THE HUMAN PAPILLOMAVIRUS TYPE 16 E5 PROTEIN: STRESS SIGNALING AND EXPRESSION IN CERVICAL CANCER CELL LINES

A Dissertation submitted to the Faculty of the Graduate School of Arts and Sciences of Georgetown University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Tumor Biology

By

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ABSTRACT

Human papillomavirus (HPV) is the causative agent of 99% of cervical cancers, approximately 60% of which are caused by the high-risk type 16. While two of the three HPV-16 oncoproteins, E6 and E7, have been well characterized, the biological functions of the E5 protein remain elusive. HPV-16 E5 protein (16E5) is thought to be disrupted during viral integration, a key event in cellular transformation, suggesting that 16E5 is not essential for carcinogenesis. Conversely, its presence has been shown to enhance E6 and E7-induced transformation, and several studies, including those by Sherman et. al. (1992) and Bauer-Hofmann et. al. (1996), have shown the presence of potentially E5-coding transcripts even in cervical cancer cell lines with integrated viral genomes. However, direct confirmation of 16E5 expression in cancer cells has not been possible, mostly attributable to the lack of specific antibodies. In this thesis, we use mass spectrometry to identify 16E5 in two HPV-16 positive cervical cancer cell lines, SiHa and CaSki. This is the first time that 16E5 has been identified in cervical
cancer lines with an integrated HPV genome, and our results demonstrate the co-expression of E5 later in malignancy when E6 and E7 are highly expressed. In addition, we analyze gene expression changes induced by the endoplasmic reticulum (ER)-resident E5 in human foreskin keratinocytes (HFKs) using microarray and real-time RT-PCR. We identify the downregulation of spliced XBP-1, IRE1α, and COX-2, key players in the ER-stress response, as biochemical markers of E5 expression. Similar studies were performed in HFKs expressing either low-risk HPV-6b E5 or a C-terminal 16E5 mutant, where the histidine and alanine residues at position 77 and 78 (conserved in high-risk HPVs) were replaced with tyrosine and isoleucine (conserved in low-risk HPVs). Importantly, neither construct affected levels of these genes, suggesting that the ability to induce these changes in stress gene expression is specific to 16E5 and might facilitate viral persistence leading to cell transformation. Overall, the data presented within this thesis suggest a potential role for E5 in productive viral infection as well as the initial and late stages of malignant transformation.
ACKNOWLEDGEMENTS

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I. INTRODUCTION
1.1 Papillomavirus Pathology

1.1.1 Cervical Cancer: Burden and Etiology

Cervical cancer is the second most common cancer in women worldwide (128). Half a million women are diagnosed with cervical cancer annually, with close to 11,300 of those women residing in the US. In fact, 250,000 women worldwide, including 4,000 Americans, are estimated to die annually (75, 120). Even though screening has cut the incidence and mortality of cervical cancer in half over the past thirty years, the economic and societal burden remains high. New cases continue to arise due to misdiagnosis, lack of patient follow-up, or especially aggressive tumors that evade screenings (186). It is estimated that 1.7 billion dollars are spent yearly in the US on treatment of cervical cancer (25).

The recognition of these burdens led to the development of a cervical cancer vaccine. Unlike most other cancers which are caused by the accumulation of random mutations over many years, cervical cancer often has a unique cause: the human papillomavirus (HPV). HPV is responsible for 99.7% of all cervical cancers, making it the target for most screening and therapeutic approaches (18, 182). One recent innovation in the fight against HPV-induced cervical cancer is the cervical cancer vaccine. This vaccine consists of the HPV structural outer capsid protein L1, which self-assembles into virus-like particles (VLPs) (24, 190). These VLPs are able to induce a high titer of neutralizing antibodies in both humans and animals, thus preventing future viral infection (190).
However, the existence of an HPV vaccine does not mean an end to cervical cancer or to other serious HPV pathologies, such as warts. There are many types of human papillomavirus, and the vaccine does not protect against all of them. In addition, the expense of the vaccine makes it unattainable for most of the developing world (190). Finally, HPV is a sexually transmitted disease (STD). Since the vaccine is preventative only, to be effective it must be administered prior to any exposure (109). Therefore, even in the developed world where the vaccine is affordable and recommended by health organizations, the ethical, social and political factors involved in vaccinating middle school children against an STD have prevented the vaccine from becoming mandatory (43).

For this reason, the study of HPV and HPV-induced cervical cancer continues to be important. Development of second generation and third generation vaccines are underway. Goals include building vaccines that are protective across many types of HPVs and vaccines that can be produced cheaply and therefore distributed worldwide (127). Vaccines aimed at preventing future cancers developing from past infections, and therapeutic vaccines against current cancers, would allow vaccination of older adults and minimize the ethical concerns currently involved (106, 190).

The clinical interest in HPV and the search for vaccines has revealed many questions yet unanswered about HPV-induced cervical cancer. These topics include areas as vastly diverse as the basic life cycle of HPV, the role of various HPV proteins in subverting the host system, host immune response to viral infection, host cellular response to DNA damage, and ultimately, the loss of cell cycle control and subsequent
development of cancer. This thesis aims to study the E5 protein of the high-risk HPV type 16 in an attempt to define those biological properties that contribute to HPV-induced tumorigenesis.

1.1.2 Human Papillomavirus Pathology

In the 1980s, new developments in molecular biology allowed for the discovery and classification of many types of HPV. The first laboratory to link HPV to genital warts was that of H. zur Hausen (66). It was soon discovered that HPV was a sexually transmitted disease and that it contributed to cervical dysplasia (56, 94, 145). Phylogenetic classification of HPV types based on HPV non-structural proteins proved to be a good predictor of the biological outcome of infection; those viruses able to cause cancer clustered away from those that could not (16, 22, 149). Analysis of natural variants of HPV types showed that even small differences in genomic DNA could correlate with differences in ability to infect host cells and cause lesions (17, 74, 119, 163). Soon, the presence of HPV and HPV-induced changes in genomic expression became important as screening tools and biomarkers for cervical cancer (18).

Cervical cancer is perhaps the most well known pathology of the human papillomavirus. However, it is not the only result of an HPV infection, and neither can it be caused by all types of HPV. There are over 100 types of HPV, over 40 of which are sexually transmitted (16). Some of these viruses infect cutaneous tissue, while others infect mucosal tissue.
Infections with cutaneous HPV are mostly non-symptomologic or cause warts. However, even these benign tumors and warts can have severe consequences. In some cases, HPV infection can been known to cause an overwhelming cutaneous growth of the hands and feet, leading to a tree-like appearance (62, 141).

For mucosal HPVs, there are at least three possible outcomes, two of which can occur after infection with any type of HPV. The first is a non-symptomologic infection. 91% of HPV infections in women are resolved to a point where the host tests negative for HPV DNA within two years (31, 149). The second potential outcome is a benign wart or benign tumor formation that stems from a persistent infection. These supposedly benign mucosal tumors can still have severe clinical consequences in terms of both quality of life and mortality, especially in the pediatric population. For example, recurrent respiratory papillomatosis caused by low-risk HPV types 6b or 11 is a condition where benign tumors arise on the larynx, affecting breathing (61). Treatment includes frequent surgery and anti-viral therapy; left untreated, the airway obstruction can be lethal (33, 162). The third possible consequence of a mucosal HPV infection is the development of a carcinoma after persistent infection. Only a few types of HPV generally cause such an outcome, and these types are labeled “high-risk” HPVs, to denote the fact that being infected by these viruses gives the host a risk of developing cervical cancer. Viruses that do not confer a risk of malignant transformation are termed as “low-risk” viruses, i.e. infection with these viruses carries a very low risk of ever developing cancer (148, 149).
Still, less than 1% of all HPV infections ever lead to cervical cancer, despite the fact that HPV infection is almost ubiquitous. The current prevalence of HPV infection is 26.8%, with 8% of the population infected with the high risk viruses HPV-16 and HPV-18. In fact, 80% of all women over 50 probably have had an HPV infection at some point in their lives, making HPV is the most common sexually transmitted infection in the US (30, 32, 55).

Other HPV-induced cancers include epidermodysplasia verruciformis (EV). HPV-5 and HPV-8 are known to cause EV, a very rare disorder presenting first with flat warts on the skin, and often followed by skin cancer later in life (113, 125). Increasingly, HPV has also found to be an etiologic agent in squamous cell carcinomas of the head and neck (65). The types of HPV that can cause head and neck cancers overlap with the types that cause cervical cancer, with HPV-16 causing the majority (90%) of these tumors (65). The mechanism of tumorigenesis is also similar (3, 134). Gene expression changes found in these cancers were found to be similar to those in cervical cancers, including cell cycle deregulation and downregulation of genes involved in viral defense and immune response (134, 150). However, there are differences between the two cancers, because for unknown reasons HPV infection does not appear to be either necessary or sufficient for head and neck cancers (65). Still, HPV-induced head and neck cancers remain an important subgroup amongst cancers not related to smoking and alcohol (64). It is estimated that around 44%-64% of tonsillar cancers, 39% of tongue cancers, and 18% of other oropharyngeal cancers are HPV-positive (138, 143). In addition, HPV-induced head and neck cancers have been
found to correlate with a positive disease prognosis and response to radiation and chemotherapy as compared to HPV-negative cancers (58, 64, 100).

1.1.3 Non-human papillomaviruses Pathology

Due to the limitations of studying the viral life cycle in-vitro, and the difficulties involved with human subjects, non-human papillomaviruses have often served as model systems for understanding human papillomaviruses. The ones that have most often been used in studies are the bovine papillomavirus (BPV), the canine oral papillomavirus (COPV), and the cottontail rabbit papillomavirus (CRPV) (29). Similar to the human papillomavirus, all three animal PVs are capable of causing malignant transformation in animals, although studies comparing individual proteins between species have revealed that some of the mechanisms can vary (169, 172). For example, the major transforming protein of BPV is BPV E5, whereas in HPV, the main oncoproteins are E6 and E7. However, the basic mechanisms of replication and cell transformation are highly related between animal and human papillomaviruses, and animal models have provided invaluable information on viral biology as well as the development of vaccines and therapeutics. The cervical cancer vaccine, as well as therapeutics such as DHA, have both been developed in part by use of the canine oral PV model (50, 173). In addition, the recent discovery that naked CRPV DNA (ie. DNA with no viral packaging directly applied to an abraded surface) induces tumors gives further promise to the rabbit model and its use in mutagenic studies (42).
1.2 Human Papillomavirus

1.2.1 Structure and Classification

Papillomaviruses are small non-enveloped DNA viruses (16). They have an outer capsid comprised of a major (L1) and a minor (L2) protein. Five L1 proteins form a structure called a capsomere. 72 capsomeres self-assemble to form the icosohedral capsid, which is held together and tethered to the DNA by the L2 protein. The final capsid is thus comprised of 360 copies of L1 and twelve copies of L2 (70, 112). Inside this structure, the virus carries its circular double stranded DNA of around 8kb, from which multiple polycistronic mRNA transcripts are made (113, 155).

The structure of this DNA is highly conserved amongst all HPVs (126). There are three “regions” of the DNA: a 4 KB early region, a 3 kb late region, and a 1kb long coding region or upstream regulatory region (LCR or URR) (60, 113). The early region has six to eight open reading frames (ORFs) for “early” non-structural proteins, including E1, E2, E4, E5, E6, and E7. The late region has the ORFs for the structural proteins L1 and L2, which are expressed later on during packaging of the viral genome (54). Each of these regions is controlled by its own promoter (60, 126). The URR has numerous promoters and enhancers that maintain a strict temporal expression of proteins (126). Protein expression can be additionally regulated by the viral proteins themselves. For example, the viral protein E2 is one of the first to be expressed. It is known to negatively regulate the expression of viral proteins E6 and E7 by binding to E2Ps (E2-responsive palindromes) in the URR upstream of the E6-E7 ORF (47). When E2 is removed as a consequence of viral genomic integration the levels of E6 and E7
increased. Such tightly controlled temporal regulation of viral proteins is crucial to the viral life cycle (126).

1.2.2 Protein expression as a function of Viral Life Cycle

The expression of viral proteins and the viral life cycle are tightly linked to the host state of cellular differentiation. HPV usually infects the host through some type of cut or microabrasion (105). The virus penetrates to the basal layer of the epithelium, which is the only layer that is actively dividing. Once there, the viral DNA is maintained episomally in a low copy (~50-100/cell) as the basal cells divide, passing into a much larger number of daughter cells (166). Viral early proteins are expressed but at low levels, perhaps in an attempt to evade immune recognition. As the infected host cells move upwards and into the suprabasal layer, the virus causes the cell to move into S phase and replicates to a much larger copy number (thousands/cell). An uninfected cell would have instead exited the cell cycle upon detachment from the basement membrane (60). Later, when the host cell starts terminally differentiating, the virus starts expressing its structural proteins, L1 and L2, and packages its DNA. Viral progeny are shed with the epithelium non-lytically to complete this productive life cycle (113).

Functions of the various HPV proteins are closely tied to this pattern of differentiation (Figure 1A, 1B, 1C). The first proteins to be expressed after infection are E1 and E2. E1 serves as a DNA helicase (76). The interaction of E1 and E2 is key for the proper recognition of the origin of replication. Together, these proteins
regulate viral DNA replication. E2 also regulates viral DNA replication by binding to the URR upstream of E1, and in keratinocytes, acting as a replication repressor (40). This keeps the levels of all early proteins, including E6 and E7, low. Additionally, E2 plays a role during cell division by tethering the viral DNA to the host chromosomes and allowing separation of viral DNA into the different daughter cells (97).

Also expressed early on are the E6 and E7 proteins. Both E6 and E7 help in maintaining the viral DNA extrachromosomally (105, 133). E6 protein from low-risk HPVs binds p53 along with E6AP and sequesters it in the cytoplasm, inducing p53-mediated apoptosis (132); high-risk E6s are capable of p53 degradation (132). E7 enables the cells to re-enter S phase even as they differentiate, allowing keratinocytes to retain their nuclei, which are normally degraded as daughter cells exit the cell cycle (105, 166). Viral proteins can often cooperate towards a specific goal. For example, E6 and E7 together increase EGFR levels, thus extending the life of human keratinocytes (2). E7 has also been shown to cooperate with HPV protein E5 to stimulate cell proliferation (19). It is thought that E5 (and also E4) are expressed early on as well along with the other early proteins. However, since they are the third and fourth ORFs on a polycistronic transcript, the level at which they are expressed is unknown (105, 126).

Through an unknown mechanism, the differentiated state of the host cell activates the late promoters, leading to the production of L1 and L2 proteins. The viral replication proteins E1 and E2 are also highly produced. E2 does not have negative regulatory control over the late promoter, so viral replication can continue unimpeded.
In this differentiated state, E4 and E5 are expressed as the first and second ORFs on a polycistronic transcript (105). In differentiated cells, E4 associates with keratin filaments and is thought to aid in viral release of progeny (183). The role of E5, like the timing and level of its expression, is mainly unidentified. E5 been implicated in the formation of koilocytes, the activation of EGFR, the downregulation of MHC, and the upregulation of gangliosides, and has been shown to interact with many different lipid raft proteins including caveolin and v-ATPase (169, 171). However, as stated by Bravo et.al, although “many disparate functions have been described for HPV16 E5, we still lack a proper hypothesis bringing them all together into a comprehensible framework” (22).

1.2.3 Functions of HPV E6 and E7 Proteins in Cancer

The HPV life cycle is selected to ensure successful replication and production of viral progeny. HPV uses host machinery for all of its DNA replication. The challenge for papillomaviruses is to maintain replication for packaging of viral progeny even when the host keratinocyte is terminally differentiated. Thus, many of the functions of the early HPV genes are to maintain the host cell in a DNA replication-competent state. In some cases, this ability goes awry. For example, in some cases the viral DNA integrates into the cellular genome rather than remaining episomal, leading to cervical cells that are continually stimulated to proliferate (Figure 1D). Indeed, it appears that a critical step in tumorigenesis is the integration of viral DNA into the host chromosome. Although there is not one defined spot for integration, there are certain
“hot spots” in the host chromosome (177). On the viral side, the integration process almost always involves the loss of the viral protein E2, which normally regulates the levels of E6 and E7. Once E2 is gone, E6 and E7 expression increases (44, 140).

It has been shown that cells that express E6 and E7 from these integrated genomes have a growth advantage over cells that maintain their DNA episomally (80).
### A.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
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<tbody>
<tr>
<td>E1</td>
<td>Viral DNA replication: DNA helicase</td>
</tr>
<tr>
<td>E2</td>
<td>Viral DNA replication: ORI recognition and tethers viral DNA to host chromosomes. Represses E6 and E7.</td>
</tr>
<tr>
<td>E4</td>
<td>Release of viral progeny; Associates with keratin filaments</td>
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<tr>
<td>E5</td>
<td>Cell proliferation, immune evasion</td>
</tr>
<tr>
<td>E6</td>
<td>Cell proliferation; degrades p53</td>
</tr>
<tr>
<td>E7</td>
<td>Cell proliferation; degrades Rb</td>
</tr>
<tr>
<td>L1</td>
<td>Viral capsid</td>
</tr>
<tr>
<td>L2</td>
<td>Viral capsid</td>
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### B.

![Uninfected Epidermis](image)

### C.

![Productive Life Cycle](image)
Figure 1. Productive vs. Abortive Life Cycle  (A) Role of viral proteins (B) Uninfected Epithelium. Cells actively divide at the basal level. As they migrate upwards, cells differentiate, losing their nuclei and their ability to replicate. (C) Productive Life Cycle. Virus must maintain replication of its genome under the changing differentiating conditions of the host. Low copies of DNA are maintained episomally at lower levels of differentiation, and viral DNA divides among daughter cells as they divide. Later on, high viral DNA copy level is maintained as the virus makes its progeny. (D). Abortive Life Cycle. Under some conditions, the viral DNA integrates into the host genome. Early proteins like E2 and E5 are lost, while E6 and E7 oncoproteins are highly expressed. Cells retain their nuclei as they are pushed into a replicative state even under differentiation conditions. Early proteins – yellow. Oncoproteins – red. Late proteins – blue. Green open circle- viral episomal DNA. Grey circles- nuclei. Green circles – Nuclei with integrated viral DNA. Intensity of color signifies estimated protein expression levels.
1.2.4 Phylogenetics of HPV Proteins as it Relates to Cancer

The original classification of papillomavirus types was based on partial L1 and E6 gene sequences, and resulted in five major clades called supergroups. (36). Supergroup A includes all 54 papillomaviruses, both human and non-human, that cause genital infections (although a few cutaneous types were also included) (36). This group includes both high risk viruses, such as HPV-16 and HPV-18 which cause the majority of cervical cancer, as well as HPV-6 and HPV-11, which are non-tumorigenic sexually transmitted pathogens (54). Supergroup B generally consists of viruses that cause latent infections in healthy individuals, but can become problematic for individuals with genetic mutations or who are immunocompromised. This includes HPV-5 and HPV-11 which cause EV (36, 54, 125). Supergroup E only consists of three HPVs, all of whom cause cutaneous lesions, COPV, and CRPV (36, 54). The other two supergroups include the ungulate papillomaviruses, one group for those causing cutaneous lesions and the other for fibropapillomaviruses (36).

In 2004, Bravo published a paper looking at the phylogenetic tree of HPVs based on their E6 and E7 proteins. He found that when HPVs were looked at in this manner, high risk and low risk viruses clustered separately from each other (Figure 2a). This new phylogenetic classification of the virus by its oncoproteins mimicked the epidemiological classifications of the virus better than the classification by supergroups (22). In addition, the phylogenetic tree of the non-oncogenic structural proteins, L1 and L2, did not correlate to oncogenic potential of the virus (Figure 2B) (22).
More interestingly, Bravo looked at the HPV protein E5, which has been postulated to be the third oncoprotein of HPV. He found that the divergence and evolutionary patterns of E5 mirrored that of E6 and E7, not those of the structural proteins L1 and L2 (Figure 2c). There was a clear correlation between the phylogenetic classification of E5 and the oncogenicity of the papillomavirus types. These two findings together led him to suggest that E5, just like E6 and E7, likely has a role in HPV-induced cellular transformation (22).
Figure 2. Phylogenetic Tree of HPV proteins. Trees constructed from protein alignments by the maximum-likelihood method. Phylogenetic tree for E6, E7, and E5 coincide with the classification of virus types as low risk of high risk. (A) E6 tree. (B) L1 tree. (C) E5 tree. E7 tree not shown, but correlates with E6 tree. Black circles – high risk E5s. White circles – low risk E5s. Adapted from Bravo and Alonso 2004.
1.3 Papillomavirus E5 proteins

1.3.1 BPV E5 and transformation

In non-human papillomaviruses, the role of E5 in tumorigenesis is already well established. BPV is able to cause malignant and benign tumors in horses and cattle (95, 118, 169). E5 is the major transforming protein of BPV-1, and can induce focus formation in mouse and human fibroblasts (15, 131). BPV-1 E5 acts both in promoting cellular proliferation at the basal cell level and is active also in differentiating cells (27). Its transformative properties are due in part to its activation of growth factors such as platelet derived growth factor receptor (PDGF-R), which can activate cell proliferation signals (174). In addition, BPV-1 E5 binds the 16kDa subunit of v-ATPase and impairs H+ pumping and alkalizing the Golgi lumen (67, 130). Alkalization of the Golgi lumen by other methods has been shown to transform fibroblasts to grow independently in soft agar (5). Similarly, v-ATPase mutants, mutated in regions responsible for binding to BPV E5, were shown to transform NIH 3T3 cells (5). BPV-1 E5 can maintain activation of c-Src, which has been shown to regulate cell proliferation (170). In addition, BPV-1 E5 is able to sequester MHCI molecules in the Golgi and prevent their transport to the cell surface, thus potentially aiding in immune evasion (9, 108, 169).

A closely related E5 is BPV-4 E5. BPV-4 E5 can also downregulate MHCI molecules at the cell surface (107). Similar to BPV-1 E5, it can also transform fibroblasts and can disrupt gap junctions (169). In addition, both E5s are capable of
disrupting the actin cytoskeleton of bovine cells, but only BPV-1 E5 can impart invasiveness to transformed cells (179).

1.3.2 CfpV2 E5 and XBP-1

The canine familiaris papillomavirus type 2 (Cfpv2) was discovered relatively recently. It is an epidermatropic virus that causes painful lesions on dog footpads, and can lead to metastatic squamous cell carcinoma if left untreated (196). Of note, CfpV2 has an E5 open reading frame that encodes a very hydrophobic 41 amino acid protein (196). Further studies revealed that the CfpV2 E5 protein, similar to the human papillomavirus E5 proteins, localized to the ER (45). In addition, the CfpV2 E5 protein was shown to decrease keratinocyte proliferation, increasing the percentage of cells in G1 and reducing the number of cells in the S phase (45). Unpublished microarray data revealed that E5 was able to reduce the expression of several genes involved with lipid, protein, and carbohydrate metabolism (Condjella, thesis).

These findings provoked an investigation on whether the ER-localized canine E5 protein was affecting the cellular ER-stress response, or the unfolded protein response (UPR). The UPR can be triggered by any major disruption to the ER and its ability to process proteins. Instigating events include a buildup of unfolded proteins in the ER, chemical or lipid imbalances, or recognition of foreign (eg. viral) proteins (20). Induction of ER-stress leads to a halting of general protein translation while attempts are made to rectify the situation; failure can lead to cell death (20).
The status of the cellular UPR can be measured in many ways, including by measuring levels of XBP-1, which has been established as an indicator of ER-stress induction (72). XBP-1 has two transcript forms, the unspliced mRNA, and a longer, more stable, spliced form in which a 26-nucleotide region has been cleaved by the endoribonuclease IRE1α, leading to alternate translation (28, 194). The spliced XBP-1 protein is a highly active transcription factor that regulates ER-chaperone proteins (194). To date, several viruses have been found to affect this pathway in varied and complicated ways. For example, the envelope glycoproteins of the hepatitis C virus (HCV) were shown to induce the UPR in cells by increasing levels of spliced XBP-1 mRNA (34, 35, 175). However, the overall effect was to decrease the transactivating function of the XBP-1 protein, thus suppressing the ER-stress response and promoting HCV expression and viral persistence (175). On the other hand, both the flavivirus and the Japanese encephalitis virus were found to activate the XBP-1 pathway in an attempt to reduce viral-induced cytotoxicity (167, 195).

To test whether the Cfpv2 E5 could alter XBP-1 splicing in any way, a real-time PCR-based assay was developed to measure the levels of spliced versus unspliced XBP-1 mRNA transcripts. It was found that the Cfpv2 E5 could consistently increase the ratio of spliced to unspliced XBP-1 transcripts in HFKs (194). This change was specific to Cfpv2 E5, since not all ER-expressing viral proteins were found to alter XBP-1. Low-risk HPV-6b E5 was found to have no change on XBP-1 splicing, as shown in Figure 3 (45). Other effects on the ER-stress pathway for Cfpv2 E5 have yet to be studied. Preliminary data gathered from microarray analysis indicate that Cfpv2
E5 may be able to downregulate COX-2, another member of the ER-stress pathway that may be downstream of XBP-1, by two fold (Condjella, unpublished).
Figure 3. HFK cells stably transduced with LXSN or HPV-6b E5; \( n = 4 \). Bars represent means ± standard errors of the mean. Adapted from Condjella, et. al. 2009.
1.3.3 Biological Properties of HPV-16 E5

Given the importance of E5 in BPV, the natural question is whether these transforming properties are shared by E5s of the human papillomavirus. Phylogenetic studies indicated that E5s of low-risk HPVs cluster together with each other and separately from E5s of high-risk E5s (22). The correlation of phylogeny with cancer risk suggested that HPV-16 E5 might also contribute to tumorigenesis.

The main oncoproteins of HPV-16 are E6 and E7, which are both necessary and sufficient for cell immortalization. E5 is neither necessary nor sufficient for immortalization. Besides E2, E5 is one of the other proteins that is assumed to be disrupted during viral integration (113, 158). Estimations for the percentage of HPV-induced cervical cancers that have integrated DNA – and therefore potentially no E5 – varies greatly, from 15-86% (6, 41, 156). One study estimated that only 60% of HPV-16 induced cervical cancers might express E5 (37, 169).

For several reasons, E5 is considered the third oncoprotein of HPV. First of all, the lack of E5 at later stages of malignant transformation does not mean that early E5 expression is not essential in establishing a successful and persistent infection (the precursor to dysplasia and cancer). It has been suggested that E5 helps to expand the initial population of HPV infected basal cells, perhaps by enhancing EGFR activation (169). Second, while E5 is present in all high risk viruses, many low risk types either lack an E5 ORF altogether or lack a translation start codon (22, 149). In addition, while E5 alone cannot induce focus formation or immortalize cells, it can induce anchorage-independent growth of immortalized cells in soft agar (96). Finally, E5 is
able to enhance the transformation of cells by E6 and E7 in-vivo. For example, it was shown that estrogen-treated transgenic mice expressing HPV-16 E5 in addition to E6 and E7 developed a larger number of tumors than mice expressing E6 and E7 alone (63, 137).

How E5 actually causes these observed phenotypes is still under debate, although there are several possibilities, including EGFR activation, activation of c-jun and c-fos, binding of v-ATPase, disruption of gap junctions, immune evasion, formation of koilocytes, and binding of nuclear transport proteins (Table 1) (90, 91, 169, 171).
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Table 1. HPV E5 affects the proliferation and structure of virally-infected cells.

Many targets for HPV E5 have been identified using various cellular and animal models. The effects on these targets can broadly be classified as contributing to cell proliferation, affecting cell transport and cell junctions/communication, and contributing to the immune evasion of HPV-infected cells.
Research on HPV-16 E5 and EGFR was stimulated by two findings. One was that 16E5 induced anchorage-independent growth in fibroblasts over-expressing the EGFR (96). The other was that 16E5, with EGF stimulation, could induce anchorage-independent growth in keratinocytes. Cells with 16E5 also showed increased recycling of the EGFR and a higher magnitude of EGFR phosphorylation, although this required ligand (EGF) binding (48, 165). It was found that rates of EGF internalization and degradation, as well as affinity levels of EGFR for EGF, were unaltered. However, 16E5 was found to increase the number of EGFR on the cell surface and delay the degradation of EGF-bound EGFR (165). In addition, it was found that E5 affects the interaction of c-cbl, the ubiquitin ligase that targets the EGFR for degradation, with EGFR (49, 98, 199). This in-vitro data was supported by a study in transgenic E6/E7 mice, where E5 was targeted to the basal epithelium. The study found that tumors induced by E5 in transgenic mice required EGFR (63).

Because of these EGFR studies, other experiments were done on related EGF-induced pathways. It was found that 3T3 cells expressing E5 had higher levels of c-fos and c-jun than non-E5 expressing cells, perhaps due to an interaction between E5 and ERB4 (19, 38). In addition, E5 also induced COX-2 expression through NF-kappaB when expressed in HaCat cells (85). E5 was also found to increase MAPK signaling, thereby potentially protecting cells from apoptosis (198).

Not all studies found a correlation between E5 and EGFR, however. Laimins et al. published a report saying that HPV-31 E5 had no effect on the growth rate of human foreskin keratinocytes (HFKs) in the presence or absence of EGF.
Additionally, the authors found no differences in surface EGFR levels between HFKs with 31E5 and HFK without (59). We have found similar results when studying HPV-16 E5 and HPV-6b E5 in HFKs (see Figure 4; unpublished data by Sudarshan; similar results by Suprynowickz et. al. (manuscript in revision; J. Virology)).
A.

B.
Figure 4. No change in surface EGFR between cells expressing 16E5, 6bE5, or pLXSN. HFKs stably expressing 16E5, 6bE5 or pLXSN were either serum starved (to increase surface EGFR) for 24 hours or grown in normal KGM. Cells were then fixed and stained with either A) anti-EGFR conjugated to PE or B) Alexa-flour 448-EGF to measure the number of surface EGFR. As expected, cells which were serum starved exhibited higher number of EGFR than did cells that were not serum starved. However, no difference was seen between cell types.
Another finding involving both HPV-16 E5 and HPV-6 E5 was that they both bind to the 16K subunit of the v-ATPase pump (5, 46). This was interesting because it seemed to explain how E5 could affect the recycling of the EGFR. V-ATPase is responsible for endosome acidification. EGFR requires endosomal acidification for dissociation of EGF from the EGFR and for receptor degradation. It was theorized that E5 binding would disrupt acidification by binding v-ATPase and as a secondary effect would increase EGFR activation. In fact, it was found that E5 disrupts endosomal acidification, even though it did not disrupt other intracellular compartments (51). However, later it was shown that the area of HPV-16 E5 that binds to v-ATPase is not the area required for EGFR activation (139). In yeast, it was shown that several E5 mutants that could still bind v-ATPase failed to disrupt acidification, suggesting that E5 binding to v-ATPase alone is not sufficient to affect the v-ATPase’s activity (1, 39). Yet other studies in yeast suggested that HPV-16E5 binding does not affect v-ATPase dependent acidification at all (7).

V-ATPase is also found in gap junction-like membrane complexes, and it was theorized that E5 could potentially effect cell-cell communication as well (67). In fact, E5-infected HaCaT cells demonstrated an impairment of dye transfer ability compared to non-E5 cells. This impairment was thought to be due to the decreased phosphorylation of connexin-43 (123). In fact, levels of connexin-43 were found to be substantially decreased in the presence of E5 (178).

HPV-16 E5 has also been suggested to have a role in immune evasion. E5 was shown to prevent the transport of MHCI to the cell surface, perhaps by interaction with
MHCI molecules through its first hydrophobic domain (8). This interaction involved a complex with calnexin, and it was found that E5 could not down-modulate MCHI levels in calnexin-deficient cells (69). Additional studies suggested that the effect of E5 on MCHI levels and EGFR activation could both be explained as consequences of E5’s initial alteration of the lipid composition of cellular membranes (23). In addition, it was suggested that due to E5’s effects on endosomal acidification, E5 is able to affect appropriate peptide loading onto MHCII molecules, further inhibiting recognition of virally infected keratinocytes (197). Another finding relating E5 to immune evasion was that E5 upregulated ganglioside GM1, a strong inhibitor of cytotoxic T lymphocytes, in keratinocytes (171). This change was found to be mediated by the last ten amino acids of E5 (171).

Another phenotype attributed to both high- and low-risk E5s is the ability to induce koilocytosis in conjunction with high or low risk E6. Koilocytes, the pathognomonic feature of HPV infection, are essentially “squamous epithelial cells that contain an acentric, hyperchromatic nucleus that is displaced by a large perinuclear vacuole” (91). It is hypothesized that the role of koilocytosis is to facilitate viral escape from keratinocytes. AU1-E5 was localized to koilocyte vacuoles, and especially to the vacuolar membranes (91). In addition, E5 was also seen to colocalize with and associate with karyopherin beta3, a nuclear transport protein. Interestingly, a mutant with decreased karyopherin binding also showed a decreased ability to cause koilocytes (90).
E5 pulls down many other proteins in co-IP experiments as well, and most of these proteins reside in lipid rafts. For example, E5 binds and upregulated caveolin-1, a component of lipid rafts that mediates cell signaling, at the cell surface (171). Also, E5 targets B cell associated protein 31 (Bap31), which was shown to be a regulator of keratinocyte proliferation following differentiation. The C-terminus of E5 was shown to be important for this interaction (135).

In 2008, Auvinen et al. published a study on the effects of HPV-16 E5 on cellular gene expression. The study aimed to resolve the disparate findings regarding 16E5 and to categorize areas of gene expression change for further analysis. They found that 16E5 affected genes pertaining to cell adhesion, cell motility and mitogenic signaling. Specifically, they were able to confirm at a protein level that 16E5 downregulated lamin A/C and upregulated protein kinase C-delta and phosphoinositol-3-kinase proteins. However, the study was performed in HaCaT cells, which are a spontaneously immortalized cell line from adult trunk keratinocytes. In addition, 16E5 was expressed under a dexamethosone-inducible promoter for 24 hours; longer times were found to be lethal. Finally, because HPV-16 E5 was compared to vector alone, it was not clear which gene changes were common across all E5s (low and high risk), which were responsible for 16E5’s oncogenic properties, and which gene changes occurred simply as a response to exogenous viral protein expression or apoptotic signaling (86).

1.3.4 Structural Properties of HPV-16 E5
Without any antibody to the native E5 protein, it has been hard to define the protein’s exact localization, structure, expression level, and interactions. In biological studies in physiologic cell lines, it has proven very difficult to visualize even epitope-tagged E5 due to low expression levels. For this reason, many E5 studies have been performed with a codon-optimized version, where codons used infrequently in mammalian are substituted with codons more frequently used in an effort to boost protein levels (52). E5 localization and structure studies are generally performed with this tagged, codon-optimized construct.

HPV-16 E5 has 83 amino acids. These amino acids are not well conserved amongst HPVs, although there are more similarities to other high-risk E5s than to low-risk E5s (4). Given that 16E5 has six cysteines, there has been much speculation on ability of 16E5 to dimerize. Several studies have shown that using a high expression vector, E5 can dimerize in COS-1 cells (52, 172). Still, others have shown that E5 cannot form oligomers (46, 79). Our lab (see Figure 5) has recently shown that when 16E5 is over-expressed in COS-1 cells using a high-expression vector, pJS55, it will dimerize. However, when expressed in the same cells using a low-expression vector, pLXSN, or when expressed in human ectocervical cells (HECs), E5 does not dimerize (92). This suggests that whether E5 dimerization is apparent or not might be a function of both the cell type studied as well as the level of E5 expression. This finding has been true for other E5 phenotypes as well. For example, when studying localization in COS-1 cells, E5 is seen both in the Golgi and in the ER (46). However, in keratinocytes, E5 localizes exclusively to the ER (52). It
is possible that over-expression in COS-1 cells leads to a backup of E5 in the Golgi.

Similarly, it is possible that E5 only dimerizes when over-expressed.
Figure 5. **16E5 dimerization is a function of the expression level.** COS cells were transfected with AU1-16E5 in the pJS55 or pLXSN expression vector. 16E5 was immunoprecipitated from lysates of these COS cells or from lysates of stable HEC lines by using a mouse anti-AU1 monoclonal antibody (Covance). The immunoprecipitates were eluted for SDS-PAGE by using 2x Laemmli sample buffer with or without 10% β-mercaptoethanol (βME). Western blots were labeled with a rabbit anti-AU1 polyclonal antibody. Since the pJS55 vector contains the simian virus 40 (SV40) origin of replication, it was extensively amplified in COS cells, and the levels of the E5 protein were greatly overexpressed, unlike the situation with the LXSN vector. Longer exposures of pLXSN-16E5-transfected COS cells and HECs (bottom) failed to reveal the presence of E5 dimers. Molecular mass markers (in kDa) are indicated on the right. From Krawcyzk, et. al (2010). Copyright by American Society for Microbiology.
A notable feature of HPV E5s is the conservation of three hydrophobic domains (26). Because of the extreme hydrophobicity E5, it has been difficult to determine its crystal structure. Still, attempts were made to detect its secondary structure using circular dichroism spectroscopy. Based on these results, it was predicted that HPV-16 E5 has three transmembrane regions (4). Immunofluorescence using differential permeabilization data demonstrated that the N-terminus is in the ER lumen, while the C-terminus is in the cytoplasm (92).

1.4 Current Questions in the HPV-16 E5 field and Statement of Purpose

Although HPV-16 E5 has been studies for many years, its role in HPV-induced carcinogenesis remains poorly defined. The lack of an E5 antibody has resulted in frequent use of over-expression systems with codon-optimized, epitope-tagged E5. Basic questions, such as when and how often E5 is present in cancer cell lines or cancer tissues, remain unanswered. Without this crucial information, it is unknown how E5 functions in the HPV life cycle and tumor progression. In addition, due to the difficulty and expense in growing primary cells, there are few studies evaluating the effect of E5 on the relevant host cell. The only previous microarray analysis of HPV-16 E5 was performed in HaCaT cells, a spontaneously immortalized adult trunk keratinocyte line with mutant p53 (86). E5 expression in these cells induces high levels of apoptosis, requiring the use of an inducible promoter.

This thesis aims to answer some questions pertaining to 16E5 biology, with the goal of identifying those properties of E5 which contribute to oncogenesis. The first
aim was to examine the gene expression profile of high-risk 16E5 by microarray and real-time RT-PCR. By comparing the gene profiles of vector control, low-risk 6b E5, and high-risk 16E5, we aimed to identify unique biochemical markers for high-risk E5. All studies are conducted in three separate strains of primary genital keratinocytes in order to best mimic natural conditions of infection. The second aim was to answer the question of when and in what background E5 is expressed. We developed a mass spectrometry method to detect the native, untagged 16E5 protein and used this method to definitively establish the presence of 16E5 protein in cervical cancer cell lines.
II. MATERIALS AND METHODS
2.1. Constructs. E5 mutant constructs were generated by Celtek Biosciences (Nashville, TN). All constructs have an N-terminal AU1 tag (DTYRYI). A Kozak sequence (CTCGAG) was also included 5’ of the start codon. For cloning purposes, constructs were built with EcoRI (5’), XhoI (5’), BamHI (3’) and SalI (3’) restriction sites flanking the E5 open reading frame. EcoRI and BamHI sites were used to clone the construct into the pLXSN vector for stable expression (Clontech, Mountain View, CA).

2.2 Cells and Cell Culture. Human foreskin keratinocytes (HFKs) were prepared from human foreskins donated by Georgetown University Hospital. Human ectocervical cells (HECs) were obtained from tissue procured from hysterectomies following benign uterine disease, cultured according to Baege et al. (2002), and immortalized with retroviruses encoding HPV-16 E6 and E7 followed by puromycin selection (10). Both HFKs and HECs were maintained in Keratinocyte Serum Free Media (Invitrogen, Carlsbad, CA), supplemented with 50 g/ml bovine pituitary hormone, 26 ng/ml recombinant epidermal growth factor, and 10 g/ml gentamycin (KGM). Cos-1, SiHa, HeLa, C33A, and SD3443 cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in complete Dulbecco’s Modified Eagle’s Medium (DMEM), with 10% fetal bovine serum (FBS), 2 mM glutamine, and penicillin/streptomycin (Invitrogen). CaSki cells were maintained in RPMI 1640 (Invitrogen), supplemented with 10% FBS, 2 mM glutamine, and penicillin/streptomycin. All cells were maintained using T75 or T175 flasks. RNA or
western blot lysates were collected from 100mm tissue culture plates, all from BD Falcon (San Jose, CA).

2.3 Creation of Phoenix Retroviral Stocks and Cell Transduction. 5x10^6 SD3443 retroviral packaging cells were plated per 100mm dish overnight in DMEM complete. After 24 hours, media was replaced with 5 ml serum-free DMEM and plates were treated with 25 uM chloroquine for at least 15 minutes. Cells were transfected with 4 µg of DNA using Lipofectamine Plus transfection reagent (Invitrogen) per manufacturer’s protocol for retrovirus packaging. After 4 hours, 5 ml complete DMEM with 20% FBS was added to the plate. The next day, the media was replaced with 5 mls fresh DMEM complete with 10% FBS. After 24 hours, retrovirus was collected by harvesting the supernatant and filtering it through a 0.22 mm filter (Millipore, Billarica, MA) to remove cell particulates and ensure sterility. Retrovirus was either used fresh or stored at -80°C until needed.

To transduce cells, 1.5 ml retroviral stock supplemented with 1.5 µl polybrene was added to cells in T75 flasks at 40-60% confluency. Cells were incubated with the retrovirus on a gentle rocker at 37°C. After 2 hours, the retrovirus was removed and replaced with media appropriate to the cell type. Cells were allowed to grow to approximately 80%, which occurred within 1-3 days. For cell selection, geneticin (G418) (Invitrogen) was used and selection was maintained until all the cells in the control (uninfected) flask died. For HFKs and HECs, G418 selection was 75-100 µg/ml.
2.4 Cos-1 Cell Transfection. For western blot, Cos-1 cells were plated in antibiotic-free OptiMem + 4% FBS (Invitrogen) onto 100 mm tissue culture dish plates (BD Falcon) at 80% of a one day split. For immunoflorescence, cells were plated instead on sterile glass coverslips (22 x 22) in 6 well plates. The next day, cells were transfected with 32 μg DNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. After 4 hours, the media was replaced. Cells were harvested or stained 48 hours after plating or at ~80% confluency.

2.5 Immunoflorescence. 24 hours after transfection (COS-1 cells) or plating (for stably-expressing cells such as HFKs and HECs), cells were washed with PBS and fixed with 4% (w/v) paraformaldehyde. After 10’ incubation on ice and subsequent 15’ incubation at RT, cells were washed 4 times with PBS. Cells were then permeabilized with 0.1% (w/v) saponin for 10 minutes, washed 2 times with PBS, and blocked for 20 minutes in a humid box with P-GelS (PBS with 0.2% gelatin and 0.1% saponin) and 20% normal donkey serum. After three PBS washes, cells were covered with primary antibody for one hour in the humid box, followed by another three washes. Primary antibodies used were rabbit polyclonal anti-AU1 (1:1500 dilution, Covance) and rabbit polyclonal anti-calnexin (1:75 dilution, Santa Cruz). Cells were incubated with a 1:400 dilution of Alexa-flour secondary antibody for one hour at room temperature. After another three PBS washes, cells were washed with PBS containing 2% gelatin. Then, nuclei were stained with 0.5 mg/ml Hoeschst stain for 3 minutes at room temperature. Coverslips were then removed and inverted over slides.
with 30 μl mounting media (Invitrogen) and allowed to rest at room temperature for several hours until the mounting media hardened. Slides were stored at 4°C overnight and viewed the next day using a Zeiss Axioskop microscope (Carl Zeiss, Inc., Thornwood, NY). Cells were imaged using a 63x objective, Hamamatsu CCD camera, and Openlab 3.0.7 software.

2.6 Cell Lysis and Protein Concentration. For direct western, whole cell lysates were made by plating cells on 100mm dishes (BD Falcon) and allowing them to grow to 80% confluence. Plates were washed with cold PBS, and cells scraped in 300μl of 2x Laemmlli buffer. Lysates were kept on ice, then boiled for 10’ at 110°C, allowed to cool for 2 minutes, and frozen on dry ice. Prior to protein assay, lysates were thawed in a 37°C water bath. Prior to loading, up to 45 μl of sample (40-60 μg protein) was mixed with a volume of β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) equal to 10% of the final loading volume. For immunoprecipitation, cells were scraped instead with 1.2 ml RIPA buffer with 12μl protease inhibitor cocktail set 1 (Calbiochem, 100X stock) and frozen on dry ice. Prior to protein assay, lysates were thawed in a 37°C water bath, DNA was sheared with a 23G needle, and lysates were spun down at 2K rpm. Protein concentration for both lysates was determined using the BioRad Dc Protein Assay (Bio-Rad Laboratories, Hercules, CA) per manufacturer’s protocol.
2.7 Immunoprecipitation. Equal amounts of protein (up to 600 μg) per sample were added to 40 μl Protein A Plus beads (Pierce, Rockford, IL). After washing with 1 ml PBS, beads were rotated for 90’ end-to-end with antibody. After being spun down for 1’ at 2k rpm, beads were washed with 1 ml cold RIPA buffer with protease inhibitors, followed by an additional 5’ rotation and 1’ centrifugation. This was repeated two times more, followed by three consecutive washes with PBS (no rotation). Beads were pelleted and then resuspended in 47 μl 2x Laemmli with 10% βme. No βme was added if reducing conditions were not to be used (as for E5 dimerization studies). After 20 minutes in a 37°C water bath, beads were boiled for 6 minutes at 110°C before being frozen on dry ice. Prior to gel loading, samples were thawed in a 37°C water bath. Antibodies used for E5 immunoprecipitation included rabbit and mouse anti-AU1 (BABCO/Covance), used at 4ul/tube (~4 ug).

2.8 Western Blot Analysis. Samples were electrophoretically separated on Tris-Glycine gels (Invitrogen) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). Membrane was blocked for 30’ in either PBS with 5% nonfat dry milk or in wash buffer with 2% BSA, depending on the antibody. Primary antibodies were left overnight at 4°C on a rocker. Primary antibodies used include: XBP-1 (1:300, Santa Cruz), COX-2 (1:200), IRE1α (1:200, Cell Signaling), PD1 (1:200, Cell Signaling), ERO1α (1:200, Cell Signaling), and BIP (1:200, Cell Signaling). β-actin (Sigma-Aldrich, St. Louis, MO) at a final dilution of 1:10,000,
served as the loading control. Membranes were washed two times for 15’ with either PBS + .05% Tween or wash buffer (Fisher Scientific, Waltham, MA). Membranes were then probed with secondary antibodies. These were either antibodies conjugated to horseradish peroxidase (HRP) (Santa Cruz) used at a 1:5000 final dilution for 30’ at room temperature, or were Tropix alkaline phosphatase conjugated antibodies used at a 1:5000 dilution for 90’ at room temperature. The membrane was rinsed again three times for 10 minutes in the appropriate buffer. For HRP secondary antibodies, the Western Blotting Luminol Reagent (Santa Cruz) was applied for less than two minutes before exposure to Kodak BioMax MR film (Eastman Kodak Company, Rochester, NY). For alkaline phosphatase secondary antibodies, membranes were washed in 1X Tropix Assay Buffer for 5’ prior to incubation with CDP Star Reagent (New England Biolabs) for 5’ before exposure to film. Blots were stripped between antibodies using Restore Western Blot Stripping Buffer (Pierce, Rockford, IL). Densitometry was performed using BioRad Quantity One imaging system (Bio-Rad Laboratories, Hercules, CA)

2.9 RNA extraction and generation of cDNA. RNA was harvested from 100 mm tissue culture dishes (BD Falcon) at 80% confluence using 1 ml TRIzol Reagent according to manufacturer’s protocol. Alternatively, the RNAqueous-4PCR kit was used to harvest RNA according to manufacturer’s protocol (Ambion, Austin, TX). For both methods, DNAse treatment was done according to the manufacturer’s protocol for the RNAqueous-4PCR Kit. RETROScript kit (Ambion) was used to perform reverse-
transcriptase polymerase chain reaction (RT-PCR). RNA was denatured for three minutes at 80° C with Oligod(T) and Random Hexamers. This was followed by the reverse-transcriptase step consisting of 60 minutes at 45 °C and 10 minutes at 92° C. cDNA samples were diluted between 25-75 ng/ul (with a 10-100x dilution for GAPDH) and stored at -20 until needed for PCR or for real-time PCR.

2.10 Microarray. cDNA microarray analysis was performed on HFKs that were retrovirally infected and selected for HPV-16 E5, HPV-6b E5, or pLXSN. Each sample from E5-expressing keratinocytes was run against the pLXSN vector in a two color Agilent whole human genome slide with a 4 x 44K format. For each E5, there were a total of six arrays, consisting of three biological replicates run in twice for dye swapping. RNA was harvested from 100 mm tissue culture dishes (BD Falcon) at 80% confluence using 1 ml TRIzol Reagent according to manufacturer’s protocol. DNase treatment was done according to the manufacturer’s protocol for the Rnaqueous-4PCR Kit (Ambion, Austin, TX). RNA was sent to MOGene, LC (St. Louis, MO) for microarray analysis. RNA was amplified using the Agilent Low Input Linear Amplification kit (Agilent Technologies, Santa Clara, CA), and then labeled with either cyanine-5 or cyanine-3 using the ULS aRNA Fluorescent Labeling Kit (Kreatech Biotechnology, Amsterdam, The Netherlands) according to manufacturer’s instructions. 825 ng each of labeled c-DNA was hybridized overnight at 65° C in an ozone-free room to protect the label. All washes and hybridization conditions followed were consistent with the Agilent processing manual (protocol version 4.0). Arrays
were scanned using Agilent scanner (G2505B) and extracted using the Agilent Feature Extraction software (Agilent Technologies, Santa Clara, CA). Analysis of data by MOGene was done using the GeneSpring software (Agilent). The Bioinformatics and Biostatistics Shared Resource at the Georgetown University Lombardi Comprehensive Cancer Center (Washington, DC) performed pre-processing and differential analysis, including calculating average fold change and p-values, using Rosetta Resolver (Rosetta Biosoftware, Microsoft).

2.11 PCR for E5 expression. cDNA was prepared as previously described and then used for PCR. This involved a preliminary denaturation step at 94°C for 4 minutes, followed by 30 cycles of: 30 seconds at 94°C, 30 seconds at 55°C, and 45 seconds at 72°C. This was followed by a final extension step of 10 minutes at 72°C. E5-specific and GAPDH primers used include:

- HPV-16 E5 and mutants-Forward: 5’- GCTGGCCTGCTTTCTGCTGT -3’
- HPV-16 E5 and mutants-Reverse: 5’- CCTAAAGGCAGAGGCTGCTG -3’
- HPV-6b E5-Forward: 5’- TGTACACATCTGTGCTAGTACT -3’
- HPV-6b E5-Reverse: 5’- GGACAGTAACACACAAAGTA -3’
- HPV-6b E5 Mutant (6bYI)-Forward: 5’-GGCACCACATCAACCTTTTAT-3’
- HPV-6b E5 Mutant (6bYI)-Reverse: 5’-TATAGACGATGAACTCGCTG-3’
- GAPDH forward: 5’-TCTCCTCTGACTTCAACACCCAAGT-3’
- GAPDH reverse: 5’-GAAATGAGCTTGACAAAGTG-3’
PCR products were run using a 1.2% gel on a Flash Gel System (Lonza, Rockland, ME), and photographed under ultraviolet light.

2.12 Quantitative Real Time PCR. cDNA was prepared as previously described and then used for real-time PCR. GAPDH served as the control. Real time reactions for E5 constructs and GAPDH (primers listed above in the section ‘PCR for E5 expression’) were 20μl, but contained 0.8 μl cDNA at 75 ng/μl, 10 μl 2x Bio-Rad IQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA), 0.125 20uM forward primer, 0.125 20uM reverse primer, and 8.95 ul dH₂O. Primers for native E5 (untagged, not codon-optimized) and E1^E4 spliced product for use in cancer cell lines include:

Native E5-Forward: 5’- CTGTGCTTTTGTGTGCTTTG -3’
Native E5-Reverse: 5’- AAGCGTGCATGTGTATGTATTAAA -3’
E1^E4 spliced transcript-Forward: 5’- TGGCTGATCCTGCAGCAGC -3’
E1^E4 spliced transcript-Reverse: 5’- AGGCGACGGCTTTGGTATG -3’

Other real time reactions were 20μl and contained 0.8 μl cDNA at 75 ng/μl, 10 μl 2x Bio-Rad IQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA), 0.125 20uM primer mix (forward and reverse primers), and 9.08 ul dH₂O. Primers using these conditions were ordered from RealTimePrimers.com and include:

GAPD- Forward: 5’- GGAAGGACTCATGACCACAG -3’
GAPD- Reverse: 5’- TTGGCAGGTTTTTCTAGACG -3’
COX-2/PTGS2-F: 5’- TCT GAA ACC CAC TCC AAA CA -3’
COX-2/PTGS2-R: 5’- AAG GCT TCC CAG CTT TTG TA -3’
IRE1α/ERN1-F: 5’- GGC GAA CAG AAT ACA CCA TC -3’
IRE1α/ERN1-R: 5’- TCA CTG TCC ACA GTC ACC AC -3’
MED26-F: 5’- AGC ATC CAT GAC CTG AAG AG -3’
MED26-R: 5’- AAG CTC TCT GGA CTC CCA CT -3’
UBE2E1-F: 5’- GCA AAC CGA GAA AGA AAC AA -3’
UBE2E1-R: 5’- GGC CCT AGA ATG GTT GAT CT -3’
GPR135-F: 5’- AGG GCT ACC GGA CTA GGA AT -3’
GPR135-R: 5’- TTA GGC TGT TTG GTC ACT GC -3’
CDK2NC-F: 5’- AAT GGA TTT GGA AGG ACT GC -3’
CDK2NC-R: 5’- CAG CTT GAA ACT CCA GCA AA -3’
MMP9-F: 5’- CTC TGG AGG TTC GAC GTG -3’
MMP9-R: 5’- GTC CAC CTG GTT CAA CTC AC -3’
PLA2G4C-F: 5’- ATC GAT TTA CCC GAC AGG AG -3’
PLA2G4C-R: 5’- GGG TAG TGT CCC TTC TTC CA -3’
SERPINA3-F: 5’- CTC AGT CTG CTG GAC AGG TT -3’
SERPINA3-R: 5’- TGA GTA TCT TGG GGG TCA AA -3’
ICAM1-F: 5’- TTT TCT ATC GGC ACA AAA GC -3’
ICAM1-R: 5’- AAT GCA AAC AGG ACA AGA GG -3’

Three biological replicates for each sample were run in triplicate on a 96-well plate and spun down for 5’ at a low RPM. Reactions were annealed and analyzed using a Bio-Rad iCycler and accompanying software (Bio-Rad Laboratories).
2.13 Protein Mass Spectrometry. Cells were plated on 100 mm tissue culture dishes (BD Falcon) and allowed to grow to 80% confluency. Cells were washed with PBS and harvested in 0.5 ml RIPA buffer with protease inhibitors (Calbiochem, 100X stock). Lysates were frozen on dry ice immediately and stored at -80°C until use. Samples were thawed quickly in a 37°C water bath, and then centrifuged at 14,000 g for 15’ to pellet any debris. 200 µL of each cell lysates were filtered using a centrifugal filter device with a molecular weight cut-off (MWCO) of 10kDa (Catalog # UFC501096, Millipore, Billerica, MA or Catalog #Z722065, Sigma) at 14, 000 × g for 5 minutes. The flow-through was collected, vacuum dried, reconstituted with 20µL of dissolution buffer composed of 0.5 M triethylammonium bicarbonate (TEAB) pH 8.5. The samples were then denatured by adding 1 µL of a 2% SDS solution. Reduction was accomplished by incubating the samples for 1 hour at 60°C with 1µL of TRIS-(2-carboxyethyl) phosphine (TCEP). Cysteines were blocked by adding 1µL of a 200 mM solution of methyl methane-thiosulfonate (MMTS) and incubating the mixture for 10 minutes at room temperature. Samples were then digested by 1µg of trypsin overnight at 37°C. 5 µL of each sample was injected and peptides were fractionated using a reversed phase column BEH C18 column (1.7 μm, 75 μm x 150 mm, Waters), on a nanoUPLC Acquity (Waters) with buffer A (2% acetonitrile, 0.1% formic acid) and buffer B (98% acetonitrile, 0.1% formic acid). Analytes were eluted over a 30 min solvent B linear gradient of 0-60% with a 300 nl/min flow rate. The nanoUPLC instrument was coupled to a 4000QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems, Framingham, MA). Eluted peptides were ionized in
the positive mode using a fused silica PicoTip emitter (New Objective, Woburn, MA) with a spray voltage of 2300 V, a curtain gas of 13, a nebulizer gas of 13, an interface heater temperature of 180°C, at unit resolution for Q1 and Q3 for MRM mode, and at low resolution in an enhanced product ion scan. Methods for 4000QTRAP mass spectrometer include dependent Enhanced Product Ion scans, which are triggered when the MRM (multiple reaction monitoring) signal exceeds a threshold (50 counts/s). A 493.3 Da primary ion was monitored with the following secondary ions: 215.1; 233.1; 261.1; and 374.2 Da.
III. THE HPV-16 E5 PROTEIN REPresses EXPRESSION OF STRESS SIGNALING GENES XBP-1 AND COX-2 IN GENITAL KERATINOCyTES
3.1 Abstract

The HPV-16 E5 protein resides in membranes of the endoplasmic reticulum (ER) and modulates cell growth and viral replication. In order to help define its biological activities, we analyzed E5-induced changes in human keratinocyte gene expression. Our studies identified the downregulation of spliced XBP-1, a key player in the ER-stress response, as a biochemical marker of E5 expression. IRE1α, the endoribonuclease responsible for XBP-1 RNA splicing, was also downregulated. Furthermore, cDNA microarray analysis revealed the repression of COX-2, another member of the ER stress pathway. In contrast, these genes were not altered either by the low-risk HPV-6b E5, or a C-terminal HPV-16 E5 mutant, in which the histidine and alanine residues (conserved in high-risk HPVs) were replaced with tyrosine and isoleucine (conserved in low-risk HPVs). HPV-16 E5 was also able to lower COX-2 mRNA levels in cells co-expressing E6/E7, suggesting that it might exert similar activity during viral replication. Interestingly, the E6/E7 genes were independently able to lower COX-2 transcripts compared to vector cells, indicating that multiple pathways of COX-2 repression exist. COX-2 downregulation by E5 could be overcome by thapsigargin or tunicamycin treatments, which initiate ER-stress via calcium fluxes and abnormal protein glycosylation respectively, making it unlikely that E5 specifically tempers these pathways. Overall, our data indicate that E5 represses the cellular ER-stress response and suggest a potential role for E5 during productive HPV infection.
3.2 Introduction

Cervical cancer is the second most common cancer in women worldwide (128) and the high-risk human papillomaviruses (HPVs) are responsible for nearly all of these malignancies (117). Approximately 60% of HPV-induced cervical cancers are caused by the high-risk type 16, which encodes three transforming proteins: E5, E6, and E7. The E6 and E7 proteins cooperate to immortalize primary keratinocytes (71, 115) and target several key cell regulatory proteins including p53 (73, 77, 147), Rb (110, 116), Myc (68, 181), hTERT (103), and a significant number of proteins with PDZ domains (73, 87, 176, 184). In contrast, HPV-16 E5 is a weak transforming protein, which localizes predominantly to the ER (171). It is postulated to expand the initial population of HPV-infected basal cells, perhaps by enhancing EGFR activation (48, 63, 169). While E5 alone cannot immortalize human primary cells, it can induce anchorage-independent growth of immortalized rodent cells in soft agar (165) and enhance cell immortalization by E6/E7 (164). In addition, estrogen-treated transgenic mice expressing HPV-16 E5 in addition to E6 and E7 developed a larger number of tumors than mice expressing E6 and E7 alone (63, 137). Most recently, our laboratory reported that E5 induces koilocytosis in collaboration with E6 (91). The mechanism behind these E5-induced phenotypes is unknown. However, the ability of E5 to bind several cellular proteins, including the 16-kDa subunit of the vacuolar H+-ATPase (1, 5, 46), BAP31 (135), HLA (8, 69), ErbB4(38), calnexin (69), and karyopherin β3 (90) might account for some of its biological activities.
We recently reported that the ER-localized E5 protein of canine familiaris papillomavirus (CfPV2) alters XBP-1 splicing in keratinocytes (45). XBP-1 is part of the ER-stress response pathway, and alteration of its splicing has been shown to assist viruses establish persistent infections and promote the viral life cycle (93, 175, 195). To determine whether our findings with the small canine E5 protein (40 amino acids) might also apply to the larger HPV E5 proteins (approximately 80 amino acids), we examined the effect of the high-risk HPV-16 E5 protein on XBP-1 splicing. In addition to analyzing the XBP-1/IRE1α pathway, we also used a gene expression microarray approach so that we would capture any additional alterations in the ER stress pathway. The only previous microarray analysis of the effect of HPV-16 E5 expression was performed in HaCaT cells, a spontaneously immortalized keratinocyte line with mutant p53 (86). E5 expression in these cells induces high levels of apoptosis, requiring the use of an inducible promoter. Consequently, analysis is temporally limited following the induction of E5 and is potentially confounded by the genetic changes present in these cells. Rather than study immortalized cells, we chose to examine gene expression changes in primary genital keratinocytes which are the host cell of high-risk HPV infection.

In brief, our results with primary keratinocytes differ very significantly from those obtained with the immortalized HaCaT cells in terms of the number and types of genes that were altered in expression. We found that 16E5 repressed the levels of spliced XBP-1 mRNA as well as the expression of IRE1α, the endoribonuclease that
cleaves XBP-1 mRNA. Furthermore, microarray analysis revealed that COX-2, which is downstream of XBP-1, was consistently reduced in E5-expressing cells.

3.3 Results

3.3.1 Downregulation of spliced XBP-1 mRNA is specific to high-risk HPV-16 E5.

Splicing of XBP-1 mRNA occurs after the induction of ER stress and results in a transcriptionally active protein that regulates the cellular unfolded protein response. Alteration of ER-stress has been shown assist viruses in establishing persistent infections and promoting the viral life cycle (93, 175, 195). We have previously published that the low-risk HPV-6b E5 protein did not significantly alter the splicing of XBP-1 mRNA (45). To determine if this was also true for an ER-localized, high-risk HPV E5 protein, we stably transduced HFKs with 16E5 or control vector (pLXSN). Using real-time RT-PCR, we quantified the ratio of spliced to unspliced XBP-1 transcripts in both cell types (Figure 6a) as well as the levels of spliced, unspliced, and total XBP-1 mRNA (Figure 6b). These experiments were repeated using three independent preparations of HFKs. We found that level of spliced XBP-1 was significantly reduced in E5-expressing HFKs when compared to vector-expressing controls, either when normalized to the unspliced transcript (Figure 6a) or to GAPDH (Figure 6b).
A. 

![Graph showing fold change in spliced XBP-1 mRNA between LXSN and 16E5.]

B. 

![Graph showing normalized fold change in total, spliced, and unspliced forms between LXSN and 16E5.]
Figure 6. (A-B). HPV-16 E5 expressing keratinocytes demonstrate reduction in spliced XBP-1 mRNA. Primary HFKs were stably transduced with either 16E5 or LXSN control vector. Real-time RT-PCR was used to measure mRNA levels. A) The ratio of spliced : unspliced transcripts of XBP-1. B) Levels of XBP-1 transcripts relative to GAPDH. n=3. Bars represent means ± SEM. ** indicates p value < 0.01 as determined by a paired student’s t-test.
3.3.2 Creation of 16E5 mutant constructs

We then generated several E5 mutants to define the protein domains that might account for the biological differences between the low- and high-risk E5 proteins. First, an alignment of the E5 amino acid sequences from several low and high-risk HPV types was performed (Figure 7a). Mutations were made in regions of the E5 protein that were highly conserved in the high-risk E5 proteins, but not conserved in the low-risk types. Four mutants were constructed with point substitutions to alanine: L32A, S35AS37A, Y39A, and Y63A. In the fifth mutant, H77YA78I, the histidine and alanine residues (conserved in high-risk HPVs) were replaced with tyrosine and isoleucine (conserved in low-risk HPVs). Immunofluorescence was used to confirm the expression and localization pattern of mutant constructs. All mutant E5 proteins merged with the ER-marker calnexin in both transfected COS-1 cells and stably-expressing HECs (Figure 7b). This pattern is similar to the previously published localization of the wild-type 16E5 protein (171).
Figure 7. (A-B). Construction of 16E5 mutant constructs. A) Alignment of low-risk (yellow) and high-risk (purple) HPV E5 protein sequences. Mutations were made in boxed regions. All mutants contained alanine substitutions except for mutant H77YA78I, where amino acids histidine and alanine (highly conserved in high-risk HPVs) were swapped for tyrosine and isoleucine (highly conserved in low-risk HPVs). B) Mutant co-localization with the ER-marker calnexin in transiently transfected COS cells and stably transduced HECs.
For XBP-1 experiments, mutant constructs were stably transduced into HFKs. Three biological replicates were included for each construct. Total RNA was harvested from these HFK strains immediately after selection. In order to confirm construct expression in these RNA samples, reverse-transcriptase PCR and real-time PCR was performed. Although levels of expression varied between biological replicates, all samples were shown to express RNA for the appropriate construct (Figure 8 (a-c)).
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200 bp 142 bp 100 bp
Figure 8. Mutant constructs express in keratinocytes. RNA was harvested from HFKs stably transduced with mutant constructs. A) RT-PCR confirms the expression of point mutants to alanine and B) mutant H77AY78I. C) Real-time RT-PCR for all constructs shows that expression level varies between constructs and between biological replicates (BR 1, 2 or 3) for each construct.
3.3.3 Downregulation of spliced XBP-1 mRNA is specific to HPV-16E5

XBP-1 levels were measured in HFKs stably expressing these mutant proteins. Two mutants, mutant H77YA78I and mutant Y63A, were defective for reducing the spliced : unspliced ratio of XBP-1 mRNA by real-time PCR (Figure 9a). Only mutant, H77YA78I, was unable to reduce the levels of spliced XBP-1 mRNA normalized to GAPDH (Figure 9b). These findings suggest that the ability of a high-risk E5 protein to reduce XBP-1 splicing is dependent upon two highly-conserved C-terminal amino acids.
Figure 9. (A-B). Keratinocytes stably transduced with 16E5 mutant, H77YA78I, fail to demonstrate reduced levels of spliced XBP-1 mRNA. C) The ratio of spliced : unspliced transcripts of XBP-1 in mutant constructs. B) Levels of XBP-1 transcripts relative to GAPDH in mutant constructs. n=3. Bars represent means ± SEM. ** indicates p value < 0.01; * indicates p value < 0.05 by student’s t-test.
3.3.4. *IRE1α, upstream of XBP-1, is also altered in 16E5-expressing keratinocytes.*

Given the consistency with which 16E5-expressing cells demonstrated a reduction in spliced XBP-1 levels, we examined whether E5 altered upstream or downstream components of XBP-1 splicing in the ER stress signaling pathway. IRE1α, immediately upstream of XBP-1 in the ER stress response pathway, is the endoribonuclease responsible for the cleavage of unspliced XBP1 mRNA into its more stable spliced form. We found that IRE1α transcript levels were also significantly downregulated in E5-expressing keratinocytes as compared to those expressing the vector-control (Figure 10).
Figure 10. IRE1a mRNA levels are reduced in HPV-16 E5 expressing keratinocytes. Primary HFKs were stably transduced with either 16E5 or LXSN control vector. Real-time RT-PCR was used to measure IRE1a mRNA transcripts relative to GAPDH. n=3. Bars represent means ± SEM. * indicates p value < 0.05 as determined by a paired student’s t-test.
3.3.5 Microarray identifies COX-2 downregulation in 16E5-expressing cells

The alteration of both XBP-1 and IRE1α in E5-transduced keratinocytes suggested that a spectrum of gene expression changes, including perhaps changes in other ER stress response genes, may occur after expression of 16E5. To address this possibility, we performed a cDNA microarray study to identify E5-induced changes in gene expression. To ensure that these changes were reproducible, we performed the microarray assays in triplicate, using three different donor pools of foreskin keratinocytes that were separately transduced with either E5 or pLXSN and selected with G418. These biological replicates included a dye swap to further validate the findings. The dye swap is a control for the microarray itself wherein the same sample is run twice, switching the dyes used to distinguish the control from the experimental sample between runs. Surprisingly, less than 25 genes were consistently regulated in the arrays (>1.5 fold change in each array, p-value < 0.01) (Table 2).
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Table 2. Microarray analysis reveals gene expression changes induced by 16E5. Microarray was performed on three different donor pools of primary foreskin keratinocytes which were stably transduced with 16E5 or LXSN. Dye swap was performed for each replicate. Less than 25 genes were found to be consistently changed (>1.5 fold in each array, p < 0.01) in 16E5-expressing cells as compared to LXSN-expressing cells.
Real-time RT-PCR was used to confirm the downregulation of four genes (Figure 11). These genes were chosen based on fold change, available literature, and relevance to cancer or the ER-stress response pathway. In addition, any relevance to genes already known to be regulated by HPV proteins was taken into account.

UBE2E1, or ubiquitin conjugating enzyme E2 E1, is part of the E2-ubiquitin conjugating enzyme family. It is also known as UBCH6. This protein has been shown to interact with E6-AP, which interacts with the HPV E6 protein to degrade p53 (122). Its homologue in yeast is known as UBC4/5, and its loss can impair growth in yeast (154). In addition, this protein is involved with degrading short-lived and abnormal proteins, and in yeast its loss induces heat shock protein expression (154). UBCH6 itself has a c-terminal hydrophobic tail that allows it to remain in the ER, where it is reported to be involved with the ERAD (ER-associated degradation) pathway (191). For these reasons, UBE2E1 was confirmed by real-time RT-PCR as being downregulated in three biological replicates of E5-expressing cells. However, this was not significant (p value of 0.07).

GPR135, or G-protein coupled receptor 135, is also known as the relaxin-receptor 3 (RXFP3). It has been described to bind both relaxin-3 (a luteal peptide hormone), and insulin-like peptide 7 (101). Relaxin promotes growth of the uterus and other tissues in an estrogen-dependent manner. Relaxin has also been suggested to induce VEGF and lead to EGFR activation (12, 88). Due to the importance of G-protein receptors in activating tyrosine kinase receptors in cancer, GPR135 was chosen for real-time confirmation. However, although 16E5 was shown to consistently
decrease its expression in our biological replicates, the decrease was not significant (p value of 0.06).

MED26, or the mediator of RNA polymerase II transcription subunit 26 also known as CRSP70, is a subunit of the cofactor required for SP1 activation (CRSP) (144). SP1 is a transcription factor that recruits histone deacetylases to regulate numerous genes, including hTERT (187). SP1 has also been shown to physically and functionally interact with E2F and act synergistically to further activate E2F targets (82, 99). MED26 was chosen for real-time confirmation because of SP1’s relation to both hTERT (which has been shown previously to be regulated by HPV-16 E6 protein) and E2F (involved in cell cycle regulation) (103, 104). MED26 was shown to be significantly (p value <0.05) downregulated in E5-expressing cells.

The fourth gene that we confirmed by real-time, COX-2/PTGS2, was perhaps the most interesting. COX-2 has been shown to be a part of the ER stress response pathway (78). COX-2 could possibly be both downstream of XBP-1 and IRE1α, as well as independently activated through another arm of the ER stress response (78). Additionally, consistent with the pattern seen with XBP-1 repression, 6bE5-expressing cells and H77YA78I E5-expressing cells were unable to repress COX-2 mRNA levels (Figure 11). Thus, our data indicated that the high-risk HPV16 E5 protein specifically and significantly altered these ER stress related genes in HFKs (p value < 0.01).
Figure 11. Real-time RT-PCR confirmation of genes discovered by microarray to be altered by 16E5 expression in keratinocytes. Four genes of the ~25 genes changed in 16E5-transduced cells were chosen for real-time RT-PCR confirmation. Three biological replicates were tested for each gene. Each gene was consistently downregulated in all replicates of 16E5-expressing cells vs. vector control. ** indicates p value < 0.01; * indicates p value < 0.05 by student’s t-test.
3.3.6 No changes seen in protein expression of ER-stress genes

Next, we wanted to see if the changes in mRNA expression were reflected in protein levels. Western blot was performed for XBP-1, IRE1α, and COX-2. In addition, we looked at the level of other ER-stress response proteins such as BIP, PDI, and ERO1α. Very surprisingly, we did not observe dramatic changes in expression between 16E5 and LXSN expressing cells for any of the proteins which were altered at the mRNA level (Figure 12(a-c)). However, if COX-2 is used as an example (Figure 12A), it is clear that there is a reduction of this target protein when E5 is expressed. At the level of mRNA there is a 75% reduction in COX-2. Due to the non-linear nature of the Western blots, the reduction of COX-2 protein might be compatible with the observed mRNA changes. Alternatively, there may also be compensatory factors increasing protein stability in these cells. Experiments to test this possibility are being pursued.
A.

COX-2
Expected ~70

B-actin

LXSN
16E5
H77YA78I

B.

XBP-1 (S)
Expected ~54 kDa

XBP-1 (U)
Expected ~33 kDa

B-actin
Figure 12. No change seen in ER-stress pathway protein levels. Western blot was done to determine levels of ER-stress pathway protein levels in keratinocytes expressing LXSN, 16E5, or H77YA78I mutant protein A) COX-2 B) XBP-1 C) other ER stress proteins. Western blots are representative of three separate experiments.
3.3.7 Co-expression of the HPV-16 E6/E7 genes partially alters the E5 phenotype.

Having discovered that 16E5 reduces ER stress response genes when expressed alone in HFKs, we then determined whether this E5 activity was still detected in cells that were expressing the early HPV-16 E6/E7 proteins. We and others have observed that the activity of one HPV early protein can often be counteracted or negated by the expression of another HPV protein (45, 81, 113, 114). To insure that the E5 effects would still be observed under conditions where the early E6/E7 genes were expressed (as observed in viral infection), we examined XBP-1, IRE1a and COX-2 expression in keratinocytes stably transduced with E6/E7 in the presence and absence of E5 expression. Interestingly, when expressed alone, the E6/E7 genes induced ER stress as evidenced by increased XBP-1 splicing, but simultaneously decreased levels of downstream COX-2 mRNA, consistent with a protective response against ER stress (Figure 13(a-e)).
A.

![Graph showing Relative Fold Change for LXSN and E6/E7](image)

B.

![Graph showing Normalized Fold Change for LXSN and E6/E7](image)
Figure 13. Keratinocytes expressing E6 and E7 demonstrate increased XBP-1 splicing, but reduced COX-2. Primary keratinocytes were transduced with either pBabe-puro vector or E6/E7. A) Real-time PCR was performed to determine the levels of spliced: unspliced XBP1 transcripts. Additionally, real-time PCR was performed to determine the levels B) COX-2, C) XBP-1 unspliced transcripts, D) XBP-1 spliced transcripts, and E) IRE1a. (B-E) are normalized to GAPDH.
We do not know the relative contributions of the individual E6 and E7 genes to these apparently counteractive responses. However, we hypothesized that the downstream reduction of COX-2 by E6/E7 might be protective and might be further augmented by 16E5. In support of this, we found that cells co-expressing E6, E7 and E5 had lower levels of COX-2 expression than cells expressing E6 and E7 alone (Figure 14a). Thus, both E6/E7 and E5 contribute to the reduction of COX-2 expression in keratinocytes. In contrast, E5 was unable to downregulate XBP-1 or IRE1a in the presence of E6 and E7 (Figure 14 (b-d)). The mechanism by which E6/E7 antagonizes the effect of E5 on XBP-1 splicing and IRE1a expression is unknown. In summary, it appears that 16E5 can downregulate several components of the ER stress response when expressed alone, but it only selectively inhibits COX-2 expression in the context of cells simultaneously expressing E6/E7.
C.

D.

E6/E7/LXSN
E6/E7/E5

Normalized Fold Change

0
0.2
0.4
0.6
0.8
1
1.2

Spliced
Unspliced
Total

0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6

*
Figure 14. Levels of COX-2, but not XBP-1 or IRE1a, are reduced in keratinocytes expressing E5, E6, and E7 as compared to E6 and E7 alone. Real-time RT-PCR was performed in E6/E7 keratinocytes additionally expressing LXSN vector control or E5. A) COX-2 mRNA levels B) Ratio of spliced: unspliced XBP-1 transcripts C) Levels of XBP-1 transcripts relative to GAPDH. D) IRE1a mRNA levels. Bars represent means ± SEM for an n=3. * indicates p value < 0.05 as determined by a paired student’s t-test.
3.3.8 HPV-16 E5 cannot inhibit chemically-induced ER stress.

To gain insight into the pathways that were being altered by E5, we tested whether E5 could inhibit ER stress genes that were induced by either thapsigargin or tunicamycin. While both chemicals are potent inducers of ER stress, thapsigargin functions by disrupting calcium homeostasis and tunicamycin by altering protein glycosylation. We treated vector and E5-expressing keratinocytes with several concentrations of thapsigargin or tunicamycin, and monitored the expression of XBP-1, IRE1a and COX-2 (Figure 15). Regardless of drug or drug concentration, E5 had no detectable effect on the induction of the ER stress genes, suggesting that E5 does not regulate the calcium- or glycosylation-dependent pathways.
Figure 15. E5 is unable to repress levels of COX-2, IRE1a, and spliced XBP-1 when challenged with chemical ER-stress inducers. Keratinocytes stably expressing either LXSN vector control or E5 protein were treated for 4 hours with different doses of tunicamycin (3 ug/ul, 0.3 ug/ul, 0.03 ug/ul) or thapsigargin (300 nM, 30 nM, 3 nM). Real-time RT-PCR was used to determine levels of selected genes. For all experiments, n = 3. Data is plotted on a log scale. A) Cox-2 relative to GAPDH B) IRE1a relative to GAPDH C) Spliced XBP-1 relative to GAPDH D) Ratio of spliced : unspliced XBP-1 transcripts. Pink = Tunicamycin I. Black lines = Thapsigargin (Thap). Triangles = E5-transduced keratinocytes. Squares = LXSN-transduced keratinocytes.
3.3.9. Expression of 6bE5 induces many changes in gene expression in keratinocytes

In all of our XBP-1, COX-2, and PTGS2 experiments, we were unable to see a change in mRNA levels when keratinocytes were transduced with 6bE5 instead of 16E5. Given that type 6b is a low-risk HPV with very different properties from the high-risk type 16 virus, it seemed possible that there might also be differences between the 16E5 and 6bE5 proteins. Since we were interested in gene expression changes, and already had identified differences between the two proteins in the ER-stress response pathway, we decided to do an explorative microarray using 6bE5. We conducted this microarray in a very similar fashion to the 16E5 microarray, using biological replicates and the Agilent microarray system to compare 6bE5 to LXSN vector control.

Statistical analysis of these six 6bE5-vs-LXSN arrays revealed upwards of 700 genes that were changed consistently across different HFK donors (>1.5 fold change and p value < 0.01 for all six arrays). This was a very large number when compared to the ~25 genes changed with the high-risk 16E5. In addition, these genes were changed with far greater fold change than those discovered for 16E5 (range of -13 to +356). The top 25 up and down-regulated genes are represented in Table 3 and Table 4. Probes for spliced XBP-1 are not represented on Agilent arrays. However, probes for COX-2/PTGS2 (A_24_P77008/NM_000963) are on these arrays, which is how we first discovered that COX-2 was altered in 16E5-expressing cells. Analysis of 6bE5 microarrays, however, revealed that COX-2 was not consistently altered, which correlates with the real-time PCR data (shown earlier in Figure 11).
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Table 3. Top 25 genes downregulated by 6bE5 in keratinocytes. Microarray was performed on three different donor pools of primary foreskin keratinocytes which were stably transduced with 6bE5 or LXSN. Dye swap was performed for each replicate. Around 600 genes were downregulated (>1.5 fold in each array, p < 0.01) in 6bE5-expressing cells as compared to LXSN-expressing cells.
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Table 4. Top 25 genes upregulated by 6bE5 in keratinocytes.  Microarray was performed on three different donor pools of primary foreskin keratinocytes which were stably transduced with 6bE5 or LXSN. Dye swap was performed for each replicate. Around 160 genes were upregulated (>1.5 fold in each array, p < 0.01) in 6bE5-expressing cells as compared to LXSN-expressing cells.

Of the many genes altered by 6bE5, five were chosen for confirmation by real-time RT-PCR. These genes were chosen based mainly on fold change (altered >8 fold), taking into account available literature and relevance to cell growth. CDK2NC, also known as p18, is a cyclin dependent kinase inhibitor in the INK4 family. It is thought to be a tumor suppressor that controls cell cycle progression, as it can interact with CDK4 and CDK6 (121). MMP9, or matrix metalloproteinase 9, is involved with breakdown and remodeling of the ECM, especially as part of an inflammatory response (121, 159). It is also upregulated in several cancers, including those of the head & neck, where its presence is indicative of a poor prognosis (142). PLA2G4C, or phospholipase A2 gamma, is an enzyme that hydrolyzes glycerophospholipids into free fatty acids that can later function as signaling molecules. High expression of PLA2 has been associated with apoptosis of human colon cancer cells (53). SERPINA3, or serpin peptidase inhibitor 3, is a plasma protease inhibitor. It is also known as alpha-1 anti-chymotrypsin and has been studied the most in the context of Alzheimer’s disease (136, 151). ICAM1, or inter-cellular adhesion molecule 1, is a molecule widely involved with leukocyte migration (192). It is not only an adhesion molecule with a
role in signal transduction, but also the entry binding site for HRV, the human rhinovirus (14, 57).

Regulated genes, four of which were upregulated and one of which was downregulated, were confirmed by real-time RT-PCR (Figure 16).
A.

CDK2NC

B.

MMP9
C.

**PLA2G4C**

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Normalized Fold Change

D.

**SERPINA3**

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Normalized Fold Change
Figure 16. Real-time RT-PCR confirmation of gene regulation discovered by microarray to be altered by 6bE5 expression in keratinocytes. (A-E) Five genes of the ~720 genes changed in 6bE5-transduced cells were chosen for real-time confirmation. Three biological replicates were tested for each gene. Each gene was consistently altered in all replicates of 6b-expressing cells vs. vector control.
3.4 Discussion

HPV-16 E5 is a small, hydrophobic, ER-localized viral protein whose biological function has remained elusive. In this study we examined primary human keratinocytes expressing either 16E5, 6bE5, or pLXSN for changes in gene expression using real-time RT-PCR and cDNA microarray. By analyzing multiple pools of primary keratinocytes, we aimed to identify gene changes that were reproducible in different donor cells.

To date, no gene expression studies have been performed on primary keratinocytes that are stably expressing 16E5. The only other gene expression study on 16E5 was conducted in HaCaT cells using a dexamethasone-inducible promoter (86). That study found that 179 genes were significantly altered (no fold change cutoff, p < 0.01) by 16E5 expression, including lamin A/C, PKC-γ, and PI3K. E5 was suggested to inhibit apoptosis by affecting pathways involved in cell adhesion, motility, and mitogenic signaling. In contrast, our analysis indicated that a far smaller subset of genes (~25) were consistently affected (fold change >1.5, p-value < 0.01) in three independent isolates of primary keratinocytes. The difference between our data and the above study may be due to site origins or genetic background (foreskin vs. adult trunk keratinocytes), cell status (primary vs. immortalized), or gene expression level (stable expression vs. transient inducible expression).

Our study indicates that HPV-16 E5 suppresses 3 key players in the ER stress pathway: COX-2, XBP-1 and IRE1α. Numerous other viral proteins have been reported to cause alterations of the ER stress response pathway, including the canine
papillomavirus E5 protein (Cfpv2 E5) (45, 167, 175, 195). It is noteworthy that hepatitis C virus (HCV) suppresses the XBP-1/IRE1α pathway and may therefore promote the expression of HCV proteins and aid in viral persistence (175). Persistent viral infection appears to be a major contributory factor to the development of cancer, especially in the case of infection by the high-risk HPVs (148, 149). It is interesting to note that the substitution of just two amino acids conserved in high-risk HPV E5 protein with amino acids conserved in low-risk E5 protein is enough to completely abrogate COX-2 suppression. In fact, in contrast to the high-risk HPV-16 E5, the low-risk HPV-6b E5 is unable to alter XBP-1 (45) and may even increase levels of COX-2, although this was not statistically significant. Indeed, previous studies have shown increased levels of COX-2 in recurrent respiratory papillomatosis (RRP) lesions which are caused by low-risk HPV type 6b and 11 (188, 189). However, the COX-2 phenotype described in RRPs has not yet been linked to a specific HPV protein. Our findings suggest that change of COX-2 levels by low risk HPVs may be modulated by a single viral gene (ie. E5).

Our data indicate that the ability to suppress XBP-1, COX-2, and IRE1α is unique to the high-risk 16E5, suggesting that these genes may be used as biochemical markers of HPV-16 E5 expression. In fact, cDNA microarray analyses of 16E5 and 6bE5-expressing keratinocytes show marked differences in gene expression profiles between both proteins, indicating that their functions in the host after viral infection may be very distinct.
The mechanism of 16E5-induced gene alteration remains unknown, although 16E5’s inability to interfere with ER stress induced by tunicamycin and thapsigargin suggests that it cannot alter the calcium or glycosylation pathways. However, it is possible that 16E5 may be able to exert an effect on ER stress induced by expression of viral proteins. In our primary keratinocyte model, data indicate that while E6 and E7 induce XBP-1, they are also able to suppress COX-2. Together, E5, E6, and E7 exhibit an additive effect on COX-2 suppression.

When studied in different cell systems, it appears that several HPV proteins can alter COX-2 expression. For example, E5, E6, and E7 have been shown to induce COX-2 expression in spontaneously immortalized lines (HaCat cells and HEK cells), and cervical cancer lines (C33A and SiHa) (85, 168). COX-2 levels were also upregulated in esophageal epithelial cells immortalized with E6, E7, and hTERT (200). Clinically, overexpression of COX-2 was inversely related to HPV-16 infection in esophageal squamous cell carcinomas (102), and inversely related to HPV load in patients with cervical intraepithelial neoplasia (161). However, other studies showed that COX-2 protein levels did not correlate with the disease severity of HPV-induced cervical lesions (146), or with HPV-positivity in primary and metastatic cervical cancer tissues (84, 160). It is possible that the observed phenotype in the above studies varies depending on factors such as cell origin, immortalization and transformation status, and the relative expression of the HPV early proteins. In many cases it appears that E5 may not be expressed in cancer.
In primary genital keratinocytes, our data show a very specific and consistent downregulation of ER stress response genes by 16E5, and suggest a potential role for E5 in repressing the cellular ER stress response following HPV infection (Figure 17). Although in vivo studies are not possible, one could speculate that the downregulation of this stress pathway would be favorable to viral replication and persistence.
ER Stress Response
IRE1α
XBP-1
COX-2
Halt protein translation
Increase protein folding
chaperones
Cell Death
Inability of Virus to Propagate

Stress Conditions
Chemical/Ion Imbalance (eg. tunicamycin, thapsigargin)
Unfolded Proteins (eg. hypoxia, nutrient deprivation)

ER Stress Response
IRE1α
XBP-1
COX-2
Halt protein translation
Increase protein folding
chaperones

E5/E6/E7
Failure
Success

Normal Conditions
Normal Cellular Protein Translation
Viral Protein Translation and Viral Replication

Stress Conditions
Biological (eg. viral proteins)

Unfolded Proteins (eg. hypoxia, nutrient deprivation)

E5

Failure
Success

Cell Death
Inability of Virus to Propagate

Normal Conditions

Viral Protein Translation and Viral Replication
Figure 17. Potential model of 16E5 ER-stress repression. Occasionally, cells are exposed to various environmental conditions that result in a disruption of ER function and buildup of unfolded proteins. The ER-stress response, or unfolded protein response, is the cell’s reaction to such insults. For a non-lytic virus, cell survival is essential for viral replication. It is possible that viral proteins may attempt to curb their own stimulation of such a host response, inducing one arm of the ER-stress response while simultaneously suppressing another. E6 and E7 induce XBP-1, but are able to suppress COX-2. E5 alone in keratinocytes is able to suppress IRE1a, XBP-1, and COX-2. Together, E5, E6, and E7 exhibit an additive effect on COX-2 suppression.
IV. THE HPV-16 E5 PROTEIN IS PRESENT IN TWO PROTOTYPE CERVICAL CANCER CELL LINES

Running Title: HPV-16 E5 is Present in Cervical Cancer Cell Lines

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4.1 Abstract

High-risk HPVs encode three transforming proteins, E5, E6, and E7. E6 and E7 are both required for immortalization of human genital keratinocytes via inactivation of tumor suppressors (p53 and Rb) and induction of cellular telomerase (21, 103, 104, 116, 147, 180, 185). The E5 protein may contribute to early steps of tumor initiation (19, 174) as well as to neoplasm formation in the transgenic mouse model (63). However, E5 is generally thought to be lost in cervical carcinoma due to frequent integration of HPV DNA into the host cellular genome. Despite several studies in HPV-16 positive cervical cancer cell lines demonstrating the presence of an E5 ORF or RNA transcripts that could potentially code for E5, the presence of the E5 protein has never been confirmed. This is largely due to the lack of an E5 antibody and/or extremely low abundance of E5 protein in cell lines. In this report, we utilized mass spectrometry (MS) to detect native HPV-16 E5 protein in two human cervical cancer cell lines (SiHa and Caski cells). Initially, we demonstrated that the four amino acid peptide at the C-terminus of E5 protein (FLIT), was a unique peptide marker for E5 expression. MS MRM (multiple reaction monitoring) could identify FLIT peptide in COS-1 cells transfected with either AU1-tagged E5 or untagged E5, but not in vector-transfected COS-1 cells. Similarly, we detected FLIT peptide in primary human keratinocytes (HFKs) stably expressing E5, but not in untransduced or vector-transduced HFKs. Surprisingly, our data reproducibly demonstrated the presence of the FLIT peptide in two HPV-16 positive cervical cancer cell lines, SiHa and CaSki cells. Confirming the specificity of the MS technique, no FLIT peptide was detected in
either an HPV-18 positive cervical cancer cell line (HeLa), or an HPV-negative cancer cell line (C33A). MS detection of E5 protein in these cell lines correlated directly with real time PCR analysis of E5 mRNA. Thus, we have identified HPV-16 E5 protein in established human cervical cancer cell lines, suggesting that E5 may contribute to both early and late stages of neoplastic progression, even in cell lines containing only integrated HPV genomes.

4.2 Author Summary

HPV is a highly ubiquitous virus with a population prevalence of approximately 30%. Although most productive infections of the cervix spontaneously resolve without symptoms, a small percentage (less than 5 percent) establish persistent infection and can eventually progress to malignancy. In many cases, HPV DNA integrates into the host genome during neoplastic progression, resulting in disruption of the E2 ORF and thereby enhancing expression of the E6/E7 oncogenes. The HPV-16 E6 and E7 proteins degrade p53 and Rb respectively, allowing for cell immortalization and transformation. The E5 protein of HPV-16 is considered to be a weak oncoprotein, since it enhances E6- and E7-induced transformation. Limited studies of HPV genome integration in cervical cancer indicate that the E5 ORF is often separated from the early viral promoter(s), suggesting that E5 is not transcribed in cervical tumors (11, 158) and therefore cannot contribute to the malignant phenotype. In contrast, there are also studies showing that some cervical cancer cell lines contain an intact E5 ORF and express E5 mRNA (11, 13, 129, 155, 157) However, since the early HPV genes are
expressed as spliced transcripts and encode the E5 ORF at their 3' terminus, it remained unclear whether the E5 protein was truly expressed in these cells. Here, we use mass spectrometry to establish the presence of E5 protein in the two commercially available, prototype HPV-16 positive cervical cancer cell lines, SiHa and CaSki. Our data indicate that E5 protein can be expressed in cell lines exclusively containing integrated copies of HPV-16 expression and in which the E5 ORF is no longer driven by the viral promoter. E5 protein, therefore, may contribute to maintenance of the tumorigenic phenotype of cervical cancer cells.

4.3 Introduction

The human papillomavirus type 16 (HPV-16) is the causative agent of approximately 60% of cervical cancers. This virus encodes three transforming proteins, E5, E6, and E7. E6 and E7 are both required for immortalization of human genital keratinocytes via inactivation of tumor suppressors (p53 and Rb) and induction of cellular telomerase (21, 103, 104, 116, 147, 180, 185). The E5 protein may contribute to early steps of tumor initiation (19, 174) as well as to neoplasm formation in the transgenic mouse model (63). Integration of viral DNA into the host chromosome has been proposed to be a key event in neoplastic progression. Integration of the HPV genome can result in rearrangement or regional loss of both host and viral genomes. Although there are no defined sites for HPV integration into the host chromosome, there are certain “hot spots” (termed common fragile sites, or CFS) into which HPV is more likely to integrate (177). Upon integration, the viral
The genome itself is almost always rearranged in the E2-coding region, resulting in either the deletion of the E2 ORF or its separation from the early viral promoter. The E2 protein, when present, regulates E6 and E7 expression, and its loss results in a rise of E6 and E7 levels (44, 140). Cells that highly express E6 and E7 from integrated genomes have a growth advantage over cells that maintain their DNA episomally (80).

The same genetic changes that alter E2 expression during viral integration are also believed to disrupt E5 protein expression (158). Thus, E5 is thought to function mainly during the productive life cycle of the virus (when the viral genome is maintained episomally) or in early phases of tumorigenesis. To date, there are many phenotypes ascribed to E5, including expansion of the population of HPV-infected basal cells (perhaps through EGFR activation) (48, 63, 169), enhancement of cell immortalization and transformation by E6/E7 (63, 164), induction of koilocytosis (91), and interference with the intracellular trafficking of proteins such as the 16-kDa subunit of the vacuolar H^+\text{-ATPase} (1, 5, 46), HLA (8, 69), BAP31 (135), karyopherin β3 (90).

Estimates of the percentage of HPV-induced cervical cancers that contain integrated DNA exclusively— and therefore potentially do not express E5 — vary greatly, from 15-86% (6, 37, 41, 156). The HPV-16 E5 ORF was first designated by Seedorf et. al. in 1985 based on homology comparison of HPV-16, HPV6b, and BPV1. It was determined to be located between nucleotides 3863 and 4096; however, no discernable translational initiation codon was noted (152). The same group was also the first to clone E5 into an expression vector and synthesize E5 protein (153). Based on their work, Pater and Pater examined the status of the HPV genome in the only two
existing HPV-16 positive cervical cancer cell lines, SiHa and CaSki, which have 1-2 and ~500 integrated copies of the HPV-16 genome respectively (111, 129, 193). They found that CaSki cells retained an intact HPV-16 genome, while SiHa cells showed disruption of the E1 and E2 ORFs (129). The presence of an E5 ORF was demonstrated by Southern blot in CaSki cells (11) and DNAse I mapping analyses of SiHa cells demonstrated an intact E5 ORF that was possibly expressed by a weak cellular promoter upstream of the viral/host junction (13). In addition, spliced mRNA transcripts that encoded the E5 ORF were identified in both SiHa and CaSki cells (155). Shirasawa et al. used gene specific hybridization to show that the E5 ORF was present in 5 out of 5 carcinomas in situ and 1 out of 2 invasive carcinomas, all of which had integrated HPV-genomes (157). Despite all these reports, the presence of the native E5 protein has never been confirmed in either cervical cancer tissue or cell lines, possibly due to its highly hydrophic, non-immunogenic nature that has made the development of an antibody difficult. In addition, E5 is growth inhibitory when expressed at high levels and its expression in cell lines is believed to be low, making detection difficult.

In this study, we demonstrate by real-time PCR that SiHa and CaSki cells express E5-coding mRNA transcripts. More importantly, we use reversed-phase nano-high-performance liquid chromatography (HPLC) mass spectrometry techniques to identify a four amino acid peptide at the C-terminal end of the E5 protein (FLIT), that can be used as a peptide marker for E5 expression. FLIT MRM was then used to detect E5 protein in cells that were transiently and stably expressing E5 protein.
Finally, we used this method to identify the E5 protein in both SiHa and CaSki cells. Previous groups have attempted to detect E5 using antibodies generated against it (37, 83). Unfortunately, these antibodies have not been made available, nor have attempts to re-create them been successful. This report is the first time that the native E5 protein has ever been detected by mass spectrometry. The discovery of E5 in cervical cancer cell lines definitively demonstrates that E5 is present beyond the initial phases of viral infection and might play a role in maintenance of the neoplastic phenotype.

4.4 Results

4.4.1 Characterization of FLIT peptide as an E5 marker.

HPV-16 E5 is an 83 amino acid, highly hydrophobic protein, which has three arginine residues but no lysine residues (Figure 18a). Thus, trypsinization is predicted to cleave E5 into 4 peptide products (Figure 18a). Based on the cleavage site location, resulting peptides can be detected by mass spectrometry with varying efficiency. We expected that the FLIT peptide (a four amino-acid sequence at C-terminus of HPV-16 E5 protein, molecular weight: 493.3 Dalton) could serve as an E5 marker when using the mass spectrometry technique combined with a molecular weight cut-off. A BLAST search for all permutations of KFLIT and RFLIT (a total of 48 possibilities) revealed no cellular proteins under 10KDa containing this unique E5 marker sequence.

FLIT peptide was synthesized by Genscript at a purity of >75%. The peptide was run through MS, where the nanocolumn was washed first with water (to elute all polar compounds) and then subjected to increasing percentages of acetonitrile. For this
run, FLIT was found to elute between 23.5 and 24 minutes (Figure 18b). We next analyzed the synthesized FLIT peptide using MS/MS to determine its secondary ion composition. Multiple reaction monitoring (MRM) was used to detect both the parental ion 493.3 Da), as well as secondary ions (breakdown products of FLIT such as FLI, FL, LIT, etc). We were able to identify secondary ions having four different molecular masses: 215.1 Da, 233.1 Da, 261.1 Da, and 374.2 Da (Figure 18c).
A.  
MTNLDTASTTLLACFLLCFCVL  
LCVCLLI RPLLLSVSTYTSLLVL  
LLLWITAASAFRCFIVYIIFVYIP  
LFLIHTHA  
RFLIT

B.  

C.  

493.0/215.1 493.0/233.1 493.0/261.1 493.0/374.2
Q1/Q3 Masses, Da
Intensity, cps
4000 8000 1.6e4 1.2e4 1.0e7 1.5e7 23.75
D.

Synthetic FLIT peptide

Run synthetic FLIT peptide thru MS

Elute first with water (all polar compounds) then an ACN gradient

Note time that FLIT elutes

MRM for test samples (more specific)

Separation of FLIT into parent ion and secondary ions
493.03 Da (parent)
374.2
261.1
233.1
215.1

Run FLIT peptide thru MS/MS

Synthetic FLIT peptide MRM for test samples (more specific)
Cell RIPA lysates

Centrifugation using Centrifugal Filter Device
(<10 kDa Molecular Weight Cut-off)

Sample Processing:
Denaturation
Reduction
Cysteine Blocking
Cleavage with Trypsin

Nano-HPLC
Fractionation and Elution of Peptides

Detection of FLIT by Q-TRAP
Figure 18. Characterization of the FLIT peptide as an HPV-16 E5 marker. (A) Amino acid sequence of HPV-16 E5. There are four possible peptides after trypsin digestion that could be used to identify 16E5 in MS analysis, including FLIT. (B) Total ion chromatogram. Synthesized FLIT peptide was run through MS and eluted with increasing percentages of acetonitrile (ACN) at a flow rate of 300 nL/Min. In this run, FLIT eluted between 23.5 and 24 minutes, using a protocol of 1% CAN for 1min, 1 to 40% ACN in 30 min, 40 to 60% ACN in 1 min, 60 to 99% ACN in 1 min, 99% ACN for 5 min, 99 to 1% ACN in 2 min, 1% ACN for 20 min. (C) MRM shows breakdown of FLIT into parental ion (493.0) and secondary ions (215.1,233.1,261.1,374.2). (D) Proposed workflow (E) Procedures for FLIT MS analysis.
4.4.2 Detection of the E5 marker FLIT in HPV-16 E5-overexpressing COS-1 cells.

Having characterized FLIT, we wondered whether FLIT could be detected in COS-1 cells where E5 could be expressed at high levels. The workflow for characterization of the synthetic FLIT peptide is outlined in Figure 18d, and the general methodology for the detection of FLIT peptide in cell lysates is outlined in Figure 18e. We transiently transfected COS-1 cells with pJS55 empty vector and E5 constructs. Since the E5 protein had never been detected in its native, untagged form, we also transfected COS-1 cells with an AU1-tagged version of the E5 construct.

Expression of constructs was verified using immunoflorescence as showed in Figure 19a. RIPA lysates were harvested 24 hours after transfection. Lysates were centrifuged through a size separation membrane to enrich for proteins less than 10kDa as described in Materials and Methods. The elutates (containing proteins under 10kDa) were then trypsinized and subjected to mass spectrometry analysis using QSTAR LC/MS. There was no specific signal in COS-1 cells transfected with the pJS55 empty vector (Figure 19b). A specific peak identified to represent FLIT E5 marker was detected in COS-1 cells transfected with both untagged and tagged E5 (Figure 19 (c-d)). The data suggested that mass spectrometry can be used to detect both tagged and untagged versions of the E5 protein, and that the peak of the FLIT peptide does not reflect any other non-specific proteins in COS-1 cells. Lysates retained from the size separation (containing proteins above 10kDa) were run through mass spectrometry with the same procedures, and no specific peak of FLIT marker was found (data not shown).
Next, we attempted to detect the FLIT peptide in E5-transfected COS-1 cells using Q-TRAP LC/MS/MS with multiple reaction monitoring (MRM). MRM provides additional specificity for the target peptide. This is because a combination of parent (FLIT, 493.3 kDa) and secondary ions (FLIT breakdown products, 215.1,233.1,261.1,374.2) can be used to minimize background. A very strong FLIT signal eluting at 21.16 minutes was detected in E5-expressing COS-1 cell using MRM (Figure 19e). This elution time was consistent with the elution time for the control synthetic FLIT peptide during this run (data not shown).
C.

D.
Figure 19. Detection of the E5 marker FLIT in HPV-16 E5 transiently transfected COS cells. COS cells were transiently transfected with vector and E5 constructs. (A). AU1-tagged E5 expression. Immunoflorescence using anti-AU1 antibody was used to detect AU1-tagged E5 in transiently-transfected COS-1 cells. DAPI was used for DNA staining. (B-D) Transiently transfected COS-1 cells were harvested in 0.5 mls RIPA and processed for QSTAR LC/MS. (B). FLIT E5 marker is not present in pJS55 vector control cells (C) FLIT E5 marker (493.3 Da) is present in AU1-tagged HPV16-E5 transfected COS-1 cells. (C) FLIT E5 marker (493.3 Da) is present in untagged HPV16-E5 transfected COS-1 cells. (D). E5-transfected COS cells were run through MRM MS. Peptide identified to be FLIT is seen to elute at 21.16 minutes. Red line represents the cutoff for positive (signal) vs. negative (noise) reads and is arbitrarily set at 45.
4.4.3 Detection of FLIT E5 marker in human keratinocytes stably expressing E5.

Since human keratinocytes are the physiologic host cells for HPV infection, we evaluated whether the above method could detect FLIT in human genital keratinocytes. HFKs were infected with retroviruses expressing either LXSN empty vector or E5 and then subjected to G418 selection. Expression of AU1-tagged E5 was confirmed by immunoflorescence with mouse anti-AU1 monoclonal antibody (Figure 20a). Triplicate cell lysates from plain HFKs, HFKs transduced with vector, or HFKs transduced with E5 retroviruses were subjected to molecular weight cut-off and MRM. Synthesized FLIT peptide was used again as a control. The intensity threshold for negative (noise) vs. positive (signal) reads was arbitrarily set at ~45 counts per second. As expected, MRM monitoring detected no specific FLIT peak in E5 negative cells, including untransduced HFKs (Figure 20b) and HFKs expressing LXSN vector (Figure 20c). A specific FLIT peak was only detected in E5-expressing HFKs (Figure 20d). Thus, HPV-16 E5 protein was detectable in both E5-overexpressing COS-1 cells and stably expressing human keratinocytes.
A. AU1

AU1-E5

pLXSN

Dapi
Figure 20. Detection of the E5 marker FLIT in HPV-16 E5 stably-transduced human keratinocytes. Human keratinocytes were transduced with retroviruses expressing either empty vector or E5. (A) Immunoflorescence for E5 expression in stably-transduced HFKs. AU1-tagged E5 protein was stained with anti-AU1 antibody and DNA was stained with DAPI. (B-D) MRM was used to detect FLIT peptide, which eluted between 21.0 and 21.5 seconds in this run. (B) FLIT E5 marker is not present in plain HFKs. (C) FLIT E5 marker is absent in LXSN transduced HFKs. (D) FLIT E5 marker is present in HPV-16 E5 transduced HFKs.
4.4.4 Detection of HPV-16 E5 protein in cervical cancer cell lines.

Previous reports indicate that the two HPV-16 positive cervical cancer cell lines, SiHa and CaSki, have either detectable E5 ORFs or potentially E5-coding mRNA transcripts (11, 13, 129, 155, 157). Although several groups over the years have attempted to generate a HPV-16 E5 specific antibody or tried to develop new techniques to detect the HPV E5 protein, there unfortunately is still not an available method to this date due mostly to the highly hydrophobic and non-immunogenic properties of this protein. Two groups have reported the successful detection of E5 through generation of an antibody; however, neither have these antibodies been available for study nor have any subsequent reports been published (37, 83). Thus, many groups, including ours, have had to use epitope tagged versions of E5 for biological studies as a routine method. Using the MS approach, we wanted to identify whether HPV-16 E5 protein is present in human cervical cancer cell lines. Before proceeding with mass spectrometry, we wanted to confirm that these cell lines have E5 mRNA transcripts. To do this, we conducted real-time RT-PCR using primers for the native, un-tagged HPV-16 E5. Additionally, primers for HPV-16 E1^E4 were also tested. As negative controls, we included two additional cervical cancer cell lines, HeLa (HPV-18 positive) and C33A (HPV-negative), as well as primary human foreskin keratinocytes (HFKs) from two separate donor pools. This presence of E5 mRNA in SiHa and CaSki cells was detected qualitatively by RT-PCR (Figure 21a) and quantitatively by real-time RT-PCR (Figure 21b). Neither HPV-16 E5 nor E1^E4 was detected in HeLa, C33A, or either strain of HFKs.
MRM was then used to determine the presence of the FLIT E5 marker in these cell lines. Our data demonstrated that the FLIT E5 marker was present in two well-defined HPV-16 positive cervical cancer cell lines, SiHa and CaSki (Figure 21(c-d)). We repeated this experiment at least five times. However, due to procedural variations, we saw a variation of copy number between runs. Therefore, we were not able to quantify the levels of E5 in these lines. However, we were able, in each instance, to qualitatively determine whether E5 was present or absent in each line tested. HeLa (HPV-18 positive) and C33A (HPV negative) were used as negative controls. In no case did we observe a specific FLIT marker peak in either the C33A cells or in HeLa cells (Figure 21(e-f)). Thus, despite our inability to quantify the number E5 molecules in SiHa and Caski cells, we were able to detect the native HPV-16 E5 protein in human cervical cancer lines for the first time by using the mass spectrometry approach.
A.

B.
Figure 21. Detection of E5 protein in cervical cancer cell lines. PCR was performed on RNA harvested from two different pools of primary keratinocytes, as well as four cervical cancer cell lines.  A) Representative RT-PCR product run on 2% agarose gel and visualized by UV light.  B) Real-time PCR demonstrate levels of E5 and E1^E4 transcripts relative to GAPDH. n=3. Bars represent means ± SEM.  (C-F) Multiple reaction monitoring (MRM) chromatogram shown for FLIT in C) CaSki (HPV-16 positive) and D) SiHa (HPV-16 positive) E) C33A (HPV-negative) and F) HeLa (HPV-18 positive). Data shown for C-F is representative of a minimum of three (and maximum of five) independently prepared samples. Red line represents the cutoff for positive vs. negative reads and is arbitrarily set at 45.
4.5. Discussion

Expression of the HPV-16 E5 protein is generally assumed to be lost during HPV genome integration. Many studies indicate that this may not be the case, since the presence of E5 ORFs and E5-coding RNA transcripts have been demonstrated repeatedly in cervical cancer cell lines with integrated DNA (11, 13, 129, 155, 157). However, due to the lack of an available E5 antibody and extremely low abundance of this protein, the actual protein has never been found in human cervical cancer cells.

Here, we characterized FLIT, a four amino-acid peptide at the C-terminal end of HPV-16 E5. FLIT was used as a peptide marker of 16E5 expression, and the method was established in transiently transfected COS cells expressing tagged and untagged 16E5, as well as pJS55 vector control. Reversed-phase nano-HPLC mass spectrometry was used to establish the presence of 16E5 protein in two HPV-16 positive cervical cancer cell lines, SiHa and CaSki, as well as in keratinocytes stably transduced with E5. Other HPV-16 negative cervical cancer cell lines, including HeLa and C33A, showed an absence of 16E5.

Published literature on 16E5 has long indicated that E5 could contribute to tumorigenesis, and defined E5 as the third oncoprotein of HPV. In fact, in-vivo studies demonstrated that transgenic mice expressing E5, E6, and E7 had larger tumors than mice expressing E6 and E7 alone (63). However, until this report, E5 had never actually been shown to be present with E6/E7 in late stages of carcinogenesis. This limited the conclusions one could draw from in-vitro and in-vivo experiments in which
E5 was expressed along with high levels of E6 and E7. Our data, which establish that E5 is present even in cancer cells that have integrated HPV genomes, lend support to such findings, and re-enforce the importance of E5 in tumorigenesis.

This study has several limitations that are currently being investigated. First, although the method was established in E5 and pLXSN-transduced COS-1 and HFK cells (ie. same background), it is difficult to have an appropriate null background for cancer cell lines. Both SiHa and CaSki have polycistronic transcripts, which means that E5 may be coded from the same mRNA that also codes for E6 or other viral proteins (155). This makes the use of siRNA a more difficult endeavor than for singularly regulated cellular proteins. Other methods of approaching this issue, such as using antibody depletion, are currently under investigation. In addition, the method currently presented is a qualitative approach to determining the presence of E5. Machine calibration and sensitivities introduce variation in actual intensity output between runs. In addition, sample processing and protein hydrophobicity might introduce additional variables. To pursue absolute quantification, FLIT-peptide conjugated with a heavy isotope (such as carbon-14 or nitrogen-15) would be required. A set amount of this conjugated-FLIT would be mixed with each sample to serve as an internal control, allowing normalization of each sample to the intensity of the conjugated-FLIT peptide.

Currently, however, we do have a successful method of qualitatively discerning whether a cell lysate has 16E5 or not. In the future, such studies could be expanded to include cervical tissue, as well as tissue from other HPV-induced tumors.
(RRP, head and neck cancers). These studies could help correlate E5 expression with the type and severity of HPV-induced tumors, as well as shed light on the true biological function of E5. Our data suggest that 16E5 might be essential for all stages of HPV-induced tumorigenesis, from initial viral infection to later stages of malignancy.
V. CONCLUSIONS
Cervical cancer is responsible for the mortality of around 250,000 women annually (75, 120). Not only does the high financial and social burden of this disease warrant studying it, but so does its unique etiology. 99% of cervical cancer is caused by the high-risk types of the human papillomavirus, which has allowed targeted therapies and vaccines to be developed (18, 182). Current research regarding cervical cancer builds upon these existing therapies with an aim of improving efficiency and availability of treatment. Basic research (including the role of HPV and epidemiological studies) must still be conducted for other HPV-induced diseases, including head and neck cancers (3, 65), respiratory papillomatosis (188, 189), and EV (36, 125). In addition, established in-vivo models (canine, rabbit, etc) can be used to study how individual HPV proteins contribute towards the final malignant phenotype.

In this thesis, we attempted to define properties associated with the E5 protein of the high-risk type 16 human papillomavirus. The first chapter focuses extensively on gene expression changes induced by stable E5 expression in primary human genital keratinocytes (HFKs), which are the host cells for HPV-infection. In conjunction, gene expression changes were studied in the low-risk HPV type 6b E5, as well as in 16E5 mutants. Studies were performed by real-time RT-PCR as well as by a human whole genome cDNA microarray across three separate preparations of HFKs. The gene expression changes between low- and high-risk E5s were remarkably different. Surprisingly, however, the high-risk 16E5 induced far fewer changes in gene expression than did the low-risk 6bE5. In fact, only around 25 genes were found to be consistently regulated across different donor pools by 16E5, whereas upwards of 700
genes were consistently regulated by the 6bE5. Of note, type 16 (HPV-16) E5 protein consistently downregulates three members of the ER-stress pathway: spliced XBP-1, IREα (upstream of XBP-1), and COX-2 (downstream of XBP-1). Interestingly, these genes were not amongst those altered by the low-risk HPV-6b E5. In addition, a C-terminal HPV-16 E5 mutant, where the histidine and alanine residues (conserved in high-risk HPVs) were replaced with tyrosine and isoleucine (conserved in low-risk HPVs) was also unable to alter these genes. This unique property of the high-risk HPV E5 protein might contribute to differences in biological behavior between low- and high-risk papillomaviruses. Specifically, it has been previously reported that the hepatitis C virus (HCV) suppresses the XBP-1/IRE1α pathway. It is suggested that this may promote both the expression of HCV proteins and aid in viral persistence (175). In the case of high-risk HPVs, persistent viral infection appears to be a major contributory factor to the development of cancer (148, 149). It is possible that, similar to HCV, E5 may support viral persistence through alteration of the ER-stress pathway. Another possibility is that the regulation of XBP-1 by 16E5 has effects on immune evasion. XBP-1 is required for the expression of certain classes of MHC-II molecules, so downregulation of XBP-1 by 16E5 may contribute to the downregulation of MHC-II molecules seen by 16E5 (124, 197).

Since E5 has been reported to be lost after viral integration, the function of E5 has always been assumed to be relevant soon after viral infection and before genomic integration. However, several reports have challenged this assumption, and demonstrated the presence of E5 ORFs and E5-coding RNA transcripts even in
cervical cancer cell lines with integrated DNA (11, 13, 129, 155, 157). However, because of the lack of an E5-antibody, definitive studies demonstrating E5 protein have never been done. Previously, it was unknown whether E5 would be present in an E6/E7 background at a late stage of carcinogenesis. However, published literature indicated that E5 could probably contribute to tumorigenesis, and E5 was classified as an oncoprotein despite its supposed loss after integration. In fact, in-vivo studies demonstrated that transgenic mice expressing E5, E6, and E7 had larger tumors than mice expressing E6 and E7 alone (137). Our data, which establish that E5 is present in cancer in the presence of high levels of E6 and E7, lend support to such findings. The second chapter of the thesis establishes the presence of E5 protein in two HPV-16 positive cervical cancer cell lines with integrated DNA. Following trypsin digestion and characterization of resulting peptides, mass spectrometry was used to identify the E5 protein in SiHa and CaSki lines. This is the first time that the native E5 protein has ever been detected, either in artificially transduced strains or naturally occurring cancer lines.

While the current thesis does not define a biological function for the described E5-induced gene expression changes, and also does not pursue E5’s roles at later stages of tumorigenesis, future studies can aim to answer these questions. All gene expression studies in this thesis were performed by stably-expressing a tagged E5 into primary keratinocytes. Now that we can discern the presence or absence E5 in cervical cancer cell lines, such as SiHa and CaSki, we can attempt siRNA experiments in an effort to knock down E5 in these lines. Real-time PCR studies can be used to study the
levels of ER-stress pathway genes, as well as microarray analysis to determine gene changes. This would allow us to study the effects of E5 in the background of other viral proteins expressed at physiologic levels in the appropriate host cell. In addition, in-vitro soft agar assays and in-vivo tumorigenicity assays can be attempted to see if the loss of E5 slows the growth of these transformed cells. If these E5-depletion experiments result in any differences, the same model systems can allow us to study the differences between low- and high-risk E5s. Given the variations in gene expression induced by 6bE5 vs. 16E5, one would expect that these proteins would have entirely different functions. This may well be true; however, these proteins do share certain characteristics (eg. koilocytosis, binding to v-ATPase). These shared characteristics may be due to protein-protein interactions as opposed to changes in gene expression. It would be interesting to determine the extent to which these proteins have shared vs. different functions. For example, after knockdown of high-risk E5, low risk 6b can be transduced in to see if the phenotype can be rescued.

In addition, the discovery of E5 in cervical carcinoma cell lines leads to the question of whether E5 is expressed in other HPV-induced diseases, such as HPV-positive head and neck cancers. In head and neck cancers, it was found that 48% had integrated forms of HPV DNA, 35% had episomal forms, and 17% had mixed forms (89). This means that over 50% of head and neck tumors have some episomal copies of the entire HPV genome, and, if our findings from cervical cancer lines hold true, an even greater percentage of head and neck cancer lines might actually express E5. Preparations for these studies are currently underway. Finally, it would be interesting
to move beyond cell lines and screen tumor tissues for E5. These studies would allow us to correlate E5 expression with the type, severity, and prognosis of HPV-induced cancers.

Using new, innovative techniques, our data establish the presence of E5 in cervical cancer cell lines, giving us in essence a new model system in which to study the effects of E5 on gene expression. If applied to tumor tissues, these proteomic techniques (combined with biologic in-vitro and in-vivo assays) can help answer some crucial questions regarding the extent of E5’s role in transformation. Our data suggest that E5 might be essential for all stages of HPV-induced tumorigenesis, from aiding with viral persistence after the initial infection to supporting later stages of malignancy.
VI. REFERENCES CITED


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