I, Monique A Morrison, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Developmental Biology.

It is entitled:
Targeting the Human Papillomavirus E6 and E7 Oncogenes by E2 promotes Cellular Motility and Invasion

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Targeting the Human Papillomavirus E6 and E7 Oncogenes by E2 promotes Cellular Motility and Invasion

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Abstract

Cervical cancer is the second most common cancer in women worldwide with over 500,000 new cases reported annually. Almost all cervical cancers are caused by the mucosatropic high risk human papillomaviruses (HPV). Integration of HPV DNA into the cellular genome is common in such cancers and results in the loss of expression of the viral E2 protein and increased expression of the E6 and E7 oncogenes. E6 and E7 are essential for the initiation and maintenance of cervical cancer. E6 is known to degrade p53, while E7 degrades members of the pRB family. E2 on the other hand is a negative transcriptional regulator of E6 and E7. Together, the oncogenes inhibit cellular senescence and apoptosis, enhance immortalization, and along with other oncogenic events promote transformation. E2 expression in cancer cells results in transcriptional E6/E7 repression, the re-activation of their respective tumor suppressor targets p53 and pRb, and subsequent senescence induction in cervical cancer cells. Consequently, viral oncogene suppression via E2 has been hailed as a promising approach for the treatment of HPV positive tumors. Herein we show that surprisingly, E2 expression in HPV positive cervical cancer cells stimulates cellular motility and invasion. Migration correlated with the dynamic formation of cellular protrusions, and was dependent upon cell-to-cell contact. While E2 expressing migratory cells were senescent, migration was not a general feature of cellular senescence or cell cycle arrest, and was specifically observed in HPV positive cervical cancer cells. Finally, E2 motility required E6/E7 repression, but E6/E7 knockdown alone was not sufficient to cause migration. Interestingly, E2-expressing cells were not only themselves motile, but conferred increased motility to neighboring HeLa cervical cancer cells. These findings may be clinically important as viral E2 expression is already in clinical trials for the treatment of cervical and other HPV
associated diseases. Taken together, our data demonstrate that repression of the viral oncogenes by E2 stimulates the motility of E6/E7-targeted cells as well as adjacent non-targeted cancer cells, thus raising the possibility that E2 expression may unfavorably increase the local invasiveness of HPV positive tumors.
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CHAPTER 1: INTRODUCTION
Human Papillomavirus and Associated Disease.

Human papillomaviruses (HPVs) are small, non-enveloped, double stranded DNA viruses consisting of an icosahedral capsid [1]. Over 120 types of HPVs have been identified based on DNA sequence analysis, with all infecting epithelial tissues [2]. HPVs can be grouped into five evolutionary genera: alpha (α), beta (β), gamma (γ), Mu (μ) and Nu (ν). The α genus represents the largest and most characterized group and it is this group that infects the cervical epithelium to cause cervical cancer. The remaining genera are mostly associated with cutaneous warts that generally do not progress to cancer. The α genus can be further classified as mucosal or cutaneous, with cutaneous types infecting the squamous epithelium to produce warts on the hands, face and feet [3]. There are approximately 40 mucosal HPV types that infect the genital and head and neck mucosa as well as the aerodigestive tract [4, 5]. Mucosal HPVs can be classified as non-oncogenic or ‘low risk’ (LR) or potentially oncogenic, ‘high risk’ (HR) depending on their abilities to cause papillomas (or warts depending on the sites of infection), or cancer respectively. It is now well established that HR HPVs including types 16, 18, 45 and 31 are the causative agents of almost all (>99.7%) cervical cancers [6]. Among the HR types HPV 16 and 18 are the most common types associated with cervical carcinomas. HPV 16 DNA is found in almost 50% of squamous cell carcinomas while HPV 18 DNA is present in almost 50% of adenocarcinomas of the cervix [7]. HPV infection of the genital tract is the most common sexually transmitted infection and approximately 11% of women in the general population are thought to harbor cervical HPV infection at any given time [8]. Additionally, cervical cancer is the second most common cancer in women [9]. Aside from cervical cancer, HR HPVs are also responsible for over 25% of head and neck
cancers as well as a portion of penile, prostate, vulvar, lung and other cancers [10-14]. Currently, HPV is responsible for approximately 4% of all cancers, and given its newly established role in head and neck cancers, this estimate is bound to increase [9].

**HPV Genomic Organization**

While HPV types are heterogeneous, they are all similar in size with very little variation. The HPV genome is approximately 8kb in size and consists of eight open reading frames (ORFs) which are transcribed from a polycistronic promoter for the HR types (Fig.1). All genomes consist of three main regions: (1) a long control region (LCR); (2) a region encoding the early genes (E) and (3) a region encoding the late genes (L). The LCR consists of sequences that control viral transcription and replication. The early region, which is about 50% of the entire genome, contains open reading frames (OFRs: E1, E2, E4, E5, E6, E7) that encode proteins involved in transcriptional activation, transformation, replication and viral adaptation to the cellular milieu. The late genes, L1 and L2, encode the two capsid proteins [15-17]. The viral E2 protein is also known to have transcriptional repressor activity on the early promoter which could be a mechanism to control copy number [18]. E4 is expressed as a fusion protein with the 5 amino acids from the N-terminal region of E1 (E1^E4) and is involved in the collapse of the cytokeratin filaments in infected cells [19]. While E5 is considered to have weak transforming activity, it is not consistently expressed in human cancers unlike the viral E6 and E7 genes. A more detailed list of individual gene functions is shown in Table1, and the E6 and E7 oncogenes as well as E2 are described in more detail below.
Table 1. Functions of HPV Proteins

<table>
<thead>
<tr>
<th>Gene</th>
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<tr>
<td>E1</td>
<td>Replication of viral DNA, DNA helicase activity, ATPase activity, maintenance of viral episome [20-22]</td>
</tr>
<tr>
<td>E2</td>
<td>DNA binding protein that recruits E1 helicase to control viral replication, controls transcription of E6 and E7 positively and negatively [18, 23-25]</td>
</tr>
<tr>
<td>E4</td>
<td>Expressed as fusion with E1 (E1^E4), involved in collapse of cytokeratin filaments in infected cells, G2/M cell cycle arrest [19, 26]</td>
</tr>
<tr>
<td>E5</td>
<td>Transforming activity, potential role in initiating carcinogenesis, binds to 16kDa vacuolar-ATPase to prevent acidification of endosomes, activates EGFR signaling transduction [27-30]</td>
</tr>
<tr>
<td>E6</td>
<td>Involved in transformation along with E7, degrades p53, activates telomerase, binds directly to several PDZ proteins [31]</td>
</tr>
<tr>
<td>E7</td>
<td>Degrades pRb family members to permit S-phase progression, inactivates p21 and p27 kinase inhibitors, induces DNA synthesis in quiescent cells [32]</td>
</tr>
<tr>
<td>L1</td>
<td>Major capsid protein, can assemble into capsomers then form virus-like particles [33]</td>
</tr>
<tr>
<td>L2</td>
<td>Minor capsid protein in the virus particle, binds to DNA and regulates viral packaging [34]</td>
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The HPV life cycle

Maintenance Phase

HPV infection is specific to the squamous epithelia; either the dry external cutaneous skin or the moist mucosal lining of most body openings [35]. HPV enters the epithelium at sites where wounding has occurred due to physical or chemical trauma. The viral capsid initially binds to the basement membrane but then infects the basal cells as the infected keratinocytes move into the wound [36]. Viral entry is thought to be mediated by heparin sulfate proteoglycans (HSPs) expressed on the cell surface receptors [37]. Attachment of the capsids results in a conformational change that ultimately triggers endocytosis and causes the viral genome to be released from its capsid, which eventually gets degraded by the lysosome [33]. The viral genome then gets transported to the nucleus of the infected cell where it either integrates into the host cell genome or is maintained extrachromosomally as an episome. In its episomal form, the HPV genome does not contain its own DNA polymerase or machinery and so must rely on the host replication machinery for DNA synthesis.

Maintenance of the virus in its episomal state requires that the viral DNA be replicated coordinately with the host cell chromosome, and that viral genomes be distributed to daughter cells. The early proteins, E1 and E2, are critically important to support DNA replication. E2, a DNA binding protein, binds to a palindromic sequence (ACC(N6)GGT) in the long control region (Fig.1) of the HPV DNA and recruits E1 helicase to the viral origin. Following E2 dissociation, E1 then binds to cellular proteins that are involved in DNA replication and assembles to form a double hexameric rings resembling those found at cellular replication origins which are built from the cellular
multicopy proteins (MCMs) [38]. Additionally, E2 anchors the viral DNA to mitotic chromosomes via its direct interaction with the cellular bromodomain containing protein 4 (Brd4) so that viral genomes are segregated equally to daughter chromosomes during mitosis [39]. E2 also acts as a transcription factor and regulates the early promoter to control the expression of the E6 and E7 viral oncogenes, E1, as well as itself. At low levels, E2 acts as a transcriptional activator of E6 and E7, while at high levels E2 represses expression of the oncogenes. Viral proteins are expressed at low levels in undifferentiated cells, usually at a genome copy number of approximately 100 copies per cell, and so in this way limited expression supports evasion of the immune system [40].

Differentiation-dependent Phase and Genome Amplification

Replication of the viral genome is coupled to epithelial differentiation. In the normal epithelium, cells of the basal and parabasal layer consist of stem cells that divide and give rise to other stem cells as well as transit amplifying (TA) cells found in the parabasal layer that are committed to differentiation. As the daughter TA cells divide, they exit the cell cycle permanently and these differentiated cells create the surface of the epithelium (Fig.2). Whereas in the maintenance phase viral proteins are expressed at low levels in undifferentiated basal cells, HPV amplification and expression in suprabasal cells causes the differentiating cells to remain active in the cell cycle. The E7 protein is the main HPV gene responsible for cell cycle progression in differentiated layers of the epidermis [41]. E7 associates with pRb family proteins causing pRb to dissociate from E2F transcription factor family members resulting in the expression of E2F transcriptional target genes
such as Cyclin A. The role of E7 in deregulating cell-cycle control in the HPV positive epithelium will be discussed in further details below.

In the spinous layer of the HPV infected epithelium the late promoter is activated and this results in high expression levels of viral proteins. The key factors that trigger the onset of late events remains unclear but is thought to be dependent, in part, on changes in the cellular environment as the infected cells move outward. Activation of the differentiation-dependent promoter leads to increased transcript levels of E1, E2, E1^E4 (a fusion of the first amino acids of E1 onto the E4 ORF) and E5 [42, 43]. This results in amplification of viral copy number from about 100 copies to several thousand copies [44]. E1^E4 and E5 play critical roles in the activation of the late promoter upon differentiation [45-48]. HPV E5 is involved in growth factor receptor recycling at the cell membrane which leads to increased EGF (epidermal growth factor) signaling that supports a replication competent environment in the upper epithelial layer [49]. While the role of E4 in genomic amplification is less understood, it is thought to accumulate in cells at the time of genome amplification and loss of E4 disrupts a number of late events [50].

**Viral Assembly and Release**

The final stage of the HPV life cycle consists of the replicated genomes being packaged into infectious particles followed by the shedding of virions (Fig.2). After genomic amplification, L1/L2 capsids accumulate followed by subsequent virion assembly. The link between genome amplification and capsid protein synthesis is not fully understood but is thought to involve changes in RNA splicing and the generation of transcripts that end at the late polyadenylation site. L2 localizes to the nucleus where it associates with
PML (promyelocytic leukemia) bodies, and while some L2 proteins can associate directly with DNA, this association is thought to require E2 which is known to bind DNA. Meanwhile in the cytoplasm, L1 is assembled into capsomers before it gets relocalized to the nucleus where it too associate with PML bodies after L2 has been displaced. L1 and L2 then associates with each other via hydrophobic interactions between capsomers and this contributes to viral maturation and stabilization as infected cells reach the epithelial surface [51]. HPV virions are resistant to dessication which facilitates HPV survival when it is shed from the epithelial surface into the environment with dried skin (Fig.2) [52].

**HPV Integration**

Viral integration into the host genome results in disruption of the viral genome architecture. In cancer cells, the genome is typically found interrupted in the E1 and E2 region, often resulting in disruption of E2 and subsequent stabilization of E6 and E7 mRNA [53]. Integrated genomes are more stable than episomal DNA over time, and endow aspiring cells with a growth advantage. While there are no hot spots, or defined insertional mutagenesis sites for HPV genomic DNA, it is thought to occur preferentially at fragile sites within the human genome. The result of these events is termination of the productive life cycle as large portions of the genome are lost, rendering the virus functionally inactive.

One hallmark of HPV integration is the overexpression of HR E6 and E7 oncoproteins. Studies have demonstrated both *in vitro* and *in vivo* that E6 and E7 cooperate to drive and maintain cellular transformation [54-56].
Functions of E7

Inactivation of Rb

HPV E7 was the first of the two viral oncogenes to be discovered. E7 proteins are small, acidic polypeptides comprised of approximately 100 amino acids in length and have three conserved regions, CR1, CR2 and CR3 that are critical for oncogenic activities (Fig.3). The amino terminus of E7 shares sequence homology with adenovirus (Ad) E1A protein and SV40 large T-antigen, both of which are known to have transforming abilities [57, 58]. The CR2 conserved region contains an LXCXE motif (where X is any amino acid) that mediates binding to the retinoblastoma protein family (pRB, p107, p130) and is required for viral DNA maintenance during HPV infection [59, 60]. In its hypophosphorylated form, Rb protein (pRB) binds to E2F family members to repress the transcription of genes involved in DNA synthesis and cell cycle progression [61]. Phosphorylation of pRb by cyclin dependent kinases releases E2F, resulting in cell cycle progression to S-phase. When E7 binds to hypo-phosphorylated pRb, it disrupts the pRB-E2F complex and induces cells to enter S-phase. The binding of E7 to pRb has been shown to require the calcium-activated cysteine protease, calpain, that cleaves pRb to promote proteasomal degradation [62]. All HPV E7 proteins bind to members of the pRb tumor suppressor family, however, high risk E7 proteins bind to pRb (p105), p107 and p130 with a higher affinity than the low risk types. This difference in affinity for binding to pRB is due to an aspartic acid versus glycine residue in position 21 (high risk) and 22 (low risk) of E7 proteins [63]. This difference in affinity for pRb between high risk and low risk HPV subtypes is the primary reason why HR HPV is much more efficient at inducing cellular transformation and eventual tumorigenesis. In addition to sequestering
Rb from E2Fs, E7 also forms complexes with Rb family members, targeting them for degradation through the ubiquitin-dependent pathway [64].

Association with Histone Deacetylase (HDAC) Complexes

It is well established that actively transcribed genes require high levels of histone acetylation. HDACs remove these acetyl groups from lysine residues on histones, resulting in chromatin compaction and ultimately transcriptional repression. It has been shown that pRb can interact with HDAC-1 to repress the E2F transcriptional machinery as yet another mechanism to prevent the transcription of S-phase genes [65]. To counteract this, E7 also interacts with HDAC-1 through the CR3 zinc-finger domain, independent of pRb binding. E7 associates with HDAC-1 indirectly through Mi2β, a component of the NURD histone deacetylase complex and in so doing, de-represses E2F target gene-transcription in order to stimulate cellular progression into S-phase [66].

G1/S Cell Cycle Checkpoint Deregulation

In proliferating cells, the G1/S-phase transition is driven by the cyclins and their respective cyclin-dependent kinases (cdks) that are subject to negative regulation by cyclin-dependent kinase inhibitors (CKIs). E7 interacts with, and disrupts, the growth inhibitory activities of the CKIs p21\textsuperscript{CIP1} [67, 68] and p27\textsuperscript{KIP1} [69]. The main target of these CKI’s is cdk2, a molecule that is critical for the G1 to S phase progression through interaction with cyclin A and E. E7 therefore maintains high levels of cdk2. Both cyclin A and E are expressed at high levels in E7 expressing cells [70]. Interestingly, E7 expression also stabilizes p21\textsuperscript{CIP1} protein levels through a non-transcriptional mechanism,
however cdk2 remains active [68, 71]. This ability of E7 to abrogate CKIs, combined with its ability to disrupt pRB/E2F resulting in increased cyclin A and E expression, allows differentiating keratinocytes to be maintained in a DNA synthesis state. High risk E7 also increases the levels of cdc25A phosphatase that is known to induce tyrosine dephosphorylation of cdk2, thus promoting its activation [72, 73].

Other targets of E7 include TATA box binding proteins (TBP), suggesting a general role in transcription. In addition, E7 targets AP-1 transcription factors such as c-Jun, JunB, JunD and c-Fos thus regulating early mitogenic effects and keratinocyte differentiation. Additionally, E7 can induce metabolic changes that occur commonly in cancers. While E7 is primarily a nuclear protein, it interacts with M2 pyruvate kinase, the key enzyme that controls exit of the glycolytic pathway. E7 also interacts with α-glucosidase and allosterically activates it. Activation of these enzymes results in depletion of intracellular glycogen stores, similar to that in human cancers. In E7 expressing cells the activation of these enzymes may drive hyper-proliferation or represent a consequence of the altered energy requirements of rapidly transformed cells [74, 75] (Fig.3).

Interestingly, a major consequence of E7 targeting of Rb-E2F is an increase in the levels of p53 tumor suppressor [76] which predisposes E7 expressing cells to apoptosis. It is thought that HR E6 proteins evolved to counteract p53 activities using several mechanisms.
Functions of E6

Regulation of p53 and Apoptosis

The HPV E6 protein consists of 151 amino acids and contains two zinc finger domains with a CXXC motif which are essential for E6 functions (Fig.4). The best characterized function of E6 is the degradation of the tumor suppressor p53 through its association with E6 associated protein (E6AP), an E3 ubiquitin ligase [77]. Together, HPV E6 and E6AP facilitates the ubiquitination and proteasomal degradation of p53. As one of the functions of p53 is to induce cell cycle arrest and apoptosis after DNA damage, the ability of E6 to degrade p53 prevents apoptosis and promotes the survival of transformed cells [78]. Degradation of p53 is specific to HR E6 proteins and is dependent upon E6 binding to both the core of p53 as well as E6AP, as E6 binding to p53 alone does not result in its degradation. While low risk HPV E6 protein can also associate with E6AP, this does not result in p53 degradation [79, 80], however it can inhibit transcriptional p53 activity [78].

Like E7-pRb, E6-p53 interaction is also critical for cell cycle progression. The p14ARF, an alternative gene product to the tumor suppressor p16INK4A, induces G1 or G2 cell cycle arrest in a p53-dependent manner. While normal cells undergo arrest, E6 expressing keratinocytes bypass arrest and continue to proliferate despite high levels of p14 [81].

E6 can also exert its anti-apoptotic effects by p53-independent mechanisms. E6 binds to pro-apoptotic proteins such as Bak (member of the pro-apoptotic Bcl-2 family) and degrades them via a ubiquitin mediated pathway [82]. Additionally, E6 can interfere with various growth inhibitory cytokines that are induced following infection. The tumor necrosis factor-α (TNFα), a pro-inflammatory cytokine, is a potent inhibitor of keratinocyte proliferation and is induced upon viral entry into the cell. These cytokines
can activate the apoptotic pathway through transmembrane cell surface receptors of the TNF receptor family, such as TNF receptor 1 (TNFR1), and FAS, the TNF-related apoptosis-inducing ligand (TRAIL) receptor. E6 blocks TNFα-1 induced apoptosis by binding directly to TNFR1 and in so doing blocks the formation of the death-inducing signaling complex, and ultimately apoptosis [83]. E6 can also interact with the adaptor protein FAS-associated protein with death domain (FAAD) and caspase 8 to block cell death in response to FAS and TRAIL [84].

**hTERT activation**

The human telomerase is a ribonucleoprotein that consists of a catalytic subunit, hTERT, as well as a RNA component hTR. In normal cells, telomeres progressively shorten with each round of cell division due to incomplete lagging DNA strand synthesis and end-processing events, ultimately leading to replicative senescence. In contrast, over 85% of human cancers have high telomerase activity, which prevents replicative senescence due to telomere shortening [85]. E6 activates transcription of *hTERT* through c-Myc dependent and independent mechanisms [86]. Additionally, E6/E6AP is known to degrade NFX-91, a known repressor of the *hTERT* promoter, allowing myc binding to the *hTERT* promoter and ultimately increased hTERT expression [87].

**Interference with Cell-Adhesion Proteins**

E6 binds to PDZ containing proteins through its C-terminal domain, resulting in their degradation. PDZ proteins are involved in cell-cell signaling, cell adhesion, tight junction integrity and possibly have tumor suppressor activity. E6 binds to and degrades the
mammalian homologue to the *Drosophila* disc large (hDLG) tumor suppressor, independently of its association with E6-AP [88, 89]. Other PDZ proteins including hScrib, and MAGI-1 are also targeted for proteasomal degradation by E6 and are involved in forming epithelial tight junctions [90, 91]. E6-induced degradation of these and other PDZ-containing proteins causes loss of cell-cell contacts mediated by tight junctions and so contributes to the loss of cell polarity that is associated with HPV-positive cancers. E6 also binds to other cellular proteins including the focal adhesion protein paxillin [92], which results in disruption of the actin cytoskeleton and may play a key role in tumorigenesis. E6 targeted protein (E6-TP1), a RhoGTPase activating protein (GAP), is also an E6 target though the physiological implications are still unknown [93].

**E6 and E7 cooperation**

The ability of E6 to degrade p53 and so inhibit apoptosis as well as activate telomerase, combined with the ability of E7 to inhibit pRb to promote immortalization, provides a critical mechanism by which HPV causes cervical and other cancers. It is thought that genomic instability plays an integral role in cellular transformation and together, E6 and E7 cause polyploidy [94]. Additionally, E6 and E7 cause deregulation of cellular proteins that control the G2/M transition and progression through mitosis [94]. Together the oncogenes overcome the cell cycle checkpoints that safeguard the cell against transformation.
Cervical Cancer

HPV causes almost all cervical cancers and while infection of the high risk HPV DNA is a necessary event, it is not sufficient for tumor progression. Mutations in cellular genes as well as chromosomal instability are other important events that contribute to the disease. Additionally recent studies using HPV E6/E7 transgenic mouse models show that estrogen is important for the initiation, persistence and continued development of cervical cancer [95, 96]. Moreover, case studies show that women who take oral contraceptives containing synthetic estrogen are at an increased risk for developing cervical cancer [97]. According to the World Health Organization (WHO) cervical cancer is the second most frequently diagnosed cancer in women worldwide and is the second leading cause for cancer associated death in women. Over 11% of women in the general population are thought to be infected with cervical HPV at any given time and approximately 70% of invasive cervical cancers in women are due to HPV 16 and/or 18 infection (WHO/ICO Information Center on HPV and cervical cancer, summary report 2010).

HPV Vaccines

Given that HPV infection is a key determinant to the development of cervical cancer, two vaccines have been developed that block infection of some high risk HPV types. Gardasil (Merck) and Cervarix (GlaxoSmithKline) are two vaccines developed to prevent infection of HPV types 16 and 18. Gardasil is also protective against low risk HPV 6 and 11 that are known to cause 90% of genital warts. Both vaccines are thought to be effective at blocking infections and preventing the development of cervical neoplasia over a 5 year period [98], however neither prevents the progression of cervical cancer for
those already infected. This highlights the need for therapeutic approaches for the treatment of cervical cancer.

**HPV Therapeutics**

Given the importance of E6 and E7 in cervical cancer, and their specific expression in cancer cells only, inhibiting the actions of these oncogenes is a promising approach for the treatment of cervical cancers. Several approaches have been described to target E6 and E7 both *in vitro* and *in vivo*, including antisense technology [99], RNA interference [100, 101], therapeutic vaccines [102] and oncolytic adenovirus [103] as well as the reintroduction of E2 protein [104]

The E2 protein consist of an N-terminal transactivation domain and a C-terminal DNA binding domain separated by a hinge region. Transcriptional repression by E2 requires both the DNA binding and transactivation domain. The protein binds as a dimer to cognate DNA sequences within the HPV genome (Fig.1) [105]. There are four conserved E2 binding sites located upstream of the initiation site of the early promoter (Fig.1), with two tandem repeats located 3 or 4 nucleotides upstream of the TATA box. In mucosal HPV genomes, the ubiquitous transcription factor SP1 binds just upstream of E2 binding sites with part of its sequence overlapping with E2 binding. This close proximity of E2 binding sites to TATA box is likely involved in the transcriptional repression as E2 sterically hinders the formation of the initiation complex by displacing TFIID and Sp1[106].

E2 represses transcription of the early genes, and its repressive effect can be lost when the genome gets integrated, as integration often involves disruption of the E2 open
reading frame. Re-expression of E2 in cervical carcinomas represses the viral promoter, inhibiting E6/E7 transcription, leading to cell cycle arrest, apoptosis and cellular senescence. Interestingly, high risk E2 protein has no transcriptional effect on episomal viral DNA [107]. In addition to this transcriptional mechanism, high risk E2 has also been shown to be able to shuttle out of the nucleus and into the cytoplasm where it can activate caspase 8 to initiate apoptosis directly in both HPV positive and negative cells [108, 109].

**Cellular senescence**

Cellular senescence describes cells that are irreversibly growth arrested and was first identified as a process that limits the proliferative ability of cells in culture [110]. Senescence can be induced *in vitro* by two distinct mechanisms. First, senescence by replicative exhaustion occurs by serial passaging of primary cells in culture. With each round of cell division, telomeres progressively shorten and eventually reach a critical length that causes the cells to enter replicative senescence [111]. Secondly, ‘premature senescence’ occurs independently of telomere shortening and can be induced by various stress signals such DNA damage [112]. Regardless of the mechanism of induction, senescent cells are viable, metabolically active and acquire a characteristic large, flattened, vacuole rich cytoplasmic morphology and stain positive for senescence associated β-galactosidase activity (SA-β-gal) at a pH of 6.0 [113].

In the cellular systems utilized here, senescence was induced either by serial passaging of primary human foreskin keratinocytes (HFKs) to replicative senescence or by the exogenous expression of the human papillomavirus (HPV) E2 protein in cervical
cancer cell lines. Most of these cell lines have lost HPV E2 expression due to disruption of the E2 open reading frame during integration of the HPV DNA into the cellular genome. Re-expression of E2 results in the repression of the HPV E6 and E7 oncogenes and the induction of p53 and pRb tumor suppressors, leading to cellular senescence and to a lesser extent apoptosis [114]. The bovine papillomavirus (BPV) E2 proteins have been studied most extensively, and are also known to be stronger transcriptional repressors and so are commonly used for this purpose [115].

Our studies examine the repression of HPV E6 and E7 in cervical cancer using both RNA interference technology and the re-introduction of the E2 protein. While it is well established that said repression results in senescence or apoptosis, we examine here another consequence of E6/E7 repression, that is, migration. Consequently we set out to determine the mechanism of E2 induced migration in HPV positive cancer cells and to elucidate the physiological relevance of this phenotype. This dissertation encompasses the outcome of these studies.
Fig. 1. Episomal genome of High Risk HPV. A representation of high risk HPV 16 is illustrated. The double stranded genome consists of three main regions: the upstream regulatory region (URR), the early region (E) and the late region (L). There are two promoters that drive viral gene expression; P₉₇ (early) and P₆₇₀ (late) depending on the differentiation stage. The P₆₇₀ promoter drives expression of L1 and L2 during the late stages of differentiation and acts as a switch from early (polyAₑ) to late (polyAₐ) polyadenylation signals. Adapted from [3].
Fig. 2. Structure of normal versus HPV infected epithelia. In contrast to uninfected epithelium (left) HPV infected epithelium contains low copy episomal HPV in the basal layer. During differentiation, cells in the spinous layer are pushed into S phase due to early gene expression, which leads to genome amplification and eventual release of virions in the uppermost layer. Adapted from [116].
**Fig. 3. HPV E7 protein.** A. **Schematic of E7.** The conserved regions of E7, CR1, CR2 and CR3, show sequence homology to adenovirus E1A. E7 dimerizes in the CR3 region via a zinc finger motif that is essential for folding and viability. **B. E7 effects on cellular targets.** E7 interacts with a host of cellular proteins to primarily promote proliferation and genomic instability while inhibiting apoptosis. Adapted from [116].
Figure 4.

A. Schematic of E6 protein. E6 protein consists of 151 amino acids and has two zinc finger domains, each consisting of two C-X-X-C motifs which are required for its functions.

B. E6 effects on cellular targets. The primary role of E6 is to interact with p53 and other pro-apoptotic proteins to inhibit apoptosis as well as promote immortalization by interacting with hTERT. Adapted from [116]
References


CHAPTER 2

Targeting the Human Papillomavirus E6 and E7 oncogenes through expression of the BPV1 E2 protein stimulates cellular motility

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Abstract

Expression of the high risk human papillomavirus (HPV) E6 and E7 oncogenes is essential for the initiation and maintenance of cervical cancer. The repression of both was previously shown to result in activation of their respective tumor suppressor targets p53 and pRb, and subsequent senescence induction in cervical cancer cells. Consequently, viral oncogene suppression is a promising approach for the treatment of HPV positive tumors. One well established method of E6/E7 repression involves the re-expression of the viral E2 protein which is usually deleted in HPV positive cancer cells. Herein we show that surprisingly, BPV1 E2-mediated, but not RNA interference mediated, E6/E7 repression in HPV positive cervical cancer cells stimulates cellular motility and invasion. Migration correlated with the dynamic formation of cellular protrusions, and was dependent upon cell-to-cell contact. While E2 expressing migratory cells were senescent, migration was not a general feature of cellular senescence or cell cycle arrest, and was specifically observed in HPV positive cervical cancer cells. Interestingly, E2-expressing cells were not only themselves motile, but conferred increased motility to E2-negative HeLa cervical cancer cells. Together, our data demonstrate that viral oncogene repression by E2 stimulates cervical cancer cell through a E6/E7 dependent, non cell autonomous mechanism. These data raise the possibility that therapeutic E2 expression may unfavorably increase the local invasiveness of HPV positive tumors.
Introduction

High risk (HR) HPV positive cervical cancer cells harbor integrated HPV genomic DNA and are uniquely dependent upon the expression of two viral oncogenes, E6 and E7, for the maintenance of the transformed phenotype [1]. HrHPV E6 is best known for its ability to target p53 for proteasomal degradation via its association with the E6-associated protein (E6-AP), a ubiquitin ligase [2-4]. Additionally, hrHPV E6 activates telomerase to extend the lifespan of primary human keratinocytes [5-7] and binds to and de-regulates several PDZ proteins that are known to regulate cell polarity, adhesion and proliferation [8-11]. This results in deregulation of tumor suppressive activity and in so doing contributes to carcinogenesis. HrHPV E7 binds to and degrades members of the retinoblastoma (Rb) family resulting in the transcription of E2F target genes such as cyclin E and A which are responsible for S-phase progression [12, 13]. Additional E7 activities include the inhibition of the cyclin dependent kinase inhibitors p21^{CIP1} and p27^{KIP1} which activate Rb [14, 15]. Together, both hrHPV E6 and E7 contribute to carcinogenesis by suppressing apoptosis and senescence, and by stimulating cellular proliferation. Consequently, a number of reports have pioneered various approaches to inhibit E6 and E7 function in HPV-positive cancer cells for the specific induction of cellular growth arrest and death [16-19]. This includes the use of oncogene-specific peptide aptamers [20, 21], antisense technology [22], RNA interference [23-25], and the expression of viral E2 protein [26-30]. Furthermore, in vivo experiments have provided proof of concept for the therapeutic targeting of E6/E7 in HPV-driven tumors in the presence and absence of conventional treatments [17, 31, 32].

Several laboratories including ours have previously described the consequences of virally delivered E2 expression in HPV positive cervical cancer cells: E6/E7 transcription ceased, followed by the re-activation of Rb and p53 tumor suppressors, cell cycle arrest and eventually the induction of a synchronized cellular senescence phenotype. This was observed following the expression of bovine papillomavirus-1 (BPV-1) E2 transactivator (E2-TA), but not the transcriptional repressor (E2-TR), in
HPV positive cancer cells [33, 34]. Similar phenotypes were observed using a temperature sensitive BPV1 E2 (E2ts) protein that is functional at the permissive temperature of 32°C, but not at 37°C and above [35, 36]. Senescence induction in cervical cancer cells was identified by the typical flat cell morphology [37], upregulation of senescence markers including SA-β-gal activity [38] and a transcriptional profile that is reminiscent of replicatively senescent primary cells [39]. Rescue of E2-expressing HeLa cells from either E6 or E7 repression further revealed that senescence was primarily dependent upon E7 repression and Rb-controlled transcription [40], whereas E6 repression resulted in a mixed phenotype that included apoptotic and senescent cells [40, 41].

Data from the DiMaio laboratory demonstrated that E2-TA expression in HeLa cell colonies results in gradual cellular dispersal over time [34]. Here we further characterize this phenotype and demonstrate that E6/E7 repression by E2, but not by shRNA or siRNA knockdown, stimulates the rapid formation of cellular protrusions, together with increased migratory and invasive cell characteristics. This form of migration was specifically observed in HPV positive, but not in HPV negative cells or in cells harboring HPV episomes. Furthermore, migration was non-directional and was dependent upon cell-cell contact. The knockdown of an important E7 target, the human DEK oncogene, induced senescence as previously published [39, 42], but did not stimulate motility, thus demonstrating that HeLa cell senescence per se was not sufficient for driving this phenotype. Finally, we show that E2-expressing cells could stimulate the migration of adjacent non-targeted HeLa cells, a finding which suggests E2-mediated E6/E7 repression in cervical cancers might support local tumor cell dissemination and invasiveness.
Materials and Methods

Cell culture. Human cervical cancer cells lines utilized were: HeLa (HPV18 positive), Caski (HPV16 positive) and C33A (HPV negative). These and the osteosarcoma cell line SAOS2 as well as IMR-90 human fibroblasts were maintained in Dulbecco’s modified Eagle medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin. Primary keratinocytes were prepared from human foreskins as described [43] and maintained in keratinocyte Epilife medium supplemented with HKGS (Cascade Biologics, Portland, OR). Near-diploid Immortalized Keratinocytes that form Skin (NIKS) as well as NIKS transfected with HPV16 [44] (kind gifts from Paul Lambert, University of Wisconsin-Madison) were cultured on irradiated J2-3T3 feeder cells as previously described [45].

Viral and plasmid constructs. The Ad and AdE2ts adenoviruses have been described previously [35], as well as the E2-TA and E2-TR expression plasmids [28]. Nontargeting and DEK specific short hairpin (sh) lentiviral vector were obtained through the Sigma™ MISSION® shRNA program (Sigma Aldrich, St. Louis, MO). The relevant product numbers were SHC 002VC for the control nontargeting control vector (NTsh) and TRCN0000013107 for DEKsh#5 targeting vector. The LXSN retroviral constructs were a generous gift from Denise Galloway (University of Washington, Seattle). The cytomegalovirus (CMV)-driven dsred lentiviral expression vector was a generous gift from the Malik laboratory (Cincinnati Children’s Hospital, Cincinnati, OH). Specific lentiviral constructs targeting the HPV16 E6 open reading frame were custom designed through Sigma™. The shRNA targeted the E6 coding region, nt 196-216: 5’-GTACTGCAAGCAACAGTTACT-3’. Double-stranded RNAi oligonucleotides designed against the HPV18 E7 coding region, nt 694-712 :5’-GGAAGAAAACGATGAAATA-3’, as well as the siGenome Non-Targeting control were purchased from Dhharmacon (Lafayette, CO).
**Viral infections.** Adenoviral infections using empty vector (Ad) and temperature-sensitive E2 expressing Ad (AdE2ts) were carried out as described previously [35]. Briefly, a total of 1x10^6 cells plated on 10cm dishes was infected on two consecutive days with either 10 Plaque Forming Units (PFUs) or 10 infectious unit (IU) of virus as indicated in the figure legends in 1ml of PBS containing 4% FBS for 1hr. The virus was then aspirated and cells were washed twice with PBS and then overlaid with medium. On the third day, cells were split and placed at 37°C for several hours to allow attachment and later shifted to the permissive temperature of 32°C for 3 days after which they were shown to be irreversibly growth arrested [35]. For lentiviral infections, cells were infected with 4mls of shRNA expressing nontargeting (NTsh), DEK-targeting (DEKsh), or HPV16 E6 targeting (HPV16 E6sh) virus for 8hrs. The cells were then washed twice with PBS and overlaid with fresh medium. One day post infection, the cells were overlaid with medium containing 650ng/ml puromycin for selection of a pure virally transduced population. For retroviral infections, cells were incubated with 4mls of viral supernatant of empty vector (LXSN), HPV16 E7 or HPV16 E6/E7 expressing vectors (LXSN E7, LXSN E6/E7 respectively), or with 2 mls of viral supernatant of HPV16 E6 (LXSN E6) expressing vector using 8µg/ml polybrene for 4hrs and then overlaid with fresh medium. The same was done using the pBabe empty vector or that expressing oncogenic H-ras. Cells were then selected and maintained in 500µg/ml G418 containing media.

**Transient transfections and viral infections.** Transient transfections were conducted as previously described [43]. Briefly, HeLa cells seeded on a 60mm plate were transfected with a total of 4µg plasmid DNA using FuGENE transfection reagent (Roche Diagnostics, Indianapolis, IN) according to manufacturer’s instructions. For cotransfections, cells were transfected with 400ng of neomycin resistance plasmid and 3.6µg DNA. Cells were then selected in medium containing 900µg/ml G418 for 8 days.
Migration and invasion assays. Migration assays were conducted using 8.0μm polycarbonate transwell inserts (Corning Inc., Corning, NY) according to manufacturer’s instructions. Briefly, inserts were first re-hydrated using 0.1mL media in the top inserts and 0.6mL in the wells of a 24-well dish for at least 1hr. A total of 1x10^5 cells were then plated in the upper inserts then allowed to migrate for at least 16hrs. Non-migrating were removed with a cotton swab, while cells that had migrated were fixed in methanol for 10mins and stained with Giemsa dye (Sigma Aldrich, St. Louis, MO) at a 1:20 dilution. Four quadrants of the membrane were then counted under a light microscope. For invasion assays, Matrigel-coated invasion inserts (BD Biosciences, Billerica, MA) were re-hydrated with DMEM containing 10% FBS for a final volume of 0.5ml in the upper inserts and 0.75ml in the wells of a 24-well dish. After incubation for a minimum of 2hrs, the media from the upper inserts was removed and replaced with 0.5mL serum free media containing 1x10^5 cells. Cells were allowed to migrate through the Matrigel for 20-22hrs and then stained and counted as described for migration assays.

Time-lapse videomicroscopy. A total 100μl of 1x10^5 cells were seeded as a colony in a 4-chambered Lab-Tek chambered coverglass slide (Nunc, Rochester, NY). The cells were allowed to attach for several hours, gently washed with PBS and overlaid with Leibovitz’s L-15 media containing L-Glutamine and 5% serum (GIBCO, Invitrogen, Carlsbad, CA) in the absence of phenol red. Control and treated groups were imaged simultaneously using a Zeiss LSM510 confocal system attached to a Zeiss axiovert 200 microscope and a heated stage. Images were captured at 10X magnification every 5mins for at least 12hrs using the LSM software with multi-time macro. Cell edges for at least 20 independent cells within the field of view were then tracked using Image J software-MTrackJ plugin, and average distance and/or velocity of each cell determined. This was repeated for at least two different field views for each cell group.
Western blot analyses. Cells were lysed using RIPA buffer (1% Triton, 1% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 0.16M NaCl, 10mM Tris [pH 7.4], 5mM EDTA) supplemented with protease inhibitor cocktail (Pharmingen, San Diego, CA). Protein concentrations were determined with Bradford reagent (Bio-Rad, Hercules, CA). Aliquots containing equal amounts of total protein were boiled in SDS sample buffer and resolved by SDS-polyacrylamide gel electrophoresis. Proteins were then transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) and probed overnight with the following antibodies used at the indicated dilutions: p53 monoclonal-1:1000, polyclonal cyclin A-1:400, HPV18 E7-1:200, (Santa Cruz Biotechnology Inc., Santa Cruz, CA), monoclonal DEK-1:1000, (BD Biosciences, San Diego, CA), monoclonal Rac1-1:1000 (Upstream Biosciences, Calgary, Alberta ), monoclonal actin (1:20 000) (Seven Hills Bioreagents, Cincinnati, OH) and GAPDH (Fitzgerald, Acton, MA). Membranes were exposed to enhanced chemilluminescence reagents (Perkin Elmer, Boston, MA) and protein bands detected by autoradiography. For HPV16 E7 detection, a mixture of 1:50 HPV16 E7 8C9 monoclonal (Invitrogen, Carlsbad, CA) and 1:200 HPV16E7, ED17 monoclonal (Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted in 3% milk was used. Signal was then detected using Super Signal West Femto substrate (Thermo Scientific, Rockford, IL).

Preparation of endogenous extracellular matrix (ECM). Ad or AdE2ts infected cells plated at a confluent density were washed twice with PBS and then once with water. Cells were then treated with 20mM NH₄OH at room temperature for 5mins, aspirated and the underlying ECM rinsed twice with PBS. Fresh Ad infected cells were then seeded onto the AdE2ts ECM and vice versa, and time-lapse migration assays were conducted as described above.

Cell cycle analyses. Cell cycle analysis was conducted as previously described in [42]. Briefly, HeLa cells were treated with medium containing the cell cycle inhibitors mimosine (0.5mM) and thymidine
(2.5mM) for 16hrs, to induce cellular arrest in the G_0/G_1 and S phase of the cell cycle respectively. Cells were then washed and pelleted by centrifugation and 1x10^6 cells resuspended in 800 μl solution containing 1% bovine serum albumin in PBS, 100μl of 500μg/ml propidium iodide in 10mM sodium citrate, pH 7.0 and 100μl of boiled RNase A (10mg/ml in 10mM Tris-HCl,pH 7.5). Cells were then incubated at 37°C for 30 minutes and cell cycle profiles obtained using flow cyometer (BD Biosciences, San Jose, CA).

**Quantitative real-time RT-PCR.** Total RNA was harvested from cells using Trizol reagent (Invitrogen, Carisbad, CA). RT-PCR was performed using Superscript II reverse transcriptase (Invitrogen, Carisbad, CA) or QuantiTect reverse transcriptase kit (Qiagen, Valencia, CA) as described by the manufacturer. Quantitative RT-PCR was carried out using the ABI 7500 system (Applied Biosystem, Foster City, CA) using SYBR green master mix (Applied Biosystem, Foster City, CA). The levels of mRNA assessed were all normalized to reference genes c-abl, GAPDH or EEFA1. For verification of gene regulation identified from meta-analysis, RT-PCR was conducted using mRNA from three independent experiments and all levels were normalized to the EEFA1 reference gene. A list of all primers used along with their respective sequences are listed in Table1.

**Immunofluorescence Microscopy.** Ad and AdE2ts infected cells were plated onto poly-D-lysine hydrobromide (Sigma, St. Louis, MO) coated coverslips, and fixed with 2% paraformaldehyde in PBS. Coverslips were incubated in 0.2% Triton X-100 in PBS for 2mins, blocked with 2% BSA in PBS for 2hrs, washed twice with PBS, and incubated with primary antibody at 4°C overnight. Antibody dilutions used were: E-cadherin, 1:50 (BD Biosciences, San Diego, CA), Vimentin, 1:300 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), Fibronectin,1:300 (Sigma, St. Louis, MO), and Rac1, 1:200 (Upstream Biosciences, Calgary, Alberta). Cells were then washed and incubated in anti-mouse, anit-rabbit or anti-goat Alexa-flour 568 or 555-conjuagted secondary antibody, 1:500 (Invitrogen,
Carlsbad, CA) at room temperature for 2hrs. Coverslips were then mounted onto glass slides with DAPI Vector Vectashield mounting media (Vector Laboratories, Burlingame, CA) and images were taken using a Zeiss fluorescence microscope.

**Rho GTPase pulldown assays.** HeLa cells infected with Ad or AdE2ts were washed with PBS and then lysed in buffer containing 20mM Tris HCl pH 7.6, 100mM NaCl, 10mM MgCl, 1% Triton X-100, 0.2% SDS, protease and phosphatase inhibitors. Cell lysates containing equal amounts of protein was then incubated with glutathione beads conjugated to GST-PAK 1 for Rac1 total levels determined by immunoblotting for using Rac1 (Upstream Biosciences, Calgary, Alberta) at 1:500 dilution.

**Senescence associated β-galactosidase assay.** Cells were fixed in 2% formaldehyde and 0.2% glutaraldehyde for 15mins, washed with PBS and incubated at 37°C for 16-36hrs in SA-β-galactosidase staining solution as described in [38]. Cells were analyzed under a light microscope.
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A list of primers and their sequences used to conduct RT-PCR analysis are listed.
Results

E2 mediated suppression of HPV E6/E7 stimulates migration in HPV positive cancer cells.

It is well established that the re-expression of either full length BPV1 or hrHPV E2 protein in HPV positive cervical cancer cell lines induces cell cycle arrest and cellular senescence through the repression of E6 and E7 [28, 34]. E6/E7 repression by RNA interference also results in cellular senescence [46] and was recently shown to decrease motility. However, E2-expression was shown to increase the dispersal of tightly packed HeLa cells over time [34]. To further characterize the effects of E2 expression on cervical cancer cell motility, both HPV18 positive HeLa and HPV16 positive Caski cells were infected with empty adenoviral vector Ad or AdE2ts virus as described previously for synchronous senescence induction [35]. Immunoblot analysis of the respective protein lysates confirmed decreased E7 levels directly, and decreased E6 levels indirectly based on p53 induction (Fig.1A). E2 expression and subsequent E6/E7 suppression was also associated with cellular flattening and positive staining for SA-β-gal activity as reported (Fig.1A). Transwell migration assays revealed that AdE2ts-infected HPV positive HeLa and Caski, but not HPV negative C33A cervical cancer cells and human foreskin keratinocytes (HFKs), showed elevated motility compared to the Ad-infected controls (Fig.1A). Importantly, the observed motility was not dependent upon the presence of FBS in the top, bottom, or both chambers (data not shown), thus ruling out serum-dependent chemotaxis as the underlying mechanism. HeLa cells were also transiently transfected with an expression vector for full-length, E6/E7-repressive E2-TA protein, or for the naturally occurring truncated E2-TR protein which lacks the N-terminal domain and is defective for oncogene repression and senescence induction [28]. Transwell migration assay showed that E2-TA expressing cells were significantly more motile compared to E2-TR expressing and control cells (Fig.1B). Western blot analysis confirmed that E2-TA but not E2-TR was effective in repressing E6 as demonstrated by upregulation of p53 protein levels, and E7 as shown by decreased levels of the upregulated E7 target cyclin A (Fig.1B).
To further analyze previous observations of E2-driven HeLa cell dispersion [34], we observed morphological changes over time by 2D time-lapse videomicroscopy. Interestingly, E2-infected HeLa cells were characterized by increased numbers of cellular protrusions when compared to their Ad-infected proliferating counterparts. Specifically, the majority of E2 expressing cells displayed greater than five protrusions per cell, while control cells mostly had 3-5 protrusions per cell (Fig.1C). Protrusions on Ad control cells resembled filopodia while those associated with AdE2ts were reminiscent of lamellipodia. Given the role of the small RhoGTPases Rac1, RhoA or Cdc42 GTPase in the formation of cellular protrusions and migration, namely Rac1 association with lamellipodia and Cdc42 with filopodia [47, 48], we examined both Ad and AdE2ts infected cells for increased activity of GTP-bound Rac1. Pull down assays from three independent experiments revealed that GTP-bound Rac1 was not only not upregulated, but was in fact downregulated in AdE2ts infected compared to control cells. Interestingly, this decrease in active Rac1 was correlated with a decrease in total Rac1 levels as measured by western blot analysis and immunofluorescence microscopy (Supp. Fig.S1). Neither the Cdc42 nor RhoA members of the family of small GTPases were regulated by E2 expression (data not shown). Targeting Rac1 in Ad or AdE2ts infected cells through expression of either a dominant negative Rac1 molecule (Rac1 N17) described in [49] or two separate shRNAs [50] did not change cellular migratory behaviour when compared to control transduced cells (data not shown). Taken together these findings suggest that E2 migration is associated with Rac1 inhibition in HPV positive cells, but that Rac1 inhibition by itself is not sufficient to stimulate motility. Finally, we analyzed the motility of AdE2ts versus Ad infected HeLa cells by 2D time-lapse videomicroscopy. Cells were infected as above, densely seeded as a colony, and allowed to migrate outwards for at least 12hrs. A comparison of total average velocity per cell revealed again that AdE2ts-infected cells were increasingly motile compared to control infected cells (Fig.1C). Taken together, these data demonstrate that E2-mediated E6/E7 repression in HPV positive cervical cancer cells stimulates dynamic protrusions and non-directional cellular motility.
**E2 expression stimulates invasion but not EMT.**

To determine whether E2-associated increased migration in HPV positive cells translated into invasive potential, we measured the ability of E2-infected cells to invade through extracellular matrix components *in vitro*. Matrigel invasion assays using AdE2ts infected HeLa and Caski cells revealed increased invasion when compared with Ad infected control cells (Fig.2A). Importantly, AdE2ts infected HPV negative C33A cervical cancer cells and primary human foreskin keratinocytes (HFKs) did not display increased invasion, demonstrating that this phenotype is specific to HPV positive cells and thus likely associated with E6/E7 repression.

Time-lapse videomicroscopy as well as a published report [34] suggested that E2 expressing cells tended to disengage from each other, indicating cell-cell contact was important for the observed motility. Furthermore, cell-cell adhesion assays where control or E2-expressing cells were placed in single cell suspension and allowed to attach to each other with gentle agitation, showed that E2-expressing cells were deficient in forming adherent cell clusters (data not shown). To determine whether cell-cell contact was important for E2ts-associated invasion, Ad and AdE2ts infected cells were seeded in an invasion chamber at increasing densities (Fig.2B). While increasing Ad control cell density stimulated migration in small increments, there was a significantly steeper increase in invasion with the corresponding E6/E7 repressed cells. Similarly, time-lapse videomicroscopy was conducted using Ad and AdE2ts infected cells at subconfluent and confluent densities. Migration was stimulated in E2 expressing cells only when the cells were confluent. This data suggests that E6/E7 repressed cells are particularly more motile and invasive when in contact with each other, but does not rule out additional mediators that are unrelated to cell-cell contact.

Given a decrease in cell-cell attachment and increased invasion, both integral features of the epithelial-mesenchymal transition (EMT), we examined EMT induction in E2-expressing HeLa cells. EMT is an evolutionarily conserved process wherein epithelial cells break down contacts with neighboring cells and the extracellular matrix, and acquire the motile and invasive phenotype of
mesenchymal cells (50). EMT is closely linked to E-cadherin suppression (19), the upregulation of ECM components such as vimentin and fibronectin, and the transcriptional induction of mesenchymal markers including twist and snail. E2 expression did not decrease E-cadherin expression which is already low in HeLa and Caski cells, or increase snail and twist transcription. Based on these markers, increased motility was therefore not associated with EMT (Fig.2C).

**HeLa cell migration is not a necessary consequence of cellular senescence but depends upon joint E6 and E7 repression.**

It is well established that E2 expression and E6/E7 repression in HPV positive cancer cells result in cellular senescence. Since the above invasion phenotypes were equally specific to HPV positive cells, we asked whether the two phenotypes might be linked. To determine whether senescence in general results in migration, human foreskin keratinocytes (HFKs) were passaged to replicative senescence and monitored for their motility. Similar to E6/E7 repressed HeLa cells, HFKs passaged to senescence showed positive SA-β-gal activity as well as the flat cell morphology which is a hallmark of senescence (Fig.3A). Transwell migration assays however revealed that replicatively senescent cells were not increasingly motile compared to their proliferating controls (Fig. 3A). Furthermore, IMR-90 fibroblasts undergoing oncogene induced senescence (OIS) by the expression of oncogenic H-ras showed a similar decrease in migration compared to proliferating controls (Fig.3B). This indicated that migration was not generally associated with senescence [51].

To next determine whether migration was a generic consequence of cell cycle arrest, which is intrinsic to the senescence phenotypes, HeLa cells were chemically arrested at various stages of the cell cycle. G1 arrest was induced by mimosine, and S phase arrest by thymidine. Flow cytometry revealed that both chemicals caused cell cycle arrest in the expected phases of the cell cycle, but neither was sufficient to stimulate cell migration (Fig.3C). Next we determined whether migration was a generic consequence of HeLa cell senescence. The human DEK oncogene is reported to block
cellular senescence in primary human fibroblasts keratinocytes and HeLa cells, and the knockdown of DEK causes senescence in HeLa cells [39, 42]. HeLa cells were transduced with a lentivirus expressing DEK-specific shRNA to induce senescence and compared to cells transduced with nontargeting shRNA. Both cell populations were subsequently used for migration assays. Unlike their E2-expressing counterparts, senescent DEK knockdown cells did not migrate at increased rates (Fig. 3D). Taken together, this data suggests that neither cell cycle arrest nor cellular senescence in HeLa cells is sufficient for stimulating cellular motility.

Having established that E2 expression leads to increased motility in HPV positive HeLa cells, we next determined whether E6 or E7 repression was required. An approach similar to that previously published by the DiMaio laboratory was utilized [40]. Briefly, HPV18 E6/E7 expression was inhibited by AdE2ts in HeLa cells, while either HPV16 E6, E7 or E6/E7 were simultaneously expressed retrovirally. Since E2 does not regulate retroviral oncogene expression, the net result of this approach is the individual repression of E7 in HPV16 E6-expressing cells, the individual repression of E6 in HPV16 E7-expressing cells or no repression in the presence HPV16 E6/E7-expressing cells. Western blot analysis verified the over-expression of the exogenous oncogenes; p53 protein levels were decreased – albeit not eliminated – in 16E6 and 16E6/E7 overexpressing cells while HPV16 E7 protein levels were upregulated in 16E7 and 16E6/E7 overexpressing cells (Fig. 3E). Of note, HPV16 E7 protein levels were further upregulated in cells that co-expressed E7 with E2 (Fig. 3E, Fig. 4C). This data may be explained by a published report wherein high risk E2 protein bound and stabilized HPV16 E7 [52]. As previously shown, the re-expression of HPV16 E7 or E6/E7 was sufficient to counteract E2 senescence in HPV positive cells [40] (Fig. 3E), whereas HeLa-16E6 cells displayed little senescence and eventual apoptosis. Using time-lapse videomicroscopy, we found that the re-expression of either E6, E7 or E6/E7 inhibited E2 motility, thus indicating that cellular migration requires the repression of both E6 and E7 by E2. Furthermore, Matrigel invasion assays showed that E2-expressing, HeLa-16 E6/E7 reconstituted cells invaded to a significantly lesser extent compared to E2-
expressing control cells (Fig. 3E). It is possible that remaining trends towards increased migration in E6- or E7-expressing cells are due to insufficient reconstitution of the oncogenes in a proportion of the polyclonal cell population. This experimental caveat is obvious in the E6/E7 reconstituted, E2-expressing cells which exhibit remaining p53 induction and thus likely insufficient E6 expression. Incomplete repression might also reflect independent E2-specific activities which contribute to the motility phenotype as shown in Fig. 4.

Given that joint E6 and E7 repression was required for significant E2-induced migration, we next sought to identify migration-associated genes that are controlled by E2 as well as E6/E7 and are thus potential mediators of this phenotype. We carried out a meta-analysis of migration-associated genes using microarray studies from several publications [35, 39, 53-55]. Several candidate genes upregulated by HPV E2 and/or downregulated by HPV E6/E7 were selected for qRT-PCR analysis in the E2ts system. A list of genes along with their relative expression in these experiments is shown in Table 2. Individual knockdowns of Notch1, Semaphorin 3F and Plexin A1 did not repress migration (data not shown), thus indicating that other mediators are responsible, or that migration requires the activities of multiple cellular E6/E7 targets.

**E2 expression, but not E6/E7 knockdown, stimulates migration in HPV positive cancer cells.**

Our data showed that E2 expression stimulated cellular motility, and that E6/E7 repression contributed to this phenotype. In contrast, E6/E7 repression by RNA interference was recently shown to repress motility [25]. In order to determine whether E2-specific activities in addition to E6/E7 suppression are important, we knocked down the HPV18 oncogenes in HeLa cells via siRNA transfection (Fig. 4A). As expected, this approach resulted in E7 repression and p53 up-regulation, together with cellular flattening and positive staining for SA-β-gal activity. In line with recently published data, migration and invasion were repressed by E6/E7 knockdown. Direct detection of E7 mRNA levels further verified that oncogene repression by siRNA was at least as efficient, if not more efficient, as that
induced by E2 expression. Similar data were obtained in Caski cells transduced with a retroviral vector delivering E7-specific as compared to non-targeting shRNA (Fig. 4B). Taken together, we concluded that oncogene repression by E2 is not sufficient for motility, and that specific E2 activities must also be involved. Since BPV1 E2 protein does not appear to transcriptionally regulate cellular targets [39], such activities are likely to involve post-transcriptional mechanisms.

HeLa and Caski cells harbor integrated high risk HPV DNA. In order to determine whether E2 was capable of stimulating migration in cells which maintain viral genomes episomally, we used Near-diploid Immortalized Keratinocytes that form Skin (NIKS), either positive or negative for HPV16 episomes. The cells were infected with either Ad or AdE2ts virus, followed by invasion assays. E2 expression did not decrease E7 expression in HPV16 positive NIKS (Fig. 4C) in line with published data from the Raj laboratory [56]. Furthermore, E2 expression did not stimulate cellular invasion. Together, these data support the notion that E2-driven motility in HPV positive cancer cells requires E6/E7 suppression in conjunction with specific E2 activities.

Senescence induction in AdE2ts infected HeLa cells is reversible up until 2 days of continuous E2 expression and becomes irreversible on day three. To determine whether E2-driven invasion is a reversible phenotype, we carried out timed E2-inactivation experiments similar to previous senescence experiments [35]. Cells were infected with Ad or AdE2ts at the permissive temperature of 32°C, and shifted to the non-permissive temperature on either day 2 or on day 3, followed by invasion assays on day 4. Control cells were kept at the permissive temperature throughout. The results show E2 associated migration is a reversible phenotype at least up until day 3 post E2 expression (Supp. Fig.S2).
E2-expressing cells stimulate the migration of neighboring cancer cells independent of secreted factors.

Based on our findings that E2 expressing cells stimulate motility, we explored the functional contribution of soluble and matrix factors in E2-associated migration. To test the role of soluble secreted factors, conditioned medium was transferred from the Ad2ts expressing cells onto the empty Ad control HeLa cells and vice versa, and then transwell migration assays were conducted (Fig.5A). The addition of AdE2ts medium to Ad infected HeLa cells did not significantly stimulate their migration above baseline levels. Conversely, the addition of control Ad supernatant onto the E6/E7 repressed cells did not decrease their migration. While these data do not rule out contributions of soluble factors, they implicate cell-intrinsic properties as the primary determinant of the observed E6/E7 repressed cellular motility. To examine possible contributions of the underlying extracellular matrix (ECM), E2-expressing HeLa cells were treated with ammonium hydroxide which removes the cells while leaving the native endogenous ECM intact [57]. Control and AdE2ts-infected cells were then reciprocally transferred onto ECM, and 2-D time-lapse videomicroscopy was conducted. ECM components of E2ts-infected cells did not stimulate the migration of control infected cells, and ECM of Ad-infected cells did not decrease E2ts-induced motility (Fig.5B). Finally, the concomitant exposure of control infected cells to E6/E7 repressed ECM plus supernatant, and exposure of E6/E7 repressed cells to control ECM plus supernatant did not change cellular migration rates (data not shown), thus further emphasizing the importance of cell intrinsic mechanisms (Fig.2B).

Targeting the HPV oncogenes is a promising approach to cancer treatment given that senescence is a barrier to tumor development once irreversibly established. The observed increased motility of E2-expressing cells would therefore appear irrelevant to cancer spread. However, senescence irreversibility depends upon prolonged E6/E7 repression [28], and additionally, not all cells in a given tumor are expected to be targeted even under ideal treatment circumstances. To determine the effect of E2-expressing HeLa cells on the motility of neighboring non-targeted cells, E2-expressing
GFP positive cells were mixed with dsRed marked HeLa cells to observe the motility of each within a 50:50 mix (Fig. 5C). Time-lapse videomicroscopy revealed as expected that E2-expressing cells were more motile as compared to their dsRed marked, E6/E7-proficient counterparts (Fig. 5C). Interestingly, E2-expressing HeLa cells were not only themselves motile, but stimulated the motility of adjacent non-targeted HeLa cells (Fig. 5C). This suggests that oncogene repression via E2 in a cervical cancer environment stimulates the migration of both targeted and adjacent nontargeted cells, thus potentially contributing to a locally invasive environment.
Discussion

The advent of new preventative HPV vaccines promises to reduce cervical cancer incidence significantly in the future [58, 59]. However, these vaccines are ineffective for the treatment of existing infections and will therefore not impact the HPV-related disease burden for years to come. Alternative treatments are needed, which might include therapeutic vaccines or gene therapy approaches. It is well established that the HPV oncogenes E6 and E7 are a necessary requirement for disease development and progression [1, 60]. As such, proof of concept for oncogene repression and resulting cell death has been published by multiple laboratories in vitro and in vivo [17, 18, 23, 46, 61], and has led to ongoing clinical trials wherein E6 and E7 are targeted by several vaccine strategies in patients affected with HPV-related malignancies including recurrent cervical cancer [62]. With regards to E2, its effectiveness in targeting E6 and E7 has been demonstrated in vitro [28, 34], and clinical trials carried out in Mexico have shown promising results in the treatment of high and low grade cervical intraepithelial neoplasia in [63, 64].

We report here that targeting E6/E7 by E2, but not by RNA interference, stimulated motility and invasion. Our data confirm a recent report wherein E6/E7 knockdown repressed migration [25], while further extending a previous published report wherein E2 expressing, densely packed HeLa cells dispersed from each other [34]. Surprisingly, however, E2 expressing cells were not only themselves motile, but conferred motility to neighboring cells. The underlying mechanism of E2-driven motility was independent of chemotaxis, dependent upon cell-cell contact and neither mediated by EMT nor accompanied by a reduction in EMT. In fact, published data suggested a repression of EMT and invasion should be expected in response to E2 expression, and subsequent E6/E7 repression. First, early studies have linked E-cadherin suppression to the invasive properties of HPV16 E6/E7 immortalized keratinocytes [65]. Second, a recent publication has implicated E6 and E7 independently in causing EMT-like phenotypes in human keratinocytes [66]. This was in line with earlier findings of EMT features in a cellular model of the HPV positive pre-cancerous stage [67]. The marked
discrepancy in phenotypes between E6/E7 repression and E2 expression is suggestive of E2-specific molecular activities that work in conjunction with E6/E7 repression. Regardless of the underlying mechanism, however, our data suggest possible risks associated with E2-based treatments of cervical cancer, and a need for long term follow-up of tumor regression and recurrence in such patients.
Acknowledgements

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References


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Figure 1.

Fig.1. E2 mediated suppression of HPV E6/E7 stimulates migration in HPV positive cancer cells.

A. HeLa and Caski cells were infected with an adenovirus expressing temperature sensitive E2 (AdE2ts) or Ad control. Western blot analyses showed decreased HPV E7 levels, and increased p53 expression. Senescent AdE2ts infected cells stained positive for SA-β-gal while Ad controls did not. HeLa and Caski cells infected with empty Ad or AdE2ts were subjected to transwell migration and both HeLa and Caski-AdE2ts infected cells showed increased migration compared to their proliferating controls. HPV negative C33A cells and primary human foreskin keratinocytes did not show an increase in migration upon AdE2ts expression. B. HeLa cells transfected with full length E2-TA or mutant E2-TR as well as empty vector control were subjected to transwell assay. E2-TA, but not E2-TR transfected cells showed increased migration. Error bars represent standard deviation from a representative experiment. Western blot analysis indicated that only full length E2-TA was effective in repressing E6, thus increasing p53 protein levels, while protein levels of cyclin A, a known target of
E7, was decreased. C. Ad or AdE2ts expressing HeLa cells were plated in a cloning ring and allowed to attach for at least three hours. Time-lapse videomicroscopy was conducted as cells were allowed to migrate outwards over the course of 12 hours. Image-J software, MTrackJ plugin, was then used to track cells. For every cell tracked by image J, the final number of cellular protrusions formed (indicated by numbers) per cell within a given field was counted. Total velocity over the course of the time-lapse was also determined by tracking at least 20 cells per field, and three field were tracked for each time-lapse. Error bars represent standard error of the mean of cells tracked from three different time-lapse experiments. A ‘*’ indicates p<0.05 and a ‘**’ indicates p<0.01.
**Fig. 2.** E2 expression stimulates invasion but not EMT.

**A.** HPV positive HeLa and Caski cells as well as HPV negative C33A and HFK cells infected with empty Ad or AdE2ts were subjected to Matrigel invasion assays. Images on left show positive staining of invaded cells. Both HeLa and Caski-AdE2ts infected cells showed increased invasion compared to their proliferating controls, while HPV negative C33A and HFK cells did not respond to E2 expression. Error bars represent standard error of the mean taken from three or more independent experiments. **B.** HeLa cells infected with AdE2ts, or Ad control were plated at increasing densities...
between $1 \times 10^4$ and $1 \times 10^5$. Invaded cells were counted and expressed as a regression graph. Increased invasion accompanied increased cell density in the senescent AdE2ts infected cells with little effect in Ad infected control. Time-lapse videomicroscopy was carried out with using Ad or AdE2ts-infected HeLa cells that were plated either subconfluently or confluent. AdE2ts expression induced motility in confluent, but not in subconfluent cells. C. Both HeLa and Caski cells infected with Ad and AdE2ts were plated on coverslips at a confluent density and then analyzed for the expression of epithelial (E-cadherin) and mesenchymal markers (Vimentin and Fibronectin). HeLa cells infected with Ad or AdE2ts were analyzed for mRNA expression of the mesenchymal markers Twist and Snail, p21 was used as a positive marker of AdE2ts expression. Relative expression levels were normalized to c-abl. Error bars represent standard error of the mean from three independent experiments.
Fig. 3. HeLa cell migration is not a necessary consequence of cellular senescence but depends upon joint E6 and E7 repression.
A. Human Foreskin Keratinocytes (HFKs) passaged to replicative senescence and stained for SA-β-gal activity showed that cells at later passage (P18) were positively senescent and showed decreased migration compared to early passage (P3) controls. Error bars indicate standard error of the mean taken from three independent experiments. (B). Similarly, decreased migration was observed with oncogene-induced senescent IMR-90 cells. Error bars indicate standard deviation of the mean from a representative experiment. Immunoblots from IMR-90 H-ras infected cells show increased p53 protein levels, indicative of senescence in this cell type. C. HeLa cells were treated with mimosine or thymidine or left untreated, and then subjected to cell cycle analyses. While the drugs were sufficient to arrest cells in their respective phases, no difference in migration was observed. Error bars represent standard deviation. D. HeLa cells were infected with lentiviral DEksh RNA or a nontargeting (NTsh) control. DEKsh cells displayed the large, flattened morphology indicative of the senescent phenotype. However, DEKsh senescent cells did not show increased motility compared to proliferating NTsh controls. Error bars represent standard error of the mean from three independent experiments. Immunoblot analysis for DEK indicated that the knockdown vector significantly down-regulated DEK protein levels. E. HeLa cells were transduced with LXSN, 16E6,16E7 or 16E6/E7 vectors and then infected with Ad or AdE2ts. As expected, HeLa-LXSN and HeLa-16E6 cells were primarily senescent as indicated by SA-β-gal activity staining and quantitation. While all cells infected with AdE2ts were increasingly motile compared to control Ad infected, only HeLa-LXSN cells co-expressing AdE2ts were significantly motile compared to controls, while 16E6/E7 co-expressing cells displayed a trend towards decreased motility. Invasion assays with LXSN versus LXSN 16E6/E7 transduced cells further confirmed decreased motility upon E6/E7 reconstitution. Immunoblot analysis showed that each vectors were effective in expressing their respective oncogenes as HeLa-16E6 cells showed little or no expression of p53 protein levels, while HeLa-16E7 and HeLa-16E6/E7 expressing cells showed increased 16E7 protein levels.
**Fig. 4. E2 expression, but not E6/E7 knockdown, stimulates migration in HPV positive cancer cells** A. HeLa cells were transfected with an siRNA targeting HPV18 E7. This approach was effective in repressing E6 and E7 and inducing cellular senescence as indicated by decreased HPV18 E7 and increased p53 protein levels, SA-βgal positivity and the typical flattened morphology. Repression of E6/E7 by siRNA resulted in decreased migration and invasion. Relative HPV18 E7 mRNA levels in E2 versus E6/E7 knockdown cells confirmed E6/E7 repression in each case. B. Targeting HPV16 E6/E7 by shRNA expression in Caski cells also resulted in decreased invasion. C. Normal immortalized keratinocytes (NIKS) as well as NIKS-HPV16 were infected with AdE2ts. Neither E7 repression nor increased invasion were observed.
Fig.5. E2-expressing cells stimulate the migration of neighboring cancer cells independent of secreted factors.

**A.** Cell-extrinsic components are not sufficient to stimulate migration in E2-expressing cells. Supernatant taken from AdE2ts infected cells was transferred onto Ad infected cells in the top chamber of the transwell insert. The transfer of supernatant from these AdE2ts cells was not sufficient to stimulate the migration of control cells significantly. Conversely, supernatant from control Ad infected cells transferred onto AdE2ts infected cells was not sufficient to significantly reduce cell migration of AdE2ts cells. **B.** A total of $1 \times 10^5$ Ad control cells were plated on the ECM of AdE2ts...
infected cells (see materials and methods). Conversely, AdE2ts infected cells were plated onto the ECM of control cells. Quantitation post time-lapse showed that there was no significant differences in migration when Ad cells were transferred onto the ECM of AdE2ts cells and vice versa. C. E2 expression confers migration to non-targeted adjacent HeLa cells. A total of 1x10^5 parental HeLa cells (HeLa-E6/E7) labeled with dsred or those infected with AdE2ts (HeLa-E2) which are GFP positive as well as 50:50 mix of both were subjected to time-lapse videomicroscopy. Images show cells used for time-lapse at the beginning (0hr) and end (12hrs) as well outline of cellular tracks made throughout the course of the time-lapse. Image-J quantitation of velocity showed HeLa-E2 cells migrated as expected. Additionally, HeLa-E6/E7 positive cancer cells became increasingly motile when mixed with HeLa-E2 cells. Error bars represent standard deviation of the mean from at least 20 cells tracked per field. Three or more field of cells was tracked per group.
Supp. Fig. S1. E2 expression attenuates Rac1 activity.

Levels of GTP bound Rac1 were determined by pull-down assays using GST-PAK1 binding domain. Western blot analyses from three independent experiments indicated decreased Rac1 activity as well as decreased total levels of Rac1 in E2-expressing cells. Immunofluorescence microscopy, in which images of Ad and AdE2ts expressing cells were captured at the same exposure, also indicated an overall decreased Rac1 expression in AdE2ts cells.
Supp. Fig. S2. E2 invasion is reversible.

Ad and AdE2ts expressing cells were infected as described in the Materials and Methods. Cells were shifted to the non-permissive temperature on day 2 and 3, respectively, and invasion was determined on day 4. The results indicate that E2 motility is a reversible phenotype.
Table 2.

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Table 2. E6/E7 repression stimulates the expression of migration-associated genes.

Gene expression levels relative to EEFA1 using Ad and AdE2ts mRNA from three independent experiments was measured by real-time PCR using primer sequences listed in table 1. A ‘+’ represents a fold change greater than 0 but less than 2. A ‘++’ represents greater than 2 but less than 4 fold expression. A ‘+++’ indicates a fold change greater than 4.
CHAPTER 3

The role of E2-associated motility in vivo: Xenografts and subrenal capsule implantation

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Abstract

We have demonstrated that high risk HPV E6/E7 repression by E2 in HPV positive cancer cells results in increased migration and invasion. This phenotype could be uncoupled from E2-associated cellular senescence, and required cell-cell contact. E2 expressing cells were not only themselves motile, but conferred motility to neighboring HeLa cancer cells in vitro. Herein we first seek to examine whether E2 expression in HPV positive cancer cells stimulates metastasis of adjacent non-targeted HeLa cells in vivo. NOD-SCID mice sub-dermally injected with a mix of HeLa parental cells or senescent HeLa cells expressing E2ts (HeLa-E2ts) resulted in the rapid growth of tumors over time, in comparison with control mice injected with the same number of HeLa:DEKsh senescent cells, which showed a dramatic delay in tumor formation. While large tumors of similar morphology were formed in both groups at different times, no metastasis was observed in lung, spleen or other tissues. Next, to examine local invasion in vivo, subrenal capsule surgery was performed using E2-expressing, GFP positive, HeLa-E2ts cells, and tdTomato labeled non-targeted HeLa cells (HeLa-tdTomatoe) either individually transplanted or combined in a 50:50 mix (HeLa:HeLa-E2ts). Hematoxylin-and eosin staining revealed that all grafts were successfully transplanted and cells were viable. There was not a universal invasion of cells into the kidney parenchyma. The use of human specific mitochondrial antibody, which recognizes the non-glycosylated protein component of the mitochondria, identified human cells in grafts. Additional studies were confounded by antibody issues which require optimization. Further analysis will be needed to confirm whether E2ts cells are indeed more invasive over control HeLa cells in
vivo and also whether E2ts cells promote the invasion of neighboring non-targeted cancer cells.

**Introduction**

According to the World Health Organization (WHO), cervical cancer is the second most common cancer among women worldwide, with over 500,000 new cases diagnosed annually [1]. Unlike most cancers in which the etiology is multifactorial and/or unknown, cervical cancer is unique in that it is known that the human papillomavirus (HPV) is the causal agent of almost all cervical cancers [2]. Integration of the viral genome is often associated with cervical cancer and results in increased expression of two critical oncogenes, E6 and E7. The main function of E6 is the degradation of the p53 tumor suppressor, as well as activation of telomerase in human keratinocytes [3-6]. Meanwhile, E7 primarily inactivates members of the pRb tumor suppressor family, and in so doing releases E2F transcription factors which regulate the transcription of many genes involved in S-phase [7, 8]. Together, the oncogenes prevent senescence and apoptosis, and mediate immortalization that leads to transformation in conjunction with other oncogenic events. The aberrant expression of E6 and E7 presents a scenario in which specific targeting of the viral oncogenes could restore tumor suppressor functions, inhibit cancer cell growth and induce senescence and apoptosis.

A well described approach of HPV oncogene repression is the re-introduction of exogenous E2 protein into cervical cancer cells which is frequently lost during cancer development. E2 is a negative regulator of E6 and E7 transcription. Integration of the HPV genome often occurs downstream of the E2 open reading frame (ORF) causing it to
be disrupted upon integration. However, re-introduction of exogenous E2 into cancer cell lines in which HPV is integrated, restores transcriptional repression of E6 and E7, and causes cellular senescence or apoptosis [9-11]. Targeting of HeLa cell tumors grown in the flanks of nude mice by the Modified Vaccinia Ankara virus expressing E2 (MVA-E2) virus, inhibited tumor cell growth [12]. Furthermore, the use of the same MVA-E2 viral vector in patients who presented with high grade cervical intraepithelial lesions (CIN II and CINII) has also shown efficacy and led to tumor regression in up to 90% of patients [13]. This report demonstrates impressive therapeutic potential for E2 in the treatment of cervical cancer.

We have recently uncovered a novel property of E2 expression in cervical cancer cells. Using an adenovirus expressing temperature sensitive E2 protein (AdE2ts) as well as full length E2 (E2-TA), we demonstrated that E2 expression in HeLa (HPV 18) and Caski (HPV 16) cells resulted in increased migration and invasion that was dependent on cell-cell contact but independent of cellular senescence. More importantly, not only were E2 expressing cells themselves motile, but they were also able to confer this motility to neighboring HeLa cancer cells in vitro. Here we determine whether E2 expressing cells could promote metastasis of neighboring cancer cells and also investigate whether E2 targeted HPV positive cells are invasive in vivo.

To determine whether E2ts cells could stimulate the migration and metastasis of adjacent HeLa cells, a mix of both HeLa and HeLa-AdE2ts were injected sub-dermally into the flanks of female NOD-SCID mice. Control mice were injected with a mix of HeLa cells and HeLa-DEKsh cells. DEKsh cells were used as controls as DEK inhibition in cervical cancer cells was previously shown to result in cell cycle arrest and senescence.
Importantly, we demonstrated that unlike HeLa-E2ts cells, HeLa cells expressing DEK shRNA were not more motile when compared to control cells. Thus we utilized DEK knockdown cells as controls for this experiment. The mix of HeLa and E2ts-targeted HeLa cells resulted in larger tumors compared to the DEKsh controls at the same time point. Lung and spleen samples were taken from mice and examined for metastases, with none noted.

Next, to determine whether HeLa-E2ts cells are locally invasive and can confer this property to adjacent HeLa cells, we conducted subrenal capsule surgery. Using HeLa cells transduced with a tdTomato expressing lentivirus, or HeLa-E2ts cells, or a 50:50 mix of both, we resuspended cells in collagen and surgically placed them under the capsule of the mouse kidney. Our findings herein show the results of this pilot experiment, which shows successful engraftment of plugs, viability of cells and the ability of these cells to minimally invade the mouse kidney. Further optimization and analysis will be needed to identify which cells were slightly invasive. The invasive effects of HeLa-E2ts cells on neighboring non-targeted cells will be determined by quantitating the percentage of tdTomato HeLa cells that invaded into the parenchyma in the presence of HeLa-E2ts cells compared to their invasiveness in the absence of E2-expressing cells.

Materials and Methods

Cell culture

HeLa cells were maintained in Dulbecco’s modified Eagle medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) and penicillin-
streptomycin. For xenograft injections, HeLa cells were adenovirally infected with the temperature sensitive E2 protein (AdE2ts) as described previously [15] or lentivirally transduced with a DEKsh#5 targeting vector (TRCN0000013107) purchased from the SigmaTM MISSION® shRNA program (Sigma Aldrich, St. Louis, MO). Cells were incubated with DEKsh#5 supernatant for at least 8hrs, washed and later stably selected in 650ng/ml puromycin containing media. For subrenal capsule surgery, HeLa cells were lentivirally transduced with tdtomatoe (a dsred variant) virus and sorted using FACS analysis. HeL-E2ts cells were also generated as described above.

**Xenograft injections**

Female NOD-SCID mice 4-6 weeks of age were injected sub-dermally with a total of $3 \times 10^6$ cells in a 4:1 mixed ratio of HeLa:E2ts or HeLa:DEKsh cells. All cells were resuspended in a final volume of 100ul of PBS. Tumors were allowed to grow for 8 weeks and tumor size of $300 \text{mm}^3$ and above was considered a tumor. At the end of 8 weeks, tumors along with lung and spleen were harvested and fixed in 4% paraformaldehyde for 16-20hrs, washed in PBS, and gradually dehydrated in 50% and 70% ethanol for subsequent paraffin embedding. Tissues and tumors harvested were sectioned and morphologically examined by hematoxylin-and-eosin staining.

**Collagen plug preparation**

A collagen mix containing approximately 1:3 ratio of rat tail collagen (a kind gift from the Kasper lab, University of Cincinnati, Cincinnati, OH) and DMEM was serially mixed until media reached a neutral salmon-pink color. Collagen mixture was then gently vortexed and kept on ice. A total of $1 \times 10^5$ HeLa-tdtomatoe, HeLa-E2ts or a 50:50 mix of
both were resuspended in 50ul of collagen mix and allowed to set for about 10 minutes in the 37°C incubator. Plugs were then overlaid with medium for approximately 16hrs prior to transplantation.

**Subrenal capsule xenotransplantation surgery**

Female NOD-SCID interleukin receptor 2 deficient mice or NSG mice (CCHMC mouse core) 4-6 weeks old were used and all animal experiments adhered to National and Institutional guidelines. Subrenal capsule surgery was conducted as previously described [16] and depicted in Fig.2. Mice were anesthetized using isoflurane gas supplied by a vaporizer to the nose. The mice were then shaved and swabbed with cotton soaked in iodine. A small anterior to posterior cut ~1.5cm was made through the skin over the kidney (Fig.2a). The dermis was then separated from the body wall, and then the mouse was placed on its side to locate the kidney by viewing through the body wall (Fig.2b). Another small incision was made through the body wall over the kidney and then slight pressure applied to move the kidney through the cut to the exterior (Fig.2c). Using a dissecting microscope, at a magnification so that the kidney occupied most of the field of view, a slight pinch was made to lift the kidney capsule from the parenchyma (Fig.2d). A small incision was made in the capsule using the needle and the area was kept moist throughout surgery by application of saline (Fig.2e). A moist fire-polished glass pipet was then used to make a pocket under the capsule. The edge of capsule was gently lifted with forceps to avoid tearing and the collagen plug was placed gently under the capsule using glass pipet. Up to two grafts were placed under a single capsule and both kidneys where needed (Fig.2f-g). Following transplantation of the plug, the kidney was gently eased back into body cavity, the body wall was sutured and mouse skin was stapled.
together (Fig.2h,j). No hormone pellet was inserted under skin as depicted in Fig.2i. Mice were then allowed to recover from anesthesia and when fully recovered were placed back into the cage and monitored regularly. One week post surgery, the staples were removed and mice were sacrificed after 2-4 weeks. The kidneys were removed and fixed in 4% paraformaldehyde for 20-22hrs, paraffin embedded, and analyzed by hematoxylin-and-eosin staining as well as immunohistochemical techniques.

**Immunofluorescence microscopy**

For analysis of tissue sections, 5-8 micron sections were cut from embedded tissue, deparaffinized and sequentially rehydrated. Sections were then boiled in 10mM sodium citrate buffer for 20mins, washed in PBS, blocked for 1hr and then incubated with anti-human mitochondria primary antibody at 1:50 dilution (Chemicon International, Billerica, MA). Sections were then washed and incubated with rhodamine conjugated secondary antibody for at least 2hrs at room temperature, and then mounted using Vectashield mounting medium. Images were then captured using Zeiss fluorescence microscope (Zeiss, Thornwood, NY) using Openlab software.

**Results**

**Summary of flank injections.**

To test whether E2-expressing cells could stimulate metastasis of neighboring cancer cells *in vivo*, flank injections were conducted. Four female NOD-SCID mice were injected sub-dermally with a mix of HeLa:HeLa-E2ts or as controls with a mix of HeLa-DEKsh cells. Tumor volume was determined weekly beginning at 3 weeks post injection
and only those measuring at least $300\text{mm}^3$ were considered tumors. While mice injected with the mix of HeLa:HeLa-E2ts gradually formed tumors, those injected with HeLa:HeLa-DEKsh did not show tumors until week 8. At this point images of tumor bearing mice in both groups were taken (Fig.1A) and tumor volume within groups is depicted (Fig.1B). Mice injected with the mix of HeLa:HeLa-E2ts formed significantly larger tumors and had to be sacrificed at week 8 in keeping with regulations from Veterinary Services. Mice injected with HeLa:HeLa-DEKsh cells were kept until week 14, at which point they formed tumors comparable to those of mice injected with HeLa:HeLa-E2ts and were sacrificed. Hematoxylin-and-eosin staining indicated that tumors formed from both groups, at sacrifice, were homogeneous, similar in morphology and vascularized (Fig.1C). All mice lungs and spleens were harvested and examined for metastases, however none were observed. Weights of all mice were recorded weekly, and the lack of weight loss suggested a lack of metastatic dissemination (data not shown).

**Schematic of subrenal capsule surgery**

A schematic depicting experimental steps of subrenal kidney surgery is shown (Fig.2). A total of seven mice and 10 kidneys were implanted. Five kidneys were transplanted with two plugs in which one plug consisted of tdtomatoe marked HeLa cells and the other of AdE2ts infected, GFP marked, HeLa cells. Four kidneys were transplanted with tdtomatoe HeLa control cells in one plug, and the mix of HeLa-tdtomaote and HeLa-E2ts cells in the other. The remaining kidney was only transplanted with one plug containing HeLa-tdtomatoe control cells.
Analysis of human HeLa-tdtomato and HeLa-E2ts cells in mouse kidney parenchyma.

Kidneys were harvested 2-4 weeks after surgery and as depicted by the photographs of whole kidneys, the plugs were successfully grafted and maintained in the kidneys (Fig.3A, left panels). Each kidney was cut diagonally so that both plugs transplanted could be observed on each kidney section. Hematoxylin-and-eosin stained sections were examined at 10X (Fig.3A, middle panel) and 20X (Fig.3A, right panel) magnification. Examination of all kidney sections indicated that overall there was no massive invasion of cells, but the cells present were viable (Fig.3A). In two of the five kidneys in which both control HeLa-tdtomato and HeLa-E2ts cells were transplanted, minimal cellular invasion from one of the two plugs was observed (Fig.3A, first row, middle and right panels). It is now critical to identify whether invaded cells are derived from the E2-positive or E2-negative population. This will be done by immunofluorescence staining using the human-specific mitochondrial antibody to confirm the presence of human cells. A representative kidney transplanted with HeLa-tdtomato cells or a mix of HeLa:HeLa-E2ts cells is depicted and showed specificity of the antibody for human cells (Fig.3C). Because GFP detection did not identify E2 expressing cells, I will proceed with the detection of p53 or p21, which should be strongly induced in E2ts expressing cells (chapter 2, Fig.1). Similarly, dsred staining did not identify HeLa-tdtomato cells and so PCNA will be used as an indirect marker.

Discussion

E2 expression is already used as an effective cervical cancer therapeuty in Mexico, and our findings in vitro that E2 expressing cells are motile and confer motility to
neighboring cancer cells is therefore of possible clinical importance. We set out to
determine whether E2-associated motility could be detected \textit{in vivo} by first using a mix of
HeLa:HeLa-E2ts cells as well as HeLa:DEKsh cells as controls. The findings from this
experiment were largely inconclusive due to differences in tumor formation between
groups. Indeed, the results indicate that mice injected with the mix of HeLa:DEKsh cells
showed significant delay in tumor formation. It remains to be seen whether this finding is
specific for the knockdown of DEK. If so, repression of neighboring cancer cells growth
through the targeting of DEK is in and of itself an important finding since DEK has not
yet been shown to have effects on the cellular microenvironment and since DEK is
already explored as a therapeutic target in cervical and other cancers. Regardless of the
rate of tumor formation, no metastases were observed in either group. This may be due to
the number of cells used, and differs from published studies similar to ours wherein all
mice injected with HeLa cells showed metastasis to lung and other tissues [17]. This
experiment will be repeated using the same criteria as in [17] to first establish whether
metastasis can be observed using HeLa cells and mice from our laboratory and then later
optimized for examining the role of E2-expressing cells in promoting metastasis of
neighboring cancer cells.

Subrenal kidney capsule transplantation turned out technically feasible, and awaits
further optimization and characterization. In light of our recent findings however, that
E2ts associated migration is reversible with temperature shift, it will be necessary to
repeat this experiment with a non-temperature sensitive E2 expressing vector to
determine whether E2 cells are indeed invasive and confers this property to adjacent
HeLa cancer cells.
References

17. Lu, J.Y., et al., *Establishment of red fluorescent protein-tagged HeLa tumor metastasis models: determination of DsRed2 insertion effects and comparison of
metastatic patterns after subcutaneous, intraperitoneal, or intravenous injection.
Figure 1.

**Summary of flank injections.** A. Mice mice injected with a mix of HeLa:E2ts formed significantly larger tumors compared to those injected with the same number of HeLa:DEKsh cells at 8 weeks. Flanks measuring 300mm³ and above were considered tumors. B. Measurements of tumors depicted in A. are graphed. At this time point mice injected with HeLa:HeLa-E2ts cells were sacrificed. C. Hematoxylin-and–eosin staining of a representative tumor from each group are depicted.
Figure 2. Schematic of subrenal capsule surgery.

The experimental approach for subrenal kidney surgery is described in the Materials and Methods. (a) A dorsal incision was made in mouse skin and then another made in the body wall (b). The kidney was gently taken out of mouse (c), and an incision made in the kidney capsule (d). Collagen plugs containing cells were then placed under the kidney capsule and manipulated away from the incision (e-g). Kidneys were then inserted into the abdominal cavity and then the incision in the body and skin were sutured together (h-j). Adapted from [16].
Figure 3.

A.

B.

h-Mitochondria

h-Mitochondria
Figure 3. Analysis of human HeLa-tdtomatoe and HeLa-E2ts cells in mouse kidney parenchyma. A. Two whole kidneys from the same mouse are shown in the left panel. Kidneys are shown from one experiment wherein engrafted collagen plugs consisted of control HeLa-tdtomatoe and mixed grafts and HeLa:HeLa-E2ts grafts. Hematoxylin-and eosin stained sections were examined at 10X (middle panel) and 20X (right panel) magnification. There was no massive cellular invasion overall, however minimal invasion was observed in one of two plugs transplanted with HeLa-tdtomatoe and HeLa-E2ts cells (first row, middle and right panels). B. Immunoflorescence staining of kidney sections using human-specific mitochondria antibody showed specificity for human cells.
CHAPTER 4: SUMMARY AND FUTURE DIRECTIONS
**E2 as a therapeutic target: from bench to bedside**

The re-introduction of the viral E2 protein into HPV positive cancer cells has been shown to be sufficient for the transcriptional repression of the E6 and E7 oncogenes, subsequent activation of the tumors suppressors p53 and pRb, and ultimately cellular senescence [1, 2]. As a result, E2 reverses the cervical cancer phenotype making it an attractive approach for the treatment of cervical cancer. The Modified Vaccinia Ankara (MVA), deemed safe for human studies, is often used as a viral vector to express recombinant proteins [3]. An MVA-E2 recombinant virus was shown to be effective in growth arrest of HeLa cell tumors grown in the flanks of nude mice [4]. Furthermore, in Mexico, where a woman dies from cervical cancer every two hours, clinical trials are underway using the MVA-E2 virus to treat high grade lesions (CIN 2 and CIN 3) associated with infection by the oncogenic papillomaviruses [5]. Results from these studies show up to 90% tumor regression in these lesions. The virus is also being used to treat men who have condylomas, genital warts associated with infection by low risk HPV types, as well as penile and bladder cancer, and other lesions that are associated with HPV infection [6]. These studies are proof of concept for E2-mediated therapeutic oncogene repression and subsequent tumor regression, demonstrating one example that it can be utilized from the bench to bedside.

**E2 repression of E6/E7 oncogenes stimulate motility *in vitro***

Our studies have clearly demonstrated the efficacy of the E2 protein as an inhibitor of tumor growth in cervical cancer cells. However, we have advanced these studies to uncover a novel, albeit, undesirable consequence of E2 expression: cellular migration and invasion. Specifically, E2 expression results in increased numbers of cellular protrusions, accompanied by stimulated
motility and invasion. This phenotype is dependent upon cell-cell contact, and ECM or secreted factors from E2-expressing HeLa cells are not sufficient to confer invasion. Furthermore, invasion is independent of and separable from cell cycle arrest and cellular senescence. The ability of E2 to stimulate motility was specific to cells with integrated HPV E6 and E7, as opposed to those harboring episomal HPV16 which were not transcriptionally repressed by E2 expression. This is in line with previous studies demonstrating that E2 does not repress E6/E7 transcription in the context of episomal viral DNA [7]. We also show that migration required both E2 presence and the repression of E6 and E7. Although E6/E7 repression was required for the phenotype, E6/E7 knockdown was not sufficient to stimulate, and in fact suppressed cellular migration, a finding which agrees with recently published data [8]. Interestingly, the reconstitution of E6/E7 in E2 expressing cells partially rescued the migration phenotype, suggesting that E6/E7 repression is important in the context of E2, but that additional E2 activities are required. This finding is intriguing as it has been shown that E2 has no known cellular transcriptional targets [9]. Thus the additional, as of yet undefined, E2 activities may involve post-transcriptional mechanisms regulating cellular migration. Identification of these relevant E2 functions and relevant cellular targets is now critical.

Rho GTPases are well established mediators of cellular migration, and we examined potential functional roles of these proteins in E2 migration. Given the increased number of cellular protrusions that resembled lamellipodia in E2 expressing cells, we focused on the role of three small Rho GTPases Rac1, RhoA and Cdc42 as all have been shown to localize to lamellipodia in other migration systems [10]. In particular, Rac1 activation appears to be sufficient to induce lamellipodia formation [11], making Rac1 an attractive E2 target. Interestingly, E2 stimulated migration did not require active Rac1. In fact, by conducting pull-
down assays we show that active, GTP-bound Rac1 was not up-regulated, but was down-regulated in conjunction with total Rac1 protein levels. This overall decrease in Rac1 expression in E2 expressing cells was additionally confirmed by immunofluorescence microscopy. Furthermore, inhibition of Rac1 activity using a dominant negative mutant of Rac1 or shRNA did not rescue the altered migration phenotype. Other Rho GTPases including RhoA and Cdc42 were not altered in E2 expressing cells, suggesting E2 migration is mediated by mechanisms independent of Rac1, RhoA and Cdc42. Other candidate mediators for E2-driven motility exist and should be pursued. Indeed, it has been demonstrated that the Rab5 protein, well known for its role in endocytosis, can induce lamellipodia formation and migration independent of Rac1 [12]. This finding is particularly interesting as the Rab family proteins were among genes that were highly upregulated in microarray studies conducted using E2 expressing HeLa cells [13]. Regulation of Rab genes by E2 will require confirmation by qRT-PCR. Subsequent targeting of individual Rab proteins will determine the role, if any, of these proteins in E2 dependent migration. Rho GTPases such as RhoG has been shown to induce lamellipodia through Rac-independent mechanisms that are yet to be characterized [14]. Along with decreased Rac1 levels, E2-expressing cells also showed decreased F-actin stain. This could be as a result of decreased Rac1 activity in the cells as well as a consequence of the senescence phenotype. Other cytoskeletal regulators namely the focal adhesion proteins pFAK and vinculin showed no differences in expression between E2-expressing cells and controls. Further examination of actin binding proteins will be needed to determine what cytoskeletal regulator(s) illicit the migration response in E2-expressing cells. Notably, profilin 2 (PFN2), a known regulator of actin polymerization was upregulated almost two fold in our microarray analysis. Targeting of this and
other genes activated by E2-expression will be critical in determining the critical players involved in E2 migration.

Regardless of the underlying mechanism, we show that E2 expressing cells were not only themselves motile, but confer motility to neighboring cancer cells. These finding may be biologically significant as the incomplete targeting of cervical cancer cells by E2 could at least in principle stimulate the invasion and metastasis of neighboring, non-targeted cancer cells. With these findings in mind, the use of E2-independent, perhaps RNAi-based mechanism for targeting cervical cancer cells, appear to be a safer approach, even though issues of efficacy, specificity and possible side effects remain to be compared on a clinical level. The transient nature of E2 on invasion as described in chapter 2 may lessen concerns about invasive side effects.

**Does E2 targeting of cervical cancer cells promote metastasis?**

The finding that E2 expressing cells can stimulate migration of neighboring cancer cells *in vitro* prompted us to examine possible physiological implications *in vivo*. We therefore injected NOD-SCID mice sub-dermally with a mix of unmodified and E2-expressing HeLa cells. Tumor formation was meant to be followed up by analyses of local invasion. Control cells comprised of a mix of uninfected HeLa and HeLa-DEKsh cells. As mentioned earlier in Chapter 2, the DEK oncogene is an upregulated HPV E7 target gene that is overexpressed in cervical cancer. Inhibition of DEK in HPV positive cells has been shown to result in cellular senescence and/or apoptosis [15]. Unlike E2-expressing senescent cells however, DEK-targeted senescent cells were not increasingly motile and so were considered an ideal control for these experiments. Mice injected with a mix of HeLa and HeLa-E2 cells rapidly formed tumors. However, mice injected
with the same ratio of HeLa and DEKsh showed delayed tumor formation, thus making the comparison between DEKsh- and E2-expressing cells difficult. However, the fact that DEK-repressed HeLa cells delayed the growth of a tumor from admixed HeLa cells argues DEK targeting affects the tumor cell microenvironment in a therapeutically important fashion. It will be important to elucidate the underlying mechanism wherein the knockdown of DEK delays the growth of neighboring tumor cells via the tumor microenvironment. Dr. Privette’s data in the laboratory suggests DEK transcriptionally stimulates the expression of the Wnt10B ligand which is known to activate beta catenin signaling and associated proliferation. It remains to be determined whether DEK loss reduces beta catenin signaling through reduced Wnt10B production and secretion, and whether this mechanism underlies the observed repression of HeLa cell growth. Given that DEK is sufficient to induce cellular senescence \textit{in vitro} without adversely stimulating migration, and further suppresses the growth of neighboring tumor cells \textit{in vivo}. The targeting of DEK may be an attractive therapeutic approach to treating cervical cancer. While the mix of HeLa and E2 cells allowed for the growth of the primary tumors, careful examination of murine lungs at 8 weeks post injection revealed no micro or macro metastases. Subsequent experiments will need to include appropriate empty vector controls for DEKsh and E2-expressing cells, and potentially a longer time frame to allow for metastatic dissemination.

In order to determine whether E2 expressing cells are locally invasive and confer invasion to neighboring cells \textit{in vivo}, we used an alternative approach, namely subrenal kidney capsule experiments. Unmodified or E2-expressing HeLa cells, or a 50:50 mix of both were embedded in collagen plugs which were then surgically placed under the kidney capsule of nude mice. Kidneys were harvested 2-4 weeks later. Future studies will include immunohistochemical analysis of kidney sections using human specific antibodies, to first determine whether HeLa
cells were able to invade the mouse kidney. HeLa-E2 cells are GFP positive, and HeLa cells were lentivirally marked with a dtomatoe (a dsred fluorescent protein variant) transgene. Detection of GFP and Tomatoe, respectively, by immunofluorescence microscopy will be conducted to determine whether E2 targeted or non-targeted HeLa cells invaded the kidney, and whether or not HeLa cells become more invasive in the presence of HeLa-E2 cells as predicted from the in vitro experiments. The results from these experiments will determine whether E2-stimulated motility in HPV positive cervical cancer cells can be recapitulated in a more physiologically significant in vivo model system.

**Identification of E2 activities and relevant cellular targets which drive motility.**

It is clear that in HPV positive cancer cells, ectopic expression of the E2 protein results in transcriptional repression of the viral E6 and E7 oncogenes. This repression is brought about by the site-specific binding of E2 protein to palindromic promoter sequences. E2 binding leads to exclusion of the Sp1 and TFIID transcription factors from the early promoter, which normally activate E6 and E7 transcription, thus resulting in transcriptional silencing [16-18]. Therefore, the re-introduction of E2 into HPV positive cancer cells drives E6/E7 repression. Repression of the oncogenes can be achieved by other means including RNA interference, peptide aptamers and antisense technology. Regardless of the approach, the ultimate result of these targeting mechanisms is cellular senescence and in some instances apoptosis [19-21]. The E2 protein is a proto-typical transcription factor that consists of three functional parts, an N-terminal transactivation domain and a C-terminal DNA binding domain connected to each other by a less well characterized hinge region. Studies have shown that repression of the E6/E7 promoter by E2 requires both the DNA and transactivation domains [22]. Several reports have transcriptionally
profiled BPV1 E2-expressing cells. E2 binds cognate motifs in the cellular genome and regulate cellular transcription. However, elegant data from the DiMaio laboratory revealed that all E2-induced cellular gene expression patterns are indirect consequences of E6/E7 repression, and that no direct transcriptional E2 targets exist within the cellular genome [9]. Furthermore, the transcriptional profile of E2-expressing cells had significant overlap with that of senescent human fibroblasts, thus likening E2-induced senescence to replicative senescence. Additionally microarray analyses were conducted using E2-expressing HeLa cells at the point of senescence irreversibility [13] [23]. Given that E2-induced migration is independent of cellular senescence it will be important to carry out additional, timed microarray analyses which separate senescence phenotypes from motility at the level of gene expression which might allow for insights into E6/E7 targets that specifically regulate cellular motility. In addition to E6/E7 repression, however, additional unknown E2 activities must be important since E6/E7 repression was not sufficient to confer motility. Initial attempts to identify such activities might involve studies of mutant E2 proteins which are competent for E6/E7 repression but deficient in other activities [24]. Identification of E2 domains which separate such important activities from transcriptional E6/E7 repression could then lead to biochemical studies to identify E2 interacting partners, followed by immunoprecipitation and mass spectrometry analysis.

The findings presented herein raise concerns about the treatment of cervical cancer through the targeting of E2. While E2 is effective in causing cancer cell arrest and regression we demonstrate potentially adverse motility. E2 motility in vitro suggests that it is now important for cervical cancer patients receiving MVA-E2 be closely monitored for local invasion and metastasis. In the future, it will be important to directly compare and contrast the risks and benefits of E2 expression versus E6/E7 knockdown as HPV-associated cancer therapeutics.


