Review Article

Basic mechanisms of high-risk human papillomavirus-induced carcinogenesis: Roles of E6 and E7 proteins

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Human papillomaviruses (HPV) are believed to be the primary causal agents for development of pre-neoplastic and malignant lesions of the uterine cervix, and high-risk types such as type 16 and 18 are associated with more than 90% of all cervical carcinomas. The *E6* and *E7* genes of HPV are thought to play causative roles, since E6 promotes the degradation of p53 through its interaction with E6AP, an E3 ubiquitin ligase, whereas E7 binds to the retinoblastoma protein (pRb) and disrupts its complex formation with E2F transcription factors. Although prophylactic vaccines have become available, it is still necessary to clarify the mechanisms of HPV-induced carcinogenesis because of the widespread nature of HPV infection. Approximately 493 000 new cases of cervical cancer are diagnosed each year with approximately 274 000 mortalities due to invasive cervical cancer. In the present article, the mechanisms of HPV16 E6- and E7-induced multistep carcinogenesis and recently identified functions of these onco-proteins are reviewed. (*Cancer Sci* 2007; 98: 1505–1511)

HPV and cancer

The genome of human papillomavirus (HPV) is a circular double-stranded DNA molecule of approximately 8000 base pairs (Fig. 1). More than 100 HPV genotypes have been described so far and approximately 40 of them infect the genital mucosa. These HPV are classified into low- or high-risk types according to their presence in malignant lesions of the cervix, and high-risk types (16, 18, 31, 33, 45, 51, 52, 58, etc.) are associated with more than 90% of cervical cancers. Of these, HPV16 accounts for approximately half of all cervical cancers while HPV18 is involved in another 10–20%,[1]. HPV infection has also been suggested to cause the majority of anal cancers as well as a subset of vulvar, vaginal and penile cancers.[2] An association between the presence of HPV and the development of head and neck cancer has also been recently established.[3]

The HPV viral oncogenes, E6 and E7, have been shown to be the main contributors to the development of HPV-induced cervical cancer and increased expression, probably due to integration of the viral DNA in the host cell genome, has been detected in invasive cancers and a subset of high-grade lesions.[4] Inactivation of tumor suppressor p53 and/or retinoblastoma protein (pRb) is a common event for the carcinogenesis of human cells. Both E6 and E7 HPV oncogenes interact with and inhibit the activities of these tumor suppressors. Furthermore multifunctional properties of the two proteins have been revealed (see below).

The HPV life cycle

The HPV life cycle is tightly linked to their host cell biology (Fig. 2). Normal squamous epithelial cells grow as stratified epithelium, with those in the basal layers dividing as stem cells or transit amplifying cells. After division, one of the daughter cells migrates upward and begins to undergo terminal differentiation while the other remains in the basal layer as a slow-cycling, self-renewing population.[5] HPV virions initially infect the basal layers of the epithelium, probably through microwounds and enter cells via interaction with certain receptors such as α-6 integrin for HPV16.[6] In infected cells at the basal layer, low levels of viral DNA are synthesized to an episomal copy number of approximately 50–100 genomes per cell. The early HPV genes *E1* and *E2* support viral DNA replication and its segregation so that the infected stem cells can be maintained in the lesion for a long period. As infected daughter cells migrate to the upper layers of the epithelium, viral late gene products are produced to initiate the vegetative phase of the HPV life cycle, resulting in high-level amplification of the viral genome. As the viral DNA replication almost totally depends on host replication factors except for viral helicase E1, other early genes *E5*, *E6* and *E7* are considered to coordinate a host cell

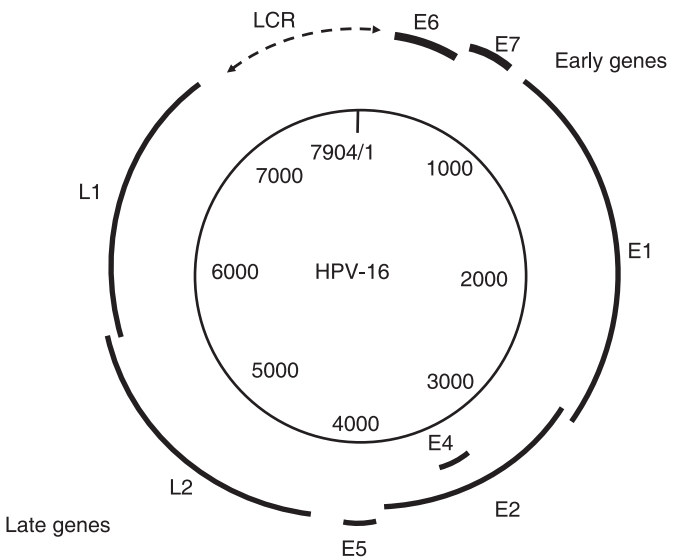

Fig. 1. The genome of human papillomavirus (HPV) is a circular double-stranded DNA molecule of approximately 8000 base pairs. The genetic map of HPV16 is illustrated. Open reading frames (ORF) are indicated by bold type. The six early ORF (E1, E2, E4, E5, E6 and E7 are expressed at different stages during epithelial differentiation. L1 and L2 ORF, grouped in late region, are expressed in cells replicating viral DNA in the upper epithelial cells.

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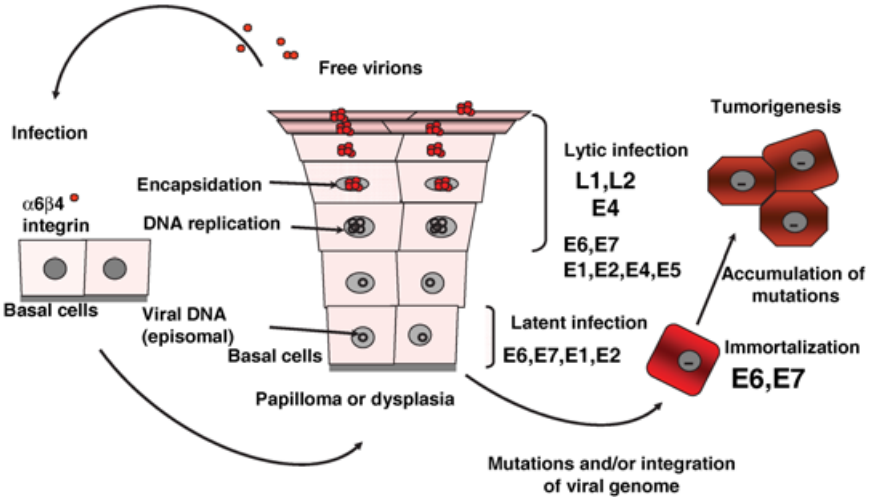

Fig. 2. Human papillomavirus (HPV) use a unique strategy for propagation, limited to stratified flattened epithelial tissue of mucosa and skin. Initially, HPV must infect stem cells or basal cells of the tissue where a phase of latent infection is established in which viral DNA replicate without making virions. In the upper layer, as cells differentiate, vegetative replication of viral DNA coordinates with expression of capsid proteins to make virions that are then freed to search for new host cells. Expression levels of E6 and E7 in basal cells are considered to be quite low. However, as such infections can continue for years and even for decades, cells may acquire high-level expression of E6 and E7 through mutations and integration of the viral genome. Such cells could become immortal and tumorigenic with further genetic and epigenetic events.

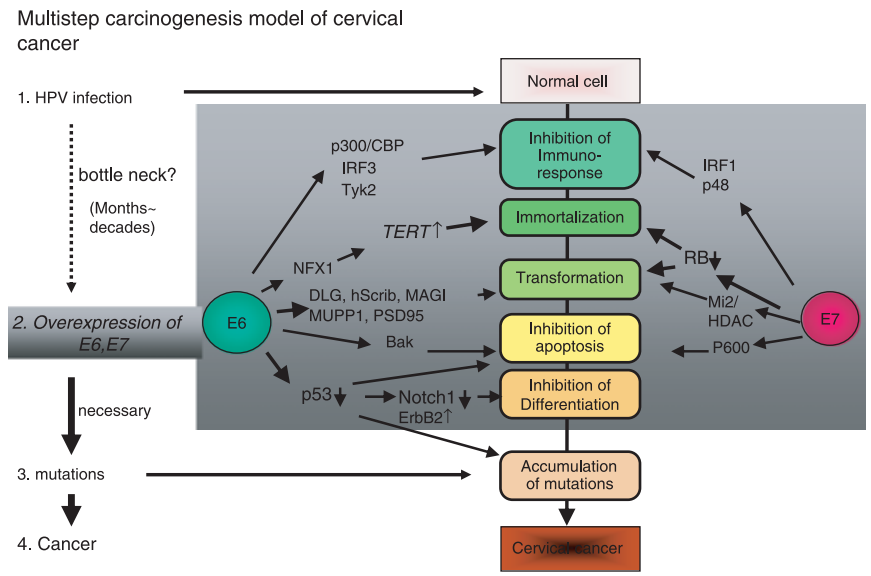

Fig. 3. E6 and E7 cooperatively function in the development of cervical cancer. Multistep carcinogenesis for human papillomavirus (HPV)-induced cervical cancer. The authors would like to emphasize that the bottleneck step to cancer is the overexpression of E6 and E7, which is usually achieved by accidental integration of a viral genome into a host chromosome. Once *E6* and *E7* genes are overexpressed, subsequent events (in the dark box) might be expected to occur within a short period of time because E6 and E7 can cooperatively induce chromosomal instability.

environment suitable for viral DNA replication, which sometimes induces host cellular DNA synthesis and prevents apoptosis. In the outer layers of the epithelium, viral DNA is packaged into capsids and progeny virions are released to re-initiate infection. Because the highly immunogenic virions are synthesized at the upper layers of stratified squamous epithelia they undergo only relatively limited surveillance by cells of the immune system. In addition, E6 and E7 inactivate interferon (IFN) regulatory factor (IRF),[7,8] so that HPV viruses can remain as persistent, asymptomatic infections.

HPV infection and HPV-induced transformation

Cervical cancers originate from the lining of the cervix, the lower part of the uterus. The squamocolumnar junction, where the stratified non-keratinizing squamous epithelium from the exocervix and the columnar epithelium from the endocervix meet, is the most important cytologic and colposcopic landmark, as this is highly susceptible to HPV infection and is the site where more than 90% of lower genital tract neoplasia arises. Infection with high-risk HPV is associated with cervical dysplasia or cervical intraepithelial neoplasia (CIN), and cervical cancers are thought to arise from these lesions after long persistent infection.[9,10] CIN I (mild dysplasia) and CIN II (moderate dysplasia) lesions show relatively low levels of E6 and E7 expression in which the viral genomes replicate episomally, whereas CIN III (severe dysplasia, carcinoma *in situ*) and invasive cancer lesions often display high-level expression of E6 and E7, in most cases with the integration of viral DNA into the host cell genome whereby neoplastic development is believed to be initiated.[11]

Although HPV infections are common and the life-time risk of infection is approximately 80% for productive women, in most cases they are resolved spontaneously by an effective immune response. The ultimate development of cervical cancer is rarely accompanied by high expression of E6 and E7 proteins. Thus the authors speculate that the integration of the viral genome into the host cell is a very rare event, but after it has happened carcinogenic transformation progresses rapidly (Fig. 3). However, epidemiological studies and experimental data indicate that the viral presence is not enough to induce cervical cancer and additional genetic and epigenetic events are

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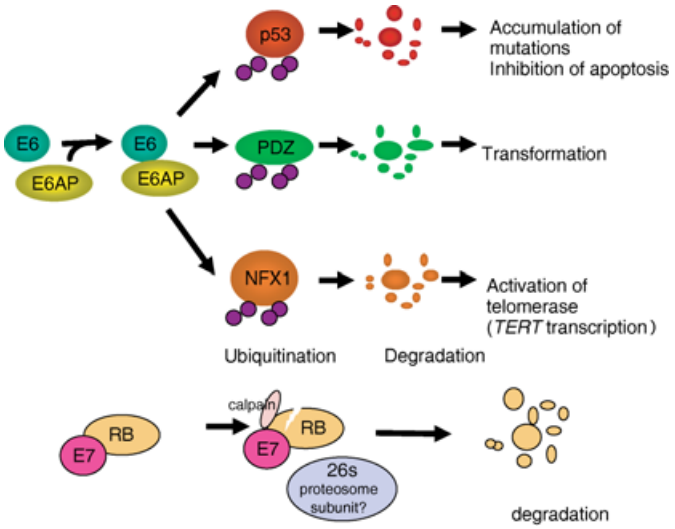

Fig. 4. E6 and E7 proteins of the high risk human papillomaviruses (HPV) can inactivate tumor suppressors p53 and retinoblastoma protein (pRb), respectively. Although E6 protein itself cannot bind to p53, it can bind to a cellular ubiquitin ligase named E6AP, and make ternary complexes with p53 so that it becomes ubiquitinated. E6 protein also has functions independent of p53 inactivation. It is likely that ubiquitin ligase E6AP is a key player not only in the degradation of p53 but also in the activation of telomerase and cell transformation by E6. Most recently, it has been found that E7 promotes C-terminal cleavage of pRb by the calcium-activated cysteine protease calpain and that this cleavage is required before E7 can promote the proteasomal degradation of pRb (A. Suhrbier, personal communication, 2007). Most biological functions of E7 are achieved by inactivating pRb family proteins.

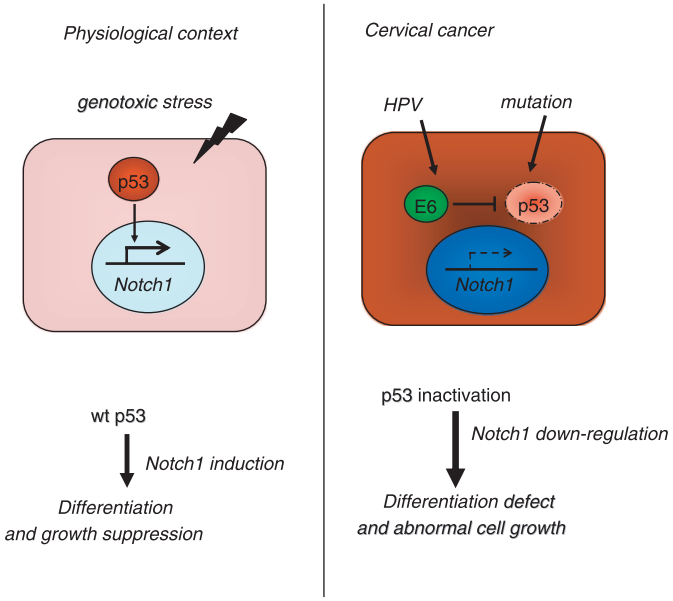

Fig. 5. The *Notch1* gene is a novel target of p53 at least in normal keratinocytes. Upon DNA damage, p53 transactivates the *Notch1* gene so as to induce differentiation. This could be a barrier function against genotoxic stress. In cervical cancer, E6 can down-regulate expression of Notch1 through inactivation of p53. As Notch1 could function as a tumor suppressor not only in skin but also in cervical keratinocytes, its down-regulation by E6 might contribute to the development of cervical cancer. In other squamous cell carcinomas, mutation of p53 may have the same consequence. Modified from the authors' latest publication.[25]

presumably required to alter the cellular factors.[9] Amplifications of *PIK3CA*,[12] *c-myc*, *ErbB2*,[13] and *cIAP1*,[14] mutation of *ras*[13] and decreased expression of PTEN,[15] and TSLC1,[16] have been reported in cervical cancers.

The functions of HPV onco-proteins E6 and E7

Both E6 and E7 proteins are essential to induce and maintain cellular transformation, due to their interference with cell-cycle control and apoptosis. Genomic instability is thought to be an essential part of the cellular transformation and it has been shown that E6 and E7 together cause polyploidy soon after they are introduced into cells. This appears to result from deregulation of Plk1 by the loss of p53 through E6, and pRb family members by E7, overcoming the safeguard arrest response.[17] Acute loss of pRb family members by E7 has also shown to induce centrosome amplification and aneuploidy,[18] In addition, E6 and E7 cause deregulation of cellular genes controlling the G2/M phase transition and progression through mitosis, such as the genes controlling centrosome homeostasis.[19,20]

The function of the E6 onco-protein

Inactivation and degradation of p53 through the E6/E6AP complex. The most manifest function of the E6 protein is to promote the degradation of p53 through its interaction with a cellular protein, E6 associated protein (E6AP), an E3 ubiquitin ligase[9] (Fig. 4). The affinity of E6AP for p53 is likely to be modified by the association with E6. The *p53* tumor suppressor gene itself regulates growth arrest and apoptosis after DNA damage. When DNA damage is moderate, a prolonged p53-dependent arrest and DNA repair are induced, but when the damage is severe, apoptosis is provoked. Although aberrant inactivation of pRb family members would also normally induce apoptosis through p53, HPV-infected cells avoid such cell death by E6 inactivation of p53. In addition, E6 interferes with other pro-apoptotic proteins, Bak, FADD and procaspase 8,[21,22] (Table 1) to comprehensively prevent apoptosis. Alternatively, the susceptibility of E6-induced degradation of p53 has been suggested to link the polymorphisms in codon 72 of *p53*.[23]

Newly identified target of p53: the *Notch1* gene. Recently, the product of the *Notch1* gene has been identified as a novel target of p53 (Fig. 5).[24,25] *Notch1* has been shown to function as an oncogene in the development of human T-cell leukemia,[26] but also acts as a determinant of keratinocyte differentiation,[27] and a tumor suppressor in the mammalian epidermis.[28] Although Notch1 expression has been found in neoplastic cervical lesions, particularly in well-differentiated squamous cell carcinomas,[29] it disappears in the late stages or poorly differentiated cervical cancer.[30] Induction of Notch1 through p53 occurs in response to genotoxic stress. Therefore, its down regulation through p53 with E6/E6AP has been revealed as a novel tumor suppressor mechanism blocking development of HPV-induced cervical carcinogenesis.[25]

The ErbB2 protein expression level is also regulated by p53 degradation and interference with this by E6/E6AP complexes contributes to cervical carcinogenesis.[31]

E6-mediated hTERT induction. Over the past dozen years or so, an increasing number of other proteins have also been revealed to be target proteins of E6 that might contribute to cellular transformation (Table 1), with telomerase as one probable important example. Human telomerase is a ribonucleoprotein complex composed of at least the reverse catalytic transcriptase (hTERT) and an RNA component (hTR). hTERT is expressed only in specific germ-line cells, proliferative stem cells of renewal tissues, and cancer cells. The expression of hTERT in

Table 1. Target proteins of E6 and E7 and their functional relevance

Target molecules of E6	Implicated/observed biological effect
E6AP/p53	Degradation of p53/suppression of apoptosis[9]
PDZ-domain-containing proteins	Degradation of PDZ proteins/loss of cell polarity[38–51]
CAL	Deregulation of the vesicular trafficking processes[71]
NFX1-91	Degradation of NFX1-91/activation of hTERT, immortalization[36]
Paxillin	Interference in the association of paxillin and focal adhesion kinase[72]
IRF3	Inhibition of IRF-3's transcriptional activity thereby inhibiting the IFN-induced signaling[7]
Bak	Degradation of Bak/suppression of apoptosis[21]
FADD	Degradation of FADD/suppression of apoptosis[22]
Procaspase 8	Degradation of procaspase 8/suppression of apoptosis[22]
GADD34/PP1	Suppression of apoptosis[73]
Tyk2	Impairment of Tyk2 activation thereby inhibiting IFN-induced signaling[74]
CBP/p300	Down-regulation of p53 activity by targeting the transcriptional coactivator[75]
MCM7	Induction of chromosomal abnormalities[76]
TSC2 (tubulin)	Activation of mTOR signaling[53]
BRCA1	Release the inhibition of ER signaling[77]
Target molecules of E7	**Implicated/observed biological effect**
pRb family proteins	Disruption of pRb–E2F complexes thereby initiating the E2F mediated transcription[9]
Cyclin A	Regulation of cell cycle (binding through pRb)[60]
Cyclin E	Regulation of cell cycle (binding through p107)[61]
p27	Binding to and subsequent inactivation of the CDK inhibitor p27[62]
p21	Binding to and subsequent inactivation of the CDK inhibitor p21[63]
AP1	Interaction with and transactivation of the AP1 family of transcription factors[78]
TBP	Deregulation of the TBP mediated transcription[79]
S4 subunit of the 26 S proteasome	Targeting of pRb for degradation[80]
MPP2	Activation of MPP2-specific transcriptional activity[81]
hTid 1	Genome replication[82]
p48	Down-regulation of IFN α-mediated signal transduction[64]
M2 pyruvate kinase	Modulation of type M2 pyruvate kinase activity[83]
p600	Contribution to anchorage-independent growth and transformation[65,66]
Mi2	Form complex with HDAC to promote the E2F2-mediated transcription[59]
IRF1	Abrogation of transactivation function of IRF1[8]

CDK, cyclin-dependent kinase; ER, estrogen receptor; HDAC, histone deacetylases; IFN, interferon; IRF, interferon regulatory factor; pRb, retinoblastoma.

normal cells reconstitutes telomerase activity and suppresses senescence. Because telomerase activity is hardly detected in most somatic tissues, telomeres shorten with each cell division, eventually leading to senescence (aging), due to incomplete lagging DNA strand synthesis and end-processing events. High telomerase activity is observed in more than 85% of human cancer cells, strongly indicating a key role in tumorigenesis.[32] E6 induces telomerase activity, thereby contributing to the immortalization of epithelial cells by maintaining telomere length.[33] E6 and Myc interaction has been shown to activate the telomerase reverse transcriptase promoter,[34] and in the presence of E6, a repressor complex of TERT promoter, containing USF1 and USF2, is replaced by Myc, which corresponds to higher levels of *TERT* transcription and consequently, telomerase activity.[35] NFX1-91 is a recently identified novel cellular repressor of the *hTERT* promoter that is degraded in a E6/E6AP dependent manner so that myc binding to the *hTERT* promoter can occur and result in increased hTERT expression[36] (Fig. 4). In contrast, NFX1-123, a splice variant of NFX1, together with cytoplasmic poly(A) binding proteins, is suggested to be critical to hTERT activity in HPV16 E6-expressing epithelial cells.[37]

Targeting of PDZ-containing proteins by E6. High-risk HPV E6 has been shown to interact with PDZ-domain-containing proteins through its *C*-terminal motif,[38] leading to their degradation (Fig. 4). This ability of E6, which is distinct from that to binding and degrading p53, is important for cell transformation because PDZ-domain-containing proteins are involved in a variety of cellular functions such as cell signaling and cell adhesion. E6 binding to these proteins appears to be particularly important for transformation and tumorigenesis in cultured cells,[39] and hyperplasia and carcinogenesis in *E6*-transgenic mice.[40] Recently, several PDZ-domain-containing proteins have been identified to be targets of E6 proteins, including mammalian homologs of DLG (DLG1/hDLG) and Scribble (Scrib/Vartul), MUPP1, MAGI-1, -2, and -3, GIPC, PATJ, PTPN3 and PSD95.[38,41–48] E6-induced degradation of these proteins potentially causes loss of cell–cell contacts mediated by tight junctions and thus contributes to the loss of cell polarity seen in HPV-associated cervical cancers.[45] The tumor suppressor properties of some of these proteins against the development of HPV-associated cancers have been reported[49–51] (Fig. 3). Meanwhile, interactions between β1-adrenergic receptor and Class I PDZ-domain-containing proteins have been uncovered, indicating the regulation of the receptor signaling and trafficking by PDZ proteins.[52] Thus E6 might affect the physiological status of G protein-coupled receptors such as β1-adrenergic receptor through the regulation of PDZ-containing-proteins.

Others. The tumor suppressor gene *TSC2* product, Tuberin, has been proposed as a possible target of E6, implying a contribution to E6-induced oncogenesis.[53] Because TSC complexes play an important regulatory role in the survival signaling involving mTOR, further exploration of this possibility is needed. More recently, induction by E6 of E2F-responsive genes, MCM7 and cyclin E, has been reported, implying the existence of dysregulation of the p16/pRb pathway with mechanisms distinct from those involving E7.[54]

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The function of the E7 onco-protein

Inactivation of pRb. E7 is a small nuclear phosphoprotein separated into three conserved regions denoted in an analogous fashion to adenovirus E1A as CR1, CR2 and CR3.[55] E7 is known to bind to the retinoblastoma tumor suppressor gene product, pRb, and its family members, p107 and p130, via a LXCXE (where X represents any amino acid) binding motif conserved in its CR2 region. In the hypophosphorylated state, pRb family proteins can bind to transcription factors such as E2F family members and repress the transcription of particular genes involved in DNA synthesis and cell-cycle progression.[56] Phosphorylation of pRb by G1 cyclin-dependent kinases releases E2F leading to cell cycle progression into the S phase. Because E7 is able to bind to unphosphorylated pRb, it may prematurely induce cells to enter the S phase by disrupting pRb–E2F complexes. Most recently, it was found that E7 promotes *C*-terminal cleavage of pRb by the calcium-activated cysteine protease calpain and that this cleavage is required before E7 can promote the proteasomal degradation of pRb (A. Suhrbier, personal communication, 2007; Fig. 4). The E7 protein function enables HPV replication in the upper layers of the epithelium where uninfected daughter cells normally differentiate and completely exit the cell cycle (Fig. 2). One cyclin-dependent kinase inhibitor, $p16^{INK4a}$, which prevents the phosphorylation of pRb family members, is overexpressed when pRb is inactivated by HPV E7.[57] Normally, overexpression of $p16^{INK4a}$ results in cell cycle arrest but with E7 expression, this is overcome. Thus overexpression of $p16^{INK4a}$ is suggested to be a useful bio-marker for evaluating HPV pathogenic activity in cervical lesions.

Others. In addition to the inactivation of pRb family members, numerous functions of E7 have been reported (Table 1). The histone deacetylases (HDAC), the transcriptional co-repressors, have been reported to associate with E7 via Mi2 to promote cell growth,[58,59] The interaction of E7 with cyclin-dependent kinase (CDK)2/cyclin A,[60] as well as CDK2/cyclin E,[61] has also been reported. These cyclin–kinase complexes exhibit kinase activities that can phosphorylate the pRb proteins. Furthermore, E7 binds the CDK-inhibitor (CKI), p27,[62] and CKI p21,[63] confirming the abrogation of cell-cycle inhibition. For evasion of the immune system, E7 also interacts with p48,[64] as well as IRF1.[8] Recently, p600 has been identified as a cellular target of E7 that contributes to anchorage-independent growth and cellular transformation.[65] Because the knockdown of p600 sensitizes cells to apoptosis induced by cell detachment irrespective of the presence of E7,[66] it is suggested as a novel target of cancer treatment. Binding of E7 with both the 35-kDa catalytic and 65-kDa structural subunits of PP2A has been reported, resulting in the sequestration of these subunits and inhibition of their interaction with PKB/Akt, thereby maintaining its signaling by blocking its dephosphorylation.[67] Despite these multifunctional properties of E7 protein, however, the expression of a mutant form of pRb (DeltaLXCXE) that is selectively defective for binding E7 revealed that most of the effects of E7 on epidermal differentiation are indeed due to pRb inactivation.[68]

Conclusions

HPV onco-proteins E6 and E7 are essential factors for HPV-induced cellular immortalization, transformation and carcinogenesis (Fig. 3). RNA interference with E6 and E7,[69] as well as their functions in inhibiting the actions of various molecules,[70] is a promising approach for the treatment of cervical cancers. Because prophylactic vaccination is still in its early stages, concentration of attention on such therapeutic measures is still warranted.

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