HPV-16 E7 Attenuates AKT Signaling

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Dedication Page

I would like to dedicate my work to my loving husband, Jared, and my wonderful parents, Bobby & Bonnie, for all their support throughout my education career.

Acknowledgements

I would like to first thank my mentor Scott, for always being available to discuss my project and life in general and for teaching me to look at science with a critical eye. He has taught me to look broadly and think about how new science fits in to the bigger picture and for that I am thankful because he has trained me to be a great scientist.

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Abstract

While the role of high-risk HPV E6 and E7 in targeting p53 and Rb has been intensively studied, how E6 and E7 manipulate cellular signaling cascades to promote the viral life cycle and cancer development is less understood. We have shown that HPV-16, specifically 16E7, attenuates pAKT, enhances IRES-dependent translation of several cellular proteins, inhibits keratinocyte differentiation and translocates YAP to the nucleus. Attenuation of pAKT was ablated by a missense mutation in the E7 carboxy-terminus, H73E, thereby defining a novel structure-function phenotype for E7. Coinciding with the pAKT attenuation, phosphorylation of S6K and 4E-BP1 was also attenuated and correlated with an increase in IRES-dependent translation of cellular proteins, including cMYC. This shift in cellular translation could lead to the enhanced expression of other cellular proteins that are essential for the viral life cycle or a novel mechanism to express other HPV proteins, like E5. The attenuation of pAKT could also represent another mechanism by which E7 induces dysplastic phenotypes typically associated with cervical cancer, as Rb degradation alone does not induce invasive cervical cancers. Keratinocytes containing the HPV-16 genome showed severe dysplasia and impaired differentiation in organotypic raft cultures, while HPV-16 genomes harboring the 16E7 H73E mutation had enhanced differentiation and markedly reduced cellular dysplasia. This demonstrates that the ability of E7 to reduce AKT activation correlates with increased dysplasia and reduced keratinocyte differentiation. The block in keratinocyte differentiation could also promote the viral life cycle, as viral amplification is dependent upon terminally differentiated keratinocytes re-entering the

cell cycle. Inhibition or delay of keratinocyte differentiation through attenuation of pAKT could promote a more “basal-like” phenotype, which is ideal for the amplification of the viral genome. We have also shown that 16E7 promotes the nuclear accumulation of YAP, where YAP acts as a transcriptional co-activator to promote the expression of genes controlling cell growth. Nuclear localization and activation of YAP have been associated with many cancers and thus, YAP has been termed an oncogene. We hypothesize that E7 could be mediating both the attenuation of pAKT and the nuclear localization of YAP through similar mechanisms involving PP2A or PDK1, both of which are involved in AKT and Hippo signaling. The attenuation of pAKT by 16E7 could be to promote a cellular environment that is ideal for viral amplification, by promoting cell cycle re-entry through inhibition of keratinocyte differentiation and activating YAP to enhance the expression of genes involved in cell proliferation.

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Chapter 1: General Introduction

Papillomaviruses

Papillomaviruses (PVs) are double stranded DNA viruses that infect cutaneous or mucosal epithelia of all vertebrates(1). Each viral type is categorized based upon their sequence homology in the L1 capsid gene and are organized into genera, then groups, and finally species. Currently, there have been over 200 types of human papillomavirus (HPV) identified, and classified into 5 genera, α, β, γ, μ and ν. The α-genus primarily infects respiratory and anogenital mucosal epithelia and are sexually transmitted (2). The β-genus primarily infect cutaneous epithelia and give rise to benign lesions, however several β-types are associated with squamous cell carcinomas in patients with epidermodysplasia verruciformis (EV) (3). The γ, μ and ν-genera infect cutaneous epithelia and give rise to warts.

The PV viral life cycle is dependent upon normal keratinocyte differentiation pathways (reviewed in (4-6)). First, an injury to the cornified epithelia is required for the viral particles to infect the basal layer of keratinocytes, which are actively progressing through the cell cycle. Once infected, these keratinocytes maintain the viral genome as an episome in low copy number. In normal keratinocyte differentiation, cells undergo cell division and a single cell moves up from the basement membrane to the suprabasal cell layer, withdraws from the cell cycle, and begins a terminal differentiation process (Fig. 1.1). However, in the presence of viral proteins, suprabasal cells reenter the cell cycle and begin to amplify the viral genome by prolonging the G2 phase (7). The terminal differentiation of suprabasal cells is not completely ablated by viral proteins as virus capsid protein transcription and virus packaging are dependent upon keratinocyte

differentiation. The final stage of keratinocyte differentiation requires the formation of tight junctions between keratinocytes in order create an efficient barrier to function as skin (granular and cornified layers). At this stage the viral episomes are packaged into viral particles, which are released after the sluffing off of dead keratinocytes from the top layer (4-6).

HPV Proteins

Each viral protein is expressed at specific times during the viral life cycle (Fig. 1.1). The early genes (E1, E2, E5, E6, and E7) are expressed within the basal cell layers and spinous cell layers to maintain and amplify the viral genome, while the late genes (E4, L1, and L2) are expressed in the granular and cornified cell layers to package the viral genome. Each viral protein plays a crucial role to the viral life cycle. E1, a helicase (8), and E2, a transcription factor (9), are both critically involved in the expression of viral proteins during the viral life cycle. E2 provides tight control of the expression of the viral early genes, keeping their expression low when the viral episome is maintained. E5, E6, and E7 are termed oncoproteins, as they alter cell cycle regulation, induce proliferation, promote survival, and block keratinocyte differentiation (reviewed in (10)). In HPV, E6 and E7 are the two major oncoproteins. E6 proteins of all genera have a highly conserved structure that allows for interaction with specific cellular proteins through LxxLL motif. α-genera E6 proteins interact with the ubiquitin ligase, E6 Associated Protein (E6AP). This interaction is required for the recruitment and degradation of p53 (4). E6 proteins from the β and μ-genera interact with MAML1, a transcriptional co-activator, rather than E6AP (11). E7 contains three conserved regions;

conserved region 1 (CR1), which interacts with ubiquitin ligases, conserved region 2 (CR2), which interacts with Rb family members (Rb, p107, and p130), and conserved region 3 (CR3), which interacts with cellular proteins like HDACs and p21 (reviewed in (12)). The CR3 domain also contains a single zinc binding domain, which provides structure to E7.

High-risk versus Low-risk HPVs

The α-genus can be further subdivided into high- and low-risk viruses. High-risk viruses, including HPV-16 and 18, cause cervical cancer (13), while low-risk viruses, including HPV-6 and 11, induce benign genital warts. High-risk and low-risk E7 proteins have varying functions that alter the overall effect of the virus upon the infected keratinocytes. High-risk E7s bind and degrade Rb, p130, and p107, while low-risk E7s bind Rb, p130, and p107 with lower affinity, but only degrade p130 (14, 15). The different affinities are associated with amino acid changes within the CR2 domain, which contains the LxCxE binding motif. Studies have shown that a single amino acid change within the LxCxE motif or within flanking amino acids can drastically alter the Rb binding affinity (16). The ability of E7 to bind Rb is necessary for viral amplification but is not required for the formation of warts or proliferation (17). This suggests there are other functions of E7 that low-risk viruses utilize to disrupt normal keratinocyte differentiation and to induce keratinocyte proliferation.

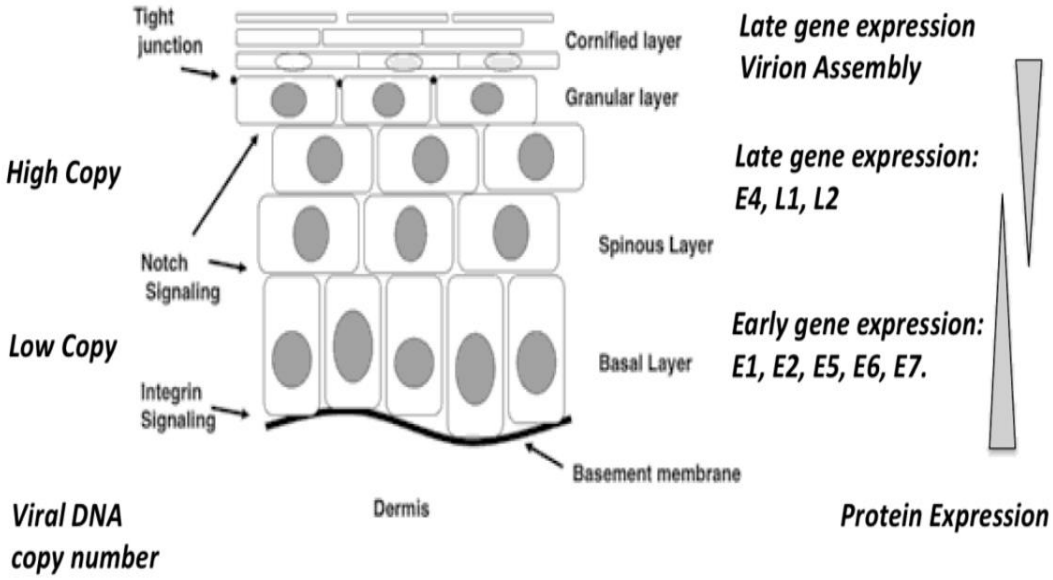

Figure 1.1: Keratinocyte differentiation and viral protein expression. Viral proteins are expressed at specific times during the viral life cycle and keratinocyte differentiation (Adapted from Vande Pol, 2012) (4).

While low-risk E7s do not degrade Rb, they retain the ability to induce DNA synthesis, through association with p107 and p130 that releases E2F transcription factors, in order to amplify the viral genome (18). However, low-risk E7s cannot bypass growth arrest, as bypassing growth arrest requires both Rb degradation and p21 inhibition (19). By comparing the unique functions of high- and low-risk HPV types, those that are specifically related to oncogenesis and those features that are conserved functions required for the viral life cycle can be discerned.

HPV-16 and Cervical Cancer

The link between cervical cancer and HPV has been clearly defined (20-23). HPV-16 DNA was present in cervical carcinoma specimens (21) and HPV-18 was identified in both cervical cancer and HeLa cell lines (20). HPV DNA was found to be integrated in cervical carcinomas within the region of E1 and E2, allowing for the enhanced expression of E6 and E7 (23); this was the first indication that E6 and E7 were playing a role in cervical cancer progression.

Over 90% of HPV infections are cleared within two years of the initial infection, while the remaining 5-10% of infections become persistent infections that potentially progress to cervical carcinoma(24-26). More than 99% of cervical cancers are caused by high-risk HPV types, including HPV 16 and 18, which account for 70% of all cervical cancers. HPV infections also account for 25% of newly diagnosed head and neck squamous cell carcinoma (HNSCC) in the United States (24-26). HPV-positive HNSCC are sensitized to radiation therapy in comparison to HPV-negative HNSCC that are caused by alcohol and tobacco use. HPV-positive HNSCC lesions are also attenuated for pAKT on

immunohistochemistry (IHC) when compared to HPV-negative HNSCC, which suggests alternate cellular pathways are involved in the progression of HPV-positive versus HPV-negative cancers.

Both E6 and E7 play a vital role in the development and progression of cervical cancer through dysregulation of the cell cycle regulators p53 and Rb, respectively (reviewed in (12), (27), and (28)). E7 is the major transforming protein of HPV (29) (reviewed in (12)) through degradation of Rb primarily, but also through other mechanisms that are still being elucidated. Mutations within the C-terminal domain of E7, for example, retain the ability to degrade Rb, but no longer transform cells, suggesting E7 interacts with other cellular proteins to induce transformation (30). The ability of E7 to promote invasive cancers could require manipulation of other key cellular signaling cascades known to be involved in cancer progression. We sought to examine the role of HPV-16, and specifically E7, in manipulating cellular signaling pathways critical to the survival of the cell and initially focused on the PI3K/AKT pathway.

PI3K/AKT Signaling

AKT was originally identified as the causative agent in the acute transforming retrovirus AKT8, which causes spontaneous lymphomas in mice (31). Human homologues of v-akt were identified as AKT1 and AKT2, and further studies found AKT1 to be upregulated in gastric adenocarcinomas, further validating the oncogenic potential of AKT (31). Taken together, these results showed that AKT alone could act as a transforming oncogene.

AKT is activated by PI3K through the activation of several upstream signaling receptors (32). Once activated, PI3K phosphorylates the inositol PIP2 to PIP3, which recruits pleckstrin homology (PH) domain containing proteins, including AKT and PDK1, to the plasma membrane. Phosphorylation of AKT on two sites is required for full activation: T308, which is phosphorylated by PDK1 and S473, which is phosphorylated by mTORC2 (Fig 1.2) (32, 33). T308 is the more critical site necessary for AKT activation; if T308 is mutated, AKT remains predominately in the inactive state (34). If the S473 site is mutated, AKT still retains some activity, but phosphorylation of this site is required for the highest amount of AKT activity (34).

AKT is also regulated by multiple phosphatases, which dephosphorylate activation sites to return AKT back to its inactive state. Two phosphatases act directly on AKT, PP2A and PHLPP, while PTEN acts upstream in opposition to PI3K. PP2A dephosphorylates AKT at the T308 site, while PHLPP dephosphorylates at the S473 site (reviewed in (32)). PTEN, while not acting directly on AKT, influences pAKT levels by attenuating PI3K signaling. PTEN is a known tumor suppressor and converts PIP3 to PIP2 to attenuate PI3K signaling (reviewed in(35)).

Once fully activated, AKT plays a role in multiple downstream cellular processes including cell survival, protein translation, metabolism, and proliferation (reviewed in (36)). AKT both activates and inhibits multiple proteins directly to alter its downstream signaling cascade. The vast array of cellular processes that AKT manipulates informs its importance in the overall fate of the cell.

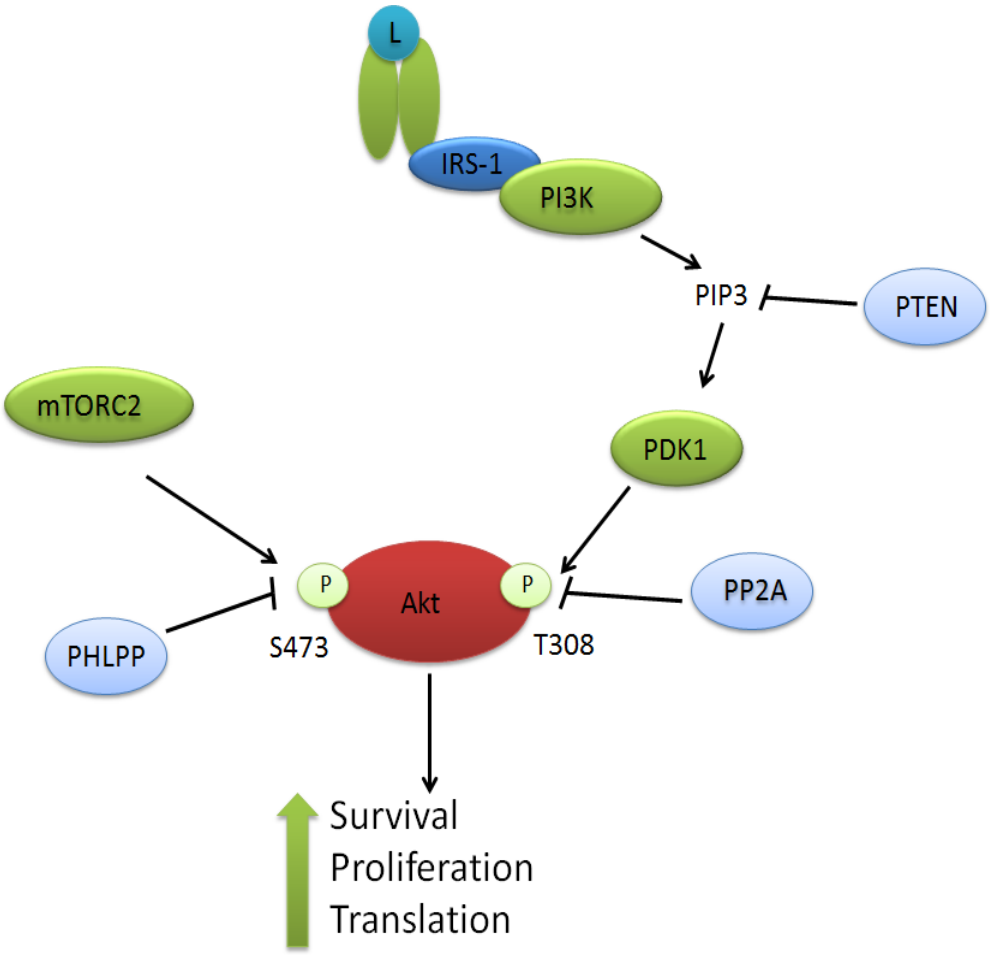

Figure 1.2: PI3K/AKT Signaling Pathway. A simplified PI3K/AKT signaling pathway as activated by the insulin signaling pathway.

Viruses and AKT Signaling

As AKT signaling is such a crucial cellular signaling pathway, several studies have examined how viral proteins alter this pathway. In HPV, both E6 and E7 have been shown to augment AKT signaling (37-39). E6 increases AKT phosphorylation (pAKT) that leads to the activation of downstream kinases involved in protein translation, thereby inducing increased binding of translation initiation factors to the mRNA cap (37). However, this study was conducted entirely in the context of keratinocyte serum free media (KSFM), which contains no serum or calcium, and prevents adherens junction formation between keratinocytes. The formation of adherens junctions by E-cadherin has been shown to regulate AKT signaling, where overexpression of E-cadherin attenuates PI3K signaling and loss of E-cadherin augments PI3K signaling (40). Thus, experiments conducted in KSFM media must be interpreted in the context of increased PI3K signaling due to the absence of adherens junctions. E7 expressed in HEK 293 cells identified an interaction between E7 and PP2A, which led to an increase in pAKT (38). However, these results should be interpreted in the context of overexpression of E7, AKT and PP2A, and co-expression of the adenoviral protein E1a and E1b transcription units. An additional study showed HPV-16 E7 (16E7) augmenting pAKT S473 in organotypic squamous cell raft cultures, with the augmentation of pAKT S473 dependent upon Rb degradation by E7 (39). However, this study was limited to only immunohistochemical (IHC) staining of a single AKT phosphorylation site (S473), which is the less critical of the two activation sites. While these studies suggest an increase in pAKT mediated by E6 and E7, limitations to each study render the effects of E6 and E7

on pAKT signaling inconclusive and the effects of the entire early region of HPV-16 unknown.

In contrast to the effects of E6 and E7 oncoproteins, the cutaneous HPV-8 E2 transactivator has been shown to down-regulate AKT1 transcription, leading to attenuated AKT activity in 8E2 expressing keratinocytes (41). This downregulation of AKT1 expression altered keratinocyte differentiation, which was proposed to weaken the cornified epithelia, thereby allowing for easier release of the viral particles (41). Adenovirus E1a, which shares similar functions with HPV E7, has been shown to attenuate pAKT upon insulin stimulation (42). Due to the attenuation of pAKT, cells transduced with E1a are sensitized to apoptosis when induced with a stimulus such as cisplatin or radiation treatment (42, 43). In contrast, other studies have shown activation of AKT by adenovirus E1a (44). More recently, it was shown that Polyoma small T (PyST) attenuates pAKT through association with and recruitment of PP2A Aβ, one of the A-subunits that makes up a small fraction of the cellular PP2A A-subunit population (45). The attenuation of pAKT by PyST is linked to a reduction of differentiation in several systems including, 3T3-L1 and C2C12 cells (45). In contrast to PyST, SV40 small T inhibits PP2A isoforms resulting in the activation of AKT (46). The aforementioned studies have shown often inconsistent results that could be the result of different experimental systems as well as possible artifacts arising from overexpression and the use of cell lines that are transformed or expressing additional oncoproteins. This suggests the necessity for further studies in biologically relevant

experimental systems to elucidate the roles HPV E6 and E7 may play in modulating the PI3K/AKT signaling pathway.

Regulation of protein translation

AKT regulates protein translation through phosphorylation of the downstream target mTORC1, and subsequent activation and inhibition of p70 S6 Kinase (S6K) and 4E-BP1, respectively (47) (Fig 1.3). S6K phosphorylates the ribosomal protein S6, which associates with the 40S ribosome (reviewed in (48)). However, recent studies have shown that S6K has other targets that affect many signaling cascades, including translation initiation. S6K has been shown to phosphorylate eIF4B, an initiation factor, that activates eIF4A, a helicase, to initiate the translation of cap-dependent mRNAs (49). However, the role of S6K in translation initiation has been highly controversial, as several of the proposed S6K substrates are also phosphorylated by other kinases, thus it is difficult to map specific functions exclusively to S6K.

While the role of S6 phosphorylation by S6K remains unclear, 4E-BP1 has a clear role in regulation of translation as it binds eIF4E to prevent its association with the translation initiation complex, thereby inhibiting cap-dependent translation (47, 50) (Fig 1.3). In the unphosphorylated state, 4E-BP1 is active and binds to eIF4E to block translation initiation. However, once hyper-phosphorylated, 4E-BP1 dissociates from eIF4E and cap-dependent translation is initiated through the interaction of eIF4E with the translation initiation complex and the mRNA cap (47) (Fig. 1.3). Cap-dependent translation is initiated when growth factors bind and activate upstream signals feeding through the PI3K/AKT/mTOR signaling cascade. In the absence of growth factors or

during stress conditions, cap-dependent translation is inhibited by 4E-BP1. However, a subset of cellular proteins is still translated during stress conditions through cap-independent mechanisms via internal ribosomal entry sites (IRES) (51). IRES elements were first described in polio virus, where viral proteins degrade translational regulators and inhibit cap-dependent translation, which then promotes the translation of viral proteins under the influence of the IRES sequence (52). Later studies utilized polio virus inhibition of cap-dependent translation to determine if there was a set of cellular proteins that is translated during polio infections. These data demonstrated cellular mRNAs associated with polysomes even in the presence of poliovirus, suggesting IRES-dependent translation was not exclusive to viral mRNAs (53). Using a cDNA microarray, mRNAs that associated with polysomes in the presence of polio virus were identified, including c-MYC. Further studies showed diverse cellular mRNAs that contain IRES elements within their 5' UTR regions, further demonstrating the potential of IRES-dependent translation to regulate cellular protein expression (54).

Keratinocytes as a model system

Within the papillomavirus literature, two model systems are used to study the viral life cycle and elucidate the effects of the viral proteins on cellular processes. The first are primary cells derived from human foreskins (HFKS), and the second is a cell line that spontaneously immortalized, Normal Immortalized Keratinocytes (NIKS). The NIKS cell line was described nearly twenty years ago to be a non-transformed cell line that spontaneously immortalized and shared similar growth and differentiation properties of primary keratinocytes (55). NIKS are useful as a model system, as we are able to

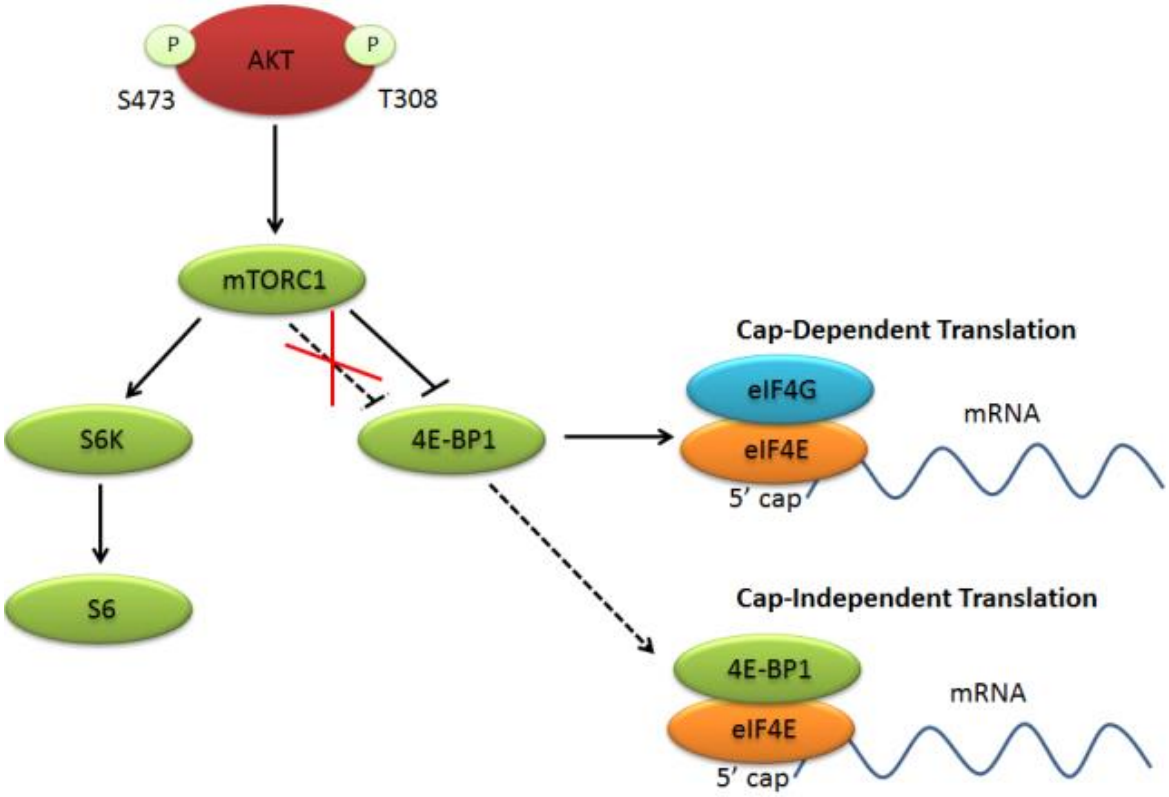

Figure 1.3: AKT regulation of protein translation initiation. mTORC1 phosphorylates S6K and 4E-BP1 after activation by AKT. The red X and dashed lines represent the interaction partner of 4EBP1 when AKT signaling is attenuated.

passage non-transformed cell lines significantly longer than non-transformed HFKs, which have a limited lifespan (56), and their uniform and consistent behavior allow for enhanced reproducibility compared to HFKs. However, some chromosomal abnormalities are present within NIKS, and how this change alters cellular signaling remains unclear.

Currently, researchers in the field use two different media formulations to culture both HFKs and NIKS: keratinocyte serum free media (KSFM) and media formulations that contain serum (E-media and F-media). F-media contains low amounts of calcium and serum, allowing for the formation of adherens junctions between keratinocytes. F-media requires co-culturing with mitotically-inactive fibroblasts that are induced either by irradiation (57) or treatment with a DNA damaging agent, such as mitomycin C (58). KSFM contains very low calcium and no serum and the keratinocytes remain more mesenchymal in nature, with no E-cadherin engagements due to the lack of calcium in the media. Keratinocytes cultured in F-media (supplemented with additional calcium) are able stratify and differentiate, while keratinocytes maintained in KSFM fail to stratify or differentiate (59). Thus, we utilize the F-media culture conditions to more closely mimic physiologic conditions of the keratinocytes. We plan to use NIKS to elucidate the effects of E7 on AKT signaling, but validate any results in HFKs.

Chapter 2: The Human Papillomavirus Type 16 E7 Oncoprotein Attenuates AKT Signaling to Promote IRES Dependent Translation and expression of c-MYC

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Abstract

While the role of high-risk HPV E6 and E7 in targeting p53 and Rb has been intensively studied, how E6 and E7 manipulate cellular signaling cascades to promote the viral life cycle and cancer development is less understood. Keratinocytes containing the episomal HPV-16 genome had decreased activation of AKT, which was phenocopied by 16 E7 (16E7) expression alone. Attenuation of phosphorylated AKT (pAKT) by E7 was independent of the Rb degradation function of E7 but could be ablated by a missense mutation in the E7 carboxy-terminus, H73E, thereby defining a novel structure-function phenotype for E7. Downstream of AKT, reduced phosphorylation of p70 S6K and 4E-BP1 was also observed in E7 expressing keratinocytes, which coincided with an increase in IRES-dependent translation that enhanced the expression of several cellular proteins, including MYC, Bax and the insulin receptor. The decrease in pAKT mediated by E7 is contrary to the widely observed increase of pAKT in invasive cervical cancers, suggesting that the activation of AKT signaling could be acquired during the progression from initial productive infections to invasive carcinomas.

Importance

HPV causes invasive cervical cancers through the dysregulation of the cell cycle regulators p53 and Rb, which are degraded by the viral oncoproteins E6 and E7, respectively. Signaling cascades contribute to cancer progression and cellular differentiation, and how E6 and E7 manipulate those pathways remains unclear. The PI3K/AKT pathway regulates cellular processes including proliferation, cell survival, and cell differentiation. Surprisingly, we found that HPV-16 decreased the phosphorylation of AKT (pAKT) and that this is a function of E7 that is independent of the Rb-degradation function. This is contrary to the observed increase in AKT signaling in nearly 80% of cervical cancers, which have typically acquired a mutation within the PI3K/AKT cascade leading to constitutive activation of the pathway. Our observations suggest that multiple changes in the activation and effects of AKT signaling occur in the progression from productive HPV infections to invasive cervical cancers.

Introduction

The causative link between HPV-16 infection and the development of cervical cancer is well established (reviewed in (13)). High-risk alpha genera HPV E6 and E7 oncoproteins interact with and degrade p53 and Rb respectively, to alter cell cycle regulation (reviewed in (12), (27), and (28)). However, less is known about the interaction of E6 and E7 with cellular proteins that manipulate cellular signaling cascades. We sought to examine the role of HPV-16, and specifically E7, in manipulating cellular signaling pathways critical to the survival of the cell and initially focused on the PI3K/AKT pathway.

AKT is activated by several upstream signaling receptors that result in the activation of PI3K (32). Full AKT activation is achieved through phosphorylation of T308 (located within the catalytic domain) and S473 (located within the regulatory domain). Two kinases are responsible for the phosphorylation of the activation sites; PDK1 phosphorylates T308 and mTORC2 phosphorylates S473 (32). Once fully activated, AKT plays a role in multiple downstream cellular processes including cell survival, protein translation, metabolism, and proliferation (reviewed in (36)). The vast array of cellular processes that AKT manipulates informs its importance in the overall fate of the cell.

AKT regulates protein translation through phosphorylation of the downstream target mTORC1, and subsequent activation and inhibition of p70 S6K and 4E-BP1, respectively (47). 4E-BP1 regulates translation through interaction with eIF4E to prevent association with the translation initiation complex, thereby inhibiting cap-dependent translation (47, 50). During periods of growth factor deprivation, such as mitosis or

other cellular stress, cap-dependent translation is inhibited. However, mRNAs are still required to be translated to enhance cellular proteins that are necessary for processes, like mitosis, to occur. This subset of cellular mRNAs is translated by cap-independent mechanisms via internal ribosomal entry sites (IRES) (51). Previous studies have identified viruses, such as polio virus, that utilize IRES-dependent translation to express viral proteins (52). Later studies identified IRES utilization was not exclusive to viruses, but rather a subset of cellular mRNAs also utilized IRES-dependent translation to enhance protein expression during cellular stress (53, 54). Cellular mRNAs that contain IRES elements within their 5' UTR include cMYC and insulin receptor.

Given the importance of the AKT signaling pathway on cellular processes, we investigated if HPV-16, and specifically E7, manipulated the AKT signaling cascade.

Materials and Methods:

Cell culture: Normal Immortalized Keratinocytes (NIKS) are spontaneously immortalized foreskin keratinocytes that are both feeder cell and growth factor dependent for proliferation and support of the complete HPV lifecycle (56). NIKS were co-cultured with mitomycin C treated 3T3 cells in F-media as described previously (56). NIKS were retrovirally transduced with replication defective murine retroviruses or transfected with the HPV-16 genome as previously described (56, 60). To investigate AKT phosphorylation, $4x10^5$ trypsinized NIKS cells were plated on 6-well plates (Day 1) together with mitomycin C treated feeder cells. Approximately 48 hours later 3T3's were removed by light trypsinization and the remaining keratinocytes were cultured in F-media for another 10 hours (Day 3, morning). The subconfluent, transduced NIKS were then placed into starvation media (F-media without FBS, EGF and insulin) for 12 hours. Transduced NIKS were then stimulated with complete F-media for 5, 10, 15, 20, 30 or 360 minutes. Cells were then washed with ice cold PBS then lysed in 1% SDS. Primary keratinocytes were obtained from neonatal foreskins collected anonymously from the University of Virginia Medical Center following the previously described protocol (61). Primary keratinocytes were cultured in F-media with mitomycin C treated 3T3 cells.

Western Blotting: SDS-lysed NIKS cell lysates were equalized for protein concentration with BioRad protein assay reagents. Equal amounts of protein normalized samples were loaded onto SDS-acrylamide gels, electrophoresed, and transferred onto PVDF membranes. Blots were probed with the indicated antibodies from Cell Signaling: pAKT T308 (#2965), panAKT (#2920), c-MYC (#2276), Insulin Receptor (#3020), Rb 4H1

(#9309), GAPDH (#3683), p70 S6K T389 (#9205), p4EBP1 S65 #9451, 4EBP1 #9644; from Sigma: tubulin (#T9026); from BD Biosciences: p16 (#550834), Bax (#610982); from Thermo Scientific: Actin (ACTN05) (MS-1295-P1), p53 (MA1-19055); and HPV 16E7 from 2 sources: Santa Cruz #sc-6981 and Invitrogen cat #28-0006. The Total S6K antibody was a gift from Janet Cross at the University of Virginia.

Luciferase Assays: Retrovirally transduced NIKS were plated as described above and transfected with the CrPV IRES Reporter (62) on Day 2. 48 hours later sub-confluent, transduced NIKS, with feeder cells removed, were starved for 12 hours and then harvested in 1x Passive Lysis Buffer; lysates were analyzed using the Promega Dual-Luciferase Reporter Assay System (Cat. #E1960).

RT-PCR: Retrovirally transduced NIKS were plated as described above, but harvested following a Trizol RNA harvest protocol (Invitrogen). cDNA was then generated via First-Strand cDNA synthesis using an M-MLV RT protocol. qPCR was prepared using the previously synthesized cDNA and SSO Advanced SYBR Green Supermix (BioRad Cat # 1725264). Primers utilized were: Bax 5'- CATGTTTTCTGACGGCAACTTC-3', 5'-AGGGCCTTGAGCACCAGTTT-3'; Insulin Receptor 5'-CTGCACAACGTGGTTTTCGT-3', 5'-ACGGCCACCGTCACATTC-3'; c-MYC 5'-TCAAGAGGCGAACACACAAC-3', 5'-GGCCTTTTCATTGTTTTCCA-3'; HPRT 5'-TGACACTGGCAAAACAATGCA-3', 5'-GGTCCTTTTCACCAGCAAGCT-3'; AKT1 5'- ATGAGCGACGTGGCTATTGTGAAT-3', 5'-GAGGCCGTCAGCCACAGTCTGGATG-3'; AKT2 5'-ATGAATGAGGTGTCTGTCATCAAAGAAGGC-3', 5'- TGCTTGAGGCTGTTGGCGACC-3'. Relative values were analyzed using the ΔΔCt method and using HPRT as a control.

7-methyl-GTP binding: The ability of 4EBP-1 to bind the mRNA cap structure was assessed through a 7-methyl GTP binding assay as described previously (37). Briefly, NIKS cells were plated as described in previous experiments, and lysed in Buffer D (50 mM HEPES pH 7.4, 40 mM NaCl, 2mM EDTA and 0.1% Triton X-100). Cell lysates were incubated with 7-methyl-GTP-Sepharose Beads (a gift from Thurl Harris at the University of Virginia) for 1 hour at 4^{O}C. 7-methyl beads were washed three times and bound proteins were analyzed via SDS-PAGE and western blotting.

Results:

HPV-16 E7 attenuates the activation of AKT

AKT signaling is typically activated in invasive squamous cell carcinomas including cervical cancer (63). Surprisingly, we observed that in normal immortalized human foreskin keratinocytes (NIKS) transfected with the episomal HPV-16 genome, phosphorylation of AKT at T308 (pAKT) was attenuated upon stimulation with F-media (Fig. 2.1A).

To elucidate if HPV-16 E7 was playing a role in the attenuation of AKT signaling, we retrovirally transduced NIKS with HPV-16 E7 (16E7) alone and found that pAKT T308 was again attenuated (Fig. 2.1B), demonstrating 16E7 alone could phenocopy the attenuation of pAKT observed in NIKS transfected with the complete HPV-16 genome. This phenotype was replicated in primary keratinocytes maintained in F-media (Fig. 2.1C). The second AKT activation site, pAKT S473, showed a similar attenuation trend (Fig. 2.1D).

Culture Conditions modulate the effect of E6 on AKT activation

The attenuation of pAKT T308 was unexpected since it was previously reported that HPV-16 E6 increased pAKT T308 (37). To examine the role of E6 and contributions that culture conditions might play in AKT activation, NIKS cells expressing 16E6 or 16E7 alone or together were examined for AKT activation in either F-media or KSFM. Our studies were performed in F-medium that contains serum, EGF, insulin, feeder cells, and higher calcium levels than KSFM (64). 16E7 attenuated pAKT T308 in both KSFM and F-media (Figs. 2.2A-F), while 16E6 only activated pAKT-T308 in KSFM and did not in F-

media and not in the presence of E7 (Figs. 2.2A-F). In NIKS cells, pAKT T308 levels remain elevated even after starvation in KSFM media (Fig. 2B), while in F-media, pAKT T308 levels are attenuated after starvation (Fig. 2A). An initial increase above the basal level of pAKT T308 is observed at 5 minutes in KSFM media (not shown in Fig. 2B); however, at 15 minutes the levels have dropped below the basal level (Fig. 2B). Thus, basal activation and the kinetics of pAKT T308 activation and subsequent inactivation are different in F-media versus KSFM. Because KSFM does not allow for adherens junction formation, keratinocyte stratification (65) or papillomavirus replication, our remaining experiments were performed in F-media.

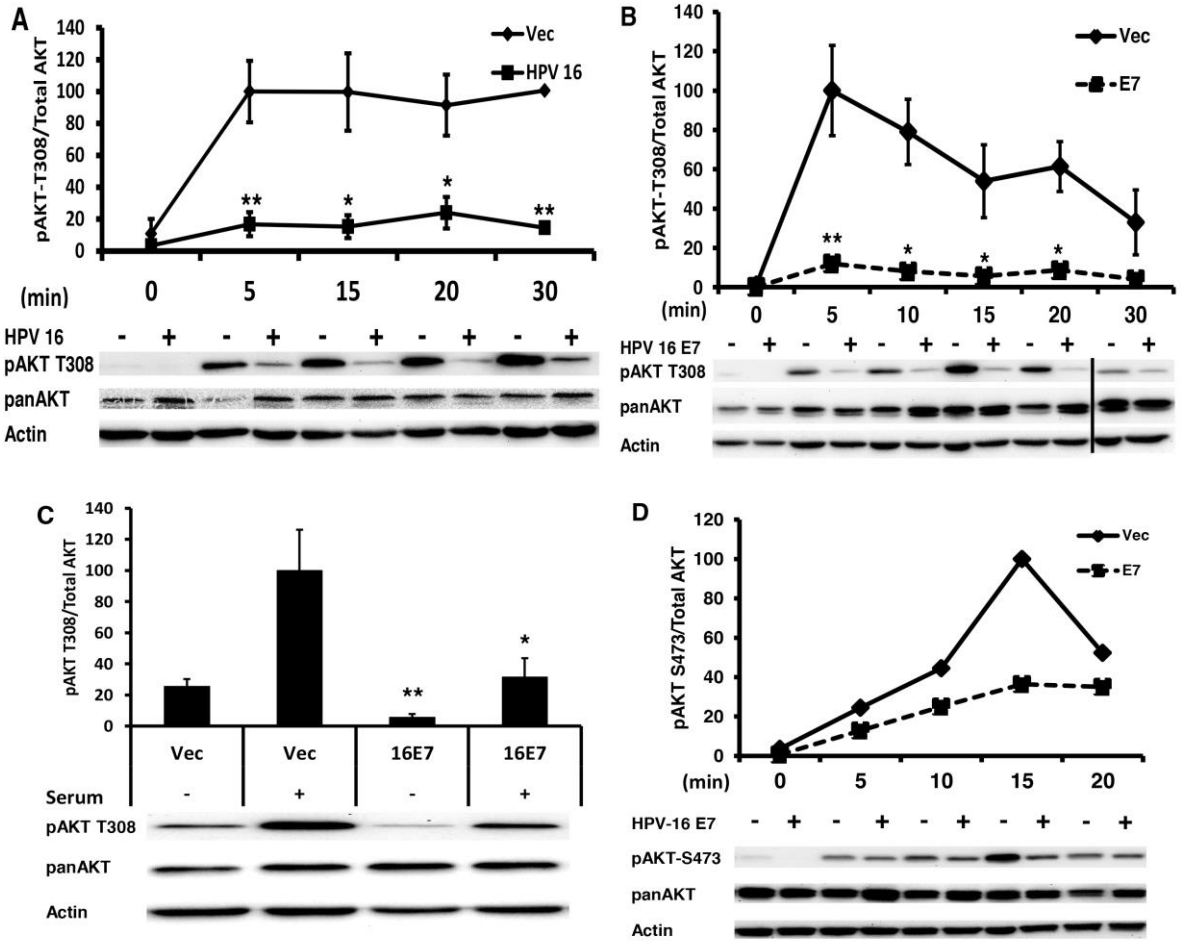

Figure 2.1: Papillomavirus E7 attenuates pAKT T308

Figure 2.1: Papillomavirus E7 attenuates pAKT T308 A: HPV-16 attenuates pAKT T308. Immortalized keratinocytes (NIKS) transfected with the HPV-16 genome were plated in co-culture with mitomycin-C treated NIH 3T3 cells, then 48 hours later NIH 3T3 cells were removed and HPV-16 NIKS were placed back in F-media. Approximately 10 hours later HPV-16 NIKS were starved in F-media lacking FBS, insulin and EGF for 12 hours and then stimulated with complete F-media for the indicated time, harvested in 1% SDS, protein normalized, and then analyzed via western blot. Samples were normalized to actin and the highest value. Statistics were calculated from four independent experiments (*=$p<0.05$ **=$p<0.01$), with western blots from a single representative experiment shown. B: NIKS retrovirally transduced with 16E7 attenuate pAKT T308. NIKS transduced with vector or 16E7 were plated and harvested as in Fig. 1A. Samples were normalized to actin and the ratio of phosphorylated AKT to Total AKT is shown. Statistics were calculated from three independent experiments by t-test and two-way Anova. The vertical bar in the western blot represents the removal of the of a redundant time point (*=$p<0.05$ **=$p<0.01$). C: E7 attenuates pAKT T308 in primary keratinocytes. Primary keratinocytes retrovirally transduced with Vec or 16E7 were plated and harvested as in Fig. 1A in F-media. Statistics were calculated from four independent experiments. *=$p<0.05$, **=$p<0.01$. D: pAKT S473 is attenuated by 16E7 in keratinocytes. Retrovirally transduced NIKS were plated and harvested as in Fig. 1A. Western blot is a representative of three independent experiments.

Figure 2.2: 16E6 does not alter E7-mediated pAKT attenuation.

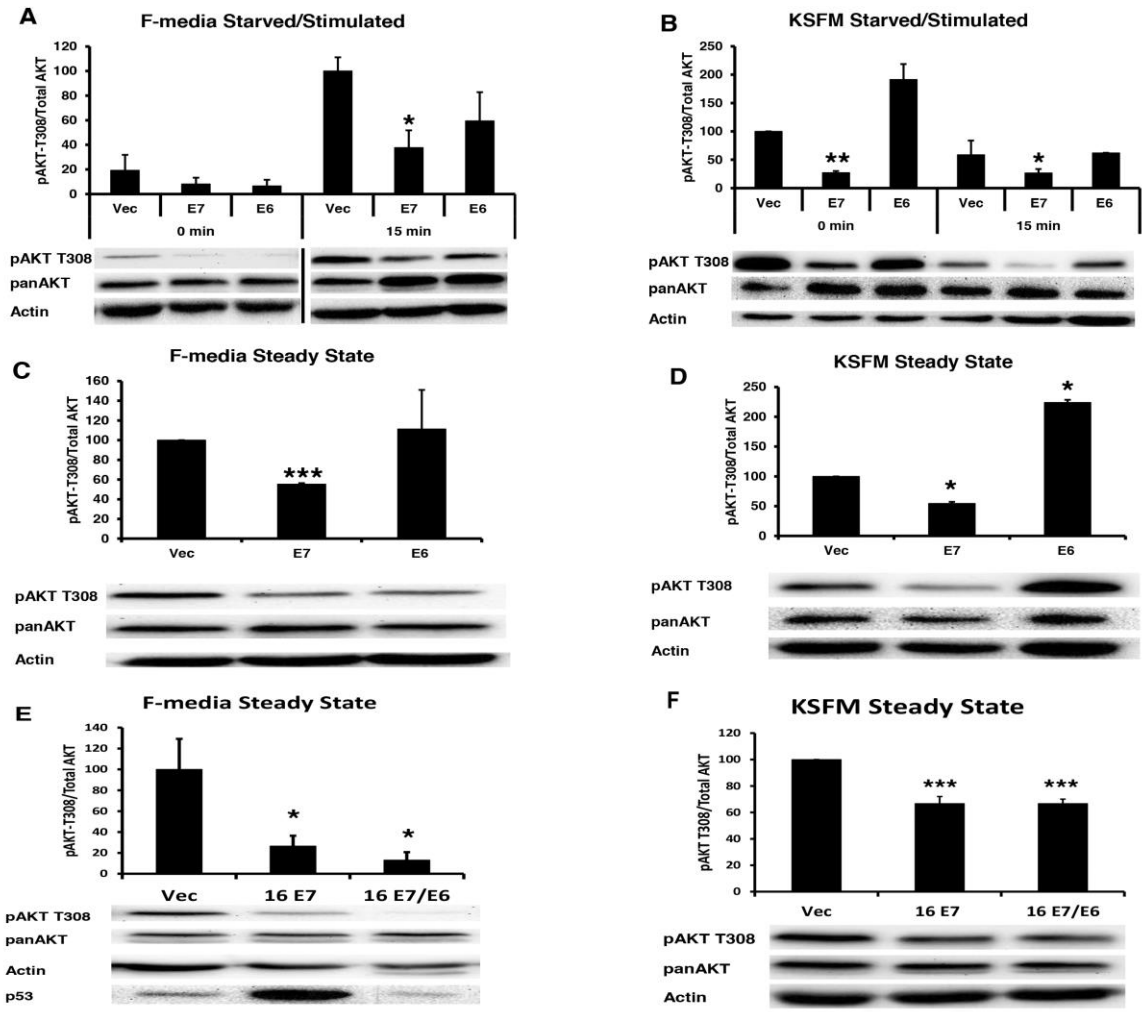

Figure 2.2: 16E6 does not alter E7-mediated pAKT attenuation. *A:* Retrovirally transduced keratinocytes with 16E7 or 16E6 were plated and harvested as described in Fig. 1A. Statistics were calculated from three independent experiments. Bar represents the removal of a redundant time point *B:* Retrovirally transduced keratinocytes with 16E7 or 16E6 were plated and harvested as described in Fig. 1A, except cells were maintained in KSFM. Statistics were calculated from three independent experiments. *C:* Retrovirally transduced keratinocytes with 16E7 or 16E6 were plated and subsequently harvested three days later in 1% SDS. Statistics were calculated from three independent experiments. *D:* Retrovirally transduced keratinocytes with 16E7 or 16E6 were plated and harvested as described in Fig. 2B. Statistics were calculated from three independent experiments. *E:* Retrovirally transduced keratinocytes with 16E7 or 16E7/16E6 were plated and harvested as described in Fig. 1A. Statistics were calculated from three independent experiments. (*=$p<0.05$, **=$p<0.01$, ***=$p<0.001$). *F:* Retrovirally transduced keratinocytes with 16E7 or 16E7/16E6 were plated and harvested as described in Fig. 2B. Statistics were calculated from four independent experiments. (*=$p<0.05$, **=$p<0.01$, ***=$p<0.001$).

E7 proteins from various papillomavirus genera attenuate AKT phosphorylation

To determine if other high risk HPV E7 proteins also attenuated pAKT-T308, we compared 16E7 with HPV-18 E7 (18E7) and found both repressed pAKT T308 (Fig 2.3A). Examining other papillomavirus genera, 16E7, HPV-1 E7 and cotton-tailed rabbit papillomavirus all significantly repressed pAKT-T308 while HPV type 6 and 8 E7 proteins, while trending down, did not reach statistical significance (Fig. 2.3B).

Attenuation of pAKT is independent of the Rb degradation function of E7

The CR2 region of E7 that binds to Retinoblastoma (Rb) family proteins is well conserved between HPV-16 and HPV-1 E7, and both demonstrate a similar binding affinity for Rb (66). However, unlike HPV-16 E7, HPV-1 E7 does not degrade unphosphorylated Rb (66). Cottontail Rabbit Papillomavirus E7, while cancer associated, has a lower binding affinity LxCxE motif compared to HPV-16E7, suggesting the Rb-binding function of E7 might be independent of the pAKT attenuation phenotype (66). To explore how E7 decreased the phosphorylation of AKT, we utilized several 16E7 mutants and determined their effect on AKT phosphorylation. 16E7 D21G converts the high affinity 16E7 Rb-binding site to the low affinity Rb-binding site found in the low-risk HPV-6 E7 (16). 16E7 D21G no longer degraded Rb, but retained the ability to decrease pAKT T308 (Fig. 2.4A). Next, we examined the effect of mutating surface exposed side chains within the carboxyl zinc-structured domain of 16E7. Three 16E7 mutants (K60E, D62K, and R66E) decreased pAKT T308 to the same extent as WT 16E7; however a single mutant, H73E, restored pAKT T308 to that of vector transduced NIKS (Fig. 2.4B). The

16E7_H73E mutant retained the ability to degrade unphosphorylated Rb, similar to WT 16E7 (Fig 2.4C).

In cervical carcinomas, p16 is utilized as a marker of the presence of high-risk HPV, as studies have shown that high-risk HPV E7 induces overexpression of p16 in keratinocytes and dependence upon p16 expression for viability of E7 expressing keratinocytes (67, 68). A recent study demonstrated that this phenotype was independent of Rb degradation by E7 and could be mapped to the E7 induction of KDM6A and KDM6B to induce demethylation at the p16 promoter, resulting in increased p16 expression (69). In order to determine if decreased pAKT T308 was associated with the induction of p16, lysates from NIKS transduced with Vec, WT 16E7 or 16E7_H73E were examined for p16 expression; both WT E7 and 16E7_H73E equally induced p16 compared to vector-transduced NIKS (Fig. 2.4D). This demonstrated that neither the degradation of Rb nor the induction of p16 was associated with the attenuation of pAKT T308.

Finally, to make sure that 16E7_H73E retained its phenotype when expressed from the complete HPV-16 genome, NIKS transfected with the HPV-16 genome harboring the 16E7 H73E mutation reversed the pAKT phenotype of WT HPV-16 and activated pAKT T308 to the levels of un-transfected NIKS upon stimulation with F-media (Fig. 2.5A). HPV-16_H73E retained Rb and p53 degradation similar to HPV-16 (Fig. 2.5B). Expression levels of 16E7 and 16E7_H73E from the complete genome were similar (Fig. 2.5A). Taken together, these results demonstrated that the attenuation of pAKT was independent of the other viral early gene products, was independent of the Rb-

degradation function of E7, independent of p16 induction, mapped to a previously uncharacterized site in the zinc-structured domain of E7, and could be specifically ablated by the 16E7_H73E mutation.

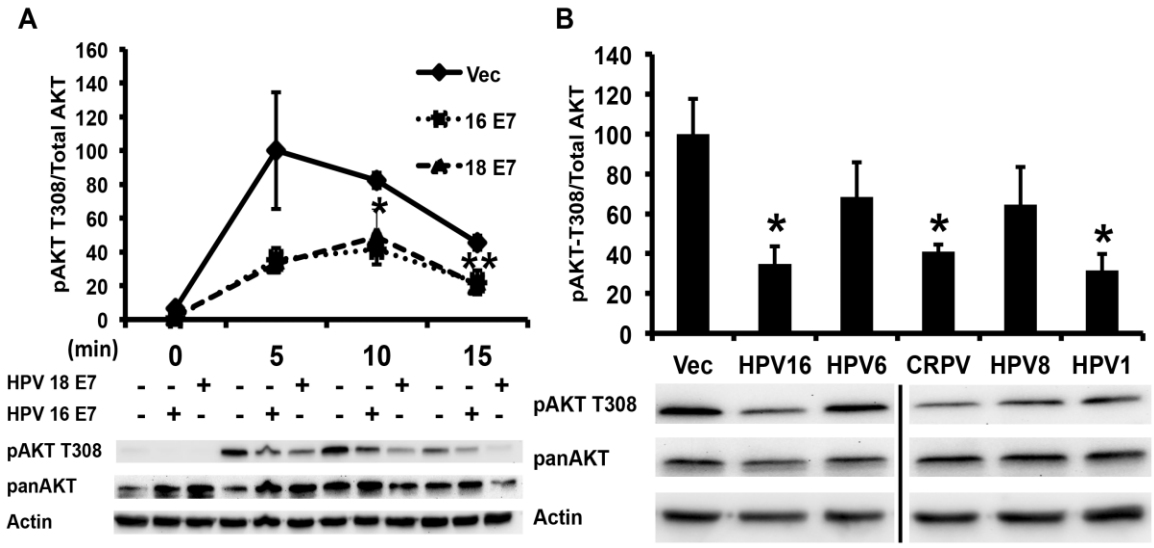

Figure 2.3: The attenuation of pAKT by E7 varies across papillomavirus genera. *A:* The AKT attenuation phenotype is conserved in high-risk 18E7. NIKS retrovirally transduced with either HPV-16 E7 or HPV-18 E7 were plated and harvested as in Fig. 1A. Samples were normalized to actin and the highest value. Statistics were calculated from three independent experiments (*=p<0.05 **=p<0.01). *B:* The AKT attenuation phenotype is not exclusive to high-risk E7 types. NIKS retrovirally transduced with the E7 genes from the indicated papillomavirus types were stimulated for 6 hours following 12 hour starvation and harvested as in Fig. 1A. Samples were normalized to actin and the highest value. Statistics were calculated from four independent experiments (*=p<0.05).

Figure 2.4: Attenuation of pAKT is independent of either p16 induction or Rb degradation by E7

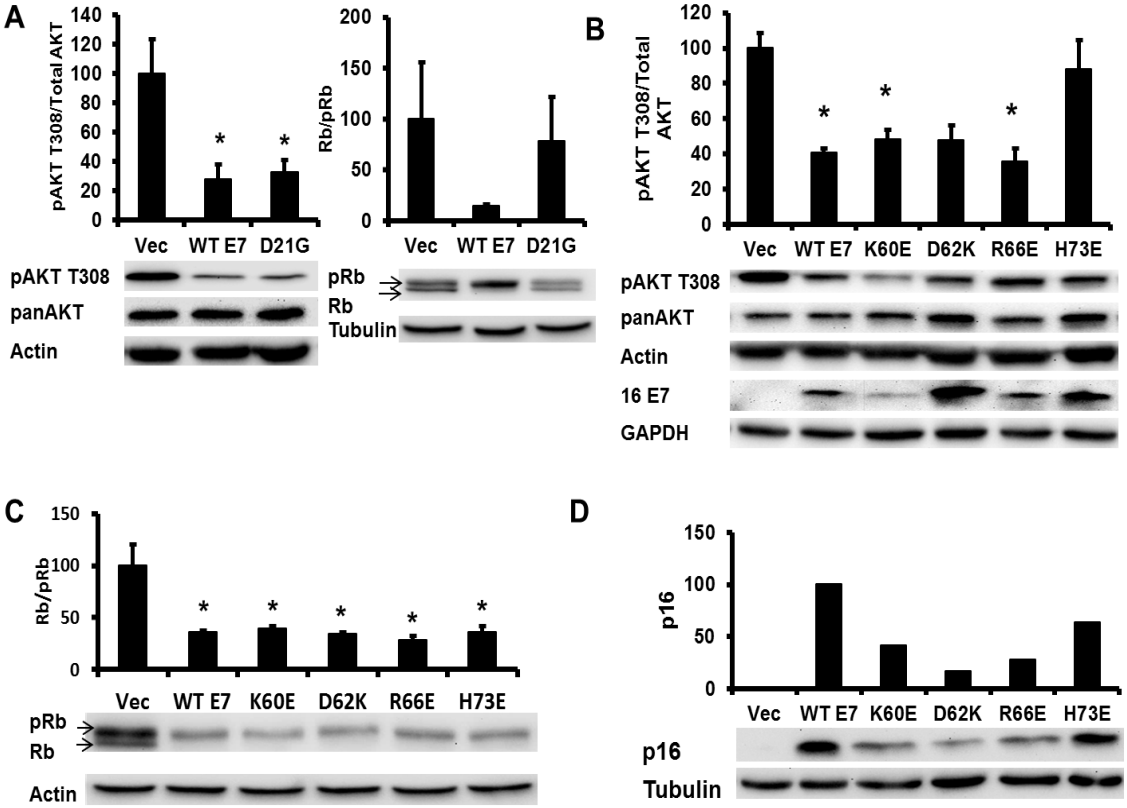

Figure 2.4: Attenuation of pAKT is independent of either p16 induction or Rb degradation by E7 *A:* The pAKT attenuation phenotype is independent of a high affinity LxCxE Rb-binding motif. NIKS retrovirally transduced with WT 16E7 or 16E7 D21G were plated, starved for 12 hours, stimulated for 6 hours, and harvested as in Fig. 1A. Samples were normalized to actin and the highest value. Statistics were calculated from four independent experiments (*=$p<0.05$). *B:* The 16E7 pAKT attenuation phenotype can be ablated by mutation of the C-terminus of E7. Results show the western blot analysis of the C-terminal domain E7 mutants retrovirally transduced into NIKS that were processed as in Fig. 1A. Samples were normalized to actin and the highest value. Statistics were calculated from three independent experiments (*=$p<0.05$). *C:* The AKT attenuation phenotype is independent of the E7 mediated degradation of Rb. Results show the western blot analysis of NIKS retrovirally transduced with E7 zinc-structured domain mutants that were plated and harvested as in Fig. 1A. Samples were normalized to actin and the highest value. Statistics were calculated from three independent experiments (*=$p<0.05$). *D:* The AKT attenuation phenotype is independent of the E7-mediated induction of p16. Results show the western blot analysis of 16E7 mutants retrovirally transduced in NIKS that were plated and harvested as in Fig. 3A. Samples were normalized to actin and the highest value. Shown is an experiment that was representative of two independent experiments.

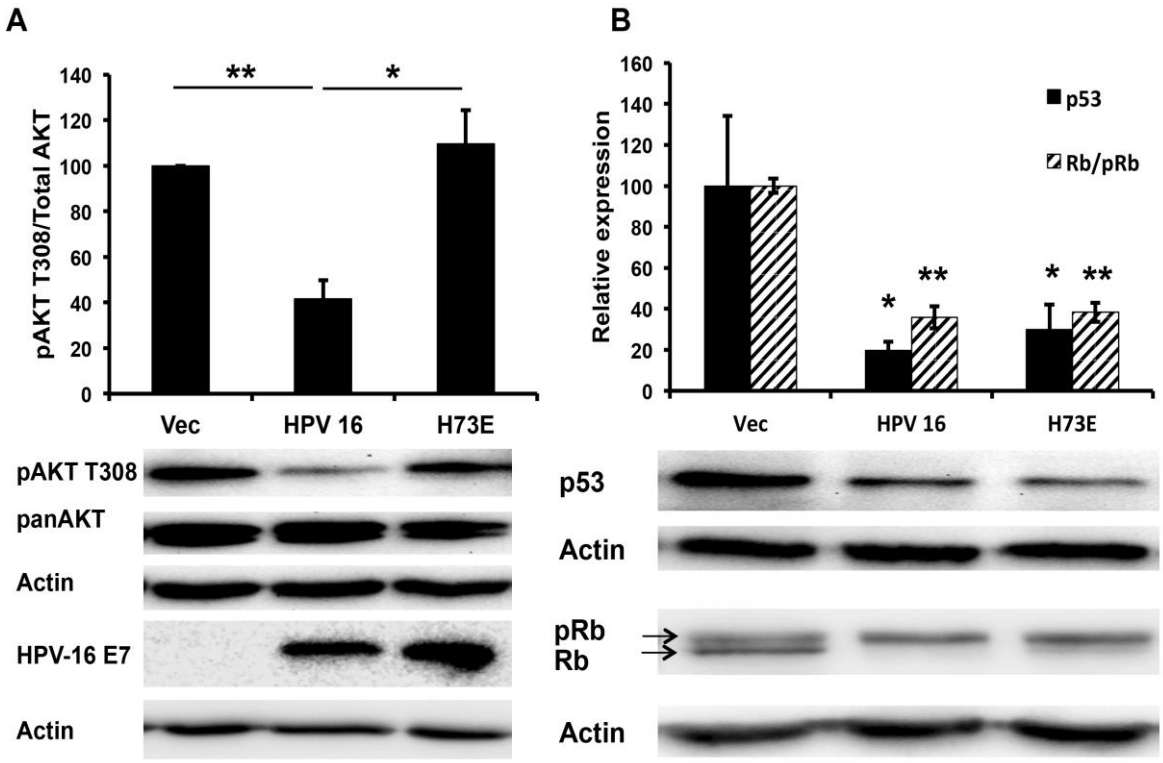

Figure 2.5: HPV-16 E7_H73E reverses pAKT attenuation mediated by HPV-16. *A:* NIKS cells transfected with the HPV-16 genome or HPV-16 E7_H73E genome were plated and harvested as in Fig. 1A. Samples were normalized to actin and the highest value. Statistics were calculated from three independent experiments (*=p<0.05). *B:* HPV-16 E7_H73E NIKS degrade p53 and unphosphorylated Rb to the same extent as HPV-16 NIKS. NIKS cells transfected with the HPV-16 genome or HPV-16 E7_H73E genome were plated and harvested as in Fig. 1A. Samples were normalized to actin and the highest value. Statistics were calculated from five independent experiments (*=p<0.05, **=p<0.01).

HPV-16 E7 alters protein translation

We next investigated the downstream effects of the E7-mediated attenuation of pAKT T308. AKT regulates protein translation through phosphorylation of the downstream effector mTORC1, which subsequently activates and inhibits p70 S6K and 4E-BP1, respectively. The activating phosphorylation site of S6K T389 and the inhibitory phosphorylation site of 4E-BP1 S64 were reduced in 16E7 expressing NIKS (Fig. 2.6). Decreased phosphorylation of 4E-BP1 results in its binding to and preventing eIF4E from associating with the translational initiation complex, resulting in the inhibition of cap-dependent translation (70). Thus, we determined if E7 altered the ratio of IRES to cap-dependent translation. To elucidate an IRES-vs cap-dependent translation ratio, we used a bicistronic reporter containing the Cricket Paralysis Virus (CrPV) IRES (62). In NIKS expressing 16E7 compared to vector transduced NIKS, the IRES to cap-dependent reporter ratio was significantly shifted towards more IRES-dependent translation (Fig. 2.7), suggesting that the decrease in AKT activation, mediated by E7, shifted cellular translation towards IRES-mediated translation.

HPV-16 E7 increases protein expression of IRES containing mRNAs

Many cellular proteins translated under the influence of IRES elements are proteins involved in processes such as apoptosis and mitosis, when signaling from growth factor receptors is low. When cap-dependent translation is decreased, translation machinery is readily available for IRES-dependent translation. IRES containing mRNAs include c-MYC and the Insulin Receptor (54), both of which were increased in 16E7 NIKS compared to vector transduced NIKS (Figs. 2.8A and 2.8B). The

levels of the c-MYC protein were also increased in NIKS containing the complete HPV-16 genome when comparing vector transduced NIKS or NIKS harboring HPV-16 E7_H73E (Fig. 2.8E). While the protein levels were significantly increased, there was no significant change in the RNA levels of Insulin Receptor or c-MYC (Fig. 2.8D). 16E7_H73E, which reversed the pAKT attenuation phenotype, did not enhance the expression levels of c-MYC (Fig. 2.8C). Taken together, these data demonstrate that the pAKT attenuation mediated by 16E7 enhanced the expression of this set of cellular proteins whose mRNAs contain an IRES element. We found that Bax, a Bcl-2 family member, was also enhanced at the translational level in E7 expressing NIKS in a similar manner to Insulin Receptor and c-MYC (Figs. 2.8A-D). Although Bax has not as yet been described as having an IRES element, our data suggest this possibility.

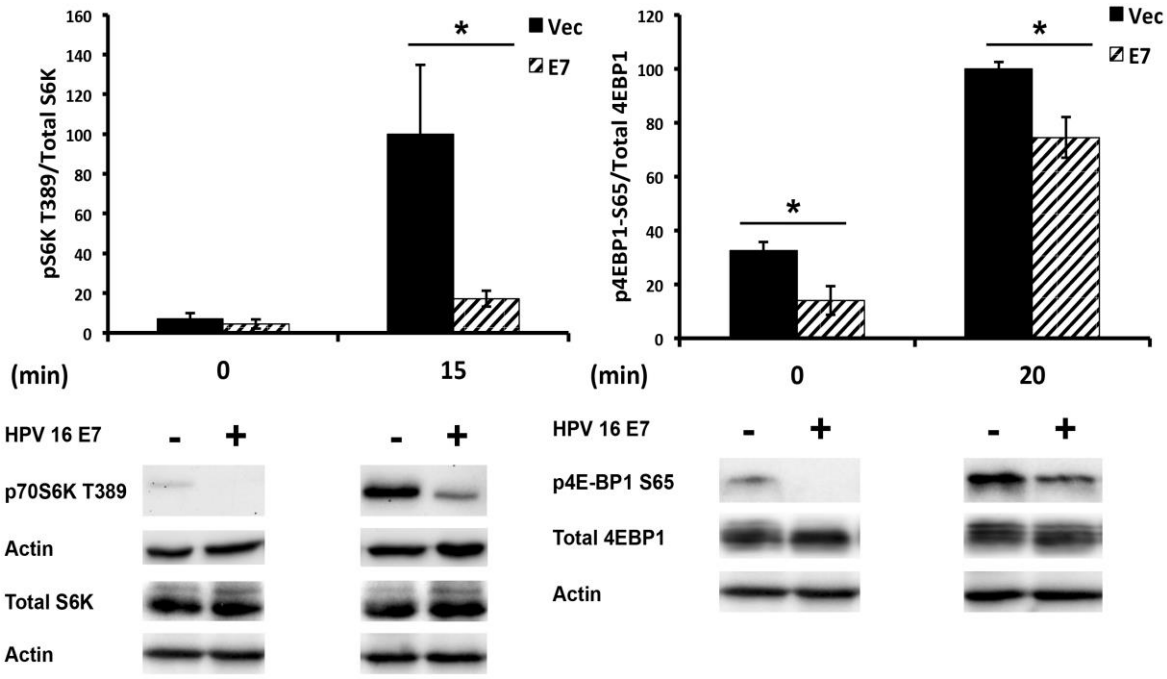

Figure 2.6: HPV-16 E7 decreases phosphorylation of downstream AKT effectors.

Results show the western blot analysis of immortalized keratinocytes retrovirally transduced with HPV 16E7. Transduced NIKS were processed as in Fig. 1A. Western blots are representative of 5 independent experiments. Western blots shown are a single blot exposure with the removal of redundant time points. Statistics were calculated from three independent experiments (*=$p<0.05$).

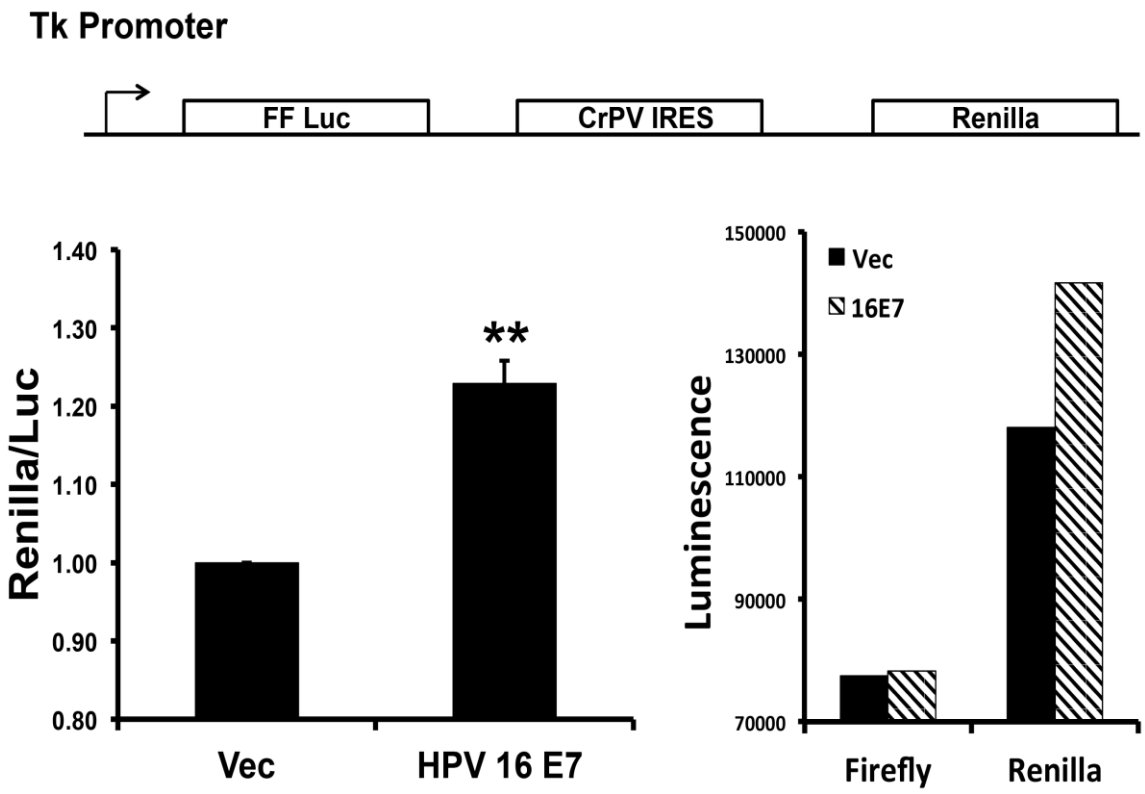

Figure 2.7: Fig. 7: HPV-16 E7 alters cap vs IRES dependent translation. NIKS that were retrovirally transduced and selected to express either vector or 16E7 were transfected with a bicistronic-reporter plasmid (shown at top) and 48 hours later were starved for 12 hours. Following starvation, transduced NIKS were assayed for Firefly and Renilla luciferase activity. Values were normalized to vector transduced cells. Statistics were calculated from three independent experiments each performed in duplicate (**=$p<0.005$).

Figure 2.8: HPV-16 E7 enhances protein expression from cellular IRES containing mRNAs

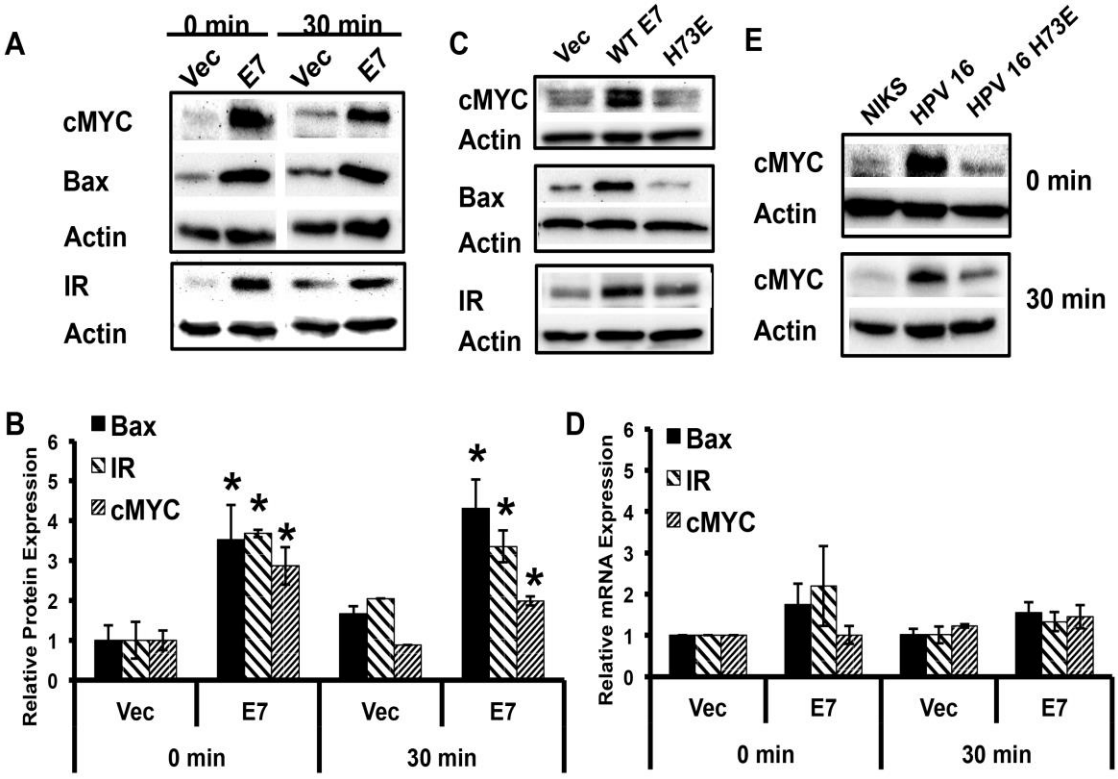

Figure 2.8: HPV-16 E7 enhances protein expression from cellular IRES containing mRNAs *A:* 16E7 enhances the protein expression from three IRES-containing mRNAs. Results show the western blot analysis of transduced NIKS plated and harvested as in Fig. 1A. A representative experiment in a cohort of three independent experiments is shown. *B:* IRES containing mRNAs have significantly enhanced protein levels in 16E7 NIKS cells. Values were normalized to vector-transduced cells. Results shown represent the quantitation of three independent experiments (*=$p<0.05$). *C:* A single point mutation in E7 reverses the enhanced expression of IRES containing proteins. Results show the western blot analysis of retrovirally transduced NIKS that were plated and harvested as in Fig. 1A. Results show a representative experiment at 30 mins from a set of three independent experiments. *D:* Relative RNA levels of IRES containing proteins remain unchanged in 16E7 NIKS. Transduced NIKS were plated as in Fig. 1A and RNA was harvested following a Trizol protocol. Values were normalized to vector-transduced cells. Statistics were calculated from three independent experiments each performed in duplicate and showed no significant differences between samples. *E:* HPV-16 E7_H73E reverses the enhancement of the c-MYC protein observed in HPV-16 transfected keratinocytes. NIKS containing the complete HPV-16 genome or HPV-16 E7_H73E genome were processed as in Fig. 1A. Results show a representative figure from two independent experiments.

Rapamycin treatment does not alter the enhanced expression of IRES-containing mRNAs

Rapamycin treatment inhibits cap-dependent translation through inhibition of mTORC1, leading to the dephosphorylation of both S6K and 4EBP-1 (71, 72). Treatment of 16E7 transduced NIKS in the presence or absence of growth factors with rapamycin did not blunt the enhanced expression of cMYC, insulin receptor or Bax (Fig. 2.9A). Of note, rapamycin treatment of vector transduced NIKS slightly induced the protein expression of cMYC and insulin receptor, over the time course of this experiment, further suggesting that inhibition of cap-dependent translation enhances IRES-dependent translation of these proteins.

When 4EBP-1 is bound to eIF4E, eIF4G is blocked from interacting with the complex to initiate cap-dependent translation. 16E7 increased 4EBP-1 binding to the 7-methyl RNA cap structure, through interaction with eIF4E (Fig. 2.9B), which corresponded to our data showing decreased p4EBP-1 S65 phosphorylation in cells expressing 16E7 (Fig. 2.6). Taken together these data suggest that E7 attenuated p4EBP-1 S65, which led to increased cap-association of 4EBP-1, inhibition of cap-dependent translation, and enhanced expression of IRES containing mRNAs.

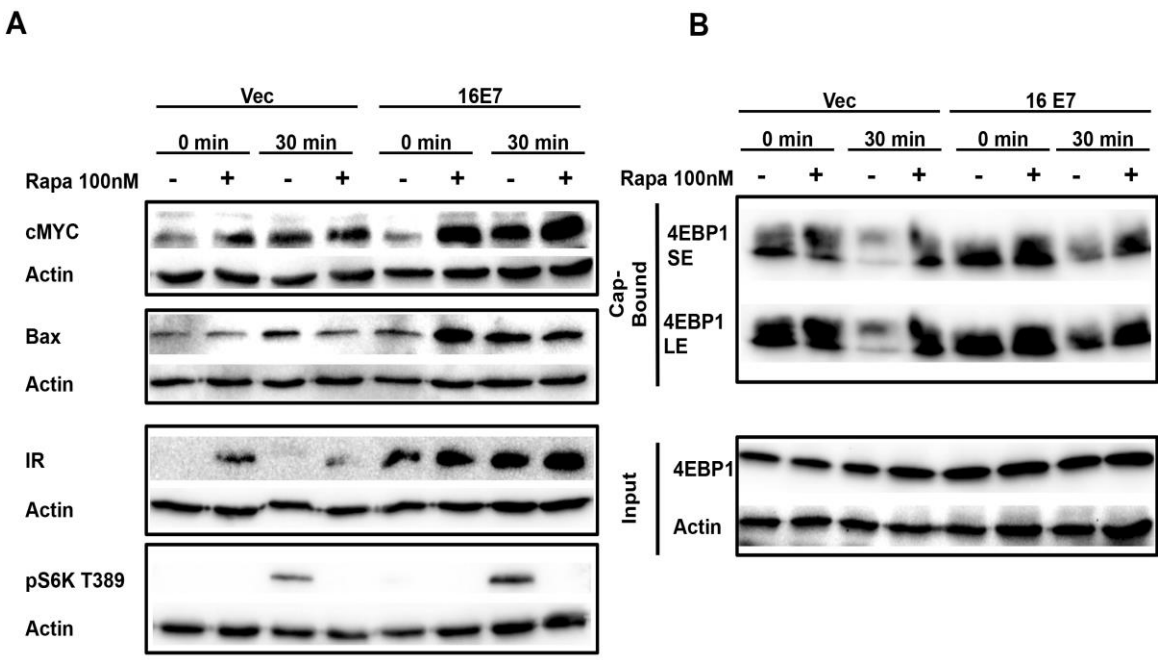

Figure 2.9: 16E7 Enhanced IRES-dependent translation of cellular mRNAs is not blunted by rapamycin treatment. *A:* NIKS cells were plated and harvest as in Figure 1A, except at 9 hour starvation cells were treated with Rapamycin for 3 hours and subsequently stimulated in the presence or absence of Rapamycin. Blots are representative of three independent experiments. *B:* 4EBP1 cap binding is increased in 16 E7 samples. NIKS cells were plated and harvested as in Figure 1A. Blots are representative of two independent experiments. SE: Short Exposure, LE: Long Exposure.

AKT transcription is unchanged in E7 expressing keratinocytes

Enhanced AKT signaling promotes keratinocyte differentiation, and pharmacologic inhibition of AKT signaling reduces keratinocyte differentiation (73, 74). Paralleling our results, another group has shown a down regulation in AKT1 mRNA mediated by cutaneous HPV-8 E2 (41). They hypothesized this decrease in AKT1 altered the normal keratinocyte differentiation pathway that weakened the top epidermal layer to promote viral release. However, we did not observe a significant decrease in the expression of AKT1 mRNAs or a significant increase in the expression of AKT2 mRNAs (Fig. 2.10).

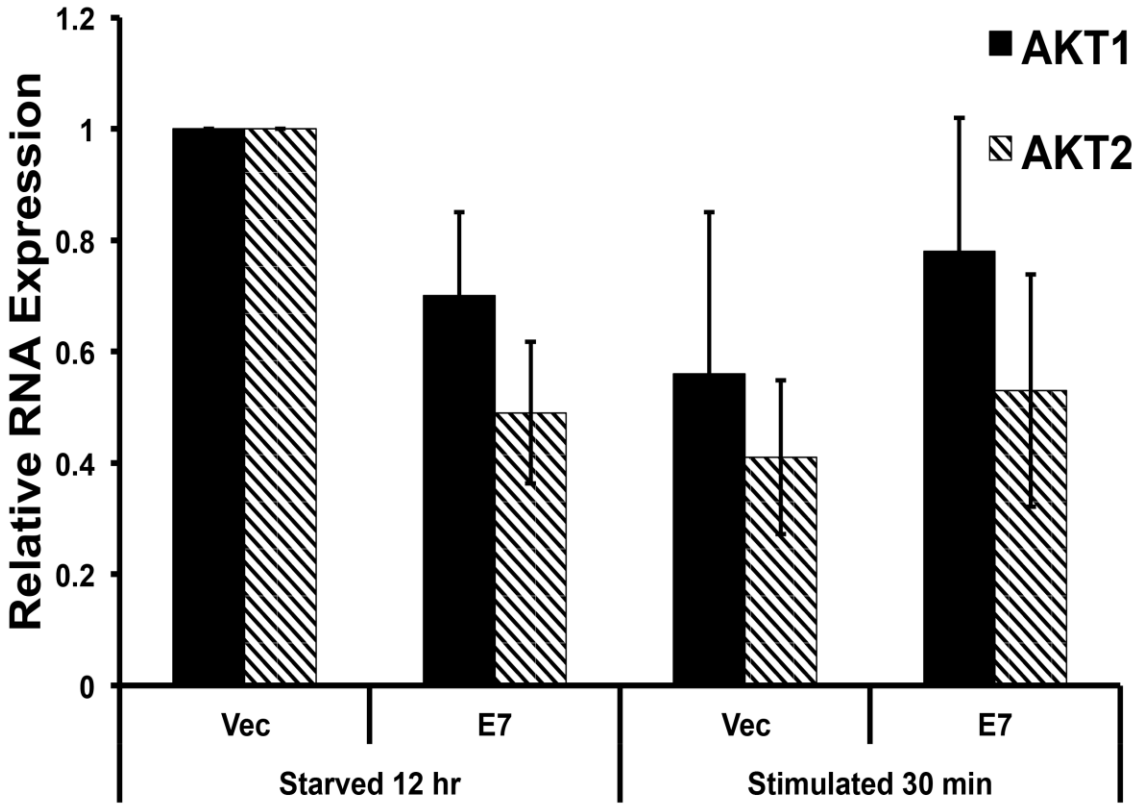

Figure 2.10: AKT RNA levels are unchanged in E7 keratinocytes. Retrovirally transduced keratinocytes with 16E7 were plated and harvested as described in Fig. 1A, except total RNA was harvested following a Trizol protocol. Statistics were calculated from three independent experiments and showed no significant difference between the samples.

Discussion

Because activated PI3K has been described as characteristic of invasive cervical cancers (75), we expected to observe activation of AKT by the HPV-16 genome. To our surprise, we have shown that HPV-16 attenuates pAKT T308 and that this phenotype is mapped to E7 alone (Fig. 1). Several other human and animal papillomavirus E7s also exhibit this phenotype including, HPV-18 E7, HPV-1 E7 and Cottontail Rabbit Papillomavirus E7 (Fig. 3).

In contrast to our results, previous studies have shown that 16E7 augments the activation of AKT, promoting an increase in downstream signaling (38, 39). However, in our hands we observe that both high- and some low-risk E7s attenuate AKT signaling. We realize that experimental differences such as culturing keratinocytes in media with serum, mitomycin C treated 3T3 cells, and calcium (F media) compared to serum-free and low calcium media formulations (KSFM) may contribute to the variance in observations. In the first study (38), the effects of E7 were accessed in HEK 293 cells transiently transfected with E7, thus the effects of E7 in that system must be interpreted in the context of E7 time course, E7 expression levels, the co-expression of adenoviral oncoproteins, and non-keratinocyte cell lines (38). In the second study (39), the effects of E7 alone in organotypic cultures were examined and pAKT was accessed using phosphospecific antibodies to serine 473 (39). Although we show parallel effects on pAKT S473 vs. T308 (Fig. 1), we chose to ascertain T308 phosphorylation because it is the initial activating phosphorylation that enables subsequent S473 phosphorylation. Since both T308 and S473 phosphorylations are required for full AKT activation (32), the

decrease of T308 phosphorylation in E7 expressing cells represents the activation state. Reinforcing our interpretation is the observed decrease in the phosphorylation of both S6K and 4E-BP1 (both targets of AKT signaling) in cells expressing 16E7 (Fig. 6). Studies by another group showed that HPV-16 E6 augments AKT and mTORC1 signaling to promote cap-dependent translation (37, 76). As noted in the results section, the ability of 16E6 to augment pAKT is manifested in low-calcium media (KSFM), a media formula that alters cell adhesion and ablates adherens junctions and stratification. In our system, the pAKT attenuation phenotype from E7 persists even in the presence of E6 (Figs. 2E and 2F) and in keratinocytes expressing the full HPV-16 genome (Fig. 1A). This suggests that any role E6 may be playing in augmenting mTORC1 signaling and cap-dependent translation can be overcome by the presence of E7 in our system.

Our mutational analysis revealed a single point mutant, H73E, that failed to attenuate pAKT and was independent of the Rb degradation function of E7 (Fig. 4). In other studies, 16E7 H73E has been shown to dimerize in vivo and transform baby rat kidney cells in cooperation with activated Ras to the same extent as WT E7, however no biologic function has been associated with this residue until our findings (30, 77). H73E is conserved only with HPV-1 E7, while HPV-18 and Cottontail Rabbit Papillomavirus E7, which also suppress pAKT, have alternate amino acids at this position. This suggests H73E could be one among several amino acids that contributes to the phenotype. Further studies are required to fully delineate the surfaces of E7 involved in the AKT attenuation phenotype.

While there are many cellular functions regulated by AKT signaling, our initial focus was on translational regulation. We showed that two indirect downstream targets of AKT, S6K and 4E-BP1, had reduced phosphorylation at their activation and inhibitory sites, respectively (Fig. 2.6). 16E7 alters the translation of a specific subset of proteins including c-MYC and insulin receptor (Fig. 2.8), which were identified as containing 5' IRES elements (54). Other groups have shown that in cells with increased PTEN activity, AKT phosphorylation is reduced, and c-MYC translation under the influence of its IRES elements is increased (78), which is consistent with our results. We hypothesize that induction of c-MYC could alter the expression of a c-MYC target gene set, which could influence metabolism, cell survival, protein translation, or proliferation (36). If metabolism genes are in fact upregulated by the enhanced protein expression of c-MYC, this could contribute to the induction of the Warburg effect mediated by E7 (79). The enhanced expression of Bax could also contribute to the known phenotype of E7 sensitization of keratinocytes to apoptosis (80). Like c-MYC, the vascular endothelial growth factor (VEGF) also contains a 5' IRES element in its mRNA. 16E7 transduced keratinocytes secrete more VEGF than vector transduced keratinocytes (81), and our work suggests a potential mechanism for increased protein translation of VEGF in 16E7 keratinocytes. Increased AKT activity has also been shown to decrease IRES-dependent translation of VEGF (82), which corresponds to our model of attenuated AKT signaling augmenting IRES-dependent translation. Taken together, these studies suggest that VEGF could also be an IRES-influenced protein augmented by the E7-mediated AKT attenuation phenotype we have described.

Beyond Papillomaviruses, there is precedence for viral oncogenes manipulating AKT activation. In Polyomavirus, Small t (PyST) has recently been shown to block differentiation by binding to and recruiting the activity of PP2A to AKT (45). In contrast, Simian virus 40 (SV40) small and large T antigens together immortalize keratinocytes, promote anchorage independent growth, and activate AKT; SV40 Large T antigen has been implicated as well in the activation of AKT (83). These disparate effects upon AKT signaling must be interpreted in light of the variable and combined effects of these viral oncoproteins. Although HPV-16 E7 has been reported to interact with PP2A leading to the activation of AKT (38), these results have been controversial, with a recent study finding no association of 16E7 with PP2A and no findings of PP2A components as E7 associated proteins (84-86). Finally, the manipulation of AKT by HPV-8 E2 has been reported (41); in that study E2 repressed the mRNA abundance of AKT. Although an interesting parallel observation, we did not observe significant changes in AKT1 or AKT2 RNA levels in E7 expressing cells (Fig. 2.10).

As noted above, PI3K is activated in most cervical cancers (87). However, our data present a paradox, in that enhanced AKT activation that is observed in invasive cancers would be predicted to drive keratinocyte differentiation. We can thus speculate that during the progression of HPV-16 infected keratinocytes from low grade to high grade lesions, which changes in the effect of AKT upon cellular differentiation might precede any enhancement of AKT activation, changing AKT from a driver of keratinocyte differentiation to a driver of cancer cell phenotypes. More experiments are required to fully develop this hypothesis.

We have shown that HPV-16 attenuates pAKT T308 and this can be mapped to 16E7. We have also shown both high and low-risk E7 types attenuate pAKT and this phenotype is independent of the Rb degradation function of E7. The AKT attenuation phenotype can be ablated by a single point mutation within the carboxy-terminus of E7 and is not dependent upon the E7 mediated induction of demethylases to induce epigenetic reprogramming. The phosphorylation of two downstream proteins of AKT (S6K and 4E-BP1) is attenuated, leading to a shift in protein translation towards IRES-dependent translation. The shift enhances the protein translation of several cellular proteins without altering their transcription, including c-MYC, insulin receptor and Bax. The AKT attenuation is a novel phenotype of E7 and demonstrates a role for E7 in the manipulation of signal transduction cascades, thereby altering the regulation of cellular translation.

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Chapter 3: Characterization of E7 H73E Mutant

Introduction

The 16E7 H73E mutation was first described in a study that was elucidating the role the C-terminus of E7 played in transformation and Rb-binding and degradation (30, 77). While these studies identified novel binding sites for Rb in the C-terminus of E7, H73E was found to have no effect on Rb-binding, E7 dimerization or transformation (30), which is consistent with our observations that Rb-degradation is unchanged when compared to WT 16E7 (Fig 2.4C). No known function has currently been assigned the H73 residue, which consists of a conserved alpha helix structure. We plan to fully characterize how this mutant is altering E7-mediated functions upon the viral life cycle and cellular signaling cascades, including PI3K/AKT signaling.

We have shown that E7 modulates the AKT signaling cascade in order to alter cellular translation (Fig. 2.6-2.8). However, AKT plays a vital role in various cellular processes that we have yet to examine. PI3K/AKT signaling plays a key role in keratinocyte differentiation, as activated AKT signaling is correlated with increased keratinocyte differentiation (73). Phosphorylation of AKT is enhanced during calcium-induced keratinocyte differentiation, while knockdown of AKT1 in keratinocytes blocks stratification and late differentiation in organotypic raft cultures, suggesting AKT is necessary for differentiation to occur. Pharmacologic blocking of PI3K activity in primary keratinocytes also inhibits the expression of late-term keratin differentiation markers (73, 74). Taken together these results suggest that AKT is necessary for and drives keratinocyte differentiation.

HPV-16 has been previously shown to delay keratinocyte differentiation through inhibition of cell cycle withdrawal in the suprabasal keratinocyte layer (88). 16E7 alone blocks expression of keratinocyte differentiation markers, keratin-10 and Involucrin (89). By delaying differentiation and the expression of keratinocyte differentiation markers, E7-expressing suprabasal keratinocytes retain the ability to transit the cell cycle, in order to amplify the viral genome. In high-risk infections, such as HPV-16, stratifying keratinocytes show a collection of visual traits in biopsies and cytologies that are referred to as dysplasia. This categorization includes features such as smaller cell size, increased nuclear-to-cytoplasmic (N/C) ratio, and increased, abnormal suprabasal mitotic figures (90, 91). Traditionally, pathologists categorize cervical lesions in three distinct categories, cervical intraepithelial neoplasia (CIN) I, II, or III (92), where the dysplastic features worsen when progressing from CIN I to CIN III. Historically, it has been shown that CIN I lesions contain episomal HPV-16 viral DNA, while CIN III lesions often contain integrated viral DNA, suggesting that the more dysplastic phenotype is a direct result of increased expression of E6 and E7 (93, 94). However, CIN III lesions and invasive cervical carcinomas have also been shown to have episomal high-risk HPV DNA as well as integrated DNA (93), suggesting there are other factors necessary to progress from a low-grade CIN I lesion to invasive cervical carcinoma other than the expression levels of E6 and E7.

Historically, the dysplastic effects seen in keratinocytes infected with HPV-16 have been linked to Rb degradation by E7, as E7 has been shown to be the main driver of transformation in transgenic mouse models (95). However, more recent work has

shown that degradation of Rb and Rb family members is not sufficient to induce cervical cancer (96). This suggests E7 has more roles in initiating cervical cancer progression than just degrading Rb, p107, and 130. We hypothesize that the attenuation of pAKT induce by 16E7 and other E7 types is contributing to the dysplastic phenotype observed in HPV-16 E7 transformed keratinocytes.

In order to investigate the role H73E is playing in AKT-induced keratinocyte differentiation and dysplastic progression, we investigated how H73E modulated keratinocyte differentiation and viral episomal maintenance, as this plays a key role in the dysplastic phenotype of keratinocytes.

Results

HPV-16 E7_H73E reverses some dysplastic features of keratinocytes infected with HPV-16

HPV-16 has been shown to inhibit keratinocyte differentiation and induce dysplastic features in stratifying keratinocytes (88). We observed similar effects where dysplasia was apparent in NIKS transfected with the HPV-16 genome (Fig. 3.1). HPV-16 organotypic raft cultures showed statistically significant increases in hyperplasia (Fig. 3.2A), the nuclear-to-cytoplasmic (N/C) ratio (Fig. 3.2B), mitotic figures (Fig. 3.2C), and a decrease in cell size in comparison to NIKS cells (Fig. 3.2D). Introduction of the 16E7_H73E mutation into the complete HPV-16 genome failed to induce the severe dysplasia observed in the HPV-16 rafts in that the N/C ratio, mitotic figures and cell size were not statistically different than control NIKS values (Fig 3.2B-D). HPV-16 also represses the expression of the early differentiation marker keratin 10 (97). Keratin 10 is normally expressed in the first suprabasal keratinocyte cell layer (97) as is seen in differentiated NIKS cells (Fig. 3.1). In keratinocytes containing HPV-16, there is a diminution in overall keratin 10 expression and a delay in expression, in that keratin 10 is not observed in the first 2-3 suprabasal cell layers (97) (Fig. 3.1). HPV-16 H73E raft cultures showed expression of keratin 10 within the first suprabasal layer, similar to NIKS raft cultures (Fig. 3.1). HPV-16 E7_H73E raft cultures also showed exaggerated stratification when compared to HPV-16 raft cultures, as demonstrated by the larger cell size and more elongated cell shape (Figs. 3.1 and 3.2D). Activated AKT has been shown to promote keratinocyte differentiation (73, 74), and our data suggest that failure of

HPV-16 H7E3 to attenuate AKT could be promoting premature keratinocyte differentiation of HPV-16 E7_H73E raft cultures when compared to the wild type HPV-16.

HPV-16_H73E is not maintained as a viral episome

In order to replicate and amplify, the viral DNA must be maintained as an episome within stratifying keratinocytes. We next examined if HPV-16 and HPV-16 E7_H73 were maintained as viral episomes. Southern blot analysis of HIRT lysates revealed that HPV-16 WT was maintained episomally, but HPV-16_H73E was not (Figure 3.3A), which suggested an integration event had occurred or the episome had been lost entirely. Genomic DNA harvested from NIKS cells containing HPV-16 E7_H73E showed no episomal or integrated DNA present in HPV-16_H73E (Figure 3.3B), but these cells expressed E7 protein (Fig 2.5A) and degraded Rb and p53 to the same extent as WT HPV-16 (Fig 2.5B), suggesting HPV DNA is present, but at very low copy number.

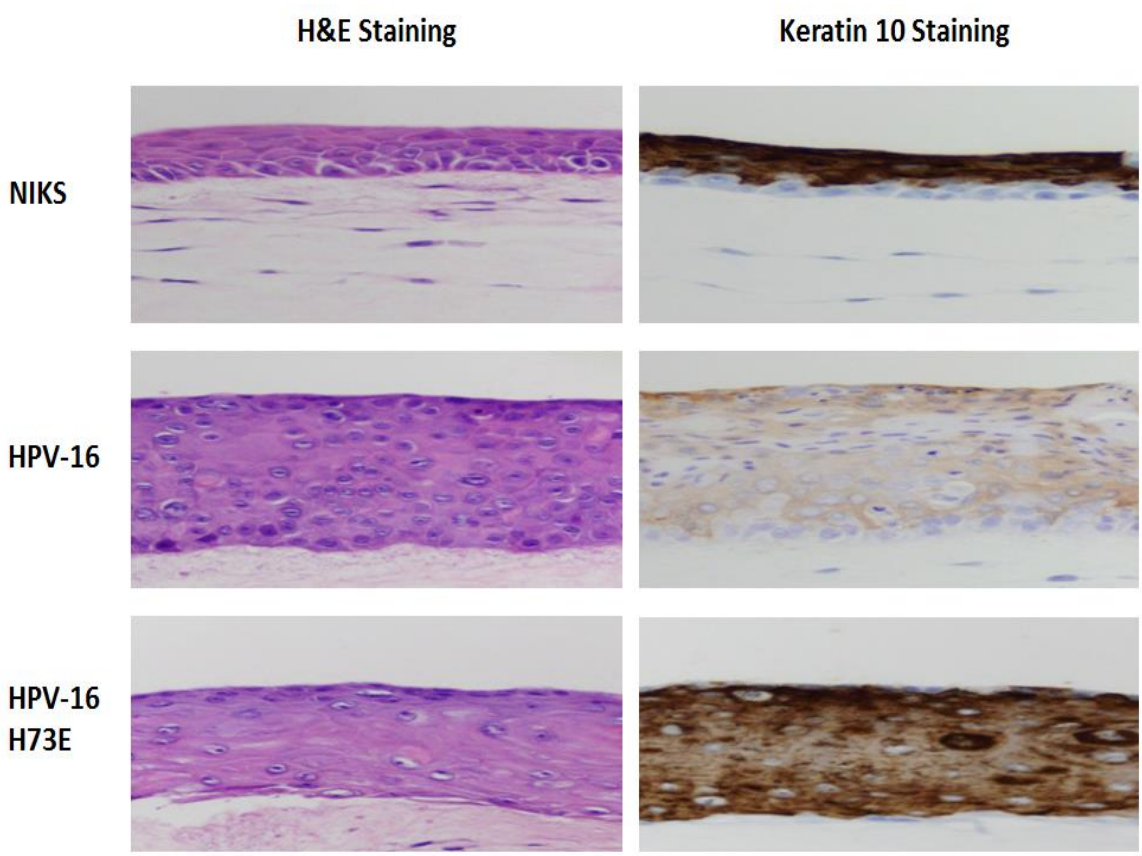

Figure 3.1: HPV-16 E7_H73E reverses dysplasia induced by HPV-16. NIKS cells transfected with the HPV-16 genome or HPV-16 H73E were plated on collagen matrix and were then raised to the air-liquid interface to induce stratification and differentiation. Fourteen days later, rafts were formalin fixed, sectioned, and stained with H&E (left) and Keratin 10 (right).

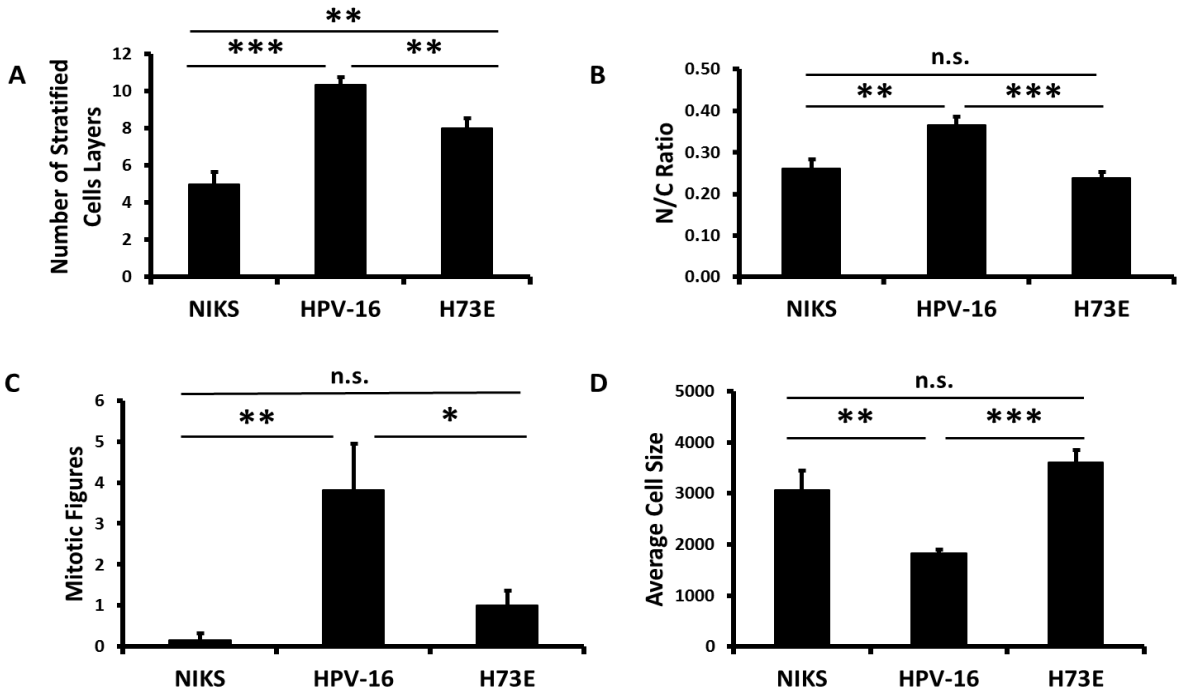

Figure 3.2: HPV-16 induces dysplasia in organotypic raft cultures that is reversed by E7_H73E mutation. *A:* HPV-16 causes epithelial hyperplasia that is reversed by the 16E7_H73E mutation. The graph quantifies the number of cell layers from the basal layer to the most stratified cells in H&E stained organotypic raft cultures (**=p<0.01, ***=p<0.001). *B:* HPV-16 enhances the Nuclear-to-Cytoplasmic ratio, which is reversed by the 16E7_H73E mutation. The graph represents the nuclear-to-cytoplasmic ratio, measured as area of the nucleus/area of the entire cell using ImageJ software, of greater than 25 cells from 3 individual sections of H&E stained organotypic raft cultures (**=p<0.01, ***=p<0.001). *C:* HPV-16 increases mitotic figures, which is reversed by the 16E7_H73E mutation. The graph counts the number of mitotic figures present in H&E stained organotypic raft cultures (*=p<0.05, **=p<0.01) from 3 separate sections and 6 fields. *D:* HPV-16 reduces cell size, which is reversed by the 16E7_H73E mutation. The area of greater than 25 individual cells from 3 independent H&E stained organotypic raft cultures were measured using ImageJ software (**=p<0.01, ***=p<0.001).

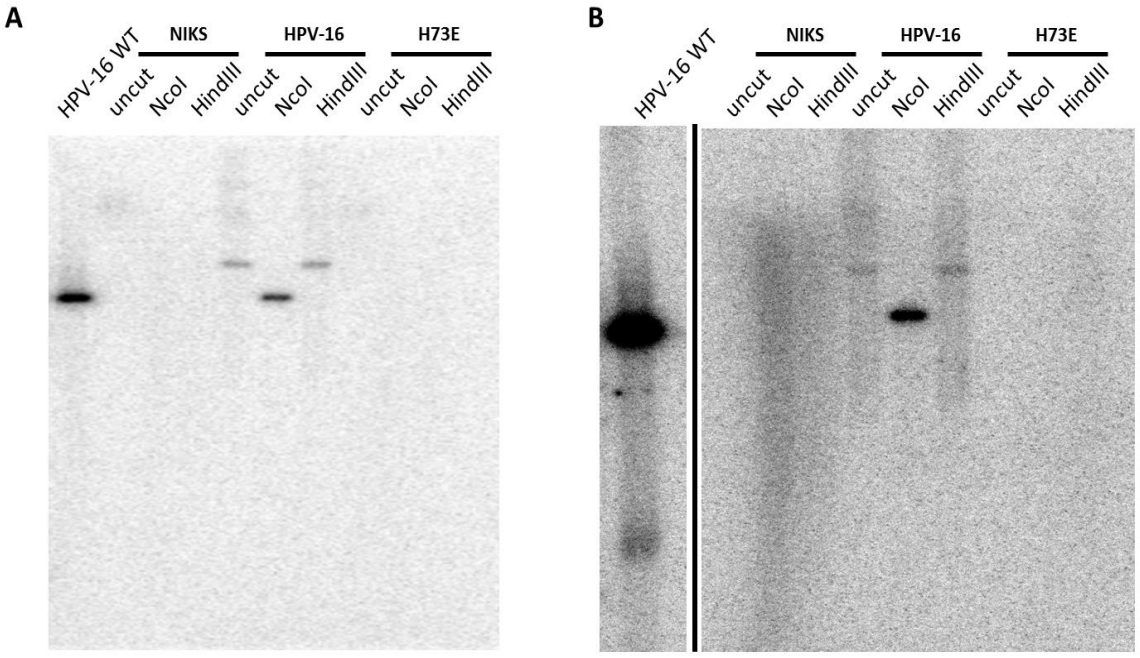

Figure 3.3: HPV-16_H73E is not maintained as a viral episome. *A:* HPV-16_H73E is not maintained episomally. NIKs cells were lysed in HIRT lysis buffer, and DNA was harvested and run on an agarose gel that was transferred and hybridized with a radioactively labeled HPV-16 probe. NcoI cuts HPV-16 DNA once giving rise to an 8kbp band, while HindIII does not cut HPV-16 DNA, which then co-migrates with uncut HPV DNA. *B:* HPV-16_H73E is not integrated at high copy number. NIKS cells were lysed and genomic DNA was precipitated with isopropanol. The black bar represents the removal of irrelevant samples. DNA was digested with Nco1 (cuts HPV DNA once), HindIII (does not cut HPV DNA) or uncut.

Discussion

HPV-16 has been shown to reduce keratinocyte differentiation (88). HPV-16 induces dysplastic features including, increased N/C ratio, increased mitotic figures and increased hyperplasia, while the HPV-16E7_H73E organotypic raft cultures have reduced dysplastic features that are not statistically different than NIKS control raft cultures (Figs. 3.1 and 3.2), despite maintaining E7 expression and the capacity to target Rb and p53 degradation (Fig. 2.5B). Our data show that the dysplasia induced by E7 expression requires the carboxy-terminus of E7 and is not exclusively the result of E7 interactions with Rb family members (98, 99); this defines a novel phenotype of the C-terminal domain of E7. It is unclear how many functions of E7 contribute to the collection of visual traits referred to historically as dysplasia. Targeted degradation of Rb is still observed in keratinocytes harboring HPV-16 where the E7 H73E mutation is present (Fig. 2.5B). Yet rafts of HPV-16 E7 H73E show reversion of several dysplastic features (Figs. 3.1 and 3.2) compared to keratinocytes harboring WT HPV-16. Rb degradation alone is not sufficient to induce dysplasia in this culture system as HPV-16 E7_H73E still degrades Rb. It has been shown previously that deletions of either CR1 or CR2 induced normal keratinocyte differentiation when compared to WT E7, which decreased the expression of the keratinocyte differentiation markers, Keratin 10 and Involucrin (99). The H73E mutation could have identified another E7 domain that is necessary to disrupt normal keratinocyte differentiation. It is as yet unclear if the effect of 16E7 upon pAKT is directly responsible for dysplasia or if the changes in pAKT enable other E7 functions that then contribute to dysplasia. Further studies are planned to elucidate the

mechanism by which E7 attenuates AKT signaling to diminish keratinocyte differentiation.

While the failure of HPV-16 E7_H73E to induce dysplasia is interesting, the fact that HPV-16 H73E is not maintained as an episome confounds these findings. While we know that the E7 protein is expressed at similar levels from HPV-16 and HPV-16 E7_H73E (Fig 2.5A), and we know that Rb and p53 is also degraded in both cell lines (Fig 2.5B), the effects of E7 expression from an episomal plasmid compared to expression from a potentially integrated plasmid are unknown, as are differences in other virally encoded functions that may be altered because of episomal compared to integrated viral genomes. However, HPV-16 E7_H73E could be episomal but at low copy number and undetectable via our current method. Other methods will be attempted, including PCR, to identify if HPV-16 H73E is episomal or integrated. A comparison where both WT E7 and E7 H73E are both expressed in a similar fashion would be ideal to elucidate the effect of E7 H73E upon keratinocyte differentiation. This could be accomplished through expression of WT E7 and E7 H73E from retroviral vectors rather than the full HPV-16 genome.

HPV-16 and 18 causes over 70% of the cervical cancers diagnosed in the US (100) and of those cervical cancers approximately 75-80% harbor integrated HPV DNA within their cellular genome (101, 102). However, the mechanism by which HPV-16 genomes integrate in cervical cancer lesions is largely unknown. In three independent experiments, HPV-16 E7_H73E was not maintained as an episomal plasmid, while WT HPV-16 was maintained episomally (Fig. 3.3). This could suggest a mutation within E7

that causes a disruption in the replication efficiency of the viral episome. However, this seems unlikely as HPV-16 harboring an E7-null mutation can still be maintained as an episome in NIKS cells (103). The loss of episomal maintenance could also be correlated with the inability of H73E to attenuate pAKT. Nearly 80% of invasive cervical cancers have a mutation within the PI3K/AKT signaling pathway that would be predicted to increase AKT activation (87) and a similar number, 75-80% of cervical cancers, harbor integrated viral episomes. It would be interesting to know whether cervical lesions that contain episomal HPV also harbor PI3K/AKT mutations. This might also be elucidated by constitutively activating AKT and observing if this was sufficient to drive integration of WT HPV-16. If so, a mutation which suppressed pAKT could potentially rescue the episomal loss observed in HPV-16 E7_H73E. Further studies are planned to elucidate these possibilities.

As noted previously, PI3K and AKT are activated in most cervical cancers (87). We have argued here that AKT activation drives keratinocyte differentiation and that E7 represses AKT activation in order to block keratinocyte differentiation. We hypothesize that the observed pAKT T308 repression by E7 is part of the productive HPV-16 life cycle. This presents a paradox, in that enhanced AKT activation that is observed in invasive cancers would be predicted to drive cellular differentiation. We can thus further speculate that during the progression of HPV-16 infected keratinocytes from low grade to high grade lesions, that changes in signaling that alter the effect of AKT upon cellular differentiation might precede the enhancement of AKT activation, changing AKT

from a driver of differentiation to a driver of cancer cell phenotypes. More experiments are required to fully develop this hypothesis.

Methods

Organotypic Raft Cultures: HPV-16 NIKS were seeded onto a collagen plug for two days and then transferred to a wire mesh for two weeks to create an air-liquid interface as described previously (56). Raft cultures were then removed from the wire mesh, formalin fixed, embedded in paraffin, sectioned, and stained with H&E or processed for immunohistochemistry. Sectioning and H&E staining was performed by the University of Virginia Research Histology Core and the immunohistochemistry was performed by the University of Virginia Biorepository and Tissue Research Facility (BTRF).

DNA Extractions: HIRT lysates were prepared following a HIRT lysis protocol. Keratinocytes were lysed with HIRT lysis buffer (0.6% SDS, 10mM EDTA pH 7.5) at room temperature and equilibrated for 20 minutes. 5M NaCl was added to lysates at ¼ volume and incubated overnight at 4^{O}C. Lysates were centrifuged 18,000 rpm for 40 minutes, iced for 30 minutes, and centrifuged again for 40 minutes. Lysates were phenol/chloroformed extracted and DNA was precipitated overnight with sodium acetate and isopropanol.

Genomic Lysates were prepared following a Fast Genomic DNA Preparation. Keratinocytes were lysed in genomic lysis buffer (100mM Tris pH 8.5, 5mM EDTA, 0.2% SDS; 200mM NaCl, 100 ug/mL Proteinase K) for 3 hours and then genomic DNA was precipitated with isopropanol.

Southern Blots: DNA harvested following HIRT or genomic lysate protocols was digested with NcoI (cuts HPV-16 once), HindIII (does not cut HPV-16), or uncut. DNA was separated via electrophoresis and transferred to a GeneScreen membrane overnight.

DNA was then crosslinked to the GeneScreen membrane and hybridized to a radioactively labeled probe specific for HPV-16 overnight at 65^{O}C. The membrane was then washed under high stringency conditions and imaged with a phosphorimager.

Chapter 4: HPV 16 and 16E7 modulation of YAP localization

Introduction

Hippo signaling is a critical pathway that controls cell proliferation, apoptosis and differentiation (reviewed in (104, 105)). The pathway was first discovered in *Drosophila melanogaster* as a regulator in body and organ size through tight control over cell proliferation mechanisms (106-109). Hippo signaling negatively regulates cell proliferation, when activated, through a kinase cascade that includes Mst1 (mammalian orthologue of hippo), Sav (mammalian orthologue or Salvador) and Lats (mammalian orthologue of Warts) (106-110). Mutations of any of these proteins results in loss of the negative regulation of cell proliferation. Inactivated Hippo signaling results in proliferative disorders, including cancer (106, 110).

One important component of the Hippo signaling pathway is the Yes-Associated Protein (YAP), a transcriptional co-activator that enhances the expression of genes necessary for proliferation and cell cycle (111-113). As a co-activator, YAP does not interact with DNA on its own, rather it relies on association with TEAD family members to bind DNA and transcriptionally regulate cell proliferation genes (112, 114). Overexpression of YAP in mammary epithelia results in the loss of contact inhibition and the induction of the epithelial-to-mesenchymal transition (EMT) (111). Based on this phenotype, YAP is an oncogene as it can drive cell proliferation in the presence of growth inhibitory signals such as cell-cell contacts. Overexpression of YAP has been found in many cancers including breast, colon and bladder cancer (115-117).

Because YAP is a transcriptional co-activator, it must be located within the nucleus to have transcriptional effects. YAP is regulated through phosphorylation by

large tumor suppressor (Lats), which is a kinase within the Hippo signaling pathway (118, 119). Phosphorylation by Lats promotes the association of YAP with 14-3-3 proteins and sequestration in the cytoplasm (120, 121). Phosphorylation by Lats and subsequent phosphorylation by a second kinase CK1, promotes the degradation of YAP, another mechanism to inhibit its translocation to the nucleus (120).

The Hippo signaling pathway is regulated by the PI3K signaling cascade (122). When growth factors are present and activating receptor tyrosine kinases, PI3K is active and coverts PIP2 to PIP3, which recruits PH domain containing proteins like PDK1 to the cell membrane. During serum starvation, PDK1 is associated with Hippo signaling proteins Mst1, Sav1 and Lats (Fig. 4.1). The association of PDK1 with this complex activates it, and Lats phosphorylates YAP, which sequesters YAP into the cytoplasm thus inhibiting its transcriptional activity. When PDK1 is recruited to the plasma membrane upon serum stimulation, the Hippo complex is inhibited, YAP is not phosphorylated, and translocates into the nucleus to associate with the TEAD family members and alter transcription of proliferation genes (122).

AKT has been shown to directly interact with and phosphorylate YAP in a similar manner to Lats (123), which resulted in the sequestration of YAP in the cytoplasm. However, this finding is controversial as more recent papers have negated the claim of AKT directly phosphorylating YAP at serine 127 (124). The role of AKT in regulating YAP specifically remains unclear and cell type dependent.

YAP has been shown to play a key role in keratinocyte differentiation (125). In this study, nuclear YAP regulates the proliferative ability of keratinocyte stem cells. YAP

must be inactive and sequestered into the cytoplasm in order for keratinocytes to terminally differentiate (125). In keratinocytes, YAP balances the growth and proliferation of the basal cell layers with the promotion of terminal keratinocyte differentiation in the suprabasal cell layers. This study utilized a YAP mutant that could not be phosphorylated and thus was unable to be sequestered in the cytoplasm. By constitutively translocating YAP to the nucleus, keratinocyte proliferation was increased and the expression of keratinocyte differentiation markers was decreased. This is very similar to the effects of HPV-16 on keratinocyte proliferation and differentiation (88, 89).

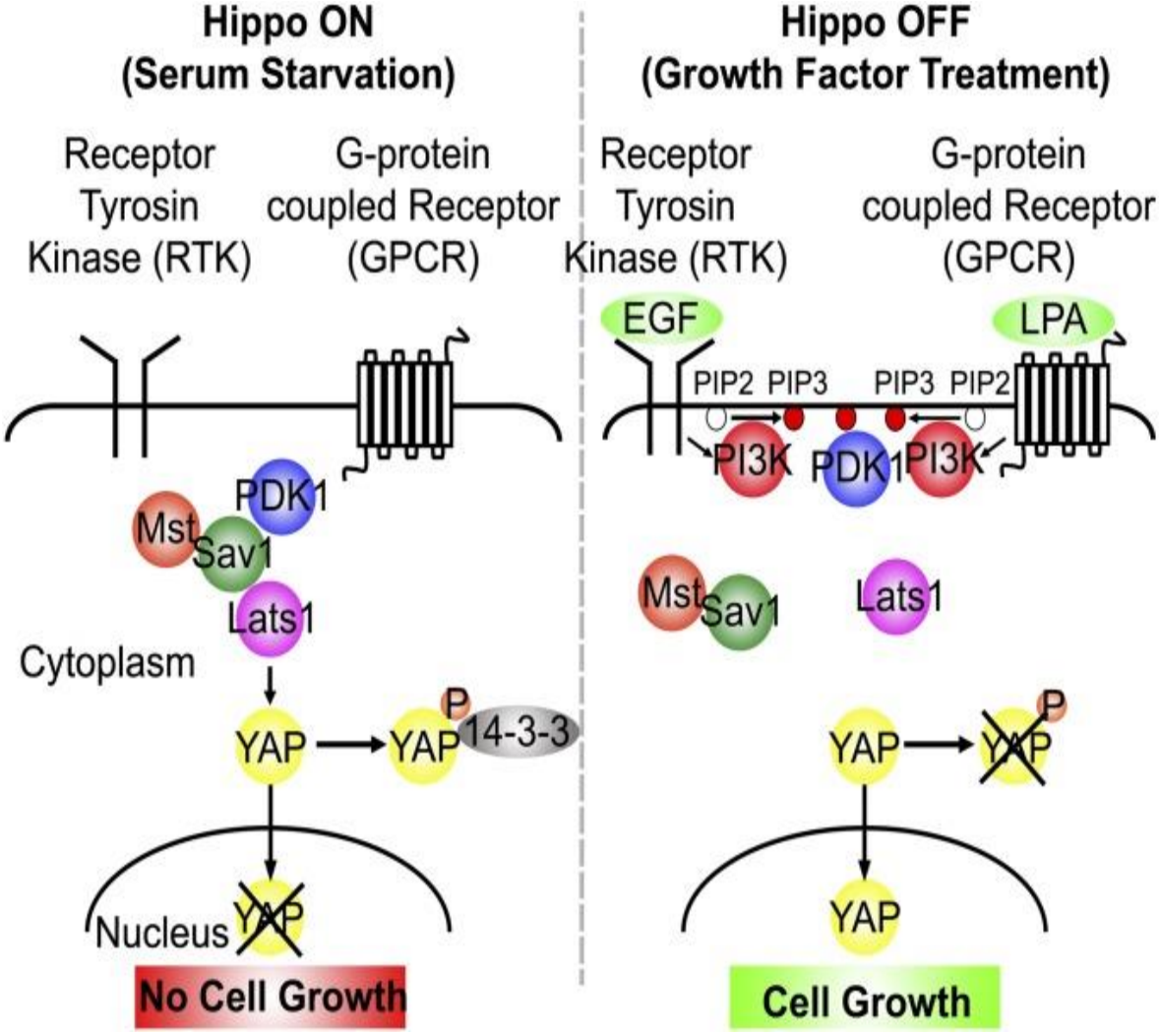

Figure 4.1: PI3K signaling regulation of YAP localization. Adapted from Fan *et al* 2013 (122).

Dysregulation of Hippo signaling through constitutive activation of YAP is an enticing target for viral proteins that rely on cellular signaling cascades for progression through the viral life cycle. Polyoma Small T (PyST) and HPV 16 E6 (16E6) dysregulate the Hippo signaling pathway through different mechanisms to induce cell proliferation and growth (126, 127). PyST directly interacts with YAP and once associated, PyST recruits PP2A to dephosphorylate YAP on serine 127 (126). Thus, PyST is driving the nuclear accumulation of YAP and increasing YAP stability by blocking degradation induced by Lats and CK1 phosphorylation (126). 16E6 does not directly interact with YAP; rather it increases the protein levels of YAP within the non-HPV infected cervical cancer cell line, HT3 (127). Upon transfection of E6 into HT3 cells, total YAP protein was increased and *AREG,* a downstream target of the Hippo/YAP pathway, was increased at the mRNA level suggesting YAP was activated and translocated to the nucleus. HPV-18 E6 endogenously expressed in HeLa cells also enhances YAP protein levels, which is specific to E6, as E6 knockdown in HeLa cells results in attenuated YAP proteins levels (127). However, more studies are needed to clearly identify the mechanism of this E6-mediated YAP stabilization.

Hippo/YAP signaling plays vital roles in cell proliferation, survival and differentiation. Thus, how viral proteins, including 16E7, modulate the activation of the pathway could provide mechanisms to known functions of 16E7 such as blocking differentiation and enhancing cell proliferation that are independent of Rb-degradation.

Results

HPV-16 enhances YAP localization to the nucleus

The localization of YAP plays a clear role in the activation or inactivation of the transcriptional co-activator (118, 119). When unphosphorylated, YAP is active and translocates to the nucleus to bind with TEAD family members and induce transcription of genes involved in cell proliferation. HPV-16 localized YAP to the nucleus under both starvation (0 min) and stimulation (15 min) conditions when compared to NIKS cells where YAP was largely excluded from the nucleus (Fig 4.2). HPV-16 containing keratinocytes had less cytoplasmic YAP staining when compared to NIKS cells. The N/C ratio of YAP was statistically increased in keratinocytes containing HPV-16 when compared to NIKS (Fig. 4.2). These data suggested that HPV-16 is either inhibiting the phosphorylation or promoting the dephosphorylation of YAP.

16E7 localized YAP to the nucleus

After demonstrating HPV-16 induced YAP translocation to the nucleus, we hypothesized that 16E7 altered the localization of YAP. We have shown that 16E7 alone localized YAP to the nucleus under starvation and stimulation conditions when compared with vector transduced keratinocytes that maintained YAP in the cytoplasm (Fig. 4.3). The N/C localization of YAP was statistically increased in 16E7 containing keratinocytes when compared to vector transduced keratinocytes (Fig 4.3). However, the cytoplasmic exclusion of YAP was not as evident in 16E7 keratinocytes (Fig 4.3) when compared to HPV-16 containing keratinocytes (Fig 4.2).

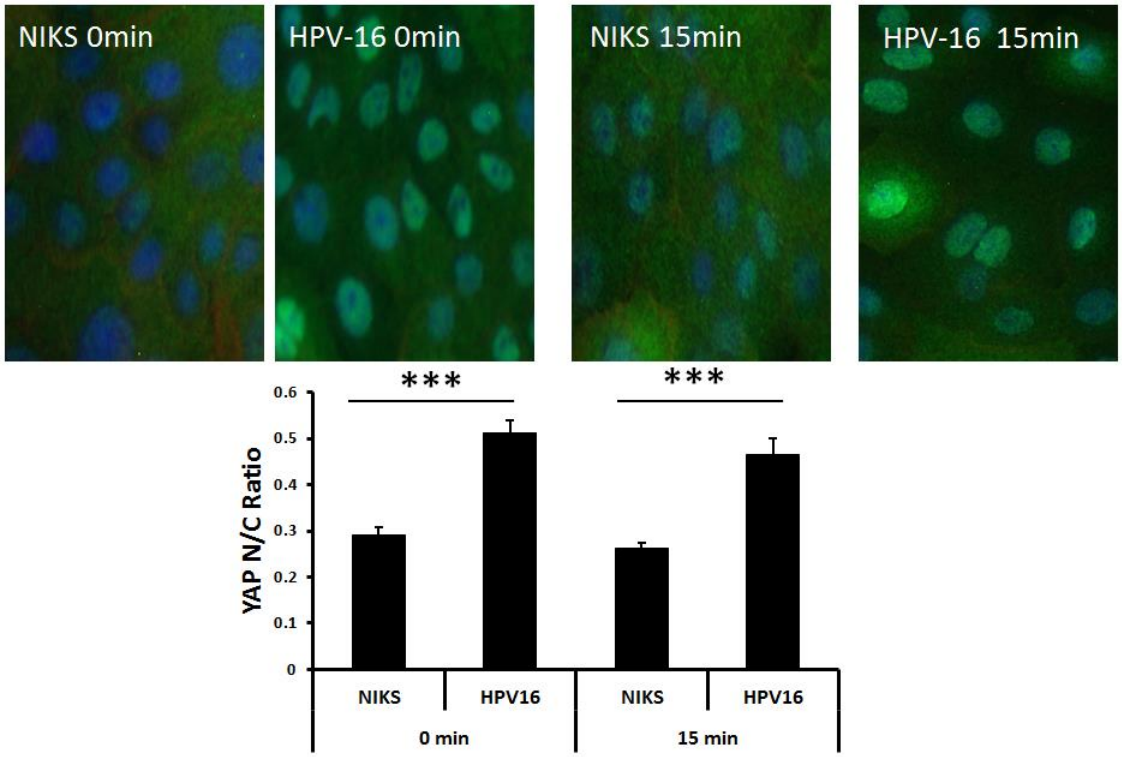

Figure 4.2: HPV-16 localizes YAP to the nucleus. NIKS cells transfected with HPV-16 were plated and maintained in F-media for 48 hours. Cells were then starved (No serum, EGF or Insulin) for 12 hours and stimulated for 15 mins with full F-media. Cells were formalin fixed and then stained for YAP (green), Phalloidin (red) and DAPI (blue). Statistics were calculated from 4 separate images on ImageJ. (***=$p<0.001$)

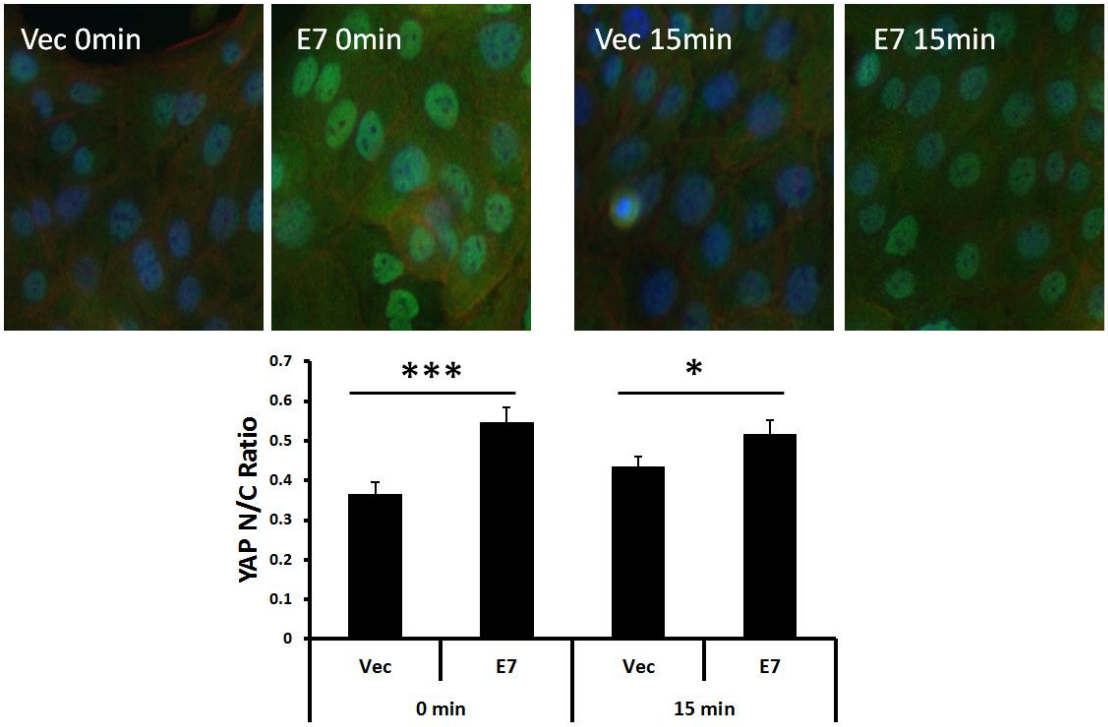

Figure 4.3: E7 localized YAP to the nucleus. NIKS cells were retrovirally transduced with vector or 16E7 and were plated and maintained in F-media for 48 hours. Cells were then starved (No serum, EGF or Insulin) for 12 hours and stimulated for 15 mins with full F-media. Cells were formalin fixed and then stained for YAP (green), Phalloidin (red) and DAPI (blue). Statistics were calculated from 4 separate images on ImageJ. (*=$p<0.05$, ***=$p<0.001$).

Discussion

Hippo signaling is a vital pathway that regulates cell proliferation and organ size in mammalian cells (106-109). YAP, the transcriptional co-activator regulating the expression of genes involved in cell proliferation, is regulated through phosphorylation that determines whether YAP translocates to the nucleus (active) or cytoplasm (inactive) (118). We have shown that in the presence of HPV-16 YAP translocates to the nucleus (Fig. 4.2) when compared to NIKS cells and this phenotype could be mapped to 16E7 alone (Fig. 4.3). Translocation of YAP to the nucleus suggests 16E7 is inhibiting Hippo signaling either at the level of YAP phosphorylation or upstream at the level of the Hippo kinases, Mst1, Sav and Lats. The upstream Hippo kinases are regulated by multiple factors including the PI3K/AKT pathway (122), which we have shown to be downregulated by 16E7 (Fig. 2.1). During growth factor deprivation PI3K is inactivate and thus PDK1 is not recruited to the plasma membrane. Hippo signaling is activated during growth factor deprivation and PDK1 is able to form a complex with Mst1, Sav, and Lats that in turn phosphorylates and sequesters YAP in the cytoplasm. However, we do not observe a sequestration of YAP in the cytoplasm in keratinocytes containing 16E7; rather we see the opposite, a translocation to the nucleus. This creates a paradigm where 16E7 attenuates pAKT while also inhibiting Hippo signaling, which seems counterintuitive. However, we can speculate that phosphatases known to regulate both AKT and Hippo signaling could be dephosphorylating both AKT and YAP simultaneously, if E7 played a role in recruiting such a phosphatase.

Recently, PP2A was identified as one of the phosphatases responsible for negatively regulating Hippo signaling (128). PP2A has been shown to act directly on MST1/2, one of the upstream kinases within the Hippo signaling pathway (129) and is well known to dephosphorylate AKT at T308 (32, 45). Two studies showed a similar phenomenon with PyST and its association with PP2A that led to reduced pAKT and reduced YAP phosphorylation (45, 126). Thus, if 16E7 associates with PP2A, it could be targeting PP2A to dephosphorylate AKT and either Mst1/2 or YAP, which is consistent with our data (Fig 2.1 and Fig. 4.3). Inhibition of AKT signaling and Hippo signaling have both been shown to block keratinocyte differentiation (74, 125); thus 16E7 inhibition of both pathways could be to block keratinocyte differentiation and promote cell proliferation via two mechanisms. Future studies are planned to fully develop this hypothesis.

Methods

Cell Culture: NIKS were maintained as described previously (Chapter 2 Methods). 400,000 NIKS cells harboring either the full HPV-16 genome, vector, or 16E7 were plated onto glass coverslips and maintained in F-media for 48 hours. NIKS were then starved (No Serum, EGF or Insulin) for 12 hours and then stimulated with full NIKS media prior to formalin fixation.

Immunohistochemical Staining: NIKS were washed with ice cold PBS once prior to formalin fixation for 10 minutes. Formalin fixative was washed out with PBS twice and cells were permeabilized with 0.1% tween for 10 minutes. NIKS were blocked with 2.5% BSA overnight at 4^{O}C. Primary antibody was added to coverslips for 1 hour. The antibody used was YAP65 (#2060-1) from Epitomics/Abcam. Coverslips were washed and then secondary antibody was added for 1 hour. The antibodies used were Alexa Fluor 488 from Pierce and Alexa Fluor 594 Phalloidin from Molecular Probes. Coverslips were washed again and DAPI stained prior to mounting the coverslips onto slides. Images were captured using the same exposure times and filter settings for each image.

Chapter 5: Future Directions

We have shown that 16E7 and other E7 types attenuate pAKT T308, resulting in altered cellular protein translation, and enhanced several cellular proteins, including cMYC. However, we currently do not understand the mechanism involved, or the rationale of how this phenotype could alter the viral life cycle or cancer progression. Thus, more studies are needed in order to address the following questions:

1. *Are there other domains of E7 involved in the pAKT attenuation?*
2. *What is the mechanism of the E7-mediated attenuation of pAKT?*
3. *Are the E7-mediated attenuation of pAKT and the E7-mediated nuclear localization of YAP connected?*
4. *Does E7 bind a cellular protein within the alpha-helix containing H73?*
5. *Does HPV-16 that harbors the H73E mutation maintain the genome episomally?*
6. *Does pAKT attenuation enhance viral genome amplification?*
7. *Is the expression of viral genes influenced by attenuated pAKT?*
8. *Are other downstream AKT targets affected by 16E7?*
9. *Is the E7-mediated keratinocyte differentiation block dependent upon pAKT?*
10. *Are other IRES-dependent proteins enhanced by 16E7?*
11. *Is cMYC protein expression enhanced in cervical carcinomas?*
12. *Are there any therapeutic implications of the E7-mediated pAKT attenuation?*

1. Are there other domains of E7 involved in the pAKT attenuation?

While we have shown that the pAKT attenuation is independent of Rb degradation, we have not determined if other domains of E7 are involved with the attenuation phenotype. CR1, which contains the PTLHE motif that interacts with UBR4 and Cullin family ubiquitin ligases, is conserved across both high and low-risk HPV types. This domain is responsible for recruiting ubiquitin ligases that facilitate the degradation of pocket proteins like Rb and Rb family members. However, the CR1 domain could be necessary to attenuate pAKT. To determine its involvement, we propose using a 16E7 deletion mutant (16E7 ΔPTLHE), which lacks the entire CR1 conserved motif, and assay if 16E7 ΔPTLHE attenuates pAKT or if the phenotype is lost. Other 16E7 mutations have been assayed for their ability to reverse the WT 16E7-mediated pAKT attenuation including C-terminal mutants, R77E and E80K/D81K. R77E showed partial reversal of the pAKT attenuation phenotype (data not shown), but was inconsistent among the four experimental replicates. R77 is highly conserved between both high and low-risk HPV types ((30) and Fig. 5.1) and thus could represent an important binding region for an E7 interaction partner. E80K/D81K also showed a partial reversal of the pAKT attenuation phenotype; however, E7 was not visible on western blot analysis suggesting this mutant may be unstable. Further studies with single mutants (E80K and D81K) are planned to

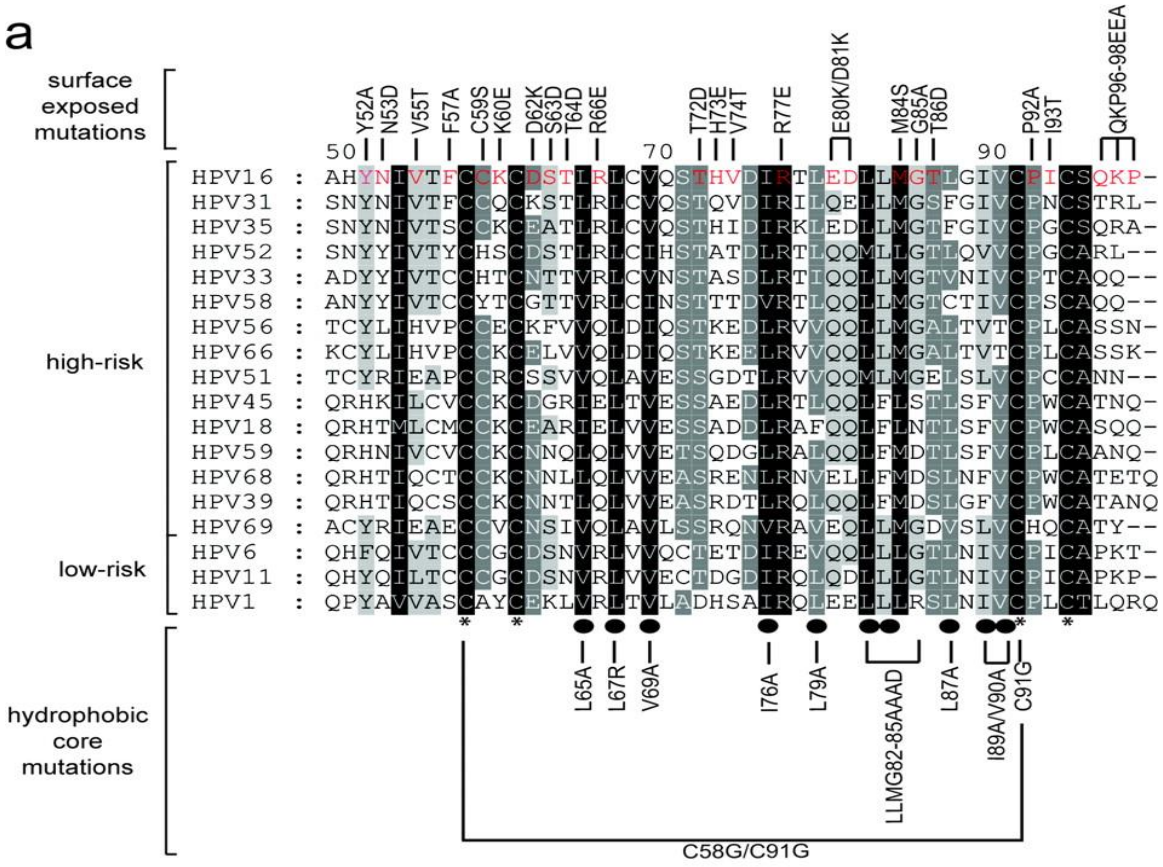

Figure 5.1: E7 C-terminal region of High and low-risk HPV types. Adapted from Todorovic *et al* 2011 (30).

elucidate the role the E7 C-terminus is playing in the pAKT attenuation phenotype. If more domains of E7 are shown to be involved in the pAKT attenuation phenotype, we could potentially identify the mechanism by which E7 attenuates pAKT T308.

2. What is the mechanism of the E7-mediated attenuation of pAKT?

While we have characterized the phenotype of the E7-mediated pAKT attenuation, we have not elucidated the mechanism by which E7 is attenuating pAKT. Studies by other groups have identified a similar phenotype in PyST, where PyST associates with PP2A Aβ to target the phosphatase to dephosphorylate pAKT T308 (45). We hypothesize that 16E7 is behaving in a similar manner and interacting with PP2A Aβ. 16E7 has been shown previously to interact with PP2A (38), but in their hands this interaction resulted in an increase in pAKT T308. However, this study should be taken in the context of overexpression of AKT, 16E7 and PP2A, and in the presence of adenoviral proteins, both of which could complicate the interpretation of the results. We propose to use immunoprecipitation assays to determine if there is an interaction between 16E7 and PP2A, specifically the Aβ subunit. If there were an interaction, the mechanism of the 16E7-mediated attenuation of pAKT would be very similar to what has been previously described for PyST (45). However, if no association exists then enhancement of upstream signaling, through knockdown of PTEN or constitutively activating PI3K, will be utilized to determine if 16E7 is still able to attenuate pAKT T308. If 16E7 still attenuates pAKT T308 in the presence of constitutively active PI3K signaling, then E7 could be interfering at the level of PDK1, recruitment of AKT to the plasma membrane, or recruitment of another non-PP2A phosphatase. 16E7 has been shown to interact with

other non-PP2A phosphatases, PTPN14 and PTPN21 (84), which could be involved in the attenuation phenotype.

3. Are the E7-mediated attenuation of pAKT and the E7-mediated nuclear localization of YAP connected?

AKT and Hippo signaling share several kinases and phosphatases including PDK1 and PP2A (122, 128). Recently PyST has been shown to augment YAP nuclear localization and attenuate pAKT through association with PP2A (45, 126). Thus, as discussed previously 16E7 could be interacting with PP2A in a similar fashion to modulate both signaling pathways. However, modulation of PDK1 activity could also lead to changes in both signaling pathways. When no growth factor signaling is occurring, PDK1 is inactive and is associated with the Hippo kinases, Mst1, Sav and Lats, which activates the complex to phosphorylate and sequester YAP in the cytoplasm (122). In this inactive state, PDK1 is not able to phosphorylate nor activate AKT and thus AKT remains in the unphosphorylated state. This is contrary to our observations of attenuated pAKT and increased nuclear YAP. However, if 16E7 was interacting with and relocalizing PDK1, both phenotypes could be explained (Fig. 5.2). The mislocalization of PDK1 could disrupt the Hippo complex, thereby inactivating it, which would allow for YAP to translocate into the nucleus. The mislocalization PDK1, away from the plasma membrane, could also prevent AKT from being phosphorylated and result in attenuation of pAKT T308. To determine if 16E7 was interacting with PDK1, we would use immunoprecipitation assays to isolate PDK1 and subsequently use western blots to identify if 16E7 is a binding partner. This assay could also be used to determine if PDK1 is in complex with the Hippo

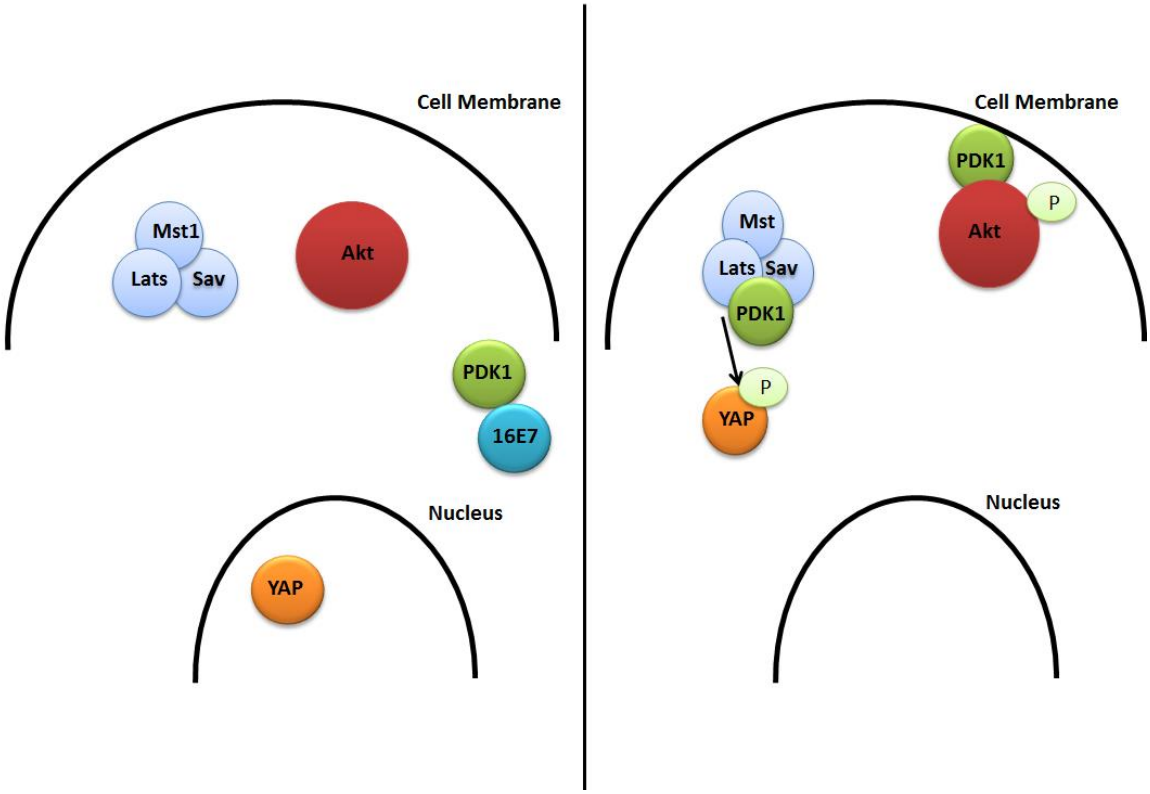

Figure 5.2: Hypothesized mislocalization of PDK1. We hypothesize that 16E7 is mislocalizing PDK1 resulting in attenuation of pAKT and inactivation of Hippo kinases (left) in comparison to keratinocytes without 16E7 where PDK1 associates with Hippo kinases and sequesters YAP in the cytoplasm and PDK1 phosphorylates AKT T308 when localized to the membrane (right).

kinases in the presence of 16E7. Since PDK1 association with hippo kinases activates the complex and sequesters YAP in the cytoplasm, we would hypothesize that PDK1 would not be associated with the hippo kinases in 16E7 keratinocytes, since YAP is localized to the nucleus in 16E7 keratinocytes (Fig. 4.3). Regardless of the ability of 16E7 to interact with PDK1, 16E7 could still be altering the localization pattern of PDK1 within the cell. We propose to utilize IHC or subcellular fractionation protocols to identify the localization pattern of PDK1 in vector transduced, 16E7 transduced or H73E transduced keratinocytes. Both of these methods would elucidate if PDK1 is mislocalized in the presence of 16E7 and could suggest that PDK1 is modulating both Hippo and AKT signaling.

4. Does E7 bind a cellular protein within the alpha-helix that contains H73?

16E7 H73E is a novel mutation within the C-terminus of E7 that fails to attenuate pAKT (Fig 2.4B). This mutation lies between the two CxxC binding motifs that make up the zinc structured domain, also known as the conserved region 3 (CR3). E7 H73 is a surface reside that is located within a conserved alpha helix structure (Fig. 5.3). Several other defined surface residue mutations have been created within this same alpha helix and create a potential binding surface that consists of H73 (yellow), R77 (red) and E80/D81 (blue) (Fig. 5.3). These residues were mutated and preliminarily characterized in a previous study for their effect on transformation and E7 dimerization (30), and do not interfere with the ability of E7 to transform BRK cells in co-transfections with activated Ras. R77E has a slightly decreased binding affinity for Rb, but H73E and E80K/D81K remained similar to WT E7. We hypothesize this

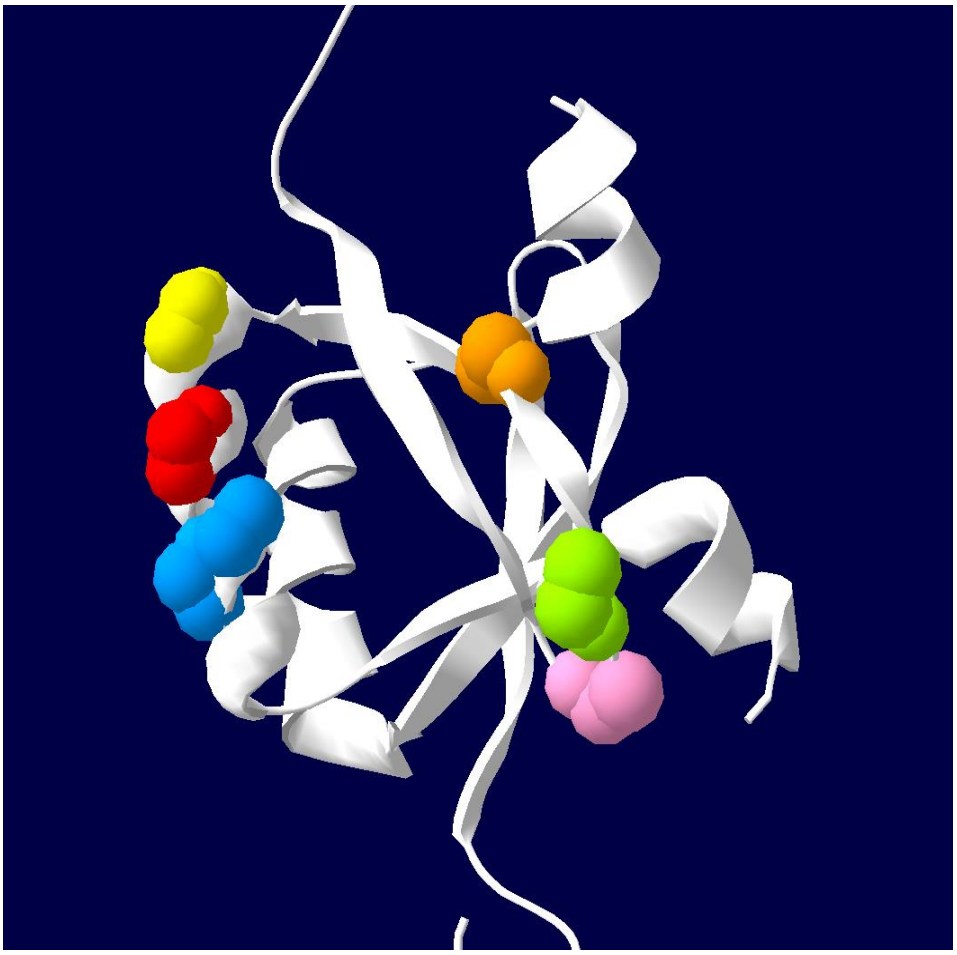

Figure 5.3: NMR Solution Structure of HPV 45 E7 dimers. HPV-45 E7 dimer with corresponding 16E7 C-terminal mutants highlighted: K60E (pink), D62K (green), R66E (orange), H73E (yellow), R77E (red), and E80K/D81K (blue). NMR Solution Structure: 2F8B.

E7 alpha helix could be interacting with a cellular protein in order to attenuate pAKT and the H73E mutant disrupts this interaction and thereby fails to reduce pAKT T308.

In order to elucidate if a binding partner is interacting at this site, we propose to utilize mass spectrometry as a way to elucidate binding partners of WT E7 compared to 16E7 H73E. We will use tandem affinity purification tags, described previously (130), fused to the N-terminus of 16E7 and 16E7 H73E, as we have found C-terminally tagged 16E7 is deficient for attenuation of pAKT. The 16E7 and 16E7 H73E fusions will be expressed in CV1 cells and clarified cell lysates will be incubated with beads to isolate 16E7 fusions and any associated proteins. TEV protease cleavage will release the 16E7 and 16E7 H73E from the beads and the fusions will be affinity purified using FLAG antibody beads, however other techniques will be pursued if a CR2 tag is used. 16E7 and 16E7 H73E associated proteins will be resolved on silver stained polyacrylamide gels. Bands that are absent in 16E7 H73E lysates, but present in 16E7 lysates will be analyzed by mass spectrometry to elucidate the identity of the protein(s) of interest. Alternatively, all of the E7-associated cellular proteins obtained from binding either 16E7 or 16E7 H73E could be sent for analysis, as silver stained band differences between the two E7 proteins may not be apparent or could co-migrate with other proteins common to both IPs. Candidate proteins will be chosen based upon unique peptide "hits" of greater than 2, which will help eliminate non-specific proteins or rare but spurious peptides. To characterize candidate proteins, we propose to use shRNA knockdowns or inhibit the protein activity using small molecule inhibitors and observe the effects on the E7-mediated pAKT attenuation phenotype. If a candidate protein is

necessary for the pAKT attenuation, then knockdown or inhibition should reverse the E7 pAKT attenuation phenotype.

5. Does HPV-16 that harbors the H73E mutation maintain the genome episomally?

We have shown that HPV-16 E7 H73E is not detectable in three independent transfections of the complete viral genome into keratinocytes (Fig. 3.3), but the presence of the HPV genome could be inferred because of the degradation of Rb by E7 and p53 in all three transfected cell lines. This suggests that H73 within the C-terminus of 16E7, and by implication the attenuation of pAKT, could be essential for the maintenance of the viral episome. However, contradicting our observations is the report that HPV-16 containing a null E7 mutation (TTL within E7) is still maintained as an episome (103). We performed our experiments using cloned HPV-16 where the bacterial vector sequences had been removed and the HPV genome recircularized and religated in vitro prior to transfection into keratinocytes. This process is somewhat inefficient and gives rise to few colonies of transfected keratinocytes that must expand to give rise to samples that are adequate for southern blot analysis. Thus, early replication and plasmid maintenance are difficult to observe. To examine HPV plasmid replication at earlier times points, we will switch to a more recent protocol to transfect HPV DNA into keratinocytes using a Cre-Lox system that does not require the recircularization of the HPV genome prior to transfection (131). This method allows a greater frequency of transfection and recircularization of the viral HPV DNA in keratinocytes, allowing for an earlier analysis of the physical state (integrated versus episomal) of the genome. In

contrast, the transfection of ex-vivo recircularized DNA as we have done is less efficient, and considerable cell proliferation is required post-transfection to obtain samples adequate for a southern blot. During that time, selection for viral integration might occur. However, if our results are consistent and HPV-16 E7 H73E is not maintained as an episome, or is rapidly lost, even in the Cre-Lox system, we will elucidate the mechanism by which HPV-16 E7 H73E ablates the ability to replicate the genome. First, we will elucidate if WT E7 (in trans) is able to compensate and restore the ability of the HPV-16 genome to replicate, which would indicate that H73E is a loss of function mutation. If this is true, we will utilize data generated from previous experiments detailed above (e.g. mass spec identification of binding partners) to identify if interaction partners of WT E7 but not H73E are involved in episomal maintenance and replication.

6. Does pAKT attenuation enhance viral genome amplification?

Both high and low-risk E7s attenuate pAKT T308, which suggests this phenotype is not linked to causing cancer, exclusively. Thus, the attenuation of pAKT could be involved in events common to both high and low risk-viral types, such as viral genome amplification and viral packaging. We hypothesize that 16E7 enhances the expression of IRES-dependent cellular protein translation in order to promote viral amplification and possibly to inhibit keratinocytes differentiation. We propose to use raft cultures to induce viral amplification of keratinocytes containing the WT HPV-16 genome and the HPV-16 E7_H73E genome. Amplification of the viral genome can be measured by Southern blot analysis by harvesting DNA from 14-day raft cultures or through DNA-

fluorescence in situ hybridization (DNA-FISH) where raft culture sections are stained with HPV-16 fluorescently labeled probes and cells with enhanced viral DNA copy number are observed within the spinous keratinocyte cell layers. If HPV-16 E7_H73E is maintained episomally but does not amplify the viral genome, HPV-16 genome amplification could be dependent upon attenuation of pAKT.

7. Is the expression of viral genes influenced by attenuated pAKT?

Other viral systems, such as poliovirus, express all of its virally encoded proteins through IRES-dependent translation (132). However, HPV viral proteins have traditionally been thought to be translated via cap-dependent processes. We hypothesize that the shift in cellular translation could also alter the expression of viral proteins, such as E5, in additional to cellular proteins. However, due to the unavailability of E5 antibodies, protein expression changes in the presence of 16E7 cannot currently be elucidated. Further studies are planned to identify novel methods to quantify E5 protein expression described previously (133) or through quantification of cytosolic mRNA and polysome-associated mRNA.

8. Are other downstream AKT targets affected by 16E7?

We have shown that the 16E7-mediated pAKT attenuation also attenuates the phosphorylation of downstream effectors S6K and 4EBP-1 (Fig. 2.6). However, AKT is involved in many cellular processes other than translation, including proliferation, survival, and glucose metabolism. AKT phosphorylates proteins involved in these processes to activate or inactivate, leading to increased survival, proliferation and metabolism. We propose to use western blot analysis with phosphospecific antibodies

to determine the phosphorylation state of downstream AKT effectors, such as FOXO, Wee1, and PIP5K, which regulate survival, proliferation and glucose metabolism, respectively. If cell survival is also attenuated, for example, this could provide a mechanism for the known E7 phenotype, where 16E7 sensitizes keratinocytes to apoptosis and autophagy (80, 134). By examining other aspects of AKT signaling, we could identify novel functions of E7 or provide mechanisms for known functions of E7.

9. Is the E7-mediated keratinocyte differentiation block dependent upon pAKT attenuation?

We have shown that 16E7 blocks keratinocyte differentiation and expression of a late differentiation marker Keratin 10 (Fig 3.1), while the HPV-16 genome harboring the H73E mutation induces premature keratinocyte differentiation and enhances Keratin 10 expression (Fig. 3.1). HPV-16 also induces dysplastic features such as increased N/C ratio, hyperplasia and increased mitotic figures, while the HPV-16 E7_H73E does not display the classic dysplasia features (Fig. 3.2). The E7 H73E mutation fails to attenuate pAKT (Fig 2.4B) and fails to induce dysplasia like the WT HPV-16 genome does. This suggests that attenuation of AKT signaling could play a role promoting dysplasia in keratinocytes. We hypothesize that attenuation of pAKT inhibits keratinocyte differentiation and thereby promotes dysplastic features independent of Rb-degradation. A triple knockout of Rb, p107, and p130 in keratinocytes of transgenic mice does not produce full dysplasia or invasive cancer (96), suggesting that degradation of Rb, p107, and p130 by 16E7 is not sufficient to cause dysplasia, thus another 16E7 phenotype is required. By blocking terminal differentiation, suprabasal cells remain in a

more basal-like phenotype continuing to transit through the cell cycle, rather than withdrawing from the cell cycle. To elucidate the role pAKT is playing in keratinocyte differentiation, we propose to constitutively activate AKT by using a mutant, such as myristoylated AKT (myrAKT), to determine if keratinocyte differentiation is induced even in the presence of WT 16E7 or HPV-16. Myr-AKT will be introduced with a tetracycline regulated lenti-virus to allow tet-regulated induction and repression of the transgene expression. If enhanced pAKT is driving keratinocyte differentiation, the E7-mediated attenuation of pAKT could represent a novel way in which HPV-16 promotes progression through the cell cycle by maintaining a more basal-like state within the keratinocytes that is independent of Rb-degradation.

10. Is the expression of cellular IRES-dependent proteins enhanced by 16E7?

We identified several proteins, cMYC, Bax, and insulin receptor that were enhanced at the translational level in 16E7 containing keratinocytes (Fig 2.8). However, there are numerous other cellular proteins that are enhanced when IRES-dependent translation is increased during stress conditions (54). Thus, it would be interesting to determine if other IRES-dependent cellular proteins such as twist and snail, which are involved in the epithelial-to-mesenchymal transition (EMT) are also enhanced by E7 expression; this could provide a mechanism to known HPV-16 functions such as inducing EMT and promoting cell survival. We propose to harvest polysome associated mRNAs and cytoplasmic mRNAs from vector, 16E7, or 16E7 H73E transduced keratinocytes to determine the translation profile of the three cell lines. RNA Seq will be utilized to identify proteins of interest that have enhanced association with polysomes in 16E7

keratinocytes, but not in vector transduced or 16E7 H73E transduced keratinocytes. Sucrose gradients will be used to separate out polysomal RNA fractions from ribosomes and free mRNA. Total cytoplasmic RNA will also be collected to identify mRNAs that are disparate in frequency between the total cytoplasmic pool and the ribosome associated pool. Identifying other 16E7 enhanced proteins could provide mechanisms for known E7 functions such as enhanced EMT and cell cycle progression.

11. Is cMYC protein expression enhanced in cervical carcinomas?

One of the proteins that 16E7 enhances the translation of is cMYC (Fig. 2.8), a known transcription factor that is upregulated in several cancer types (135). However, only 7% of cervical cancers have an amplification of cMYC (87). cMYC transcriptionally regulates many cellular processes such as proliferation, cell cycle, and cell survival (136). While 16E7 and HPV-16 enhance cMYC in culture, it would be interesting to know if cMYC is also enhanced at the translational level in cervical carcinomas. One study showed that nearly 25% of low grade cervical lesions were positive for cMYC on IHC staining (137), suggesting HPV-16 could enhance cMYC expression CIN I lesions. We propose to harvest cervical cancer specimens for both RNA and histology to observe the transcriptional and translational state of cMYC within these specimens. We propose to use IHC with a cMYC antibody to determine protein levels of HPV positive cells in comparison to HPV negative cells within the same tissue sample or a separate benign, non-HPV positive cervical specimen. HPV positivity will be determined by DNA-FISH using HPV-16 probes. RNA samples will be harvested from the same paraffin embedded tissue to quantify the gene expression of cMyc. If the transcriptional levels of cMYC are

the same, but the protein levels are elevated that would confirm our findings in cell culture. If cMYC is also enhanced in cervical specimens it would be of interest to determine if cMYC was transcriptionally active. However, the effects of enhanced cMYC expression are complicated and context dependent, thus not all cMYC target genes will be enhanced in response to increased cMYC. Activation of cMYC target genes is dependent on the availability of cMYC binding partners and activation of other signaling cascades. Therefore, determining if cMYC is transcriptionally active may not be feasible, as 16E7 alone also alters the transcription of several cMYC target genes.

12. Are there therapeutic implications of the E7-mediated pAKT attenuation?

The therapeutic potential of the E7-mediated attenuation of pAKT are complex. While the induction of dysplastic features by HPV-16, but not by HPV-16 E7_H73E, suggest that attenuation of pAKT could induce cell cycle progression, the correlation between pAKT attenuation and inhibition of keratinocyte differentiation remains unclear. Both phenotypes could be independent functions of E7 and not related to one another. To elucidate if these two phenotypes are connected we will constitutively activate AKT in keratinocytes with HPV-16 or 16E7 and observe if keratinocyte differentiation proceeds (as discussed previously). If these two phenotypes are linked, this could identify a novel way HPV-16 induces dysplasia independent of Rb-degradation.

Conversely, the attenuation of pAKT has been shown to increase apoptosis and sensitize cells to cell death, as has been described for Adenovirus E1a (42, 43). This would suggest that 16E7 could increase the susceptibility of cancer cells to

chemotherapy and radiation protocols. In head and neck squamous cell carcinoma (HNSCC), HPV-positive lesions are more sensitive to radiation therapy when compared to HPV-negative lesions (138, 139); however the mechanism for this increased sensitivity is unknown. In a recent study, HPV-positive HNSCC were shown to have lower pAKT T308 levels by IHC when compared to HPV-negative HNSCC (140). Thus in HPV positive HNSCC, 16E7 could be attenuating pAKT T308 (in the absence of a PI3K CA mutation), which could then sensitize the cells to radiation therapy. We propose to treat NIKS cells transduced with vector, 16E7 or H73E, with a DNA damaging agent or irradiation to induce apoptosis as measured by cleaved caspase 3 production. If pAKT attenuation sensitizes keratinocytes to apoptosis then 16E7 containing keratinocytes should have increased cleaved caspase 3, while H73E should be protected and have less cleaved caspase 3. This could provide a mechanism for the increased sensitivity observed in HPV positive HNSCC lesions.

Final Summary

We have shown that HPV-16 attenuates pAKT T308 and this can be mapped to 16E7. We have also shown both high and low-risk E7 types attenuate pAKT and that this phenotype is independent of the Rb degradation function of E7. The AKT attenuation phenotype can be ablated by a single point mutation within the carboxy-terminus of E7 and is not dependent upon the E7 mediated induction of Histone H3K27 demethylases to induce epigenetic reprogramming. The phosphorylation of two downstream proteins of AKT (S6K and 4E-BP1) is attenuated, leading to a shift in protein translation from cap-dependent to IRES-dependent translation. The shift enhances the protein translation of several cellular proteins without altering their transcription, including c-MYC, insulin receptor and Bax. The enhanced IRES-dependent translation could be promoting the expression of a specific set of cellular proteins that will augment virus amplification and virus packaging. The enhanced IRES-dependent translation could also induce the expression of HPV proteins, similar to other viral systems which utilize IRES-dependent translation to express viral proteins. The attenuation of AKT mediated by E7 is also correlated with inhibition of keratinocyte differentiation, as 16E7 H73E does not attenuate pAKT and induces premature keratinocyte differentiation. The pAKT attenuation is a novel phenotype of E7 and demonstrates a role for E7 in the manipulation of signal transduction cascades, thereby altering the regulation of cellular translation and differentiation.

We have also shown that HPV-16 translocates YAP to the nucleus and this could be mapped to 16E7. YAP nuclear localization induces the association with TEAD family

members to enhance the expression of genes regulating cell growth and proliferation (112, 114). PI3K and AKT have both been shown to regulate the Hippo signaling pathway and there are kinases and phosphatases that act on proteins involved in both signaling pathways. This phenotype is consistent with the observed hyper proliferation induced by 16E7 and could be functioning in conjunction with transcriptional changes induced by the E2F family members to drive cell cycle progression.

The mechanism by which 16E7 attenuates pAKT T308 is currently unknown; however, potential mechanisms are being considered. One current hypothesis is that 16E7 might be interacting with PP2A similar to what has been shown for PyST which attenuates pAKT T308, differentiation, and YAP localization, in an analogous manner as 16E7 (45, 126). Studies are underway to determine if 16E7 interacts with PP2A or another phosphatase in order to dephosphorylate both pAKT and pYAP.

We have defined a novel function of E7 that could play a role in creating the ideal cellular environment to amplify the viral HPV genome. Attenuated pAKT prevents terminal differentiation of keratinocytes, which promotes continued transit through the cell cycle in suprabasal cell layers. Cell cycle progression is essential to amplify the viral genome to produce infectious virions. Enhanced nuclear accumulation of YAP mediated by E7 could also promote keratinocytes to continue to transit through the cell cycle by enhancing the expression of genes involved in cell proliferation. Attenuation of pAKT could be a novel mechanism by which E7 promotes the progression through the cell cycle that is independent of Rb-degradation in order to promote viral amplification.

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