin lesions. HPV 1 E7 binds Rb and cyclin A with affinity similar to the high-risk HPVs, yet this protein is neither a transactivator of the AdE2 promoter nor an oncoprotein (Ciccolini et al., 1994). Further, unlike either high-risk HPV E7 or low-risk HPV E7, HPV 1 E7 does not abrogate C/EBP-mediated growth arrest, an activity dependent upon the CKII site present in the alpha-HPV E7s but not HPV 1 E7 (Muller et al., 1999) (see next section).

E7 activates other promoters/cis elements and may participate in chromatin remodeling through Rb-dependent and Rb-independent activities

In rodent cells, high-risk HPV E7 but not low-risk HPV E7 transactivates the c-fos promoter and does so through the cyclic AMP responsive element (Morosov et al., 1994). High-risk HPV E7 activates ATF, Oct 1, MPP2 and AP-I (Antinore et al., 1996; Luscher-Firzlaff et al., 1999; Wong and Ziff, 1996). Expression of E7 also alters C/EBPα function. In rodent fibroblasts both high-risk and low-risk HPV E7 abrogate the growth inhibitory effects of C/EBP (Muller et al., 1999). This activity requires the CKII site but not the Rb binding site. Further, high-risk HPV E7 enhances the differentiation promoting activity of C/EBP. The activity of low-risk HPV E7 was not determined (Muller et al., 1999). High-risk HPV E7s bind E2F1 and transactivate E2F-responsive promoters more efficiently than low-risk HPV E7s (Hwang et al., 2002). Further, high-risk HPV E7 binds BRCA1 and blocks its ability to repress hTERT; this activity is dependent upon the C-terminus of E7 (Zhang et al., 2005b). Finally, both low and high-risk HPV E7s bind TBP and this binding is enhanced when E7 is phosphorylated by CKII (Massimi et al., 1996; Phillips and Vousden, 1997). Amino acids within the zinc binding site are required for binding of E7 to TBP (Massimi et al., 1997) but not for transactivation of the AdE2 promoter. Massimi et al. suggest that E7 may pull TBP away from a p53 complex, thereby inhibiting p53 transactivation activity (Massimi et al., 1997). However, a more recent report indicates that binding to TBP decreases its ability to interact with DNA (Maldonado et al., 2002). The potential implication of binding of E7 to TBP on viral replication will be discussed in the viral life cycle section below.

High-risk HPV E7 can also repress at least one promoter, the E-cadherin promoter, by binding to and activating DNA methyl transferase 1, Dnmt-1, (Laurson et al., 2010). This binding is Rb-independent, requires E7 C-terminal amino acids 84–96 (Burgers et al., 2007; Laurson et al., 2010). The E-cadherin promoter itself is not methylated suggesting that Dnmt-1 is affecting the expression of another gene whose product then represses E-cadherin. The identity of this other gene(s) is not yet established nor has the region of

E7 required for this activity been delineated; however, the activity seems to be independent of Rb degradation (Laurson et al., 2010).

High-risk HPV E7 interacts with both histone acetyltransferases (HAT) and histone deacetylases (HDAC), as well as coactivators through sequences in CR1, CR2 and the C-terminus (Avvakumov et al., 2003; Baldwin et al., 2006; Bernat et al., 2003; Brehm et al., 1999; Huang and McCance, 2002). High-risk HPV E7 upregulates expression of cdc25a and this activity requires both binding to Rb and binding to HDAC (Nguyen et al., 2002). Expression of HPV 16 E7 results in a global increase in the level of acetylation of histones and specific increase on E2F-regulated promoters. Increased expression from these promoters is lost when cells express high-risk HPV E7 mutated in either the Rb binding site or the HDAC binding site (located in the C-terminus (Zhang et al., 2004)). In contrast, increased transcription from HIF-1α-responsive promoters is Rb-binding independent but HDAC-binding dependent (Bodily et al., 2011b). The effect of low-risk HPV E7 on HATs and HDACs has not been reported. Low-risk HPV E7 also binds HIF-1α but whether it can displace HDAC was not established.

High-risk HPV E7 further induces chromatin remodeling by the induction of EZH2, a histone methyl transferase, and of two histone demethylases (KDM6A and KDM6B) (Holland et al., 2008; McLaughlin-Drubin et al., 2011). Transcriptional regulation of EZH2 is presumed to be mediated by Rb because an E7 mutant that fails to bind Rb also fails to induce EZH2. Induction of transcription of KDM6A and KDM6B, in contrast, is Rb-independent. Interestingly, this latter activity provides a means by which high-risk HPV E7 can increase expression of the cell cycle inhibitor, $p16^{INK4A}$. Whether these demethylases are also induced by low-risk HPV E7 proteins has not been reported but EZH2 is induced by low-risk HPV E7. This shared E7 activity is likely related to the virus life cycle since EZH2 plays a role both in inducing proliferation and in inhibiting apoptosis (see below).

Cross talk between the Rb and p53 pathways

Although high-risk HPV E6, but not E7, targets p53 for degradation (see E6 section), E7 does affect p53 expression and function. High-risk HPV E7 abrogates p53-mediated growth arrest; low-risk HPV E7 does not (Demers et al., 1994b; Hickman et al., 1994; Vousden et al., 1993). High-risk HPV E7 increases the steady state level of p53 (Demers et al., 1994b; Jones et al., 1997b) through an ARF-independent mechanism (Seavey et al., 1999). In some cell types and model systems this elevated p53 is transcriptionally active: MDM2 and $p21^{CIP1}$, for example, are induced (Jones and Munger, 1997; Morozov et al., 1997; Pei et al., 1998; Ruesch and Laimins, 1997; Seavey et al., 1999; Wang et al., 1996; Zerfass-Thome et al., 1996). In others, the elevated p53 is not transcriptionally active and E7 increases the steady state level of $p21^{CIP1}$ protein (Eichten et al., 2002; Jones et al., 1999). Notably, high-risk HPV E7 disrupts the interaction of mdm2 with p53, abrogates the growth inhibitory effect of $p21^{CIP1}$ and $p27^{KIP1}$ and targets Rb family members (the downstream targets of cyclin-dependent kinase inhibitors) for degradation, releasing E2Fs (Jones and Munger, 1997; Seavey et al., 1999; Wang et al., 1996; Zerfass-Thome et al., 1996). The need to inactivate both the p53 and Rb pathways has become clearer through a set of experiments that showed that both the ability of E7 to target Rb for degradation and to inhibit $p21^{CIP1}$ are required for bypass of growth arrest (Helt et al., 2002; Helt and Galloway, 2001). Yet, in several model systems, E7 cannot block p53-mediated apoptosis (Jones et al., 1997b; Stoppler et al., 1998; Wang et al., 1996). In contrast to high-risk HPV E7, low-risk HPV E7 is much less efficient at binding $p21^{CIP1}$ and abrogating its ability to inhibit cdk2 activity (Funk et al., 1997; Jones et al., 1997a). As noted earlier, the high-risk HPV E7s transactivate the p73 promoter in HFKs; a low-risk HPV E7 does not. This transactivation is dependent upon the Rb binding site and suggested to be dependent upon E2F1 (Brooks et al., 2002). An isoform of p73, p73Δ2, inhibits the ability of both p53 and p73 to induce apoptosis (Fillippovich et al., 2001). It is possible that whether E7 causes apoptosis or not reflects differences in the relative abundance of the p73 isoforms.

E7 and apoptosis, senescence, autophagy

It is generally accepted that all viruses have to inhibit apoptosis, a host response to infection, in order for the virus to have sufficient time to produce more virus. However, the response of the cell to the induction of unscheduled S phase entry necessary to this productive cycle, may also send host death response signals. While certain E7 functions send pro-apoptotic signals, these are countered not only by E6 functions but also by other E7 functions. As noted in a previous section, E7 can stabilize p53, resulting in some cases, in an apoptotic response. Further, as noted above, release of E2F may upregulate p73, another inducer of apoptosis. Both low and high-risk HPV E7s increase AKT activity that may inhibit the apoptosis induced when E7 maintains an S phase environment within the differentiated compartment (Menges et al., 2006; Pim et al., 2005). High-risk HPV E7 may also inhibit apoptosis by binding glutathione S-transferase P1 and inhibiting the phosphorylation of JNK; whether this is also true for low-risk HPV E7 was not examined (Mileo et al., 2009). Several point mutations within the high-risk HPV E7 zinc binding site result in loss of binding to GST P1 and loss of the ability of E7 to abrogate UV-induced apoptosis in HaCat cells (Mileo et al., 2009). Finally, high-risk HPV E7 increases the level of SIRT1, an NAD-dependent deacetylase, in HFKs. Knock-down of this deacetylase results in apoptosis of SiHa cells (Allison et al., 2009).

Expression of high-risk HPV E7 in HeLa cells, after silencing the endogenous high-risk HPV E6 and E7 genes, results in some cells proliferating, some undergoing senescence and some undergoing apoptosis (DeFilippis et al., 2003). Mutations in CR1 or CR2 of E7, which cause an inability to inactivate Rb, resulted in greater levels of senescence but neither proliferation above background nor apoptosis (Psyrri et al., 2004). One negative mediator of senescence that is upregulated in high-risk HPV E7 but not low-risk HPV E7-expressing keratinocytes is DEK (Wise-Draper et al., 2005). A positive mediator of senescence, promyelocytic leukemia protein IV (PML IV) can, at least in fibroblasts, be bound and its activity inhibited by both high-risk and low-risk HPV E7. This requires both Rb-dependent and Rb-independent activities since this activity is lost when high-risk HPV E7 is mutated in either the Rb binding site or in the C-terminal half of HPV 16 E7 (Bischof et al., 2005). E7 appears to disrupt the PML:p53:CBP complex that is responsible for increasing p53-mediated transcription (Bischof et al., 2005).

Most recently, high-risk HPV E7 has been shown, in the absence of E6, to induce autophagy in response to growth factor deprivation (Zhou and Munger, 2009). An activity in the C-terminus of high-risk HPV E7, not shared with the low-risk HPV E7, is the interaction with M2 pyruvate kinase, M2-PK. This interaction results in a less active form of the enzyme, reducing the cell's requirement for oxygen and increasing glycolysis (Zwerschke et al., 1999). Combining these two sets of data, the authors suggest that autophagy may be the cellular response to enhanced proliferation in the absence of appropriate nutrients or the E7-induced increased requirements for energy (Zhou and Munger, 2009; Zhou et al., 2009).

Genomic instability: aberrant centrosome duplication, mitotic abnormalities, and bypass of DNA damage checkpoints

In contrast to the shared ability to disrupt the G1/S phase checkpoint regulated by Rb family members, high-risk HPV E7 but not

low-risk HPV E7 can cause genomic instability (Duensing et al., 2000). Some bypass of checkpoints is due to Rb-dependent and other to Rb-independent activities of E7. Abnormal centrosome duplication is not dependent upon inactivation of Rb family members (Duensing and Munger, 2003). Rather centrosome amplification may, in part, be due to binding of E7 to gamma-tubulin and disruption of its interaction with centrosomes (Nguyen et al., 2007). Further, centriole multiplication correlates with E7-induced increased expression of Polo-like kinase 4 (Korzeniewski et al., 2011). High-risk HPV E7 upregulates several proteins which target claspin, a positive regulator of the mitotic checkpoint, for degradation, thereby allowing bypass of this checkpoint (Spardy et al., 2009). This E7 activity is presumed to be Rb-dependent because several cellular proteins involved in this degradation are encoded by genes regulated by E2F transcription factors (Spardy et al., 2009). High-risk HPV E7 can also induce polyploidy through abrogation of the mitotic spindle checkpoint and/or the post-mitotic checkpoint (Heilman et al., 2009; Thomas and Laimins, 1998). Further, activation of the ATM pathway by high-risk HPV E7 may contribute to genomic instability (Moody and Laimins, 2009).

Both low and high-risk HPV E7 is required for the life cycle of the virus

As noted above, HPVs infect the basal cells of the epithelium through microabrasions. These cells are the site of the non-productive stage of the viral life cycle where genomes are maintained at approximately 50 copies per cell. When the cells move off the basement membrane and into the suprabasal compartment, the virus enters the permissive stage of the viral life cycle. Since cells in this compartment have normally exited the cell cycle, and HPVs cannot replicate in such cells, the virus has to create an environment consistent with replication of its genome. Interestingly, in some HPV types, E7 is required for viral maintenance in the basal cell compartment; in others it is required for viral amplification in the differentiated compartment (Flores et al., 2000; Oh et al., 2004; Thomas et al., 1999). The necessity for E7 in the different compartments does not completely correlate with the risk level of the virus type: E7 is not required for maintenance of two high-risk genomes, HPV 16 and 18, in undifferentiated basal cells (Flores et al., 2000; McLaughlin-Drubin et al., 2005) but is required for the maintenance of another high-risk HPV, HPV 31, and low-risk HPV 11 (Oh et al., 2004; Thomas et al., 1999). Regardless, this links the E7 protein to the virus life cycle as well as to oncogenic transformation.

Mutational analysis identified amino acids required for episomal maintenance and further defined differences between high-risk HPV types. While HPV 16 E7 deleted in CR1 for PTLHE or deleted in the Rb binding site in the context of the HPV genome could be maintained episomally, HPV 31 mutated to encode a low affinity Rb binding site was greatly compromised with respect to episomal maintenance (Flores et al., 2000; Thomas et al., 1999). Differences in the requirement for functions in the C terminus of E7, required for HDAC binding, have also recently been reported (Bodily et al., 2011a). Further, when the HPV 31 genome carrying the Rb binding site mutant was introduced in the context the HPV 16 genome carrying a mutated E6 gene which could neither target p53 for degradation nor be maintained episomally, the double mutant was maintained (Park and Androphy, 2002). The authors comment that this indicates that the E6 and E7 genes have evolved to balance each other. Interestingly, this chimeric genome would, at least in two respects (inability to target p53 for degradation and having a low affinity Rb binding site) be similar to a low-risk genome.

As noted in an earlier section, E7 binding to TBP decreases its ability to interact with DNA (Maldonado et al., 2002). For both low and high-risk HPVs, competition for binding to the viral origin of replication and the early promoter might determine whether viral DNA synthesis or transcription occurs. The proteins of interest include the viral replication proteins E1 and E2, the cellular inhibitor of viral transcription and replication CCAAT displacement protein, and TBP (Ai et al., 1999; Maldonado et al., 2002; Narahari et al., 2006; O'Connor et al., 2000). Thus, it is possible that E7 contributes to maintenance of the viral genome not only by enhancing S phase entry but also by decreasing the amount of available TBP. Why this should be important for viral maintenance of some viral genomes but not others is unclear.

Insight into the mechanism by which the high-risk HPV E7 protein is required for genome amplification in the differentiated compartment has recently been provided. E7 binds to and activates the ATM DNA repair pathway, possibly resulting in activation of caspase 3/7 and cleavage of the viral replication protein E1 which enhances viral genome amplification (Moody et al., 2007; Moody and Laimins, 2009). Binding to ATM requires the LXCXE motif. If indeed ATM activation is linked to caspase activation, it is reasonable to expect that low-risk HPV E7 will also bind and activate ATM to enhance viral genome amplification (Moody et al., 2007). However, as noted earlier, this activation of ATM may contribute to high-risk-induced genomic instability. Finally, down-regulation of transcription of STAT-1 by high-risk HPV E7 is also required for viral amplification (Hong et al., 2011). Presumably this activity is not shared by low-risk HPV E7 since prior microarray data from this laboratory indicated that STAT-1 was, in fact, slightly elevated (Thomas et al., 2001). However, it is possible that STAT-1 is not as efficiently induced in low risk HPV infections.

The E7 protein is necessary and sufficient for reprogramming the differentiation compartment to allow virus replication. There are several ways to induce differentiation of keratinocytes: growth of confluent monolayers of HFKs in 2 mM calcium, suspension of HFKs in methylcellulose, and growth of HFKs in organotypic raft cultures. Early experiments showed that both low-risk and high-risk HPV E7 were sufficient to reprogram the differentiation compartment of the organotypic rafts to support DNA synthesis and delay differentiation (Cheng et al., 1995; Chien et al., 2000; Collins et al., 2005; Flores et al., 2000). More recent studies showed that the ability of high-risk HPV E7 to induce cell proliferation and delay differentiation was separable (Collins et al., 2005). The wild-type high-risk HPV genome and HPV deleted in the E7 CR1 PTLHE sequence could reprogram suprabasal HFKs to proliferate but could not delay differentiation. Both activities required the Rb binding site. Thus, while the induction of unscheduled DNA synthesis (i.e., DNA synthesis in the suprabasal compartment) required binding to the Rb family, delayed differentiation also required degradation of family members. Both low and high-risk HPV E7 can delay differentiation (Jones et al., 1997a; Zhang et al., 2006). It is important to emphasize that both low and high-risk HPV E7s can bind Rb, albeit with different affinities, but the only degradation function they share is to target p130 for degradation (Zhang et al., 2006). Thus degradation of p130 may be critical for completion of the virus life cycle and degradation of the other Rb family members may give high-risk HPV E7s their oncogenic potential. As will be described below, upon differentiation, high-risk HPV E7 decreases the level of miRNA203 that, in turn, increased the level of ΔNp63, thereby promoting cell proliferation. The regions of high-risk HPV E7 needed for this activity have not been defined, nor is it know whether low-risk HPV E7 also decreases miRNA203.

Uncoupling of proliferation and differentiation, a requirement for completion of the viral life cycle but also for immortalization

There are a variety of pathways whereby E7s ensure a proliferative state in the differentiated compartment of the epithelium. The ability to bypass growth arrest, induced by differentiation correlates with the ability to abrogate $p21^{CIP1}$ activity (Jones et al., 1997a). Additionally, E7 can abrogate the ability of C/EBP to block proliferation while not abrogating its ability to induce differentiation (Muller et al., 1999). As noted above, common to both low and high-risk HPV E7s is the ability to target the Rb family member p130 for degradation, suggesting that its degradation may be critical for the viral life cycle

(Genovese et al., 2008; Zhang et al., 2006). Consistent with this, recent data indicate that reformation of the p130/DREAM complex, in an HPV-positive cancer cell line, restored growth arrest (Nor Rashid et al., 2011). Further, both high and low-risk HPV E7 bind to and inactivate E2F6, an Rb-independent repressor of E2F-responsive promoters which might serve to maintain an S-phase-like environment (McLaughlin-Drubin et al., 2008). And both high and low-risk HPV E7 can prolong the G2 phase of cells in the differentiated compartment (Banerjee et al., 2011). Recently, a positive role for the CKII site in increased expression of S phase proteins in the differentiated compartment has been reported (Bodily et al., 2011a). A critical difference, in addition to the binding affinity for Rb family members, in the ability of low-risk versus high-risk E7 to induce unscheduled DNA synthesis has recently been mapped to conserved lysines present in the low-risk E7s only (Genovese et al., 2011). Further, both high-risk and low-risk HPV E7s bind nuclear mitotic apparatus protein 1 (NuMA) and delocalize dynein from mitotic spindles. The authors suggest that this might negatively affect the segregation of daughter cells into one basal and one suprabasal cell (Nguyen and Munger, 2009). Via AKT activation, high risk E7 upregulates nucleophosmin, a positive regulator of proliferation and negative regulator of differentiation (McCloskey et al., 2010). Although the authors previously showed that Rb was required for E7-induced AKT activity (Menges et al., 2006), knocking down Rb did not result in increased nucleophosmin. Thus, it is unclear whether the ability to upregulate nucleophosmin might be shared by low-risk HPV E7.

p600 binds both low-risk and high-risk HPV E7 (Huh et al., 2005). Silencing of p600 in a cervical carcinoma cell line expressing high-risk HPV E6 and E7 results in loss of function with respect to growth in soft agar, a marker for transformation (Huh et al., 2005). However, the fact that low-risk HPV E7 can also bind p600 would suggest that this interaction may also be relevant to the virus life cycle.

Regulation of miRNAs by E7

In contrast to E6, there is much more limited information about the regulation of miRNAs specifically by E7. Interestingly, high-risk HPV E7 has been reported to decrease expression of miR-203, a cellular microRNA that is normally increased in differentiating cells and downregulates a p53 family member p63 (in particular the ΔNp63 isoform) and cell proliferation (Melar-New and Laimins, 2010). This E7-mediated downregulation of miR203 appears necessary for robust amplification of viral genomes, at least in part, by creating a replication-competent cellular environment. In contrast, high-risk HPV E7 upregulates the tumor suppressive miR16-1 in rafts cultures (Zheng and Wang, 2011). The implications of this for the viral life cycle are unclear. One might expect these E7 activities to be shared with low risk E7; unfortunately, however, there are no comparable studies with low risk E7.

Summation of activities of E7 related to life cycle versus oncogenicity

Given that both high and low-risk HPV E7s have to create an environment in the suprabasal compartment conducive to completion of the virus life cycle and that this involves an uncoupling of cell proliferation from differentiation, the oncogenic activities of high-risk HPV E7 can be discerned after separating out the activities used by low-risk HPV E7 to complete the virus life cycle. It is not necessary that both use the same tactic, only that the end result is the same. Further, the issue of whether both high and low-risk HPV E7 proteins have the same affinity for a cellular protein may be misleading since the expression of these proteins may be regulated differently (certainly the case with respect to their transcription) and affinity can be overcome with quantity. Unfortunately, a number of activities identified in high-risk HPVs have yet to be tested for low-risk HPV E7. However, the compilation of current data suggests that the key E7 activities pertinent to the virus life cycle are: 1) binding of Rb, albeit with different affinities to produce an S-phase like environment; 2) degradation of p130 to delayed differentiation; 3) subtle disruption of p53 activity with respect to inhibiting acetylation of p53; and 4) transactivation of a subset of E2F-responsive genes. On the other hand, key activities pertinent to the oncogenic activity of E7, particularly given that E6/E7 immortalized HFKs are not tumorigenic, are 1) the ability of high-risk HPV E7 to promote genomic instability through a variety of mechanisms; 2) degradation of Rb and p107 which may promote certain aspects of genomic instability; 3) degradation of all three Rb family members which may result in induction of a wider range of E2F responsive genes; and (4) a greater number of mechanisms through which expression of other cellular genes is modified. A further take-home message would be that in addition to designating E7 activities as Rb-dependent or Rb-independent, it is important to make a distinction between Rb family members. Finally, since some of the mutations in the C-terminus are overlapping but have not been consistently tested for the full range of E7 functions attributed to this region, further work is needed to establish which E7 functions correlate with which E7 binding activities.

The beta-HPV E6 and E7 proteins

In contrast to the high-risk alpha-HPV genomes, in vivo analyses indicate that the beta-HPV genomes are rarely integrated in the cancer cells and the copy number per cell is usually significantly below 1 (Feltkamp et al., 2008; Forslund et al., 2007). However, tumors with higher-copy number of beta-HPV genomes per cell are often observed in patients who suffer from epidermodysplasia verruciformis (EV), a rare genetic disease that confers susceptibility to HPV infection (Orth, 2006). Further, studies with transgenic mice indicate that HPV 8, regarded along with HPV 5 as a high-risk HPV in EV patients, can cause non-melanoma skin cancer (Schaper et al., 2005). Because of the prevalence of multiple beta-PV types in normal skin and in non-melanoma skin cancers of non-EV patients, the distinction between high-risk and low-risk beta-HPVs in non-EV patients is not clear (Caldeira et al., 2003; Dong et al., 2005; Forslund et al., 2007). These confounders have led to the possibility that, in contrast to the alpha-PVs, the beta-PVs may play a "hit and run" role in cancer development. The activities of beta-HPV E6 and E7 proteins will be briefly reviewed here.

Cellular transformation studies on the beta-HPVs, implicated in non-melanoma skin cancer, have been much less extensive than those conducted on the alpha-HPVs. However, certain transforming functions of beta-HPV E6 and E7 have been demonstrated. HPV 38 E7 cooperates with HPV 38 E6 to increase the life span of human fibroblasts and keratinocytes (Caldeira et al., 2003). Further, HPV 38 E6 and E7 together activate NFκB and protect cells against TNFalpha and UV-mediated apoptosis (Hussain et al., 2011). In addition, HPV 38 E6 and E7 perturb the interferon pathway, with most of this effect being mediated through E6 (Cordano et al., 2008). HPV 38 E6 has been demonstrated to disrupt p53 signaling through its interaction with deltaNp73 (Accardi et al., 2006) and with the p300/CBP complex (Muench et al., 2010). HPV 38 E7, like high-risk alpha HPV E7, binds to and targets Rb for degradation, abrogates $p21^{CIP1}$-mediated growth arrest, transforms rodent cells and overcomes *ras*-induced senescence (Caldeira et al., 2003). In contrast, the E7 protein of HPV 20, like low-risk alpha HPV E7, binds Rb with lower affinity and neither targets it for degradation nor transforms rodent cells.

The E6 and E7 proteins of HPVs 5 and 8, which are found in squamous cell carcinomas of EV and immunosuppressed patients, and therefore might be designated high-risk based on epidemiology, also have transforming properties. For example, E6s from HPV 5 and 8 activate telomerase (Bedard et al., 2008), a function that is associated with high-risk alpha-PVs. HPV 5 and 8 E6s also degrade Bak and

protect keratinocytes from UVB irradiation (Underbrink et al., 2008). While HPV 5 and 8 E6s are not thought to bind with high affinity to p53, transcriptional regulation by p53 may be abrogated by other mechanisms. In this regard, HPV 8 E6 can interact with TIP60 which, as discussed for high-risk HPV E6, is involved in modifying p53 through acetylation (Jha et al., 2010). In addition, both HPV 5 and HPV 8 E6s interact with and promote the degradation of p300 (Howie et al., 2011). E7s from HPV 5 and 8 bind Rb with lower affinity than the high-risk alpha-E7 (e.g., HPV 16 E7), even though each contains what would, based on the alpha-E7s, be considered a high affinity Rb binding site (DLXCXE). Both HPV 5 and HPV 8 E7 cooperate with *ras* to transform primary rodent cells, albeit less efficiently than HPV 16 E7 (Yamashita et al., 1993). Further, HPV 8 E7 fails to morphologically transform rodent cells, is a poor transactivator of the AdE2 promoter, and fails to immortalize primary human keratinocytes (Schmitt et al., 1994). Yet HPV 8 E7 can alter the proliferation and differentiation program of primary adult epidermal cells grown in organotypic raft cultures and even invade the dermis of these cultures (Akgul et al., 2005). Most recently, HPV 38 E7 has been shown to have a more profound effect than HPV 5 and 8 E7 on the differentiation of organotypic raft cultures, including decreasing expression of the differentiation marker keratin 10 and increasing the percentage of cells replicating their DNA. Yet even for HPV 38 E7, these proliferating cells are not throughout the differentiated layers as they are with the high-risk alpha-HPV E7. In contrast, HPV 5 and 8 E7-expressing cells in the differentiated layers, like the alpha-HPV E7 expressing cells, express both cyclin E and $p16^{INK4a}$ (Westphal et al., 2009). HPV 20 E7 expression results in raft cultures that share with HPV 8 E7 histological appearance, keratin 10 expression and the location of cells replicating their DNA.

The alpha HPV E5 proteins

High-risk HPV E5s are considered minor oncogenes based on their absence in carcinomas in which the HPV genome is integrated (Schwarz et al., 1985; Yee et al., 1985). However, episomal copies of the HPV genome are sometimes seen in carcinomas and the transgenic mouse model has revealed the oncogenic potential of E5 in both the skin and cervix, the latter in conjunction with estrogen treatment (Genther Williams et al., 2005; Maufort et al., 2010; Maufort et al., 2007). Hence a brief summary of HPV E5 in vitro activity follows.

High-risk HPV E5 can induce anchorage independent growth of rodent cells and cooperate with E7 to stimulate proliferation and extend the life of primary rodent cells; these activities are enhanced in the presence of EGF (Bouvard et al., 1994; Leechanachai et al., 1992; Valle and Banks, 1995). Most biologically relevant, high-risk HPV E5 can stimulate primary human keratinocytes to proliferate and can enhance the efficiency of immortalization of keratinocytes by E6 and E7 (Stoppler et al., 1996; Straight et al., 1993).

High-risk HPV E5 binds the 16 kDa component of the vacuolar ATPase, decreases the acidity of endosomes, decreases trafficking through the endocytic pathway and increases ligand-dependent signaling through the EGF receptor (Conrad et al., 1993; Hwang et al., 1995; Straight et al., 1995; Thomsen et al., 2000). While this latter may be due to the decreased degradation of the EGFR in the endosomes or to decreased trafficking through the endosome pathway, it may also be due to E5-mediated disruption of the interaction of c-Cbl, an ubiquitin ligase, with the EGFR (Straight et al., 1995; Thomsen et al., 2000; Zhang et al., 2005a). Alternatively, the increased signaling may be due to E5-mediated upregulation of surface gangliosides (Suprynowicz et al., 2008). These results now appear to be clarified by the recent development of new reagents allowing a specific analysis of endogenous 16 kDa protein, the pH of early endosomes containing EGF, endocytic trafficking and endosome fusion. High-risk HPV E5 enhances EGF signaling by inhibiting the fusion of EGF-containing early endosomes with acidic vesicles (Suprynowicz et al., 2010).

As with the other high-risk alpha-HPV oncogenes, high-risk HPV E5 has recently been reported to alter cellular gene expression by regulating cellular miRNAs. Notably, like high-risk HPV 16 E7, high-risk HPV E5 down regulates miR-203 (Greco et al., 2011).

E5 activities pertinent to the life cycle of the virus can be gleaned from three types of experiments: 1) activities of low-risk HPV E5, 2) the effect of mutated high-risk HPV E5 in the context of the intact genome on the viral life cycle, and 3) the ability of both low- and high-risk HPV E5 to contribute to a morphological change typical of HPV-infected epithelium. Limited experiments with low-risk HPV E5 have shown that it too can induce anchorage independent growth of rodent cells, co-operate with high-risk HPV E7 to stimulate colony formation of primary rodent cell, and bind the 16 kDa component of the vacuolar ATPase (Chen and Mounts, 1990; Conrad et al., 1993; Valle and Banks, 1995). Life cycle experiments conducted with high-risk HPV 16 or HPV 31 genomes indicate that E5 does not play a role in viral maintenance in the non-productive stage of the viral life cycle (Fehrmann et al., 2003; Genther et al., 2003). In contrast, E5 does play a role in optimizing the differentiated environment for completion of the viral life cycle, although knocking out E5 in the context of the HPV 31 genome has more profound effects than when the experiment is conducted in the context of the HPV 16 genome (Fehrmann et al., 2003; Genther et al., 2003). For both high-risk HPV E5s, binding of E5 to B cell receptor-associated protein 31 (Bap31) correlates with the ability of an E5-proficient HPV genome to amplify its viral genomes (Regan and Laimins, 2008). Finally, when expressed in primary cervical or foreskin epithelial cells, both low- and high-risk HPV E5, in conjunction with either low- or high-risk HPV E6, induce the formation of koilocytes, a cellular manifestation in the differentiated epithelium characteristic of HPV infection (Krawczyk et al., 2008).

In sum, both in vitro and in vivo data indicate that E5 has oncogenic activity. However, the paucity of data with low-risk HPV E5 and the relatively early stage of understanding of the role of HPV E5 in the viral life cycle preclude definitive identification of the HPV E5 oncoprotein-specific activities. Further, it is interesting to note that the beta-HPVs do not encode an E5 protein.

Final comments

This review has taken the position that a comparison of activities of low and high-risk HPVs will allow the identification of common activities, presumably needed for the viral life cycle, and additional activities of high-risk HPVs, presumably essential for transformation/immortalization. However, it should be acknowledged that another review has suggested that there may be a fundamental difference in the cells infected within the basal layer: with high-risk HPVs infecting stem-like cells where the genome persists and eventually leads to malignancy and low-risk HPVs infecting transient amplifying cells giving rise to virus factories in hyperplastic cells. This hypothesis suggests that the replication cycles of the two groups of viruses are quite different and therefore one can't use low-risk HPV activities to sort out life cycle events from transformation/immortalization events (Munger et al., 2004). Later experiments, however, indicate that both low and high-risk HPV E7s may have the ability to alter the segregation of daughter basal cells, resulting in delayed differentiation and maintenance of viral genomes (Nguyen and Munger, 2009). This shared activity plus others discussed in this review suggest that there is a significant overlap in the pathways altered by low and high-risk HPV E7 validating the comparative approach. Finally, carcinomas arise in the transformation zone of the cervix where the columnar and squamous epithelium meet. It has been postulated that the intracellular environment of the basal cells in this region, or the reserve cells which give rise to these basal cells, may somehow limit completion of the productive life cycle (Doorbar, 2006). Why this would be more true for high-risk than low-risk HPVs is not clear.

However, if the productive life cycle cannot be completed, transformation might be favored, consistent with the polyomavirus literature cited in the Introduction. Finally, one should also bear in mind that most experiments are conducted using the reductionist approach where, for example, the activities of E6 and E7 alone or together are studied. In a primary infection, these genes would be expressed in the context of the intact genome where other PV replication proteins, for example E1 and E2, might tip the balance with respect to the outcome of infection.

The limited activities of the low-risk PVs, e.g., targeting neither Rb nor p53 for degradation, may have allowed these PVs to send fewer distress signals and thereby to better coexist with their host cell. In contrast, the high-risk viruses, by targeting all Rb family members for degradation have raised more distress signals, necessitating more activities, e.g., degradation of p53, to counter the normal host cell response. The more limited interaction with p53, blocking its acetylation by p300, and with the Rb family members, targeting only p130 for degradation and perhaps activating only a subset of E2F-responsive promoters, for example, appear to be sufficient for the HPVs to complete their life cycle.

An analysis of the activities of oncoproteins encoded by different PVs indicates that they have evolved such that the relative oncogenicity of E5, E6 and E7 varies depending upon the PV type. This may reflect the balance of activities of proteins within a particular PV type as well as the particular squamous epithelium they infect. Further, an analysis of PV functions indicates that when the expression or function of a given cellular protein must be altered, PVs use overlapping or redundant approaches to succeed in this endeavor. As examples, both E6 and E7 alter hTERT expression; and high-risk HPV E6 alters p53 function both by targeting it for degradation and by inhibiting its activation.

Finally, while the number of cellular binding partners of high-risk PV oncoproteins is ever increasing, determining whether these binding partners also bind low-risk PV proteins continues to be very limited. Expanding this comparison will enhance the interpretation of the importance of these different interactions to cellular transformation versus viral life cycle. Further, given that a particular region of a PV protein is required for binding to multiple cellular proteins (Fig. 1 and Fig. 2), it is imperative that not only the ability of low-risk PV proteins to bind be determined but also that the significance of any particular cellular protein to PV function be established by silencing that cellular protein.

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