EXPRESSION OF HUMAN PAPILLOMAVIRUS TYPE 16 E7 IS SUFFICIENT TO SIGNIFICANTLY INCREASE EXPRESSSION OF ANGIOGENIC FACTORS BUT IS NOT SUFFICIENT TO INDUCE ENDOTHELIAL CELL MIGRATION

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DEDICATION

This thesis is dedicated to my wonderful parents, Guy and Margie Walker, for their love, support, encouragement, prayers and imparting strength and wisdom from which I drew from daily during the pursuit of my Ph.D. To my siblings, Angie, Zakee, Fat Daddy, Kemmy Pooh and Big Boy, thank you for your love and encouragement. To my Aunt Ardean who is no longer with me on Earth but in spirit, thank you for getting me off to a great start so that I can fly beautifully.

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ABSTRACT

Joanna Antigone Walker

EXPRESSION OF HUMAN PAPILLOMAVIRUS TYPE 16 E7 IS SUFFICIENT TO SIGNIFICANTLY INCREASE EXPRESSION OF ANGIOGENIC FACTORS BUT IS NOT SUFFICIENT TO INDUCE ENDOTHELIAL CELL MIGRATION

Human papillomavirus 16 (HPV 16) causes cancer. Two viral oncoproteins of HPV 16, E6 and E7, are consistently expressed in these cancers. HPV 16 E6 and E7 proteins target p53 and Rb family members, respectively, for degradation thus inactivating the functiond of these tumor suppressor proteins. Tumor development requires the acquisition of a blood supply, a process known as angiogenesis. Tumor suppressors negatively regulate angigogenesis. Expression of HPV 16 E6 and E7 together in human foreskin keratinocytes (HFKs) increases the level of angiogenic inducers vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8). Further, conditioned media from such cells are sufficient to alter endothelial cell behavior both *in vitro* and *in vivo*. To determine the individual contributions of HPV E6 and E7 to angiogenesis, translational termination linkers (TTLs) were inserted into the coding region of E6 or E7. Following retroviral transduction of the mutated cassette into HFKs, the ability of E7 in the context of the E6TTL mutation (E6TTLE7) and E6 in the context of the E7TTL mutation (E6E7TTL) to induce VEGF and IL-8 was compared to the LXSN control retrovirus. E7 and, to a lesser extent E6, increased the expression of VEGF and IL-8. Migration of human microvascular endothelial cells was not induced using conditioned media from either E6 or E7 expressing cells. Since the increased levels of VEGF and IL-

8 induced by HPV 16E6ETTLE7 were not sufficient to alter endothelial cell behavior, immunological depletion experiments were used to determine whether either angiogenic factor was required for HPV 16E6 and E7 together to induce HMVEC migration. Only VEGF was required. Preliminary data suggest that the ability of HPV 16 E7 to induce angiogenic factors is dependent upon degradation of a specific Rb family member.

Ann Roman, Ph.D., Chair

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ABBREVIATIONS

AP-1 Activator protein 1
ATCC American Type Culture Collection
bFGF Basic fibroblast growth factor
BM Basal Media
BM Basement membrane
BPE Bovine pituitary extract
CAFs Carcinoma-assoicated fiboblasts
CDK2 Cyclin-dependent kinase 2
CDK4 Cyclin-dependent kinase 4
CDK6 Cyclin-dependent kinase 6
C/EBP CAAT/ enhancer-binding protein
CKSFM Complete-keratinocyte serum free media
CM Conditioned media
CS Calf serum
cDNA complementary deoxyribonucleic acid
DFO Deferoxaminemesylate
DMEM Dulbecco's modified eagle's medium
DMSO Dimethyl sulphoxide
E6-AP E6 associated protein
EBV Epstein-Barr virus
ECM Extracellular matrix
EDTA Ethylenediaminetetraacetic acid
EGF Epidermal growth factor
EGM-2 Endothelial cell growth medium 2
ELISA Enzyme-Linked Immunosorbent Assay
EtBr Ethidium bromide
FADD Fas-associated death domain
FBS Fetal bovine serum
FCS Fetal calf serum
FGF Fibroblast growth factor
HDACs Histone deacetylases
HFKs Human foreskin keratinocytes
HIF-1 Hypoxia-inducible factor 1
HIF-1α Hypoxia-inducible factor alpha
HIF-1β Hypoxia-inducible factor beta
HIF-2 Hypoxia-inducible factor 2
HMVEC Human Microvascular endothelial cell
HPFs High power fields
HPV Human papillomavirus
HPV-16 Human papillomavirus 16
HRP Horse peroxidase
HUVEC Human umbilical vein endothelial cell
H Hypoxia
IAP-2 Inhibitor of apoptosis 2

IGF-1 Insulin-like growth factor 1
IL-8 Interleukin-8
K14 Human keratin 14
LAT Linker for activation of T cells
LCR Long control region
LMP-1 Latent membrane protein 1
LTR Long terminal repeat
MCP-1 Monocyte chemotactic protein 1
MMLV Moloney Murine Leukemia Virus
MHC Major Histocompatability Complex
MMP-2 Matrix metalloproteinase 2
MMP-9 Matrix metalloproteinase 9
mRNA Messenger ribonucleic aci
NDFs Normal diploid human fibroblasts
Neo Neomycin
NFκB Nuclear factor kappa B
N Normoxia
NZB Obstructive airway disease
OD Optical density
ORFs Open reading frames
PBS Phosphate buffered saline
PDGF Platelet-derived growth factor
PIGF Placenta growth factor
PyMT Polyoma virus middle T oncoprotein
Rb Retinoblastoma protein
RT-PCR Reverse transcription polymerase chain reaction
SEM Standard error of mean
SDF-1 Stromal cell derived factor 1
SDS Sodium dodecyl sulfate
SRC-1 Steroid receptor coactivator 1
SV40 Simian virus 40
TAE Tris-acetate-ethylenediamine tetraacetic acid
TAFs Tumor-assoicated fibroblasts
TAM Tumor-assoicated macrophage
TBSt Tween-20
TGFβ Transforming factor beta
TNFα Tumor necrosis factor alpha
TNFR1 Tumor necrosis factor receptor 1
TGF-β Transforming Growth Factor beta
TTLs Translation termination linkers
TNF-α Tumor necrosis factor alpha
TSP-1 Thrombospodin 1
VEGF Vascular endothelial growth factor
VHL von-Hippel-Lindau
VPF Vascular permeability factor

INTRODUCTION

I. Human Papillomaviruses

Human papillomaviruses (HPVs) are small double-stranded DNA viruses that infect mucosal and cutaneous stratified epithelia of the anogenital tract, head and neck, hands or feet (1, 106). The genomes of these viruses, which are approximately 8 kb, have virtually the same genomic organization, which can be divided into three main regions: the early, late and long control regions (1). The early region of the genome contains the open reading frames (ORFs) for the early proteins E1-E7, which are necessary for viral replication, transcription and cellular transformation (1). The late region, encodes the viral capsid proteins, L1 and L2 (Figure 1). The long control region (LCR), is neither transcribed or translated but contains a number of cis elements which are responsible for regulating viral transcription and replication (37). E5, E6 and E7 are considered the oncogenes although E5 is deleted in cervical cancer cells (149).

The HPV replication cycle is differentiation dependent and can be divided into a productive and non-productive stage. (37,106). Normally proliferation occurs in the basal cells and as they begin to migrate upwards these cells undergo differentiation and eventually exit the cell cycle. On the other hand, HPV infected suprabasal cells fail to exit the cell cycle. HPV genomes are established as extrachromosomal genomes, which replicate in synchrony with the cellular DNA. After cell division, daughter cells migrate into the suprabasal compartment, where uninfected keratinocytes undergoes terminal differentiation, while HPV-infected cells enter the S-phase of the cell cycle resulting in the production of infectious viral progeny (164).

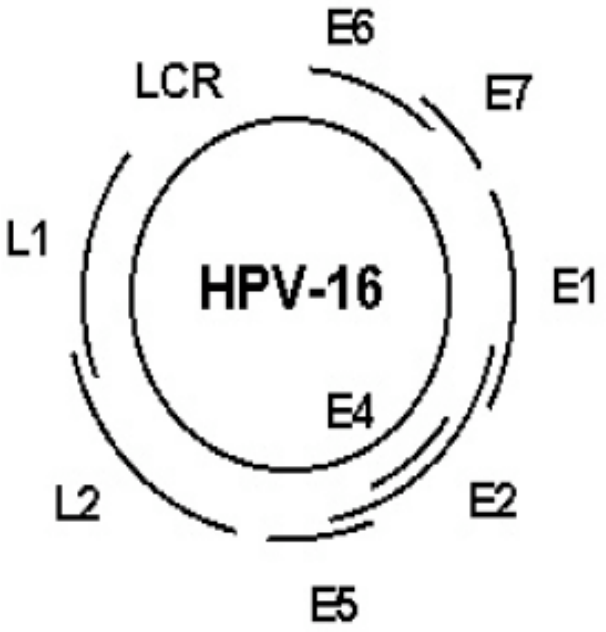

Figure 1. Organization of the HPV 16 Genome.

HPV 16 contains an approximately 8 kb circular double stranded DNA genome. The genome is divided into three basic sections: early (E1-E7), late (L1 and L2) and long control region (LCR). Adapted from (1).

II. Human Papillomaviruses and Cervical Cancer

To date there are more than 200 types of HPVs that have been identified. These viruses can cause a wide range of diseases from benign genital warts to invasive cancer. There are 40 types that infect the mucosa and based on their oncogenic potential these viruses can be divided into two groups: high-risk HPV or low-risk HPV (117). The high-risk HPV types such as HPV 16, 18, 31, 33 and 45 are mainly associated with the development of high-grade intraepithelial neoplasias of the anogenital tract, whereas low-risk HPV types such as HPV 6 and 11 are mainly associated with low-grade abnormalities such as benign condyloma acuminatum or anogenital warts. The high-risk HPV type 16 and 18 are responsible for more than 70% of cervical cancer cases (120). Persistent infection with these high-risk types is thought to be a precursor to neoplastic transformation (120).

III. Biological Functions of E6 and E7

The two early proteins of HPV 16, E6 and E7, play a significant role in tumor formation. E6 and E7 proteins are involved in cellular transformation (39, 140). Both *in vitro* and *in vivo* studies show that the functions of high-risk E6 and E7, of are necessary for the induction and maintenance of the transformed phenotype of cervical cancer cells by hijacking cellular pathways (13, 79, 113). The transforming properties of E6 and E7 proteins result, in part, from their ability to modulate many host proteins that regulate cell growth and differentiation.

HPV 16 E6, a small protein of approximately 150 amino acids, can localize to both the nucleus and cytoplasm of HPV infected keratinocytes (61). E6 contributes to cellular transformation by binding to and targeting p53 for ubiquitin-mediated

degradation (29, 147). p53 functions as a checkpoint for the integrity of cellular DNA before S-phase entry of the cell cycle. Normally, p53 is maintained at low levels and is inactive. Upon DNA damage or unscheduled induction of DNA replication, p53 is activated and this sends signals to the cell to undergo cell cycle arrest or apoptosis at the G1/S and G2/M checkpoints. The ability of p53 to initiate signaling pathways to trigger cell cycle arrest or apoptosis is thus lost in the presence of E6 and this allows for unscheduled replication of damaged DNA.

E6 mediated degradation of p53 involves the E6 associated protein (E6-AP) (Figure 2). E6-AP functions as an E3 ubiquitin ligase and only in the presence of E6 can E6-AP ubiquitylate p53 so it can be recognized by the proteasome. E6 mutants, defective for binding to E6-AP but retaining p53 binding, are incapable of mediating p53 degradation. Low-risk HPV E6 proteins bind p53 with a reduced affinity and do not participate in its degradation. Low-risk HPV E6 can bind to the p53 C-terminus but only high-risk HPV E6 has the ability to bind to the core region. Binding of the core region is required for p53 degradation by high-risk HPV E6 (30, 101).

High-risk E6 proteins also interact with a number of other host proteins. For example, E6 has been reported to prevent apoptosis by inhibiting *Bax* gene expression in human keratinocytes (110). Inhibition of the *Bax* protein results in inhibition of apoptosis and therefore cells accumulate mutations in their DNA. High-risk E6 proteins also prevent apoptosis by binding to tumor necrosis factor receptor 1 (TNFR1) and inhibiting TNFR1 apoptotic signaling (46). Other cellular targets include Bak, Fas-associated death domain-containing protein (FADD) and procaspase 8, which is degraded by E6/E6AP

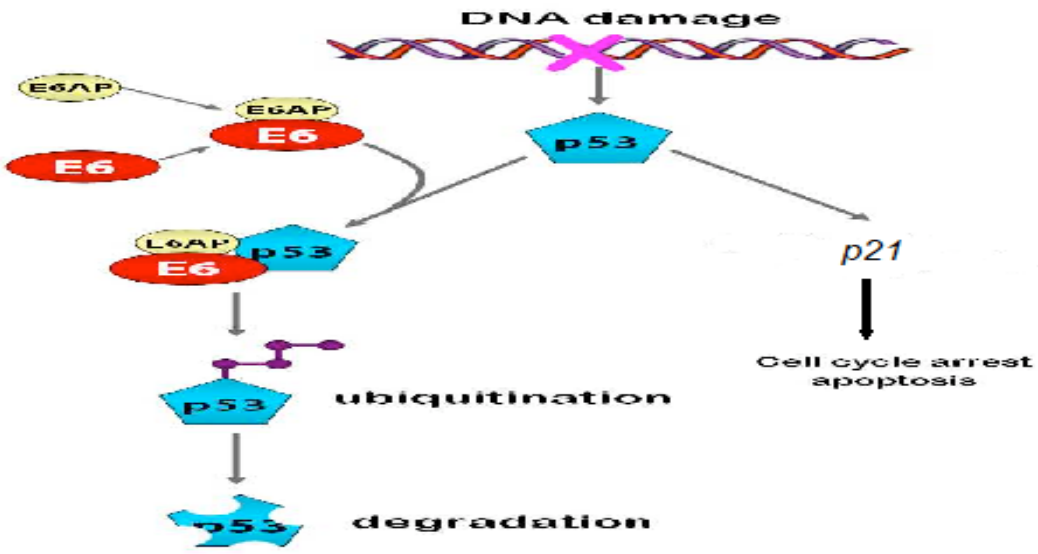

Figure 2. High-risk E6 targets p53 for degradation.

Normally, the level of p53 in normal cells is very low. In response to DNA damage, the level of p53 increases, induces downstream target p21 and sends signals to the cell to undergo cell cycle arrest or apoptosis. Degradation of p53 occurs through a complex containing E6 and E6-associated protein (E6AP). E6AP acts as an E3 ubiquitin ligase that ubiquitinates p53 and targets it for degradation by proteasome.

causing inhibition of apoptosis (56, 171). HPV E6 also downregulates the expression of Notch1 (167, 188). Decreased expression of Notch1 in cervical cancer cells contributes to tumorigenesis. HPV 16 E6 also activates nuclear factor kappa B (NF-κB) leading to enhanced expression of inhibitor of apoptosis 2 (IAP-2), which prevents apoptosis of epithelial cells (123). HPV E6 also binds and inactivates the transcriptional coactivators CBP and p300 (133, 195) which results in downregulation of transcription from the interleukin-8 promoter (77).

A. E6 Interaction with PDZ Domain proteins

E6 interaction with PDZ-domain-containing proteins, which are involved in cellular functions such as cell signaling and cell adhesion, leads to their degradation (81, 162, 173, 184). Low-risk HPV E6 does not contain the PDZ-binding motif and therefore cannot PDZ-domain-containing proteins (172). Mutations within this motif result in a loss of the PDZ-binding ability of E6. Conversely, introduction of the PDZ-binding motif from a high-risk E6 into the C-terminus of a low-risk E6 protein results in gaining the ability to target and degrade PDZ domain proteins (55, 135). Interaction of E6 with numerous proteins contributes to E6 effectiveness in the promotion of HPV oncogenicity (178).

B. E7 Protein

HPV 16 E7, a small nuclear protein of 98 amino acids, is best known for its association with the pocket proteins retinoblastoma protein (Rb), p107 and p130 (197) which regulate E2F transcription factors (51) (Figure 3). E2F transcription factors are heterodimers composed of E2F1-8, which are vital for regulation of G1 exit and S-phase

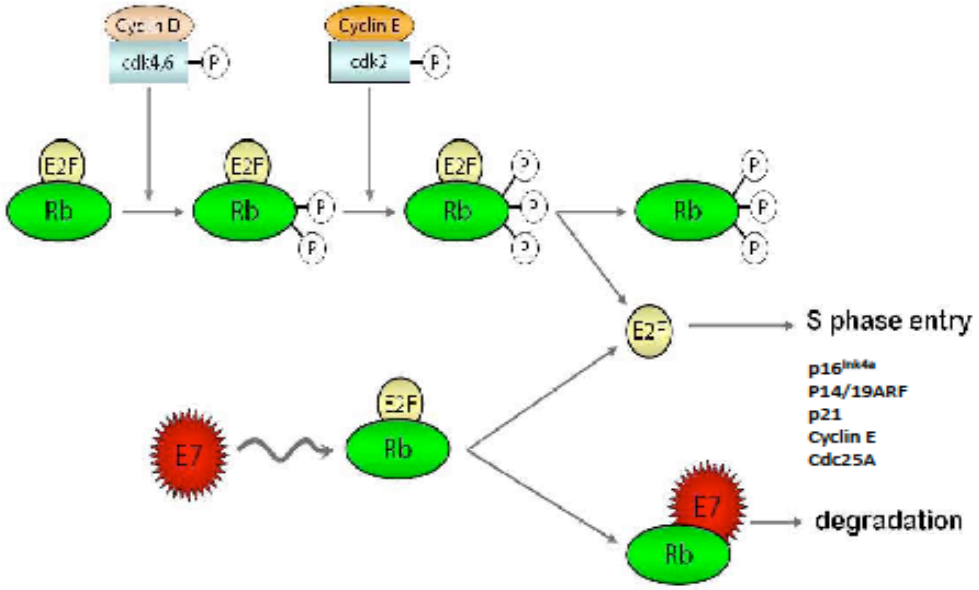

Figure 3. High-risk E7 targets Rb for degradation.

Rb is active in its hypophosphorylated form and binds to E2F transcription factors to prevent S-phase entry. During the normal cell cycle, pRb is phosphorylated by cyclin D1/cyclin-dependent kinase 4 (CDK4) and cyclin E/CDK2 complexes causing dissociation of pRb from E2F thereby allowing S-phase progression. HPV E7 binds to the hypophosphorylated pRb, preventing its interaction with E2F. Therefore, the checkpoint Control at G1/S transition is lost and the cells continually traverse the cell cycle leading to uncontrolled proliferation.

progression. In uninfected cells, Rb is hypophosphorylated in early G1 and bound to E2F transcriptional factors 1-3, repressing their transcriptional activity. Upon phosphorylation triggered by CDK4/6 and CDK2, the complex dissociates, allowing these E2Fs to act as a transcriptional activators. Association of E7 with hypophosphorylated Rb, prevents its binding with E2F, thereby promoting uncontrolled cell cycle progression (21). Additionally, E7 ubiquitin-mediated degradation of Rb involves association and reprogramming of the cullin-2 ubiquitin complex (78). Low-risk HPV E7 binds to Rb family members with lower affinity than the high-risk HPV 16 E7 (22, 54, 119). For Rb binding, this difference is due to an Asp 21 in HPV 16 E7 compared to a Gly 22 in HPV 6E7 (69, 144).

C. E7 Interaction with Cyclin-dependent Kinase Inhibitors

E7 has been shown to interact with various cellular targets. For example, E7 interaction with cyclin-dependent inhibitors $p21^{Cip1}$ and $p27^{KIP1}$ abrogates their growth inhibitory activities (53, 82, 190), which are induced in various ways such as through activation of p53 and anti-proliferative signals (41). Interestingly, the steady state levels of $p21^{CIP1}$ are increased during differentiation of keratinocytes and, through some unknown non-transcriptional mechanism in the presence of E7.

D. E7 Interaction with Histone Deacetylases (HDACS)

E7 interactions with HDACs, which function as corepressors by chromatin remodeling, results in an increase in the level of E2F2- mediated transcription in differentiating cells (107). Other E7 binding partners include members of the AP1 family, which play a role HPV transcription (58, 74, 169). AP-1 binding sites are found in the

regulatory regions of both high-risk and low-risk HPVs. Also, E7 can directly or indirectly interact with histone acetyl transferases (86), including p300, pCAF and steroid receptor coactivator-1 (SRC1) (7, 77).

IV. RNA Splicing Regulates E6 and E7 Expression

RNA splicing is necessary for gene expression and vital during the papillomavirus life cycle. In a natural infection, HPV 16 E6 and E7 are transcribed as a single bicistronic transcript (Figure 4). The p97 promoter is positioned upstream of the E6 open reading frame and its role is to initiate transcription of E6 and E7. Within the E6 coding region there is one 5' splice donor (226) and three alternative 3' splice acceptors (409, 526, and 742). Unspliced mRNA is translated into E6. mRNA spliced from nt 226 to nt 409 E6 is referred to as E6*I and encodes a truncated E6 protein called E6*I. Splicing from nt 226-526 created truncated E6*II and splicing from nt 226 to 742 creates truncated E6*III (Figure. 4). The majority of transcripts within cancer tissues and cervical cancer cell lines produce E6*I (15, 38, 152, 193, 194). Splicing depends on the distance of the splice donor from the promoter (194). Translation of E7 is dependent upon splicing (194). The distance between the termination of E6 translation and the reinitiation of E7 translation is 2 nucleotides. E6*I contains a premature codon immediately downstream of the splice junction creating enough space for termination of E6 translation and the reinitiation of E7 translation (93) therefore benefiting the expression of E7.

Splicing is inefficient in the context of the retrovirus, L(16E6E7)SN, encoding HPV 16E6E7 within the LXSN retrovirus. E6 and E7 expression is dictated not by the

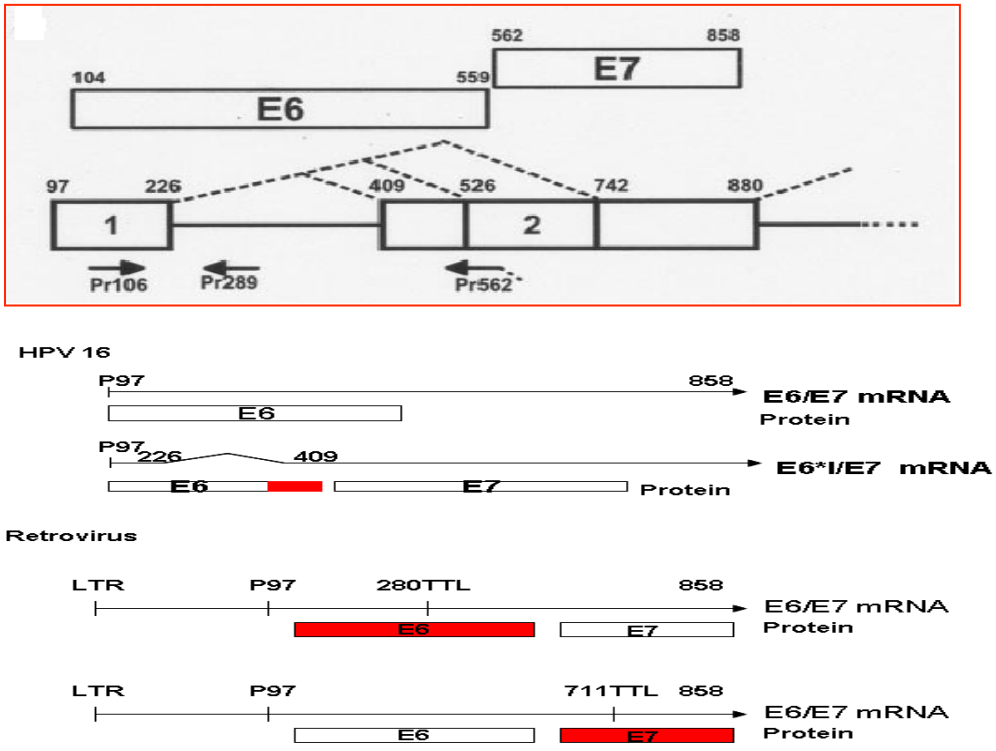

Figure 4. Diagram of splice message within HPV 16 versus unspliced message within the retrovirus HPV 16E6E7.

Exons are represented as boxes and introns are lines between the boxes. Numbers above the ORFs are nucleotide positions for each viral gene. Dashed lines from 5' splice site indicate the splice acceptor for each 3'splice site. The arrows below the bicistronic transcript are primers used for RT-PCR analysis in Chapter 1. The lower schematic shows the unspliced and spliced mRNA and corresponding encoded protein.

P_{97} promoter but by the retroviral long terminal repeat (LTR). The mRNA in cells infected with the retrovirus undergoes splicing less frequently due to the distance between the LTR and initiation of E6 transcription (194).

V. Angiogenesis and Tumor Suppressors

The establishment and maintenance of a vascular supply is required for growth of malignant tissue. Consistent with this, progression of the pre-malignant stages to invasive cervical cancer is associated with the development of new blood vessels. Angiogenesis is the recruitment of new blood vessels by extension of preexisting vasculature (16, 48). Angiogenesis is crucial for a developing tumor to acquire nutrients and oxygen for sustained growth and expansion (16).

Angiogenesis is tightly regulated by several secreted factors. According to the current model of angiogenesis, vessels seen in normal tissue are quiescent due to the presence in the microenvironment of elevated levels of angiogenic inhibitors such as thrombospondin-1 and maspin, relative to angiogenic inducers, such as VEGF and IL-8 (16, 67)(Figure 5). As cells progress towards tumorigenicity, the balance between angiogenic inducers and angiogenic inhibitors switches, such that angiogenic inducers dominate. This may occur by several mechanisms: cells may decrease the amounts of angiogenic inhibitors being secreted while continuing to secrete the same amounts of inducers, cells may increase the amounts of angiogenic inducers being secreted while continuing to secrete the same amounts of inhibitors, or both events may occur.

In tumor cells, a link between the functions of tumor suppressors and angiogenesis has long been established (59). Following HPV 16 infection, the E6 and E7 oncoproteins are consistently expressed. This results in disruption of the normal function of tumor suppressors, p53 and pRb, leading to unrestricted cell cycle entry and deregulated proliferation of the infected cell but also alteration in expression of angiogenic factors and induction of angiogenesis (23, 177).

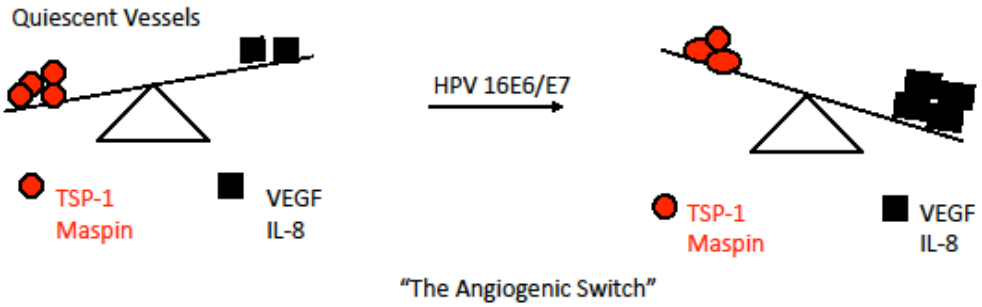

Figure 5. Current Model of Angiogenesis.

In quiescent or normal tissue, inhibitors of angiogenesis predominate and the angiogenic switch is off. Angiogenesis can be achieved by increase of inducers and/or decrease of inhibitors such that the balance is tipped in favor of a pro-angiogenic phenotype i.e angiogenesis is turned on. The circles represent angiogenic inhibitors and boxes represent angiogenic inducers.

VI. Regulation of Angiogenic Factors

There are a number of factors that have been known to induce angiogenesis (16, 25). Among the known factors, vascular endothelial growth factor (VEGF) serves as a major regulator of the angiogenic processes and has been the center of numerous investigations. The VEGF family of glycoproteins is composed of VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PIGF). VEGF-A, also known as vascular permeability factor (VPF), has four main isoforms ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$) produced by alternative splicing (75, 175). A fifth isoform $VEGF_{145}$is found within the reproductive tract of females, and has also been found in placenta and carcinoma cells. $VEGF_{189}$ and $VEGF_{206}$ are the most abundant forms. $VEGF_{189}$ is present throughout the tissues, whereas $VEGF_{206}$ is only found in embryonic tissue. Both are sequestered in the extracellular matrix. $VEGF_{165}$ and $VEGF_{121}$ are the most frequently secreted, however, $VEGF_{165}$ is the mature isoform (98).

VEGF stimulates migration and proliferation of microvascular endothelial cells derived from arteries, veins, and lymphatic vessels (45) as well as angiogenesis *in vivo* and *in vitro*. For example, Phillips, et al. reported (134) that VEGF stimulated direct angiogenesis in the rabbit cornea. Similarly, Tolentino, et al. (176) reported that VEGF was sufficient to produce iris neovascularization and neovascular glaucoma in primates. VEGF also induces lymphangiogenic responses in mice (122). When serum is lacking, VEGF prevents apoptosis of endothelial cells *in vitro* (57). Cells of many human solid tumors express large amounts of VEGF protein, thus stimulating the development of new blood vessels within the developing tumor.

Hypoxia plays a major role in regulating the expression of VEGF in both malignant and normal cells (114, 154). The response of VEGF to hypoxia is primarily regulated through the activity of the transcription factor hypoxia-inducible factor-1 (HIF-

1). HIF-1 is composed of two subunits, HIF-1α and HIF-1β; only HIF-1α is regulated by low oxygen tension (10, 18). Under normoxia, two prolyl residues (Pro^{402} and Pro^{562}) of the HIF-1α subunit are hydroxylated by oxygen-dependent prolyl hydroylases. The hydroxylated protein is targeted for degradation through its interaction with the von-Hippel-Lindau (VHL) proteasome pathway (42).

During hypoxia, prolyl hydroxylation is inhibited, blocking VHL-mediated degradation, resulting in the accumulation of HIF-1α within the cell. This in turn activates expression of HIF-1 target genes including VEGF (10, 179). Knocking out HIF-1α results in embryonic lethality due to impaired vascular formation (143).

The expression of VEGF is also enhanced by a number of factors independent of hypoxia and HIF-1α. For example growth factors such as epidermal growth factor (71), transforming growth factors (TGFα and TGFβ), insulin-like growth factor 1 (IGF-1), fibroblast growth factors (FGF), platelet-derived growth factors (PDGF) all may regulate VEGF expression possibly through an autocrine or paracrine effect (43, 125). In addition to growth factors, oncogenes have also been known to induce expression of VEGF. Oncogenic ras and myc, major regulators of angiogenesis, regulate expression of VEGF (12, 138, 182) under *in vitro* and *in vivo* conditions.

A link between VEGF and the progression of disease in HPV 16 transgenic mice and in cervical carcinogenesis has been shown (155). E6 and E7 proteins of high risk HPVs affect proteins known to regulate angiogenesis. E6 under some conditions can increase expression of c-myc and under other conditions can target c-myc for degradation (87). Both E6 and E7 are required for maintenance of c-myc expression (33). E6 and E7 can enhance the expression of VEGF (23) through many possible mechanisms. VEGF expression can be regulated by NFκB and activator protein -1 (AP-1). E6 has been

reported to transactivate genes via NFκB and E7 can enhance AP-1 mediated transactivation (3, 36).

Interleukin-8 (IL-8), which is produced by macrophages, is another important mediator of tumor-derived angiogenesis. IL-8 is normally secreted in response to growth factors, inflammatory cytokines, and pathophysiologic conditions (94, 183, 186). The increased expression of IL-8 correlates with amplified neovascularization density (109, 187)as well as an increase in tumor growth (109). IL-8 expression was first discovered in malignant melanoma cell lines, and IL-8 expression is considered to play a role in regulating the growth and metastasis of melanoma (189). The ability of IL-8 to increase levels of matrix metalloproteinase-2 (MMP-2), which degrades the extracellular basement membrane and remodels the extracellular matrix (ECM), initiates the early phase of tumor angiogenesis (89). IL-8-transfected melanoma cells displayed increased levels of MMP-2, while transfection of identical melanoma cells with VEGF and bFGF did not affect MMP-2 levels (88), which demonstrated that IL-8 is important for tumor-induced angiogenesis. Endogenous expression of IL-8 has also been detected in numerous human cancers including breast cancer (62), colon cancer (19), ovarian cancer (11), prostate cancer (181) and cervical cancer. IL-8 was found to be upregulated in oral squamous cell carcinomas and to be affected by HPV gene expression (105, 177).

IL-8 has been shown to induce proliferation and chemotaxis of human umbilical vein endothelial cells (HUVEC) and human aortic endothelial cells (91, 166). IL-8 has also been shown to stimulate both endothelial proliferation and capillary tube formation *in vitro*; the effect can be reversed using neutralizing antibodies to IL-8 (153, 180).

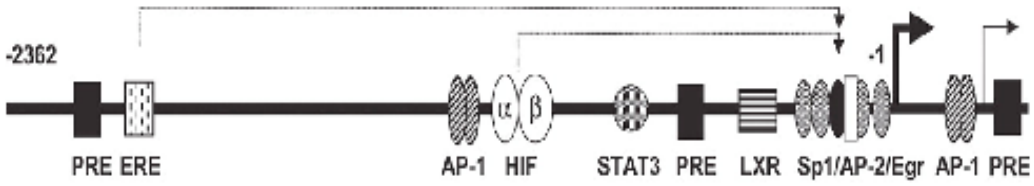

Figure 6. The VEGF promoter: response elements to trans-activating factors.

The entire region of the VEGF promoter is represented. The heavy arrow indicates the classical initiation site of transcription, whereas the light arrow indicates the position of the cryptic promoter present in a domain of the gene described as the 5′ untranslated region of the VEGF mRNA.

The 5' flanking region of the IL-8 gene is sufficient for transcriptional regulation of IL-8 gene expression (68, 115). The IL-8 promoter contains binding sites for AP-1, nuclear factor kappa B (NFκB) and CAAT/ enhancer-binding protein (C/EBP). NFκB induction is necessary for the induction of IL-8 expression and both NF-κB and AP-1 sites have been shown to be responsible for expression of IL-8 in certain cancers (96). As noted earlier, E6 and E7 can activate these transcription factors (3, 36). In all, IL-8 expression in cancer cells and the tumor microenvironment plays a vital role in enhancing tumor progression.

VII. Tumor Microenvironment and Angiogenesis

Angiogenesis is crucial for tumor development. Solid tumors cannot grow beyond a few millimeters without a sufficient blood supply (49). As stated previously, angiogenesis depends on a balance between angiogenic inhibitors and inducers. During tumor progression, this balance is tipped favoring proangiogenic events and the angiogenic switch is turned on.

The tumor microenvironment contains a mixture of extracellular matrix (ECM) proteins, tumor cells, endothelial cells, fibroblasts and immune cells that turn the angiogenic switch on. The extracellular matrix provides structural support to cells and tissues, transmits signals through receptors, and binds and stores growth factors and other active molecules. Basement membranes (BM) are ECM that function as barriers, polarize cells, shape tissue structures and support migrating cells (65). Vascular BM support blood vessel endothelial cells (84) and modulate endothelial cell behavior (31).

Figure 7. The IL-8 promoter.

The entire region of the promoter is represented. The heavy arrow indicates the classical initiation of transcription.

Many ECM proteins, such as collagens and laminins, have proangiogenic properties. They help promote endothelial cell survival, proliferation and migration (127). Many pro-angiogenic factors such as VEGF, basic fibroblast growth factor (bFGF) and transforming factor beta (TGF-β) are sequestered in the ECM and can be mobilized during ECM degradation by proteases secreted by tumor or stromal cells (159).

Fibroblasts regulate ECM degradation, epithelial differentiation and behavior, inflammation, wound healing and cancer progression. Stromal fibroblasts, carcinoma-associated fibroblasts (CAFs) or tumor-associated fibroblasts (TAFs) have the ability to promote tumor growth (85, 131). Olumi, et al. showed that CAFs have tumor promoting ability in prostate carcinomas (129). CAFs from invasive breast carcinomas promote the growth of mammary carcinoma cells and enhanced tumor angiogenesis (130).

CAFs secrete elevated levels of stromal cell-derived factor (SDF-1), which plays a vital role in the promotion of tumor growth and angiogenesis. SDF-1 produced by immune cells also induces VEGF in endothelial cells (73). VEGF induces vascular permeability resulting in an influx of endothelial cells, fibroblasts and inflammatory cells (20). Fibroblasts are the main source of VEGF in tumors (52). Fibroblasts are involved in the production and storage of latent VEGF in the extracellular environment.

Chronic inflammation is associated with most cancers. The immune cells play a dual role in cancer progression (96). On the one hand the immune system is capable of recognizing and attacking cancer cells. On the other, evidence suggests that cancer associated inflammation promotes tumor growth and progression. The cancer associated inflammation starts with migration and infiltration of leucocytes from the blood stream to the site of inflammation followed by the infiltration of monocytes, plasma cells and

lymphocytes. Tumor cells produce a variety of growth factors, cytokines and chemokines, such as VEGF, bFGF and PDGF that attract leucocytes and stimulate migration of mast cells.

The link between chronic inflammation, premalignant progression, tumor growth and metastasis has been shown. Arbeit, et al. and Coussens, et al. utilized a transgenic mouse model of multistage epithelial carcinogenesis. In this model the early genes of HPV 16 were expressed under the control of the human keratin 14 (K14) promoter to produce (K14-HPV 16) transgenic mice(5, 25). Despite transgene driven oncogene expression in keratinocytes, the absence of mast cells or the lack of innate immune cell recruitment to premalignant skin prevents neoplastic progression. Secretion of IL-8 by xenograft tumor cells is required for RasV12-dependent tumor-associated inflammation, tumor vascularization and tumor growth (161). Depletion of granulocytes abolishes angiogenesis of RasV12-expressing tumors, therefore suggesting that the recruitment of granulocytes by neoplastic cells facilitates tumor growth (161).

Macrophage infiltration is critical in the wound healing process. When tissue damage occurs, macrophages infiltrate the wound to participate in the repair process. Macrophages aid in tissue matrix remodeling, cell growth and angiogenesis (112). One important inflammatory cell type found in the stroma of the tumor is the tumor-associated macrophage (TAM) (8). TAMs have an important role in tumor progression by exerting their immune functions in tumor cell proliferation, tumor cell invasion and tumor angiogenesis. TAMs are found in precursor lesions of cutaneous melanoma, squamous and cervical carcinoma, and mammary adenocarcinoma (27, 90, 104). Precursor lesions containing elevated numbers of TAMs may sabotage normal macrophage associated

processes and aid with the invasion of neoplastic cells into the surrounding stroma (104).

The proangiogenic function of TAMs has been demonstrated in vivo. In polyoma virus middle T oncoprotein (PyMT)-induced mouse mammary tumors, macrophages were recruited to the lesions (100). Depletion of macrophages in PyMT-induced tumors decreased vascular density(100). Bingle et al. showed that by using a mouse dorsal skinfold chamber model, which allowed in situ visualization and monitoring of implanted tumor spheroids and the surrounding blood vessels in vivo, macrophages in tumor spheroids increased VEGF release and significantly enhanced angiogenesis (14).

TAMs serve as the main source for chemokines, growth factors, cytokines and proteases that regulate endothelial cells in the tumor microenvironment (28, 136). Studies using MCP-1-expressing tumor cells indicate that low levels of monocyte chemotactic protein-1 (MCP-1) have a modest effect on macrophage recruitment and enhanced angiogenesis and tumor growth in melanoma xenograft models (124). In contrast, elevated levels of MCP-1 induce more extensive macrophage infiltration, resulting in robust angiogenic reponses, enhanced tumor growth and tumor regression (124).

Growth factors, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), epidermal growth factor (71)(71), and transforming growth factor-α (TGF-α), are known to be produced by TAMs. Among them, VEGF can promote endothelial cell growth, maturation and survival (44). VEGF functions as a monocyte chemotactic factor to attract TAMs to the hypoxic areas of tumor (99, 121).

Inflammatory cytokines have been known to participate in macrophage-associated angiogenesis during wound healing (95, 165). IL-8 is a mitogen of endothelial cells and stimulates angiogenesis (76). The conditioned media of macrophages has been shown to

have similar effects and the effects can be abolished by neutralizing antibodies to IL-8, suggesting that IL-8 produced by macrophages plays a role in macrophage-associated angiogenesis (91). Tumor necrosis factor-α (TNF-α) is also involved in macrophage-associated angiogenesis. When overexpressed in endothelial cells, TNF-α was growth-inhibitory and cytotoxic to endothelial cells (141, 146). In contrast, exogenous TNF-α promoted formation of new blood vessels in several *in vivo* models (50). Leibovich, et al. (97) further showed that anti-TNF-α antibodies neutralized angiogenic activity in the conditioned media of activated macrophages, supporting the role of TNF-α as an angiogenic molecule from macrophages (97).

Other inflammatory cells such as mast cells, neutrophils and eosinophils contribute to angiogenesis (28). Mast cells are able to promote angiogenesis in squamous cell epithelia (26). In a mouse model of pancreatic islet carcinogenesis, neutrophils and macrophages are the main source of matrix metalloproteinase-9 (MMP-9) and thus mediate the angiogenic switch. Furthermore, depletion of neutrophils affected the association of VEGF with its receptor. Thus neutrophils play a crucial role in activating the angiogenic switch during early carcinogenesis (126).

VIII. Present Studies

The Roman laboratory has shown that expression of HPV 16E6E7 together in human foreskin keratinocytes (HFKs) increases the level of two angiogenic inducers VEGF and IL-8(177). Further, conditioned media from such cells are sufficient to alter endothelial cell behavior(23). Experiments were designed to determine the individual contributions of E6 and E7 to this process. Translational termination linkers (TTLs) were introduced into the coding region of E6 or E7. The ability of E7 in the context of the E6TTL mutation (E6TTLE7) and E6 in the context of the E7TTL mutation (E6E7TTL) to induce VEGF and IL-8 was examined. Both VEGF and IL-8 were significantly enhanced in HFKs expressing either HPV 16E6TTLE7 or HPV 16E6E7TTL. Migration assays revealed that neither E6 nor E7 induced migration of human microvascular endothelial cells (HMVECs). Immunological depletion experiments showed that only VEGF was required for HPV 16E6E7 together to induce HMVEC migration. These studies shed light on the contribution of the E6 and E7 viral oncoproteins and the angiogenesis required for the development of cervical cancer and may further help us better understand how to manage HPV-induced disease.

MATERIALS AND METHODS

I. Cell Cultures

A. Human Foreskin Keratinocytes (HFKs)

Primary human foreskin keratinocytes were prepared from newborn male foreskins obtained from Wishard Hospital. Keratinocytes from individual foreskins were plated onto mitomycin C (Sigma)-treated 3T3-J2 fibroblasts 10 cm plates. The cells were maintained in E medium [3 parts Dulbecco's modified Eagle's medium (DMEM)/1 part Ham's F-12 supplemented with 10% fetal bovine serum (FBS) (Thermo Scientific), (50 mg/ml, Sigma) gentamicin, (100 U/ml, Invitrogen) penicillin/streptomycin, (24 μg/ml, Sigma) adenine, (.4 μg/ml, Sigma) hydrocortisone, (50 μg/ml, Sigma) transferrin, (2 nM, Sigma) 3,3'-5-triodo-L-thyronine, (5 μg/ml, Sigma) insulin, (10 μg/ml, Invitrogen) EGF and (10 μg/ml, Sigma) cholera toxin. At approximately 80% confluence, the cells were trypsinized and frozen with DMEM + 20% fetal calf serum (FCS) + 10% dimethyl sulphoxide (DMSO) at one plate/vial at -80^{o}C.

To make pooled keratinocyte cultures, one vial offrozen foreskin keratinocytes was thawed and plated in complete-keratinocyte serum free media (C-KSFM). After reaching 80% confluency, the cells were trypsinized, counted and an equal number of cells from foreskins of 5 individuals vials (HFK pool 13: 602, 603, 607, 608 or HFK pool 14: 605, 612, 613, 614, 615) were mixed and refrozen at 1.28 x $10^{6}$and 1.8 x 10^{6} cells/vial in C-KSFM. Hypoxia was mimicked by addition of 100 μM deferoxamine mesylate (DFO, Sigma).

B. Human Microvascular Endothelial Cells (HMVECS) Culture

Human dermal microvascular endothelial cells (HMVECS) (Cambrex) were obtained at passage 7 or 8. Cells were seeded in tissue culture flasks coated with type 1 rat tail collagen (BD Biosciences) in complete endothelial cell growth medium -2 (EGM-2) (Lonza) supplemented with 10% FBS (Hyclone) and 1% antibiotic/antimicotic (100U/ml penicillin/streptomycin and 25 μg of amphotericin B) (Invitrogen) for passage. Cells were collected for passage with trypsin (Invitrogen), the trypsin was neutralized with EBM-2 and cells were centrifuged at 500 x g for 5 minutes. Cells were resuspended in 5 ml EBM-2 and 20 μl was used to count live cells by trypan blue exclusion on a hemo-cytometer. 3-5 x 10^5 live cells were seeded in 75 cm^2 flasks pre-coated with type 1 rat tail collagen for passage.

II. Generation of Mutants

A. Generation of HPV 16E6E7

HPV 16E6E7 was generated by cloning into the BamHI and EcoRI sites of the pLXSN vector. Oligonucleotides EcoRI (forward CCGGAATTCACCGGTTAGTA TAAAAGC and BamHI (reverse CGCGGATCCGGATCAGCCATGGTAGATTATGG) was used to amplify the E6E7 cassette. PCR was performed as follows: 35 cycles using the following conditions: denatured for 30 seconds at 95^{o}C, annealed for 30 seconds at 60^{o}C and elongated 7 minutes at 72^{o}C. The PCR product was then separated on a 1% agarose gel and the band was excised and purified using PCR Purifican Kit (Qiagen). 2 μl of E6E7 were digested with 0.5 μl of BamHI (10U/μl) and EcoRI (10U/μl)(Invitrogen) for 1 hour at 37^{o}C and analyzed by DNA gel electrophoresis. Digestion with BamHI and EcoRI allowed for positive identification due to BamHI and EcoRI sites outside of the

E6E7 cassette. T4 DNA ligase was used to ligate the DNA with the pLXSN vector. Although the HPV E6E7 cassette is immediately downstream of the p97 promoter, E6E7 transcription was directed from the Moloney murine leukemia virus promoter-enhancer sequences (MMLV). The vector also contained a gene conferring neomycin resistance (137), which was transcribed from the simian virus 40 (SV40) promoter. Three mls of NZB media containing amplicillin were inoculated with a single colony and incubated at 37^{o}C for >16 hours with shaking (225-250 rpm). The Qiagen Mini prep kit (Qiagen, Valencia, CA) was used according to manufacturer's instructions to isolate plasmid DNA. After the sequence was confirmed by the DNA Sequencing Core Facility (Indianapolis, IN), positive clones were inoculated into 250 ml of NZB media containing ampicillin (0.1 g/L) for >16 hours at 37^{o}C. The Qiagen Maxi kit was used according to manufacturer's instructions to extract and purify plasmid DNA on a larger scale. The plasmid DNA was then subcloned back into a clean pLXSN vector by digestion with restriction enzymes BamHI (10U/µl, Invitrogen) and EcoRI (10 U/µl, Invitrogen), transformed into competent cells, purified by Qiagen Maxi kit and the desired product confirmed again by sequencing the DNA Core Sequencing facility.

B. Generation of HPV 16E6TTLE7 and HPV 16E6E7TTL

HPV 16 E6 and E7 mutants, HPV 16E6TTLE7 and HPV 16E6E7TTL, were generated using the Quick-Change Site-Directed Mutagenesis kit (#200521, Stratagene) according to manufacturers instructions. Oligonucleotides HPV 16E6TTLE7 (forward GTATATAGAGATGGGAATCCTGAGTTAACTGAATATGCTGTATGTG; reverse CACATACAGCATATTCAGTTAACTCAGGATTCCCATCTCTATATAC); HPV 16E6E7TTL (forward CCGGACAGAGCCTTAGTTAACTAACATT

ACAATATTGTAACC; reverse GGTTACAATATTGTAATGTTAGTTAACTAA GGCTCTGTCCGG) were designed based on the published sequence (118) and purchased from Invitrogen Custom Primers. The oligonucleotides (125 ng) were denatured for 50 seconds at 95^{o}C, annealed for 50 seconds at 60^{o}C and elongated for 7 minutes at 68^{o}C. One microliter of *Dpn I* (10 U/µl) restriction enzyme was used to digest parental methylated and hemimethylated DNA for 1 hr at 37^{o}C. Forty-five microliter of XL10-Gold ultracompetent *Escherichia coli* (#200314, Stratagene) were incubated with 1 µl of the *Dpn I*- treated DNA on ice for 30 minutes. The transformation mixture was then heated for 30 seconds in 42^{o}C water bath and then placed on ice for 2 minutes. Then 500 µl of preheated (42^{o}C) NZY^{+} broth (supplemented with 1 M $MgMCL_2$, 1M $MgSO_4$, and 20 ml of 20% glucose) was added to each tube and incubated at 37^{o}C for 1 hour with shaking (225-250 rpm). Two-hundred and fifty microliter of each transformation mixture was plated onto individual NZB agar + (100 µg/ml, Sigma) amplicillin plates and placed upside down in a 37^{o}C incubator for >16 hours. Three mls of NZB media containing amplicillin was inoculated with a single colony and incubated at 37^{o}C for >16 hours with shaking (225-250 rpm). The Qiagen Mini prep kit (Qiagen, Valencia, CA) was used according to manufacturer's instructions to isolate plasmid DNA. Two microliter of L(16E6TTLE7)SN and L(16E6E7TTL)SN were digested with 0.5 µl of Hpa I (5 U/µl, Invitrogen) for 1 hour at 37^{o}C and analyzed by DNA gel electrophoresis. Digestion with Hpa I allowed for positive selection due to Hpa I site within the TTL. After the appropriate sequence was confirmed by the DNA Sequencing Core Facility (Indianapolis, IN), positive clones were inoculated in 250 ml of NZB media containing ampicillin (0.1 g/L) for >16 hours at 37^{o}C. The Qiagen Maxi kit was used according to manufacturer's

instructions to extract and purify plasmid DNA on a larger scale. The plasmid DNA was then subcloned back into a clean pLXSN vector by digestion with restriction enzymes BamHI (10 U/µl, Invitrogen) and EcoRI (10 U/µl, Invitrogen), transformed into competent cells, purified by Qiagen Maxi kit and confirmed again by the DNA Core Sequencing facility.

C. Generation of HPV 16E6E7C24G

HPV 16E6E7C24G Rb-binding deficient mutant was generated using the Quick-Change Site-Directed Mutagenesis kit (#200521, Stratagene) according to manufacturers instructions. Oligonucleotides HPV 16E6E7C24G (forward CAGAGACAACTGA TCTCTACGGTTATGAGCAATTAAATGAC; reverse GTCATTTAATTGCTCATA ACCGTAGAGATCAGTTGTCTCTG) were purchased from Invitrogen Custom Primers based on the published sequence (60). The oligonucleotides (125 ng) were denatured for 50 seconds at 95^{o}C, annealed for 50 seconds at 60^{o}C and elongated for 7 minutes at 68^{o}C. One microliter of *Dpn I* (10 U/µl) restriction enzyme was used to digest parental methylated and hemimethylated DNA for 1hr at 37^{o}C. Forty-five microliter of XL10-Gold ultracompetent *Escherichia coli* (#200314, Stratagene) were incubated with 1 µl of the *Dpn I*- treated DNA on ice for 30 minutes. The transformation mixture was then heated for 30 seconds in at 42^{o}C water bath and then placed on ice for 2 minutes. Then 500 µl of preheated (42^{o}C) NZY^{+} broth (supplemented with 1 M $MgMCL_2$, 1M $MgSO_4$, and 20 ml of 20% glucose) was added to each tube and incubated at 37^{o}C for 1 hour with shaking (225-250 rpm). Two-hundred and fifty microliter of each transformation mixture was plated onto individual NZB agar + (25 mg/4 µl, Sigma) amplicillin plates and placed upside down in a 37^{o}C for >16 hours. Three mls of NZB media containing amplicillin

was inoculated with a single colony and incubated at 37°C for >16 hours with shaking (225-250 rpm). The Qiagen Mini prep kit (Qiagen, Valencia, CA) was used according to manufacturer's instructions to isolate plasmid DNA. Two microliter of HPV 16E6E6C24G was digested with 0.5 µl of BamHI (10 U/µl, Invitrogen) and EcoRI (10 U/µl, Invitrogen) for 1 hour at 37°C and analyzed by DNA gel electrophoresis. After the mutated sequence was confirmed by the DNA Sequencing Core Facility (Indianapolis, IN), positive clones were inoculated in 250 ml of NZB media containing ampicillin (0.1 g/L) for >16 hours at 37°C. The Qiagen Maxi kit was used according to manufacturer's instructions to extract and purify plasmid DNA on a larger scale. The plasmid DNA was then subcloned back into a clean pLXSN vector by digestion with restriction enzymes BamHI (10 U/µl, Invitrogen) and EcoRI (10 U/µl, Invitrogen), transformed into competent cells, purified by Qiagen Maxi kit and confirmed again by sequencing the DNA Core Sequencing facility (Indianapolis, IN).

III. Production of Retrovirus Stocks

One vial of SD3443 Phoenix cells obtained from the American Type Culture Collection (ATCC) was thawed 1:2 onto 10 cm plates in DMEM + 10% FBS for 2-3 days and then split 1:10. After the cells were about 70-80% confluent, media were aspirated and 5 ml of DMEM (Lonza) was used to rinse the cells. The cells were then incubated in 5 ml of DMEM and chloroquine (3.2 µl/5 ml) for approximately 15 minutes at 37°C, 5% CO_2. Four micrograms of DNA from plasmids HPV 16E6E7, HPV 16E6TTLE7, HPV 16E6E7TTL and HPV 16E6E7C24G were added to 20 µl of plus reagent and 730 µl of DMEM and allowed to sit at room temperature for 15 minutes. After 15 minutes, the complex was added to 30 µl of Lipofectamine (Invitrogen) in 720 µl of DMEM for an

additional 15 minutes. One and a half ml of the complex were added to each plate and incubated for at 37°C, 5% CO_2. After 3.5 hours, 6.5 ml of DMEM + 20% FBS were added and incubated overnight at 37°C, 5% CO_2. Within 24 hours, media were aspirated and 5 mls of complete DMEM (10% FBS + gentamicin) was added. The following day the retrovirus was harvested and frozen in 5 ml aliquots at -80°C. Another 5 mls of complete DMEM was added to each plate and then harvested the next day. The retroviruses were stored in 5 mls aliquots at -80°C until further use.

A. Titering Retrovirus

Retroviral titers were obtained by seeding 0.4 x 10^6 NIH 3T3 fibroblasts (ATCC) onto 6 cm tissue culture plates in 4 ml of DMEM + 10% calf serum (CS). The next day the cells were fluid changed with 2 ml of DMEM + 10% CS, 8 μg/ml polybrene (3 mg/ml) and 20 μl of diluted retrovirus (1 x 10^{-2}, 1 x 10^{-3}, 1 x 10^{-4}, 1 x 10^{-5}). After 3 hours, 6 ml of DMEM + 10% FBS were used to dilute the polybrene to 2 μg/ml. After two days, 1 mg/ml G418 (50mg/ml, Sigma) selection media were added and the cells were fluid changed every 3 days with selection media until colonies were visible. The media were aspirated from the plates and the colonies were fixed and stained with Diff-quick kit (Invitrogen). The plates were then rinsed with distilled water and allowed to dry overnight. The following formula was used to calculate the viral titer.

$$\text{Infected virus/ml} = \frac{\text{number of colonies}}{\text{Virus volume (ml)} \times \text{dilution factor}}$$

IV. Infection of pooled Human Foreskin Keratinocytes

One vial of pooled human foreskin keratinocytes (HFKs) was thawed, plated in complete-serum free keratinocyte media (C-KSFM) (Invitrogen) supplemented with epidermal growth factor (71) and bovine pituitary extract (BPE) (Invitrogen) and grown to about 40% confluence. The cells were infected with 5 ml of parental virus LXSN, recombinant retrovirus HPV 16E6E7, HPV 16E6TTLE7, HPV 16E6E7TTL, HPV 16E6E7C24G or HPV 6 E7 and 8 μg/ml polybrene as previously described (23, 177). Mock infected cells were generated by incubating HFKs with 5 ml of DMEM + 10% FCS and 8 μg/ml polybrene. After 6 hours the culture media was aspirated, fresh C-KSFM was added and incubation continued for 48 hours. The cells were then split 1:4 onto 10-cm dishes (passage 1). After selection, the antibiotic resistant cells were expanded in CKSFM for 2-3 days and used for further experiments as described in the next section.

V. Protein Analysis

A. Secreted Proteins VEGF and IL-8

Following selection, retrovirally-transduced HFKs were transferred 1:4 onto mitomycin- C (4 μg/ml in ddH_2O, Sigma) treated J2-3T3 fibroblasts and grown in complete E media until 80% confluence (passage 2). At passage 3, $2.5x10^6$cells were plated in E media in 10 cm plates with $2x10^5$ feeder cells for 24 hours. Serum-free conditioned media (CM) was generated by rinsing the cells three times with 4 ml of basal media (BM) (3 parts DMEM/1 part Ham's F-12 with 100 U/ml of penicillin and 100 μg/ml of streptomycin), incubating the cells for 4 hours in BM, replacing this media with 8 ml of the same media and harvesting CM 24 hours later. Typically, 5 ml of

unconcentrated CM was frozen at -80^{o}C in 1 ml aliquots and used for ELISA. For functional assays, CM was concentrated 30x using 15 ml Amicon Ultracel (Millipore) centrifugal filter devices (10000 MWCO) at 4^{o}C for 45 minutes at 3000 rpms. Protease inhibitor cocktail (Sigma) and phosphatase inhibitors (20 μM Na_3VO_4 and 100 mM NaF, Sigma) were added prior to freezing at -80^{o}C. Protein concentrations were determined using the Bio-Rad DC protein assay kit.Concentrated CM was frozen at -80^{o}C in 1 ml aliquots and used for migration assays.

B. Intracellular Proteins: p53, pRb, HIF-1α, HIF-2α, GAPDH, Tubulin and p130

In parallel with harvesting conditioned media, whole-cell lysates of the retrovirally-transduced HFKs were obtained by incubating two minutes on ice with 600 μl of 1x lysis buffer [20 mM Tris, pH 6.8, 1% sodium dodecyl sulfate (SDS), 1 mM ethylenediamine tetraacetic acid (EDTA)] plus protease and phosphatase inhibitor cocktail (20 μM Na_3VO_4 and 100 mM NaF, Sigma) per 10 cm plate after rinsing the plate with PBS three times. Each cell lysate was harvested by scraping the plates; the lysate was placed in1.5 ml eppendorf tubes. The recovered lysate was boiled for 5 minutes to denature proteins and then centrifuged for 15 minutes at 4^{o}C at 13,000 rpm to pellet any cellular debris. Lysate was placed into a new eppendorf tube and frozen at -80^{o}C in 200 μl aliquots. Protein concentrations were determined using the Bio-Rad DC protein assay kit.

C. Western Blot Analysis

Proteins (50 μg) were separated on 8% or 10% sodium dodecyl sulfate (SDS) polyacrylamide gels. The gels were run at 130 V for 1-2 hours and then removed and

proteins were transferred to a nitrocellulose membrane (0.45 μm, BioRad) at 100 V for 1 hour. Following transfer, the membrane was stained with Ponceau red (BioRad), rinsed with 5% acetic acid (Fisher) and photographed (HP Photosmart Premier). The membrane was blocked in 1x Tris-buffered saline 0.1% Tween-20 (TBS-t) supplemented with 5% nonfat dry milk as previously described (23, 177).

Primary antibodies were diluted in TBS-t supplemented with 5% nonfat dry milk used at the following dilutions as previously described (23, 177): 1:1000 for 1 hr at room temperature, p53 (DO-1 Sc-126, Santa Cruz), pRB (554136, BD Pharminogen); 1:500, HIF-1α (610958, BD Pharminogen), HIF-2α (NB100-132, Novus Biologicals); 1:1000 overnight at 4°C, Rb2 (p130) (610262, BD Bioscience); and loading controls 1:30000 for 1 hr at room temperature, GAPDH (MAB 374, Chemicon) and 1:5000 for 1 hr at room temperature, Tubulin (Sigma). Secondary goat anti-mouse horseradish peroxidase (HRP) (BioRad) was diluted 1:5000 into TBS-t for 1 hr at room temperature. The Pierce ECL Western Blotting substrate (Thermo Scientific) protein detection system was used to visualize proteins. Densitometry was performed using Quality One software (BioRad).

VI. Neutralization Studies

Concentrated CM (500 μg) from LXSN and L(16E6E7)SN was incubated with 10 μg of mouse monoclonal neutralizing antibodies (Abs) directed against IL-8 (R&D Systems, MAB208), VEGF (R&D Systems, MAB 293), or isotype controls (IgG_1 for IL-8 (R&D Systems, MAB002) or IgG_{2B} for VEGF (R&D Systems, MAB 004) and rotated for 2 hours at 4°C. Twenty μl of 50% vol/vol of protein G sepharose beads (Roche, #11719416001) in 1 x phosphate buffered saline (PBS) was added and rotated for 2 hours. The beads were then centrifuged at 4,000 rpm for 30 seconds and the supernatants

were collected into microfuge tubes. The protein concentration of the supernatants was determined using the Bio-Rad DC protein assay kit. The supernatants were then frozen at -80^{o}C and used for ELISA and migration assays.

VII. Enzyme-Linked Immunosorbent Assay (ELISA)

A. VEGF Analysis

$VEGF_{165}$ secretion was detected in unconcentrated cell supernatants using the Quantikine Human VEGF ELISA kit (R&D systems). Monoclonal antibodies specific for VEGF were pre-coated onto the microplates. To generate standards, human VEGF was diluted with Calibrator Diluent RD5K (supplied by the manufacturer). The undiluted standard served as the highest standard (1000 pg/ml) and two-fold dilutions were made until the lowest concentration (15.6 pg/ml) was obtained. The calibrator diluent served as the zero standard (0 pg/ml). Fifty microliters of diluent RD1W, a buffered protein base supplied by the manufacturer, was added to each well and 200 µl of standards and samples were then added. Each sample was assayed in triplicate. The plate was incubated at room temperature for 2 hours and then washed three times with wash buffer. Two hundred microliters of VEGF conjugate antibody, a polyclonal antibody against VEGF conjugated to HRP, was added for 2 hours at room temperature. The plate was washed three times and 200 µl of the substrate solution was added to each well for 20 minutes at room temperature with protection from light. Adding 50 µl of 2N sulfuric acid stopped the reaction. The optical density (OD) of each well was measured within 30 minutes, using the Spectra Max Plus (Molecular Devices) with the plate reader set to 450 nm.

B. IL-8 Analysis

IL-8 secretion was detected in unconcentrated cell supernatants using the Quantikine Human CXCL8/IL-8 ELISA kit (R&D systems). Monoclonal antibodies specific for IL-8 were pre-coated onto the microplates. To generate standards (2000 pg/ml to 31.2 pg/ml), two-fold dilutions of human IL-8 were made in Calibrator Diluent RD5P supplied by the manufacturer. The calibrator diluent served as the zero standard (0 pg/ml). One hundred microliters of diluent RD1-85, a buffered protein base supplied by the manufacturer, was added to each well and 50 μl of standards and samples were then added. Each sample was assayed in triplicate. The plate was incubated at room temperature 2 hours and then washed four times with wash buffer. One hundred microliters of IL-8 conjugate antibody, a polyclonal antibody against IL-8 conjugated to HRP, was added for 1 hour at room temperature. The plate was washed four times and 200 μl of the substrate solution was added to each well for 30 minutes at room temperature protected from light. The reaction was stopped and results determined as for VEGF.

VIII. RNA Analysis

Total RNA was isolated from the retrovirally-transduced keratinocytes using 1 ml Tri Reagent (Molecular Research Center) according to the manufacturer's instructions. During isolation, contaminating DNA was removed by digestion using the RNase-free DNase kit according to manufacturer's instructions (Qiagen). After isolation, the quality of the RNA was determined by electrophoresis through a 2% tris-acetate-ethylenediamine tetraacetic acid [TAE] agarose gel containing ethidium bromide (EtBr). The products

were visualized by Quality-One software (BioRad). TheRNA was quantitated by Beckman Coulter (Schaumburg, IL).

A. Semi-quantitative limiting cycleRT-PCR

Semi-quantitative reverse transcriptase polychain reaction (RT-PCR) was used to amplify 1 μg of RNA from retrovirally-transduced HFKs as previously described (23, 177). Amplification of the cDNA was stopped at 22 cycles for GAPDH, 26 cycles for VEGF and 28 cycles for IL-8. The products were visualized by EtBr staining and analyzed with Quality-One software (Bio-Rad). All primers were designed by Invitrogen Custom Primers. Reverse transcription was done for 30 minutes at 50^{o}C followed by 2 minutes at 94^{o}C. Denaturation was done for 15 seconds at 94^{o}C, annealing for 30 seconds and elongation for 1 minute at 72^{o}C. The following primers (10 μM) and annealing temperature were used as previously described (23, 177)- GAPDH forward GAAGGTGAAGGTCGGAGTCA; reverse GAAGATGGGATGGGATTTC annealing temperature 55^{o}C; VEGF forward CGAAGTGGTGAAGTTCATGGTG; reverse TTCTGTATCAGTCTTTCCTGGTGAG annealing temperature 55^{o}C; IL-8 forward ACATACTCCAAACCTTTCCACCC; reverse CAACCCTCTGCACCCA GTTTTC annealing temperature 55^{o}C.

IX. *In vitro* Migration Assay

The endothelial cell migration assay was performed as previously described (23, 116)with minor modifications. The upper and lower chamber of the transwell inserts with 8 μm pores (3422, Costar) were coated with 60 μl of type I rat tail collagen 50 μg/ml (BD Pharmingen) and incubated for no more than 90 minutes at 37^{o}C, 5% CO_2. The collagen

was aspirated and the transwell inserts were placed into individual wells of a 24 well tissue culture plate. 2 x10^4 human dermal microvascular endothelial cells (HMVECs) were suspended in 100 µl of EBM-2 (Cambrex) supplemented with 0.5% BSA and added to the top of each transwell. Six hundred microliters of EBM-2 supplemented with 0.5% BSA, VEGF (100-20, 50 ng/ml, Peprotech) or conditioned media (50 µg) from retrovirally-transduced HFKs was added to the lower chamber of each transwell. Cells were incubated for 3.5 hrs at 37^{o}C, 5% CO_2. Non-migrating cells were removed using a cotton swab; migrating cells were fixed with 600 µl of methanol and stained with Diff-quick kit (Invitrogen). Six high power fields (HPFs) of cells on the bottom of the membrane were counted in each individual well with an inverted microscope at 20x magnification. Assays were performed in triplicate. Results are reported as average number of migrated cells $\pm$ standard error of the mean (SEM).

X. Statistical Analysis

Student unpaired two tailed t-test (Prism GraphPad 4.0) was used for statistical analysis. P values less than 0.05 were regarded as significant.

RESULTS

Chapter I. Construction and Characterization of HPV 16E6TTLE7 and HPV 16E6E7TTL

A. Construction of HPV 16E6E7

The HPV 16 E6E7 cassette was previously cloned into the retrovirus LXSN by the Galloway laboratory (66). The cloning strategy, however, did not allow manipulation of the cassette. Therefore, the same E6E7 cassette was amplified using primers containing EcoRI (forward) and BamHI (reverse) sites. BamHI and EcoRI were also used to isolate the E6E7 cassette from HPV16. The PCR products were then ligated into pLXSN vector (ATCC) (Figure 8.). Transcription of HPV genes within the vector is directed from the Moloney murine leukemia virus promoter enhancer sequences. The vector also contains a gene conferring neomycin resistance, which is transcribed from the simian virus 40 promoter (Figure 8). HPV 16E6E7 retrovirus was generated according to previously described procedures in Materials and Methods. Briefly, plasmid DNA was transfected into SD3443 Phoenix packaging cell line using Liptofectamine. The virus produced was used to infect pooled human foreskin keratinocytes (HFKs).

B. Validation of HPV 16E6E7

Expression of HPV 16 E6 results in degradation of p53, while expression of HPV 16 E7 results in degradation of Rb (17, 147). Thus to confirm that E6 and E7 proteins

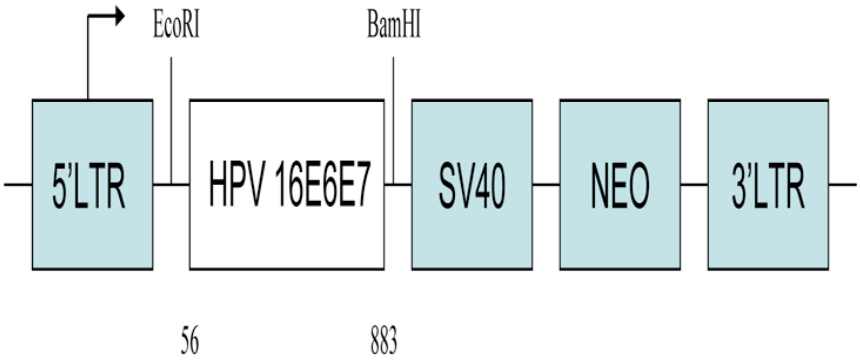

Figure 8. Schematic of DNA encapsidated in the retrovirus used to transduce human foreskin keratinocytes with HPV 16 E6 and E7 (the HPV 16 E6E7 cassette).

Recombinant retrovirus containing the coding sequence for HPV 16E6E7 under the control of the viral Long Terminal Repeat (80). The neomycin resistant gene was expressed under the control of the SV40 promoter, allowing selection of infected cells with G418. Numbers under the HPV 16E6E7 cassette indicate the nt numbers at the beginning and end of the cloned fragment. The ATG for E6 is at nt 83; for E7 is at nt 562.

were functioning as expected, using the Galloway virus HPV 16E6E7as the control, western blot analysis was performed on whole cell lysates to detect levels of p53 and Rb. There was at least a 2-fold decrease in p53 expression in cells expressing HPV 16E6E7 and HPV 16E6E7 relative to LXSN transduced HFKs (Figure 9). The levels of Rb protein in cells infected with HPV 16E6E7 was not decreased relative to LXSN transduced HFKs (Figure10). However, this result is consistent with that of the Galloway laboratory (Figure 10). These results demonstrate that the new retrovirus HPV 16E6E7 was performing identically to that of the Galloway laboratory. Rb may not be decreased because the mRNA is mostly unspliced, favoring increased expression of E6, which stabilize Rb (see Discussion).

C. Transfected HPV 16 genome in pooled human foreskin keratinocytes expresses more spliced message than retrovirally transduced HPV 16 E6 and E7.

As discussed in the Introduction, splicing is important for the regulation of E6 and E7 gene expression in the intact HPV 16 genome (38, 151)(156)(168). In HPV16, E6 and E7 are transcribed as a single bicistronic transcript using a common promoter, p97. Within the E6 ORF there is one 5' splice site (ss) at nucleotide (nt) 226 and two alternative 3' ss at nt 409 and 526 in the virus genome. Splicing of nt 226 to 409 creates E6*I, and splicing to nt 526 produces E6*II. Studies from various laboratories have demonstrated that the majority of the transcripts in cancer tissues and cervical cancer cell lines are E6*I. (156)(24)(152). In contrast, splicing is inefficient in the retrovirus, HPV 16E6E7, and, as a result, full-length unspliced message is made (96). To confirm that splicing is inefficient in cells infected with the retrovirus HPV 16E6E7, semi-quantitative

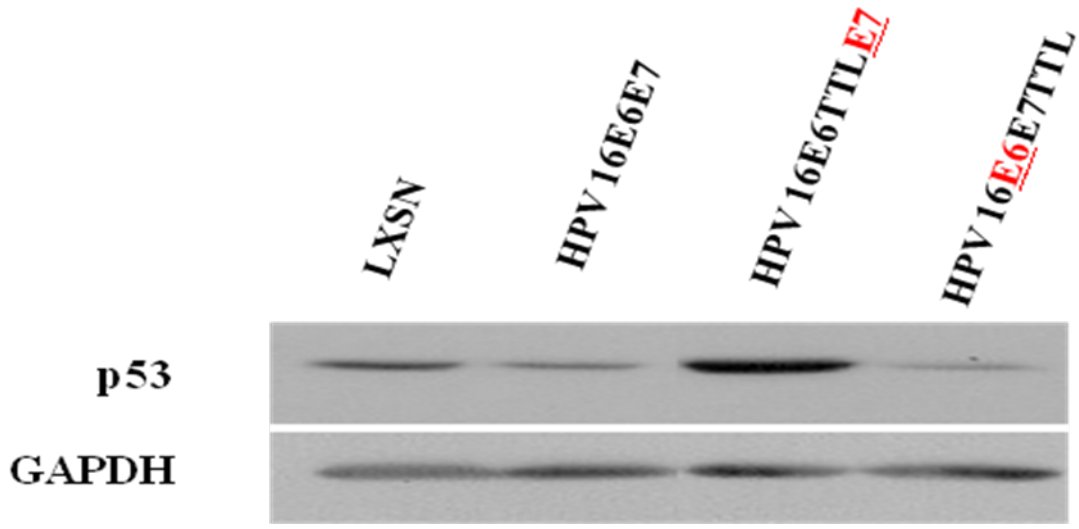

Figure 9. The effect of expressing HPV 16E6E7, HPV 16E6TTLE7 or HPV 16E6E7TTL in pooled HFKs on the steady state level of p53.

Thirty microgram of protein in whole cell lysate from pooled HFKs transduced with LXSN, HPV 16E6E7, HPV 16E6E7, HPV 16E6TTLE7 or HPV 16E6E7TTL expressing cells, were separated on a 10% gel. Proteins were transferred to nitrocellulose membrane and probed with antibody to p53 and GAPDH. The results shown are representative of three independent experiments. GAPDH serves as a loading control.

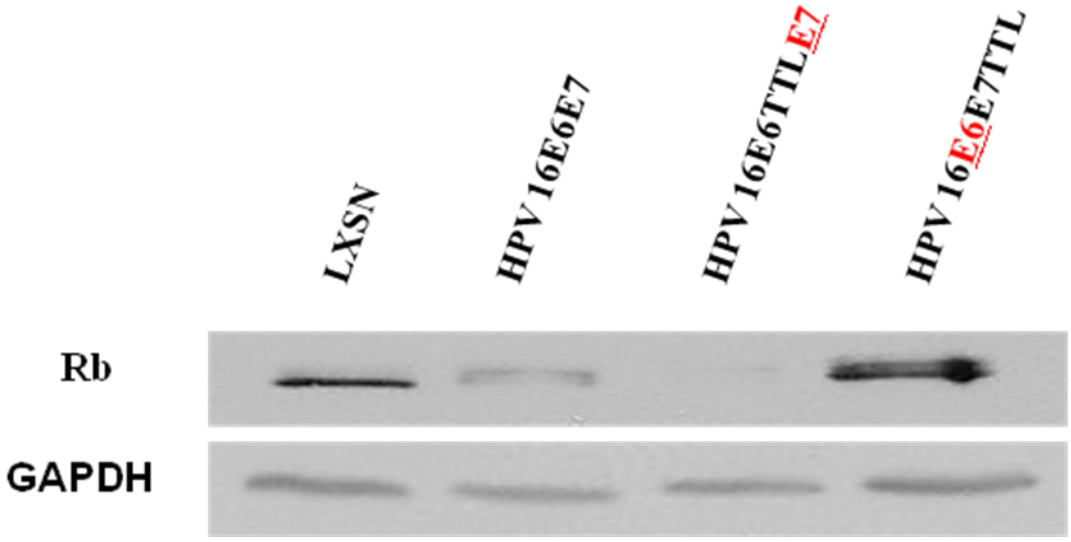

Figure 10. The effect of expressing HPV 16E6E7, HPV 16E6TTLE7 or HPV 16E6E7TTL in pooled HFKs on the steady state level of Rb.

Thirty microgram of protein in whole cell lysate from pooled HFKs transduced with LXSN, HPV 16E6E7, HPV 16E6E7, HPV 16E6TTLE7 or HPV 16E6E7TTL expressing cells, were separated on an 8% gel. Proteins were transferred to nitrocellulose membrane and probed with antibody to Rb and GAPDH. The results shown are representative of three independent experiments. GAPDH serves as a loading control.

RT-PCR was performed on RNA isolated from HFKs expressing LXSN, HPV 16E6E7 and HPV 16E6E7. Primers 106 and 562 were used to amplify E6 and E7. As anticipated, unspliced message was expressed in HFKs retrovirally transduced with HPV 16E6E7 and HPV 16E6E7, relative to HPV 16, which expressed more spliced message as shown in Figure 11.

D. Transduced HPV 16 E6TTLE7 in pooled human foreskin keratinocytes expresses more spliced message than HPV 16 E6 and E7 or HPV 16 E6E7TTL.

To determine the individual contributions that E6 and E7 play in inducing angiogenesis, translational termination linkers (TTLs) were placed within the open reading frames of either the E6 coding sequence at nt 280 or the E7 coding sequence at nt 711 and spliced versus unspliced message was examined. Semi-quantitative RT-PCR was performed on RNA isolated from HFKs expressing LXSN, HPV 16E6E7, HPV 16E6TTLE7 or HPV 16E6E7TTL. Primers 106 and 562 were used to amplify mRNA across the nt 226 through nt 409 splice junction. HFKs retrovirally transduced with HPV 16E6E7 and HPV 16E6E7TTL expressed predominantly unspliced message. Surprisingly, more spliced message was expressed in HFKs retrovirally transduced with HPV 16E6TTLE7 compared to HPV 16E6E7 or HPV 16E6E7TTL (Figure 11). In addition, placement of the TTL at nt 280 within E6 creates a premature stop codon therefore providing more E7 RNA templates and translation of E7.

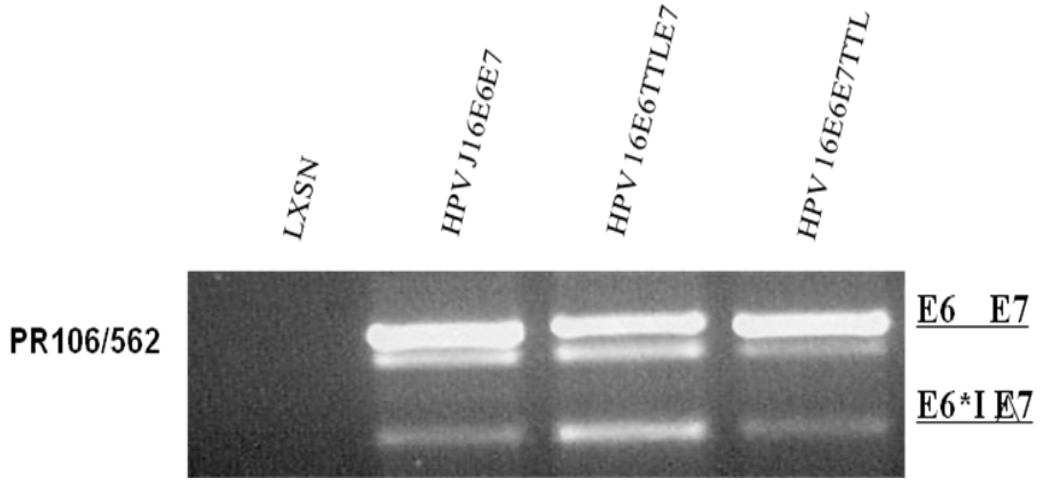

Figure 11. HPV 16E6TTLE7 produces more spliced message relative to HPV 16E6E7 or HPV 16E6E7TTL. Semi-quantitative RT-PCR was performed using 0.5 μg of RNA isolated from HFKs expressing LXSN, HPV 16E6E7, HPV 16E6TTLE7 or HPV 16E6E7TTL. Primers 106 and 562 were used to amplify E6 and E7.

E. Expression of HPV16E6TTLE7 in pooled human foreskin keratinocytes does not decrease the tumor suppressor p53 relative to LXSN transduced HFKs.

Expression of HPV 16 E6 results in degradation of p53, (147). If disruption of the E6 gene is successful, p53 will not be degraded. Thus, to confirm that insertion of a TTL results in loss of function of E6, the expression level of p53, a surrogate marker for E6 protein, was determined by Western blot analysis. As expected, relative to LXSN, p53 protein was not decreased in cells infected with HPV 16E6TTLE7 (Figure 9). In fact, the levels of p53 were increased in the presence of E7 relative to LXSN. This is consistent with previously published data which indicate that expression of HPV 16 E7, in the absence of E6, increases the steady state level of p53 protein, (34, 35)(150). These results demonstrate that the mutant is indeed null for E6.

F. Expression of human papillomavirus HPV16E6E7TTL in pooled human foreskin keratinocytes does not decrease the tumor suppressor, Rb, relative to LXSN transduced HFKs.

Expression of HPV 16 E7 results in degradation of Rb, (17). Thus, to confirm that insertion of a TTL results in loss of function of E7, the level of Rb, a surrogate marker for E7 protein, was monitored by Western blot analysis. If disruption of the E7 gene is successful, Rb will not be degraded. As expected, relative to LXSN, Rb protein expression was not decreased in cells infected with HPV 16E6E7TTL (Figure 10). In fact, the levels of Rb were increased in the presence of E6 relative to LXSN. These results demonstrate that the mutant is indeed null for E7. This is also consistent with published data which indicate that expression of HPV 16 E6 in the absence of E7

increases the steady state level of Rb protein (111). Taken together the data in (Figures 9 and 10) indicate that the E6 and E7 mutants are functioning as expected.

Chapter II. Establish whether HPV 16 E6 and/or E7 is required for increased expression of VEGF and/or IL-8 and to induce an angiogenic response.

The results of the previous chapter indicate that HPV 16E6TTLE7 and HPV 16E6E7TTL are loss of function with respect to targeting p53 or Rb, respectively, for degradation. Additionally, HPV 16E6TTLE7 increased the steady state levels of p53 and HPV 16E6E7TTL increased the steady state levels of Rb. In this chapter the hypothesis that increased expression of VEGF and IL-8 requires expression of E6 and/or E7 and whether E6 and/or E7 is sufficient for an angiogenic response will be tested. To examine the ability E6 and/ or E7 loss of function mutants to affect expression of the angiogenic inducers VEGF and IL-8, ELISAs were used. To characterize the ability of conditioned media from HFKs expressing HPV 16E6TTLE7 and or HPV 16E6E7TTL to alter endothelial cell behavior, endothelial cell migration assays were used.

A. Expression of HPV 16 E6TTLE7 and HPV 16 E6E7TTL in pooled human foreskin keratinocytes significantly upregulates IL-8 secretion.

To determine whether E6 and E7 each contributes to the increase of secreted IL-8, HFKs were transduced with control retrovirus LXSN, HPV 16E6E7, HPV 16E6TTLE7 or HPV 16E6E7TTL. Conditioned media from the cells expressing HPV 16E6E7TTL had a 2.9 +/- 0.3 fold increase in the level of secreted IL-8 relatively to LXSN ($p<0.001$). Cells expressing HPV 16E6TTLE7, however, had an 11.7 +/- 0.6 fold increase in the level of secreted IL-8 relative to LXSN ($p<0.0001$) (Figure 12).

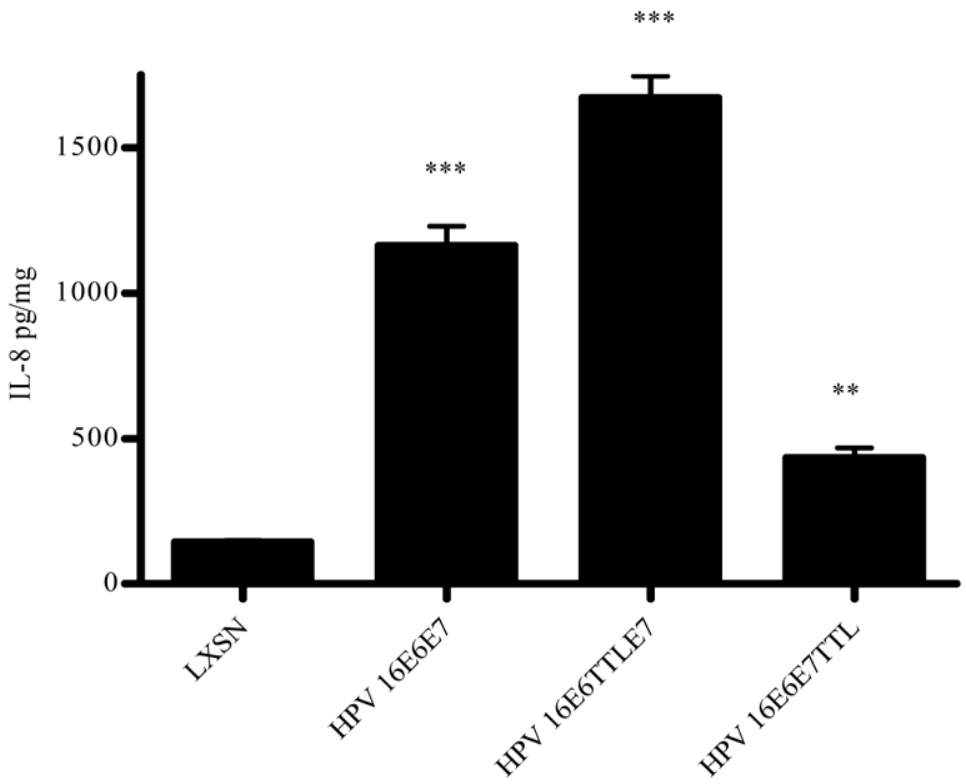

Figure 12. Levels of IL-8 in conditioned media were increased in cells expressing either E6 or E7.

The quantity of IL-8 was determined by ELISA and normalized to total protein in the conditioned media. The data represent mean ± SEM and are representative of three experiments each conducted in triplicate. $p< 0.0001$for LXSN vs 16E6E7 and LXSN vs HPV 16E6TTLE7; $p< 0.001$ for LXSN vs HPV 16E6E7TTL; $p< 0.001$ for HPV 16E6E7 vs HPV 16E6TTLE7 and $p< 0.0005$ for HPV 16E6E7 vs HPV 16E6E7TTL.

Conditioned media from the cells expressing HPV 16E6TTLE7 also significantly enhanced the expression of IL-8 relatively to HPV 16E6E7 ($p < 0.0001$). In contrast, although, relative to LXSN, the level of IL-8 was significantly increased in HPV 16E6E7TTL-expressing cells, the level of increase was much less than that seen with HPV 16E6E7. Therefore, E7 seems to play a greater role in upregulating IL-8 than E6.

B. Expression of HPV 16E6TTLE7 and HPV 16E6E7TTL in pooled human foreskin keratinocytes significantly upregulates VEGF secretion.

To determine whether E6 and E7 each contributes to the increase of secreted VEGF seen when HPV 16E6E7 are expressed together in HFKs (23, 177), HFKs were transduced with control retrovirus LXSN, HPV16E6E7, HPV 16E6TTLE7 or HPV 16E6E7TTL. Conditioned media from the cells expressing HPV 16E6TTLE7 or HPV 16E6E7TTL caused an 4.8 +/- 0.3 fold and 2 +/- 0.1 fold ($p < 0.0001$ and $p < 0.001$) increase in the expression of VEGF relatively to LXSN (Figure 13). Conditioned media from the cells expressing HPV 16E6TTLE7 also caused a 3.3 +/- 0.04 fold ($p < 0.0001$) increase in expression of VEGF relatively to HPV 16E6E7. In contrast, although, relative to LXSN, the level of VEGF was significantly increased in HPV16E6E7TTL-expressing cells, the level of increase was only slightly greater than that seen with HPV 16E6E7. Therefore, E7 seems to play a greater role in upregulating VEGF than E6.

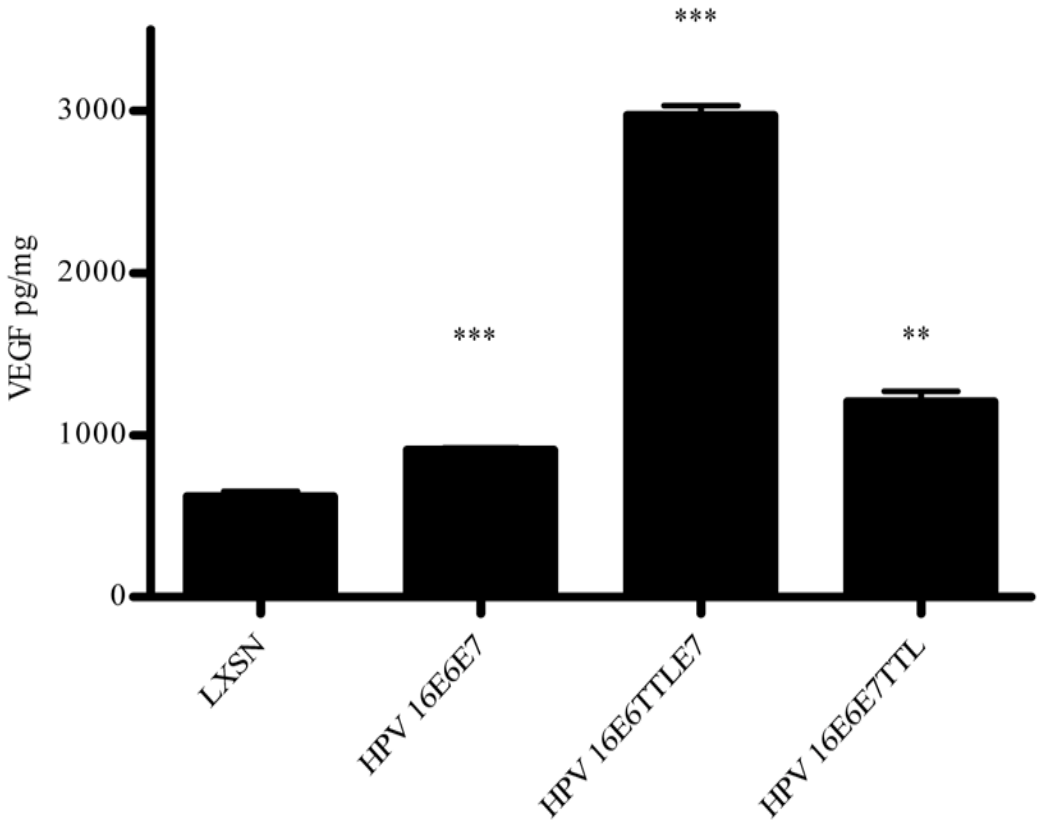

Figure 13. Levels of VEGF in conditioned media were increased in cells expressing either E6 or E7.

The quantity of VEGF was determined and normalized to total protein in the conditioned media. The data represent mean ± SEM and are representative of three experiments each conducted in triplicate. $p < 0.001$ for LXSN vs HPV 16E6E7; $p < 0.0001$ for LXSN vs HPV 16E6TTLE7; $p < 0.001$ for LXSN vs HPV 16E6E7TTL; $p < 0.0001$ for HPV 16E6E7 vs HPV 16E6TTLE7 and $p < 0.001$ for HPV 16E6E7 vs HPV 16E6E7TTL.

C. Conditioned media from pooled HFKS transduced with HPV 16E6TTLE7 or HPV 16E6E7TTL did not induce migration of human microvascular endothelial cells (HMVECs) *in vitro*.

To determine whether of E6 or E7 alone contribute to the altered endothelial cell behavior seen when HPV 16E6E7 are expressed together in HFKs (23, 177), migration assays were used. Human microvascular endothelial cells (HMVECs) were placed in the top of a transwell plate and concentrated media from control HFKs or HFKs expressing HPV gene products were placed in the bottom well and migration was monitored as described in Materials and Methods. Consistent with previous results, conditioned media from HPV 16E6E7-expressing cells increased the migration of HMVECs (Figure 14). In contrast, concentrated conditioned media from retrovirally transduced cells expressing HPV 16E6TTLE7 or HPV 16E6E7TTL did not induce migration of HMVECs relative to LXSN (Figure 14). Consistent with these observations, the level of migration of endothelial cells seen in the presence of conditioned media from HPV 16E6E7 expressing cells was statistically different from that seen with either HPV 16E6TTLE7 or HPV 16E6E7TTL (0.8 +/- .12 fold and 0.9 +/- 0.13 fold respectively). These results indicate that both E6 and E7 are required to alter endothelial cell behavior.

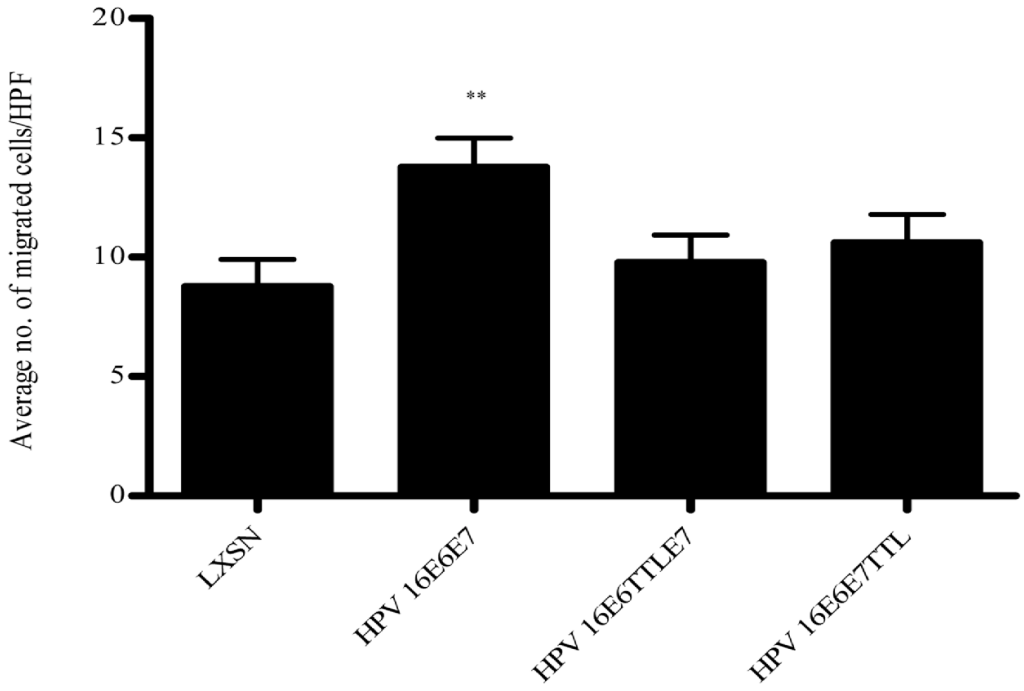

Figure 14. Conditioned media from pooled HFKs transduced with either E6 or E7 did not induce migration of HMVECs *in vitro*.

HMVECs were plated on a collagen-coated transwell membranes. Conditioned media from HFKs retroviral transduced with LXSN, HPV 16E6E7, HPV 16E6TTLE7 or HPV 16E6E7TTL were added to the bottom chamber. After 3 hrs, 6 high power fields (HPF) of cells on the bottom were counted. The data represent mean number of migrated cells per HPF± SEM and are representative of three experiments each conducted in triplicate. $p< 0.005$ for LXSN vs HPV16E6E7 and $p< 0.02$ for HPV 16E6E7 vs HPV 16E6TTLE7 or HPV 16E6E7TTL.

Chapter III.

A. HPV 16 E7 enhancement of VEGF is independent of HIF-1α and HIF-2α expression under normoxic conditions.

Angiogenesis is induced in response to hypoxia. The cellular response to hypoxia is regulated by HIF-1α. As stated previously in the Introduction, HIF-1α is regulated by oxygen (10, 18). In normoxia, constitutively expressed cytosolic HIF-1α subunits are hydroxylated on two proline residues and targeted for degradation by their interaction with the VHL proteasomal pathway (23). In hypoxia, VHL-mediated degradation of HIF-1α is inhibited, HIF-1α is stabilized and translocates to the nucleus where it activates HIF-1 target genes (42, 179).

VEGF, a HIF-1 target gene, is an important angiogenic inducer. Our previous publication has shown that HPV 16E6E7 is capable of enhancing the expression of VEGF in HFKs under normoxic conditions (177). However, whether HIF-1α activation is responsible for this increased expression was unknown. A precedent for this possibility was provided in a publication by Kondo, et al. 2006, which showed that Epstein-Barr Virus (EBV) latent membrane protein-1 (LMP1) induces stabilization of Siah1, a ubiquitin ligase, which, in turn destabilizes the prolyl hydroxylases, thereby stabilizing HIF-1α under normoxic conditions (92).

To determine whether expression of HPV 16E6E7 had any influence on HIF-1α activation in normoxia and hypoxia, we first examined whether HIF-1α could be induced in hypoxia in the presence of HPV 16E6E7. We used the hypoxic mimicking drug, deferoxamine mesylate (DFO), which has previously been shown to enhance the levels of

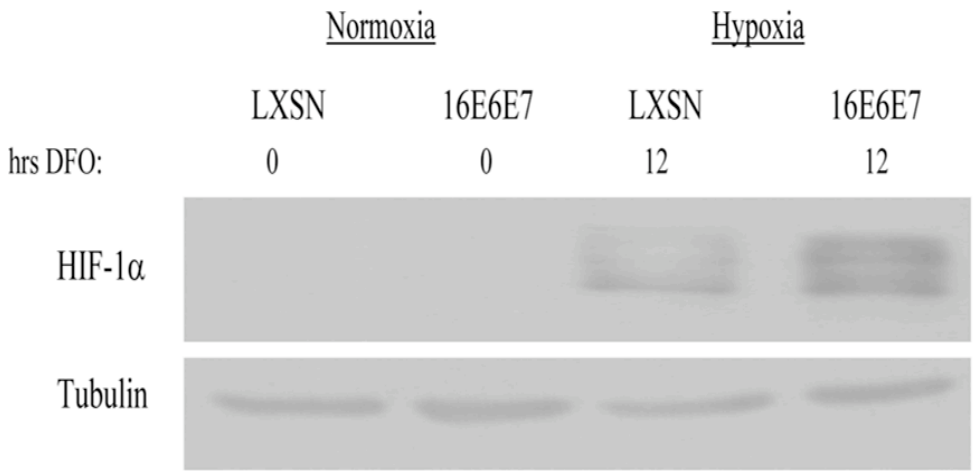

Figure 15. Enhanced induction of HIF-1α in HFKs expressing HPV 16E6E7 only in the presence of DFO.

HFKs transduced with HPV 16E6E7 or LXSN were incubated in the presence of 100 μm DFO for 12 hrs. Fifty micrograms of whole cell lysate were separated on an 8% SDS polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane and probed with antibodies to HIF-1α and Tubulin. The results are representative of three experiments. Tubulin serves as a loading control.

HIF-1α (2) as a positive control. As seen in Figure 15, treatment with DFO for 12 hrs induced HIF-1α in control retrovirus, LXSN or HPV 16E6E7 transduced HFKs. Although there was induction of HIF-1α in the presence of LXSN there was a greater increase in the presence of HPV 16E6E7.

We next examined whether the HPV 16E6E7, HPV 16E6TTLE7 or HPV 16E6TTLE7 expressing cells had any influence on HIF-1α in normoxic conditions. As seen in Figure 16, HIF-1α was not detected above background in either HPV 16E6E7, HPV 16E6TTLE7 or HPV 16E6E7TTL expressing cells in the absence of DFO.

Like HIF-1α, HIF-2α also has been shown to correlate with VEGF expression (47, 160). We used renal carcinoma cells (786 wt), which have been previously shown to enhance levels of HIF-2α under hypoxic conditions, as a positive control (Figure 16). HIF-2α was not detected above background in either HPV 16E6E7, HPV 16E6TTLE7 or HPV 16E6E7TTL expressing cells in the absence of a hypoxic environment (Figure 16). These results indicate that the presence of HPV 16E6E7, HPV 16E6TTLE7 or HPV 16E6E7TTL is not sufficient to induce HIF-1α and HIF-2α expression under conditions of normoxia. Therefore the enhanced level of VEGF seen in the presence of HPV 16E6E7 is due to HIF-1α and HIF-2α- independent mechanisms.

B. VEGF is required for the increased migration of human microvascular endothelial cells (HMVECs) in vitro

Angiogenesis involves activation of endothelial cells. IL-8 and VEGF have been reported to regulate angiogenesis and affect endothelial cell migration and proliferation (70, 102, 128, 142). However, the direct role of IL-8 and VEGF in HPV-mediated

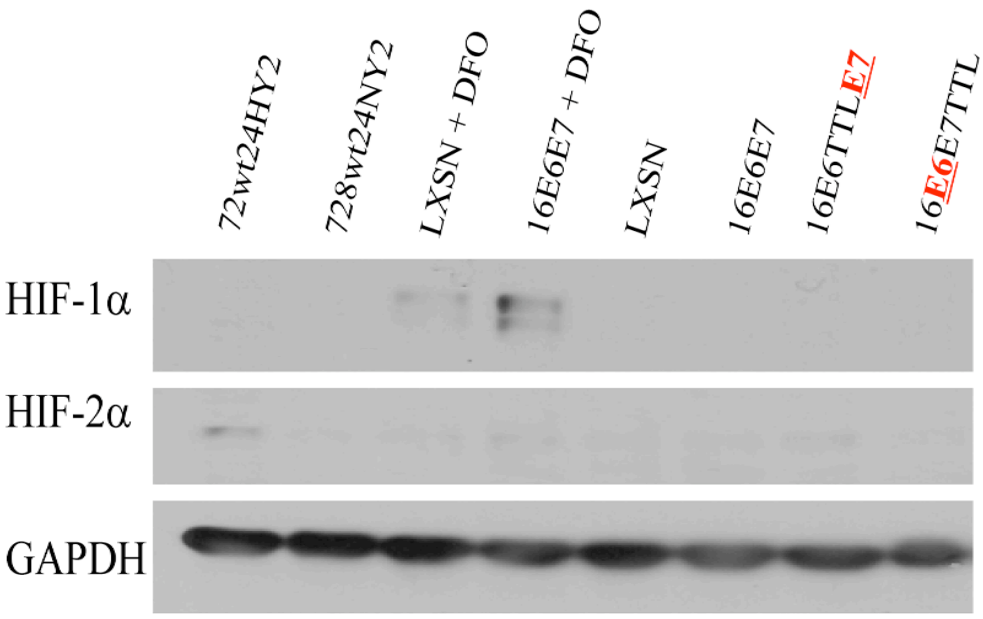

Figure 16. Enhanced expression of neither HIF-1α nor HIF-2α was detected above background in HPV 16E6E7, HPV 16 E6 or HPV 16 E7 expressing cells.

HFKs transduced with HPV 16E6E7 or LXSN were incubated in the presence or absence of 100 μm DFO for 12 hrs. 786 wt renal carcinoma cells were treated with normoxia (N) or hypoxia (H) for 24 hrs before lysis for Western blot analysis. Fifty micrograms of whole cell lysate were separated on an 8% SDS polyacrlyamide gel. Proteins were transferred to nitrocellulose membrane and probed with antibodies to HIF-1α, HIF-2α and GAPDH. GAPDH serves as a loading control.

angiogenesis remains unclear. To determine whether IL-8 and/or VEGF is required for increased migration of endothelial cells when such cells are exposed to conditioned media from HPV 16E6E7 expressing cells, neutralizing Abs directed against IL-8, VEGF or isotype controls (mouse IgG_1 or mouse IgG_{2B} respectively), were used to deplete conditioned media of IL-8 or VEGF. If IL-8 is successfully depleted and the depletion is specific, the level of IL-8 following incubation with antibodies to IL-8 should decrease but the level of VEGF should not. Similarly, if VEGF is successfully depleted and the depletion is specific, the level of VEGF should decrease following incubation with antibodies to VEGF but the level of IL-8 should not. The levels of neither IL-8 nor VEGF should decrease following incubation with isotype controls. ELISAs documented that IL-8 (Figure 17) and VEGF (Figure 18) were successfully and specifically depleted from conditioned media expressing HPV 16E6E7.

Conditioned media from cells expressing HPV 16E6E7 induced migration of HMVECS relative to LXSN, as shown in Figures 14 and 19, consistent with the literature (23). As seen in Figure 19, IL-8 depleted conditioned media from cells expressing HPV 16E6E7 retained its ability to increase migration of HMVECs when compared to IL-8 depleted conditioned media from LXSN transduced cells. However, VEGF-depleted conditioned media from cells expressing HPV 16E6E7 did not induce migration of HMVECs when compared to VEGF-depleted conditioned media from LXSN transduced cells in Figure19. These results indicate that VEGF, but not IL-8, is required to increase endothelial cell migration in response to conditioned media from HPV 16E6E7 expressing cells.

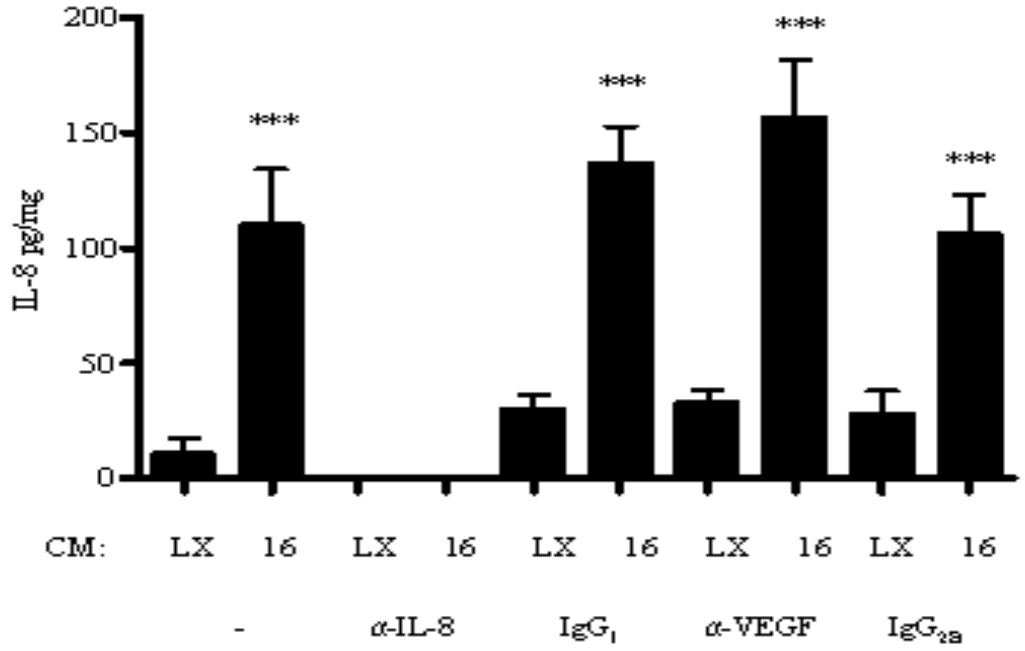

Figure 17. Specific depletion of IL-8 in conditioned media from pooled HFKs expressing HPV 16E6E7.

Conditioned media from HFKs retroviral transduced with LXSN and 16E6E7 were depleted of IL-8 or VEGF. The data represent mean ± SEM and are representative of three experiments each conducted in triplicate. $p< 0.0001$ for LXSN vs HPV 16E6E7; LXSN vs HPV 16E6E7 (isotype control IgG_1); LXSN vs HPV 16E6E7 (depleted of VEGF) and LXSN vs 16E6E7 (isotype control IgG_{2B}).

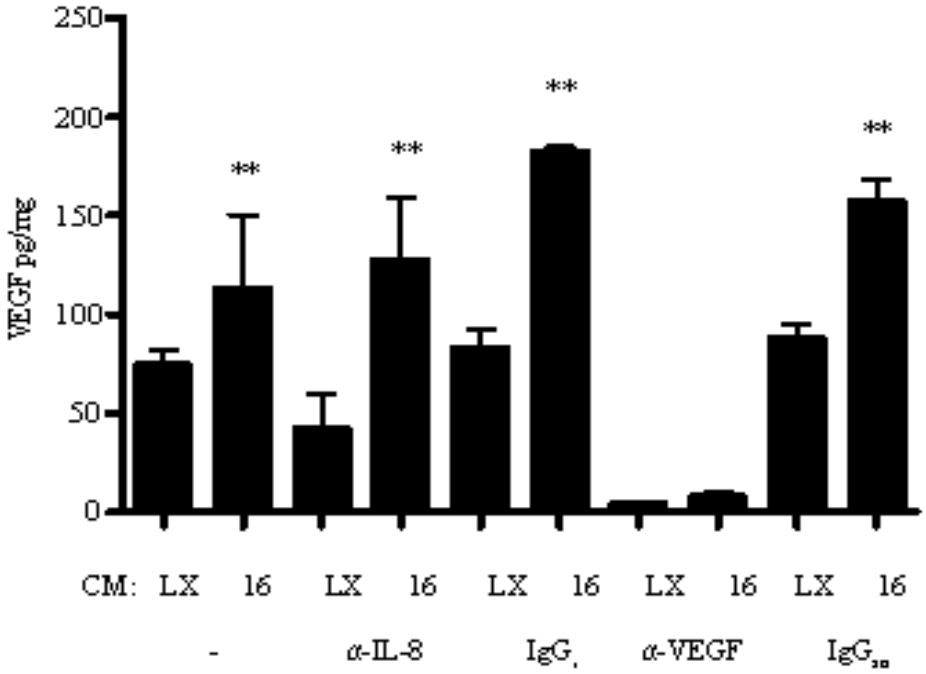

Figure 18. Specific depletion of VEGF in conditioned media from pooled HFKs expressing HPV 16E6E7 from pooled HFKs.

Conditioned media from HFKs retroviral transduced with LXSN and HPV 16E6E7 were depleted of IL-8 or VEGF. The data represent mean ± SEM and are representative of three experiments conducted in triplicate. $p<0.02$ for LXSN vs HPV 16E6E7; $p<0.001$ for LXSN vs HPV 16E6E7 (depleted of IL-8); LXSN vs HPV 16E6E7 (isotype control IgG_1); and LXSN vs 16E6E7 (isotype control IgG_{2B}).

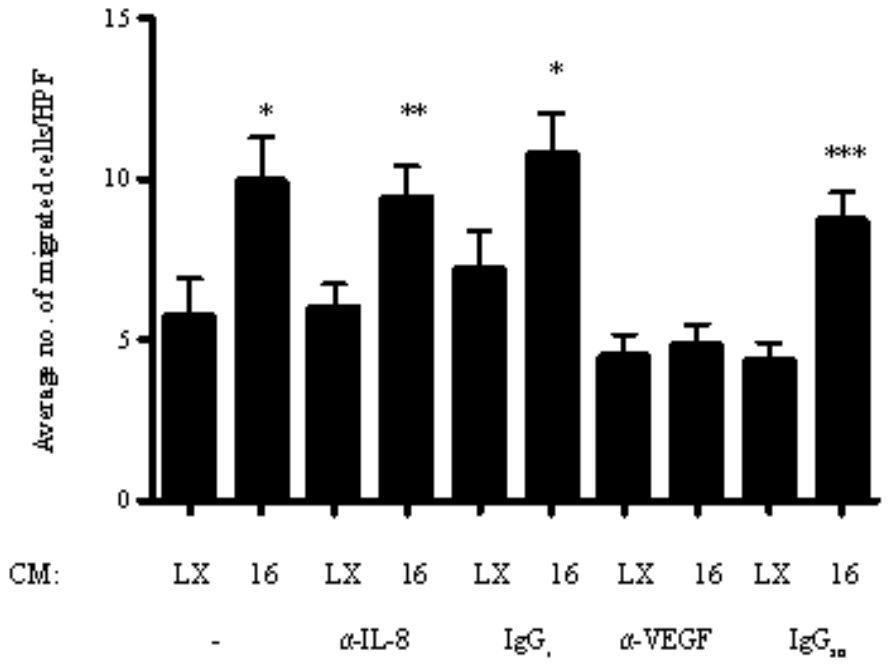

Figure 19. VEGF, but not IL-8, is required for the increased migration of HMVECS *in vitro*.

Conditioned media from HFKs retroviral transduced with LXSN and 16E6E7 were depleted of IL-8 or VEGF. The data represent mean number of migrated cells ± SEM and are representative of three experiments conducted in triplicate. $p< 0.0001$ for LXSN vs 16E6E7 and LXSN vs 16E6E7 (isotype control IgG_{2B}), $p< 0.002$ for LXSN vs 16E6E7 (depleted of IL-8) and LXSN vs 16E6E7 (isotype control IgG_1).

C. HPV 16E6E7C24G retains its ability to target p130 for degradation

HPV 16 E7 interacts with Rb as well as its family members, p107 and p130, through the LXCXE motif (aa 21-26). The motif contains the core for Rb pocket binding (40, 119, 185). Mutations in this region, especially changes to C_{24}, result in a substantial loss of affinity for Rb (9, 83, 119). Data in Figures 12 and 13indicate that expression of HPV 16 E7 is required for the enhanced expression of IL-8 and VEGF, equivalent to or greater than that seen with HPV 16E6E7. Therefore, experiments were designed to test the hypothesis that Rb binding and degradation is required for that enhancement.

To test this hypothesis, HPV 16E6E7C24G was constructed, to render E7deficient in binding to and targeting Rb for degradation, and was used to infect pooled HFKs. If the mutation is successfully engineered, the level of Rb in these infected cells should not be decreased relative to LXSN transduced cells. In fact, as was shown in Figure 10, Chapter I, the level of Rb may be increased relative to LXSN and HPV 16E6E7 due to the presence of E6. To confirm this, we monitored expression levels of pRb, as well as p53, in the retrovirally-transduced cells by Western Blot analysis. As expected, relative to LXSN and HPV 16E6E7, Rb protein expression was increased in cells infected with HPV 16E6E7C24G (Figure 20). Relative to LXSN, as expected, p53 expression was decreased in cells infected with HPV 16E6E7C24G, similarly to the decrease seen with HPV 16E6E7 (Figure 21). These results demonstrate that the mutant is indeed deficient for targeting Rb for degradation.

Although the C24G mutant has been reported to continue to target another Rb family member, p107 for degradation (60), its ability to target p130 for degradation has

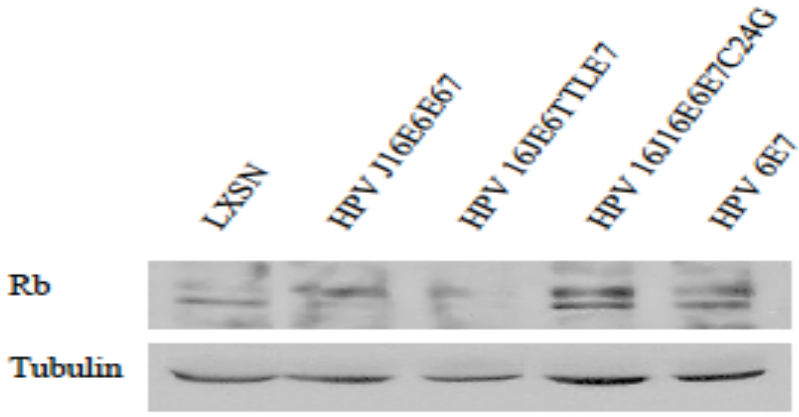

Figure 20. The steady state level of Rb is increased in pooled HFKs expressing HPV 16E6E7C24G relative to LXSN.

Fifty micrograms of whole cell lysate from pooled HFKs transduced with LXSN, HPV 16E6E7, HPV 16E6TTLE7, HPV 16E6E7C24G or HPV 6 E7, were separated on an 8% SDS polyacrylamide gel. Proteins were transferred to nitrocellulose membrane and probed with antibodies to Rb and Tubulin. Tubulin serves as a loading control.

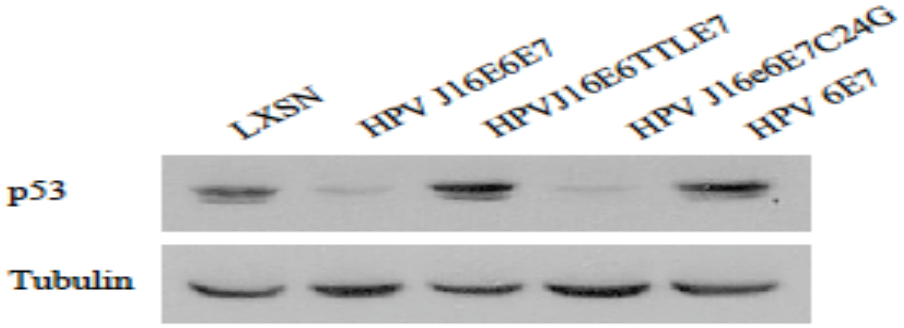

Figure 21. Expression of HPV 16E6E7C24G in pooled HFKs does not affect the ability of E6 to target p53 for degradation.

Fifty micrograms of whole cell lysate from pooled HFKs transduced with LXSN, HPV 16E6E7, HPV 16E6TTLE7, HPV 16E6E7C24G or HPV 6E7, were separated on an 8% SDS polyacrylamide gel. Proteins were transferred to nitrocellulose membrane and probed with antibodies to p53 and Tubulin. Tubulin serves as a loading control.

not been reported. Therefore, the effect of HPV 16E6E7C24G on p130 expression was determined by Western Blot analysis. As expected, the level of p130 in cells expressing wt E7 (HPV 16E6E7 or HPV 16E6TTLE7) was decreased relative to LXSN (Figure 22). Further, even in the E7C24G mutant (HPV 16E6E7C24G), p130 was decreased in cells expressing HPV 16E6E7C24G relative to LXSN (Figure 22). These results demonstrate that HPV 16E6E7C24G, which does not destabilize Rb (Figure 22) (60), retained the ability to decrease the steady state level of p130.

D. Rb binding and degradation is not required for the enhanced expression of IL-8 and VEGF.

To determine whether degradation of Rb was required for increased expression of IL-8 or VEGF, conditioned media from retrovirally transduced HFKS, were analyzed by ELISA to quantitate the levels of secreted IL-8 and VEGF. As in Figures 12 and 13, expression of HPV 16E6E7 or HPV 16E6TTLE7 significantly increased the levels of both angiogenic inducers relative to LXSN (Figures 23 and 24). Conditioned media from cells expressing HPV 16E6E7C24G had a 2.2 +/- 0.10 fold increase ($p < 0.0001$) in the level of secreted IL-8 compared to retrovirus control LXSN, although not to the same extent as seen with conditioned media from HPV 16E6E7 and HPV 16E6TTLE7 expressing cells (Figure 23). However, VEGF expression was increased 8.7 +/-1.6 fold ($p < 0.0001$) in cells expressing HPV 16E6E7C24G compared to retrovirus control LXSN and to a greater extent than conditioned media from HPV 16E6E7 or HPV 16E6E7TTL expressing cells (Figure 24). These preliminary data suggest that HPV 16 E7 binding to Rb is required for maximally enhanced IL-8 and VEGF expression. Further experiments

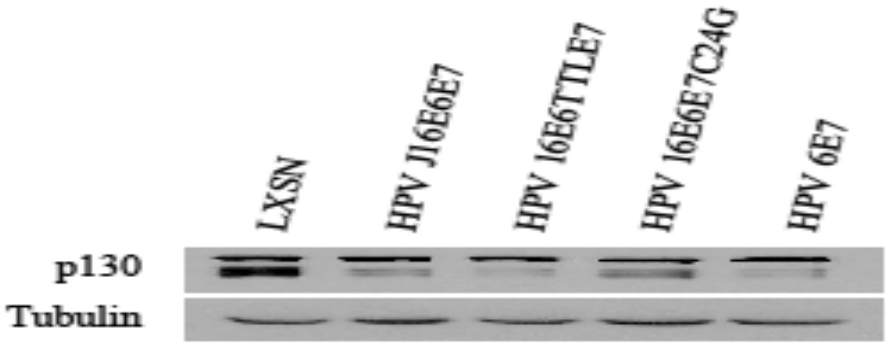

Figure 22. HPV 16E67C24G, which does not associate with Rb, is still capable of targeting p130 for degradation.

Fifty micrograms of whole cell lysate from pooled HFKs transduced with LXSN, HPV 16E6E7, HPV 16E6TTLE7, HPV 16E6E7C24G or HPV 6 E7, were separated on an 8% SDS polyacrylamide gel. Proteins were transferred to nitrocellulose membrane and probed with antibodies to p130 and Tubulin. Tubulin serves as a loading control.

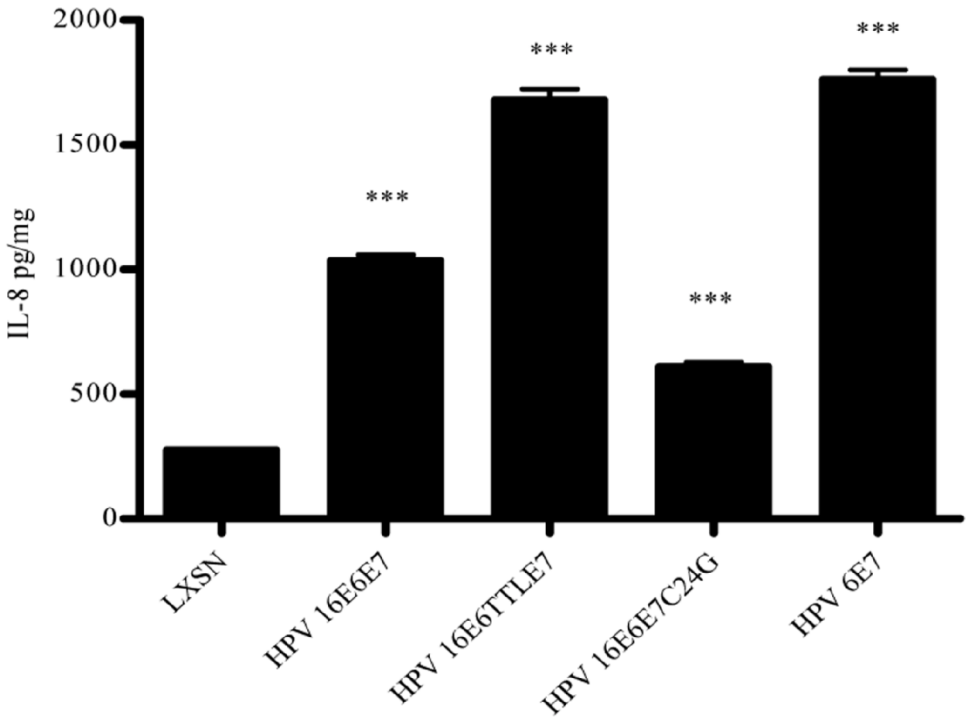

Figure 23. Levels of IL-8 in conditioned media were increased in cells expressing HPV 16E6E7C24G.

The quantity of IL-8 was determined and normalized to total protein in the conditioned media. The data represent mean ± SEM and are representative of two experiments each conducted in triplicate. $p < 0.0001$ for LXSN vs HPV 16E6E7; LXSN vs HPV 16E6TTLE7; LXSN vs HPV 16E6E7C24G and LXSN vs HPV 6 E7.

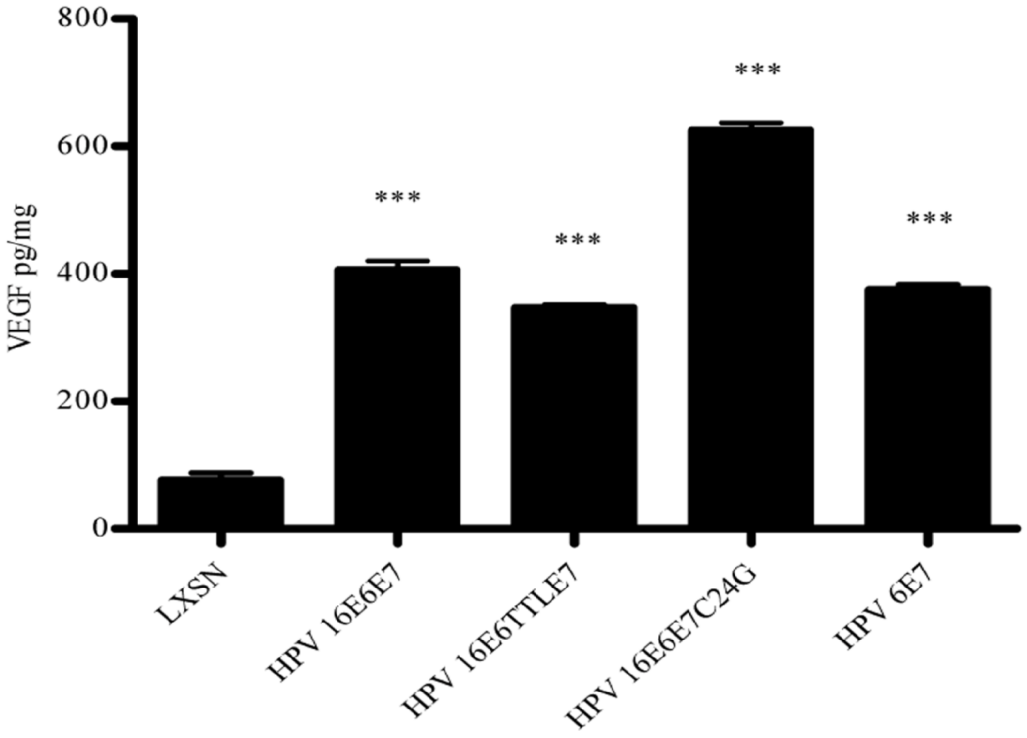

Figure 24. Levels of VEGF in conditioned media were increased in cells expressing HPV 16E6E7C24G.

The quantity of VEGF was determined and normalized to total protein in the conditioned media. The data represent mean ± SEM and are representative of two experiments each conducted in triplicate. $p < 0.0001$ for LXSN vs HPV 16E6E7; LXSN vs HPV 16E6TTLE7; LXSN vs HPV 16E6E7C24G and LXSN vs HPV 6 E7. $p < 0.0003$ for HPV 16E6E7 vs HPV 16E6E7C24G and $p < 0.02$ for HPV 16E6E7 vs HPV16E6TTLE7.

need to be conducted in order to validate these results.

E. Low risk HPV 6 E7 also increases IL-8 and VEGF expression

Low-risk virus HPV 6 E7 has been shown to target Rb family member p130 for degradation (191). VEGF and p130 expression are inversely correlated (145). ELISAs were used to determine the ability of HPV 6E7 to induce VEGF and IL-8 expression. Conditioned media from cells expressing HPV 6E7 caused a 5.2 +/- 1.0 fold and 6.4 +/- 0.10 fold ($p < 0.0001$) increase in the level of secreted VEGF and IL-8 compared to retrovirus control LXSN (Figures 23 and 24); comparable to the levels seen in conditioned media from cells transduced with HPV 16E6TTLE7.

DISCUSSION

The study described in this thesis were undertaken to determine the individual contributions of human papillomavirus type 16 E6 and E7 oncoproteins in regulating the angiogenic response seen in the early stages of cervical cancer. To do so, translation termination linkers were introduced into the coding region of E6 or E7. Human foreskin keratinocytes were retrovirally transduced with the mutated cassette; the ability of E7 in the context of the E6TTL mutation (E6TTLE7) and E6 in the context of the E7TTL mutation (E6E7TTL) to induce VEGF and IL-8 was analyzed. We showed that the expression of E6 or E7 in human foreskin keratinocytes significantly increased the expression of both VEGF and IL-8. E7, however, is the major contributor. We have shown that the effect on VEGF was independent of HIF-1α and HIF-2α. Here we have shown that conditioned media from either E6 or E7 expressing cells does not induce migration of human microvascular endothelial cells (HMVECs). In addition, immunological depletion experiments showed that VEGF is required for HPV 16E6 and E7 together to induce HMVEC migration. Data further show that the ability of HPV 16 E7 to induce IL-8 and VEGF may be dependent upon degradation of the Rb family member p130.

A. Transduced HPV 16E6TTLE7 in pooled human foreskin keratinocytes expresses more spliced message than HPV 16 E6 and E7 or HPV 16E6E7TTL.

As discussed in the Introduction, splicing is important for the regulation of E6 and E7 in the intact genome. During a natural infection, HPV 16 E6 and E7 are transcribed as a single bicistronic transcript (Figure 4). Splicing occurs within the E6 coding region

from nt 226 to nt 409 creating truncated E6*I. The majority of transcripts within cancer tissues and cervical cancer cell lines are E6*I (15, 38, 152, 192, 194). Translation of E7 is dependent upon splicing (193). Splicing depends on the distance of the splice donor from the promoter (96). The distance between the termination of E6 translation and the reinitiation of E7 translation is approximately 2 nucleotides. E6*I contains a premature codon immediately downstream of the splice junction creating enough space for termination of E6 translation and the reinitiation of E7 translation (93) therefore benefiting the expression of E7. Unspliced mRNA is translated into E6.

In the context of the retrovirus, splicing is inefficient. E6 and E7 expression is dictated not by the P_{97} promoter but by the retroviral long terminal repeat (LTR). Cells infected with the retrovirus undergo splicing less frequently due to the distance between the LTR and the splice donor at nt 226 (96).

We have shown that more spliced message was expressed in HFKs retrovirally-transduced with HPV 16E6TTLE7 compared to HPV 16E6E7 or HPV 16E6E7TTL (Figure 12). Even without the enhanced splicing, placement of the TTL at nt 280 within E6 should increase E7 mRNA templates therefore favoring expression of E7. The splicing within this mutated sequence appears to mimic splicing seen within the natural infection. This is in contrast to the TTL placed at nt 711 within the E7 coding region, this does not affect the translation of E6. This increase in E7 mRNA template may explain why an even greater increase in the level of secreted VEGF and IL-8 seen in cells expressing HPV 16E6TTLE7 compared to HPV 16E6E7 or HPV 16E6E7TTL in Figure 13 and 14.

B. Expression of HPV 16E6TTLE7 in pooled HFKs reduces the levels of Rb while the levels of p53 are increased relative to LXSN transduced HFKs

E6 and E7 target the tumor suppressors p53 and Rb, respectively, for degradation. In contrast, we have shown that expression of E7 in the absence of E6 (HPV 16E6TTLE7) leads to stabilization of p53 as well as the induction of p21 (data not shown), which is consistent with the literature (35, 82, 150, 170). However, the mechanism underlying this stabilization of p53 by E7 remains unclear.

One possible mechanism for the increased steady state level of p53 function by E7 is through p53 interaction between E2F and MDM2. E7 degradation of Rb liberates E2F from Rb and overexpression of E2F1 is known to increase both p53 and MDM2 (196). This result was shown to be independent of $p19^{ARF}$. Seavey et al. 1999 showed that E7, like E2F1, could increase the levels of both p53 and MDM2 in normal diploid human fibroblasts lacking $p19^{ARF}$(150). Therefore, E7 and E2F1 may stabilize p53 through a mechanism independent of $p19^{ARF}$. Another possible mechanism for E7-mediated increase in the steady state level of p53 is that E7 interferes with the function of p300, which serves as a platform for the stimulation of degradation of p53 by MDM2 (63).

We have also shown that expression of E6 in the absence of E7 leads to stabilization of Rb, which is consistent with the literature (111). However, the mechanism underlying this stabilization of Rb by E6 also remains unclear. A possible mechanism for E6 dependent stabilization of Rb may be through the activities of the cyclin dependent kinases (CDKs), which are involved in the phosphorylation of Rb (cyclin A/CDK4, Cyclin D/CDK6 and cyclin A/CDK2). Degradation of p53 in the presence of E6 and inhibition of p53-induced expression of cyclin-dependent kinase inhibitor p21 may lead

to increase activity of CDKs, leading to increased stability of Rb. Therefore, E6 could stimulate phosphorylation of Rb by enhancing levels of CDK4/6 (111). However, how E6 stimulates levels of CDK4/6 is unknown and needs to be further explored.

C. Possible Mechanism of HPV 16 E6 and HPV 16 E7 Enhancement of IL-8.

The Roman laboratory has previously shown that expression of HPV 16E6 and E7 together is sufficient to cause an increase in the angiogenic inducer IL-8 (177). The ability of E6 and E7 alone to alter the expression of IL-8 was not investigated. Experiments were therefore designed to test the hypothesis that E6 or E7 alone is sufficient to alter the expression of IL-8. Human foreskin keratinocytes (HFKs) were infected with recombinant retrovirus expressing HPV 16E6TTLE7 or HPV 16E6E7TTL. ELISAs was used to analyze IL-8 expression. In cells expressing HPV 16E6TTLE7 or HPV 16E6E7TTL, the levels of secreted IL-8 were upregulated relative to control LXSN transduced HFKs.

As discussed in the Introduction, IL-8 expression is controlled at the transcriptional and the posttranscriptional level (72). IL-8 expression is regulated in cooperation with three inducible transcription factors, NFκB, AP-1 and C/EBP depending upon cell type and stimulus (72). Desaintes et al. showed that HPV 16 E6 is able to transactivate promoters containing NFκB sites (Figure 25) (36). One possible mechanism for 16 E7's ability to enhance IL-8 expression is through C/EBPα. CEB/Pα is a positive regulator of IL-8 expression. Timchenko et al. found that C/EBPα regulates E2F/p107 and E2F/p130 complexes (174). Specifically, C/EBPα disrupts E2F/p107 complexes, while E2F/p130 complexes are induced (174). We propose that HPV 16 E7 ability to enhance IL-8 expression is due to HPV 16 E7's ability bind and target p107 and p130 for degradation

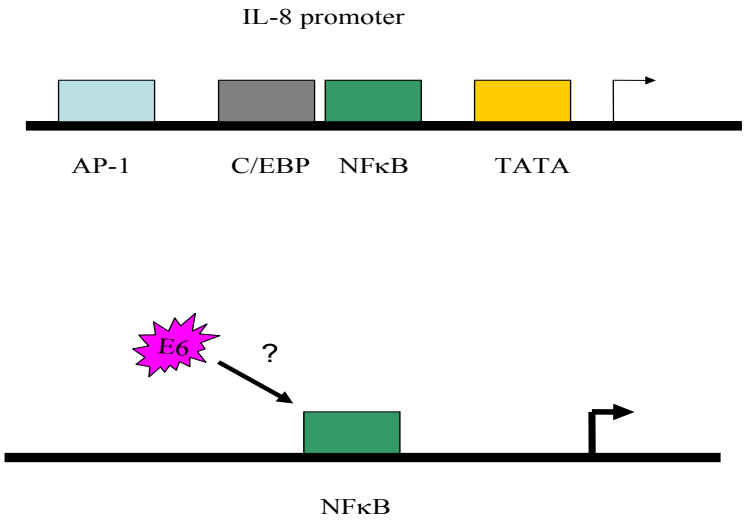

Figure 25. Possible Mechanism of HPV 16 E6 Enhancement of IL-8.

HPV 16 E6 transactivates NFκB thereby leading to the enhancement of IL-8.

thereby leading to the positive regulation of C/EBP and enhancing IL-8 expression (Figure 26). HPV 16 E7 is also able to transactivate promoters containing AP-1 sites (3). Perhaps HPV 16 E7's ability to transactivate this additional site may result in the greater increase in IL-8 expression.

D. Possible Mechanism of HPV 16 E6 and HPV 16 E7 Enhancement of VEGF

Previous data from the Roman laboratory has determined that expression of both E6 and E7 are sufficient to enhance VEGF. Whether E6 or E7 alone was sufficient was examined. Conditioned media from cells expressing HPV 16E6E7TTL or HPV 16E6TTLE7 increased the levels of secreted VEGF relative the control LXSN transduced HFKs. One possible mechanism for the ability of HPV 16 E6 to increase expression of VEGF is through the coactivator p300, which is important for cell cycle progression and differentiation. Both HIF-1α and p53 bind to distinct regions of p300 and p300 is required for full activity of both transactivators (4, 6, 64, 103, 157). Recent data proposed a competition between p53 and HIF-1α for p300 (32, 148). Negative regulation of HIF-1 by p53 was evident as levels of wt p53 increased upon cellular stress such as prolonged or severe hypoxia, thereby leading to repression of HIF-1 stimulated transcription of a number of genes including VEGF (139) and HIF-1 destabilization (148). We propose that HPV 16 E6's ability to bind and target p53 for degradation reverses this effect thereby allowing interaction between HIF-1α and p300 resulting in the enhanced expression of VEGF (Figure 27). Another mechanism by which HPV 16 E6 may increase expression of VEGF is through the transcriptional factor Sp-1 located on the VEGF promoter. It has been demonstrated that p53 complexes with SP-1 and inhibits Sp-1-mediated VEGF transciption under normoxic and hypoxic conditions (132). E6 has been shown to

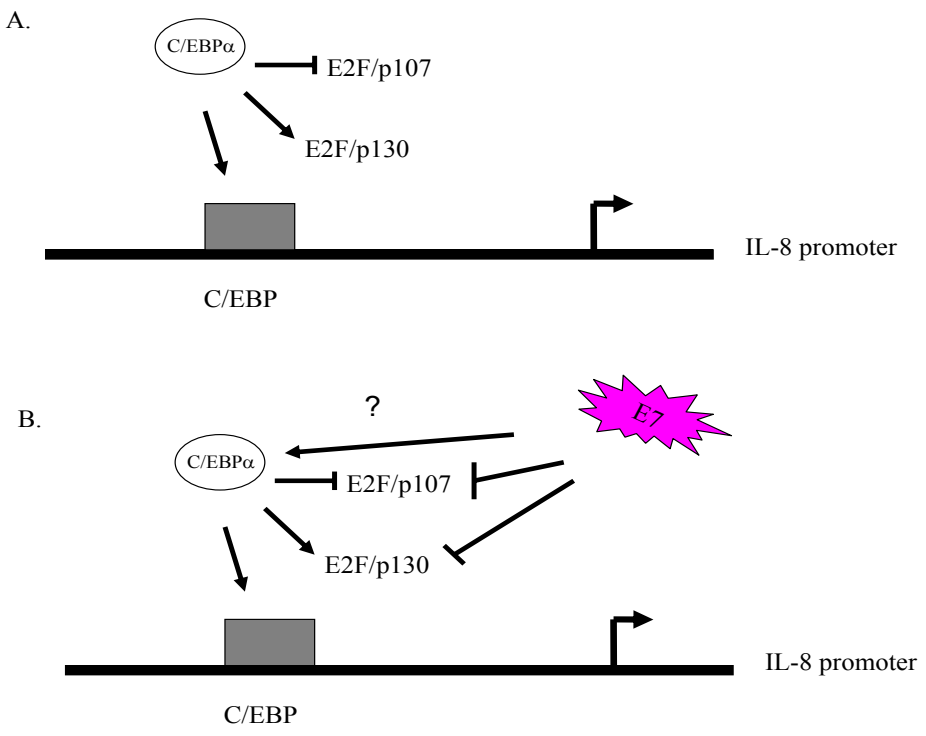

Figure 26. Possible Mechanism of HPV 16 E7 Enhancement of IL-8.

(A) C/EBPα regulates E2F/pl07 and E2F/p130. (B) HPV 16 E7's ability to bind and target p107 and p130 for degradation leads to the enhancement of IL-8.

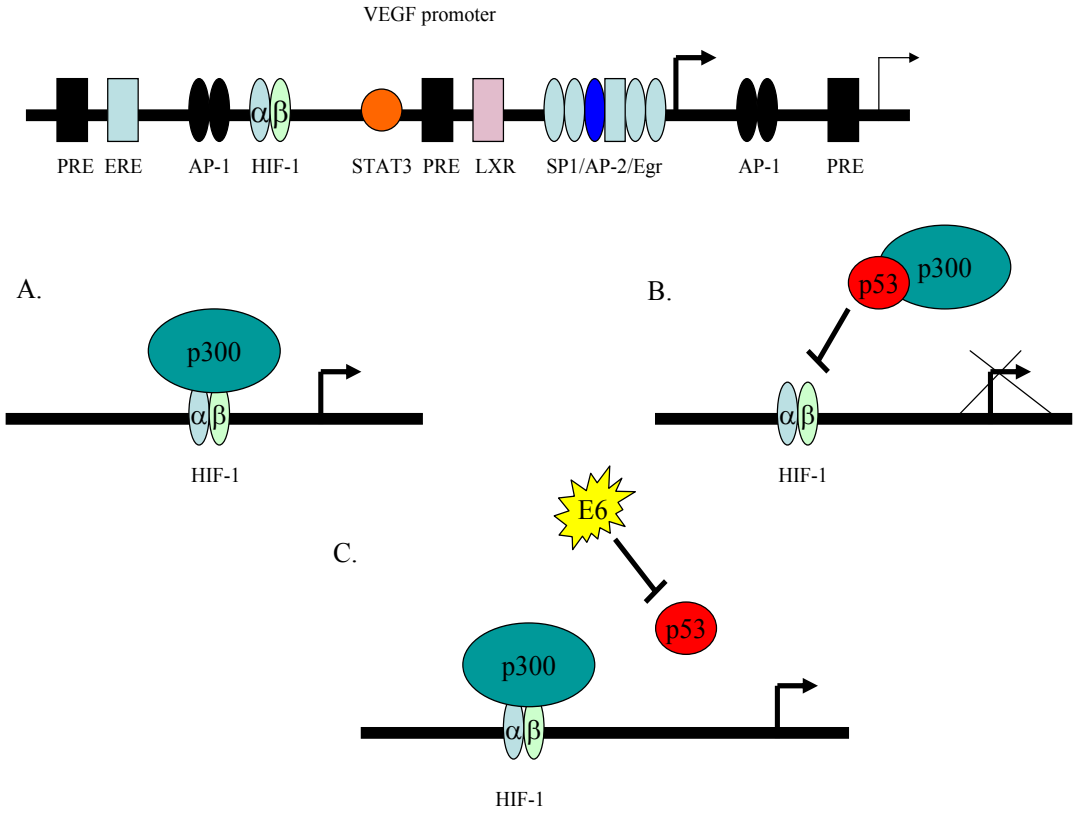

Figure 27. Possible Mechanism of HPV 16 E6 Enhancement of VEGF.

(A) HIF-1α binds to p300 and enhances VEGF. (B) Negative regulation of HIF-1α by p53. (C). Degradation of p53 by HPV 16 E6 leads to the enhancement of VEGF.

regulate VEGF though SP-1 (108). We propose that E6 ability to bind and target p53 for degradation increases binding of Sp-1 to the VEGF promoter thereby increasing VEGF transcription (Figure 28). Further experiments could distinguish between each of these possible mechanisms.

The enhanced levels of HIF-1α in HPV positive cells were found to activate genes such as VEGF. VEGF is strongly induced by hypoxia. Our previous work has shown that expression of high-risk E6 and E7 can enhance the expression of angiogenic factors such as VEGF even in non-hypoxic conditions (177). However, it was unclear if HIF-1α activation was responsible. Additionally, another putative mechanism based on the EBV literature, is that HPV 16 E7 may interfere with the ability of prolyl hydroxylases to hydroxylate proline residues on HIF-1α under normoxic conditions thus stabilizing HIF-1a. As seen in Figure 14, HPV 16 E7, as well as HPV 16 E6 or HPV 16E6E7, have no detectible effect on HIF-1α levels in normoxia thus suggesting that the induction of HIF-1α occurs specifically under hypoxic conditions. Therefore, HIF-1α and HIF-2α-independent mechanisms maybe responsible for E7-mediated activation of VEGF.

HIF-1α independent mechanisms have been reported responsible for the activation of VEGF. One possible mechanism for HPV 16 E7 ability to increase VEGF expression is through AP-1. E7 can transactivate AP-1 sites on the VEGF promoter (Figure 28). Further experiments are needed to determine how VEGF expression is regulated by E6 and E7.

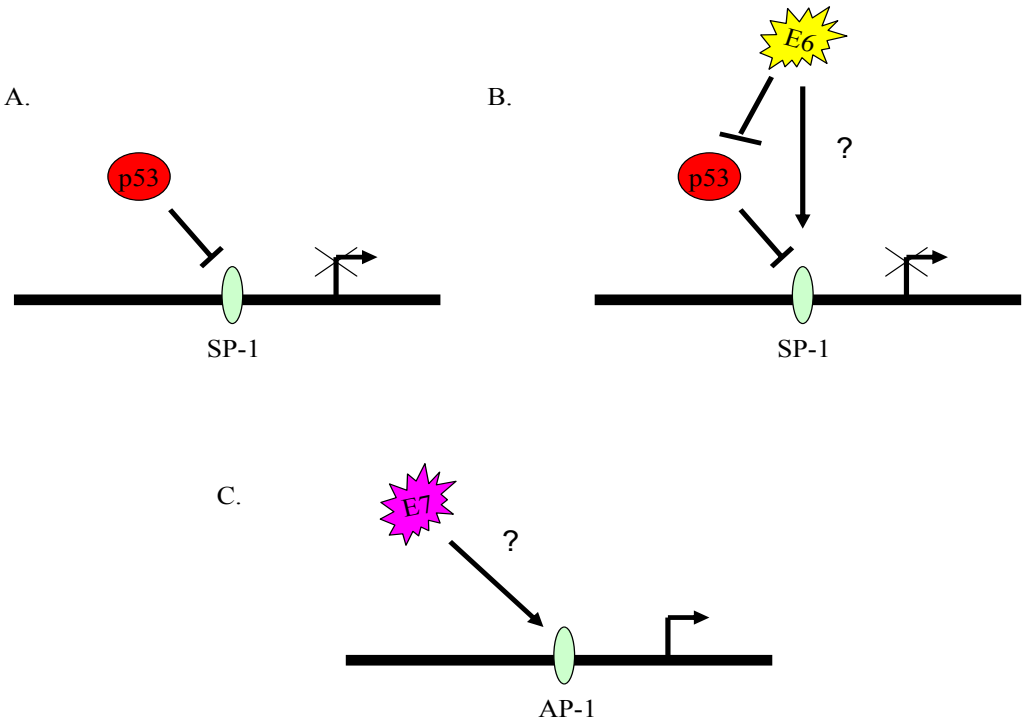

Figure 28. Possible Mechanism of HPV 16 E6 and 16 E7 Enhancement of VEGF.

(A) Negative regulation of SP-1 by p53. (B) HPV 16 E6 degradation of p53 leads to the enhancement of VEGF. (C) HPV 16 E7 transactivates AP-1 leading to the enhancement of VEGF.

E. Conditioned media from pooled HFKS transduced with HPV 16E6TTLE7 or HPV 16E6E7TTL did not induce migration of human microvascular endothelial cells (HMVECs) *in vitro.*

We have shown that conditioned media from cells expressing HPV 16E6TTLE7 or HPV 16E6E7TTL do not induce migration of HMVECs. This is in contrast to cells expressing HPV 16E6E7. These results are consistent with a series of transgenic experiments from the Lambert laboratory. Lambert et al. showed that E7 played a major role in promotion, an early step in tumorigenesis (158). In contrast, E6 was shown to contribute weakly to the promotion stage to benign disease. E6 in addition to E7 led to an increase in the formation of large and more invasive cancers (158). Based on the above data we conclude that the combination of E6 and E7 is required to induce migration of HMVECs which may lead to a higher grade of disease.

F. VEGF, but not IL-8, is required for the increased migration of HMVECs *in vitro*.

We have shown that IL-8 depleted conditioned media from cells expressing HPV 16E6E7 retained its ability to increase migration of HMVECs. This is in contrast to VEGF-depleted conditioned media from cells expressing HPV 16E6E7, which does not induce migration of HMVEC. There are a number of different factors within the tumor microenvironment that are regulators of angiogenesis (fibroblasts and mast cells), which combine to recruit endothelial cells from the local vasculature (130). Fibroblasts are known to produce VEGF. These factors in addition to VEGF and HPV16 E6 and E7 may result in the increase of endothelial cell migration.

G. Low risk HPV 6 E7 also increases IL-8 and VEGF expression

We have shown that conditioned media from cells expressing HPV 6 E7 increased the level of secreted VEGF. One possible mechanism for the increase in VEGF expression is through Rb family member p130. Sanseverino et al. found that VEGF and p130 expression were inversely correlated in endometrial cancers (145). Specifically, immunohistochemical staining showed low VEGF staining correlated with high expression of p130. Vice versa, higher levels of expression of VEGF correlated with low expression of p130 (145). Further examination of VEGF upregulation by HPV 6 E7 in normoxic conditions needs to be explored.

We further show conditioned media from cells expressing HPV 6 E7 increases the level of secreted IL-8. The mechanism for this increase remains unknown. Further examination of IL-8 upregulation by HPV 6 E7 needs to be explored.

FUTURE DIRECTIONS

The work in this thesis has begun to address the contributions of E6 and E7 to an angiogenic switch, which is important for the development of HPV-mediated disease. There are a number of questions still to be answered and observations to be investigated because of results presented in Chapters I-III and discussed above.

For future investigations, it is important to first study the individual contribution of E6 and E7 in an *in vivo* system. To test whether conditioned media from HPV 16 E6TTLE7 or HPV 16E6E7TTL transduced pooled human foreskin keratinocytes (HFKs) could induce an angiogenic response *in vivo*, the Matrigel plug assays could be utilized. This assay is an excellent method for determining the angiogenic potential of different compounds and would provide valuable information on the individual role E6 and E7 in the recruitment of a blood supply. We have shown that neither E6 nor E7 alone is sufficient to induce migration of human microvascular endothelial cells (HMVECs). Based on those findings we predict that neither E6 nor E7 alone would be sufficient to elicit an angiogenic response *in vivo*.

We have verified that angiogenic inducer VEGF in conditioned media from HPV 16E6E7 expressing cells influenced migration of human microvascular endothelial cells (HMVECs) *in vitro* using neutralizing antibodies against VEGF. Further investigations will determine if VEGF contributes to the recruitment of a blood supply to the Matrigel plug. Matrigel plug assay would be used to assess whether conditioned media from HPV 16E6E7 expressing cells depleted of VEGF could induce an angiogenic response.

Low-risk virus HPV 6 E7 has been shown to target p130 for degradation (191). VEGF and p130 expression have been shown to be inversely correlated (145). Based on

these findings, we hypothesized that low-risk HPV 6 E7 would able to increase expression of VEGF. We have preliminary data showing that HFKs expressing HPV 6 E7 increased the levels of secreted VEGF as well as IL-8. Since there was variability between experiments, these experiments need to be repeated.

We have preliminary data showing that p130 may regulate the expression of VEGF as well as IL-8. The HPV 6 E7C25A mutant, can inhibit E7 binding to p130, could be used to determine if p130 binding is required for the increased expression of angiogenic inducers VEGF and IL-8.

Finally, it needs to be investigated whether expression of E6 and E7 proteins of low-risk HPVs are able to alter the expression of angiogenic inducers, VEGF and IL-8 as well as angiogenic inhibitors, thrombospondin-1 (TSP-1) and Maspin similar to the affect observed upon expression of high-risk E6 and E7. Pooled HFKs would be co-infected with two retroviruses HPV 6 E6 and neo^r and the other expressing HPV 6 E7 and $hygromycin^r$. This will ensure that the cells are infected with both viruses. Semi-quantitative RT-PCR would be used to analyze VEGF, IL-8, Maspin and TSP-1 transcripts. Conditioned media from these retrovirally-transduced cells would be monitored using ELISA for VEGF and IL-8 and Western blot analysis for TSP-1 and Maspin. To characterize the ability of conditioned media from cells expressing HPV 6 E6 and 6 E7 to alter endothelial behavior migration assay would be used. Additionally, the Matrigel plug assay would be used to assess whether conditioned media from HPV 6 E6E7 could induce an angiogenic response.

These experiments would provide additional mechanisms as to how angiogenesis is regulated in HPV-mediated disease.

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CURRICULUM VITAE

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EDUCATION

2010 **Ph.D. Microbiology and Immunology**, Indiana University, Indianapolis, IN, U.S.A.

2003 **B.Sc Biology (Magna Cum Laude)**, Grambling State University, Grambling, LA, U.S.A.

RESEARCH AND TRAINING EXPERIENCE

2003-2004 **Research Associate**
Principal Investigator: David Wilkes, MD.
Division of Pulmonary, Allergy, Critical Care and Occupational Medicine, Wishard Hospital, Indiana University School of Medicine, Indianapolis, IN, U.S.A.

2003 **Summer Research Fellow**
T-35 NIH Training Program for Minority, Indiana University School of Medicine, Indianapolis, IN, U.S.A.
Mentor: Randy Brutkiewicz, Ph.D.

2002 **Summer Research Fellow**
T-35 NIH Training Program for Minority Students, Indiana University School of Medicine, Indianapolis, IN, U.S.A.
Mentor: David Wilkes, MD

2001 **Summer Research Fellow**
CIC Summer Research Opportunities Program, University of Illinois School of Medicine, Champaign, IL, U.S.A.
Mentor: Naveen Manchanda

2000-2001 **Laboratory Assistant**
Department of Biology, Grambling State University, Grambling, LA, U.S.A.
Mentor: Waneene Dorsey, Ph.D.

TEACHING EXPERIENCE

2008-09 **Camp MD-Medical Detectives**
Indiana University School of Medicine, Indianapolis, IN.
Invited Guest Speaker- Topic: "What are the advantages of graduate school?"
Developed biomedical basics module curriculum for minorities at early stages (prior to college)

2005 **Teaching Assistant**
Department of Microbiology/Immunology
Indiana University School of Medicine, Indianapolis, IN.
Conducted practicals and tutorials in Microbiology (Micro J210) for Nursing school students
Gained experience in basic microbiology techniques and developed communication skills

HONORS, AWARDS AND FELLOWSHIPS

2009-2010	Southern Regional Educational Board Doctoral Scholar (SREB)
2008	IUSM Ambassador Travel Award
2007-2010	T32 CURE NIH Supplement to NIH T32 CA111198 Cancer Training Program
2004-2010	IUSM Graduate Division Student Ambassador
2004-2010	IUSM Graduate Division Student Mentor
2003	Graduated Magna Cum Laude from Grambling State University
2000-2003	Louisiana Alliance for Minority Participation Scholarship
2001	Black Voices Quarterly Top 100+ Academic Excellence Award
2001	Golden Key National Honor Society
2000	Earl Lester Cole Honors College
1999	Tuition Opportunity Program Scholarship
1999	Academic Achievement Scholarship

PUBLICATIONS

1. Chen, W., Li, F., Mead, L., White, H., Walker, J.A., Ingram, D. A., Roman, A. Human Papillomavirus Causes an Angiogenic Switch in Keratinocytes Which is Sufficient to Alter Endothelial Cell Behavior. Virology 367:168-174 (2007). (PMID: 17602722).

2. Thiele, A.T., Sumpter, T.L., Walker, J.A., Xu, Q., Chang, C.H., Bacallao, R.L., Wilkes, D.S. Pulmonary Immunity to Viral Infection: Adenovirus Infection of Lung Dendritic Cells Renders T Cells Non-Responsive to IL-2. J Virol 80:1826-1836 (2006).

3. Walker, J., Smiley, L., Ingram, D.A., Roman, A.
Expression of Human Papillomvirus Type 16 E7 Is Sufficient to Significantly Increase Expression of Angiogenic Factors But Is Not Sufficient to Induce Endothelial Cell Migration. Virology (In progess).

CONFERENCES ATTENDED

1. International DNA Tumor Viruses Conference. Oxford, UK.
Walker JA and Roman A "Expression of Human Papillomavirus Type 16 E7 but not HPV16 E6 is Sufficient to Significantly Increase Expression of Angiogenic Factors". July 2009. Poster Presentation.

2. DNA Tumor Viruses Conference. Madison, WI.
Walker JA and Roman A "Investigating the Role of Human Papillomavirus 16 E6 and E7 Oncoproteins in Angiogenesis". July 2008. Poster Presentation.

3. Indiana University Melvin and Bren Simon Cancer Center Cancer Research Day. Indianapolis, IN.
Walker JA and Roman A "Investigating the Role of Human Papillomavirus 16 E6 and E7 Oncoproteins in Angiogenesis". May 2006-2009. Poster Presentation.

4. NOBCCHE Biotech Conference, Eli Lilly. Indianapolis, IN.
Walker JA and Roman A "Investigating the Role of Human Papillomavirus 16 E6 and E7 Oncoproteins in Angiogenesis", October 2008. Poster Presentation.

5. CIC Summer Research Opportunities Program National Conference. Minneapolis, MN. Walker JA, Venkataraman S and Brutkiewicz R "Structural Requirements for the Functional Expression of Mouse CD1d1 Molecules". August, 2003. Poster Presentation.

6. CIC Summer Research Opportunities Program National Conference. University Park, PA. Walker JA and Wilkes DS "Cytokine Induced Regulation of Indoleamine 2,3-dioxygenase Expression in Dendritic Cells". August, 2002. Poster Presentaion.

7. CIC Summer Research Opportunities Program National Conference. Anarbor, MI. Walker JA and Manchunda N "Elucidation of the molecular determinants responsible for interactions between urokinase-type plasminogen activator and plasminogen activator inhibitor-1 versus plasminogen". July, 2001. Oral Presentation.