Cutaneous Papillomavirus E6 Inhibit NOTCH and TGF-Beta Signaling to Disrupt Keratinocyte Differentiation.

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Cutaneous papillomavirus E6 inhibit NOTCH and TGF-beta signaling to disrupt keratinocyte differentiation.

A dissertation presented

by

Jordan Michael Meyers

to

The Division of Medical Sciences

in partial fulfillment of the requirements

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Cutaneous papillomavirus E6 inhibit NOTCH and TGF-beta signaling to disrupt keratinocyte differentiation.

Abstract

Cutaneous beta-papillomaviruses are associated with non-melanoma skin cancers that arise in patients who suffer from a rare genetic disorder, Epidermodysplasia Verruciformis or after immunosuppression following organ transplantation. We along with others have shown that the E6 proteins of the cancer associated beta human papillomavirus (HPV) 5 and HPV8 inhibit NOTCH and TGF-beta signaling. However, it is unclear whether disruption of these pathways may contribute to cutaneous HPV pathogenesis and carcinogenesis. A recently identified papillomavirus, MmuPV1, infects laboratory mouse strains and causes cutaneous skin warts that can progress to squamous cell carcinoma. To determine whether MmuPV1 may be an appropriate model to mechanistically dissect the molecular contributions of cutaneous HPV infections to skin carcinogenesis, we investigated whether MmuPV1 E6 shares biological and biochemical activities with HPV8 E6. We report that the HPV8 and MmuPV1 E6 proteins share the ability to bind to the MAML1 and SMAD2/SMAD3 transcriptional cofactors of NOTCH and TGF-beta signaling, respectively. Moreover, we demonstrate that these cutaneous papillomavirus E6 proteins inhibit these two tumor suppressor pathways and that this ability is linked to delayed differentiation and sustained proliferation of differentiating keratinocytes. Furthermore, we demonstrate that the ability of MmuPV1 E6 to bind MAML1 is necessary for papilloma formation in experimentally infected mice. Our results, therefore, suggest that
experimental MmuPV1 infection in mice will be a robust and useful experimental system to model key aspects of cutaneous HPV infection, pathogenesis and carcinogenesis.
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Chapter One: Introduction

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Jordan M. Meyers wrote the first drafts of these reviews.
1.1 Organization and classification of papillomaviruses

Papillomaviruses (PVs) are a large, diverse, group of DNA viruses. PVs are species specific and only infect squamous epithelium. The double stranded circular genome generally encodes eight open reading frames (ORFs) which can be subdivided into early and late genes. All PVs encode an Early Protein 1 (E1), 2 (E2), and 4 (E4) and most encode an E5, E6, and E7. PVs also encode two structural proteins Late Protein 1 (L1) and 2 (L2); these proteins form the viral capsid. All known viral genes are encoded in the same direction and on the same strand. The region after the L2 stop codon and the start codon of the E6 ORF is named the long control region (LCR) which contains an origin of replication, transcription regulatory elements, and the early viral promoter (1). A second, epithelial-differentiation dependent promoter or late viral promoter is imbedded in the E7 ORF and regulates transcription of L1 and L2 (2). Viral transcripts are spliced and polyadenylated with host machinery from signals encoded on the viral genome (3).

Papillomavirus classification is based on sequence identity of the major capsid protein L1 and viruses are broadly subdivided into genera and type. Members of a genera share around 60% or greater nucleotide sequence identity in the L1 gene. Types, which are not recognized by the International Committee on Taxonomy of Viruses, are used extensively in the field and distinguish between viruses whose L1 sequence is at least 10% dissimilar from any other PV (4). The diversity of PVs can be appreciated in the variety of species they can infect. PVs have been isolated from vertebrates such as reptiles to aquatic and terrestrial mammals. Of the 30 characterized PV genera only five contain PV types capable of infection of humans and include Alpha-, Beta-, Gamma-, Mu-, and Nu-. The majority of human PVs (HPVs) belong to either the Alpha or Beta genera. One difference between these two groups is the epithelial tissue that they
infect. Most members of the alpha-papillomaviruses only infect anogenital and oral mucosal epithelia while beta-papillomaviruses infect the keratinized cutaneous epithelia. Other genera of note include delta papillomaviruses whose members infects ungulates such as cows, kappa papillomaviruses whose members infect rabbits, and pi papillomaviruses whose members can infect mice.

E1 and E2 proteins are essential for origin recognition and PV genome replication. Hexameric E1 is recruited to bind the viral origin through interactions with E2 and subsequently, E1 helicase activity unwinds the origin allowing for DNA replication (5-7). In addition to being necessary for viral genome replication, E2 is involved in genome maintenance and viral gene expression. E2 tethers the viral genomes to the host chromosome during mitosis to ensure genome maintenance (8, 9). Full-length E2 functions as a transcriptional transactivator capable of inducing mRNA synthesis of both the early and late viral promoter. Truncated isoforms of E2 generally act as transcriptional repressors in concert with other cellular repressors (10, 11). The functions of E4 are not well characterized but evidence suggests it has a role late in viral replication as its expression greatly increases late in infection (12). E5 proteins, while not encoded by all PV types, are small transmembrane proteins. Bovine papillomavirus (BPV) E5 dimerizes and activates the platelet-derived growth factor receptor independent of ligand binding (13). E5 proteins from other diverse PV types, including HPV 16 E5, are able to interact with growth factor receptors like epidermal growth factor receptor (EGFR), but only E5 proteins from BPV1 or related types is highly oncogenic (14). E6 and E7 will be discussed in greater length in section 1.2, but they represent the major oncogenes of human papillomaviruses and function to target and inactivate key cellular tumor suppressors.
Viral capsids are approximately 55 nm in diameter and assume a T=7 icosahedral symmetry which requires 360 copies of the major capsid protein L1 forming 72 pentamers (15, 16). Up to 72 molecules of the minor capsid protein L2 can be incorporated into a virion and its role in infection is dependent on PV type. L2 is involved in processes such as DNA encapsidation (17-20), intracellular transport, and nuclear delivery of viral DNA (21-24).

1.2 Viral entry and life cycle events

PV life cycle begins with infection of basal epithelial cells, although the biology of stratified epithelium will be discussed in greater detail in section 1.4, it is important to note that only the basal cells are capable of DNA replication and cell division in differentiated epithelium (Figure 1.1). This requirement is likely due to the fact that post entry the virus requires cell division to establish infection (25). Initial binding of the virus occurs between the major capsid protein L1 and heparin sulfate proteoglycans (26). The requirement for these proteoglycans may be both PV type-specific and dependent on the method of virus production (27, 28). Viruses that require binding of glycosaminoglycans undergo conformational changes upon binding that expose a N-terminal portion of the minor capsid protein L2. L2 is then cleaved by cell surface furin-like proteases (29). This cleavage seems to be necessary for downstream events including virus uptake. Viruses which do not require glycosaminoglycan binding, can undergo furin-mediated L2 processing intracellularly during particle assembly (30). Identification of a bonafide second receptor that mediates uptake subsequent to protease cleavage has remained elusive but potential receptors include annexin A2 (31, 32), alpha6 integrin(33), and tetraspanin CD151 (34, 35). These receptors are all expressed on keratinocytes, but cells deficient in individual receptors
Figure 1.1. Life cycle of papillomavirus. (1) Virus enters replication competent basal epithelial cell. (2) Virus is maintained as a stable episome in the nucleus. (3) Differentiation induces viral genome amplification and late structural genes. (4) Virion assembly occurs in terminally differentiated keratinocytes. (5) Infectious particles accumulate until dead cells slough off.
still allow for infection indicated that there are either multiple entry receptors or additional required receptors. It has also been shown that soluble growth factors are capable of binding to virus particles and allow for viral uptake after growth factor receptor binding and endocytosis (36). There is a lack of a consensus on the endocytosis pathway but multiple reports suggest that virus uptake may involve a novel pathway that is actin-dependent, caveolin-independent, and clathrin-independent (37). Activation of the PI3 kinase signaling cascade through RAC-alpha serine/threonine-protein kinase (Akt) and mechanistic target of rapamycin (mTOR) leads to inhibition of autophagy (38). This inhibition may be crucial to infection as it has been shown that autophagy can restrict PV infection (39).

Retrograde endosomal transport of virus particles results in virus particles accumulating in the trans-Golgi network and requires penetrance of L2 into the endosomal membrane and sorting nexin 17 (SNX17) (22, 40-43). Viral genomes associated with L2 remain inside vesicles and attach to mitotic chromosomes; they remain attached until after the nuclear membrane reforms in these transient vesicles to allow for nuclear entry (43). This model explains the requirement of proliferation-competent basal keratinocytes for productive viral replication (25).

After the viral genome accesses the nucleus, it is replicated and maintained at stable copy numbers as an episome. The copy number appears to be determined by a number of factors including PV type, severity of disease, and host determinants but can range from a few copies to several thousands (44, 45). Keratinocytes harboring high-risk HPV genomes can maintain viral genomes indefinitely. During asymmetric cell division of basal keratinocytes, cells containing viral episomes travel with daughter cells into the suprabasal layers. As these cells differentiate, it is thought that cellular transcription programs facilitate a viral switch from genome maintenance to genome amplification. This switch to genome amplification may involve a
switch from theta circle replication to rolling circle mode (46-48). Genome amplification is accompanied by a switch to late viral promoter activity and the transcription of the two viral capsid genes L1 and L2 (2, 49). At sufficiently high concentrations, L1 and L2 are capable of self-assembly into viral particles which encapsidate the newly synthesized viral genomes (17, 50). Maturation of these viral particles, such as via furin processing occurs in certain PV types (30). Papillomaviruses are incapable of inducing cell lysis and infectious viral particles can be observed inside cells by electron microscopy. Dissemination of these virions is thought to occur during the natural desquamation of cornified cells. HPV virions are extremely resistant to chemical disinfection, suggesting that HPV particles are likely extremely stable between transmission to new hosts (51).

1.3 Historical discoveries of PV and cancer

1.3.1 Animal PVs

The concept that viruses may be etiological agents of cancers has existed since the discovery of the some of the first animal viruses. In 1908, three years before Peyton Rous was passaging what later became to be known as Rous Sarcoma Virus (RSV) in chickens, two Danish scientists, Vilhelm Ellerman and Olaf Bang, were characterizing a transmissible filtrate that reproducibly caused leukemia in chickens. These findings were received with harsh skepticism and the scientific community did not universally accept the concept that tumors could be caused by transmissible agents. Richard Shope, a colleague of Peyton Rous at the Rockefeller Institute identified an infectious agent that infected cottontail rabbits. It caused cutaneous papillomas that could grow to be quite large and are likely the basis of sightings of the mystical and ravenous “Jackelope” of southwestern American lore. Shope later collaborated with Rous to demonstrate
that exposure of these papillomas to coal tar or infection of a host that does not support viral replication caused malignant progression to skin cancers. This infectious agent, the cottontail rabbit papillomavirus (CRPV) or *Sylvilagus floridanus* Papillomavirus 1 (SfPV1), was the first virus linked to a cancer in a mammalian host (52-54).

Discovered after CRPV, bovine papillomaviruses (BPVs) were shown in 1959 to induce hyperplastic growth cows both in cutaneous skin and in bladders (55, 56). These viruses, unlike many other PV types, have expanded tropism and can infect mucosal and cutaneous epithelial cells as well as mesenchymal cells and can cause hyperplastic fibropapillomas (57). The epithelial and adjacent mesenchymal cells are positive for BPV DNA but only the epithelial cells are positive for the L1 structural gene suggesting that productive replication only occurs in epithelial cell. Differing from both CRPV and high-risk HPVs, the major transforming oncogene of BPV1 and BPV2 is E5 and not E6 and E7. This has limited the usefulness of BPV and related types from being animal models to study human disease.

1.3.2 Beta-HPVs

Eleven years before Shope and Rous discovered that CRPV caused skin cancers in rabbits, Felix Lewandowsky and William Lutz described a rare skin disorder that would come to be known as epidermodysplasia verruciformis (EV) (58). EV patients develop widespread wart-like lesions that can cover entire portions of their skin and frequently develop malignant skin tumors particularly at sun-exposed areas. Seminal work by Stefania Jablonska and Gerard Orth linked HPV infections to skin lesions and cancers in EV patients (59). EV patients suffer from a deficiency that prevents effective clearance of beta-HPV infections. Interestingly, however, EV
patients do not seem to be at a higher risk for bacterial or other viral infections, including alpha-HPV infections (60).

The genetic basis of EV was discovered in 2002 when Michel Favre and colleagues discovered that EV patients harbored mutations in either one of two adjacent genes, \textit{TMC6} or \textit{TMC8}, on chromosome 17 (61). These genes encode the transmembrane proteins, EVER1 and EVER2, which localize to ER membranes and are involved in intracellular zinc transport. How this relates to susceptibility to persistent cutaneous HPV infections remains to be fully delineated.

Beta-HPV genomes can readily be detected in tumor cells of EV patients and are likely etiologic agents of non-melanoma skin cancers (NMSCs) that arise in chronically immune suppressed patients (62-65). Whether or not beta-HPV infections also contribute to NMSCs in other patients has been a matter of debate, mostly because subclinical beta-HPV infections are very widespread and not every tumor cell in these patients contain HPV sequences (66). As detailed below, this does not rule out, however, that infections with some beta-HPVs may be drivers of NMSC initiation in the general population.

Most of the fundamental concepts of how HPVs contribute to human cancer formation have been established by studies with alpha-HPVs, which preferentially infect mucosal epithelia. These HPVs have been studied extensively and they fall into “high-risk” and “low-risk” groups based on their propensities to cause lesions that can undergo malignant progression. Notably, high-risk alpha-HPV infections cause almost all cases of cervical carcinomas, a significant fraction of other anogenital tract tumors as well as oropharyngeal cancers. Approximately 5% of all human cancers, overall, are caused by high-risk alpha-HPV infections. These cancers regularly maintain viral gene expression; every tumor cell generally contains and expresses HPV
sequences and they remain “addicted” to expression of the E6 and E7 oncogenes. The high-risk alpha-HPV E6 and E7 proteins target and functionally compromise the p53 and retinoblastoma (pRB) tumor suppressors, respectively, which are frequently mutated in non-HPV associated cancers.

It has been proposed that beta-HPVs may be similarly classified into “high-risk” and “low-risk” groups. HPV5 and the phylogenetically related HPV8 were originally isolated from NMSCs arising in EV patients (67, 68). These viruses may be considered “high-risk” for NMSC development in EV patients. Early research demonstrated that HPV8 E6 but not E7 was capable of morphological transformation of C127 and Rat 1 cells (69). Later experiments performed with transgenic mice corroborated these findings. Expression of the early genes of HPV8 from the basal keratinocyte-specific promoter, keratin 14 (K14), causes spontaneous development of malignant skin tumors in transgenic mice (70). Additional studies revealed that HPV8 E6 and, surprisingly, E2, scored as the major transforming proteins in this model (71, 72). While these tumors will arise spontaneously, UV irradiation dramatically accelerates carcinogenesis, thereby recapitulating a key risk factor of EV-associated cancers.

1.3.3 Alpha-HPVs

Early research was highly suggestive of a viral agent as the etiological cause of cervical cancer. Strong epidemiological evidence had found correlation between herpes simplex type 2 (HSV-2) and uterine cancers (73, 74). Later epidemiological evidence demonstrated that cervical intraepithelial dysplasia (CIN) were the results of papillomavirus infection and that papillomavirus particles could be detected in the nuclei of these cells (75, 76). Harald zur Hausen later characterized and isolated papillomavirus DNA in cervical cancer cells which were
designated HPV16 and HPV18 (77, 78). Importantly, sequences from HPV18 were found in cell lines derived from cervical cancer specimens such as HeLa cells (78). With these new sequences, cervical cancers were screened to identify the prevalence of papillomavirus DNA and approximately 70% of cervical cancers were identified as HPV positive. It was observed in malignant tumors that HPV DNA was often integrated into the host genome (79). The establishment of high-risk HPV types as the causative agent of cervical cancer coincided with the identification that oncogenes from high-risk HPV types shared transformation functions with other DNA tumor viruses (80, 81). In contrast to BPV1 E5, E6 and E7 of high-risk HPVs were determined to be responsible for maintenance of the tumor phenotype (82) and were shown to transform cells (83, 84). With advanced detection techniques, HPV DNA is now detected in virtually all cervical cancers (85).

In addition to cervical cancer, high-risk mucosal HPVs are associated with carcinomas of the anogenital tract and the head and neck. Unlike cervical cancer, where HPV DNA is detected in nearly all cancers, identifying HPV DNA in these cancers is not universal (86, 87). Head and neck cancers among certain populations such as young nonsmokers are on the rise, and a large proportion of these new cases are HPV-positive (88). In all HPV infection represents a significant cause of cancer worldwide and is responsible for around 5% of all new cancer cases each year (89).
1.4 Functions of E6 and E7

1.4.1 Alpha-HPVs E6 and E7 functions

HPV E6 and E7 encode low molecular weight proteins of ~150 and ~100 amino acids, respectively. They each contain zinc-binding domains consisting of two copies of CXXC separated by 29 amino acid residues and this similarity may be due to gene duplication in a common ancestor (Figure 1.2). Interestingly, some animal papillomaviruses contain only E6 and no E7 or vice versa. There is also evidence for domain shuffling between E6 and E7 as the PDZ binding motif found on high-risk HPV E6 proteins is at the carboxyl termini of the Rhesus macaque papillomavirus (RhPV) and some cynomolgus macaque papillomavirus E7 proteins (90). The recently identified Mus musculus papillomavirus 1 (MmuPV1) E6 protein contains an LXCXE sequence similar to the core pRB-binding site in many HPV E7 proteins (91) but it is not functional as an RB binding site (Miranda Grace and Karl Munger, unpublished).

Structures of several E7 proteins have been reported (92-94), and a structure of BPV1 and HPV16 E6 has also been published (95, 96). Both E6 and E7 can form dimers and, despite the conserved CXXC domain architecture, the 3 dimensional structures of these domains in E6 and E7 are quite distinct (93, 94, 97). The E7 amino terminus is an intrinsically unstructured region, and a peptide corresponding to the HPV16 E7 core pRB-binding site assumes an extended structure when bound to pRB (92).

HPV E6 and E7 proteins lack intrinsic enzymatic activities and function by associating with and thus function by associating and reprogramming key components of host cellular signal transduction networks. The most extensively studied cellular E6 and E7 targets are the p53 and retinoblastoma (pRB) tumor suppressors, respectively. High-risk HPV E6 and E7 proteins do not inactivate these tumor suppressors by stoichiometric sequestration but target them for ubiquitin-
Figure 1.2. Organization of generic PV E6 and E7 proteins. E6 and E7 are small nonenzymatic proteins of around 150 and 100 amino acids respectively. Note that most E6 proteins contain two paired CXXC motifs and E7 proteins contain only one set.
mediated proteasomal degradation. The inactivation of p53 and pRB lead to aberrant and extended proliferation and the extent to which PV types affect these two pathways is the basis of distinction between high and low-risk alpha-PVs (Figure 1.3).

High-risk HPV E6 proteins target p53 for ubiquitination by associating with and reprogramming the UBE3A (E6AP) ubiquitin ligase (98). High-risk HPV E6 proteins also contain a type I PDZ binding motif (X-X-S/T-X-I/L/V) at their carboxyl termini, and E6 associated PDZ proteins can also be targeted for degradation through UBE3A (99). PDZ protein binding is critical for E6 transforming activities. Even though the physiologically relevant cellular PDZ target(s) have not yet been identified, association of E6 with the discs large homolog DLG1 causes constitutive activation of the RAS homolog gene family, member G (RHOG), which contributes to the invasive properties of cervical carcinoma cells (100). Low-risk HPV E6 proteins also form complexes with UBE3A. However, low-risk HPV E6 proteins do not associate with p53 and lack carboxyl terminal PDZ binding domains; low-risk HPV E6/UBE3A complexes do not target p53 or cellular PDZ proteins for degradation (101). Ubiquitination substrates of low-risk HPV E6/UBE3A complexes remain to be determined.

High-risk HPV E6 proteins can also induce degradation of cellular proteins through UBE3A independent mechanisms (102). HPV E6 modulates cell adhesion and focal adhesion pathways through UBE3A-independent of TAp63beta thereby contributing to anchorage independent growth and cellular transformation (103).

While pRB-mediated activation of E2F factors is observed for many PV types including high-risk and low-risk, E7-mediated pRB degradation has been mechanistically elucidated only for HPV16 E7. HPV16 E7, similar to E6, associates with a ubiquitin ligase, in this case ZER1 containing cullin 2 (CUL2) complex, and targets pRB for ubiquitination and subsequent
Figure 1.3. Model of alpha-HPV-induced tumorigenesis.
proteasomal degradation (104). Whereas pRB degradation is shared with other high-risk HPV E7 proteins, this is accomplished by alternative ligases as they do not involve the same CUL2 complex. Other targets of many papillomavirus E7 proteins include UBR4 ubiquitin ligase, a 600 kDa protein that may regulate anoikis (98). Association with UBR4 is independent of pRB and the potential degradation substrates of the E7/UBR4 complex is relatively unknown. One target of the complex is the protein tyrosine phosphatase PTPN14. Many alpha and beta-papillomavirus E7 are capable of interacting with PTPN14 but only high-risk alpha-PVs can destabilize PTPN14 through UBR4 ubiquitination (105). Furthermore, PTPN14 degradation is important for pRB-independent transformation functions of high-risk E7. An additional function of high-risk E7 is the ability to inhibit TGF-beta signaling. Tumor virus oncogenes capable of binding pRB are able to inhibit TGF-b1-mediated G1 growth arrest and suppression of c-myc transcription (106). Additional studies suggested that HPV16 E7 inhibits TGF-beta signaling by preventing DNA binding of the SMAD2/3/4 complex (107).

1.4.2 Beta-HPVs E6 and E7 functions

Similar to what has been reported for high-risk alpha-HPVs, the beta-papillomavirus types HPV5 and HPV8 E7 proteins can activate E2F-mediated transcription but can only weakly associate with and do not destabilize pRB (104, 108, 109). In contrast to high-risk alpha-HPVs, E6 proteins from HPV5 and HPV8 do not directly inhibit p53 activity (110). HPV5 and HPV8 E6 proteins have been reported to inhibit pro-apoptotic factors activated during UV damage and impair DNA damage response pathways. Several groups have reported that beta-HPV E6 proteins can trigger the degradation of the pro-apoptotic BCL2 family member BAK through a proteasome dependent pathway (111, 112). BAK is normally retained in the mitochondria but is
released and induces apoptosis following UV exposure. BAK degradation in beta-HPV infected cells may, therefore, blunt the apoptotic response to UV irradiation and allow survival of cells that have suffered extensive DNA damage and possibly acquired oncogenic mutations.

There is evidence that the repair of UV-induced DNA damage is inhibited in HPV8 E6 expressing cells (112, 113). HPV5 and HPV8 E6 proteins also inhibit double strand DNA break repair by associating with and destabilizing the histone acetyl transferase, p300 (114, 115), which can regulate activity of the ATM/ATR kinases by acetylation. Similar to subverting apoptosis signaling though BAK degradation, blunting DNA break repair may allow for accumulation of mutations in beta-HPV infected cells, thereby facilitating malignant progression. According to such a model, beta-HPV infections contribute to cancer initiation in non-EV patients through a “hit-and-run” mechanism, and since viral gene expression may not be necessary for the maintenance of the transformed state, it might explain why the viral genome is not detected in all tumor cells (66).

HPV5 or HPV8 E6 expression in transgenic mice or in organotypic tissue culture models of skin dramatically inhibits epithelial differentiation (71, 116). This ability of E6 to uncouple the processes of epithelial differentiation and proliferation may be relevant to the viral life cycle, since viral genome synthesis and progeny formation is restricted to terminally differentiated cells that have normally withdrawn from the proliferative pool. Since HPVs require cellular DNA synthesis for the replication of their genomes, it is essential that cell cycle proficiency be maintained during differentiation. One of the critical regulators of epithelial differentiation is NOTCH signaling. Several recent studies have shown that the HPV5 and HPV8 E6 proteins inhibit NOTCH signaling by interacting with MAML proteins, critical co-activators of the NOTCH transcription complex (109, 117-120). NOTCH has tumor suppressor activities in
epithelia and loss-of-function NOTCH pathway mutations are highly prevalent in squamous cell carcinomas (SCC) (121, 122). Whereas alpha-HPVs do not directly target MAML, high-risk alpha-HPV E6 proteins inhibit Notch indirectly through p53 inactivation (123) and degradation of TAp63beta (103). HPV5 E6 has also been shown to inhibit TGF-beta signaling in keratinocytes through destabilization of the SMAD3/4 transcriptional complex (124). Similar to NOTCH, TGF-beta signaling can be oncogenic or tumor suppressive in different tissues and/or at different stages of carcinogenesis.

While beta-HPV5 and HPV8 E6 and E7 proteins do not target p53 or pRB tumor suppressors for degradation, some other beta-HPVs have been reported to cause pRB and p53 degradation (125) and/or to inhibit p53 functions indirectly by causing accumulation of the dominant negative form of p73, ΔNp73 (126). Moreover, HPV5 and HPV8 E6 proteins associate with and may induce degradation of p300 (115, 127), a critical co-activator for many transcriptional programs, including p53.

The E6 and E7 proteins of other beta-HPVs, including HPV types 20, 27, and 38 also exhibit carcinogenic activities in transgenic mouse models, although, and in contrast to the HPV8 model, tumor formation was strictly dependent on UV exposure (120, 128, 129). HPV38 has been studied in some detail, and in contrast to many other beta HPVs, HPV38 can immortalize primary human epithelial cells and has transforming activities in vitro. Unlike HPV5 and HPV8, HPV38 E6 has been reported to cause p53 inactivation and HPV38 E7 has been shown to efficiently associate with pRB and trigger its degradation (108, 126). These activities of the HPV38 E6 and E7 proteins mirror the function of the mucosal high-risk alpha-HPV E6 and E7. Whether humans infected with HPV38 are at a particularly high risk for NMSC development remains to be determined.
Despite the strong association with SCCs in EV and immune compromised patients, the mechanistic role(s) of beta-HPVs in human cancers is not clearly defined. Unlike cancers caused by alpha-HPVs, beta-HPV associated SCCs represent productive infections. Mutations of the EVER1 and EVER2 genes on 17q25 have been linked to EV development but how defects in these zinc-binding proteins mechanistically contribute to HPV-associated SCC development is yet to be determined (130). There are no sensitive cell based transformation assays for beta-HPVs, but E6, E7 and E2 score as tumorigenic when expressed in basal epithelial cells of transgenic mice (71, 72).

1.5 Systems for studying virus life cycle and virus production.

Productive infection by PVs only occurs in differentiating epithelium. These cells express the receptors necessary for entry by the virus. Unlike many viruses, species specificity of various PV types is not based on differences in receptors. Though incapable of productive infection, human PVs are capable of entry in mouse models but are unable to initiate early viral gene expression (131). This suggests species restrictions are not due to entry defects. Despite these host restrictions all PVs investigated require differentiation of epithelial tissues for full productive life cycle. This requirement has made life cycle studies of the virus difficult as simple tissue culture of undifferentiated epithelial cells does not support full replication.

Animal models have proved useful but are limited to large and genetically intractable mammals. Due to this obstacle, two different systems were developed that can produce infectious viruses to use in in vitro studies. PV pseudoviruses are made synthetically and rely on the ability of the viral capsid genes to self-assemble into particles when expressed at high levels. These methods require the capsid genes to be expressed from a plasmid containing a highly
active promoter and particles are made in non-epithelial cells such as HEK293 (132, 133), insect (134, 135), or yeast (136, 137) systems. This method is capable of generating high titers of viral particles either containing native viral genomes or more commonly containing reporter plasmids capable of expressing fluorescent proteins or other trackable elements. They have been very useful in studying immunity, antibody neutralization, and viral entry. The downside to these systems is that due to the artificial expression of late viral genes, they are incapable of studying viral events that are dependent on epithelial cells and their differentiation.

So-called native virions can be made utilizing organotypic culturing methods originally designed to grow fully differentiated epithelium (138-140). This method originally required epithelial cells to be seeded onto a dermal equivalent comprised of a collagen matrix and fibroblasts and floated on top of culture medium. These “rafts” would allow the epithelial cells to be exposed to air and be fed underneath through the dermal equivalent. Polarization and differentiation progresses and leads to fully differentiated skin similar in thickness and physiology to normal skin. To adapt this system to PV production, keratinocytes harboring intact viral genomes are seeded onto the collagen matrix (141, 142). As the tissue differentiation progresses, the stably maintained viral genomes switch from maintenance to amplification phase replication and capsid gene expression and assembly of virions can be observed in the uppermost layers. The benefits of this system are that it utilizes the natural host cells of the virus and the natural processes required for productive infection. Organotypic “raft” culture systems can also produce relatively high titers of virus but are technically difficult and resource intensive in comparison to synthetic methods.
1.6 Regulation of epithelial regulation of differentiation and proliferation

1.6.1 PV effect on differentiation

Keratinocyte differentiation is a highly regulated and multifunctional program that results in both the death of the keratinocyte and the formation of a highly resilient skin barrier (143). Epithelial cells need to be constantly replenished as the outermost layers are lost and this is achieved through a variety of stem cell progenitors mainly found in the hair follicle (144). These progenitors give rise to the basal keratinocytes, which asymmetrically divide resulting in one daughter cell that remains in the basal layer and one suprabasal daughter cell. As they are pushed outward, these cells experience a gradient of increasing calcium concentration that is important for reinforcement and maintenance of differentiation. Calcium is necessary for activation of pro-differentiation cellular signaling and transcriptional regulation including desmosome formation and is required for transglutaminase activities required for the formation of the cornified cell envelope (145). Due to the relevance of calcium levels to differentiation, keratinocytes are routinely maintained in media containing low levels of calcium and the addition of calcium alone to the media is sufficient to induce terminal differentiation. Of particular interest in relation to PV, downstream signaling pathways involved in differentiation include the NOTCH and TGF-beta pathways.

As mentioned, PV replication requires differentiating keratinocytes. There are many potential benefits of coupling late viral life cycle events to the more differentiated layers of the epithelium. One likely benefit is delaying expression of the immunogenic capsid genes (146) until the host cells exit normal immune surveillance. This protection comes at a cost as differentiation induces strong signals to fully withdraw from cell cycle and proliferation (147). All PVs, outside of replication functions provided by E1 and E2, require host machinery to
complete their life cycle. Thus, the role of PV early proteins is to establish an environment conducive to DNA replication in cells which are actively exiting proliferation. To accomplish this PVs have developed strategies that uncouple cell-cycle from differentiation effectively allowing unlicensed suprabasal proliferation of keratinocytes. These activities must be balanced by the virus and their ability to do so largely determines their oncogenic potential.

1.6.2 NOTCH signaling and differentiation

NOTCH signaling is a highly-conserved pathway and involved in a multitude of processes during embryogenesis and extending to the adult organism. Of particular interest to PV biology, NOTCH is a key driver of epithelial differentiation and is required for proper formation of the skin barrier (148). As a key regulator of diverse developmental processes, NOTCH signaling occurs through cell-to-cell contact where membrane anchored receptors of the Delta or Jagged family bind to the NOTCH receptor. In humans there are four NOTCH receptors (NOTCH1-4) which exhibit some overlapping functions, though this may context specific (149). Receptor-ligand binding induces conformational changes in NOTCH that result in a series of cleavages ultimately releasing an intracellular portion of the NOTCH receptor termed ICN (150). ICN localizes to the nucleus where it associates with Recombination Signal Binding Protein for Immunoglobulin Kappa J Region (RBPJ) which is bound to DNA. It is thought that in the absence of NOTCH activity, RBPJ is bound to the DNA in association with co-repressors inhibiting target gene expression (151). ICN displaces these corepressors and the ICN-RBPJ complex recruits the transcription co-activator mastermind-like (MAML) (152). This represents the core NOTCH transcriptional complex and MAML then recruits additional co-activators to allow for transcription of target genes (Figure 1.4) (153). Classical NOTCH targets include
**Figure 1.4. Schematic of NOTCH target gene expression.** In the absence of ligand binding the NOTCH receptor remains inactive and RBPJ exists in a complex with repressors such as N-Cor and histone deactylases (HDACs). Ligand binding leads to the cleavage and release of ICN which enters the nucleus and displaces repressors and recruits MAML and other coactivators.
genes like Hairy and Enhancer of Split (HES), which belongs to a family of basic helix-loop-lineage specific so it is important to validate both context dependent expression and relevance of target genes in any system. Though critical for epithelial differentiation, critical NOTCH targets have not been well-characterized for keratinocytes.

NOTCH has been implicated as an oncogene in a number of cancers, particularly hematopoietic cancers such as T-cell acute lymphoblastic leukemia (T-ALL). NOTCH is an oncogenic driver in these cancers as gain of function mutations are commonly observed in patients (154). In contrast, SCCs frequently harbor loss of function mutations in the NOTCH pathway implicating NOTCH activity as tumor suppressive in this context (121, 122, 155, 156). The tumor suppressive effects of NOTCH in the context of keratinocytes is largely thought to be due to differentiation induced block of cell proliferation and the activation of keratinocyte cell death (157).

1.6.3 TGF-beta signaling and regulation of keratinocytes

Similar to NOTCH, transforming growth factor (TGF) beta signaling is important pathway for many developmental and cellular processes. A simple model of the pathway consists of heterotetrameric kinase receptors, receptor associated SMAD (R-SMADs) proteins, and coactivator SMADs (co-SMADs). The receptor consists of one dimer of the ligand interacting TGF-beta type II receptor, and one dimer of TGF-beta type I receptor. Ligand binding results in a phosphorylation cascade from the type II receptor to the type I receptor to phosphorylation of the R-SMADs (158). R-SMADs can be placed into one of two groups dependent on the TGF-beta type I receptors substrate specificity. Activin/Nodal and TGF-beta type I receptors phosphorylate the R-SMAD pair SMAD2 and SMAD3, whereas the BMP type I
**Figure 1.5. Schematic of TGF-beta signaling.** Ligand binding to the TGF-beta receptor induces phosphorylation cascade resulting in phosphorylation of R-SMADs such as SMAD2 and SMAD3. Phosphorylated R-SMADs disassociate from the receptor and complex with SMAD4 to enter the nucleus. This complex then binds to SMAD binding elements to stimulate target gene transcription.
receptors activate the R-SMADs SMAD1, SMAD5, and SMAD8 (159). After phosphorylation, R-SMADs complex with co-SMADs, of which mammals only encode one SMAD4, and enter the nucleus, bind DNA, and activate target gene transcription (Figure 1.5). Similar to NOTCH, the R-SMAD pair SMAD2/3 can directly activate the transcription of the cell-cycle regulators CDKN1A and CDKN2B underlying the role of TGF-beta signaling in cell-cycle exit and growth arrest (159). Additionally, TGF-beta signaling induces the expression of factors involved in epithelial to mesenchymal transition (EMT) which results in morphological changes and increased production of matrix metalloproteases leading to enhanced migration and invasion (160, 161). Also like NOTCH loss of function mutations, TGF-beta loss of function mutations occur frequently in cancer (162-164), but conversely activated TGF-beta signaling is often seen in late in cancer progression (165, 166). This somewhat paradoxical finding can be resolved in a model where early inhibition of TGF-beta results in increased proliferation, but later progression is aided by the inflammation and invasive properties of TGF-beta signaling and EMT transition.
Chapter Two: HPV8 E6 inhibits NOTCH and affects epithelial differentiation

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Jordan M. Meyers performed all experiments except for the creation of the HPV8 E6 expressing keratinocyte cells.
Abstract

Cutaneous beta-HPV E6 proteins inhibit NOTCH signaling by associating with the transcriptional co-activator MAML1. NOTCH has tumor suppressor activities in epithelial cells and is activated during keratinocyte differentiation. Here we report that HPV8 E6 subverts NOTCH activation during keratinocyte differentiation by inhibiting RBPJ/MAML1 transcriptional activator complexes at NOTCH target DNA. NOTCH inhibition impairs epithelial differentiation and may thus contribute to beta-HPV replication and viral oncogenesis.
Results

Papillomaviridae are a large family of epitheliotropic viruses with double-stranded circular ~8kb DNA genomes. More than 150 human papillomaviruses (HPVs) have been isolated (167). Some HPVs infect mucosal epithelia whereas other HPVs infect cutaneous epithelial cells. HPV infections can be non-symptomatic or cause hyperplastic skin lesions, commonly referred to as warts. Mucosal alpha-HPVs have been studied in great detail since some of these viruses, the “high-risk” HPVs, are the causative agents of cervical carcinoma as well as other anogenital cancers and oropharyngeal carcinomas. Cutaneous beta-HPVs are less well studied. While beta-HPVs appear to be part of the normal “skin flora” and infections are often asymptomatic, they can cause cutaneous warts, which undergo malignant progression in patients suffering from the rare genetic disease epidermodysplasia verruciformis (EV) or in systemically immune suppressed organ transplant patients particularly in sun-exposed areas of the body (130, 168-170). Beta-HPVs may also contribute to non-melanoma skin cancers (NMSCs) in patients with a normal immune system but this has not been formally proven.

Cutaneous β-HPV E6 proteins have intrinsic transforming activities (71, 171) but do not associate with known cellular target proteins of high-risk mucosal beta-HPV E6 proteins, including p53, UBE3A and cellular PDZ proteins (172). Several groups have recently reported that the E6 protein from HPV5, HPV8, and other beta-HPVs can bind the NOTCH co-activator mastermind-like 1 (MAML1) thereby inhibiting NOTCH transcriptional programs (109, 118, 173). This is particularly interesting since NOTCH functions as a tumor suppressor in epithelial cells and mice with expression of a dominant negative MAML in basal epithelial cells develop squamous cell carcinoma (174). Since NOTCH signaling is important for cellular differentiation, we wanted to determine whether HPV8 E6 inhibits NOTCH signaling during
keratinocyte differentiation. To experimentally address this issue, we generated populations of hTERT-immortalized human keratinocytes (cl398) (175) with stable expression of amino-terminally HA/FLAG epitope tagged HPV8 E6 (8E6-iHFKs) by lentiviral transduction. Vector infected cells were generated as controls (C-iHFKs). To confirm the interaction of HPV8 E6 with components of the NOTCH transcriptional complex in these cells, we prepared lysates of 8E6-iHFKs and C-iHFKs in mammalian cell lysis buffer (MCLB, 50 mM Tris [pH 7.5], 150 mM NaCl, 0.5% NP-40) supplemented with one complete EDTA protease inhibitor cocktail tablet (Roche) per 50 ml and performed HA immunoprecipitations. As expected, MAML1 (#4608 Cell Signaling, 1:1000) and intracellular NOTCH (ICN, D3B8 Cell Signaling, 1:1000) were found in complex with HA/FLAG-tagged HPV8 E6 (Figure 2.1A). To confirm the significance of this interaction in iHFKs, we harvested total RNA and determined relative mRNA levels of the prototypical NOTCH target gene HES1 as well as MAML1 in comparison to GAPDH by first synthesizing cDNAs using the Quantitect Reverse Transcription kit (Qiagen) followed by quantitative PCR using the Quantitect SYBR Green PCR kit (Qiagen) using HES1, MAML1, and GAPDH qSTAR qPCR primer pairs (Origene). In agreement with published studies in other cell types, we observed a reduction in HES1 mRNA levels in 8E6-iHFKs compared to C-iHFKs (0.59 ± 0.11; P = 0.0001; Student’s t-Test) (Figure 2.1B) (109, 118, 173).

Next, we tested whether HPV8 E6 can interfere with NOTCH activation during epithelial differentiation. Calcium treatment of keratinocytes has been widely used to study keratinocyte differentiation (148, 176). Therefore we tested if calcium treatment of iHFKs resulted in a NOTCH activity profile similar to that of differentiating skin, namely, a gradual but robust increase of signaling during differentiation (148). 8E6-iHFKs and C-iHFKs were grown to confluence in standard keratinocyte serum free media (KFSM) supplemented with bovine
Figure 2.1. HPV8 E6 biochemically and functionally interact with the NOTCH signaling pathway in immortalized human foreskin keratinocytes (iHFK). (A) HPV8 E6 complexes with NOTCH transcription components in iHFK cells. Lysates from control and HPV8 E6 expressing iHFKs (C-iHFK and 8E6-iHFKs, respectively) were collected after 6 days calcium treatment. Input corresponds to 5% used for immunoprecipitations. HPV8 E6 immunoprecipitated with HA antibody associates with both MAML1 and intracellular NOTCH (ICN). (B) RNA isolated from C-iHFKs and 8E6-iHFKs grown in Keratinocyte serum free medium (KSFM) was analyzed for expression of MAML1 and HES by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Expression of the NOTCH target gene HES1 was lower in 8E6-iHFKs than in C-iHFKs (0.60 ± 0.09; \( P = 0.0001 \)) despite the fact that the NOTCH co-activator MAML1 was expressed at higher levels in 8E6-iHFKs than in C-iHFKs (1.38±0.12; \( P = 0.0006 \)). The graph depicts averages and standard deviations of 4 independent experiments, each performed in duplicate.
pituatory gland extract and epidermal growth factor (GIBCO) and then switched to calcium containing Dulbecco’s modified Eagle medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum. Total RNA was harvested with RNAeasy Plus Mini kit (Qiagen) at days 0, 3, 4, 5, and 6 post calcium treatment and relative mRNA levels of \textit{HES1} and \textit{MAML1} were determined as described in Figure 2.1B. In C-iHFKs, we observed a steady increase in \textit{HES1} and \textit{MAML1} transcripts over the course of the experiment (Figure 2.2A). In contrast, however, \textit{HES1} mRNA levels were lower in 8E6-iHFKs as compared to C-iHFKs even at late time points, while \textit{MAML1} transcript levels over the same period of time are only modestly decreased as compared to C-iHFKs (Figure 2.2B). If the differences in \textit{HES1} mRNA expression in C-iHFKs and 8E6-iHFKs were caused by HPV8 E6 mediated inhibition of NOTCH signaling, then chemical inhibition of NOTCH activation in C-iHFKs would similarly interfere with stimulation of \textit{HES1} mRNA expression during keratinocyte differentiation. To explore this possibility, we treated C-iHFKs with 2 µM Compound E (CpdE) (Millipore), a gamma secretase inhibitor (GSI). Gamma secretase cleaves membrane bound NOTCH and causes release of the intracellular NOTCH (ICN) fragment. ICN then relocalizes to the nucleus where it assembles with the DNA binding protein recombination signal binding protein for immunoglobulin kappa J (RBPJ), MAML family member coactivators, and other coactivators such as p300/CBP into a transcriptional activator complex. Vehicle (DMSO) treated C-iHFKs and 8E6-iHFKs were used as controls. Cells were calcium differentiated as before and total RNA collected at days 0, 3, and 6 and analyzed by qRT-PCR (Figure 2.2C). Both 8E6-iHFKs and C-iHFKs treated with GSI showed a similar reduction in \textit{HES1} mRNA levels at days 0 and 6 ($P = 0.0003$ and 0.0001 at day 0 and 0.031 and 0.049 at day 6 for GSI-treated C-iHFKs and 8E6-iHFKs, respectively). At day 3 only 8E6-iHFKs were significantly different from vehicle treated C-iHFKs ($P = 0.0026$).
Figure 2.2. Calcium induced differentiation of iHFKs activates NOTCH signaling, which is inhibited in HPV8 E6 expressing iHFKs. C-iHFKs (A) and 8E6-iHFKs (B) were grown to 80% confluence in KSFM and then switched to Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum. RNA was isolated at various times and HES1 and MAML1 mRNA levels were measured by qRT-PCR. HES1 and MAML1 mRNA levels dramatically increase upon calcium treatment of C-iHFKs, whereas there is only a slight increase in HES1 and MAML1 mRNA expression in 8E6-iHFKs. A representative experiment is shown; similar results were obtained in 2 additional experiments. Standard deviation is shown from technical replicates of the shown qRT-PCR experiment. (C) NOTCH inhibition by a gamma secretase inhibitor (2 μM CpdE - GSI) abrogates induction of HES1 mRNA expression similar to HPV8 E6 expression at both day 0 and day 6 after calcium addition. Cells were prepared similar to (A) and (B) except for CpdE and DMSO (vehicle) addition as shown. * indicates P<0.05 compared to C-iHFK (DMSO). Data represent averages and standard deviations from two independent experiments.
Figure 2.2 (Continued).
We next analyzed levels of proteins participating in NOTCH signaling during keratinocyte differentiation and determined whether NOTCH inhibition by GSI in C-iHFKs had similar effects as HPV8 E6 expression. Lysates were prepared in MCLB from 8E6-iHFKs and C-iHFKs treated with either DMSO or GSI at days 0, 3, 6, after calcium differentiation. In addition to MAML1 (4608 Cell Signaling, 1:1000), we examined levels of ICN1 (D3B8 Cell Signaling, 1:1000), the stem cell marker p63 (H-137 Santa Cruz, 1:1000), the NOTCH transcriptional target p21\(^{CIP1}\) (148), (DCS60 Cell Signaling, 1:2000), HDAC1 (10E2 Cell Signaling, 1:1000), and the epithelial differentiation marker involucrin (SY5 Thermo Scientific, 1:100) by immunoblot. Signals were quantified by densitometric analysis of X-ray film using ImageJ software. ICN1 levels were initially high in both DMSO treated C-iHFKs and 8E6-iHFKs and progressively decreased over time. GSI-treated C-iHFKs and 8E6-iHFKs showed no detectable ICN1 confirming that CpdE potently inhibited gamma secretase activity (Figure 2.3). MAML1 levels increased in C-iHFKs during calcium treatment but lower MAML1 levels were detected in 8E6-iHFKs and GSI-treated cells. In DMSO treated C-iHFKs, involucrin levels steadily increased during the time course of calcium differentiation. At each time point involucrin levels were similarly lower in DMSO treated 8E6-iHFKs and GSI treated C-iHFKs. GSI treatment of 8E6-iHFKs caused a further decrease of involucrin. These results suggest that HPV8 E6 impairs epithelial differentiation similar to inhibition of NOTCH processing but not necessarily by completely overlapping mechanisms. Levels of the p53 family member and epithelial stem cell marker p63 remained higher only in differentiating 8E6-iHFKs and were only modestly affected by GSI suggesting that p63 expression in differentiating 8E6-iHFKs may not be strictly NOTCH dependent. The two forms of p63 at day 0 may represent isoforms either lacking (\(\Delta Np63\)) or containing (Tap63) the transactivation domain (177). The levels of HDAC1,
Figure 2.3. Immunoblot analysis of NOTCH signaling components and bona fide NOTCH regulated genes. Protein extracts from C-iHFKs and 8E6-iHFKs treated with either DMSO or 2 µM CpdE (GSI) were prepared at indicated times from calcium differentiated C-HFKs and 8E6-iHFKs. Steady state levels of various NOTCH signaling associated proteins were quantified using ImageJ software. Data shown is representative of two independent experiments.
a corepressor found in repressive RBPJ complexes, were reduced in 8E6-iHFKs as well as cells treated with GSI. Lastly, the levels of p21^{CIP1}, which in previous studies was identified as a NOTCH-induced mediator of G1/S cell cycle arrest during keratinocyte differentiation (148), increased during early stages of C-iHFK differentiation. Overall levels of p21^{CIP1} were decreased in 8E6-iHFKs but not in cells treated with GSI. This indicates that HPV8 E6 may be able to retard cell cycle exit of differentiating epithelial cells but that the increase in p21^{CIP1} during keratinocyte differentiation is largely independent of NOTCH signaling. These results suggest that inhibition of NOTCH signaling by HPV8 E6 expression or GSI treatment has overlapping yet distinct effects on NOTCH pathway protein expression.

To more globally explore HPV8 E6 mediated inhibition of NOTCH signaling during keratinocyte differentiation and differences between HPV8 E6 and GSI-mediated NOTCH inhibition, we utilized a qRT-PCR based NOTCH pathway array (SABiosciences). The array analyzes the transcript levels of 84 genes associated with NOTCH signaling. Total RNA was prepared from untreated C-iHFKs, 6 days calcium differentiated C-iHFKs, 6 days calcium differentiated GSI-treated C-iHFKs, and 6 days calcium differentiated 8E6-iHFKs. Relative mRNA levels were determined by comparing levels detected in calcium differentiated 8E6-iHFKs (Figure 2.4A) and in calcium differentiated GSI-treated C-iHFKs (Figure 2.4B) to those in calcium differentiated C-iHFKs. We focused this analysis on genes represented in the Kyoto Encyclopedia of Genes and Genomes (KEGG) NOTCH signaling pathway and a few additional NOTCH pathway genes (Figure 2.4). Comparing HPV8 E6 NOTCH inhibition to C-iHFKs with GSI-mediated NOTCH inhibition, we detected decreases in expression of direct NOTCH target genes consistent with inhibition of NOTCH signaling by both HPV8 E6 and GSI treatment. As we had observed in our analysis of NOTCH pathway protein expression (Figure 2.3), HPV8 E6
Figure 2.4. HPV8 E6 induced NOTCH pathway changes. Comparative analysis of NOTCH signaling pathway components in calcium differentiated 8E6-iHFKs (A) and GSI-treated C-HFKs (B) by qRT-PCR. 8E6-iHFKs grown in DMSO and C-iHFKs grown in either DMSO or CpdE (2 µM) for 6 days in calcium differentiation medium and total RNA was collected and analyzed using the Human NOTCH Signaling Pathway PCR Array (SABiosciences). Genes corresponding to those identified in the KEGG NOTCH pathway are displayed with relative mRNA levels as compared to differentiated C-iHFKs with DMSO are indicated using the color scheme shown at the bottom of the figure. Results represent averages from duplicate PCR arrays.
Figure 2.4 (Continued).
expression and GSI treatment have overlapping but not identical effects on NOTCH pathway gene expression (Figure 2.4; Table 3.1). This suggests that inhibition of NOTCH signaling by HPV8 E6 through MAML co-activator binding is different than global inhibition of NOTCH activation at the level of NOTCH receptor processing.

Hence, we sought to elucidate the mechanism by which HPV8 E6 may be functioning to impair NOTCH signaling. Previous work has shown that HPV8 E6 binds to an LXXLL motif within the carboxyl terminal region of the poorly characterized MAML1 transactivation domain 2 (TAD2) (118, 173). However, the mechanism by which HPV8 E6 inhibits NOTCH signaling through this interaction is unknown. One possibility may be that E6 binding displaces MAML1 from DNA bound RBPJ. In this case, HPV8 E6 expression, similar to GSI treatment, would prevent formation of the MAML1/p300 containing transcriptional activator complex on target DNA bound RBPJ and NOTCH target genes would remain in a repressed state. Alternatively, E6 may inhibit binding of one or more proteins necessary for MAML1 coactivator activity and would inhibit or inactivate the DNA bound RBPJ/MAML1/p300 transcriptional activator complex (Figure 2.5A). To distinguish between these two possibilities we performed Chromatin immunoprecipitation (ChIP). Briefly, we grew cells as described below and investigated binding of HA/FLAG-tagged HPV8 E6 to functional RBPJ binding sites of the HES1 gene using EZChIP (Millipore). The HES1 promoter has two well-characterized RBPJ binding sites adjacent to the promoter that are required for NOTCH mediated transcription (178). An additional RBPJ binding site important for HES1 expression in response to NOTCH activation has also been described approximately 5000 base pairs upstream of the promoter (179). These sites in the HES1 enhancer (Forward: 5’ CCTCCCAGGATAGCTCTTG 3’, Reverse: 5’ TTTGCCTGAGGACTTTTG 3’) and promoter (Forward: 5’
Table 2.1: Effects of calcium differentiation on NOTCH pathway components. To determine changes induced by differentiation NOTCH pathway transcript levels obtained from PCR arrays in Figure 4 were compared to transcript levels from C-iHFKs treated with DMSO but no calcium (Day 0). Genes involved in NOTCH signaling occurring at the cell surface such as ligands, receptors, gamma secretase complex components are distinguished from genes involved “nuclear” signaling such as coactivators and direct NOTCH transcriptional targets.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>C-iHFK with DMSO</th>
<th>C-iHFK with GSI</th>
<th>8E6-iHFK with DMSO</th>
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Table 2.1 (Continued).
Figure 2.5. HPV8 E6 accesses regulatory regions of NOTCH target genes and is dependent in part on ICN. (A) Schematic of the different transcriptional states of NOTCH target genes (NTG). In the repressed state, NOTCH responsive elements are occupied by RBPJ bound to co-repressors (left panel) and in the active state ICN is bound to RBPJ, a MAML family members and other co-activators such as p300/CBP (middle panel). Association of HPV8 E6 to MAML may prevent formation of the activator complex, or bind to the activator complex and interfere with its transcriptional activation activity (right panel). The results shown in the figure are consistent with the second model. (B) Schematic of RBPJ binding sites in the HES1 regulatory region. Primers amplify tandem RBPJ binding sites in a promoter proximal region (P) as well as a single RBPJ binding site upstream of the promoter (E). (C) C-iHFKs and 8E6-iHFKs were harvested and fixed with formaldehyde. Sonicated chromatin was immunoprecipitated with anti-HA antibody (Abcam) and after reversing cross-links purified DNA was analyzed by qPCR. HA signal is represented as fold increase over control IgG antibody with (P) and (E) primer sets. (D) ChIP was performed on 8E6-iHFKs treated with either DMSO or GSI (2 µM CpdE) and analyzed by qPCR. HA signal decreases with GSI treatment along with modest reductions in Pol II and RBPJ. Results shown are representative of two independent experiments.
Figure 2.5 (Continued).
were assessed using specific primer pairs (Figure 2.5B). The HA signal was higher at both the HES1 enhancer and promoter sites in 8E6-iHFKs compared to C-iHFKs (Figure 2.5C). This suggested that HPV8 E6 is can bind to NOTCH responsive RBPJ target sites. MAML binding to RBPJ requires a composite ICN/RBPJ binding groove and thus cannot bind either of the factors independent of the other (18). It has been reported that HPV8 E6 binds to MAML directly and subsequently indirectly to RBPJ and ICN (116). To address whether HPV8 E6 requires ICN to access DNA, we performed ChIP on 8E6-iHFKs with or without GSI (2 µM CpdE) treatment for occupancy of HA/FLAG-tagged HPV8 E6, RBPJ, and Pol II. As expected, GSI treatment reduced occupancy of HPV8 E6 at both regulatory sites (Figure 2.5D). The apparent residual binding of HPV8 E6 to RBPJ in GSI-treated cells may either reflect unspecific DNA binding by the HA antibody, the presence of ICN below the level of detection by Western blotting, or that HPV8 E6 may interact with RBPJ through MAML independent mechanisms, such as association with p300/CBP (180). GSI treatment of 8E6-iHFKs also reduced the signal of RBPJ and Pol II at both HES1 sites (Figure 2.5D).

In summary, we report that HPV8 E6 inhibits NOTCH activation during keratinocyte differentiation. This may provide a mechanistic explanation of earlier studies in organotypic raft cultures that showed that HPV8 E6 expression causes hyperproliferation and delayed differentiation (116). Moreover, HPV8 E6 perturbs NOTCH target gene expression through a mechanism causing inhibition of RBPJ/MAML1 transcriptional activator complexes at NOTCH target DNA. This is distinct from the effects of GSIs, which inhibit ICN production and hence cause persistent formation of DNA bound RBPJ transcriptional repressor complexes (Figure 5A). These mechanistic differences may account for the differences that we have observed in
expression NOTCH target genes in calcium-differentiated HPV8 E6 expressing and GSI-treated HFKs. Further studies will be focused on a detailed analysis of the mechanism of NOTCH inhibition by beta-HPV-encoded E6 proteins and the biological implications of inhibition.
Acknowledgements

We thank Dr. Al Klingelhutz for his generous gift of the hTERT immortalized human keratinocytes (cl398), Dr. Jon Aster for reagents and advice, Drs. Bo Zhao and Daniel Portal for their help with chromatin immunoprecipitations, and Miranda Grace for technical assistance. This work was supported by Public Health Service grants CA081135, CA066980 and CA141583 from the National Cancer Institute/National Institutes of Health.
Chapter Three: Cutaneous papillomaviruses inhibit the NOTCH and TGF-beta tumor suppressor pathways and effect keratinocyte differentiation and survival

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Jordan M. Meyers performed all experiments except for the animal experiments performed by Aayushi Uberoi.
Abstract

Cutaneous beta papillomaviruses are associated with non-melanoma skin cancers that arise in patients who suffer from a rare genetic disorder, Epidermodysplasia verruciformis (EV) or after immunosuppression following organ transplantation. Recent studies have shown that the E6 proteins of the cancer associated beta-human papillomavirus (HPV) 5 and HPV8 inhibit NOTCH and TGF-beta signaling. However, it is unclear whether disruption of these pathways may contribute to cutaneous HPV pathogenesis and carcinogenesis. A recently identified papillomavirus, MmuPV1, infects laboratory mouse strains and causes cutaneous skin warts that can progress to squamous cell carcinoma. To determine whether MmuPV1 may be an appropriate model to mechanistically dissect the molecular contributions of cutaneous HPV infections to skin carcinogenesis, we investigated whether MmuPV1 E6 shares biological and biochemical activities with HPV8 E6. We report that the HPV8 and MmuPV1 E6 proteins share the ability to bind to the MAML1 and SMAD2/SMAD3 transcriptional cofactors of NOTCH and TGF-beta signaling, respectively. Moreover, we demonstrate that these cutaneous papillomavirus E6 proteins inhibit these two tumor suppressor pathways and that this ability is linked to delayed differentiation and sustained proliferation of differentiating keratinocytes. Furthermore, we demonstrate that the ability of MmuPV1 E6 to bind MAML1 is necessary for papilloma formation in experimentally infected mice. Our results, therefore, suggest that experimental MmuPV1 infection in mice will be a robust and useful experimental system to model key aspects of cutaneous HPV infection, pathogenesis and carcinogenesis.
Author Summary

The lack of a genetically tractable small animal model to study viral infection and pathogenesis has significantly hindered papillomavirus research. The recent discovery of Mus musculus papillomavirus 1 (MmuPV1), which can replicate and form skin warts and cancers in experimentally infected laboratory mouse strains, has the potential to be transformative to the field. However, it is important to determine whether MmuPV1 targets some of the same cellular signaling pathways as the skin cancer-associated human papillomaviruses (HPVs). We show that MmuPV1 E6 shares with the skin cancer-associated HPV8 E6 protein the capacity to inhibit NOTCH and TGF-beta signaling. Importantly, MmuPV1 E6 expression and specifically the ability of E6 to inhibit NOTCH signaling are necessary for wart formation in mice. Hence, MmuPV1 will be an excellent animal model to study key aspects of the life cycle and pathogenesis of skin cancer-associated HPVs.
Papillomaviruses (PVs) represent a large, diverse group of DNA viruses that infect squamous epithelia of many animals. Genetically, these viruses are grouped into genera based on diversity of their major capsid protein. Biologically, PVs can be stratified according to the type of epithelium that they can productively infect, either mucosal or cutaneous tissue. Infections with cutaneous human PVs (HPVs) is associated with a wide range of pathologies from asymptomatic infection to benign warts, actinic keratosis to squamous cell carcinomas (181). Two HPV types in particular, beta-HPV5 and beta-HPV8, were found to be associated with lesions and tumors in patients suffering from a rare hereditary disease, epidermodysplasia verruciformis (EV) (59). A majority of these patients harbor genetic mutations in TMC6 (EVER1) or TMC8 (EVER2) genes on chromosome 17, which encode putative transmembrane channel proteins that may be involved in cellular zinc and calcium homeostasis (61, 182). HPV-associated warts in EV patients have a high risk for progression to squamous cell carcinomas (SCC), and these tumors are positive for viral DNA (183). Further studies provided evidence that SCCs that arise in immunosuppressed individuals such as organ transplant patients are also associated with cutaneous HPV infections (184, 185). HPV-associated SCCs often arise in sun-exposed areas of the skin, implicating UV exposure as a key risk factor for malignant progression (186). However, beta-HPV sequences are not maintained in every cancer cell. The association of cutaneous HPV infections with SCC in immunocompetent patients is less clear. One study showed a positive serological connection between HPV8 and SCC (187), but other studies have not shown a link (188). This has led to a gradated model of beta-HPV association with human skin cancers. Cancer development is highly associated in the case of EV patients, correlated in the case of immune suppression, and only slightly or sporadically associated in
immune competent patients. However, in transgenic mouse models skin restricted expression of the beta-HPV oncogenes E6 and E7 are capable of tumorigenesis suggesting that oncogene expression may play an important role (70, 71, 189).

In order to assess HPV contributions to skin cancer, it is important to define the effects of cutaneous HPVs on host cell pathways. UV exposure is an important risk factor for skin cancer, and several reports suggest that cells expressing cutaneous HPV E6 proteins can tolerate or survive UV exposure and UV-induced DNA damage better than normal cells. It has been shown that HPV5 E6 can inhibit apoptotic cell death in response to UV through degradation of the pro-apoptotic BCL2 family member, BAK1 (112, 190). Moreover, activation of the ATM/ATR kinases that play an important role in UV induced DNA damage signaling is also inhibited in cutaneous HPV E6 expressing keratinocytes, and this has been linked to E6 mediated EP300/CREBBP degradation (114, 191).

Beyond modulation of the cellular UV response, cutaneous HPVs have additional oncogenic activities, and our group along with several others discovered that HPV8 E6 can bind to MAML1 and inhibit NOTCH signaling (117-119). NOTCH is an important driver of keratinocyte differentiation, and defects in the pathway are highly associated with cutaneous SCCs (155, 156). Additionally, HPV5 E6 was shown to interact with SMAD3 causing its destabilization and subsequently inhibiting TGF-beta signaling (124). SMAD2 and SMAD3 are TGF-beta receptor associated factors that are activated by phosphorylation upon TGF-b1 ligand binding and translocate to the nucleus. They form complexes with non-receptor associated SMADs and transcriptional co-activators to bind and activate expression of TGF-beta target genes. TGF-beta also plays an important, albeit complicated role in skin carcinogenesis, as it has both tumor suppressive and tumor promoting activities; loss of cytostatic TGF-beta signaling is
important during the early phase of carcinogenic progression, whereas TGF-beta signaling may drive late stage carcinogenic events including invasion and metastasis through activation of the epithelial to mesenchymal transition (EMT) (192, 193).

In addition to understanding the effects of beta-HPV oncogenes on host pathways, it would be beneficial to have a robust model system to study viral pathogenesis and oncogenesis. One principal hurdle in investigating HPV pathogenesis and oncogenesis has been the difficulty to study these viruses in appropriate experimental model systems. The exquisite host specificity of papillomaviruses has precluded experimental infections of heterologous hosts and the viral life cycle cannot be fully studied in conventional tissue culture experiments. Organotypic “raft” culture systems have been developed to study aspects of the productive life cycle of high-risk mucosotropic HPVs (141), but cutaneous HPVs such as HPV5 and HPV8 have not been studied in this system. Moreover, raft cultures do not faithfully recapitulate the steady state physiology of the skin, and thus they cannot be used to examine long-term persistent HPV infections. To circumvent some of these issues, researchers has resorted to growing HPV genome expressing human keratinocytes in implantation chambers on the backs of immunodeficient mice (194) or culturing them in the renal capsule (195). The recent isolation of a mouse PV, MmuPV1, from warts developed in a colony of NMRI-Foxn1nu/Foxn1nu (nude) mice (196) has provided an important breakthrough and now allows PV pathogenesis studies in a genetically tractable animal model. Infectious MmuPV1 quasivirions that are synthesized in vitro can be used for experimental infections that result in warts (197). Alternatively, circularized viral genomes directly applied to scarified skin regions also lead to wart formation (198). Recent reports have shown that lesions arising due to MmuPV1 infection have malignant potential and in some cases, can progress to SCC (198). Moreover, experimental MmuPV1 infection causes papillomas
associated with SCC in UVB-irradiated immunocompetent strains of mice. Importantly, these studies showed that MmuPV1 mediated tumor formation was a consequence of UV-induced immunosuppression (199). Given these biological similarities to cutaneous HPVs, there is an exciting probability that MmuPV1 is a biologically relevant animal papillomavirus model that will be useful in determining whether and how cutaneous HPVs mechanistically contribute to skin carcinogenesis. Therefore, we hypothesized that MmuPV1 gene products must share biochemical properties and biological activities with those of cutaneous HPV gene products. The goal of this study was to identify host cell signal transduction pathways targeted by the MmuPV1 E6 protein, to compare and contrast them with those targeted by HPV8 E6, to determine their effects on proliferation and survival of terminally differentiated keratinocytes, and to assess their role in wart formation in mouse infection models.
Results

HPV8 E6 and MmuPV1 share the ability to bind MAML1 and SMAD2/3.

MmuPV1 infections cause skin warts (196-198) that can progress to cancers in conjunction with UV irradiation (199). To determine whether MmuPV1 may be used to model pathogenesis and carcinogenesis of human cutaneous HPVs, we investigated whether it targets similar cellular signaling pathways as cutaneous HPV5 and 8. We focused on the MmuPV1 E6 protein, because there are marked differences between the protein interactomes of mucosal versus cutaneous HPV E6 proteins (104, 110). Mucosal HPV16 E6 proteins interact with the LXXLL (L, leucine; X any amino acid) domain containing ubiquitin ligase UBE3A, the TP53 tumor suppressor, and cellular proteins containing a PDZ (post synaptic density protein-PSD95, Drosophila disc large tumor suppressor-Dlg1, and zonula occludens-1 protein-zo-1) domain (109, 110, 200). In contrast, cutaneous HPV5 and HPV8 E6 proteins interact with the LXXLL domain protein, MAML1, as well as SMAD3, which lacks an LXXLL motif, and they do not bind to PDZ domain proteins because these E6 proteins lack the appropriate C-terminal binding site (109, 117, 118, 124, 180). To determine whether MmuPV1 E6 interacts with cutaneous HPV5 and HPV8 specific cellular interactors, we infected normal human oral keratinocytes (NOKs) with lentiviral vectors expressing MmuPV1 FLAG/HA-E6, HPV8 FLAG/HA-E6 as a positive control, or GFP as a negative control. Lysates from cell populations with stable expression of the corresponding epitope tagged E6 proteins or GFP were then subject to HA immunoprecipitation followed by immunoblot. These experiments show that similar what we previously observed with HPV8 in immortalized foreskin keratinocytes, HPV8 and the MmuPV1 E6 protein interact with MAML1 as well as with intracellular cleaved NOTCH1 (ICN1) (Figure 3.1A). Like HPV8 E6, MmuPV1 E6 binds SMAD2 and SMAD3. MmuPV1 E6 preferentially
Figure 3.1. HPV8 and MmuPV1 E6 interact with components of NOTCH and TGF-beta signaling pathway and inhibit these pathways. (A) Whole cell extracts (WCE) from human keratinocytes stably expressing FLAG/HA-tagged HPV8 E6 (8), MmuPV1 E6 (M), or GFP (C) were subjected to HA immunoprecipitation (IP) and analyzed for associated EP300, MAML1 and ICN1 (three components of NOTCH transcriptional complex) and SMAD2 and SMAD3 (receptor SMADs of TGF-beta signaling) as well as immunoprecipitated E6 by immunoblot. Expression of the various proteins was assessed by immunoblotting of 1% input of WCE. GAPDH expression is shown as a loading control. (B) Effects of HPV8 and MmuPV1 E6 on SMAD responsive reporter activity using either TGF-b1 (10 ng/ml) or constitutively active TGF-beta Receptor 1 (TGFBR1-T204D) as agonists in U2OS cells. (C) Effects of HPV8 and MmuPV1 E6 on NOTCH responsive reporter activity using ICN1 as an agonist in U2OS cells. Data shown is from a representative experiment from three independent experiments. P-values were calculated with unpaired t-test with Welch’s correction; * = P \leq 0.05, ** = P \leq 0.01. (D) Expression level of HES1 transcripts in GFP-control, GSI treated GFP-control, HPV8 E6, and MmuPV1 E6 expressing iHFKs as measured by RT-qPCR.
Figure 3.1 (Continued).
interacts with SMAD2 whereas HPV8 E6 preferentially interacts with SMAD3. Unlike what had been observed with HPV5 E6(124), we did not observe any differences in steady state levels of SMAD2 or SMAD3 in HPV8 E6 or MmuPV1 E6 expressing cells. MmuPV1 E6, unlike HPV8 E6, does not detectably interact with EP300/CREBBP. These results suggest that MmuPV1 E6 does not modulate EP300 activities as has been reported for HPV8 E6 (114, 115, 201) but that MmuPV1 and HPV8 E6 share the capacity to associate with components of the NOTCH and TGF-beta tumor suppressor pathways.

**HPV8 and MmuPV1 E6 share the ability to inhibit NOTCH and TGF-beta transcriptional activity.**

It was previously shown that HPV8 can inhibit NOTCH signaling (109, 117-119) and the highly-related HPV5 E6 protein was shown to inhibit TGF-beta signaling (124). When active, both of these pathways are known tumor suppressors in the skin (156, 193). Hence, we determined whether MmuPV1 E6 inhibited these two tumor suppressor pathways. To assess the effect of HPV8 and MmuPV1 E6 on TGF-beta signaling, we performed luciferase assays in U2OS cells using a SMAD responsive luciferase reporter that can monitor transcriptional activity of SMAD2 and SMAD3 after TGF-beta stimulation. HPV8 E6 and MmuPV1 E6 expression vectors or empty vector were cotransfected with a SMAD3 expression plasmid. Signaling was activated by adding exogenous TGF-b1, co-transfection of a vector expressing a constitutively active mutant of the TGF-beta receptor 1 (TGFBRI-T204D), or both. Both TGF-b1 treatment and expression of TGFBRI-T204D led to greater than 20-fold increases (23.6±2.7, 20.6±1.9, respectively) in reporter activity as compared to control transfected cells (Figure 3.1B). Co-transfection of HPV8 or MmuPV1 E6 expression plasmids significantly inhibited the ability to activate this response. To rule out any effects of epitope tags, N-terminally-tagged and untagged
constructs of HPV8 E6 and MmuPV1 E6 were assessed side by side and no difference was observed in their ability to inhibit NOTCH and TGF-beta reporter induction (Figure 3.2A and 3.2B). Additionally, we repeated these reporter experiments in iHFKs and we obtained similar results as previous obtained with U2OS cells (Figure 3.2C). These results indicate that although we do not observe destabilization of SMAD2 or SMAD3 (Figure 3.1A), we do observe inhibition of TGF-beta activity by HPV8 E6 and show that cutaneous MmuPV1 E6 shares this ability.

To investigate inhibition of NOTCH signaling we co-transfected a NOTCH responsive luciferase reporter with an expression plasmid encoding the active, cleaved NOTCH fragment (ICN1) and HPV8 E6, MmuPV1 E6 expression vectors, or empty vector into U2OS cells. ICN1 transfection in combination with empty vector yielded a 69.4 (±2.3)-fold increase in NOTCH reporter activity compared to cells transfected with the reporter alone (Figure 3.1C). Cotransfection of HPV8 E6 inhibited ICN induced reporter activity (10.1±0.4 fold). Cotransfection of MmuPV1 E6 similarly inhibited ICN induced reporter activation (10.15±0.3 fold). Similar to the TGF-beta reporter studies, we tested the effect of HPV8 E6 and MmuPV1 E6 on NOTCH inhibition in iHFKs. Similar to what we observed with U2OS cells, ICN1 transfection alone resulted in an increase in reporter activity (46.7±2.7 fold), which was inhibited upon HPV8 E6 or MmuPV1 E6 cotransfection (3.6±0.1 and 5.0±0.1 respectively) (Figure 3.2D). To confirm the ability of HPV8 E6 and MmuPV1 E6 to block expression of direct NOTCH transcriptional targets we assayed the mRNA levels of HES1 a canonical NOTCH regulated target. Similar to the gamma secretase inhibitor compound E (GSI) treated control cells, HPV8 E6, or MmuPV1 E6 expressing cells showed reduced HES1 mRNA compared to untreated control cells. This demonstrates the ability of HPV8 E6 and MmuPV1 E6 to inhibit expression of endogenous NOTCH target genes (Figure 3.1D). Since ICN1 transfection bypasses regulatory steps at the
Figure 3.2. Inhibitory effects of tagged and untagged E6 and HPV8 E6 and MmuPV1 E6 in iHFKs on NOTCH and TGF-beta reporter activity. Effects of N-terminally tagged and untagged HPV8 E6 and MmuPV1 E6 on TGF-beta (A) and NOTCH (B) reporter activity in U2OS cells. (C) The activity of SMAD responsive promoter when induced by the constitutively active receptor TGFBR1 T204D in iHFK cells. (D) The activity of the NOTCH responsive promoter when induced by ICN in iHFK cells.
membrane such as ligand binding and NOTCH cleavage, we conclude that, similar to what we have shown for HPV8 E6, MmuPV1 E6 inhibits NOTCH signaling downstream of early receptor proximal events, presumably through association with MAML1 in the nucleus.

These results demonstrate that MmuPV1 E6 can inhibit NOTCH and TGF-beta signaling and that this may be a conserved function of skin cancer-associated PVs including HPV5 and HPV8 (117, 118, 124). We do not observe SMAD destabilization but show evidence that inhibition of these pathways is downstream of ligand binding or other receptor proximal events.

**HPV8 E6 and MmuPV1 E6 abrogate TGF-beta induced inhibition of keratinocyte growth.**

TGF-beta induces cell-cycle arrest in keratinocytes (202). Since HPV8 and MmuPV1 E6 can inhibit TGF-beta signaling in a reporter assay (Figure 3.1B), we predicted that HPV8 and MmuPV1 E6 could modify the TGF-beta induced growth arrest response in human keratinocytes. We treated iHFKs stably expressing either HPV8 E6 or MmuPV1 E6 or GFP with TGF-b1 (10 ng/ml). Cell proliferation/viability was measured every 24 hours for 5 days using resazurin, a redox-sensitive dye that interrogates redox fitness of cells reduction as a proliferation/viability indicator. As expected, control iHFKs treated with TGF-b1 showed a significant decrease in proliferation/viability compared with untreated cells (p-value = 0.313) (Figure 3.3A, top left). Concurrent treatment with the TGF-beta receptor 1 (TGFBRI) inhibitor SB-431542 (TGF1) abrogates TGF-beta growth inhibition (p-value = 0.1563). As expected, treatment with compound E (GSI), a NOTCH inhibitor, did not rescue TGF-beta-induced growth arrest (Figure 3.3A, top right). In contrast, iHFKs expressing either HPV8 E6 (Figure 3.3A
Figure 3.3. HPV8 and MmuPV1 E6 inhibit TGF-beta signaling and activity. (A)
Proliferation/viability of GFP-control, HPV8 E6, and MmuPV1 E6 expression iHFKs after TGF-
 b1 (10 ng/ml) and TGF-beta inhibitor (SB-431542) treatment over five days as measured by
resazurin reduction. P-values (see text) were calculated using Wilcoxon matched-pairs test. (B)
Accumulation of phosphorylated SMAD2/3 (pSMAD2/3) and expression of CDKN1A (p21^{CIP})
following TGF-b1 treatment in iHFKs by immunoblot. (C) Expression level of CDKN2B
(p15^{INK4B}) transcripts in GFP-control, HPV8 E6, and MmuPV1 E6 expressing iHFKs after TGF-
b1 treatment as measured by RT-qPCR. P-values were calculated with unpaired t-test with
Welch’s correction where \( * = P \leq 0.05 , ** = P \leq 0.01 , *** = P \leq 0.001 \).
Figure 3.3 (Continued).
bottom left) or MmuPV1 E6 (Figure 3.3A, bottom right) are largely resistant to TGF-beta growth inhibition and proliferate similarly to untreated control iHFKs (p-values = 0.8438 and 0.4375, respectively). These results indicate that HPV8 and MmuPV1 E6 render keratinocytes insensitive to TGF-beta induced growth arrest and this ability correlates with their capacity to interact with SMADs (Figure 3.1A).

**HPV8 E6 and MmuPV1 E6 abrogate TGF-beta-induced transcriptional responses.**

Since HPV8 and MmuPV1 E6 expression inhibits reporter assay activity, we hypothesized that expression of these proteins would inhibit endogenous TGF-beta responsive transcriptional targets. To test this, we treated GFP expressing iHFKs with TGF-b1 and monitored phosphorylation of SMAD2 and SMAD3 over a time course of 72 hours by immunoblot. Increase of phosphorylation as well as expression of CDKN1A, a TGF-beta target gene, was detected within minutes of treatment and increased to maximal signal by two hours (Figure 3.3B). Consequently, we analyzed TGF-beta transcriptional responses at two hours after TGF-b1 treatment for the remainder of our studies. One important transcriptional target of TGF-beta is CDKN2B (p15\(^{INK4B}\)), a CDK4/CDK6 inhibitor, that blocks G1 progression by inhibiting cyclin D binding. We first verified that CDKN2B expression is dynamically regulated in iHFKs. Cells were treated with TGF-b1, and RNA was harvested two hours after treatment. Expression of CDKN2B was assessed by RT-qPCR. TGF-b1 treatment increased abundance of CDKN2B mRNA in GFP control iHFKs, which was blocked by the (Figure 3.3C) TGF-beta inhibitor SB-431542 (TGF1). Similar to inhibitor treated cells, HPV8 and MmuPV1 E6 iHFKs also failed to induce CDKN2B expression after TGF-b1 treatment. As expected based on our previous reporter assays, HPV8 and MmuPV1 E6 can inhibit the expression of critical TGF-beta targets genes following stimulation by TGF-b1.
**HPV8 E6 and MmuPV1 E6 do not interfere with TGF-beta mediated SMAD2/3 phosphorylation and nuclear translocation.**

We sought to better understand the step at which E6 inhibits TGF-beta signaling. Normally, receptor-mediated phosphorylation of SMAD2 and SMAD3 leads to their translocation to the nucleus. In the nucleus SMAD2 and SMAD3 complex with SMAD4, a TGF-beta coactivator. Contrary to previous studies with HPV5 E6 (124), we did not observe consistent changes to SMAD2 or SMAD3 steady state levels in HPV8 E6 or MmuPV1 E6 expressing iHFKs (Figure 3.1A). We hypothesized that E6 may block phosphorylation or nuclear translocation of SMADs to inhibit TGF-beta signaling. We then examined phosphorylation of SMAD2/3 during TGF-b1 treatment and its subsequent nuclear translocation. To do so, we treated iHFKs with TGF-b1 and harvested the cells 2 hours post treatment, prepared nuclear and cytosolic fractions and performed immunoblot analyses. We observed similar levels of phosphorylated SMAD2 and SMAD3 in the cytosolic and nuclear fractions of HPV8 and MmuPV1 expressing cells as in control iHFKs (Figure 3.4A). As expected, treatment with the TGF-beta inhibitor SB-431542 abrogated phosphorylation and nuclear translocation of SMAD2 and SMAD3. These results indicate that E6 inhibition of TGF-beta is downstream of nuclear translocation of phosphorylated SMADs.

**HPV8 E6 and MmuPV1 E6 interfere with TGF-beta nuclear complex assembly.**

Since E6 does not prevent phosphorylated SMADs from entering the nucleus, we predicted it may prevent transcriptional complex formation. After phosphorylation and transport into the nucleus, SMAD2 and SMAD3 proteins form complexes with SMAD4 and assemble at
Figure 3.4. Effects of HPV8 and MmuPV1 E6 on TGF-beta nuclear events. (A)

Immunoblot analysis of TGF-beta signaling components following TGF-b1 treatment in cytoplasmic and nuclear fractions of GFP (C), HPV8 E6 (8E6) or MmuPV1 E6 (ME6) expressing iHFKs. Data shown is representative of three independent fractionation experiments.

(B) Occupancy of SMAD2 and SMAD4 at the TGF-beta regulatory site of the CDKN2B gene in GFP-control, HPV8 E6, and MmuPV1 E6 iHFKs as measured by ChIP-qPCR. Data shown is displayed as percent input after subtraction of isotype matched IgG signal. Similar results were obtained from two independent experiments. P-values were calculated with unpaired t-test with Welch’s correction where * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001.
Figure 3.4 (Continued).
regulatory sites to induce transcription of target genes. The CDKN2B promoter contains a well-defined SMAD binding element (SBE) (203). We determined whether HPV8 or MmuPV1 E6 disrupt SMAD association with DNA, thereby blocking transcriptional activity. We treated iHFKs with TGF-b1, performed chromatin immunoprecipitations (ChIPs) and measured occupancy of SMAD2 and SMAD4 at the SBE of the CDKN2B promoter. As expected, there was an increase in occupancy of both SMAD2 and SMAD4 after TGF-b1 treatment of control iHFKs (Figure 3.4B). In contrast, HPV8 E6 and MmuPV1 E6 expressing iHFKs showed reduced SMAD2 and SMAD4 occupancy at the CDKN2B promoter even after TGF-b1 treatment. Surprisingly, HPV8 and MmuPV1 E6 were both detected at the SBE of the CDKN2B promoter.

Given that HPV8 and MmuPV1 E6 proteins bind to SMAD2/3 and are not known to directly bind DNA, it is conceivable that E6 binding may interfere with binding of the SMAD2 antibody used for the ChIP assays and occlude the SMAD4 binding site. Another possibility is that E6 may prevent stable SMAD complexes from forming leading to decreased signal of both SMAD2 and SMAD4.

Since we had not been able to observe interaction of either HPV8 E6 or MmuPV1 E6 with SMAD4 by immunoprecipitation (Figure 3.5), we hypothesized that one way that E6 may prevent stable SMAD complexes from forming would be that E6 may prevent SMAD4 from interacting with phosphorylated SMAD2/3. To test whether E6 binding to SMAD2/3 precludes SMAD4 binding, we transfected U2OS cells with plasmids encoding a FLAG-tagged SMAD2 and plasmids encoding untagged versions of either HPV8 E6 or MmuPV1 E6. Two days after transfection we treated these cells with TGF-b1 and prepared lysates at two hours after treatment. After immunoprecipititating SMAD2 using FLAG antibodies we analyzed co-precipitated SMAD4 by immunoblot analysis. We observed that SMAD4 association with SMAD2
Figure 3.5. Characterization of HPV8 E6 and MmuPV1 E6 interactors. WCE of TGF-β1 treated iHFKs expressing GFP, HPV8 E6, or MmuPV1 E6 were immunoprecipitated with HA antibody beads and analyzed for association with p300, SMAD2, SMAD3, pSMAD2, pSMAD3, and SMAD4.
following TGF-β1 treatment was decreased upon HPV8 or MmuPV1 E6 co-transfection (Figure 3.6A). Similarly, formation of the SMAD3/SMAD4 complex was also inhibited upon HPV8 or MmuPV1 E6 expression (Figure 3.6B). These data support a model whereby HPV8 and MmuPV1 E6 inhibit TGF-beta signaling by disrupting transcription factor complex formation at regulatory sites by excluding SMAD4 association with the receptor SMADs 2 and 3. Our results demonstrate that HPV8 E6 and MmuPV1 E6 can inhibit TGF-beta signaling, including the induction of keratinocyte growth arrest and supports a model wherein E6 blocks the formation of DNA bound SMAD complexes by preventing SMAD4 from interacting with SMAD2/3.

**MAML1 binding defective MmuPV1 and HPV8 E6 mutants do not inhibit NOTCH signaling.**

The ability of HPV8 E6 to bind MAML1 has been suggested to be required for NOTCH inhibition and we predict that this interaction would also be required for MmuPV1 E6 inhibition of NOTCH signaling. We sought to directly test this hypothesis. HPV8 E6 interacts with an LXXLL motif in MAML1. Structural studies have identified the amino acid residues that make direct contact between BPV1 E6 and an LXXLL containing peptide derived from Paxillin (PXN) (95). Based on these insights we set out to identify amino acid residues in HPV8 and MmuPV1 E6 that are necessary for MAML1 binding. In addition to PXN, BPV1 E6 can also interact with MAML1. There are key differences in the amino acid sequences surrounding the core LXXLL motifs of the HPV8 or MmuPV1 E6 binding PXN and MAML1, and UBE3A (E6AP) that binds to HPV16 E6 (95) but not to HPV8 or MmuPV1 E6 (Figure 3.7A). Therefore, we hypothesized that HPV8 and MmuPV1 E6 bind MAML1 using similar amino acid contacts as those required in BPV1 E6 for PXN binding. Hence we mutated putative LXXLL contact residues in HPV8 E6.
Figure 3.6. SMAD4 association with SMAD2/3 is abrogated in the presence of HPV8 and MmuPV1 E6. Immunoprecipitation of FLAG-tagged SMAD2 (A) or FLAG-tagged SMAD3 (B) with co-transfection of either HPV8 E6 or MmuPV1 E6 after TGF-b1 treatment followed by SMAD4 immunoblot. Data shown is representative of three independent experiments.
that are conserved with BPV1 E6 and tested them for MAML1 binding. The following residues were targeted by mutation: leucine (L) 59, lysine (K) 64, arginine (R) 138, and K142. To test whether any of these mutations led to dramatic changes in E6 conformation, we measured binding to EP300, which does not contain an LXXLL motif. We used previously published EP300 binding defective mutants HPV8 E6 Δ132-136 and K136N mutants (180, 204) as controls and expected these to retain MAML1 binding. U2OS cells were transfected with plasmids expressing either wild type HPV8 E6 or HPV8 E6 mutants and analyzed for E6 binding to endogenous EP300 and MAML1 by immunoblot. The HPV8 E6 L59D, K64A, and K142A as well as the EP300 binding deficient Δ132-136 and K136N mutants were all defective for MAML1 binding (Figure 3.7B). However, the L59D mutant was also defective for EP300 binding, similar to the previously described EP300 binding deficient Δ132-136 mutant; hence, these mutations likely result in global structural alterations. Contrary to what was previously published, the K136N mutant retained EP300 binding (180, 204). The R138S mutant retained EP300 and MAML1 binding, whereas the K64A and K142A mutants were defective for MAML1 binding while retaining EP300 association.

Alignment of the HPV16, BPV1, HPV8, and MmuPV1 E6 protein sequences (Figure 3.7C), revealed that the positively charged K64 residue in HPV8 E6 corresponded to the positively charged R52 in MmuPV1, and the positively charged K62 in HPV16 E6. In contrast, the positively charged K142 of HPV8 E6 corresponded to the positively charged R130 in MmuPV1, but corresponded to an uncharged T140 residue in HPV16. We mutated these residues in MmuPV1 E6 to create MmuPV1E6-R52A and MmuPV1E6-R130A and tested them for their ability to bind MAML1 and SMAD2/3 by immunoprecipitation followed by immunoblot (Figure 3.7D). Similar to the HPV8 E6 K64A and K142A mutants the MmuPV1 E6 R52A and R130A
Figure 3.7. HPV8 and MmuPV1 E6 require interaction with MAML1 for NOTCH inhibition. (A) Alignment of LXXLL motifs of Paxillin, MAML1 and UBE3A. (B) Lysates of U2OS cells expressing FLAG/HA tagged versions of wildtype or mutant HPV8 E6 proteins were subjected to HA immunoprecipitation. Immunoprecipitated HPV8 E6 and associated EP300 and MAML1 were detected by immunoblotting. Expression levels of the various proteins were assessed by immunoblotting of whole cell extracts (WCE). GAPDH expression is shown as a loading control. (C) Alignment of amino acid sequences of HPV16, BPV1, HPV8 and MmuPV1 E6 proteins. E6 proteins contain two pairs of CXXC motifs that are highly conserved and essential for E6 structure and function(95), and therefore they are useful for aligning diverse E6 proteins. Shown are the regions between the first CXXC pair (top) and the start of the second paired CXXC through the rest of the sequence (bottom). (D) Lysates of U2OS cells expressing FLAG/HA tagged versions of wildtype or mutant HPV16, HPV8, and MmuPV1 E6 proteins were subjected to HA immunoprecipitation. Immunoprecipitated HPV8 E6 and associated EP300 and MAML1 were detected by immunoblotting. Interaction between HPV16 E6 and UBE3A is shown as a control. Expression levels of the various proteins were assessed by immunoblotting of whole cell extracts (WCE). GAPDH expression is shown as a loading control. (E) Effects of wild type and mutant HPV8 and MmuPV1 E6 on NOTCH responsive reporter activity using ICN1 as an agonist in U2OS cells. Data shown is from a representative experiment from three independent experiments (F) Effects of mutating the MAML1 LXXLL motif (MAML1-LHHLL) on HPV8 and MmuPV1 E6 mediated inhibition of an ICN1 activated NOTCH responsive luciferase reporter. Data shown is from a representative experiment from three independent experiments. P-values were calculated with unpaired t-test with Welch’s correction where * = P ≤ 0.05, ** = P ≤ 0.01.
Figure 3.7 (Continued).
Figure 3.7 (Continued).
mutants showed diminished binding for MAML1 and retained the ability to bind to SMAD2/3. Given that the HPV8 E6 K64A and the MmuPV1 R52A mutants retained higher MAML1 binding than the HPV8 K142A and the MmuPV1 E6 R130A (Figures 3.7B and 3.7D) we chose the HPV8 K142A and MmuPV1 E6 R130A mutants for further analysis. The previously described NOTCH reporter system was used to assess the ability of HPV8E6-K142A and the corresponding MmuPV1E6 R130A mutant to inhibit NOTCH signaling (Figure 3.7E). Consistent with decreased MAML1 association, these mutants were unable to fully inhibit NOTCH signaling. Lastly, to further confirm that NOTCH inhibition by MmuPV1 E6 was due to MAML1 binding, we mutated the two aspartate residues of the LDDLL motif in MAML1 to histidines (LHHLL). Using the NOTCH reporter assay we verified that the MAML1 LHHLL mutant was not dominant negative and that cotransfection of the MAML1 LHHLL mutant was able to partially block E6 mediated inhibition of NOTCH signaling (Figure 3.7F). Thus, either mutation of papillomavirus E6 residues that disrupt LXXLL binding or mutations in the MAML1 LXXLL motif interfere with E6 inhibition of NOTCH signaling. This shows that the ability of the HPV8 and MmuPV1 E6 proteins to inhibit NOTCH requires the interaction with MAML1 and does not occur through an indirect mechanism.

**HPV8 and MmuPV1 E6 inhibit differentiation and prolong survival of differentiated keratinocytes.**

After determining that MmuPV1 E6 and HPV8 E6 share the capacity to inhibit NOTCH and TGF-beta signaling, we predicted that, similar to HPV8 E6 (119), MmuPV1 E6 can inhibit keratinocyte differentiation. NOTCH signaling is a critical driver of keratinocyte differentiation and negatively regulates proliferation (205). Similarly, TGF-beta has been implicated in maintaining epithelial stemness and controlling proliferation competency in differentiating
keratinocytes (206). We have previously shown that HPV8 E6 can inhibit differentiation of
keratinocytes, a process at least partially dependent on NOTCH inhibition (119). HPV8 and
MmuPV1 E6 expressing telomerase immortalized oral keratinocytes (NOKs) were grown to
confluency in low calcium containing, serum free medium and then switched to calcium
containing DMEM supplemented with 10% FBS to induce differentiation for up to 6 days. RNA
was isolated at days 0, 2, and 6, and expression of involucrin and filaggrin were measured by
RT-qPCR. GFP expressing cells showed a marked increase in the levels of involucrin, a marker
of intermediate stage keratinocyte differentiation, by day 6 (Figure 3.8A) which was absent from
HPV8 E6 or MmuPV1 E6 expressing cells. Treatment of control keratinocytes with the gamma
secretase inhibitor compound E (GSI), which blocks NOTCH cleavage, or the TGF-beta receptor
1 inhibitor SB-431542 (TGFI) either alone or in combination, similarly blocked the
differentiation induced increase of involucrin mRNA. Next, we measured the expression of
filaggrin, a marker of later stages of keratinocyte differentiation (Figure 3.8B). GFP expressing
cells showed increased filaggrin expression after 6 days of calcium induced differentiation, but
there was no similar increase in HPV8 E6 or in MmuPV1 E6 expressing cells. Treatment with
TGFI but not GSI blocked filaggrin expression. Filaggrin transcript levels were even higher
during GSI treatment as compared to control cells, an observation that has also been seen in
differentiated keratinocytes harboring heterozygous NOTCH1 deletions (148). These authors
suggested that NOTCH regulates intermediate differentiation and in the absence of signaling,
differentiating keratinocytes prematurely initiate the late differentiation program including
filaggrin expression. Additionally, we analyzed involucrin protein levels at days 0, 2, and 6
post-calcium induction by immunoblot. As observed in Meyers et al (119), control cells show
Figure 3.8. Expression of epithelial differentiation markers. GFP control telomerase immortalized oral keratinocytes (NOKs), control NOKs treated with GSI, TGFI, or a combination of both inhibitors, as well as HPV8 and MmuPV1 E6 expressing NOKs were differentiated in calcium containing media for six days and analyzed for expression of involucrin (A) and filaggrin (B) by RT-qPCR. The results of a representative experiment are shown with standard error of mean. Similar results were obtained in two additional experiments (C) Involucrin protein levels measured by immunoblot of NOKs during calcium induced differentiation.
robust induction of involucrin expression after 2 and 6 days of calcium treatment but this increase is not observed in HPV8 E6 and in MmuPV1 E6 expressing cells (Figure 3.8C).

Calcium treatment of keratinocytes causes a decrease in proliferation and eventually death of terminally differentiated cells. We hypothesized that HPV8 and MmuPV1 E6 expressing cells would be resistant to these effects of terminal differentiation. GFP-control, HPV8 E6 or MmuPV1 E6 expressing NOKs were grown to confluency and switched to calcium-containing media. The medium was changed regularly, and the cells were observed for 32 days. Pictures were taken every two days until day 16 (Figure 3.9A-C) and at day 32 (Figure 3.9D). Before calcium addition, all cell populations had similar morphologies (Figure 3.9A-C). Most of the calcium treated control cells had expired and detached from the plate by day 32. In contrast, the HPV8 and MmuPV1 E6 expressing keratinocyte populations remained attached to the plate. In parallel we directly measured proliferation/viability of these cells using resazurin. Consistent with the observed changes in morphology, proliferation/viability of control cells started to decline after 4 days of calcium treatment and continued to decrease over the entire time period observed (Figure 3.9E). In contrast, HPV8 and MmuPV1 E6 expressing keratinocytes compared to control cells remained metabolically active and survived throughout the 32 days of differentiation (p-values 0.0012 and 0.0028 respectively). Interestingly, however, chemical inhibition of NOTCH and/or TGF-beta signaling in control keratinocytes or HPV16 E6 expressing keratinocytes did not significantly differ from control cells (p-values GSI 0.8203, TGFI 0.4363, dual 0.2973, HPV16 E6 0.2136). This indicates that HPV8 or MmuPV1 E6 expression prolongs the survival of differentiating keratinocytes, but that TGF-beta and/or NOTCH inhibition does not account for these effects.
Figure 3.9. Survival of calcium differentiated keratinocytes. NOK cells expressing GFP (A), HPV8 E6 (B), or MmuPV1 E6 (C) were differentiated in calcium for 16 days and pictures were obtained every two days. (D) Pictures of GFP-control (left), HPV8 E6 expressing (middle), and MmuPV1 E6 expressing (right) NOKs after 32 days in calcium differentiation medium. (E) Proliferation/viability of keratinocytes over 32 days of calcium differentiation measured by resazurin reduction. P-values (see text) were calculated using Wilcoxon ranked sum-test.
Figure 3.9 (Continued).
Figure 3.9 (Continued).
Figure 3.9 (Continued).
Figure 3.9 (Continued).
MAML1 binding defective MmuPV1 E6 mutants do not cause papillomas in nude mice

MmuPV1 provides the ability to assess the biological importance of individual viral gene products and their biochemical activities to viral pathogenesis in vivo. To assess a role for E6 in virally induced pathogenesis, tails of 8-10 week old FoxN1
\(^{nu/nu}\) mice were infected MmuPV1 quasivirions encapsulating wild type, E6-null (E6\(^{STOP}\)) or E6\(^{R130A}\) mutant MmuPV1 genomes following topical scarification of the epidermis at the designated doses of virus (Table 3.1). Previous experiments indicated that infections with these doses of wild type viruses are sufficient to induce papillomas at 100% of sites infected (199). Consistent with these findings, we found that infections with quasiviruses carrying wild-type MmuPV1 genomes induced papillomas at 100% efficiency. Unencapsidated, wild type MmuPV1 DNA is also infectious (198). While 10 \(\mu\)g of wildtype MMuPV1 DNA induced warts at 100% of sites, the same amount of MmuPV1 E6\(^{STOP}\) or E6\(^{R130A}\) mutant DNA did not cause any papillomas (Table 3.2). To confirm that quasiviruses carrying E6\(^{STOP}\) or E6\(^{R130A}\) MmuPV1 mutant genomes were indeed infectious, we performed in vitro infections of mouse keratinocytes and tested for the transcription of E1\(^{E4}\) spliced products by RT-PCR (Figure 3.10A). We found that E1\(^{E4}\) spliced products could be detected post-infection with wild type as well as the mutant quasivirions, suggesting that mutant quasivirions are infectious but are defective for papilloma formation in immunodeficient mice.

MmuPV1 induced papillomas show evidence of disrupted epithelial differentiation and increased cellular proliferation

Our previous in vitro experiments suggested that the ability of MmuPV1 E6 to inhibit NOTCH and TGF-beta signaling would impair differentiation and increase cellular proliferation in MmuPV1-induced papillomas. Immunohistochemical analysis of differentiation layer markers cytokeratin 14 (K14) and cytokeratin 10 (K10) (Figure 3.10B) was used to assess the
Table 3.1. Papilloma incidence in FoxN1\textsuperscript{nu/nu} mice infected with MmuPV1 quasivirions.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Genome</th>
<th>VGE</th>
<th># Sites infected</th>
<th># Sites w/ papilloma (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>MmuPV1-WT</td>
<td>2.48×10(^7)</td>
<td>16</td>
<td>16 (100)</td>
</tr>
<tr>
<td></td>
<td>MmuPV1-E6\textsuperscript{Stop}</td>
<td>2.24×10(^7)</td>
<td>16</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>MmuPV1-E6\textsuperscript{R130A}</td>
<td>0.98×10(^6)</td>
<td>16</td>
<td>0 (0)</td>
</tr>
<tr>
<td>2.</td>
<td>MmuPV1-WT</td>
<td>2.11×10(^8)</td>
<td>16</td>
<td>16 (100)</td>
</tr>
<tr>
<td></td>
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<td>1.25×10(^8)</td>
<td>16</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>MmuPV1-E6\textsuperscript{R130A}</td>
<td>2.26×10(^8)</td>
<td>16</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>
Table 3.2. Papilloma incidence in FoxN1nu/nu mice infected with MmuPV1 DNA.

<table>
<thead>
<tr>
<th>Genome</th>
<th># Sites infected</th>
<th># Sites w/ papilloma</th>
</tr>
</thead>
<tbody>
<tr>
<td>MmuPV1-WT</td>
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<td>16 (100)</td>
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<tr>
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<td>16</td>
<td>0 (0)</td>
</tr>
<tr>
<td>MmuPV1-E6&lt;sup&gt;R130A&lt;/sup&gt;</td>
<td>16</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>
Figure 3.10. Infectivity of MmuPV1 quasivirus and histology and analysis of MmuPV1-induced papillomaviruses. (A) Quasiviruses were used to infect mouse keratinocytes and total RNA was harvested, cDNA was synthesized and assayed for the presence of E1^E4 spliced transcript. PCR products were visualized on agarose gel and compared to GAPDH amplification. (B) Immunohistochemistry analysis of differentiation markers in normal uninfected skin compared to MmuPV1 induced skin papillomas. Immunohistochemistry analysis of Cytokeratin14 (left), Cytokeratin10 (right) indicates delay in terminal differentiation program of epithelia in the papillomas. (C) Immunohistochemistry analysis of BrdU incorporation in cells of normal uninfected skin and papillomas. Quantification of the total percentage of BrdU-positive cells in papillomas (n=3 mice) versus normal uninfected skin (n=5 mice) from BALB/c-\textit{Foxn1}^nu mice cells is shown in the bar graph (Error bars, SD. ** P < 0.05 using a two-sided Wilcoxon rank-sum test). The dotted lines indicate basement membrane of tissue.
Figure 3.10 (Continued).
differentiation state of papillomas in comparison with normal uninfected skin. The papillomas showed expansion of basal-like epithelial cells as evidenced by immunohistochemical (IHC) analysis of the basal epithelial marker K14 (Figure 3.10B left panel). K10 expression is found in suprabasal compartment of normal, uninfected murine skin. MmuPV1-induced papillomas showed a delayed staining pattern of K10 in the papilloma (Figure 3.10B right panel).

To assess cellular proliferation in papillomas we evaluated incorporation of bromodeoxyuridine (BrdU) following intraperitoneal injection of the drug one hour prior to harvesting tissue. IHC was performed to identify BrdU-positive cells in normal epithelium or papillomas and the percentage of BrdU-positive cells was calculated. Approximately 9% of cells in normal uninfected tail skin of BALB/c-Foxn1nu were BrdU-positive (Figure 3.10C), and positive cells were restricted to the basal layer of the epithelia. Due to the mildly hyperproliferative nature of nude mouse epithelia (207) the percentage of BrdU positivity is somewhat higher than in the skin of normal immunocompetent mice. Nevertheless, there was a significantly higher percentage of BrdU-positive cells (24%, p = 0.036) in the papillomas and BrdU positivity was not confined to basal cells (Figure 3.10C). This demonstrates that there is both an increase in cellular proliferation and licensing of DNA synthesis in suprabasal cells within MmuPV1-induced papillomas similar to what we observed in MmuPV1 E6 expressing iHFKs (Figure 3.9).
Discussion

Cutaneous papillomaviruses such as HPV5 and HPV8 have long been associated with SCCs in EV patients and long-term immunosuppressed individuals (59, 185, 208). Unlike cancers associated with mucosal HPV infections, cutaneous HPV associated SCC arise in sun exposed areas, and hence these viruses are thought to cooperate with UV to induce SCCs (209). HPV8 E2, E6 and E7 each have oncogenic activities when expressed in basal epithelial cells of transgenic mice, but the molecular mechanisms by which these HPVs contribute to cancer development have remained enigmatic. Moreover, HPV sequences are not maintained in every cancer cell, and hence cutaneous HPVs are not necessary for the maintenance of the transformed state (188).

Given the well documented interaction with UV exposure, most molecular studies with cutaneous HPVs have focused on modulation of cell cycle arrest, apoptosis and DNA repair after UV irradiation (210). HPV8 E6 has been reported to interact with the pro-apoptotic BCL2 family member BAK thereby inhibiting apoptosis in response to UV (111). In addition, cutaneous HPV E6 proteins have been reported to inhibit ATM and ATR activation through an EP300 dependent mechanism, thereby inhibiting DNA repair in response to UV exposure (115, 191). Interactions with BAK and EP300 have also been reported for mucosal HPV E6 proteins (112, 211-213).

Several studies, however, have identified cellular proteins that specifically interact with cutaneous HPV5 and HPV8 E6 but not mucosal HPV E6 proteins. These include members of receptor regulated SMADs (R-SMADs) 2 and 3, which are key to TGF-beta signaling (109, 110, 124). In addition, HPV5 and 8 E6 bind to MAML1, an essential co-activator of NOTCH signaling (109, 117, 118). HPV8 and MmuPV1 E6 proteins inhibit these pathways through stoichiometric interactions and they do not appear to destabilize these proteins by co-opting the
cellular ubiquitin conjugation machinery. While mucosal HPV E6 proteins do not detectably interact with MAML1 or SMAD2 and SMAD3, they may target these pathways through other mechanisms. High-risk HPV E6 proteins may inhibit some aspects of NOTCH signaling indirectly by targeting the TP53 tumor suppressor for degradation (123) and high-risk HPV16 E7 can inhibit TGF-beta mediated growth inhibition (106) and interact with SMAD3 (107, 214). Even though TGF-beta and NOTCH are important tumor suppressor pathways in keratinocytes, it is unclear whether and how inhibition of these pathways by cutaneous HPV E6 proteins contribute to induction of lesions and cancer.

The lack of an animal system where the biological relevance of specific viral host interactions can be investigated with respect to the viral life cycle and pathogenesis has greatly hindered papillomavirus research. While early studies with bovine papillomavirus 1 (BPV1) and cottontail rabbit papillomavirus 1 (CRPV1; more recently referred to as *Sylvilagus floridanus* Papillomavirus 1 - SfPV1) enabled infections of autologous hosts, the respective host animals are not genetically tractable. The isolation of MmuPV1 from cutaneous warts of immunodeficient nude mice and the fact that it can be used to experimentally infect laboratory mice have been important steps towards enabling viral pathogenesis studies. Moreover, the recent discovery that MmuPV1-induced warts can undergo malignant progression when subjected to UV (199) suggests that experimental MmuPV1 infections may allow modeling some aspects of SCC formation by cutaneous HPVs.

As a first step towards determining whether MmuPV1 may be a useful pathogenesis model of cutaneous HPV infections, we investigated whether MmuPV1 E6 shared cellular interactors with HPV5 and HPV8 E6 proteins. We initially focused on MAML1, the R-SMADs SMAD2 and SMAD3 that are required for TGF-beta signaling and EP300. We found that similar
to what has been reported for HPV5 and 8 E6 (109, 110, 124), MmuPV1 interacts with MAML1 and TGF-beta R-SMADs (Figures 3.1A), thereby inhibiting these two important tumor suppressor pathways (Figures 3.1B and 3.1C). Our results suggest that similar to what we previously reported for HPV8 E6, MmuPV1 E6 inhibits NOTCH by interacting with a nuclear transcription factor complex that contains MAML1 and cleaved, active intracellular NOTCH (ICN) (119). HPV8 and MmuPV1 E6 also share the ability to associate with TGF-beta R-SMADs, but HPV8 E6 appears to preferentially associate with SMAD3, whereas MmuPV1 E6 associates preferentially with SMAD2 (Figure 3.1A). This was somewhat surprising given the high degree of sequence identity (83.9%) between the two proteins. However, in both cases, E6/R-SMAD associations inhibit transcriptional responses to TGF-beta (Figure 3.1B). Our results do not support earlier studies that reported SMAD3 destabilization in HPV5 E6 expressing cells (124) (Figure 3.1A and Figure 3A). Moreover, HPV8 and MmuPV1 E6 do not appear to markedly affect R-SMAD phosphorylation and nuclear translocation (Figure 3.4A). Our results (Figure 3.4B, Figure 3.5, and Figure 3.6) suggest a model whereby E6 R-SMAD binding inhibits SMAD4 binding and thus formation of an active transcriptional complex. Cancer-associated SMAD2 mutations are also defective for SMAD2/SMAD4 complex formation (162), and cutaneous papillomavirus E6 proteins seem to functionally mimic SMAD2 mutations. Since MmuPV1 and HPV8 E6 can be detected at the SRE of the CDKN2B promoter (Figure 3.4B), we propose that similar to what has been observed in their inhibition of NOTCH signaling, these E6 proteins interfere with the transcriptional activity of a DNA bound SMAD2/SMAD3 containing transcription factor complex. Additional experiments are required, however, to carefully test this model. SMAD2 and SMAD3 do not contain recognizable LXXLL motifs and hence they are expected to bind different E6 sequences than the LXXLL containing
MAML protein. Consistent with that notion, we found that the MAML binding defective HPV8 and MmuPV1 E6 mutants retained SMAD2/3 binding (Figure 3.7D).

In contrast to HPV8 E6, MmuPV1 E6 proteins does not detectably bind EP300 (Figure 3.1A and Figure 3.7D). We also tested MmuPV1 E6 EP300 binding in murine cells and did not detect an association. Similar to SMAD2 and SMAD3, EP300 does not have an LXXLL motif. EP300 is an important co-activator for many different transcriptional programs including NOTCH signaling. EP300 associates with a C-terminal sequence of MAML1 that is referred to as Transcriptional Activation Domain (TAD)1 (215), whereas MmuPV1 and HPV8 E8 associate with a separate LXXLL motif containing domain referred to as TAD2 and hence do not directly compete for EP300 binding to TAD1. Based on our finding that MmuPV1 does not co-precipitate EP300 and that MAML1 defective HPV8 E6 mutants retain EP300 association we conclude that EP300 binding to HPV8 E6 is not mediated through MAML1 and moreover, that E6 binding to TAD2 may prevent EP300 binding to TAD1. This may provide a molecular mechanism for inhibition of NOTCH transcription by cutaneous papillomavirus E6 proteins. In addition, given that MmuPV1 associated warts can progress to SCCs, it would appear that EP300 binding is not strictly required to cooperate with UV for SCC formation. Many studies that have implicated EP300 as a major cellular effector of cutaneous HPV E6 activities have been based upon the use of the HPV8 E6 Δ132-136 and K136N mutants (180, 204). Our experiments unexpectedly revealed that the Δ132-136 mutant is also defective for MAML1 binding, whereas the K136N mutant did not exhibit any overt defects for EP300 binding (Figure 3.7B). Caution should be taken when interpreting data obtained with these mutants.

The NOTCH and TGF-beta tumor suppressors are critical determinants of differentiation and cell fate in keratinocytes and act by coordinating cell-cycle withdrawal and driving
keratinocytes toward terminal differentiation and ultimately, cell death (216, 217). Our results show that similar to MmuPV1 induced cutaneous warts, HPV8 and MmuPV1 E6 expressing human keratinocytes are differentiation resistant and remain proliferatively active (Figures 3.8 and 3.9). The differentiation process is largely TGF-beta and NOTCH dependent as TGF-beta and/or NOTCH inhibitor treatment of normal keratinocytes mimics the effects of E6 expression, but extended survival of differentiated keratinocyte was independent of these two pathways (Figure 3.9E). In addition, we also observed that HPV8 and MmuPV1 E6 expressing keratinocytes remain viable for extended periods of time under conditions that induce differentiation (Figure 3.9). Interestingly, TGF-beta and/or NOTCH inhibition in normal keratinocytes was not sufficient for this phenotype (Figure 3.9E).

Taken together, our results suggest HPV8 and MmuPV1 E6 allow infected cells to remain proliferatively active and not only resist differentiation cues but also remain viable over extended periods of time. This would be manifested by an expansion of basal-like, proliferatively active cells as is seen in MmuPV1 induced skin lesions (Figure 3.10).

The number of cutaneous HPVs that have been isolated and characterized has dramatically increased over the last few years. In addition to HPV5 and HPV8, a large number of beta genus HPVs and more recently also gamma genus HPVs have been detected in cutaneous lesions and SCCs (188, 218). Proteomic studies of E6 associated cellular proteins have started to shed some light on similarities and differences of cellular pathways that may be targeted these by the various cutaneous HPVs (110).

The next important challenge will be to determine how subversion of these various pathways contributes to the pathogenesis and oncogenicity of these viruses. Are there low-risk
and high-risk cutaneous HPVs? If so, is the oncogenic potential dependent on inhibition of specific cellular pathways as has been shown for mucosal HPVs?

Our results provide evidence that MmuPV1 will be an important, biologically relevant model to address some of these issues, particularly as they relate to NOTCH and TGF-beta inhibition by E6. Our experiments show that MmuPV1 E6 expression is necessary for wart formation and that a MmuPV1 quasivirus carrying a genome encoding a MAML1 binding defective E6 mutant does not cause wart formation (Tables 3.1 and 3.2). Using recently published structures of papillomavirus E6 proteins bound to cellular targets we may be able to generate MmuPV1 mutants that are defective for binding to R-SMADs or other associated cellular target proteins and test these both *in vitro* and *in vivo*.

Even without such an E6 mutant we can perform additional experiments with small molecule inhibitors and/or by infecting mouse strains, even immune competent mice (199), that carry mutations in specific signaling pathways to conclusively assess the importance of E6 mediated NOTCH and TGF-beta inhibition for MmuPV1 replication and pathogenesis *in vivo*. 

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Materials and Methods

Cell Culture.

U2OS human osteosarcoma and HCT116 human colon carcinoma cells were obtained from ATCC and grown in Dulbecco’s Modified Eagle Medium (DMEM) with high glucose (Gibco) supplemented with penicillin-streptomycin and 10% fetal bovine serum (FBS). Tert-immortalized human foreskin keratinocytes Cl398 (175) (iHFKs) (obtained from Al Klingelhutz, University of Iowa) or normal oral keratinocytes (NOK) (219) were maintained in keratinocyte serum free media (KSFM) (Gibco) supplemented with 0.2 ng/ml EGF, 25mg/mL bovine pituitary extract, and penicillin-streptomycin. Cells were differentiated by switching KSFM to DMEM/10% FBS. Recombinant human TGF-b1 (Millipore) was used at a final concentration of 10 ng/ml in all experiments. Compound E (Millipore) and SB-431542 (Sigma) were used at 2 µM and 10 µM respectively.

Plasmids, transfections, and lentiviral transduction.

Plasmids used in transient transfections were pCMV BamNeo vectors with Flag-hemagglutinin epitopes fused to the amino termini of HPV E6 proteins: pNCMV (vector) HPV16 E6, HPV8 E6, MmuPV1 E6. Lentiviral plasmids used were generated through cloning of pLenti6.3 /V5 TOPO Gateway compatible vector (Invitrogen): GFP (control), HPV8 E6, HPV16 E6, and MmuPV1 E6. Notch reporter construct, HES1-luc (220), HA-tagged ICN1 (221), and MAML1 full-length(221) expression plasmids (obtained from Jon Aster, Harvard Medical School) were used as previously described (222). TGF-beta reporter construct, (CAGA)_9-MLP-Luc (obtained from
Jennifer Pietenpol, Vanderbilt University School of Medicine) was used as previously described (223). SMAD2 and SMAD3 expression plasmids were obtained from Michael Hoffman (University of Wisconsin). All mutations were created using QuikChange II site-directed mutagenesis kit (Agilent). Transient transfections of U2OS cells was performed using Polyethylenimine (PEI) (Polysciences) as described (224) and analysis of transfected cells was performed at 48 hours post-transfection. Transient iHFK transfections were performed using Fugene 6 (Promega) and were analyzed at 48 hours post transfection. Preparation of and infection with recombinant lentiviruses was as previously described (225). Selection of infected cells using Blasticidin (10 µg/ml) began two days post infection and was maintained for seven days. Cells were then maintained as described in KSFM.

**Luciferase reporter assays.**

Reporter assays were performed using Dual-Luciferase Reporter Assays System (Promega). Lysates of cells transfected with the appropriate plasmids (200 ng reporter, 200 ng vector or E6, 10 ng renilla, and 200 ng ICN1 or 200 ng MAML1 where appropriate) were prepared in 100 µl of passive lysis buffer at 48 hours after transfection and 20 µl of lysate was used for each reading. Readings were done in triplicate using a LMax II plate reader (Molecular Devices) and values normalized for transfection efficiency using the co-transfected renilla luciferase expression plasmid.
RNA isolation and real-time quantitative PCR analysis.

RNA was isolated using Quick-RNA MiniPrep (Zymo Research). cDNA was synthesized using Quantitect Reverse Transcription Kit (Qiagen). Quantitative PCR (qPCR) was performed in triplicate on a StepOne Plus (Applied Biosystems) thermocycler using SYBR Green PCR Master Mix (Applied Biosystems) reagents. PCR primers used are listed in Supplemental Table a. Data shown was calculated using ΔΔCT method and normalized to expression of the RPLP0 as the housekeeping gene.

QPCR primers

Involucrin Forward 5’ TGC CTG AGC AAG AAT GTG AG -3’
Involucrin Reverse 5’- TGC TCT GGG TTT TCT GCT TT -3’
Filaggrin Forward 5’- AAA GAG CTG AAG GAA CTT CTG -3’
Filaggrin Reverse 5’- AAC CAT ATC TGG GTC ATC TGG -3’
HES1 Forward 5’-GGA AAT GAC AGT GAA GCA CCT CC-3’
HES1 Reverse 5’-GAA GCG GGT CAC CTC GTT CAT G-3’
CDKN2B Forward 5’- AGA TCC CAA CGC CCT GAA -3’
CDKN2B Reverse 5’- CCC ATC ATC ATG ACC TGG ATT -3’
RPLP0 Forward 5’- ATC AAC GGG TAC AAA CGA GTG -3’
RPLP0 Reverse 5’- CAG ATG GAT CAC CCA AGA AGG -3’
E1^E4 Forward, 5’- CAT TCG AGT CAG TGC TTC TGC -3’
E1^E4 Reverse, 5’- GAT GCA GGT TTG TCG TTC TCC -3’

GAPDH Forward, 5’- ACC ACA GTC CAT GCC ATC AC -3’

GAPDH Reverse, 5’- TCC ACC ACC CTG TTG CTG TA -3’

ChIP primers

CDKN2B (p15^INK4B) promoter Forward 5’- CAT GAT TCT CGG GAT TTT TCT C -3’

CDKN2B (p15^INK4B) promoter Reverse 5’- GCG ACA GCT CTG CAC C -3’

Immunoprecipitations, fractionation and immunoblotting.

Cells were lysed in 1% NP40 buffer (1% Nonidet P-40 (NP40), 120mM NaCl and 50mM TrisHCl (pH 8.0). Immunoprecipitations of HA epitope tagged proteins were performed using HA antibodies coupled to agarose beads (Sigma). Samples were run on NuPAGE® 4-12% Bis-Tris Gels (Invitrogen) according to manufacturer’s instructions. Proteins were electrotransferred to Polyvinylidene fluoride (PVDF) membranes (Immobilon-P; Millipore). The membranes were blocked in 5% nonfat dry milk in TBST (137 mM NaCl, 2.7 mM KCl, 25 mM Tris [pH 7.4], 0.1% Tween 20) and probed with the appropriate antibody. Primary antibodies (1:1000 dilution) used for immunoblots are listed in supplemental table b. Secondary anti-mouse and anti-rabbit antibodies conjugated to horseradish peroxidase (Amersham) were used at dilutions of 1:10,000. Subcellular fractionation was done using the REAP method (226). In brief, cell pellets were washed with phosphate buffered saline (PBS) and repelleted. The cytoplasmic fraction was isolated by tituration of the pellet in PBS containing 0.1% NP40. The nuclear pellet was washed
and resuspended in 1% NP40 lysis buffer followed by sonication and treatment with Pierce Universal Nuclease (Thermo Fisher Scientific).

**Antibodies**

EP300, clone RW128 (Millipore 05-257)

MAML1, D3K7B (Cell Signaling Technology 12166)

Cleaved NOTCH1, Val1744, D3B8 (Cell Signaling Technology 4147)

SMAD2, D43B4 (Cell Signaling Technology 5339)

SMAD3, C67H9 (Cell Signaling Technology 9523)

SMAD2/3, D7G7 (Cell Signaling Technology 8685)

pSMAD2, 138D4 (Cell Signaling Technology 3108)

pSMAD3, C25A9 (Cell Signaling Technology 9520)

SMAD4 for ChIP (Cell Signaling Technology 9515)

SMAD4, 10HCLC (ThermoFisher 710714)

HA-tag for ChIP, C29F4 (Cell Signaling Technology 3724)

HA, Y-11 (Santa Cruz Biotech sc-805)

FLAG, M2 (Sigma F1804)

GAPDH, G9 (Santa Cruz Biotech sc-365062)

Involucrin, SY5 (Santa Cruz Biotech sc-21748)

CDKN1A1 (p21\(^{CIP}\)), F-5 (Santa Cruz Biotech sc-6246)
IgG for ChIP (Cell Signaling Technology 2729)

Histone H3 for ChIP, D2B12 (Cell Signaling Technology 4620)

Histone H2A, D603A (Cell Signaling Technology 12349)

BrdU (Oncogene NA20)

Keratin K10 (Covance PRB-159P)

Keratin K14 (Covance PRB-155P)

**Resazurin-based viability/proliferation assay.**

Resazurin was used at 25 µg/ml in PBS to assess redox fitness (227). Cells were incubated with dye for one hour and then sample fluorescence was read in triplicate using 560 nm excitation and 590 nm emission filters on a Synergy H1 microplate reader (BioTek).

**Chromatin Immunoprecipitation.**

Chromatin was prepared using SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology) according to manufacturer’s instructions and approximately 1*10^6 cells were used for each assay. Antibodies used are listed in supplemental table b. Co-precipitated DNA was isolated according to manufacturer’s protocol and quantitative PCR was performed as described (119). Data is expressed as compared to percent input after subtraction of isotype matched IgG signal.
Animals

Immunodeficient euthymic BALB/c FoxN1nu/nu (Harlan) were used in this study. All infected mice were housed in aseptic conditions in micro-isolator cages. Animals were handled only by designated personnel and personal protection gear was changed between cages to prevent any cross contamination from virus.

Ethics Statement

All animal experiments were performed in full compliance with standards outlined in the “Guide for the Care and Use of Laboratory Animals” by the Institute of Laboratory Animal Resources (ILARC) of the Commission on Life Sciences (CLS), National Research Council (NRC) as specified by the Animal Welfare Act (AWA), associated Animal Welfare Regulations (AWRs), Public Health Service (PHS) Policy and Office of Laboratory Animal Welfare (OLAW) and approved by the Governing Board of the National Research Council (NRC), whose members are drawn from the councils of the National Academy of Sciences (NAS), National Academy of Engineering (NAE), and Institute of Medicine (IM). Mice were housed at McArdle Laboratory Animal Care Unit in strict accordance with guidelines approved by the Association for Assessment of Laboratory Animal Care (AALAC), at the University of Wisconsin Medical School. All protocols for animal work were approved by the University of Wisconsin Medical School Institutional Animal Care and Use Committee (IACUC, Protocol number: M02478).

Infection of nude mice with MmuPV1 wild type or mutant quasivirions

Infections were performed using quasivirions containing MmuPV1 wild type or mutant genomes as described previously (25, 228). Briefly, 293FT cells (ATCC) were cotransfected with a
MmuPV1 capsid protein expression plasmid (pMusSheLL- a gift from Chris Buck, National Cancer Institute) (197, 229) and MmuPV1 wild type or mutant DNA for encapsidation. After 48 h at 37°C, cells were harvested and virions were purified using Optiprep gradient centrifugation. The generated quasivirions were quantified and used to infect of FoxN1\textsuperscript{nu/nu} mice (Harlan) as described (199). Briefly, \textit{in vivo} infections with purified MmuPV1 quasivirions were performed on scarified skin of the animals’ tails. Animals were anesthetized and four spots on tails were scarified using a 27-gauge syringe needle to scrape the epithelia (not sufficient to cause bleeding) followed by pipette delivery of virus solution using a siliconized pipette tip.

\textbf{Infection of nude mice with wild type or mutant MmuPV1 genomes}

\textit{In vivo} infection with wild type or mutant MmuPV1 genomes was as previously published reports (197, 198, 229, 230) with some modifications. The viral genomes were recovered by excision from the plasmid backbone using the restriction enzyme XbaI, followed by intramolecular religation using T4 DNA ligase, as detailed on the website of the Laboratory of Cellular Oncology (http://home.ccr.cancer.gov/Lco). Animals were scarified as described above and four days post-scarification inoculated with 10 µg recircularized viral DNA (in a 10 µl volume) by injecting with a 30-gauge needle into the scab.

\textbf{Detection of MmuPV1 E1\textsuperscript{E4} spliced transcripts}

Mouse keratinocytes JB6-clone 41 (gift from Dr. Nancy H. Colburn, NCI) were maintained in modified Eagle’s Medium MEM containing 5% FBS and infected with MmuPV1 mutant or wild-type quasivirions at a multiplicity of infection of 10. Forty-eight hours post-infection, total RNA was isolated using the RNeasy kit (Qiagen) and reverse-transcribed into cDNA using
SuperScript III (LifeTechnologies) as per manufacturers’ instructions. E1^E4 transcripts were analyzed by PCR. GAPDH was used as a positive control. Primer sequences for the detection of MmuPV1 E1^E4 spliced transcripts and GAPDH have been published previously (229) and are listed in supplementary table above. PCR products were resolved by Agarose gel electrophoresis.

**Tissue procurement and histochemical analysis**

Skin was harvested, fixed in 4 % paraformaldehyde, and embedded in paraffin. Serial sections (5 µm thick) were analyzed for Keratin markers and BrdU. For immunohistochemistry, sections were deparaffinized and rehydrated with xylenes and graded ethanol, respectively. Endogenous peroxidase activity was quenched with 3% H₂O₂ in methanol and followed with heat-induced antigen retrieval in 10 mM citrate, pH 6.0. Antigen antibody complexes were detected with biotinylated horse anti-mouse/rabbit IgG (Vector Laboratories) and were visualized with 3,3′-diaminobenzidine (Vector Laboratories). Tissues were counterstained with hematoxylin. All images were taken with a Zeiss AxioImager M2 microscope using the AxioVision software version 4.8.2.

**Bromodeoxyuridine incorporation**

To assess cellular proliferation, we evaluated incorporation of bromodeoxyuridine (BrdU) (203806, Calbiochem) at one hour after intraperitoneal injection. Tissue was harvested and processed for immunohistochemistry using a BrdU antibody as described above. For each experimental group (normal skin and papillomas), three slides, each derived from an individual animal, were analyzed by microscopy. Ten random fields of normal skin or the papilloma were
selected on each slide and the total number of epithelial cells and the number of BrdU-positive cells were manually counted. The percentage of BrdU-positive cells was calculated. A two-sided Wilcoxon rank-sum test was used to compare the average percentage of BrdU-positive cells between the two groups.
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Chapter Four: Discussion
The earliest associations of HPV with malignancy was seen with viruses that infected cutaneous skin. Two beta-papillomaviruses, HPV5 and HPV8, were discovered to be found in SCCs. However, the percentage of HPV-associated SCCs compared to the total number of SCCs was extremely low. In fact, only patients suffering from the rare genetic disorder epidermodysplasia verruciformis (EV) were routinely found to have HPV-associated skin cancers (61). Only a few years later the identification of high-risk mucosal HPVs fundamentally altered the field. As detection methods advanced nearly all cervical cancers were found to harbor HPV. The virus genome is commonly found integrated into the host genome and leads to the continuous and dysregulated expression of E6 and E7. E6 and E7 of high-risk mucosal HPVs have become the standard for studying HPV oncogenesis. Much like adenoviruses and polyomaviruses, the viruses capable of transformation were studied more extensively than the viruses that could not transform cells. Research then focused on the activity and functions of high-risk HPV E6 and E7 oncoproteins and how these functions were involved in tumorigenesis. Research of HPV5 and HPV8 and other cutaneous PVs took a back seat to high-risk mucosal types such as HPV16, HPV18, and HPV31 as cutaneous E6 and E7 failed to immortalize or transform tissue culture cells to the extent of the mucosal high-risk HPV types. Study of the high-risk HPVs was nonetheless very fruitful and helped to identify the retinoblastoma protein (RB) and p53 as important tumor suppressors, E6AP, the first E3-ubiquitin ligase with an associated target (231, 232), along with many other important discoveries. This established the model that HPV-induced tumorigenesis was primarily driven by inactivation of tumor suppressors (p53 and RB) through hijacking of host ubiquitin ligases that targeted these factors for proteasomal degradation.
This model of HPV-induced oncogenesis was hard to apply to the beta-HPVs. HPV5 and HPV8 E6 were unable to interact with p53, and had weak to no binding to the E3 ubiquitin ligase E6AP or any other cellular ubiquitin ligase. While E7s from beta-HPVs could activate E2Fs, similar to the low-risk alpha-HPV E7s, they could not destabilize pRB. Despite the lack of the high-risk HPV specific functions, it was demonstrated in mouse models that E6 from beta-HPVs was capable of inducing skin tumors. In the absence of interacting with the p53 pathway or cellular E3 ubiquitin ligases, does beta-HPV E6 interact with other cellular tumor suppressors and inactivate them? The first major focus of my research was to determine the interactome of beta-HPV8 E6 in order to explain their contributions to skin cancers.

No known HPV E6 proteins have intrinsic enzymatic activities and thus alter the cell through disruption and reorganization of host protein complexes. Advancements in mass spectrometry now allow us to interrogate the composition of viral-host protein complexes. This has allowed researchers to explore the interactomes of virus proteins. Several groups have surveyed various PV E6 proteins using mass spectrometry for novel cellular interactors (109, 110, 115). Our group (109, 119) along with two other groups (110, 117) identified that MAML1 interacts with HPV8 E6. BPV1 E6, a cow papillomavirus and member of the delta genus, was also capable of interacting with MAML1 (117, 118). These groups showed that this interaction led to the repression of NOTCH signaling. The NOTCH pathway is critical in regulating epithelial differentiation and its tumor suppressive role in cutaneous carcinomas and its inactivation could help explain HPV8 E6 role is SCCs.

Chapter two seeks to explore the effects of HPV8 E6 on NOTCH signaling in keratinocytes. HES1 is a canonical NOTCH target gene and its mRNA expression increased during calcium differentiation indicative of NOTCH activity. Using calcium differentiation,
HPV8 E6 expressing cells as compared to control keratinocytes failed to increase HES1 levels during six days of differentiation indicating that NOTCH was inhibited (Figure 2.2).

Furthermore, I observed that HES1 levels with the use of a NOTCH inhibitor mirrored the levels seen in HPV8 E6 expressing cells (Figure 2.2C). I also investigated downstream effects of HPV8 E6 inhibition of NOTCH. Involucrin forms part of the cornified cellular envelope and is dynamically expressed in terminally differentiating keratinocytes. It is also positively regulated, although not directly, by NOTCH. Keratinocytes show a dramatic increase in involucrin protein levels during six days of differentiation. This upregulation is largely blocked by both NOTCH inhibition and HPV8 E6 expression (Figure 2.3). This is the first evidence that HPV8 effects keratinocyte differentiation through blockade of NOTCH by E6. Notably the effect on involucrin expression in HPV8 E6 expressing cells is increased when also treated with NOTCH inhibitors. I also observed increased p63 levels in HPV8 E6 expressing cells compared to both control and NOTCH inhibitor treated control cells (Figure 2.3). Taken along with the involucrin data, this suggests that NOTCH inhibition alone does not explain all of HPV8 E6 effects on keratinocytes.

To further explore the effects of HPV8 E6 on NOTCH signaling I utilized a PCR-based pathway analysis. Analysis of NOTCH pathway changes revealed many differentially regulated components between the HPV8 E6 expressing cells and the control cells. Most striking was that HPV8 E6 expressing cells showed lower levels of NOTCH target genes (Figure 2.4). Also of note was the similarity between HPV8 E6 and NOTCH inhibitor treated cells in expression of NOTCH pathway components. This demonstrates that though NOTCH inhibition alone cannot account for all of HPV8 E6 effects, NOTCH inhibition closely resembles HPV8 E6 effects focused on NOTCH pathway and direct target expression (Figure 2.4).
Tan et al (118) suggested a model in which HPV8 E6 was bound to MAML1, NOTCH, and RBPJ the core components of the active NOTCH transcriptional complex. They observed these three components to all be high confidence interacting partners to HPV8 E6. Furthermore along with Brimer et al (117) they suggest that HPV8 E6 requires the C-terminal LXXLL motif of MAML1 for interaction. I sought to further investigate the model and assess if HPV8 E6 interacted with the DNA bound NOTCH transcriptional complex. RBPJ is the only component of the core complex capable of direct DNA binding ability. Regardless of NOTCH activity, RBPJ is bound to DNA, but in the absence of cleaved NOTCH DNA-bound RBPJ associates with co-repressors to silence chromatin and block transcription. Cleaved NOTCH localizes to the nucleus and binds RBPJ displacing co-repressors. The NOTCH-RBPJ complex creates a composite binding surface that recruits MAML, specifically N-terminal alpha-helical domain comprised of the first 1-75 amino acids (152). If MAML requires both NOTCH and RBPJ for recruitment, then HPV8 E6 should be present at NOTCH regulatory sites in seemingly active transcriptional complexes. I tested this hypothesis by assessing the occupancy of HPV8 E6 at two characterized (179) HES1 regulatory sites and found that it occupied both the upstream enhancer and promoter proximal RBPJ binding sites (Figure 2.5C). This places HPV8 E6 in active DNA-bound NOTCH complexes and suggests that HPV8 E6 blocks NOTCH signaling not through sequestration of MAML1 from either NOTCH and RBPJ but inhibits NOTCH through inactivation of the “active” complex perhaps by preventing recruitment of other coactivators (Figure 2.5A). Preliminary efforts to identify C-terminal interactors of MAML1 did not yield any potential co-activators but a more thorough examination of MAML interactors or NOTCH complex components in the presence or absence of E6 is possible.
PV research has long been stymied by the lack of virus that can replicate in genetically tractable laboratory animal. In 2011 a group identified a novel papillomavirus that is capable of infecting laboratory mice (196). Reports indicate that MmuPV1 can productively infect both cutaneous and mucosal tissues (198, 233, 234). Papillomas caused by MmuPV1 infections are capable of progressing to SCCs (199, 235). Ability to cause papillomatosis occurs in most mouse strains that have T-cell deficiencies or are temporarily immune suppressed, but MmuPV1 infections fail to cause papillomatosis in wildtype mouse strains such as C57BL/6 (199, 230, 236). Because of its ability to infect cutaneous skin and cause lesions capable of malignant progression, MmuPV1 may be an important model for determining the role of beta-HPVs in skin cancer.

Early research into MmuPV1 did not focus on molecular functions of viral proteins, so I began to analyze MmuPV1 E6 cellular interactors and functions. Notwithstanding there existing some debate about whether MmuPV1 preferentially targets mucosal or cutaneous tissues (198, 233, 234), the data supports that MmuPV1 can productively infect both tissues. To address this dual tropism, I first sought to compare protein interactors of MmuPV1 E6 to those of the high-risk mucosal HPV16 E6 and the cutaneous HPV8 E6. MmuPV1 E6 interacts with MAML1 and the two receptor SMADs: SMAD2 and SMAD3, consistent with the interactors of the cutaneous beta-PVs (Figure 3.1A). From these simple proteomic studies, it appears that MmuPV1 shares a distinct pool of interactors that can be considered cutaneous-specific. Based on a model that PVs target and inactivate tumor suppressor pathways, I hypothesized that MmuPV1 E6 and HPV8 E6 inhibit these crucial tumor suppressive pathways in keratinocytes and in the case of MmuPV1 this leads to the formation of papillomas that can progress to squamous carcinomas. This leads to a model where mucosal and cutaneous PVs interact with distinct tumor suppressors and
Figure 4.1. Mucosal and cutaneous PVs interact with distinct tumor suppressors. The current model suggests that mucosal PVs inhibit tumor suppressors distinct from those targeted by cutaneous PVs. For example, the ability to directly target p53 is shared by mucosal PVs is a critical distinction between high and low-risk PVs. The cutaneous PVs HPV8 and MmuPV1 target the NOTCH and TGF-beta pathways. Is the ability to inhibit these two pathways necessary for all cutaneous PVs are is this ability only associated with those types capable of carcinogenesis?
inactivate these pathways in order to replicate and explain their contributions to cancer (Figure 4.1.) First, I tested the ability of MmuPV1 E6 to inhibit NOTCH and TGF-beta signaling as compared to HPV8 E6. By luciferase-based reporter assays (Figure 3.1B and Figure 3.1C) and qPCR of endogenous targets of each respective pathway (Figure 3.1D and Figure 3.3C) I demonstrate that MmuPV1 represses NOTCH and TGF-beta transcriptional activity, similar to HPV8 E6. TGF-beta signaling is a potent inhibitor of keratinocyte proliferation and, consequently many cutaneous tumors are TGF-beta resistant (202). If cutaneous E6 proteins are able to inhibit TGF-beta signaling previously suggested (124), then it would be expected that cells expressing these viral oncogenes would be resistant to TGF-beta anti-proliferative effects. I observed that cells expressing E6 from either HPV8 or MmuPV1 were resistant to TGF-beta induced viability changes (Figure 3.3A). This resistance supports a model in which expression of E6 renders the infected cell unable to initiate proper or full withdrawal of cell cycle after activation of TGF-beta signaling.

The TGF-beta pathway is targeted by many HPVs including both alpha and beta-HPVs. This suggests that inactivation of this pathway may be conserved across diverse PV types. HPV16 can inhibit TGF-beta through either E5 (237) or E7 (214) but not E6. HPV5 E6 was shown to target SMAD3 for proteasomal degradation through an unidentified ubiquitin ligase (124). After identifying a functional consequence of TGF-beta inhibition, I was interested in characterizing the mechanism by which cutaneous E6 proteins blocked this pathway. I first sought to confirm that HPV8 E6, similar to HPV5 E6 could destabilize SMAD3. In my experiments, we were never able to observe destabilization of SMAD2, SMAD3, or SMAD4 by either HPV8 E6 or MmuPV1 E6 in either basal or TGF-beta activated conditions (Figure 3.5). I had observed that both HPV8 E6 and MmuPV1 E6 could interact with SMAD2 and SMAD3 but
was never able to detect any binding between E6 and SMAD4 (Figure 3.5). I hypothesized that cutaneous E6 protein interaction with SMAD2 and SMAD3 prevents SMAD4 association, and that consequently the SMAD2/3/4 transcriptional complex fails to stably occupy DNA regulatory sites. I tested this hypothesis by interrogating the occupancy of both SMAD2 and SMAD4 at the SMAD binding element in the regulatory region of CDKN2B and found reduced levels of both SMAD2 and SMAD4 in cells expressing either HPV8 E6 or MmuPV1 E6 (Figure 3.4B). Additional evidence for this model was found in experiments where I immunoprecipitated N-terminally tagged SMAD2 and SMAD3 in cells treated with TGF-b1. I found that transfection of cutaneous E6 expressing vectors diminished the amount of SMAD4 detected (Figure 3.6).

Considering the effects of cutaneous PVs on both NOTCH and TGF-beta it is interesting that the proposed mechanism does not involve ubiquitin ligases or destabilization of host proteins. This is at odds for the main functions of E6 from mucosal types, where oncogenic activities seem to stem from the ability to hijack the cellular E3 ligase E6AP and retarget it to degrade critical tumor suppressor pathways (238). Our proteomic experiments, supported by other studies (110, 115), yielded no good candidate ligases as interactors for either HPV8 E6 or MmuPV1 E6. Cutaneous E6 disruption of NOTCH and TGF-beta signaling expands our understanding of the diversity of how PV oncogenes work. Though HPV8 E6 and MmuPV1 E6 do not appear to be involved in protein turnover and degradation, they still function through reorganization of host protein complexes, specifically key cellular transcriptional complexes. It should be noted that the downstream effects of these signaling pathways including transcriptional targets include many of the same factors downstream of the pathways that high-risk alpha-PVs disrupt. Cell-cycle regulators such as p15 and p21 are inhibited by both mucosal and cutaneous PV types. Activation of apoptotic cell death signaling is downstream of both p53 and NOTCH
both of which are targeted by PV E6 proteins. This demonstrates that though the viruses differ on how they manipulate the host, there seems to remain a core set of targets that are essential for viral replication and, perhaps, tumorigenesis. My current model of HPV8 E6 and MmuPV1 E6 is summarized in Figure 4.2. I next wanted to test whether inhibition of NOTCH by cutaneous E6 required the direct interaction with MAML. All human and many animal PV E6 have been shown to interact with at least one LXXLL containing protein. Many such LXXLL containing proteins have been found to interact with various E6 proteins including E6AP (231, 239, 240), paxillin (241), the related HIC5(242), AP-1(243), IRF3 (244), MAML1 (117-119). The preference and ability to bind specific LXXLL proteins is based both on the composition of the motif and surrounding residues as well as contact residues in the viral protein. BPV1 E6 is capable of binding both E6AP and MAML1. This shared interaction with MAML along with a published structure (95) allowed me to identify a residue that was conserved in both HPV8 E6, MmuPV1 E6, and BPV1 E6, not present in HPV16 E6, and also reported to be a direct contact residue. Using protein structure prediction software I was able to confirm that the mutants were surface exposed (Appendix A). I verified that these mutants were deficient only in MAML1 binding (Figure 3.7A). The HPV8 K142A E6 mutant was still able to retain interaction with p300 and both HPV8 K142A E6, and MmuPV1 R130A E6. These mutants fail to inhibit NOTCH reporter activity as compared to wildtype E6 (Figure 3.7E). I also made mutations in the LXXLL motif found in the extreme C-terminus of MAML1 and show this mutant is resistant to E6 mediated reporter inhibition (Figure 3.7F). These data along with previous studies describing the necessity of the C-terminus of MAML1 for HPV8 E6 interaction (117, 118), demonstrate that E6 inhibition of NOTCH signaling occurs through a direct interaction with MAML proteins.
Figure 4.2. Mechanism of HPV8 E6 and MmuPV1 E6 inhibition of NOTCH and TGF-beta signaling. Cutaneous E6 inhibit the NOTCH and TGF-beta signaling pathways by preventing transactivation of the “active” NOTCH complex and disrupting ability of SMAD4 to stably assemble on the chromatin respectively.
I hypothesized that the cumulative effects of NOTCH and TGF-beta inhibition by either HPV8 E6 or MmuPV1 E6 would result in impaired differentiation of keratinocytes. If this hypothesis were true, I could test in an animal model the consequences of E6 function on replication and pathogenesis. I induced differentiation of the keratinocytes with calcium containing media and tracked the expression of two genes: involucrin and filaggrin. While both genes are expressed during terminal differentiation involucrin is specific to intermediate differentiation and filaggrin is tied to late events. Expression of either cutaneous E6 resulted in lack of expression of both markers as compared to control keratinocytes (Figure 3.8). This impairment was also seen with either treatment with a NOTCH inhibitor, TGF-beta inhibitor, or dual treatment. This suggests that activity of both pathways is involved in commitment to differentiation. Calcium-induced differentiation mimics physiological differentiation in many aspects. Importantly, calcium treatment leads to keratinocyte death after six days of differentiation, which is similar to that seen in organotypic raft cultures. I measured viability of TERT-immortalized keratinocytes and monitored the cells over a 32 day time course. Strikingly HPV8 E6 and MmuPV1 E6 expressing keratinocytes remained viable for 32 days. Control cells showed near complete loss of viability over the time course, which correlated with clearing of cells from the tissue culture plate (Figure 3.9). Previous studies indicate that unlike HPV16 E6, HPV8 E6 failed to extend the lifespan of keratinocytes (245). However, under differentiating conditions HPV16 E6 expressing cells were unable to extend keratinocyte lifespan, but the HPV8 E6 and MmuPV1 E6 expressing cells have a profound survival advantage. This suggests that cutaneous E6 proteins may confer growth advantages only during specific cellular contexts, like differentiation, which are not tested with traditional assays.
NOTCH and TGF-beta activity are both stimulated from extracellular signals. In the case of NOTCH, signaling occurs through cell-to-cell contact from neighboring cells. TGF-beta signaling initiates from binding of soluble extracellular ligands. This is especially relevant in the context of epithelial differentiation as skin differentiates in a directional manner from the proliferative basal layer outward towards the cornified layer. Calcium gradients and extracellular signals such as TGF-beta as well as cell-to-cell contacts are a key mechanism of coordinating and maintaining orderly differentiation (246-248). Reinforcement of differentiation program requires the constant integration of these extracellular stimuli. Cutaneous E6 effectively blocks the integration of two major pathways for maintaining cell identity and driving terminal differentiation. As infected cells leave the basement membrane, they are resistant to extracellular signals and continue to proliferate in a semi-undifferentiated state despite constant signaling to do so. Expanded proliferation and impaired differentiation can be quite evidently seen in papillomas such as those caused by MmuPV1 (Figure 3.10B and Figure 3.10C).

The discovery of MmuPV1 opened the door to many new questions that were as of yet difficult or impossible to explore. The two best studied animal PVs, BPV1 and CRPV replicated in hosts not readily amenable to genetic manipulation. High-risk mucosal PVs can be grown in organotypic raft skin cultures but researchers have been to isolate infectious particles from patient lesions. Cutaneous PVs can be readily obtained from patient materials, but their contributions to human cancers have been harder to identify. MmuPV1 has the potential to possibly overcome these two issues. The virus is able to replicate in lab strains of mice (196). Infection of mice leads to papilloma formation and these lesions can progress to SCC (198, 199, 236). MmuPV1-induced squamous cell carcinomas only seem develop near or within papilloma lesions. Many groups have focused on identifying the preferred tropism of the virus, as reports
indicated that it is able to infect cutaneous as well as mucosal anatomical sites (234, 249). Depending on the preferred tissue the virus infects, may determine whether MmuPV1 better models mucosal or cutaneous PV replication and pathogenesis. I decided to take a different approach and sought first to identify the cellular targets and function of MmuPV1 E6 and to use that information to predict which viruses (mucosal versus cutaneous) this model may better simulate. My research demonstrates that in terms of interactors MmuPV1 E6 appears to be more similar to HPV8 and other cutaneous PVs. Beyond interactors, MmuPV1 E6 functionally inhibits the same pathways that HPV8 E6 does. These effects cumulate in impaired differentiation and increased lifespan of differentiating keratinocytes. I sought to address whether the effects of MmuPV1 E6 on NOTCH and TGF-beta pathways were required for papilloma formation as a precursor to squamous carcinoma. To this end I engineered mutations into the E6 coding region of the MmuPV1 genome. The first mutation introduced a stop codon at the start of the E6 open reading frame eliminating E6 expression while maintaining normal genome size. The second mutation was to introduce the R130A MAML binding mutant. These constructs were incorporated into synthetic MmuPV1 virions and used to infect mice. The results show that papilloma formation requires E6 expression and also the ability to of E6 to interact with MAML1 as no papillomas developed with either mutant (Figure 3.10). This implicates that inhibition of NOTCH is a required step in both viral replication and pathogenesis. Future studies will be necessary to directly test this hypothesis. If true, then replication and papilloma formation of the R130A mutant should be rescued by inhibition of NOTCH. This can be accomplished through chemical inhibition using NOTCH inhibitors, genetically engineered mice that have skin specific NOTCH defects such as RBPJ deletion (250), NOTCH deletions (251, 252), or dominate negative MAML1 expression (253), or perhaps expression of HPV8 E6.
Mice encode for four NOTCH genes (NOTCH1-4) all of which have been implicated to have a role in epithelial differentiation. While NOTCH1 has the strongest phenotype on skin differentiation, the role of each individual NOTCH receptor on MmuPV1 replication can be determined. Since humans also encode four NOTCH genes with high similarity, these data are likely to be applicable to understanding host determinants of human cutaneous PV replication and disease.

My studies identified mutants that specifically abolished binding between cutaneous E6 and MAML1, but were not able to identify SMAD binding defective mutants. These mutants will be beneficial to performing studies in mice similar to those to determine the role of NOTCH, but to determine the role of TGF-beta signaling in MmuPV1 replication and disease. MAML binding mutants were informed by structural information defining residues between the LXXLL region of paxillin and BPV1 E6. Paxillin and MAML1 share a similar LXXLL region based on amino acid sequence (Figure 3.7A), but no structure between E6 and SMAD proteins exist. One strategy that may prove effective in yielding SMAD mutants, however, is based on lessons learned from high-risk alpha-papillomaviruses. Co-crystal structures of HPV16 E6, E6AP, and p53, identified residues of HPV16 E6 necessary for p53 binding (96). Crystal structures indicate that when bound to an LXXLL peptide, E6 adopts a “kidney bean” shape with the LXXLL peptide cradled in the crease of the kidney bean. The two paired CXXC domains lie on opposite ends of the kidney bean. In the case of HPV16 E6 this allows for substrates to be bound on either side of E6AP and to be subsequently ubiquitinated. The N-terminus contained residues necessary for p53 interaction while the C-terminus is known to contain the PDZ binding domain (254). For E6AP-mediated ubiquitination to occur, it is likely that HPV16 E6 simultaneously interacts with both E6AP and p53 and possibly additional proteins such as MAGI-1, an HPV16
degraded PDZ protein. HPV8 E6 and MmuPV1 E6 may similarly simultaneously bind MAML1 and other proteins such as SMAD2 and SMAD3. This would suggest that regions of E6 necessary for their binding would lay outside of the central LXXLL binding pocket.

One frequent hallmark of high-risk mucosal HPV driven cancers is the integration of the viral genome into the host chromosomes. This is a key difference between cutaneous PV association with cancer and mucosal types as integration of cutaneous PV genomes is not often seen even in squamous cell carcinomas of EV patients who have extremely high rates of beta-HPV infection (255). Integration as seen in high-risk PVs leads to dysregulated constitutive expression of the E6 and E7 oncogenes. Transformed cells are addicted to E6 and E7, knockdown or repression of either of these genes activates rapid apoptosis (256, 257). This response is seen with other cancers driven by factors such as RAS (258), MYC (259), or BCL-2 (260). To explain the difference in oncogenic activity between cutaneous and mucosal HPVs it has been proposed that cutaneous PVs are not required for maintenance of the tumor phenotype but are responsible for initiation of the tumor. A so-called “hit and run” model posits that cutaneous HPV types allow for the accumulation of mutations as well as increasing the survivability of keratinocytes after insults such as UVB damage. Once driver mutations accumulate, the viral oncogenes are no longer needed and viral DNA can be lost, explaining their absence in skin cancers. Cutaneous E6 has been heavily implicated to increase survival through inhibition of apoptosis after UVB irradiation (111, 112, 114). My research demonstrates that HPV8 E6 confers enhanced survival of keratinocytes also during terminal differentiation. Loss of function NOTCH and TGF-beta mutations are commonly seen in SCCs and are suggested to be particularly important for initiation steps (157, 193) Moreover, I have shown that the functionally similar MmuVP1 E6 also enhances this survival. 
Additionally, the interaction between HPV8 E6 and p300 has been suggested to allow persistence of UV-induced DNA damage and may contribute to a hit and run model of oncogenesis (114, 115, 191). These studies demonstrate that cutaneous HPV E6 destabilize p300 by blocking p300 association with AKT (115). The decrease in p300 levels correlates with a decrease in the levels of ATM (191) and ATR (114) and this results in the disruption of downstream p53 signaling. Though HPV degradation of p300 was suggested to cause these effects, some caution is warranted as the HPV8 E6 mutant utilized was the same Δ132-136 mutant I tested for MAML binding (Figure 3.7B). While this mutant was not detectably able to interact with p300, it was also unable to bind MAML. This may be due to this particular stretch of amino acids being critical to proper structure and stability (Appendix A1.1). HPV8 E6 and other cutaneous HPVs may alter DNA damage signaling but more specific mutants are necessary to access the role of p300 in these functions. As I have not observed an interaction between MmuPV1 E6 and p300, if MmuPV1 E6 similarly alters ATM and ATR levels as compared to HPV8 E6, then disruption of DNA damage signaling may be both shared among cutaneous PVs and independent of p300. Alternatively, MmuPV1 E6 may utilize a p300-independent mechanism and suggest that inhibition of DNA damage pathways is a critical target of all cutaneous PVs.

The similarities between HPV8 E6 and MmuPV1 E6 suggests that MmuPV1 induction of SCCs may be a relevant model to understand cutaneous HPV contributions to analogous human cancers. In particular, the mouse model can be used to test directly if MmuPV1 contributes to skin cancer in either a direct, “driver”, or indirect, “hit and run,” manner. Since no integration occurs, viruses, such as MmuPV1 which alter the host to provide a conducive environment for early tumorigenesis, can be easily lost as they exist in the cell as extrachromosomal DNA.
Papillomas induced by MmuPV1 are positive for both viral DNA and late gene expression indicative of active replication (199), but it remains to be seen if carcinoma cells retain MmuPV1 genomes. If no viral DNA or viral gene expression is observed, then a “hit and run” model implies the cells were previously infected and the virus was lost during malignant progression. This can be directly tested using mice engineered to respond to the viral transcription factor E2. Synthetic promoter consisting of tandem E2 binding elements driving expression of a recombinase like Cre could be introduced into mice. When E2 is expressed early during viral infection, then Cre is activated, and recombination would lead to expression of a marker such as GFP, marking cells that were infected with MmuPV1. A mouse like this would allow researchers to identify if cancer cells were ever infected by MmuPV1 and also to analyze the surrounding tissue for evidence of infection. Experiments such as these would also be valuable in understanding the role of E6 inhibition of NOTCH, TGF-beta, or other described functions in viral replication, papilloma formation, and tumorigenesis.

Our early hypothesis was that cutaneous E6 proteins serve to alter the host cell environment, in order to allow for genome amplification. I have identified two pathways that when active coordinate cell-cycle exit. Cell cycle inhibitors such as CDKN1A are regulated by a variety of signaling pathways and include p53, NOTCH, and TGF-beta. Many PV types inactivate CDKN1A. Mucosal types target p53 and thereby indirectly downregulate CDKN1A. My research suggests cutaneous PV types target both NOTCH and TGF-beta to down-regulate CDKN1A. Are factors like CDKN1A universally targeted and are there additional novel strategies that different PVs utilize to block these factors? If so, then it would suggest that, regardless of which host factors a particular PV type hijacks, the end goals are similar and predictable. It may then be possible to replace E6 from one PV type to that of another and retain
viral replication. For example, one can test if replication defective MmuPV1 virus with the E6 stop mutation can be rescued with E6 from HPV8 E6 or the even more dissimilar HPV16 E6. Or more directly, is inhibition of CDKN1A alone sufficient to restore viral replication? Outside of viral replication, it would be interesting to see if there are any differences in pathogenesis and oncogenesis.

Many hundreds of PV types have been described that specifically infect humans (261). While animal PVs have been discovered in over 50 different hosts (262), only MmuPV1 is capable of infecting lab strains of mice. Potentially there remain many additional mouse-specific PV types. Of these undiscovered mouse viruses, are there mucosal PVs with similar interactors and pathogenesis of mucosal HPVs? With discovery of more mouse viruses, we may find that they mirror human viruses and group into high-risk and low-risk. What is the difference in cellular interactors of high and low risk mouse PVs? If the interactors are the same, similar to high and low-risk mucosal HPVs, is it the strength that they inhibit tumor suppressors crucial in understanding their oncogenic potential? Evidence for high and low-risk cutaneous HPV types has begun to accumulate, but new HPV types still need to be evaluated. For example a recent study discovered HPV197 as the most commonly present HPV type in skin disease biopsies ranging from actinic keratosis to squamous cell carcinomas (218). These new types may have been missed in previous studies because the methods used were insufficiently sensitive to detect the presence of divergent PVs. As more unbiased approaches using next-generation deep sequencing are performed, it is likely more links between PVs and human disease will be found. The HPV197 E6 interactome has recently been described and identified E6AP, MAML1, Paxillin, and IRF3 as interactors of HPV197 E6 (263). This suggests that HPV197 E6, much like BPV1 E6, is able to bind a diverse set of LXXLL-containing proteins blurring the lines
between cutaneous and mucosal specific E6 binding partners. It will be interesting to see the full extent of activities of the HPV197 oncogenes and how they compare and contrast to both my and other studies of cutaneous PV types and the extensively characterized functions of mucosal PV types.
Appendices
Appendix A. HPV8 E6 and MmuPV1 E6 predicted structures.

**Figure A1.1.** HPV8 E6 predicted structure with mutants labeled. Structure of HPV8 E6 using structure prediction software (264) to model HPV8 E6 sequence on BPV1 structure (PDB accession number: 3PY7) (95). Residues chosen for mutation shown in blue.
**Figure A1.2. MmuPV1 E6 predicted structure with mutants labeled.** Structure of MmuPV1 E6 using structure prediction software (264) to model MmuPV1 E6 sequence on BPV1 structure (PDB accession number: 3PY7) (95). Residues chosen for mutation shown in blue.
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