VISUALIZING THE PRODUCTIVE PROGRAM OF HPV IN DIFFERENTIATING SQUAMOUS EPITHELIAL TISSUE

by

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A DISSERTATION

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VISUALIZING THE PRODUCTIVE PROGRAM OF HPV IN DIFFERENTIATING SQUAMOUS EPITHELIAL TISSUE

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BIOCHEMISTRY AND MOLECULAR GENETICS

ABSTRACT

The human papillomavirus (HPV) establishes persistent infections in the basal stratum of squamous epithelia, while productive amplification of viral DNA occurs in differentiated keratinocytes prior to virion assembly in the superficial strata. Until recently, only in situ hybridization (ISH) of low-grade HPV lesions could be used to reveal a snap shot of the viral life cycle. There has been a critical need to reproducibly propagate HPV infections in culture for consistent genetic analyses. Organotypic raft cultures recapitulate a differentiated squamous epithelium. Our lab utilized in vivo Cre-mediated recombination to reconstitute the entire HPV-18 genome in primary human keratinocytes (PHKs). My fluorescent in situ hybridization (FISH) to examine HPV DNA and RNA revealed robust differentiation-dependent viral activities and enabled a redesign that ultimately generated abundant progeny virus. Crucially, harvested virus elicited a new cycle of the productive infectious program in PHKs.

The contextual progression of raft cultures containing HPV-18 replicons was consistent with known patterns of viral DNA amplification. Importantly, a detailed view of virus-host interactions was achieved by simultaneous in situ probing for viral DNA, viral proteins and host cell biomarkers. S-phase reentry was induced in numerous differentiated suprabasal epithelial cells on days 8 and 10. Unexpectedly, the patterns of HPV DNA amplification did not parallel those of the stochastic S phase cells. Rather, I show that viral DNA amplification initiated from G2 arrested on days 10 and 12 in the
spinous strata. These cells accumulated high levels of cytoplasmic cyclin B1. While ectopic expression of HPV E1^E4 leads to G2 arrest in cell lines, I demonstrate that this viral protein accumulated following cyclin B stabilization and coincidental with the high amplification of viral DNA. Interestingly, such differentiated cells with high copies of viral DNA lost HPV E7 activity and transitioned to express capsid proteins for virion morphogenesis. An immortalization-null HPV-18 E6*I mutant genome was deficient in viral DNA amplification and failed to express the major capsid protein. These observations emphasize the value of simultaneous multiplexed detections in discerning the subtle interplay between naturally progressing HPV pathogenesis within its differentiating environment.

Keywords: Human Papillomavirus (HPV) life cycle, HPV DNA amplification, primary human keratinocyte (PHK) organotypic raft cultures, tyramide signal amplified (TSA) DNA fluorescent in situ hybridization (DNA FISH), cell cycle arrest
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TABLE OF CONTENTS

CHAPTER ..................................................................................................................... Page

ABSTRACT .................................................................................................................. ii

ACKNOWLEDGEMENTS ......................................................................................... iv

LIST OF TABLES ..................................................................................................... viii

LIST OF FIGURES ..................................................................................................... ix

LIST OF ABBREVIATIONS ...................................................................................... xi

1 HUMAN PAPILLOMAVIRUS (HPV) ................................................................. 1

Transmission and Pathogenesis ................................................................................. 2
Pathology and Therapeutics ...................................................................................... 5
Genome Organization ................................................................................................ 6
  Upstream Regualtory Region .............................................................................. 8
  Early Region ......................................................................................................... 12
  Late Region ......................................................................................................... 25
HPV Vaccination ..................................................................................................... 27
  Prophylactic Vaccine ........................................................................................... 27
  Therapeutic Vaccine ........................................................................................... 29
The Integument ........................................................................................................ 29
Keratinocytes ........................................................................................................... 31
  Epidermis ............................................................................................................. 31
  Cell Cycle ............................................................................................................ 32

2 IN VITRO MODEL SYSTEMS TO STUDY HPV ........................................... 37

HPV Cell Lines and Exogenous Gene Expression .................................................. 37
Organotypic (Raft) Cultures of Primary Human Keratinocytes .............................. 38
Introduction of HPV DNA into PHKs ................................................................. 38
Assays to Investigate HPV Activities .................................................................... 40
  In Situ Hybridization (ISH) ................................................................................. 41
  Tyramide Signal Amplification (TSA) ................................................................. 43
Preliminary Studies: Illuminating HPV Activities in Squamous Epithelia by
  Tyramide Signal Amplified Fluorescent In Situ Hybridization (TSA-FISH) ....... 45

3 HPV-18 RAFT CULTURES RECAPITULATE THE PRODUCTIVE PROGRAM ....... 50
Efficient In Vivo Generation of HPV-18 Genomes in Cotransfected PHK Raft Cultures .................................................................50
HPV-18 Raft Cultures Recapitulate Early HPV Pathogenesis ...............51
Determination of HPV DNA and mRNA Content by TSA-FISH ..........52
Infection of Naïve PHKs ..................................................................55
Conclusions to Chapter 3 ..................................................................56
Rationale and Objectives ..................................................................57

4 NOVEL TEMPORAL AND SPATIAL INTERACTIONS DURING THE HUMAN PAPILLOMAVIRUS PRODUCTIVE PROGRAM WITHIN EPITHELIAL TISSUE AS REVEALED BY MULTIPLEXED FLUORESCENT IN SITU DETECTIONS ..................................................59

Robust Cellular DNA Replication Precedes HPV-18 DNA Amplification ....59
S Phase Progression Is Distinct From HPV-18 DNA Amplification ..........61
HPV-18 Genomic Amplification from the G2 Phase .........................63
Cessation of HPV-18 E7 Activity in Cells With Abundant Viral DNA ......65
An Essential Role of E6 in Viral DNA Amplification and Virion Production ..67
Conclusions to Chapter 4 ..................................................................71

5 A HIGHLY EFFICIENT SYSTEM TO PRODUCE INFECTIOUS HUMAN PAPILLOMAVIRUS: ELUCIDATION OF NATURAL VIRUS-HOST INTERACTIONS .................................................................73

6 DISCUSSION ..................................................................................87

Productive HPV Raft Cultures ..............................................................87
HPV Infection of Naïve PHKs ...............................................................89
Viral DNA Amplification in G2 Arrested Cells ......................................89
E1^E4 Protein Does not Induce G2 Arrest ...........................................91
A Critical Role for E6 in Viral DNA Amplification ..............................93
A Switch to the Late Phase .................................................................95
Spontaneous HPV Regression ..........................................................96
Summary .........................................................................................96

7 FUTURE DIRECTIONS AND PERSPECTIVES ..................................98

8 MATERIALS AND METHODS ..........................................................109

Construction of HPV-18 E6*I parental plasmid ..................................109
Cell Culture ......................................................................................110
Oligonucleotide probe 3'-end labeling ..............................................110
Biotin nick translated FISH probe synthesis ......................................111
Tyramide signal amplified fluorescent in situ hybridization (TSA-FISH) ..112
Immunofluorescence (IF) .................................................................113
Multiplexed RNA-DNA-FISH or DNA-FISH and IF .........................114
Image acquisition and processing ..................................................114
LIST OF TABLES

Table ............................................................................................................................................ Page

MATERIALS AND METHODS

1  PCR primer sequences to clone HPV-18 E6*I. ................................................................. 110
2  Complimentary sequences used in RNA-TSA-FISH. ......................................................... 111
3  Primers to PCR amplify template for short nick-translated RNA-FISH probes............ 112
# LIST OF FIGURES

*Figure* .............................................................................................................................................. *Page*

**HUMAN PAPILLOMAVIRUS (HPV)**

1. The HPV life-cycle requires squamous epithelium ................................................................. 3

2. The HPV-18 genome .................................................................................................................. 7

3. Organization and splicing of HPV-18 transcription ................................................................. 11

**IN VITRO MODEL SYSTEMS TO STUDY HPV**

1. HPV DNA and mRNA FISH in HeLa, SiHa or CaSki cells ...................................................... 46

2. HPV-11 DNA and mRNA detections in a laryngeal papilloma .............................................. 48

**HPV-18 RAFT CULTURES RECAPITULATE THE PRODUCTIVE PROGRAM**

1. *In vivo* generated 8 kb HPV-18 genomes in PHKs for raft culture ..................................... 51

2. HPV-18 DNA amplification and mRNA expression in PHK raft cultures .......................... 53

3. HPV-18-DNA amplification in PHK raft cultures ................................................................. 54

4. Infectivity assay of HPV-18 virions in PHK Raft Cultures ..................................................... 56

**NOVEL TEMPORAL AND SPATIAL INTERACTIONS DURING THE HUMAN PAPILLOMAVIRUS PRODUCTIVE PROGRAM WITHIN EPITHELIAL TISSUE AS REVEALED BY MULTIPLEXED FLUORESCENT IN SITU DETECTIONS**

1. HPV-18 DNA amplification lags behind cellular DNA replication prior to L1 expression ........................................................................................................... 60

2. HPV-18 DNA amplification follows S phase dependent cyclin A expression of BrdU incorporation .......................................................................................................................... 62

3. HPV-18 DNA amplification at G2, relative to cytoplasmic cyclin B1 detection ................. 64

4. HPV-18 DNA amplification relative to the cell cycle ............................................................ 66
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Complementation of HPV-18 E6*I genome by a retrovirus delivering HPV-18 URR-E6</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>A HIGHLY EFFICIENT SYSTEM TO PRODUCE INFECTIOUS HUMAN PAPILLOMAVIRUS: ELUCIDATION OF NATURAL VIRUS-HOST INTERACTIONS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Time course of HPV-18 E1^E4 protein expression, viral DNA amplification and cyclin B1 accumulation or L1 expression</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>FUTURE DIRECTIONS AND PERSPECTIVES</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>HPV-18 does not delay squamous epithelial differentiation</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>HPV-18 does not delay differentiation dependent Involucrin expression</td>
<td>101</td>
</tr>
<tr>
<td>3</td>
<td>The p21CIP1 protein is stabilized in normal PHKs differentiation</td>
<td>103</td>
</tr>
<tr>
<td>4</td>
<td>Normal PHK differentiation</td>
<td>105</td>
</tr>
<tr>
<td>5</td>
<td>HPV-18 amplified in terminally differentiating epithelia</td>
<td>107</td>
</tr>
</tbody>
</table>
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>a.a.</td>
<td>amino acid</td>
</tr>
<tr>
<td>APC</td>
<td>anaphase-promoting complex</td>
</tr>
<tr>
<td>BPV</td>
<td>bovine Papillomavirus</td>
</tr>
<tr>
<td>BrdU</td>
<td>bromodeoxyuridine</td>
</tr>
<tr>
<td>BS</td>
<td>binding site</td>
</tr>
<tr>
<td>C-</td>
<td>carboxyl-terminus</td>
</tr>
<tr>
<td>CAK</td>
<td>cdk-activating kinase</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>CIN</td>
<td>cervical intraepithelial neoplasia</td>
</tr>
<tr>
<td>CK</td>
<td>cytoplasmic keratin</td>
</tr>
<tr>
<td>CRPV</td>
<td>cottontail rabbit PV</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA binding domain</td>
</tr>
<tr>
<td>ddATP</td>
<td>dideoxy-ATP</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>ds</td>
<td>double-stranded</td>
</tr>
<tr>
<td>E</td>
<td>Early</td>
</tr>
<tr>
<td>E1BS</td>
<td>E1 protein binding site</td>
</tr>
<tr>
<td>E2BS</td>
<td>E2 protein binding site</td>
</tr>
<tr>
<td>E2N</td>
<td>E2 protein amino-terminus</td>
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<td>Description</td>
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<tr>
<td>E6AP</td>
<td>E6 associated protein</td>
</tr>
<tr>
<td>E6TP1</td>
<td>E6-targeted protein 1 polymerase</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FFPE</td>
<td>formalin-fixed paraffin-embedded</td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescent <em>in situ</em> hybridization</td>
</tr>
<tr>
<td>Flg</td>
<td>filaggrin</td>
</tr>
<tr>
<td>GFR</td>
<td>growth factor receptor</td>
</tr>
<tr>
<td>GKLF</td>
<td>gut-enriched Krüppel-like factor</td>
</tr>
<tr>
<td>H2O2</td>
<td>hydrogen peroxide</td>
</tr>
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<td>HR</td>
<td>high risk</td>
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<tr>
<td>HPV</td>
<td>human papillomavirus</td>
</tr>
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<td>HRP</td>
<td>horseradish peroxidase</td>
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<td>IF</td>
<td>immunofluorescence</td>
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<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
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<td>IRF-3</td>
<td>interferon regulatory factor-3</td>
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<td>IRMAAs</td>
<td>immunoradiometric assays</td>
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<td>ISH</td>
<td><em>in situ</em> hybridization</td>
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<tr>
<td>IVL</td>
<td>involucrin</td>
</tr>
<tr>
<td>KLF4</td>
<td>Krüppel-like factor 4</td>
</tr>
<tr>
<td>L</td>
<td>Late</td>
</tr>
<tr>
<td>LCR</td>
<td>long control region</td>
</tr>
<tr>
<td>LEEP</td>
<td>loop electrocautery excision procedure</td>
</tr>
<tr>
<td>Lor</td>
<td>loricrin</td>
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**LIST OF ABBREVIATIONS (Continued)**

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<tr>
<td>LR</td>
<td>low risk</td>
</tr>
<tr>
<td>LSIL</td>
<td>low grade squamous intraepithelial lesion</td>
</tr>
<tr>
<td>M</td>
<td>mitosis</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>MPF</td>
<td>mitosis, or maturation, promoting factor</td>
</tr>
<tr>
<td>N-</td>
<td>amino-terminus</td>
</tr>
<tr>
<td>NCR</td>
<td>non-coding region</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>Ori</td>
<td>origin of replication</td>
</tr>
<tr>
<td>P</td>
<td>promoter</td>
</tr>
<tr>
<td>pAE</td>
<td>early polyadenylation</td>
</tr>
<tr>
<td>pAL</td>
<td>late polyadenylation site</td>
</tr>
<tr>
<td>PBS</td>
<td>pBlueScript II</td>
</tr>
<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PHK</td>
<td>primary human keratinocyte</td>
</tr>
<tr>
<td>pol</td>
<td>polymerase</td>
</tr>
<tr>
<td>polyA</td>
<td>poly-adenylation</td>
</tr>
<tr>
<td>pRB</td>
<td>retinoblastoma susceptibility protein</td>
</tr>
<tr>
<td>PV</td>
<td>papillomavirus</td>
</tr>
<tr>
<td>RIAs</td>
<td>radioimmunoassays</td>
</tr>
<tr>
<td>RRP</td>
<td>recurrent respiratory papillomatosis</td>
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<tr>
<td>SA-HRP</td>
<td>streptavidin-conjugated horseradish peroxidase</td>
</tr>
<tr>
<td>ss</td>
<td>single-stranded</td>
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<td>Description</td>
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<tr>
<td>TA</td>
<td>transactivation</td>
</tr>
<tr>
<td>TSA</td>
<td>tyramide signal amplification</td>
</tr>
<tr>
<td>TSA-FISH</td>
<td>tyramide signal amplified fluorescent <em>in situ</em> hybridization</td>
</tr>
<tr>
<td>TSA-IF</td>
<td>tyramide signal amplified immunofluorescence</td>
</tr>
<tr>
<td>URR</td>
<td>upstream regulatory region</td>
</tr>
<tr>
<td>VLP</td>
<td>viral-like particle</td>
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<td>wt</td>
<td>wild-type</td>
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CHAPTER 1

HUMAN PAPILLOMAVIRUS (HPV)

The papillomavirus (PV) is a small, nonenveloped DNA virus (~55 nm) that replicates as an extrachromosomal plasmid in the nuclei of differentiating epithelia (Fig. 1). The large family of over 120 PV genotypes is tropic to either mucosal or cutaneous squamous epithelia (Roland et al. 1997; de Villiers et al. 1999, 2004; zur Hausen 2002). Wounding down to basal epithelia is requisite for the virus to establish a primary infection, after which it can remain subclinical. HPVs can persist asymptomatic by maintaining low genomic copies in the lower undifferentiated or less differentiated squamous epithelia (Broker and Botchan 1986). Expression of viral early genes reestablishes a cellular environment permissive to amplify the extra-chromosomal plasmids in the nuclei of differentiating epithelia, inducing benign hyperproliferation. The newly amplified HPV DNA becomes encapsidated into viral particles as the epithelium undergoes the final stages of terminal differentiation (Chow and Broker 2006a).

A small fraction of mucosotropic high-risk (HR) HPV infections (e.g. HPV-16, -18, -31, -33 and related types) are associated with neoplastic progression to high grade intraepithelial lesions and cancers; specifically anal, cervical, laryngeal, nasopharyngeal, penile and tonsillar cancers (Durst et al. 1983; Liaw et al. 2001; Munoz et al. 2003; de Villiers et al. 2004). Conversely, low-risk (LR) HPV-6 and -11 pathogenesis (and related types) generates predominantly benign exophytic lesions on genitalia and only rarely
become carcinogenic (Stoler 2003; de Villiers et al. 2004). Exploring HPV pathogenesis and its associated cancer biology has generated potential therapeutic strategies. This relatively small DNA virus has also provided valuable insights into eukaryotic DNA replication, gene regulation and cell cycle progression.

Transmission and Pathogenesis

HPV is transmitted through direct contact with infected tissue, sexual contact in the case of genitalia (Koutsky 1997). The extremely stable papillomavirus virions particles can also persist on surfaces for very long times and infect epithelia opportunistically. U.S. women have a 70–80% likelihood of HPV infection during their lifetimes. 20-46% of sexually active individuals (20-25 years old) are infected (Lajiness 2007). Mechanical abrasion stresses can cause micro-fractures that expose uninfected basal epithelia while also liberating viral particles from infected desquamating superficial epithelia (Bryan and Brown 2001). HPV infects basal cells; after uncoating in the endosomes, the DNA genome traffics to the nucleus and commences expressing early region viral genes. Infected differentiating epithelial cells stochastically reenter the cell cycle to amplify viral DNA in some of upper spinous cells. Transition from early to late region gene transcription commences in the upper spinous cells and viral DNA packaging occurs in koiocyte nuclei (Almeida et al. 1962; Hills and Laverty 1979; Stoler et al. 1990, 1992; Evander et al. 1997; Joyce et al. 1999; Giroglou et al. 2001). Indeed, a cytologic indicator for productive HPV infection is koiocytotic atypia (Koss and Durfee 1956; Meisels 1983).

HPV-6 and -11 cause approximately 90% of benign genital warts that can produce progeny virions, whereas high-grade dysplasia and cancers do not produce virions (Stoler...
et al. 1989, 1992). Cervical basal cells may remain latently infected for years, or they can expand and proliferate (1-3 months) to a benign, low grade squamous intraepithelial lesion (LSIL), also known as low-grade cervical intraepithelial neoplasia (CIN I). Most low-grade CINs regress and are cleared by the host 1-2 years following incidence (< 30 years old). However, some LSIL associated with the high-risk HPV types remains chronic, with 10-15% progressing to moderate or severe dysplasia (carcinoma in situ) (Cullen et al. 1991; Stoler et al. 1992). Benign lesions contain extrachromosomal viral plasmids, whereas cervical and other ano-genital carcinomas contain multimeric circular molecules with occasional deletions or, more frequently, the viral DNA is found to be integrated into host chromosomal DNA (Vinokurova et al. 2008). In cancers, integration disrupts viral dependent autoregulation resulting in over-expression of viral oncogenes (Stoler et al. 1992; Van Tine et al. 2004b). The malignant transformation achieved through constant expression of HR HPV oncogenes E6 and E7 has permitted the
derivation and propagation of cell lines from cervical carcinomas. In vitro, ectopic
expression of HR HPV oncogenes is sufficient to immortalize primary epithelial cells
(Schwarz et al. 1985; Yee et al. 1985; Schneider-Gadicke and Schwarz 1986; Smotkin
and Wettstein 1986; Baker et al. 1987; Shirasawa et al. 1987; Inagaki et al. 1988; Pater et

The apparent chronic progression of dysplasias caused by HR HPVs to cervical
cancer (>10-15 years) may imply several possibilities: 1) HPV is not sufficient to
induce/sustain neoplasms alone. Cellular factors are dysregulated by environmental
cofactors (such as smoking) and/or internal genetic insults until neoplastic progression is
favored. 2) A persistent incubation increases the likelihood that HR HPV integrants will
express viral oncogenes sufficient for transformation. 3) Depreciation of the CMI due to
age no longer holds neoplastic progression in check; underscored by an elevated
incidence for cutaneous and anogenital HPV-associated disease in patients with genetic
or acquired CMI deficiencies. Specifically, individuals with both HPV and HIV
infections have extensive warts and progression of SIL to high grade lesions or cancers is
accelerated. High grade ano-genital dysplasias and cancers in young adults are regarded
as warning signs of AIDS. 4) HR HPV infections likely employ diverse molecular
mechanisms to maintain latent persistence and which inadvertently empowers malignant
transformation. Significant factors include the multiplicity of infection (MOI) and the
multiplicity of different infecting HPV genotypes; influenced by multiple sexual partners
and the age at first intercourse. Yet, advances and guidelines in collecting and screening
exfoliated cervical cells for cytologic malignancy has made invasive cervical cancer one
of the most preventable types of cancer (Ponten et al. 1995; Evander et al. 1997).
Unfortunately, there is an increasing incidence of cervical cancer among older women, largely due to the gradual implementation of regular Pap screens. In the U.S., all women are recommended to receive annual cervical cytology screening beginning no later than the age of 21 (or 3 years after initial intercourse) up to ages ~65-70. Across the divide is a generation of older women, as well as socio-economically disadvantaged and recent immigrants from underdeveloped countries, who do not choose to access such preventative measures because of the stigma surrounding sexually transmitted diseases. As a result, each year half of the cervical cancer patients worldwide (approximately 270,000 women) will die from cervical cancer.

Pathology and Therapeutics

Pap tests score atypical squamous cells as an indicator of HPV associated diseases. This is insufficient to definitively diagnose the nature and extent of diseases. Therapeutic intervention first must assess CIN stage/grade and then devise an ideal treatment to save the infected tissue. No clinically available treatment specifically targets the virus. Women with an abnormal Pap smear initially undergo a colposcopically directed biopsy to diagnose the stage/grade of infection. This can be followed by any of the following procedures: loop electrocautery excision procedure (LEEP) (excising the infected site, most common), laser (burning off the lesion), cryosurgery (freezing the lesion - application of Imiquimod to induce interferon production and boost the immune response, or general surgery (hysterectomy, rarely vulvectomy) (Kalliala et al. 2007; Winters et al. 2008).

HPV is associated with 99.7% of >1,000 cervical cancer biopsy screens performed across 22 countries: HPV-16 (57.4%), -18 (16.6%), -45 (6.8%), -31 (4.3%)
and -33 (3.7%) being the most prevalent (Bosch et al. 1995; Walboomers et al. 1999). Additional studies have confirmed types-16 and -18 are found in >70% cervical cancer (Goldie et al. 2004; Giles and Garland 2006). Among women worldwide, cancer of the cervix is the second most common cancer (after breast); in developed countries, it is seventh in frequency (following lung, breast, colon and rectum, stomach, prostate and liver). Within the U.S., ~$6 billion spent annually on clinical screening and intervention have reduced HPV associated cancer incidence by ~80% and deaths to ~3,700 (Singer 1995). However, 75% of the 15-50 year old population become genital HPV infected, with 60% transiently infected, 10% persistently infected (viral DNA-positive), 4% with mild cytological signs and 1% with advanced clinical lesions over their lifetime (La Torre et al. 2007; WHO position…2009). HPV infection can occasionally lead to rampant papilloma growths in the oral cavity and upper respiratory tract (Hu and Goldie 2008). In total, an estimated 20 million Americans are currently infected with at least one HPV type while ~5.5 million people will become infected with various types each year.

Genome Organization

All papillomavirus genomes are double-stranded (ds), closed-circular DNA plasmids (7400-8200 bp) that encode nine open reading frames (ORFs) on the same strand (Fig. 2). The last and first ORFs are separated by a non-coding region (NCR) known as the long control region (LCR) or upstream regulatory region (URR). The URR contains elements regulating amplification of viral DNA and expression of viral genes transcribed in the differentiating epithelial cell (Auborn et al. 1989; Auborn and Steinberg 1991; Dong et al. 1994; Demeret et al. 1997; Parker et al. 1997; Zhao et al. 1997; Swindle et al. 1999). The nine HPV ORFs are divided into “Early” (E) and “Late”
Fig. 2. The HPV-18 genome. All HPV genomes are roughly 8 kb in length and contain nine ORFs. The origin of replication and early and differentiation dependent promoters reside within the URR.

(L) regions as defined by their expression relative to the productive infection. The E proteins establish a persistent infection and amplify the viral genome: (5′-3′) E6, E7, E1, E8 (which overlaps E1), E2, E4 (which overlaps E2), E5 and the L region genes produce the structural capsid proteins that package the new viral genomes and produce HPV progeny: L1 encodes the major capsid protein and L2 the minor capsid protein (Boshart et al. 1984; Danos et al. 1984; Howley 1996; Chow and Broker 2006a). While early stages traverse the suprabasal to spinous strata, late events culminate in the production of viral particles in the terminally differentiating and desquamating superficial strata (Chow and Broker 2007).

The rather simple appearing HPV genome belies a complexity that is differentially regulated through multiple promoters and multiple splice sites that generate a myriad of polycistronic viral transcripts. Three putative promoters are fairly well conserved among HPVs: P1 is located in the 3′ end of the URR, immediately upstream of
E6, P2 active in LR genotypes within E6, giving rise to E7 messages and P3 within E7, from which the E1, E2, E4 and E5 messages are generated. Mapping the 5'-ends of HPV mRNAs has identified additional putative minor promoters, including within the E1 ORF. The URR contains late 5'-UTR, many enhancer and repressor protein binding sites, the viral origin of replication (Ori) and the Early promoter (P1). Transcripts are alternatively spliced to generate various polycistronic mRNAs, sometimes fusing portions of different ORFs (Rotenberg et al. 1989b; Doorbar et al. 1990; Chiang et al. 1991; Rohlfs et al. 1991; Sherman et al. 1992a, 1992b; Tan et al. 2003; Chow and Broker 2007). The majority of P1 messages terminate at a small NCR between the E and L regions containing the Early polyadenylation (pAE) site. P3 is responsible for both early gene and late gene expression. The 3'-UTR of all late HPV transcripts utilizes the late polyadenylation site (pAL) located in the 5'-portion of the URR.

**Upstream Regulatory Region**

The URR is the least conserved region among PV genotypes and varies between 400 bp - 700 bp in length. It spans the NCR between the pAL and the E6 AUG, the first early gene. Within the URR are differentiation-dependent cis-elements that regulate mRNA transcription and DNA replication. The HPV origin comprises 3 (sites #2, #3 and #4) of the 4 conserved E2 protein binding sites (E2BS) in the URR and a series of overlapping E1 binding sites located between E2BS #2 and #3 (Chiang et al. 1992a, 1992b; Kuo et al. 1994; reviewed by Chow and Broker 2006a), E2BS #4 at the viral Ori is located immediately upstream of the TATA boxes of the P1 promoter whereas E2BS #3 is located immediately downstream of the binding site for a key transcription factor Sp1. E2 binding to these sites interferes with the binding of these transcription factors.
and represses E6-E7 transcription (Thierry and Howley 1991; Romanczuk and Howley 1992; Dong et al. 1994; Taylor et al. 2003).

Viral transcription is also regulated by negative and positive cis-elements that recruit host-derived machinery and factors: Sp1, GT1, AP1, Oct-1, C/EBP, YY1, AP2 and NF1 and nucleolin (Chong et al. 1990; Hoppe-Seyler and Butz 1994; O'Connor and Bernard 1995; Zhao et al. 1997; Grinstein et al. 2002; Tan et al. 2003). AP1, Oct-1 and Sp1 activate viral transcription in the upper epithelia, whereas C/EBP is a repressor in proliferating basal cells (Parker et al. 1997; Zhao et al. 1997). AP1 and JunB/Fra2 heterodimers further regulate HPV expression as well as epithelial cell genes important to differentiation (Bouallaga et al. 2003; Mehic et al. 2005). AP1 and HMGI/Y bind within the HPV-18 URR and form an enhanceosome, initially described for IFNβ and TCRα, that recruits CBP/p300 histone acetylase coactivator (Falvo et al. 1995; Giese et al. 1995; Thanos and Maniatis 1995; Bouallaga et al. 2000, 2003).

**HPV Transcription**

The E6 promoter (P1) contributes only a minor portion of the total HPV mRNA expressed. Transcription initiation begins immediately upstream of the E6 AUG start codon and contains virtually no 5′-UTR (Hou et al. 2000). The majority of the transcripts initiated from this promoter encode both E6 and E7, then are spliced from an E1 donor site to an E4 acceptor site (E1^E4) and continue through E5 to terminate at the pAE (E6-E7-E1^E4-E5). Unspliced E1 mRNA is rare (E6-E7-E1-E5), but a minority of transcripts joins a splice donor located about 5 amino acids into the E1 ORF to a splice acceptor near the 3′-end of E1 and about 100 bases before the beginning of the E2 ORF (E6-E7-E1^E2-E5) to produce a full-length E2 protein (Chow and Broker 2007). The 3′-end of the E1
ORF overlaps the 5’-end of the E2 ORF in all papillomavirus genomes. The immediate proximity and often overlapping *E6* and *E7* ORFs among HR and LR HPVs suggested that the sudden hand-off from E6 translation termination to E7 initiation is frequently fumbled. LR HPVs utilize P2, typically within *E6*, to transcribe a polycistronic message with a sufficiently long 5′ UTR for efficient *E7* translation (*E7*-E1^E4-E5). For HR HPVs, it was believed that intragenically spliced *E6* mRNA facilitates translation of E7 protein by providing a longer 5′ UTR following the translation termination codon of *E6*I or *E6*II peptides (Smotkin et al. 1989; Zheng et al. 2004; Tang et al. 2006). However, *in vitro* translation of E7 protein initiated efficiently due to an inefficient ribosome scanning mechanism skipping the full-length E6 ORF (Stacey et al. 1995, 2000; Remm et al. 1999; Zheng et al. 2004; Tang et al. 2006). Similarly a bicistronic *E6*-E7 expression vector, containing an *E6* splice donor site mutation, promoted efficient S phase entry similar to WT (Cheng et al. 1995). This does not rule out that E7 translation is affected by splicing of *E6*.

The E1^E4 message comprises >90% of all viral transcripts and the E1^E4 protein represents the most abundant viral protein in productive infections (Chow et al. 1987a; Nasseri et al. 1987; Brown et al. 1988). It is the major P3 transcript and becomes greatly upregulated upon differentiation (E1^E4-E5) (Chow et al. 1987a; Stoler et al. 1989, 1990; Brown et al. 1996, 1998). In the upper spinous cells, P3 derived transcripts can also traverse through pAE to terminate at pAL. A twice spliced mRNA encodes the E1^E4 and L1 proteins, whereas those with only the E1^E4 splice encodes E1^E4 and possibly L2 as well. (Fig. 3). Transcripts with unique 5′ ends suggestive of additional promoters have been described for various HPVs, but they have yet to be fully
characterized for function (Chow et al. 1987b; Chiang et al. 1991; Karlen and Beard 1993; Ozbun and Meyers 1998; Stubenrauch et al. 2000; Zobel et al. 2003).

LR and HR HPVs exhibit similar mRNA expression patterns, while some novel mRNA combinations have been detected in HPV-16 precancerous lesions and cell lines (Doorbar et al. 1990; Rohlf s et al. 1991; Sherman et al. 1992a). The differentiation-dependent up-regulation of P670 in HPV-16 is equivalent to P3 of LR HPV. But there is no P2 promoter (Crum et al. 1988a; Higgins et al. 1992; Stoler et al. 1992; Grassmann et al. 1996). While P2 in LR produces a more efficiently translated E7 transcript than P1, evidence suggests intragenic E6 splicing may be important for HR E7 translation (Stacey et al. 1995; Tang et al. 2006). While these scenarios benefit E7 protein, as well as further
downstream early genes, such proximal P1 dependent transcription and E6 intragenic
splices undoubtedly seriously disadvantage E6 translation (Chow and Broker 2006a).
Additional HPV-16 Early transcripts originating from the 5′-end of the URR or in L1 may
improve E6 translation initiation (Glahder et al. 2003; Tan et al. 2003). Similar
compensatory mechanisms have been implicated by minor 5′UTRs from HPV-18 in
HeLa cells and HPV-31b (Thierry et al. 1987; Ozbun and Meyers 1999).

Early Region

The HPV E region is ~4 Kb long and encodes nonstructural proteins required for
successful viral genome replication, RNA transcription as well as immune evasion: E6,
E7, E1, E8, E2, E4 and E5. Differential promoter usage and alternative splicing regulates
mRNA expression, thereby modulating viral protein synthesis and ratios to ensure
optimal support for viral DNA maintenance and amplification. Importantly, most cervical
cancers contain integrated HR HPV DNA from which the viral oncogenes E6 and E7 are
overexpressed.

E6

Expression of the ~151 amino acid (a.a.) E6 protein is required to maintain viral
plasmids in submerged primary epithelial cultures (Thomas et al. 1999; Park and
Androphy 2002; Oh et al. 2004). E6 interacts with numerous cellular proteins via two
known binding regions, the LXXLL motif and the PDZ domain, though others exist
(Tungteakkhun and Duerksen-Hughes 2008). In a ternary complex with the host protein
E6 associated protein (E6AP), the E6 protein contributes to HPV pathogenesis by
targeting diverse host proteins as an ubiquitin E3 ligase (Werness et al. 1990; Huibregts
et al. 1991; Scheffner et al. 1993). Their subsequent proteasomal degradation alters signal
transduction, transcription regulation and inhibits apoptosis. A principal target, inactivated by other DNA tumor viruses (adenoviruses, polyomaviruses and SV40), is the tumor suppressor protein p53 (\(TP53\)) (Scheffner et al. 1990, 1993).

The p53 protein plays a central role in suppressing carcinogenic events that result from chromosomal insult. Depending on the type and severity of DNA damage, p53 becomes stabilized by posttranslational modifications and functions as a transcription factor to activate or repress target genes. Canonically, DNA damage-dependent stabilization of p53 transactivates the Cdk inhibitor p21\(^{CIP1}\) (\(CDKN1A\)) to arrest proliferating cells at G1 and promote their repair or caspase-mediated apoptosis (Kastan et al. 1995; Sherr and Roberts 1999; Garner and Raj 2008). Loss of p53 alone does not significantly affect normal proliferation or epidermal differentiation in p53-null mice or organotypic cultures of p53-knockout PHKs (Donehower et al. 1992; Weinberg et al. 1995; Truong et al. 2006). However, HR HPV E6 is neither necessary nor sufficient to induce the cell cycle in differentiated epithelia (Halbert et al. 1992; Cheng et al. 1995).

Although initiatory events remain speculative, HR E6 is one of two major viral oncoproteins that are consistently expressed at high levels in HPV associated carcinomas. The other HR viral gene \(E7\) targets the pRB/E2F pathway and promotes dysplasia and loss of epithelial cell differentiation in concert with E6 (Bosch et al. 1990; Hudson et al. 1990). Interestingly, HR HPV \(E7\)-dependent unscheduled S phase entry induces p53, independent of DNA damage, in cell cultures and in organotypic PHK raft cultures (Jian et al. 1998; Jones et al. 1999). The dramatic increase in E2F1 levels has been implicated in promoting the post-translational modifications that stabilize p53 and additionally activate Chk2, which together mediate growth-arrest and apoptosis (Adams and Kaelin...
1996; Rogoff et al. 2004). Critically, elevated levels of p53 inhibit HPV DNA replication in transient replication assays in cell lines (Lepik et al. 1998). Therefore E6 protein could counteract E7-induced p53 (Demers et al. 1994; Jian et al. 1998; Lepik et al. 1998; Eichten et al. 2002). However, the role of E6 during productive infections has been elusive because E6 mutants do not persist in transfected PHKs (Thomas et al. 1999; Park and Androphy 2002; Oh et al. 2004; Wang et al. 2009).

The importance of HR HPV E7 and E7 in neoplastic progression is underscored by cervical cancer derived cell lines that require their constant expression (Francis et al. 2000; DeFilippis et al. 2003). pRB and p53 pathways can be reactivated when the expression of E7 and E6 genes is inhibited, leading quickly to senescence or apoptosis (Desaintes et al. 1997; Goodwin and DiMaio 2000; Wells et al. 2000; DeFilippis et al. 2003; Psyrri et al. 2004; Horner et al. 2004; and refs. therein). Organotypic cultures of primary human keratinocytes (PHKs) with constitutive expression of HR HPV E6 and E7 resemble a high-grade CIN. Moreover, constitutive expression of these two viral oncogenes is sufficient to immortalize primary cells, while E6 alone can only immortalize human mammary epithelial cells (Münger et al. 1989; Band et al. 1991; Halbert et al. 1992; Liu et al. 1995b).

PV E6 proteins associate with a conserved α-helical domain in additional target proteins to mediate cellular apoptosis, tumor suppressive gene transcription, epithelial organization and maintenance and cellular proliferation (Chen et al. 1998; Be et al. 2001; Mammas et al. 2008). Among these proteins are E6-BP (a calcium binding protein) (Chen et al. 1995), Bak (a proapoptotic member of the Bcl-2 family) (Thomas and Banks 1998), the multicopy maintenance protein 7 (MCM 7) (a subunit of the replication
licensing factor M) (Kuhne and Banks 1998), paxillin (focal adhesion protein) (Tong and Howley 1997), the interferon regulatory factor-3 (IRF-3) (a virus-activated transcription factor) (Ronco et al. 1998) and E6-targeted protein 1 (E6TP1) (a Rap GTPase activating protein homologue) (Gao et al. 2001). The LR HPV E6 proteins have lower affinity for p53 and do not efficiently degrade p53 nor affect such a myriad of effectors. Thus LR HPVs are unable to achieve the level and spectrum of inactivation analogous to HR HPVs.

HR HPV E6 contains one splice donor and two intragenic splice acceptor sites. A translation termination codon immediately follows the intra- and intergenic splice junctions to generate four truncated E6 proteins (E6*I, II, III, IV). Each E6* protein contains the same N-terminus and is deleted of the C-terminal portion of the protein, including the PDZ binding domain. The E6*I intragenic splice is the predominant E6 transcript in HPV-16 or HPV-18 associated cancers and cancer cell lines and HPV-18 E6*I protein reportedly antagonizes full-length E6, but also shares some of the same properties as the full-length protein (Shally et al. 1996; Pim et al. 1997, 2009; Pim and Banks 1999; Guccione et al. 2004). Thus the viruses appear to limit the activities of the full-length E6 protein, at least under certain conditions, although it is not known whether and where it occurs in a differentiating squamous epithelium.

\(E7\)

Replication of HPV genomes requires the E7 phosphoprotein (98 a.a.) to induce differentiating epithelial cells to reenter the cell cycle and produce all proteins and substrates requisite for viral DNA amplification synthesis (Cheng et al. 1995). Specifically, HPV DNA replication requires the host DNA replication machinery,
including DNA polymerase (pol) α/primase, DNA pol δ, PCNA (the processivity factor or sliding clamp for DNA pol δ), RFC (the PCNA clamp loader), RPA (single-stranded DNA binding protein) and topoisomerases I and II (Yang et al. 1991; Kuo et al. 1994; Müller et al. 1994; Chow and Broker 2006a). However, the productive phase of HPV infections occurs in suprabasal keratinocytes that normally exit the cell cycle. The retinoblastoma susceptibility tumor suppressor protein (pRB) (a.k.a. the G1 restriction point gate keeper) and related pocket proteins control the differentiation and homeostasis of the squamous epithelia. In this state, hypophosphorylated pRB remains bound to E2F transcription factors (acting as repressors) on the cell cycle genes (Frolov and Dyson 2004; Cobrinik 2005; Sun et al. 2007). In the context of the viral productive program in the differentiating squamous epithelium, p130, a pRB-related pocket protein, is likely to be the primary target of E7.

Under optimal growth conditions, mitogens induce expression of cyclin D. Cyclin D forms a complex with cyclin-dependent kinase 4 or 6 (cdk4 or cdk6) which phosphorylates pRb. This releases the E2F/DP heterodimeric transcription factor, which induces early responsive promoters, in particular cyclin E (CCNE1). Cyclin E forms an active complex with cdk2 that irreversibly hyperphosphorylates pRB to induce the genes preparatory for S phase. At this point, the cell is resistant to mitogenic depletion and commits to transition into and propagation through DNA synthesis (S phase) (Herwig and Strauss 1997; Sherr and Roberts 2004).

E7 proteins associate with pRB through a highly conserved LxCxE motif (Dyson et al. 1989; Münsterg et al. 1989; Scheffner et al. 1992). HR HPV E7 proteins bind pRb and related pocket proteins (p107 and p130) and accelerate their degradation, whereas LR
E7 proteins bind pRb with reduced affinity and destabilize it inefficiently (Cheng et al. 1995; Chien et al. 2000; Banerjee et al. 2006). In spite of this, PHKs raft cultures expressing HR or LR HPV E7 alone induces differentiated nuclei to enter S phase stochastically, albeit at different efficiencies (Banerjee et al. 2006; Cheng et al. 1995). This establishes the cellular milieu necessary to support viral DNA amplification (Flores et al. 2000; McLaughlin-Drubin et al. 2005). Additionally, deletion or mutation of E7 fails to maintain HPV genomes, suggesting an E7 induced cell cycle allows proper viral DNA maintenance (Heck et al. 1992; Sang and Barbosa 1992; Scheffner et al. 1992; Thomas et al. 1999; Flores et al. 2000; Oh et al. 2004; McLaughlin-Drubin et al. 2005; Zhang et al. 2006).

As with E6/p53 interactions, LR HPV E7/pRb or p130 affinity is much lower than with HR types. In addition, it promotes significantly less destabilization (Woodworth et al. 1992; Lochmuller et al. 1999; Collins et al. 2005; Zhang et al. 2006; Genovese et al. 2008). The differences in both E6 and E7 affinity/destabilization activity further demonstrate why LR HPV infections do not undergo neoplastic progression. E7 exerts additional control over the cell cycle beyond interrupting pRb, p107 and p130 associations by interacting with cyclin A, cyclin E, p21 and p27. E7 further binds TAF110, a TATA-binding protein, hTid-1, a Dna J protein that affects protein folding and assembly of large protein complexes and TAP-1, which affects antigen presentation. HR E7 expression has been reported to delay squamous epithelial differentiation.

\textit{E1}

E1 is a multi-functional phosphoprotein (68 kDa) containing both DNA-dependent ATPase and ATP-dependent DNA helicase activity; the only enzyme encoded
by HPV (Hughes and Romanos 1993; Seo et al. 1993; Yang et al. 1993; Sedman and Stenlund 1998; Lin 2002). Because E1 is virally derived, it is not bound by the same restrictions as the host replication origin licensing factors (Chow and Broker 2006a). E1 is required for both the initiation and elongation phases of viral DNA replication, but only binds the viral origin weakly (Kuo et al. 1994). Ori-bound E2 recruits and stabilizes E1 association at the E1 binding site (E1BS) in the URR but is not present during elongation (Kuo et al. 1994; Titolo et al. 2000; Stenlund 2003). Additional E1 recruitment produces a hexameric or dihexameric ring complexes and this is facilitated by the presence of heat shock/chaperone proteins Hsp70 and Hsp40 (Sedman and Stenlund 1995; Liu et al. 1998; Fouts et al. 1999; Liu et al. 1999). To initiate replication, E1 unwinds the duplex DNA spanning the ori sequences, recruits RPA and interacts directly with the p180 and p70 subunits of DNA polymerase α/primase to initiate viral DNA replication (Park et al. 1994; Bonne-Andrea et al. 1995; Masterson et al. 1998; Conger et al. 1999; Han et al. 1999; Amin et al. 2000; Lin et al. 2002; Loo and Melendy 2004). Moreover, the cyclin E- cdk2 complex is also necessary for efficient HPV ori replication (Lin et al. 2000).

**E2 and E8**

Full-length E2 is a multifunctional protein (43 kDa) that regulates viral replication and early gene transcription when bound to consensus palindromic E2BS within the URR (ACCN₆GGT) (Hirochika et al. 1988; Chiang et al. 1992b; Dong et al. 1994; Liu et al. 1995a). Three distinct functional domains comprise the E2 protein: the amino-terminus (E2N) harbors the transacting (TA) domain; the hinge (H) localizes E2 to the nucleus (as shown for HPV-11) and the nuclear matrix; and the carboxyl-terminus (C) contains the
DNA binding domain (DBD) and is responsible for dimerization (Giri and Yaniv 1988; McBride et al. 1988; Zou et al. 1998; Zou et al. 2000; Bellanger et al. 2001). Both termini are required for HPV DNA replication in that only E2C homodimers recognize E2BS and E2N recruits the E1 helicase domain (Lusky and Fontane 1991; Chiang et al. 1992b; Liu et al. 1995a; Amin et al. 2000; Abbate et al. 2004). Indeed, nuclear foci of replicating HPV ori-containing plasmids colocalize with and require both E1 and E2 proteins (Swindle et al. 1999).

While full-length E2 is vital to initiate Ori-dependent HPV DNA replication, the E2BS are also utilized to regulate viral transcription. Within the ori, there are three E2BS that overlap P1 and its regulatory elements. In transient assays, low amounts of E2 protein slightly stimulate P1, but overexpressed E2 has significant repressive effects (Chin et al. 1988, 1989; Bernard et al. 1989; Romanczuk et al. 1990; Dostatni et al. 1991; Thierry and Howley 1991; Dong et al. 1994; Tan et al. 1994; Strappe et al. 1997; Alloul and Sherman 1999b; Hou et al. 2000, 2002; Stubenrauch et al. 2000). Bovine PV type-1 (BPV-1) E2 activates the transcription of the BPV-1 E region transcription (apparently because its binding sites are not in close proximity to the TATA motif), yet functions as a potent repressor of HPV P1 promoter (Spalholz et al. 1985; Thierry and Yaniv 1987; Chin et al. 1989; Dostatni et al. 1991; Thierry and Howley 1991; Hwang et al. 1993; Francis et al. 2000; Goodwin and DiMaio 2000; Wells et al. 2000; Lee et al. 2002; DeFilippis et al. 2003; Horner et al. 2004). Interestingly, ectopic HPV E2 expression in cervical cancer derived cell lines that harbor integrated HR HPV induces senescence or apoptosis (Bernard et al. 1989; Dowhanick et al. 1995; Demeret et al. 1997; Nishimura et al. 2000; DeFilippis et al. 2003). This has been similarly attributed to E2 protein
dependent $E_{6-7}$ repression. Bound E2 proteins at the tandem E2BS occludes Sp1 and TATA cis promoter elements and further forms an E2/Brd4 transcriptional silencing complex (Romanczuk and Howley 1992a; Dong et al. 1994; Wu and Chiang 2007).

Truncated E2 proteins result from alternative mRNA splicing and alternate promoter usage: E2C and E1M^E2C (Spalholz et al. 1985; Chow et al. 1987a; Phelps and Howley 1987; McBride et al. 1988, 1991; Rotenberg et al. 1989a; Chiang et al. 1991; Stubenrauch et al. 2000). The 5′-end of E2C is transcribed from within $E1$ at a P4 promoter, which is located upstream of $E8$. HPV-18 $E8$ is only 39 nts and splices from SD3 to SA4, to generate the E8^E2C transcript (Fig. 3). HPV-18 $E8$ is highly homologous in length and sequence to HPV-31, from which E8^E2C was found requisite to maintain HPV-31 genomes in human keratinocytes (Stubenrauch et al. 2000). Only full-length E2 recruits E1 helicase to viral replication Oris, whereas truncated E2s lack the TA domain and modulate full-length E2 activity by competing for E2BS. Interestingly, the TA domain E2 is also required to effect HPV transcriptional repression (Thierry and Yaniv 1987; Goodwin et al. 1998; Nishimura et al. 2000).

Elevated ectopic HPV E2 protein causes chromosome polyploidy or cell death in cells independent of HPV oncogenes (Frattini et al. 1997a; Webster et al. 2000; Demeret et al. 2003). It should be noted that endogenous E2 levels are low in productive infections and unlikely ever to achieve ectopically expressed levels. The abundance of full-length HPV E2 protein translated from its alternatively spliced mRNA pool is regulated by the differentiation state of the epithelial cell (Alloul and Sherman 1999a, 1999b; Deng et al. 2003).

There is also evidence that E2 does not function as a transcription factor during
the productive program (Stubenrauch et al. 1998; Bechtold et al. 2003). Yet an enticing proposal upon viral DNA amplification is for Ori bound E2 to repress the E6-E7 expression to facilitate a transition to late region expression of the capsid proteins requisite for subsequent infectious HPV particle formation. Thus, the primary function of E2 during productive infections may still be the initiation of viral DNA replication (Stenlund 2003; Chow and Broker 2006b). E2 may play an additional role in dividing basal cells by conferring viral plasmid persistence. Ectopic E2 (HPV-11, -16, -18) associates with mitotic spindles whereas BPV-1 E2 associates with mitotic chromosomes. These mechanisms would tether viral plasmids to ensure proper segregation following successful mitotic events (Lehman and Botchan 1998; Skiadopoulos and McBride 1998; Ilves et al. 1999; Van Tine et al. 2004a; You et al. 2004; Baxter et al. 2005; Dao et al. 2006).

**E4**

E4 protein is the most divergent protein in sequence and length among HPVs. The entire E4 ORF is contained within E2H and typically lacks its own translation initiation codon. Thus E4 protein expression requires the splicing from a donor site immediately downstream of the E1 ATG to an acceptor site within the 5′ E4 ORF (Chow et al. 1987a, 1987b; Nasseri et al. 1987). Translation of E1^E4 mRNA yields a fusion protein wherein the first five amino acids of the E1 N-terminus continues in-frame to produce E1^E4 protein. As discussed earlier, the E1^E4 transcript utilizes either pAE or pAL. The pAE terminated E1^E4 messages are among the earliest and most abundant RNAs detected immediately following infection, despite the comparatively low protein levels in lower epithelia (Stoler et al. 1990; Egawa et al. 2000; Nicholls et al. 2001; Nakahara et al.)
Significant upregulation of E1^E4 protein in the upper spinous cells can be visualized by in situ hybridization of the mRNA and immunostaining of the protein in association with cytokeratin bundles in the cytoplasm (Stoler et al. 1989, 1990). In these cells, transcription from the P3 promoter is elevated to encode E1^E4 from both pAE and pAL terminated messages (Chow et al. 1987a, 1987b). This shift from E to L gene expression correlates with colocalization of E1^E4 proteins with replicated viral DNA and L1 capsid in superficial epithelial cells (Brown et al. 1988, 1994, 1995a; Crum et al. 1990; Sterling et al. 1993).

E1^E4 associates with cytoplasmic keratin (CK) bundles in cell lines and natural lesions through a highly conserved amino-terminal leucine rich motif, LLXLL (Brown et al. 1988; Peh et al. 2002). Cell lines ectopically expressing HPV-16 E1^E4 collapsed or rearranged CK networks due to carboxyl-terminal residues (NSVVVTL) (Doorbar et al. 1991; Roberts et al. 1994, 1997; Wang et al. 2004). In vivo studies have been unable to substantiate this conclusion since CK networks remain intact in various differentiated epithelial cultures, suggesting mucosotropic HPVs do not naturally express comparable E1^E4 levels and possibly because different keratins are expressed in these cells (Roberts et al. 1993; Pray and Laimins 1995). Interestingly, ectopic expression of E1^E4 in a non-CK expressing cell line induced apoptosis by an LLXLL motif-dependent localization to mitochondria (Raj et al. 2004). No such apoptosis is exhibited in papillomas from benign patient lesions, in nude mouse foreskin xenografts (Stoler and Broker 1986; Stoler et al. 1989, 1990), or in productive organotypic cultures (Wang et al. 2009). Alternatively, E1^E4 accumulation may facilitate virion release by increasing cornified envelope fragility, but again it occurs independent of CK collapse (Doorbar et al. 1991; Roberts et
Recent studies have found that over-expression of E1^E4 interfered with cellular proliferation at the G2/M transition. Expression of cyclin B1 (CCNB1) at late S phase forms a complex with cytoplasmic cdc2 (cdk1) to form the mitosis, or maturation, promoting factor (MPF). To ensure daughter cells do not perpetuate deleterious genotypes, MPF sits at the crux of a highly regulated checkpoint. Multiple posttranslational modifications must occur to overcome potent nuclear export signals that maintain MPF’s continual nucleo-cytoplasmic shuttling with a default position predominantly in the cytoplasm. MPF activation requires full cdk1 activity and localization to the nucleus to promote the cell out of G2 and into mitosis (Smits and Medema 2001; Porter and Donoghue 2003).

Ectopic E1^E4 expressed in monolayer cell cultures establishes a pronounced G2 arrest by binding to and preventing cyclin B/cdk1 nuclear localization. This property is conserved between LR and HR HPV E1^E4 proteins (Davy et al. 2002, 2005, 2006; Nakahara et al. 2002; Knight et al. 2004, 2006). The G2-arrest domain of HPV-16 E1^E4 (TPPRP) is a consensus cdc2 binding and phosphorylation site (S/TPXP+) (Davy et al. 2002). Prolonged G2/M arrest increases >4N DNA replication; thus E1^E4 may contribute to a cellular environment conducive to viral genome replication in suprabasal epithelia (Nakahara et al. 2002; Knight et al. 2004). Unfortunately, genetic analyses of E4 among HPV-11, -16, -18, -31 and cottontail rabbit PV (CRPV) genomes either contradict or fail to definitively demonstrate a consistent role of the E1^E4 in the productive program (Peh et al. 2004; Nakahara et al. 2005; Wilson et al. 2005, 2007; Fang et al. 2006a). Why are there such different phenotypes among very similar E4 mutations? As
HPV is highly dependent on splicing, most early and late transcripts encode \textit{E4}. The introduced premature termination codon may affect polycistronic mRNA stability, translatability of adjacent 5′- or 3′-cistrons or interrupt the transition from early to late gene expression.

\textit{E5}

\textit{E5} proteins (~82 a.a., but like E4 variable in length and sequence) are small transmembrane proteins which associate with the Golgi, ER, nuclear and cell membranes. E5 can interfere with endocytic trafficking of growth factor receptors (GFRs) (i.e. Epidermal GFR (EFGR)) to enhance signal transduction (Crusius et al. 1997, 1998, 2000; Thomsen et al. 1999; Tomakidi et al. 2000; Venuti et al. 2002; Cartin and Alonso 2003; Disbrow et al. 2005; Zhang et al. 2005; Kim and Yang 2006; Chen et al. 2007). E5 also down-regulates class I and II HLA molecules, while HPV-16 E5 can overcome TRAIL and FasL mediated apoptosis (Zhang et al. 2003; Kabsch et al. 2004; Ashrafi et al. 2006a, 2006b). In raft cultures, E5 mutations reduced HPV-31 DNA amplification and late gene expression, whereas neither was greatly changed with HPV-16 E5 mutants, though the number of S phase cells diminished (Fehrmann et al. 2003; Genther et al. 2003). Additionally, growth-arrested mouse cells containing BPV-1 \textit{E5} distinguished its functions to induce BPV-1 DNA amplification (Alderborn and Burnett 1994). In addition, E5 is the primary BPV-1 transforming protein in mouse cell lines (Schiller et al. 1986). The differences between these PV types are not understood and the mechanisms by which E5 modulates viral DNA amplification in differentiated cells remain to be elucidated.

\textit{E5} is present at the 3′-end of all the early mRNAs as it is immediately upstream of
the pAE site. Thus one unaccounted consequence of the aforementioned premature E1^E4 termination experiments could have been the loss of E5 protein expression. While E5 translation for HPV-11 can occur from E1^E4 mRNA and BPV-1 can utilize E2 mRNA, it is not known whether HPVs translate E5 from E1^E4 or E2 mRNA (Yang et al. 1985; Brown et al. 1998). If E2 mRNA is the source of E5 translation, E5 protein may be low, since the E2 message is of low abundance relative to E1^E4-E5 mRNA. Thus, E5 protein expression could be deleteriously affected in some of the early E4 termination mutants. This possibility may offer a possible explanation of these conflicting phenotypes of E4 mutants.

Late Region

The L region expresses L2 and L1, the minor and major capsid proteins respectively, which encapsidate newly amplified viral DNA to form icosahedral, nonenveloped progeny virions (~55 nm by negative staining and about 42 nm by positive staining for transmission electron microscopy) in a small number of terminally differentiated, superficial epithelial cells (Stoler and Broker 1986; Crum 1988a; Stoler 1989, 1992; Higgins 1992; Chow and Broker 2006). Multiple mechanisms have been identified in vitro to modulate HPV L region transcripts including transcription, nuclear retention, splicing, polyadenylation and RNA stability as mediated by cellular proteins bound to UTRs or coding sequences (Dietrich-Goetz et al. 1997; Terhune et al. 1999, 2001; Koffa et al. 2000; Collier et al. 2002; Cumming et al. 2002; Wiklund et al. 2002; Zhao et al. 2003, 2005; Zhao and Schwartz 2008). Importantly, differentiation-dependent promoter upregulation drives pAE read-through to encode either L2 or L1 translatable mRNAs that conclude at pAL.
**L2**

*L2* precedes *L1* as the first ORF in the L region and encodes the minor capsid protein (49-60 kDa). The ability of L2 to bind DNA makes it requisite to generate infectious progeny. Further, L2 can be phosphorylated and can neither self-assemble into a virion nor be linked to itself (Xi and Banks 1991, Day et al. 1998). Recently, L2 monomers were shown to localize and associate with the interior surface of the L1 capsid (Buck et al. 2008). With no detectable L2 localizing to the exterior of the capsid, three-dimensional reconstructions suggest L2 juts out at the axis of each L2 pentavalent L1 vertex (see below) (Trus et al. 1997). Despite uncertainty as to L2’s CMI exposure, vaccination against L2 may diminish HPV transmission (Stauffer et al. 1998).

**L1**

*L1* encodes the major capsid protein (56-60 kDa) and is relatively well conserved among all PVs. 72 pentamers of L1 can self-assemble to form viral-like particles (VLPs), though they are empty due to inability of L1 to specifically bind HPV DNA (Baker et al. 1991; Hagensee et al. 1994; Volpers et al. 1994; Belnap et al. 1996; Roden et al. 1996). Further, L1 can be glycosylated, weakly phosphorylated and undergoes disulfide cross-linking (Xi and Banks 1991). The implications for these modifications suggest that the viral capsid may require specific maturation events to realize its full infectious potential (Kirnbauer et al. 1992, 1993).

Recombinant expressed L1 alone or L1 and L2 expressed together from baculovirus (SF9 insect cells), vaccinia virus (human cell lines), or plasmids (in yeast) can self-assemble into VLPs (Zhou et al. 1991; Kirnbauer et al. 1992; Rose et al. 1993; Carter et al. 1994; Volpers et al. 1994; Sasagawa et al. 1995). VLPs that contain HPV or
a reporter plasmid DNA can bind and be taken up by epithelial cells, though infectivity is appreciably less than that of natural HPV virions (Kirnbauer et al. 1993; Hagensee et al. 1994; Roden et al. 1994; Müller et al. 1995). Importantly, L1 is the predominant PV protein exposed to the environment and is the most antigenic (Christensen et al. 1994; Le Cann et al. 1994). While HPVs are not classified according to serology, in fact the different genotypes induce type specific immune responses.

HPV Vaccination

Prophylactic HPV vaccines have been approved and are widely available in developed countries. The vaccines consist of VLPs assembled from recombinant L1 protein and elicit a neutralizing antiviral antibody response. Therapeutic vaccines based on early viral antigen-induced CMI are still in early stages of clinical trials.

Prophylactic Vaccine

Development of vaccine strategies against HPV have been hindered due to HPV’s dependence upon epithelial differentiation for virion morphogenesis. The advent of VLPs have allowed the development of highly immunogenic prophylactic HPV vaccines (Kirnbauer et al. 1992, 1992; Breitburd et al. 1995). As mentioned earlier, VLPs are empty capsids and contain no viral or cellular DNA (Moscarini et al. 2004). The recognition of VLPs by human anti-sera from HPV infected individuals suggested their antigenicity is similar to authentic virions (Rose et al. 1993). Animal model systems validated that VLP antigenic presentation can generate a sufficient humoral immune response to prevent PV infections (Lin et al. 1992; Jansen et al. 1995; Suzich et al. 1995; Kirnbauer et al. 1996; Stanley 2003). VLP administration elicits high levels of PV-neutralizing HPV type-specific IgG antibodies (Moscarini et al. 2004).
There are currently two prophylactic vaccines available in the U.S.:

GARDASIL® (Merck and Co., Inc., Whitehouse Station, NJ), a tetravalent VLP vaccine (HPV-6, -11, -16 and -18) synthesized by using *Saccharomyces cerevisiae*-recombinant technology, whereas Cervarix® (GlaxoSmithKline (GSK) Biologicals, Rixensart, Belgium) is a bivalent HPV-16 and -18 VLP vaccine based on baculovirus technology. Phase III clinical trials for the currently available vaccines yielded 95-99% protection against the targeted HPV types (Harper et al. 2004; Villa et al. 2006; Quadrivalent Vaccine…2007; Ault 2007; Paavonen et al. 2007).

The high immunogenicity of L1 VLPs induces a potent antibody response even in the absence of adjuvant by activating innate and adaptive immune responses (Harro et al. 2001). Myeloid dendritic cells and B lymphocytes bind VLPs and activate B cells to commence generating antibodies (Yang et al. 2004; Yan et al. 2005). Multiple VLP epitopes will generate a polyclonal response representative of all stimulated B cells, but the most antigenic will generate a more acute immune response than the rest. While these antibodies are largely HPV type specific, subpopulations cross react with certain highly related HPV types with lower avidity (i.e. the antibodies induced by HPV-18 VLPs cross-react with HPV-45 and antibodies to HPV-16 cross-react with HPV-33) (Heino et al. 1995; Hong et al. 1997; Smith et al. 2007; Stanley 2009).

Critically, VLP based vaccines are only efficacious until free viral particles penetrate epithelial basal cells (Stanley 2003; Schiller and Davies 2004). VLP CMI is irrelevant due to minimal or lack of L1 epitopes as seen in infected basal epithelial cells of benign lesions or abnormal proliferative cells of premalignant or malignant lesions (Galloway 2003; Roden and Wu 2003; Jansen and Shaw 2004; Schiller and Davies...
2004). Though CMI could have detected an early post-infection epitope, the humoral response is the more relevant mechanism for the prophylactic HPV vaccines (Roden and Wu 2003; Jansen and Shaw 2004).

**Therapeutic Vaccine**

Prophylactic vaccinations do not offer any relief to current infections and will require significant time to reduce cervical cancer incidence. Therapeutic vaccines attempt to induce HPV-specific antitumor immune responses against dysregulated $E6$ and $E7$ expression in pre-existing lesions. Strategies typically involve the introduction of modified $E6$-$E7$ proteins fused to an immunogenic protein that will generate a response specific for tumor cells expressing these HPV oncoproteins. While vaccine development has been a challenge, some have induced HPV-specific antitumor immune responses in preclinical animal models and even been applied in clinical trials (Hung et al. 2008; Kanodia et al. 2008). It will be interesting to follow the efficacy of these strategies against lesions of transformed cells overexpressing $E6$-$E7$.

**The Integument**

All HPVs exhibit explicit tropism for specific regions of the integumentary system. As the largest human organ (~15,000-20,000 cm$^2$ and ~5-10 lbs), skin functions as both a protective barrier for its internals as well as a direct interface to the surrounding environment. Maintaining such a balanced homeostasis against diverse insults (i.e. infection, temperature changes, dehydration, etc.) are three distinct layers: hypodermis (adipose), dermis (connective tissue) and epidermis (stratified squamous epithelium). The subdermal layer is primarily adipose cells and connective tissue that insulate, store energy, cushion muscles and internal organs from trauma, as well as provide anchorage
for the outer layers. The middle of the integument is extensively vascularized so as to
diffuse nutrients, immune responses and repairs to the epidermal layer. The flexible
dermis also contains loosely woven bundles of elastic connective tissue (collagen, elastin,
fibronectin, laminin and vitronectin) that endure and restore stretching, distortions and
wrinkling. Despite its varying roles, the dermal layer maintains a hydrous environment
with only a sparse population of cells: fibroblasts that produce connective tissue are the
most represented, whereas mast cells generate immune and inflammatory responses.
Additionally, hair follicles and sweat ducts originate deep within the dermis and traverse
the epidermis to terminate outside of the skin.

The outermost integumentary layer is ectodermally derived to overlay the dermis.
The epidermis is the major target of all infectious HPVs. The epithelia, thinnest on
eyelids (~0.05 mm) and thickest on palms and soles (~1.5 mm), is primarily a matrix of
keratinocytes interspersed with three additional cell types (<5%): melanocytes,
Langerhans cells and the Merkel cells. Adjacent to the dermis, proliferating keratinocytes
self-renew and/or differentiate to ascending strata: the stratum basale, the stratum
spinosum, the stratum granulosum, the stratum lucidum and the stratum corneum. Except
for the monolayer stratum basal and the layer of cycling transit amplifying parabasal
keratinocytes immediately above it, the other epithelial zones can be one to several cells
thick, depending upon body site. A host of foreign substances can divert stratifying
keratinocytes off course either spontaneously or by design (Green 1980; Eckert et al.
1997). Fortunately, such adverse events are often bypassed and cleared amidst the
continually shed superficial keratinocytes. Some pathogens persist simply because their
growth rate either exceeds that of keratinocyte shedding or they directly retard terminal
differentiation. Further, the epidermis is largely avascular and therefore inaccessible to
humoral immune surveillance, underscored by a diverse range of annoying, debilitating
and terminal diseases that perturb this immense bodily surface, often with subtle nuances.

Keratinocytes

The productive phase for both LR- and HR-HPV types exploits differentiating
keratinocytes. HPV infects the proliferative layer but amplifies its genome in
differentiated strata upon inducing host proteins necessary to support viral DNA
replication. With such specific requirements, HPV is intimately dependent on its
successful interpolation between keratinocyte proliferation and differentiation.

Epidermis

Squamous differentiation is a multi-step process that forms and maintains a self-
perpetuating barrier to protect the dermis by keeping harmful microbes out and essential
body fluids in (Rice and Green 1979; Steinert and Marekov 1995). Terminal
differentiation involves a complex and highly coordinated program wherein keratinocytes
undergo two major transitions that alter their transcription, morphology and function. The
first occurs when daughter basal cells lose contact with the basal lamina and cease
division and DNA synthesis as they differentiate into the spinous strata, though some
parabasal cells can continue to proliferate as the epidermis is being established (Fuchs
and Raghavan 2002). The second essentially terminates granular cells by packing these
enucleated cellular skeletons with keratin filament bundles prior to being encased by a
cross-linked protein-lipid envelope to establish the stratum corneum (Dai and Segre
2004).
Cell Cycle

The duplication and distribution of genetic information is required to perpetuate most life and the primary determinants for the two major cell cycle phases: 1.) Interphase includes all events prior to, during and following cellular DNA synthesis, whereas 2.) Mitosis (M) partitions synthesized genomes between daughter cells until their reentry into interphase.

Interphase

Interphase generally lasts at least 12 to 24 hours in proliferating cells, during which they generate copious amounts of RNA and proteins to either accommodate a tissue dependent function or prepare for cellular division. These molecular events further characterize four sequential, yet distinct phases: Gap 0 (G0), Gap 1 (G1), S (DNA synthesis) and Gap 2 (G2). Most gene expression occurs from extended, loosely-coiled chromosomes in G1 ("gap in DNA synthesis"). Robust mitogenic signaling is required to promote S phase entry, though continual discrete signals may ensure its completion. At G2 ("second gap in DNA synthesis"), the cell verifies DNA synthesis is successfully completed and finalizes any repairs to the genome. G2 also serves as preparatory phase wherein decondensed chromatin, requisite for gene expression, begins tightly coiling and the nucleus reorganizes as the cell prepares for transition into M. As cell division concludes, the chromatin decondenses and disperses throughout the nucleus conducive to inducing transcription and reentry into G1.

Mitosis

At M phase, the ordered partition of equal sets of chromosomes between daughter
nuclei requires coordinate and distinct phases: prophase, metaphase, anaphase, telophase and cytokinesis. The compacting of chromatin continues until dense DNA rods mark entry into prophase. The nuclear envelope disassembles and spindle fibers radiate from polar centrosomes and attach to chromosomal centromeres. At metaphase, the attached centromeric fibers pull in a kind of 'tug of war' to position the condensed chromosomes equidistant between the opposite centrosomal poles. During anaphase, the daughter chromosomes are separated and pulled along spindle fibers towards each centrosome. In telophase and cytokinesis, nuclear envelopes reassemble around each set of chromosomes and the plasma membrane pinches off to generate two independently functioning daughter cells. The reorganization of each nucleus allows normal cellular functions to resume as the cell assesses its environment and reenters the cell cycle, becomes quiescent, or commits to differentiate.

Cyclin Dependent Kinase

The cell cycle functions as a clock wherein the timing of each event (G0, G1, S, G2 and M) is interdependent upon defined periods of Cdk activity. This oscillation of Cdk serine/threonine protein kinase activity and specificity requires heterodimerization with cyclically expressed regulatory subunits, the cyclins (Rao and Johnson 1970; Krek and Nigg 1991b; Hoffmann et al. 1993; Dynlacht et al. 1994; Hayles et al. 1994; Cobrinik 2005; Malumbres and Barbacid 2009). Since free Cdk is prohibited from substrate binding due to a portion of its C-terminal lobe (T loop) occupying the active site, all Cdks are broadly regulated through the synthesis and destruction of their cyclin partner(s) (De Bondt et al. 1993). The first of two 5-helix bundles (cyclin box) within the cyclin central domain binds Cdk and provides a rigid framework against which conformational changes
mold the pliable kinase to expose the active site (Nugent et al. 1991; Kobayashi et al. 1992; Brown et al. 1995b). Activation of cyclin-cdk complexes (the D type cyclins-Cdk4/Cdk6, cyclin E/Cdk2 and cyclin A/Cdk2) controls the rate limiting step in mid- to late-G1, at G1-S and in S phase progression respectively. The cyclin A/Cdk1 and cyclin B/Cdk1 activities function at S-G2 and G2-M, respectively (Olashaw and Pledger 2002). Thus, cyclin-Cdk complexes do not simply trigger, but interconnect cell cycle events by ensuring their own inactivation and initiating the activation of the ensuing cyclin-Cdk complex.

All cyclin/cdk complexes phosphorylate and inactivate pRb, though S phase genes are activated at G1 due to the inactivation of pRB by kinase activities of Cdk4 or Cdk6 (DeGregori et al. 1995; Weinberg 1995; Lundberg and Weinberg 1998). Cyclin E/Cdk2 irreversibly promotes S phase entry while cyclin A/Cdk2 activity plays some poorly defined role in S phase progression. Cyclin A/Cdk1 complexes begin to appear in early to mid S, concurrent with cyclin B/Cdk1. The cyclin B component of MPF (cyclin B-Cdk1) is synthesized throughout S phase, but is constantly turned-over. Post-translational modifications beginning late in S begin to stabilize cyclin B and allows MPF to accumulate in the cytoplasm throughout G2. Full activation and nuclear translocation of cyclin B/Cdk1 transitions cells through G2/M and stimulates downstream proteins involved in chromosome condensation and mitotic spindle assembly. As mitosis progresses into anaphase, MPF ensures the irreversibility of telophase and cytokinesis by activating a ubiquitin ligase, the anaphase-promoting complex (APC), which promotes the degradation of structural kinetochore proteins as well as cyclin B (Nigg 1995; Edgar and Lehner 1996).
The cyclin B/Cdk1, cyclin A/Cdk2 and cyclin E/Cdk2 complexes readily form without other components or modifications, whereas cyclin A/Cdk1 and cyclin H-Cdk7 both require phosphorylation of an activating threonine residue (Ducommun et al. 1991; Desai et al. 1995; Fisher et al. 1995). Even the spontaneously formed cyclin/Cdk complexes are not fully active until this conserved threonine (Thr160) on their T loop is phosphorylated by the Cdk-activating kinase (CAK); composed of Cdk7, cyclin H and a RING-finger protein MAT1 (Adamczewski et al. 1996). Cyclin binding initially displaces the T loop to expose Thr160 for CAK phosphorylation in vitro and tends to parallel cyclin levels in vivo (Krek and Nigg 1991a; Gu et al. 1992). Thr160 phosphorylation flattens and further displaces the T loop through increased interactions with the bound cyclin. This yields subtle, yet significant changes that enhance protein-substrate binding independent of the active site (Goda et al. 2001). Specifically, CAK dependent flattening of the C-terminus allows substrates containing SPXK consensus sequences to be coordinated within the catalytic kinase residues such that the serine hydroxyl oxygen more efficiently interacts with the ATP γ-phosphate (Krek and Nigg 1991a; Gu et al. 1992; Adamczewski et al. 1996; Drapkin et al. 1996; Nigg 1996; Reardon et al. 1996; Svejstrup et al. 1996).

Multiple cellular pathways converge to ensure Cdk activity only occurs within an allotted cell cycle window. For example, the availability of critical substrates mediating Cdk-substrate phosphorylation is tightly regulated (Leopold and O'Farrell 1991; Lew et al. 1991; Amon et al. 1994; Hayles et al. 1994; Correa-Bordes and Nurse 1995; Dahmann et al. 1995; Stern and Nurse 1996). Following CAK activation, removal of Wee1 (Tyr15) and/or Myt1 (Thr14 and Tyr15) inhibitory phosphorylations from the Cdk active site
requires upregulation of cdc25 phosphatase(s). Other regulatory kinases can further phosphorylate the Cdk subunit at inhibitory sites near the N-terminus. Detection of cellular irregularities such as growth inhibition, DNA damage, or poor spindle body assembly can divert cell cycle phases by altering or eliminating Cdk activity (Elledge 1996). For example, inappropriate growth factor concentrations induce the INK4 family of Cdk inhibitors (p16INK4a, p15INK4b, p18INK4c and p19INK4d) which inhibit only the assembly of D type cyclin and cdk4, or Cdk6. G1 progression arrests by reducing cyclin D transcription (Sherr and Roberts 1999). DNA damage induces p53-dependent transcription of p21^{CIP1} and leads to G1 or G2 arrest (Sherr and Roberts 1995; Bagui 2000). DNA damage at G2 delays mitotic entry by enhancing MPF inhibitory phosphorylations. Defects in spindle assembly can delay the progression beyond metaphase by blocking APC. Therefore, the unique sequence of cell cycle events is not simply a programmed cascade of specific cyclin-Cdk activities, but the culmination of positive and negative stimuli directing cellular activities to the most appropriate outcome. Inherently, this simultaneous priming by competing stimuli ensures that Cdks are regulated rapidly.
CHAPTER 2

IN VITRO MODEL SYSTEMS TO STUDY HPV

HPV Cell Lines and Exogenous Gene Expression

A recurring theme across biomedical research has been the failure to successfully extrapolate findings from model systems into clinically relevant treatments. For HPV, research has been hampered by the lack of a conventional culturing system to propagate the virus. Partly because PHKs rapidly senescence in culture (5-6 passages), by the time the recombinant viral genomes are introduced by transfection and transfected cells are selected, PHKs cease to divide. Thus, only the wild type HR HPVs that can extend PHKs proliferation capacity or immortalize them can be analyzed in differentiated epithelium in vitro. However, their capacity to recapitulate the productive program is limited.

Cell lines can be clonally derived from cancer tissues or primary cells transformed or immortalized by dysregulated or exogenously overexpressed viral oncogenes (i.e. HR HPV E6-E7). Most cancer cell lines have been expanded from a single aneuploid cell that survived genomic catastrophe and adapted to long-term cell culture. Consequently, different cell lines originating from the same tissue could be distinct in terms of genomic structure, gene regulation, signaling and biological behavior. Similarly, the process of immortalizing primary cells for extensive culturing often alters or limits the extent of differentiation requisite to reconstitute the architecture of the original three-dimensional tissue in vitro. Murine studies utilizing transgenic and knockout mice, while extremely powerful to study HPV oncogenes and the oncogenic mechanisms, cannot recapitulate
viral productive program. Adaptation of organotypic epithelial cultures has provided a means whereby consequences of HPV activities can be modeled and manipulated.

Organotypic (Raft) Cultures of Primary Human Keratinocytes

PHKs or cervical cancer cell lines generate organotypic tissue cultures that regulate gene expression and signaling pathways veridical to human skin or cancerous lesions, respectively (Lambert et al. 2005; Fang et al. 2006b). Essentially, cell isolates, in this case PHKs, are seeded onto a dermal equivalent consisting of mouse fibroblasts embedded within a rat tail type I collagen matrix. The overlying PHK culture is raised to the medium:air interface where they stratify and differentiate into a squamous epithelium within two weeks (see Chapter 3, Fig. 1) (Prunieras et al. 1983; Wilson et al. 1992). This system has enabled studies on epithelial stem cell biology, cancers and therapeutics (Khavari 2006). This culture system quickly became a crucial technique for HPV research since the entire viral productive infection may finally be achievable in culture (Asselineau and Prunieras 1984; Broker and Botchan 1986). Indeed, two seminal studies reported completion of the viral productive phase by growing explants of HPV infected tissue or cell lines in raft cultures (Dollard et al. 1992; Meyers et al. 1992).

Introduction of HPV DNA into PHKs

Despite these advances, genetic dissection of the HPV life cycle has been difficult and slow. PHK cultures senesce after a few passages and are less susceptible to transfection agents than cell lines, making HPV genomes difficult to introduce and maintain. Alternatively, PHKs can be readily transduced with retroviruses that deliver an HPV gene or genes and developed into raft cultures. LR or HR HPV E7 proteins promote S-phase reentry in post-mitotic, differentiated PHKs whereas E6 is neither necessary nor
sufficient to promote S-phase (Halbert et al. 1992; Cheng et al. 1995; Chien et al. 2000; Genovese et al. 2008). Consequently, E7 activity is required for the amplification of viral DNA in differentiated keratinocytes (Flores et al. 2000; McLaughlin-Drubin et al. 2005). The E6 protein is also essential for efficient viral DNA amplification, but the mechanism is not understood (Wang et al. 2009).

The susceptibility of epithelia to HR HPV-induced neoplasm has been exploited to increase PHKs capacity for passage in culture in that HR HPV E7 and E6 can immortalize PHKs. Previous work utilized this rationale to introduce 8 kb HR HPV genomes (excised from recombinant plasmid with or without \textit{in vitro} recircularization) along with a plasmid encoding drug resistance into PHKs. Following drug selection, clonal cell lines were expanded (over weeks or months) and found to contain varying populations of extra-chromosomal or integrated HR HPV genomes. The introduction of linearized or \textit{in vitro} recircularized, non-supercoiled DNA alone is highly inefficient and this approach increases the propensity that the foreign DNA will integrate. When such cell lines with extra-chromosomal viral genomes were grown into raft cultures and stimulated to differentiate with chemicals such as phorbol esters or matrices such as soft agar (McLaughlin-Drubin and Meyers 2005; Wilson and Laimins 2005), only a limited productive program was achieved.

The resulting inefficient productive programs explain inconsistent results for E5 and E4 mutants regarding their roles in viral DNA amplification (Fehrmann et al. 2003; Genther et al. 2003; Nakahara et al. 2005; Wilson and Laimins 2005; Fang et al. 2006a). This reliance upon HR immortalization means that the rationale is insufficient to study immortalization-defective HR-E6 or -E7 mutant genomes or the immortalization
incompetent LR HPVs (Thomas et al. 1999; Oh et al. 2004). Spontaneously immortalized keratinocyte cell lines have been used to circumvent these problems, but display similar deficiencies in their inability to support a highly productive program (Flores et al. 1999; Lambert et al. 2005). Thus, there has been a critical need to introduce or generate efficiently the HPV genome in PHKs suitable for raft cultures free from immortalizing requirement or consequences.

Assays to Investigate HPV Activities

Several assays have proven invaluable in assessing HPV infections. The highly sensitive polymerase chain amplification reaction (PCR) is used to determine rapidly the HPV types, whereas Southern blots, though less sensitive, can determine viral load and distinguish extrachromosomal from integrated vDNA. Accurately assessing infection requires both methods to disrupt a significant amount of tissue to acquire the nucleic acid target sequences (Macnab et al. 1986; Van den Berg et al. 1989). On the contrary, in situ assays such as immunofluorescence (IF) and immunohistochemistry (IHC) to detect viral or host proteins enables the precise and accurate measurement of multiple phenotypic analytes with minimal perturbation of the tissues. In preserving tissue morphology, the localization and relative quantification of targets can be determined within an individual cell, its neighboring cells and throughout the tissue architecture.

Unfortunately, many HPV early proteins are poorly detected in situ, because they are expressed at low levels and there are no antibodies suitable for in situ assays. In lieu of antibodies specific to E6 and E7 products, a gamut of cellular biomarkers are indicative of early viral gene expression. As discussed above, the targeting of p130 by E7 protein to reactivate S phase in differentiated epithelia, necessary for viral DNA
amplification, correlates with a myriad of late G1 and S markers downstream of E2F activation (i.e. BrdU incorporation into host chromosomal DNA, PCNA, cyclin E, cyclin A, cyclin B1, MCM, etc.) (Cheng et al. 1995; Jian et al. 1998, 1999; Noya et al. 2001; Flores et al. 2000; McLaughlin-Drubin et al. 2005; Doorbar 2007). For late region gene expression, detection of the highly immunogenic L1 antigen in superficial epithelia correlates to the completion of HPV DNA amplification and the packaging of viral particles (zur Hausen and de Villiers 1994; zur Hausen 1996).

In Situ Hybridization (ISH)

In situ hybridization (ISH) to detect either DNA or RNA offers superior phenotypic sensitivity than immunoassays on infected tissues. ISH does not consistently detect low copies of HPV DNA or DNA in basal cells of productive infections and cannot identify latently infected cells. Similar to IHC with antibodies, detection of modified nucleotides for ISH can either be direct (i.e. $^{35}$S, $^{3}$H or fluorophore) or indirect via moieties (i.e. biotin or digoxigenin) bound by reactive agents (i.e. streptavidin, anti-digoxigenin, or even anti-fluorescein) which is conjugated to an assayable label (i.e. fluorophore or enzyme/substrate) (Van den Berg et al. 1989; Warford and Lauder 1991).

Following its inception, ISH was broadly applied in identifying genomic, viral and mRNA sequences in frozen tissue sections or formalin-fixed paraffin-embedded (FFPE) tissue sections for light microscopy or in ultra-thin sections for electron microscopic (EM) (Gall and Pardue 1969; John et al. 1969; Jacob et al. 1971; Orth et al. 1971; Buongiorno-Nardelli et al. 1972; Harrison et al. 1973; Pardue and Gall 1975). It also provided valuable information regarding DNA amplification and mRNA expression in the spectrum of diseases associated with viral infections (Sequiera et al. 1979; Blum et

A myriad of caveats affecting sensitivity and background staining have generated diverse ISH methodologies for specific probes, targets and tissue types. For example, the stability of a desired target/probe duplex depends on either polynucleotide’s base composition (annealing strength decreases: RNA:RNA > DNA:RNA > DNA:DNA). The $T_m$ for these reactions can be fine tuned by directly adjusting the temperature or the hybridization buffer composition (i.e. formamide and salt concentrations) (Wetmur et al. 1981). Taken together, ISH requires significantly more technical perseverance and invested time than IHC and, especially in the case of RNA-ISH, in the preparation and maintenance of RNase-free solutions and glassware to prevent degradation of the nucleic acid targets and probes. Yet, iterations of the ISH protocol can be refined to five essential steps: preparation of sections, tissue unmasking, hybridization, post-hybridization washes and detection (Van Tine et al. 2001, 2004).
Briefly, tissue preparations are immediately frozen or fixed to minimize degradation of target nucleic acids, especially critical when detecting mRNA (Allan et al. 1989; Pringle et al. 1989). While antigens are more stable and antisera from multiple hosts permits simultaneous detections with minimal cross-reactivity, the detection of novel antigens or acquiring antisera from a distinct host may require a significant investment of time and money. Conversely, any probe for ISH involves a simple labeling reaction that polymerizes nucleic acids to a known target sequence in the presence of a modified nucleotide (i.e. X-NTP): ds DNA probes from nick-translation, PCR or random primer labeling; single-stranded (ss) RNA probes from in vitro transcription of a recombinant plasmid; or synthesized ss oligonucleotide probes can include nucleic acid labels or be in vitro 3’-end-labeled (single X-ddNTP or tail with X-dNTP) (Rigby et al. 1977; Hudson et al. 1981; Langer et al. 1981; Lewis et al. 1985, 1986). Sequential rounds of ISH, utilizing distinctly detectable probes, can illustrate relationships pertinent to the entire tissue morphology otherwise irreplaceably destroyed by conventional biochemical assays (Hopman et al. 1988, 1998; Speel et al. 1998).

Tyramide Signal Amplification (TSA)

Radioimmunoassays (RIAs) and immunoradiometric assays (IRMAs) displayed sensitivity and accuracy in measuring analytes that was paralleled by radioisotopic labeling of ISH probes (Lo 1986; Crum et al. 1988b; Weiss and Movahed 1989; Barksdale and Baker 1993, 1995). Alternatively, to avoid the hazards of isotopic labels, investigators have employed enhanced fluorescent labels or enzymes to catalyze hydrogen-donating substrates that became chromogenic or fluorescent when oxidized. Products of these amplification reactions either remain soluble or become insoluble.
precipitates on the solid phase. Horseradish peroxidase (HRP) has become one of the preferred enzymes to catalyze reporter molecules due to its high turnover rate, stability and availability. Eventually, optimized biotin-streptavidin-HRP catalysis of chromogens proved faster, easier, safer, equally sensitive and less dependent upon expensive, sophisticated instrumentation to homologous radiolabels (Nuovo and Richart 1989). Recently, novel HRP substrates, tyramide conjugates, were introduced and found to significantly increase the sensitivity, speed and focal resolution of ISH detection beyond those previously achieved by chromogens and autoradiography.

Tyramide signal amplification (TSA) irreversibly deposits detectable tyramide conjugates within the immediate solid phase surrounding the reaction. HRP catalysis, or an equivalent peroxidase, yields a highly reactive tyramide intermediate that covalently binds a virtually unsaturatable receptor, electron-rich amino acids (i.e. tyrosine), at or very near the bound HRP (Bobrow et al. 1989). Immobilized precipitates of tyramide conjugates can be detected directly or indirectly, as an analyte for subsequent labeling, to dramatically amplify the primary probe signal. TSA has been coupled to FISH (TSA-FISH), IF (TSA-IF) or in tandem with other methods to detect DNA, RNA and proteins individually or simultaneously (Kerstens et al. 1994; Raap et al. 1995; Shindler and Roth 1996; Macechko et al. 1997; Speel et al. 1997; Strappe et al. 1997; van Gijlswijk et al. 1997; Teramoto et al. 1998; Van Tine et al. 1998, 2001; Jian et al. 1999; Schriml et al. 1999; Swindle et al. 1999; Wang et al. 1999; Buki et al. 2000; Ma et al. 2000; Zaidi et al. 2000; Bobrow and Moen 2001). Sequential rounds of tyramide reactions can also be performed for multiple targets and detected with distinct conjugates (different fluorophores) (Hopman et al. 1998; Speel et al. 1998; Van Tine et al. 2001, 2004). The
first round of HRP labeling must be inactivated by hydrogen peroxide (H$_2$O$_2$), then followed by subsequent probing and HRP labeling, each with an alternatively labeled tyramide (Speel et al. 1997; Hopman et al. 1998; Brouns et al. 2002). Especially pertinent to this study, following TSA-FISH or -IF deposition and H$_2$O$_2$ inactivation, labeled tissue sections can be heat treated to remove the previous probing(s), unmask antigens, denature nucleic acids targets or all three to facilitate successive detections. Since each the tyramide conjugate remains covalently bound until detected, the only limitation is the capacity of the microscope camera and filter systems to distinguish multiple labels of different colors (Van Tine et al. 2005).

**Preliminary Studies:**

**Illuminating HPV Activities in Squamous Epithelia by Tyramide Signal Amplified Fluorescent In Situ Hybridization (TSA-FISH)**

TSA-FISH was performed on submerged cultures of HPV-containing cervical carcinoma cell lines CaSki, SiHa and HeLa, on organotypic cultures of CaSki and a laryngeal papilloma to ascertain that the probe technology on formalin fixed tissues works in my hands. CaSki was established from a metastatic cervical epidermoid carcinoma and contains ~600 copies of integrated HPV-16 DNA scattered into six major tandem arrays across ~8-10 minor loci (Pattillo RA 1977; Pater and Pater 1985; Yee et al. 1985; Baker et al. 1987). Remarkably, the transcription of HPV-16 E6-E7 was dynamically selected to occur from a single dominant transcription center, independent of integrated viral DNA copies and loci (Van Tine et al. 2004b).

Oligonucleotides (40-mers) complimentary to either full-length (i.e. spans $E6$ splice donor site or sense and anti-sense to $E7$) or alternatively spliced (i.e. spans E6*I splice junction) HPV-16 messages were designed (Table 2 in Chapter 6). To enable
Fig. 1. HPV DNA and mRNA FISH in HeLa, SiHa or CaSki cells. (A) Dot blot to determine the efficiency of either 3’-biotin end-labeling or tailing of HPV-16 oligomer probes (Table 2). (B-E) CaSki, SiHa or HeLa cells were grown on chamber slides and subjected to TSA-FISH (green). Detection of (B) HPV-16 E7 (sense), E7, E6*I or full-length E6 mRNA duplexes in CaSki cells. (C-E) HPV-16 DNA detection in (C) CaSki and (D) SiHa, or HPV-18 DNA in (E) HeLa cells. (F-G) 4μm sections of (F) PHK only or (G) CaSki raft cultures subjected to TSA-FISH (green) to detect (G left-most panel) total HPV-16 mRNA, (F&G right-most panels) full-length E6 message and (F left & G middle panels) all E6-E7 messages. Total DNA was detected by DAPI (blue).

subsequent detections of the 40-mer probes by TSA, terminal transferase was used to either 3’-end label biotin-dideoxy-ATP (ddATP) or tail with a biotin-dATP/dATP mix. A dot blot determined the tailed probes were detectable from 10 fmol down to 3 fmol (see 16 E6*I tailed oligo) whereas the end-labeled oligos were much less sensitive, ranging from 100 fmol to 30 fmol for 16 E6 and 16 E7 respectively (Fig. 1A). TSA-FISH of CaSki cell cultures with either HPV-16 E6, E6*I and E7 tailed 40-mer probes detected distinct populations of viral mRNA (Fig. 1B). E7 and E6*I were expected to be the most abundant messages, but full-length E6 message was also detected in nuclei and cytoplasms. Additionally, a negative control E7 (sense) tailed probe did not yield any signal.

While every CaSki cell expresses abundant E6-E7 mRNA due to integration of the HPV DNA, patient lesions span diverse tissue architecture that can include infected or
uninfected cells. To establish regions of interest, tissue sections were first assayed using entire HPV genomes to generate biotin-nick-translated probes with or without denaturation to detect viral DNA and total mRNA, respectively, by TSA. To determine the sensitivity of entire biotinylated HPV-16 or -18 genomic probes, additional cervical cancer cell lines SiHa (HPV-16) and HeLa (HPV-18) were hybridized, along with CaSki, because their genomes contain fewer copy numbers and chromosomal loci of integrated HPV DNA. While the ~600 copies of HPV-16 DNA was readily detected in CaSki cells (Fig. 1C), both integrated copies of HPV-16 DNA were detected in SiHa cells (Fig. 1D) as were the few HPV-18 integrations in HeLa (Fig. 1E) (Heiles et al. 1988; Van Tine et al. 2004b).

To emulate raft tissue, raft cultures of CaSki cells were prepared. As with CaSki cell cultures, the tailed oligonucleotide probes detected nuclear as well as cytoplasmic HPV-16 RNA E6*I and E7 mRNA, whereas unspliced E6 message was rare and difficult to detect (data not shown). To increase the sensitivity for the E6 message, a biotin-nick-translated cDNA probe was generated to the E6*I “intron” (nt225-nt410) (Table 3 in Chapter 6?). The entire E6-E7 region (nt104-nt655) as well as entire genomic HPV-16 were also used as probe template to detect total HPV-16 mRNA. While the entire HPV-16 and E6-E7 biotin-nick-translated probes confirmed HPV message abundance (Fig. 1G left two panels), low-level, punctate signals representing full-length E6 messages were also detected in CaSki cytoplasms using the E6*I intron as a probe (Fig. 1G). Neither E6-E7 nor E6*I intron probes were detected on PHK only rafts (Fig. 1G). Because transformation disables epithelial differentiation, there is no spatial localization to any of the detected HPV-16 messages in CaSki rafts (Fig. 1G).
Serial sections of a productive HPV-11 laryngeal papilloma patient lesion was used to ascertain the relationships between viral RNA expression and viral DNA replication, since comparable HR HPV tissues are not as readily available (Wiatrak et al. 2004). The entire HPV-11 genomic labeled probes detected low-level amplification of HPV-11 DNA, which also correlated to increased detection of total transcripts (Fig. 2E&F). Care was taken in the design of LR-HPV-11 E6 and E7 probes due to their respective intergenic promoters that partially transcribes noncoding E6 or E7 at the 5’-UTR of down-stream polycistronic messages. The increased sensitivity from biotin-nick-translation was exploited using template for probes either a 177 bp E6 fragment (nt94-nt270) or a 169 bp E7 fragment (nt500-nt668). Serial sections of the HPV-11 papilloma were used to detect total (Fig. 2F), E7 (Fig. 2G) or E6 (Fig. 2H) messages.

As mentioned above, the increase in total HPV-11 message correlated to the
amplification of viral DNA, though the DNA-FISH to this serial region was lost (data not shown). Few, nonspecific signals were detected in uninfected, neonatal foreskin tissue sections, but were typically in the below the epidermis (Fig. 2A-D). Further, detection of both HPV-11 E6 and E7 messages was found in mid-epithelia immediately prior to significant increases in total viral mRNA, to enable viral DNA amplification (Compare Fig.2F-H).
CHAPTER 3

HPV-18 RAFT CULTURES RECAPITULATE THE PRODUCTIVE PROGRAM

Efficient In Vivo Generation of HPV-18 Genomes in Cotransfected PHK Raft Cultures

The temporal and spatial viral-host interactions critical to achieve the HPV productive program are poorly understood without the means to follow and manipulate the viral life cycle (Taichman et al. 1984). Very few mucosal HPV particles can be isolated in vivo sufficient to establish an infection in PHKs (Kreider et al. 1987). Alternatively, assorted cultures expressing HPV genes continue to yield novel and pertinent findings regarding Early protein functions, but never the entire process. The lack of protocols that consistently recapitulate the full spectrum of HPV infectivity are largely due to the reliance of the viral life cycle upon epithelial formation and difficulties artificially introducing entire HPV genomes into suitable cells (Taichman et al. 1984).

Emulating the correct differentiating environment in organotypic cultures of PHKs that express HPV genes is resolving errors perpetuated in differentiation insensitive cell lines and immortalized cells. Recently, our lab developed a simple, yet highly efficient and reproducible method that generates the entire 8 kb HPV genome in PHKs via in vivo Cre-LoxP mediated recombination (data not shown, but see Wang et al. 2009. Fig. 1). Independent of extensive passaging, such PHKs were suitable to generate organotypic raft cultures that recapitulate all aspects of the HPV life cycle.

Fresh PHKs were cotransfected with two expression plasmids: a 12 kb vector encoding neomycin resistance and the entire HPV-18 genome flanked by two LoxP sites
(floxed) at the 5′-end of the URR. The second plasmid, pCAGGS expresses Cre-
recombinase, to promote LoxP-mediated recombination (Feil et al. 1997; Hardouin and
Nagy 2000; Metzger and Chambon 2001). The Cre/LoxP recombination system was
discovered in bacteriophage P1 and utilizes Cre-recombinase (343 a.a.) to mediate site-
specific DNA recombination between two LoxP sites (34-bp DNA sequences containing
two 13-bp inverted repeats and an asymmetric 8-bp spacer region) (Sternberg and
Hamilton 1981). Efficient in vivo Cre-LoxP recombination of the 12 kb parental vector
generates two independent, circular plasmids, each with a single LoxP insertion (34 bp):
pNeo and a virtually wild-type 8 kb HPV-18 genome (Fig. 1). While the reconstituted
HPV-18 genomes persist in dividing PHKs, both pNeo and Cre expressing plasmids are
transient, thus reducing potential toxic repercussions (Wang et al. 2009).

**HPV-18 Raft Cultures Recapitulate Early HPV Pathogenesis**

Reproducibly, greater than 30% of cotransfected PHKs survived acute G418-
selecton. The first iteration of the parental vector underwent nearly 100% Cre-LoxP
recombination to generate HPV-18 genomes with a LoxP site between nucleotides 7361-7362 (similar to Wang et al. 2009. Fig. 1). The drug-selected PHKs were used to develop into raft cultures that exhibited a hyperplastic histology (similar to Wang et al. 2009. Fig. 2D). On day 10, BrdU positive-S phase nuclei were detected stochastically distributed throughout the epithelium (similar to Wang et al. 2009. Fig. 2E). IHC to L1 antigen was undetectable (H.K. Wang, unpublished results). The absence of L1 antigen indicated that these raft cultures were not undergoing a full productive program. Without detectable HPV-18 L1 antigens, FISH for viral DNA and mRNA offered an alternative phenotypic assays.

_Determination of HPV DNA and mRNA Content by TSA-FISH_

The specificity of the HPV-18 DNA probe was confirmed on raft cultures of untransfected PHKs or PHKs transfected with parental plasmid only, which further bolstered the Southern and PCR data that the ~12 kb parental plasmid does not replicate and is lost without Cre dependent recombination/excision (Fig. 2A&B and data not shown). Unexpectedly, HPV-18 FISH revealed a large fraction of spinous nuclei of day 12 raft cultures containing intense viral DNA signals that tapered into the lower strata (Fig. 2C). Such a high fraction of differentiation dependent HPV DNA amplification has only been observed in HPV-infected xenografts in nude mice (Stoler et al. 1990). Importantly, throughout HPV-18 raft sections of day 10 harvests, similar patterns of intensifying viral DNA amplification were always found, though most nuclei were less intense.

Since amplification of viral DNA and L1 protein expression are often linked, raft cultures were harvested at later times. Prolonging the growth of rafts until day 12 and day
Fig. 2. HPV-18 DNA amplification and mRNA expression in PHK raft cultures. (A–F) 4 μm sections of day 12 (A) control as well as (B–F) HPV-18-containing PHK raft cultures (B) without or (C–F) with Cre and (A–D) subjected to TSA-FISH (green) to detect (A, B and D) total viral RNA (C) HPV-18 DNA, (E) HPV-18 E6-E7 message and (F) HPV-18 Late message. Total DNA was stained with DAPI (blue).

14 significantly increased the fraction of differentiated cells with intense viral DNA, yet L1 antigen was still undetectable (data not shown). Ultimately, only RNA-FISH divulged the reason for the lack of detectable L1 antigen. For the cotransfected HPV-18 rafts, both transcription of total viral RNA and probe generated to E6-E7 messages were cytoplasmic in the lower strata, but became increasingly nuclear upon differentiation (Fig. 2D&E). Detection of HPV-18 L1 mRNA yielded negligible cytoplasmic signals, whereas large nuclear foci appeared in the superficial strata (Fig. 2F). This intense nuclear retention of L1 message inferred that the LoxP insertion may have been too proximal to the pAL and interfered with Late transcript processing.

From my preliminary observations, subsequent floxed parental vectors were designed to preserve L1 gene expression by inserting LoxP in the 3′-URR downstream of
pAL at an unimportant site to HPV transcription regulation or DNA replication (nt7473-nt7474) (Bernard 2002). The new HPV-18B parental vector (hereafter referred to as HPV-18 whereas the previous iteration will be referred to as HPV-18A) was cotransfected into PHKs and assayed as before. PCR and Southern blot demonstrated only Cre recombination liberated the viral plasmid and allowed persistent replicative maintenance in PHKs (See Wang et al. 2009. Fig. 1). The raft cultures of PHKs cotransfected with this new HPV-18 transfer vector were found to exhibit virtually identical levels of dysplasia by H&E staining, stochastic nuclei labeled with BrdU and TSA-FISH detected amplified HPV DNA within nuclei of numerous differentiated mid-to upper-spinous cells (Fig. 3A-C, See Wang et al. 2009. Fig. 2A-F). The incredible number of apical nuclei that amplified HPV DNA confirmed that the cotransfection and selection had generated a near ubiquitous population of PHK that harbored HPV-18 plasmids independent of HR HPV immortalization keratinocytes (Fig. 3A-C, See Wang et al. 2009. Fig. 2C&F). Importantly, L1 antigen was abundantly observed in the superficial cells and in numerous cornified envelopes (see Wang et al. 2009. Fig. 2H).
Infection of Naïve PHKs

Virus particles were harvested and titered by real time PCR (Wang et al. 2009) To ensure that they were fully mature and infectious, PHKs were infected with a MOI ranging from 5200, 1040, 208, 42, 10, 2, 1 or 0. Contrary to earlier findings, PHKs were susceptible to the virus down to the MOI of 2, as determined by PCR to reverse transcribed E1^E4 cDNA. No PCR occurred from uninfected rafts (See Wang et al. 2009. Fig. 3A& B).

Various raft cultures, developed from PHKs infected at various MOIs were harvested on day 14 and assayed to determine whether the virus elicited productive infection. At MOI of 800 or higher, cultures contained widespread L1 antigen positive cornified envelopes. Unexpectedly, at MOIs of 400 and lower, the cultures had little or no L1 antigen. Interestingly, regardless of L1 productivity, all infected cultures were mildly hyperplastic relative to uninfected controls (See Wang et al. 2009. Fig. 3C and data not shown), indicative of early viral gene expression.

To verify this interpretation, sections were probed by DNA-FISH and IF for viral DNA and PCNA, respectively (Fig. 4). E7 induces PCNA in suprabasal cells, irrespective of BrdU incorporation (S phase), thus the induction of PCNA signifies HPV infected cells (Halbert et al. 1992; Cheng et al. 1995; Jian et al. 1998, 1999; Noya et al. 2001; Wang et al. 2009). Uninfected PHKs in raft cultures can be estimated by those fractions of cells lacking PCNA. As expected, only basal cells in control PHK cultures were PCNA positive (Fig. 4 or See Wang et al. 2009. Fig. 3D). In contrast, even at the MOI of 50, most differentiated cells were positive for PCNA, indicating that most if not all cells were successfully infected. Further, DNA-FISH demonstrated occasional pockets of superficial cells in these nonproductive cultures had low levels of amplified viral DNA (Fig. 4;
56

Fig. 9. Infectivity assay of HPV-18 virions in PHK Raft Cultures. HPV virions were titred by quantitative real-time PCR. 4 μm sections from the raft cultures above were probed for PCNA (Alexa Fluor 488, green) and viral DNA (Cy3, red). Total DNA revealed by DAPI (blue).

Conclusions to Chapter 3

In summary, this highly reproducible methodology offers several advantages over previous studies. In vivo Cre-LoxP recombination requires the cotransfection of PHKs with the floxed HPV-18 parental vector and a Cre-expression plasmid. Avoiding in vitro linearization and/or recircularization ensures recombinant vectors retain their super-coiled form to maximize the subsequent transfection efficiency. This clever exploitation
of Cre-recombinase ensured every surviving PHK contained HPV-18 DNA by virtue of the parental vector encoding neomycin resistance. Efficient cotransfection and Cre-recombination of the parental vector mechanistically coalesced under acute G418 selection where, ideally, every surviving PHK reconstituted an 8kb HPV-18 genome. Further, this rapid selection (2-3 days) was such that the organotypic cultures were developed from transfected PHKs without extensive passages. In so doing, the need for HR immortalization was obviated, as evidenced by the dramatic viral DNA amplification and infectious HPV production (Wang et al. 2009).

For my part, both RNA- and DNA-FISH detections unequivocally demonstrated that HPV DNA was not only maintained in raft cultures of the cotransfected PHKs, but also revealed its potential to robustly recapitulate the viral productive program. Further, some areas of the HPV-18 rafts had more viral DNA signal than others, suggesting that perhaps harvesting the rafts beyond day 10 would permit HPV DNA to more fully amplify and cause L1 to be detected. While this eventually was the case, it was only the second generation of HPV-18 parental vector that achieved this feat. These initial detections established the need to implement sequential harvests and permitted the snapshots of the viral productive program that will constitute Chapters 4-7.

Rationale and Objectives

The Cre-Loxp methodology which produces entire HPV-18 genomes within PHKs finally permits extensive studies to decipher the dependence and effects of viral gene expression on keratinocytes throughout epithelial differentiation. The goal of the remainder of this thesis research is to expand the knowledge of molecular interactions of HPV within differentiating epithelia throughout the productive infection. In particular,
with the advent of TSA-FISH or TSA-IF, multiple targets can be assayed in a single section. The following chapters will discuss the extensive TSA-FISH and TSA-IF detections to characterize HPV-18 containing PHK raft cultures.

- HPV-18 raft cultures harvested over a time course revealed the full gamut of early to late gene expression (Chapter 4).
- HPV DNA amplification was delayed relative to cellular DNA replication and was first detected in G2-arrested cells (Chapter 4).
- Viral DNA amplification always preceded L1 antigen detection (Chapters 4).
- The E7 activity diminished as viral DNA amplified (Chapter 4).
- The E1^E4 protein does not stabilize cyclin B1 to induce G2 arrest (Chapter 5).
CHAPTER 4

NOVEL TEMPORAL AND SPATIAL INTERACTIONS DURING THE HUMAN PAPILLOMAVIRUS PRODUCTIVE PROGRAM WITHIN EPITHELIAL TISSUE AS REVEALED BY MULTIPLEXED FLUORESCENT IN SITU DETECTIONS

Robust Cellular DNA Replication Precedes HPV-18 DNA Amplification

Intriguingly, in productive raft cultures initiated by cotransfection, the stochastic distribution of BrdU incorporation in cells of all strata did not reflect the pattern of amplified viral DNA in the more differentiated strata (Wang et al. 2009. Fig.2). Simultaneous visualization of BrdU and viral DNA revealed that the majority of cells intensely positive for HPV-18 DNA were negative for strong BrdU and vice versa. Only a very small fraction of cells had colocalized signals, regardless of the order in probe application or an additional denaturation step (data not shown). These results were inconsistent with the expectation that viral DNA amplifies concurrently with host DNA replication.

To investigate this dichotomy, HPV-18-containing PHK raft cultures were exposed to BrdU for 12 h as before and harvested on days 8, 10, 12 and 14 and analyzed by DNA-FISH and IF simultaneously (Fig. 1A). A large fraction of nuclei on days 8 and 10 was intensely positive for BrdU, with virtually no detectable HPV DNA on day 8. A low level of amplified viral DNA was typically detected in a small percentage of superficial cells on day 10. On day 12, BrdU-positive spinous cells decreased while those with intense HPV DNA signals markedly increased. By day 14, few spinous cells incorporated BrdU, while the majority of mid- and upper spinous cells were strongly
positive for viral DNA. The distinct focal FISH signals in the stratum corneum are likely due to virions containing packaged viral DNA (see below). These inverse patterns of host DNA replication to viral DNA amplification were reproducible across multiple experiments, with the majority of nuclei containing strong viral DNA signals beginning on day 12 ± 2. The fraction of spinous cells in S phase or with elevated viral DNA was

Fig. 1. HPV-18 DNA amplification lags behind cellular DNA replication prior to L1 expression. (A) HPV-18-containing PHK raft cultures were harvested on days 8, 10, 12 and 14, each following a 12-h incubation with BrdU. 4 μm sections were probed for HPV-18 DNA (red) and incorporated BrdU (green). DNA revealed by DAPI (blue). (Left column) For better visualization of tissue morphology, DAPI staining is also presented in a black-and-white images. (B) Detection of HPV DNA (red) and the major capsid protein L1 (green) in a day 14 culture.

not maintained in cultures older than day 14 suggesting a periodicity or one-time wave of replication. Regardless of culture age, there was little colocalization of intense BrdU and strong HPV DNA signals. Occasionally, distinct HPV DNA foci were seen in spinous cells with intense BrdU signals, suggestive of initiation of viral DNA amplification. However, these cells were not in S phase, as they were negative for cyclin A (see Figs. 2,4A&D). Thus, BrdU signals are specific to host DNA replication. As HPV DNA only amounts to ~1% of host DNA by mass even, at several thousand copies per cell, the amount of BrdU incorporated into HPV DNA is clearly not sufficient to be visualized by such metabolic labeling.

Punctate dots or streaks of viral DNA signals were observed in the stratum corneum, consistent with DNA packaged in virions as presented in Fig. 1A, day 14. Indeed, some of the viral DNA colocalized with L1 signals (Fig. 1B; Wang et al. 2009 Fig. 4B). Factors attributed to the incomplete colocalization of L1 and viral DNA may include the accessibility of packaged viral DNA as well as the degradation of any unpackaged viral DNA during cornification. An apparent reduction of DNA signals in live cells transitioning into the cornified strata may suggest that viral DNA condensation and packaging occurs parallel to cornification. Similarly, the L1 signals revealed by this double labeled IF image were reduced relative to those detected by IHC without the denaturation step (Wang et al. 2009. Fig.2).

S Phase Progression Is Distinct From HPV-18 DNA Amplification

To address the earlier counterintuitive results regarding viral and host DNA synthesis, the raft cultures were probed for S phase cyclin A by IF relative to HPV DNA and BrdU detection. Control PHK sections demonstrated a few cycling cells within basal
Fig. 2. HPV-18 DNA amplification follows S phase dependent cyclin A expression of BrdU incorporation. 4 μm thin sections from the same day 8, 10, 12 and 14 raft cultures as shown in Figure 4 were subjected to sequential triple fluorescence detection of cyclin A (Alexa Fluor 488, green), HPV-18 DNA (Cy3, red) and BrdU (Alexa Fluor 647, yellow). DNA revealed by DAPI (blue).


nuclei that contained both cyclin A and BrdU (Fig. 4A; Wang et al. 2009. Fig. 5A).

Consistent with earlier BrdU labeling, both day 8 and day 10 HPV-18 raft cultures contained numerous cells were positive for cyclin A (Fig. 2 top panels; Wang et al. 2009 Supplemental Fig. 2). Again, the number of positive S phase cells decreased by day 12 until very little cyclin A was detected in nuclei on day 14 (Fig. 2 bottom two rows; Wang et al. 2009 Supplemental Fig. 2). At all time points, nuclei positive for cyclin A were also positive for BrdU, indicative of cells well into S phase. Certain discrete populations of BrdU-negative cells containing cyclin A in the nucleus may have recently entered S
phase. An additional subset of cells that contained cytoplasmic or no cyclin A, but were BrdU positive, may have been transitioning from S to G2 or already in G2. Importantly, suprabasal cells with varying HPV signal intensities rarely contained detectable cyclin A. Thus, the lack of both BrdU and cyclin A supports the observation that amplification of HPV genomes proceeded outside of cellular S phase.

**HPV-18 Genomic Amplification from the G2 Phase**

Occasionally, punctate HPV DNA signals in BrdU positive nuclei were suggestive that the virus initiated amplification in cells that recently completed S phase. Can viral DNA amplify in G2 phase? Similar simultaneous probe analyses relative to the MPF component cyclin B1 were conducted. Nuclear import of the cyclin B1-cdk1 complex is essential for the conclusion of G2 and initiation of mitosis (Smits and Medema 2001; Porter and Donoghue 2003). PHK raft cultures contained pre-mitotic, BrdU-positive basal cells that occasionally exhibited weak cyclin B1 signals (Fig. 4B; Wang et al. 2009 Fig. 5B). Day 8 HPV-18 raft cultures revealed intense, cytoplasmic cyclin B1 in a fraction of spinous cells, which modestly increased by day 10. The signals typically surrounded intense BrdU-positive nuclei (Fig. 3; Wang et al. 2009 Supplemental Fig. 3). On day 12, there was a more dramatic increase in the signal strength and in the number of cytoplasmic cyclin B1-positive spinous cells (Figs. 3,4E; Wang et al. 2009 Fig. 5E; Supplemental Fig. 3). Such an increase reflects the reduction in cyclin A- and BrdU-positive cells relative to earlier time points (compare to Fig. 2; Wang et al. 2009 Supplemental Fig. 2). On day 14, when amplified or packaged HPV DNA filled most of the mid- to upper differentiated nuclei and extended into the stratum corneum, only a few cyclin B1-positive cells were observed in the lower spinous
Fig. 3. HPV-18 DNA amplification at G2, relative to cytoplasmic cyclin B1 detection. 4 μm thin sections from the same day 8, 10, 12 and 14 raft cultures as those shown in Figure 4 were subjected to sequential triple fluorescence to detect cyclin B1 (Alexa Fluor 488, green), HPV-18 DNA (Cy3, red) and BrdU (Alexa Fluor 647, yellow). DNA revealed by DAPI (blue).


epithelia. Critically, it was in the nuclei of many cyclin B1-positive mid-spinous cells that low to moderate HPV DNA signals was first detected (Figs. 3,4E; Wang et al. 2009 Fig. 5E; Supplemental Fig. 3).

To investigate whether the amplification of viral DNA indeed occurred in post-S phase nuclei, raft cultures were pulsed with BrdU for 6 h on day 10, then harvested on day 12. Indeed, this pulse-chase experiment demonstrated that an increased population of nuclei with intense viral DNA colocalized with strong BrdU signals (Fig. 4F; Wang et al. 2009 Fig. 5F). Therefore, within 48 hrs of fixation, the stochastically labeled S phase
cells on day 10 transitioned into a G2 environment permissive to HPV DNA amplification. Similar triple fluorescent assays on day 14 raft cultures of PHKs productively infected by HPV-18 virions (MOI=800) verified that the observed delay in viral DNA amplification is not an artifact of the cotransfection and acute selection that generated the sequentially harvested raft cultures above (Fig. 4G; Wang et al. 2009 Fig. 5G). Collectively, these observations show that viral DNA amplified in G2 arrested cells following cellular DNA replication and underscores the necessity to perform multiplexed probing by in situ methods in a system which closely resembles the natural infection.

Intriguingly, spinous cells with high HPV DNA were negative for cyclin B1 signals in cultures of any age (Figs. 3,4E&G; Wang et al. 2009 Fig. 5E&G, Supplemental Fig. 3). Possible explanations considered include: 1.) Cyclin B1 was degraded as cells transitioned through mitosis. This is unlikely because their enlarged nuclei is consistent with a greater than 2n DNA content (Chien et al. 2002). 2.) Cyclin B was degraded to allow for cellular DNA re-replication. This is also unlikely, as cells with high viral DNA were negative for either nuclear cyclin A or intense BrdU (Figs. 2,4D; Wang et al. 2009 Fig. 5D, Supplemental Fig. 2) Viral DNA amplification was paralleled by a loss of E7 activity, which subsequently increased the turnover of cyclin B1 and promoted cells to exit the cell cycle. Evidence is consistent with this last interpretation (see below).

Cessation of HPV-18 E7 Activity in Cells With Abundant Viral DNA

Our laboratory has recently shown that p130, not pRB, is readily detected in differentiated cells of normal squamous epithelia from diverse body sites and of PHK raft cultures. Expression of HR or LR HPV E7 proteins destabilizes p130 to enable S-phase re-entry by spinous cells (Genovese et al. 2008). Thus, the loss of p130 is an excellent
Fig. 4. HPV-18 DNA amplification relative to the cell cycle. (A-C) Day 10 raft cultures of normal PHK raft cultures. (D-F) Day 12 raft cultures initiated from PHKs transfected with HPV-18 DNA. Sections were analyzed for cyclin A (green in A & D) or cyclin B (green, B & E), HPV-DNA FISH (red, D-F) and for BrdU (red in A & B, green in C, or yellow Alexa Fluor 647 in D & E). (F) An HPV-18-containing raft culture was pulsed with BrdU for 6 h on day 10 and then chased for 48 h prior to harvest. (G) Day 14 of PHK raft cultures infected with HPV-18 virus at MOI of 800. The section was probed for viral DNA (red), cyclin B1 (green) and BrdU (gold). (H) Patterns of PCNA (red), HPV-18 DNA (gold) and p130 (green) in day-14 raft cultures of PHKs initiated after HPV-18 DNA transfection (top row) or HPV-18 virus infection at MOI of 800 (bottom row). DNA revealed by DAPI (blue).


indicator of E7 activity. As before, p130 was detected in the differentiated strata of normal PHK raft cultures, while BrdU signals were observed in cycling basal cells (Fig. 4C; Wang et al. 2009 Fig. 5C). The cells in the lower strata of HPV-18 DNA transfected
raft cultures with no amplified viral DNA were positive for PCNA but lacked p130, indicative of functional E7 (Fig. 4H, top row; Wang et al. 2009 Fig. 5H). In contrast, moderate to high p130 signals were observed in cells with moderate to high HPV DNA signals. In addition, cells with high p130 were negative for PCNA. Both these latter observations are consistent with a reduction and eventual loss in E7 activity upon viral DNA amplification. This extinction of E7 activity explains why cells with amplified viral DNA do not re-enter additional rounds of S phase and thus cease to incorporate BrdU or to express cyclin A and cyclin B1. Similarly, this loss of PCNA and reappearance of strong p130 signals was also observed in cells with high viral DNA in PHK raft cultures productively infected with HPV-18 virus (Fig. 4H, bottom row; Wang et al. 2009 Fig. 5H).

An Essential Role of E6 in Viral DNA Amplification and Virion Production

To demonstrate that this experimental system indeed obviates a dependence on HPV E6 oncoprotein for stable viral plasmid maintenance and PHK immortalization while permitting analyses in PHK raft cultures, we prepared an HPV-18 mutant in which the E6 gene (nt105-nt579) was deleted of the E6*I intron coding sequence (nt234-nt415). In natural infections, the great majority of HR HPV E6 transcripts are alternatively spliced intragenically, abrogating the ability to encode the full-length E6 protein. The predominant HPV-18 E6*I mRNA contains a frameshift that terminates translation shortly after the splice. The resulting E6*I peptide, although sharing some of the capability to degrade certain host proteins (Pim et al. 2009), has been reported to bind to and antagonize the E6 protein, preventing it from functioning as a ubiquitin ligase in conjunction with E6AP to degrade p53 (Pim and Banks 1999). Due to their low
abundance, E6 and E6* transcripts and their encoded peptides have not been localized within the stratified epithelium in naturally infected patient specimens. We harvested raft cultures containing either HPV-18 E6*I mutant genome or the wild-type HPV-18 genome over a time course extending out to 22 d. The wild-type HPV-18 cultures were highly productive as before (data not shown) whereas the mutant HPV-18 cultures were essentially negative for L1 (Data not shown; see Wang et al. 2009. Fig. 6A, left panel). The histology of the mutant-containing cultures was in between that of the control PHK raft cultures and those containing the HPV-18 plasmid, except in older cultures, when the epithelia were thin and similar to the control cultures. To examine whether the cells have lost the HPV-18 mutant DNA, raft cultures were probed for PCNA by IHC. A great majority of the suprabasal cells were positive. Relative to cultures containing the wild-type genome, a few pockets in the epithelium were negative for PCNA (Wang et al. 2009). Thus the majority of the cells still harbored the mutant plasmid DNA. However, BrdU-positive cells were reduced relative to raft cultures harboring the wild-type HPV-18 (data not shown). Indeed, most of the spinous cells did not have the enlarged nuclei typical of tetraploid cells. Raft cultures harboring E6*I mutant accumulate high levels of p53 protein without experiencing apoptosis.

To determine the defects of E6*I, we probed for viral DNA amplification and p53 protein. We demonstrated previously that p53 is induced by E7 in a fraction of spinous cells in PHK raft cultures, but it is degraded when E6 is also expressed (Jian et al. 1998). The induction of p53 in cultures with E6*I mutant genomes would therefore provide another marker for E7 activity. Further, a comparison of p53 distribution between raft cultures containing the wild-type and E6*I mutant genomes would shed light on the
Fig. 5. Complementation of HPV-18 E6*I genome by a retrovirus delivering HPV-18 URR-E6. Raft cultures were harvested over a time course. (A) IHC to detect L1 (reddish brown) on day-18 raft cultures. (Left panel) HPV-18 E6*I-containing cultures. (Middle panel) HPV-18 E6*I-containing PHKs infected with the empty pLC retrovirus. (Right panel) HPV-18 E6*I-containing PHKs trans-complemented with the pLJ HPV-18 URR-E6 retrovirus. Arrowhead points to the boundary between the upper cornified strata and live epithelium below. (B–E) Double-fluorescence detection of p53 (green) and HPV-18 DNA (red) in raft cultures. (B) Day-10 normal PHKs. (C) Day-10 and day-12 wild-type HPV-18-containing PHKs. (D) Day-12 and day-16 HPV-18 E6*I-containing PHKs. (E) Day-16 HPV-18 E6*I-containing PHKs trans-complemented with pLJ HPV-18 URR-E6. DNA detected by DAPI (blue).

normal localization for E6 and E6* peptides.

In untransfected PHK cultures, p53 was detected in a few BrdU-positive and BrdU-negative basal cells but not in suprabasal cells (Fig. 5B; Wang et al. 2009 Fig. 6B). Day 10 cultures harboring wild-type HPV-18 genomes had low viral DNA, which increased by day 12, as described previously. Weak p53 signals were detected in some basal as well as mid- and upper spinous cells. On days 12 and 14, weak p53 was also detected in a fraction of cells in the lower strata, but not in mid- and upper spinous cells with high viral DNA (Fig. 5C and data not shown; Wang et al. 2009 Fig. 6C). In contrast, numerous basal and suprabasal cells in raft cultures harboring the E6*I mutant genomes were moderately or strongly positive for nuclear p53 at all time points. Importantly, only a small number of differentiated cells were positive for very low levels of amplified viral DNA and these cells were negative for strong p53 (Fig. 5D; Wang et al. 2009 Fig. 6D). This pattern did not change out to 22 d. Poor amplification of HPV-18 E6*I mutant genomes would account for the scarce L1 signals.

Despite the high p53 protein, the cells did not undergo apoptosis based on histology of the tissues. Had extensive apoptosis taken place, we would not have been able to have raft cultures for >3 wk. To verify the absence of apoptosis, raft cultures harboring E6*I mutant or the wild-type genome for one of the effector caspases, the cleaved caspase 3 (Taylor et al. 2008). There was little or no signal in either culture (Wang et al. 2009). In contrast, the antibody yielded widespread signals in drug treated tissues with apoptotic histology (E.-Y. Kho, H.-K. Wang, T.R. Broker and L.T. Chow, unpubl.).

To ascertain that the defective phenotypes were not attributable to unintended
mutations in E6*I genomes, the E6*I DNA-containing PHKs were transduced with a retrovirus expressing HPV-18 E6 or E6–E7 controlled by the contiguous viral URR. With either retrovirus, 30% of the cornified envelopes across the day 18 and older rafts were abundantly positive for L1 (data not shown; see Wang et al. 2009. Fig. 6A right panel). Empty vector-only retrovirus had no effect on L1 synthesis (data not shown; see Wang et al. 2009. Fig. 6A, middle panel). Areas of URR-E6-complemented day 16 HPV-18 E6*I cultures exhibited a pattern of p53 loss and HPV DNA amplification similar to day 12 wild-type cultures (Fig. 5E; Wang et al. 2009 Fig. 6E).

The slower kinetics of L1 synthesis can be attributed to the delay in E6 expression from the complementing retrovirus versus wild-type HPV-18 cultures. In regions where complementation was not observed, the provirus may have integrated in chromosomal locations from which E6 expression was inadequate to overcome the dominant negative E6*I suppression of the E6 function. Indeed, raft cultures of PHKs transduced with a retrovirus harboring HPV-18 or HPV-11 URR-βgal also exhibited patchy patterns of reporter gene expression (Parker et al. 1997). However, we cannot rule out that the mutant plasmid might have been lost in some of the cells, as suggested by the pockets of PCNA-negative cells (Wang et al. 2009). E6 trans-complementation was reproducible with three batches of PHKs. These results demonstrate that E6 plays a critical role in viral DNA amplification and virion production, possibly implicating p53 in inhibiting viral DNA amplification.

Conclusions to Chapter 4

Performing these dual-detections to HPV-18 DNA and BrdU on sequentially harvested rafts are the first definitive evidence that amplification of HPV-DNA does not
occur simultaneously with S phase nuclei. Indeed, prior to performing the HPV-18 DNA- and L1 RNA-FISH in Chapter 2, the drops in BrdU detected in later harvest were interpreted to mean that the viral DNA had been lost despite the earlier, robust DNA amplification. The second HPV-18 parental vector confirmed the time-course of this phenotypic drop in E7 induced cellular proliferation was typical of productive infections and dependent upon the initial concentration of HPV-18 DNA as was evident in raft cultures of infected PHKs at increasing MOIs. It was also revealed that only those spinous cells with amplified viral DNA expressed the L1 capsid protein. Thus the entire range of early to late gene expression is recreated in this raft culture system, independent of the consequences of immortalizing PHKs. For this reason, we mutated HPV-18 E6 to only express E6*I and found that the widespread catastrophes associated with ablating full-length E6 expression in monolayer cultures was not evident in raft culture, despite readily detected p53 in nuclei. While the lack of viral DNA amplification may denote direct effects from mutating the E6 splice donor site, rather than the loss of full-length E6 protein, trans-complementation with E6 was sufficient to partially restore the productive program.
CHAPTER 5
A HIGHLY EFFICIENT SYSTEM TO PRODUCE INFECTIOUS HUMAN PAPILLOMAVIRUS: ELUCIDATION OF NATURAL VIRUS-HOST INTERACTIONS

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Abstract

A simple, efficient system has been developed to produce high titers of infectious human papillomavirus type 18 (HPV-18) in organotypic raft cultures of primary human keratinocytes (PHKs). Molecular characterization elucidated key early and late events in the reproductive program. The system obviates the need for immortalized cells and allows the analyses of mutant HPV genomes not previously possible. An E6 deletion mutant incapable of causing p53 degradation is defective in viral DNA amplification and capsid protein production. The high levels of p53 protein which accumulated in numerous cells did not lead to apoptosis over a prolonged duration. Time course and metabolic labeling experiments revealed novel interactions with the host. Notably, post-mitotic, differentiated cells are induced by HPV E7 expression to reenter S phase, whereupon host chromosomes replicate, but HPV DNA does not amplify until the cells have progressed to and are arrested in G2 phase. Here, we present data that strongly suggest that the abundant cytoplasmic viral E1\^E4 protein is not responsible for this G2 arrest, as described in the literature upon ectopic expression in cell lines. We provide additional insights into the viral life cycle and contrast them to conclusions derived from experiments in cell lines.
Small DNA tumor viruses such as adenoviruses, SV40, polyomaviruses and papillomaviruses rely heavily on cellular machinery and pathways for their propagation and have been invaluable tools in our efforts to probe and dissect all aspects of eukaryotic cell processes. Among them, human papillomaviruses (HPV) are ubiquitous pathogens of major medical concern and have attracted sustained attention of clinicians, basic scientists and epidemiologists for three decades. The knowledge gained has contributed to the dramatic advances toward improving domestic and global public health. The development of a variety of diagnostic probe technologies now forms the basis of molecular epidemiological assessments of HPV infections, diseases and their management. Active infections normally manifest as warts, condylomata or papillomas. Benign Infections by oncogenic HPV genotypes can progress to cervical, penile, anal, tonsillar, pharyngeal and laryngeal cancers (for a review see ref. 1) and oncogenic HPV DNA has also been detected non-melanoma skin cancers.

Basic studies of HPVs have had a transformative impact on our understanding of cell cycle regulation and human cancers as two major tumor suppressor proteins, p53 and pRB, are targets of viral oncoproteins E6 and E7, respectively (for a review see ref. 2). Impressively, within 23 years of the first isolation of oncogenic HPV-16 and HPV-18, prophylactic vaccines have been developed, approved and widely applied in developed countries. Although the HPV vaccines have been 100% effective in preventing new infections for 8 years and counting, they do not cure preexisting infections. Surgical excision remains the treatment of choice. However, latently infected basal keratinocytes remaining at the surgical margin are stimulated by wound healing and proliferate into the wound bed, whereupon the lesion typically recurs, most evident in recurrent respiratory
papillomatosis (RRP) patients. There are no consistently effective drugs for direct or post-operative treatment of lesions. Rational drug discovery requires a thorough understanding of their pathobiology. However, as viruses, HPVs have been very difficult to study in cultured cells.

To begin with, mucosotropic HPVs generally produce relatively few virions, as determined from biopsies of immune-competent patients, quite likely a strategy to evade host immune surveillance and achieve long-term persistence. Secondly, none of the HPV genotypes can be propagated in conventional cell cultures because their RNA transcription and DNA amplification take place only in differentiated strata of squamous epithelia (for a review see ref. 3). Rather, viral genomes are recovered from patient specimens as cloned recombinant DNAs. The 8-kb double-stranded circular DNA, which replicates as multi-copy nuclear plasmids, encodes only one enzyme, the E1 replicative ATP-dependent DNA helicase, which shares common functional motifs with other helicases. All additional functions necessary to support viral DNA amplification rely on interactions between viral and host proteins. These properties make it extremely difficult to study the virus life cycle and to discover drugs selective against HPV infections.

Numerous investigations of the oncogenic HPV E6 and E7 genes have established their crucial roles in viral carcinogenesis\(^1,2\). However, to examine their functions in the virus life cycle, researchers have been forced to adopt and adapt organotypic epithelial (raft) culture techniques.\(^4\) Briefly, when normal PHKs are plated on a dermal equivalent (comprised of type 1 rat tail collagen seeded with mouse 3T3 J2 fibroblasts) and cultured at the medium/air interface for 10 or more days, the keratinocytes proliferate, stratify and differentiate into a human skin equivalent, virtually identical to the native tissue of origin.
by histological and molecular criteria. Wicking of the tissue culture media through the collagen delivers growth factors and cytokines secreted from the fibroblasts to the keratinocytes, thereby sustaining keratinocyte growth and differentiation. This raft culture system is more time consuming and expensive than conventional cell cultures. Following tissue embedding and sectioning, *in situ* assays afford the opportunity to visualize events in individual cells in the context of the squamous epithelium. It is with these raft cultures that most of the novel insights have been gained.

The next task has been the delivery of individual viral gene(s) efficiently into PHKs in such a way that expression is maintained for the entire duration of the raft culture. Retrovirus-mediated gene transfer has served this purpose well. Our lab has shown that the E7 protein encoded by either oncogenic or non-oncogenic HPV genotypes can promote S phase reentry in the differentiated keratinocytes in raft cultures. Each of the E7 proteins examined targets p130, which is a member of the pRB tumor suppressor family, for binding and turnover. These studies additionally demonstrated that p130 normally maintains the homeostasis of differentiated cells that have withdrawn from the cell cycle. The loss of p130 due to E7-mediated destabilization then allows S phase reentry by the differentiated cells. HPV E6 protein is neither necessary nor sufficient to promote S phase reentry.

The overarching challenge has been to examine the complete HPV productive program in organotypic cultures. Efforts have been ongoing since 1996 in several laboratories. In those studies, wild type or mutant recombinant HPV DNA was excised from the vector and transfected into PHKs. Due to a low transfection efficiency and the few passages that PHKs can undergo before senescence, only cells immortalized by the
transfected oncogenic HPV DNA could be recovered and examined in raft cultures. Alternatively, a few selected immortalized epithelial cell lines were used as recipient cells. However, in raft cultures of immortalized cells, only a small fraction of the suprabasal cells supported a low level of viral DNA amplification and virus production. Passaging of progeny virions in raft cultures has not been achieved. Because of these limitations, investigations of virus-host cell interactions have been compromised. In particular, various E6 mutants that do not immortalize PHKs have not been studied and the function of E6 protein in the productive program remains unclear.

In Wang 2009, we reported a simple yet efficient and reproducible approach which overcame the limitations of the methods heretofore used by other investigators in the HPV field. We have recapitulated an exceptionally productive program in PHK raft cultures. The high titers of infectious HPV-18 particles are passaged successfully in naïve PHK raft cultures. The keys to the success are two-fold: first, the efficient transfection of supercoiled plasmids into PHKs, as opposed to the very poor transfection of non-supercoiled DNA; second, the efficient generation of HPV genomic plasmids via Cre-LoxP mediated recombination in vivo. Together, they obviate the need for immortalized cells. In our design, the parental bacterial plasmid harbors the HPV genomic sequence flanked by LoxP sites, along with the bacterial Neomycin-resistance gene. The placement of the LoxP site in the HPV genome has been experimentally chosen in order not to disrupt the regulation of viral transcription, protein expression and DNA amplification. Upon co-transfection with a separate nls-Cre expression plasmid, recombination generates an HPV-18 genomic plasmid and a residual vector plasmid, each with a 34 bp LoxP insertion. The resulting plasmids presumably maintain a physiologically
appropriate degree of negative supercoiling critical for the initiation of viral genome transcription and replication. Within a week of DNA transfection, drug-selected keratinocytes are used to develop organotypic cultures. By 2 to 2 ½ weeks at the medium-air interface, a very high percentage of the differentiated keratinocytes support robust viral DNA amplification and the major capsid protein L1 is widely detected in the superficial and cornified strata. In characterizing this system, we have gained many novel and intriguing insights into virus-host interactions in the differentiated squamous epithelium as described below.

Electron microscopy has shown that progeny virion assembly takes place in superficial cells, but virions mature in the cornified strata in the oxidizing atmosphere of dying cells, resulting in the formation of paracrystalline arrays of mature particles. The virus yields are orders-of-magnitude higher than those produced in immortalized cell lines. We attribute the superior ability of PHKs to produce HPV particles to proper squamous differentiation (our unpublished observations), while immortalized cells apparently are impaired in this process. It is intriguing that, only at a multiplicity of infection (MOI) of 800 or higher, do the progeny viruses undergo an efficient productive infection. At lower MOIs, there is little or no viral DNA amplification or late protein expression. Yet at an MOI of 50, PHKs were clearly infected, as the early protein E7 induced PCNA in virtually all the differentiated cells. Thus, late gene expression is dependent on viral DNA amplification. This conclusion is contrary to a previous report that late gene expression is dependent on squamous differentiation but does not require viral DNA replication.9

As a routine, we expose the raft cultures to bromodeoxyuridine (BrdU) 6-12 hrs
immediately prior to harvest so that cells in S phase can be identified by the incorporation of BrdU into chromosomal DNA. Using tissue sections from time course experiments, we have determined a temporal order of the HPV productive program by fluorescence in situ hybridization detection of viral DNA amplification (DNA-FISH), concurrently with antibody detection of BrdU incorporation and E7-induced cellular proteins or viral proteins, as well as by DAPI staining of nuclei. These studies show that viral DNA amplification initiates in differentiated cells that are experiencing a prolonged G2 arrest. There was no significant colocalization of BrdU positivity with amplified viral DNA signals. Rather, viral DNA signals first appeared in cells with high levels of cytoplasmic cyclin B1, signifying G2 arrest. This conclusion is further substantiated by an increased colocalization of BrdU and viral DNA upon a 2-day long chase following a BrdU pulse. Furthermore, as viral DNA signals intensify, the viral E7 activity wanes and then ceases, as deduced from the concomitant reappearance of increasing signals of the p130 pocket protein, the target of E7. We deduce that this loss of E7 function initiates a switch from the early viral replicative phase to the late phase, during which the capsid proteins are expressed for virion morphogenesis. The loss of E7 activity also accounts for the loss of cyclin B1 in cells harboring amplified viral DNA and the inability of these cells to re-enter into another round of S phase and become positive for BrdU. This molecular portrait is in complete agreement with observations of patient lesions using one or two probes at a time. Without the ability to perform time course experiments and metabolic labeling of the specimens, characterizations of biopsies can only provide a snap shot at the time of harvest (for a review see ref. 10).

Why does viral DNA amplify in G2-arrested cells? We suggest that the cells
developed a strategy of just-in-time-delivery to maximize their resources such that at least some of the host DNA replication machinery or substrates are not in excess to support viral DNA amplification while cellular DNA replication is taking place. Viruses adapt to this constraint by causing G2 arrest, thus creating a window of opportunity for their own amplification. Our observation might well explain why a number of other viruses also arrest cells in G2 phase (for a review see ref. 11).

Several publications have reported that ectopic expression of the abundant cytoplasmic E1^E4 protein (derived from a spliced mRNA) of several HPV genotypes causes G2 arrest in cell lines grown in monolayers. G2 arrest is attributed to the sequestration of cyclin B1/cdk1 to the cytokeratin filaments by the E1^E4 protein, which also collapses the intermediate filaments into a perinuclear aggregate. Additionally, mutants of several HPV types affect adversely viral DNA amplification. Another study indicates that ectopic E1^E4 protein causes the detachment of mitochondria from the microtubules, leading to apoptosis.11 Here, we put these hypotheses to a test in our productive raft cultures. The time course experiments demonstrated unequivocally that cytoplasmic accumulation of cyclin B1 in the lower and mid spinous strata occurred days ahead of the appearance of abundant E1^E4 protein and of viral DNA amplification. There was little colocalization of cyclin B1 and E1^E4 proteins. Rather, strong E1^E4 protein signals always coincided with amplified viral DNA in cells in the upper strata of the squamous epithelium and there were no perinuclear E1^E4 aggregates (Figure A; Chow et al. 2009 Figure A). We conclude that E1^E4 is a consequence of increased transcription from the abundant DNA template post-amplification, rather than the basis for G2 arrest, leading to viral DNA amplification. Furthermore, the major capsid protein
L1 was first detected at time points later than the E1^E4 protein and only in more superficial cells. Both proteins then stably accumulated in the cornified envelopes (Figure B and data not shown; Chow et al. 2009 Figure B). Indeed, a dedicated mRNA encodes E1^E4 at early times while a bicistronic mRNA encodes both E1^E4 and L1 at late times. The delayed expression of L1 relative to E1^E4 would suggest that high E1^E4 protein could not have caused cell death. In agreement, we did not detect apoptotic cells based on tissue histology, nor did we detect cleaved caspase 3 in these raft cultures. The
various properties attributed to the E1^E4 protein are likely due to its over-expression in cycling, undifferentiated cells that express low molecular weight keratins as opposed to the high molecular weight forms in differentiated keratinocytes. Our Cre-LoxP system will be ideal to elucidate the function of E1^E4 protein during the productive phase of the viral life cycle.

Wang (2009) have also shown that the Cre-LoxP system facilitates genetic analyses of HPV mutants not previously possible. An E6 mutation (deleted of the intron coding sequence in the spliced E6*I transcript), which cannot be stably maintained in transfected cells, has been successfully analyzed in PHK raft cultures following Cre-LoxP excision recombination to generate the mutant plasmid. The mutant is severely impaired in viral DNA amplification and L1 synthesis. It is, however, complemented in portions of the raft cultures by a retrovirus which expresses the wild type E6 from the HPV-18 enhancer and promoter, restoring viral DNA amplification and capsid protein synthesis. Thus, we conclude that E6 is essential for efficient viral DNA amplification. In the absence of viral DNA amplification, late proteins are not expressed. This conclusion is consistent with the results of low MOI infection experiments to which we referred earlier. The ability to trans-complement the E6 mutant with wt protein paves the way for a comprehensive characterization of E6 functions using additional E6 mutations. Unlike raft cultures harboring the wild type HPV-18 plasmid, a large fraction of basal and suprabasal cells in the E6 mutant-containing raft cultures accumulate high levels of the p53 protein, the target of E6 for degradation, as a result of E7 expression. Despite the sustained high p53 levels over 3 weeks, there is no apoptotic cells, nor cleaved caspase. We conclude that the role of E6 is not to overcome p53-induced apoptosis, as previously
proposed from studies in cell lines. In this respect, HPV oncoproteins have been reported to induce activated caspase 3 which then cleaved the amino terminus of the E1 helicase for efficient viral DNA replication.\(^\text{13}\) However, we detected no cleaved caspase 3 in our raft cultures containing wild type HPV-18, where viral DNA amplification is exuberant.\(^\text{8}\) The issue concerning E1 cleavage to enhance viral DNA amplification remains to be investigated.

In retrospect, we were extremely lucky in our experiments. It is important to realize that HPV genomes are initially cloned from DNA extracted from patient specimens rather than from purified virions. It was good fortune that the HPV-18 isolate cloned in 1983 by scientists at the German Cancer Research Center was a functional wild type and that it did not suffer any disabling mutations after repeated transfections into \textit{E. coli} as a recombinant plasmid. Indeed, some HPV-16 isolates have been reported unable to amplify in raft cultures of immortalized cells. Thus, to any lab which wishes to but is unable to produce their favorite HPV particles using our system, we would suggest that viral DNA should be recloned from benign patient lesions or from purified virions isolated from productively infected human epithelial xenografts in SCID mice.\(^\text{14}\)

In summary, the effective generation of HPV genomic plasmids via recombination \textit{in vivo} allows us to produce abundant infectious HPV-18 virions in raft cultures of acutely transfected primary human keratinocytes. By meticulous simultaneous \textit{in situ} probing for multiple targets in organotypic culture sections, this new system has allowed us to elucidate the infectious program while gaining many novel insights into the virus-host interactions during squamous differentiation. It will continue to provide opportunities to explore the roles of additional viral proteins in the viral life cycle and to
serve as an *in vitro* model system to verify potential therapeutic agents.

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References


CHAPTER 6
DISCUSSION

The *in vivo* Cre-LoxP generation of the entire HPV-18 DNA plasmid in PHKs is simple, expedient, highly efficient and reproducible. Organotypic cultures support exceptional viral DNA amplification and produce high titers of infectious HPV-18 virus (Wang et al. 2009). Critical factors include the efficient cotransfection of supercoiled plasmids and a virtually complete excision of floxed HPV from the parental vector by Cre recombinase expressed in PHKs. Under acute G418 selection, a pure population of cells containing HPV genomic plasmids is quickly obtained and ready for raft culturing within a few days (Ch. 3. Fig. 1). The extensive viral DNA amplification and virion morphogenesis resembled HPV-11 infected foreskin xenografts in nude mice (Stoler et al. 1990). Moreover, the progeny virions were able to elicit a complete round of reproduction in naïve PHK raft cultures, the first time that papillomaviruses have been passaged in tissue culture (Ch. 3. Fig. 4.; Wang et al. 2009. Fig. 3.).

Productive HPV Raft Cultures

Organotypic cultures are a staple in the genetic analysis of HPV. Without a means to propagate natural HPV, laborious and time consuming protocols to introduce extrachromosomai HPV genomes were reliant on immortalization of keratinocytes by HR papillomaviruses or starting with pre-immortalized cell lines (Frattini et al. 1996). Several research groups introduced the entire HPV genome and generated epithelial tissue that produced viral particles: 1.) Both HPV-11 and HPV-16 infected human tissue,
grafted under the renal capsules of athymic or on the skin of SCID mice produced a large of progeny virus (Kreider 1987; Bonnez 2005). However this system is not amenable to HPV genetic studies. Further, these mice are expensive and production of HPV takes several months. 2.) An excised recombinant HR-HPV genome was recircularized and cotransfected with a neomycin resistance plasmid into PHKs. Under prolonged selection, immortalized colonies carrying episomal HPV were expanded. Raft cultures were able to support a low level of viral DNA amplification and virus production (Frattini et al. 1996; Flores et al. 1999; Meyers et al. 2002). Unlike the first system, mutant genomes can be introduced to examine viral gene functions, but neither LR-HPVs nor HR HPV mutant in E6 can be explored because they fail to immortalize PHKs and they senesce. 3) The first use of a Cre-LoxP strategy relied on two chimeric adenoviruses containing either floxed HPV-16 genome or expressing Cre. Coinfected PHKs generated recombined HPV-16 genomic plasmid, but also required extensive selection to expand immortalized colonies (Lee et al. 2004). While construction of such a virus can be time-consuming, adenoviruses themselves are highly infectious to humans, thus the chimera would require greater care in handling. Importantly, adenoviruses interact with many of the same host target proteins and pathways as do papillomaviruses, so the contribution of the papillomavirus would be hard to decipher in the presence of adenovirus E1A and E1 B activities. Overall, with the exception of athymic mice or SCID mouse, these several procedures for introducing HPV into cells to establish raft cultures were found incapable of supporting high levels of viral DNA amplification or virion morphogenesis, underscoring severe limitations as to the nature and scope of these studies (Meyers 1992, 2002, Frattini 1996, 1997b). When virus was isolated, infectivity assays required
immortalized cell lines (i.e. HaCaT or SiHa) for their relatively higher efficiency of infection over PHKs (Evander et al. 1997). Further, infection was verified by the detection of viral mRNA required detection by nested RT-PCR or RT-PCR followed Southern blot hybridization. These low efficiencies begin to explain minimal and inconsistent phenotypes observed when attempting to analyze HPV mutants.

**HPV Infection of Naïve PHKs**

Using the infectious HPV recovered, naïve PHKs were productively infected at high MOI’s, generating abundant viruses as determined by the wide-spread L1 antigen detection, similar to the raft cultures of DNA cotransfected PHKs (Wang et al. 2009). At low MOI’s, the cultures were negative for L1 even out to 22 day (data not shown, but see Wang et al. 2009. Fig. 3). However, the majority of the PHKs were infected as revealed by the mild dysplastic histology relative to uninfected PHK raft cultures of the same age, as well as by their E7 activities (Ch. 3. Fig. 4.; Wang et al. 2009. Fig. 3D). Furthermore, low MOI rafts exhibited occasional cells with low levels of HPV plasmid amplification (data not shown). Although the nonproductive infections could still be a limitation of the duration of the raft cultures beyond three weeks, it nevertheless resembles many laryngeal papillomas and low grade cervical lesions with few cells harboring amplified viral DNA or being positive for the L1 antigen, unless the patient is immunodeficient. The requirement for concentrated MOIs to effect a productive infection in PHKs might explain why the low virus yields from immortalized cells have never been shown to elicit a new round of productive infection.

**Viral DNA Amplification in G2 Arrested Cells**

Most intriguing was my observation that viral DNA amplification initiated in cells
following S phase, at G2 (Ch. 4. Figs. 1-4; Wang et al. 2009. Figs. 4,5,S2,S3).

Consistently, more intense viral DNA signals in nuclei were virtually always distinct from intense BrdU detection throughout the spinous strata (Ch. 4. Fig. 1). Repeated experiments demonstrated incorporated BrdU represented cellular DNA replication during the labeling period, some still at S phase when fixed, as verified by colocalization with cyclin A (Ch. 4. Fig. 2). Distinct viral DNA foci were occasionally detected in BrdU-positive cells, though they rarely also contained cyclin A. Equally compelling is the observation that these low HPV signals can be first observed in cells with elevated cytoplasmic cyclin B1, a hallmark of G2 arrest (Ch. 4. Fig. 3). Taking this a step further, using a pulse chase experiments-we verified HPV DNA amplification occurred in cells that largely or completely finished replicating DNA forty-eight hours earlier (Ch. 4. Fig. 4F). Collectively, our evidence strongly suggests that viral DNA amplification initiated after host DNA replication and in the G2 phase.

There have been hints that cellular DNA replication and viral DNA amplification may not exactly coincide. Transient replication assays in transfected C33A cells suggested that replication of HPV origin-containing plasmids took place late relative to host DNA replication. In another study, a delay in HPV-16 DNA amplification was suggested, though not detected, in raft cultures of immortalized epithelial cells. The authors showed that BrdU-positive cells occasionally colocalized with E1^E4 protein in the upper spinous layer following an 8 h BrdU exposure two days prior to fixation (Nakahara et al. 2005). However, in the absence of simultaneous in situ detecting viral DNA in relation to other viral or cell cycle markers and BrdU incorporation, in a time course as we did in this work, no definitive temporal interpretations could be derived.
Why would HPV DNA amplification lag behind host DNA replication until G2?
The simplest and most straightforward explanation is that the host replication machinery
is optimized to replicate its own chromosomes. Thus, attempts by HPV to initiate viral
DNA amplification are futile while the cell has engaged all the necessary replication
protein to replicate its own DNA. If this is the case, virally induced G2 arrest creates an
uncontested window of opportunity for a highly efficient and rapid amplification of HPV
DNA. In fact, a surprisingly large number of DNA and RNA viruses arrest cells in the G2
phase, including parovirus, hepatitis viruses B and C, baculovirus, Herpesviruses (type-
1, -4, -6, -7 and -8), coronavirus and HIV-1 (Oleksiewicz and Alexandersen 1997;
Braunagel 1998; Alisi 2005; Zhao and Elder 2005; Davy and Doorbar 2007). While some
reports note amplification of viral genomes arising from >4N nuclei, others found G2
arrest was required to express capsid protein(s) and form virions. Interestingly, BPV-1
DNA was observed to amplify in quiescent mouse cells with >4N DNA content (Burnett
1989; Alderborn and Burnett 1994). In light of my current observations in HPV-18 raft
cultures, their results may be reinterpreted that BPV-1 DNA amplified following S phase
and in G2. Perhaps this mechanism to replicate viral DNA is not novel, just not
previously revealed. My observations reported in Wang et al. (2009) and Chow et al.
(2009) are the first that clearly establish the sequential events of virally induced cellular
DNA replication and viral DNA amplification.

E1\(^\text{E4}\) Protein Does not Induce G2 Arrest

It has been reported that ectopic expression of the abundant cytoplasmic HPV
E1\(^\text{E4}\) proteins causes G2 arrest in cell lines grown in monolayers. G2 arrest is attributed
to the sequestration of cyclin B1/cdk1 to the cytokeratin filaments by the E1\(^\text{E4}\) protein,
which also collapses the intermediate filaments into a perinuclear aggregate.

Additionally, E1^E4 mutants of several, not all HPV types affect adversely viral DNA amplification. Another study indicated that ectopic E1^E4 protein expression causes the detachment of mitochondria from the microtubules, leading to apoptosis (Roberts et al. 1994; Davy et al. 2002, 2005, 2006; Nakahara et al. 2002; Knight et al. 2004, 2006; Peh et al. 2004; Wang et al. 2004; Wilson et al. 2005; Davy and Doorbar 2007). Our time course experiments demonstrated unequivocally that cytoplasmic accumulation of cyclin B1 in the lower and mid spinous strata occurred days ahead of the appearance of abundant E1^E4 protein and of viral DNA amplification (Ch.5. Figure A; Chow et al. 2009. Figure A). There was little colocalization of cyclin B1 and E1^E4 proteins. Rather, strong E1^E4 protein signals always coincided with amplified viral DNA in cells in the upper strata of the squamous epithelium. Thus, E1^E4 is a consequence of increased transcription from the abundant DNA template post-amplification, rather than inducing G2 arrest to enable viral DNA amplification. Moreover, there were no perinuclear E1^E4 aggregates. Furthermore, the major capsid protein L1 was first detected at time points later than the E1^E4 protein and only in more superficial cells. Both proteins then stably accumulated in the cornified envelopes (Ch. 5. Figure B and data not shown; Chow et al. 2009. Figure B). Indeed, a dedicated mRNA encodes E1^E4 at early times while a bicistronic mRNA encodes both E1^E4 and L1 at late times (Chow and Broker 2007). The delayed expression of L1 relative to E1^E4 would suggest that high E1^E4 protein could not have caused premature cell death other than the program cell death associated with terminal differentiation. In agreement, we did not detect apoptotic cells based on tissue histology nor did we detect cleaved caspase 3 in these raft cultures (Wang et al.
The various properties attributed to the E1^E4 protein are likely due to its over-expression in cycling, undifferentiated cells that express low molecular weight keratins as opposed to the high molecular weight forms in differentiated keratinocytes.

What then causes G2 arrest? The expression of E2 protein (Frattini et al. 1997a; Fournier et al. 1999; Thierry et al. 2004; Bellanger et al. 2005), E1 protein (Belyavskyi et al. 1994a, 1994b), E6 or E7 proteins alone (Thomas and Laimins 1998) have each been reported to affect G2/M progression. In PHK raft cultures expressing only HPV-18 E6 and E7 genes, there is an elevated transcription of cdc2, cyclin B, other G2/M genes and, most notably, the parallel up-regulation of the cyclin B/cdc2 inhibitor Wee1 gene, as determined by microarray assays (Garner-Hamrick et al. 2004). Our recent results in PHK raft cultures demonstrate that the expression of E7 alone causes cytoplasmic accumulation of cyclin B1 as well as cytoplasmic cdc2, which bore inactivating phosphorylations (N.S. Banerjee, T.R. Broker and L.T. Chow, unpublished observation). Presently, we have no data to indicate whether the E2 protein also plays a role, as suggested by other investigators.

A Critical Role for E6 in Viral DNA Amplification.

Using our efficient method to generate HPV genomic plasmids in PHKs, we were able to analyze, for the first time, the phenotypes of an HPV-18 E6^I mutant genome. The cultures have high level p53 protein induction, a result of E7 expression in the absence of E6, not observed in wild type HPV-18 cultures, but there was little viral DNA amplification and virtually no L1 antigen. Importantly, we were able to trans-complement this mutant with a wild type E6 expressed from a retrovirus which abolished p53 accumulation and restored the production of L1 synthesis in 30% of the cells. These
results implicate p53 in suppressing viral DNA amplification and emphasize that a key function of E6 is to eliminate this blockade. This interpretation agrees with transient replication (Lepik et al. 1998; Massimi et al. 1999) in which ectopic p53 inhibits HPV DNA replication. The very important conclusion that trans-complementation is possible will facilitate future analyses additional E6 mutations to dissect this multi-functional E6 protein.

The results with the E6*I mutant have several additional implications. First, the accumulation of E7-induced p53 did not lead to apoptosis and hence, the function of E6 is not to counter E7-induced apoptosis. Second, transcriptional repression of integrated URR is conditional. Ectopic expression of BPV-1 and HPV E2 proteins from a strong surrogate promoter represses the expression of integrated HPV E6 and E7 genes from the viral URR in cervical carcinoma cell lines such as HeLa and CaSki. These cancer cell lines then went into senescence or apoptosis (Desaintes et al. 1999; Francis et al. 2000; Goodwin and DiMaio 2000; Wells et al. 2000; DeFilippis et al. 2003; Teissier et al. 2007). In contrast, in our system, the integrated URR-E6 delivered by the retrovirus appeared to be regulated in a manner to permit successful trans-complementation. Had it been silenced by the E2 family of proteins generated by the E6*I mutant plasmids, p53 would have reappeared. This is clearly not the case. Third, ectopic expression of E1 and E2 proteins in cervical cancer cell lines cause repeated reinitiation of DNA replication from the integrated viral origin. Such endo-reduplications lead to DNA translocations and cause extensive cell death (Kadaja et al. 2007). Although we did not examine possible initiation of replication from the integrated URR-E6, we detected no abnormality in the raft cultures suggestive of extensive cell death. The differences between our results in raft
cultures and those in cervical cancer cell lines in vitro likely reside in the vastly different levels of viral proteins in these two systems. In the productive infection in PHK raft cultures, the levels of autoregulated viral protein expression never reaches to those achieved by ectopic expression from strong promoters.

A Switch to the Late Phase

The mid- and upper-spinous cells that contain abundant viral DNA were devoid of cyclin B and cyclin A and incorporated little or no BrdU, but they were positive for the p130 pocket protein, a target of E7 (Ch. 4. Fig. 4H; Wang et al. 2009. Fig. 5H) (Genovese et al. 2008). Moreover, as in patient specimens, only the live superficial cells and cornified envelopes were positive for the L1 capsid protein. Individually and collectively, these parameters are consistent with a loss of E7 activity and a switch to the late phase. What turns off the E7 activity? The viral E6 promoter responsible for the expression of both E6 and E7 gene might be inactivated by the increased levels of viral E2 protein (Dong et al. 1994; Wu et al. 2006). Alternatively, the E2 protein directly binds to and inactivates the E7 protein (Gammoh et al. 2006).

We propose the following scenario for the sequence of events in the productive phase. The viral E7 gene is expressed in the differentiated cells, resulting in p130 destabilization and S phase reentry. Following host chromosome duplication, the cell transits into G2 phase and is arrested. Viral DNA amplification then takes place explosively in the G2-arrested, mid-spinous cells. E7 expression then diminishes and ceases. In the absence of E7, cyclin B accumulation is no longer sustained. Meanwhile, the cells are no longer capable of destabilizing p130 and cannot enter into another round of S phase. Consequently, cells with high viral DNA are not strongly positive for cyclin
A, cyclin B or BrdU. Upon cessation of viral DNA amplification, an early to late promoter switch would then produce more E1^E4 mRNA, thereby dramatically increasing E1^E4 protein levels (Ch. 4. Fig. 4., Ch. 5. Figure; Wang et al. 2009. Fig. 5., Chow et al. 2009. Figure). Finally the capsid proteins are made in the superficial cells for virion morphogenesis (Ch. 4. Fig. 1B; Wang et al. 2009. Fig. 4B).

Spontaneous HPV Regression

In patients, lesions typically last for some 6-15 months and then resolve spontaneously. This clearance has been attributed to immune surveillance of the host. It is thus intriguing to note that, in our raft cultures which lack any immune system, an apparent regression of viral activity appeared to take place around day 14 or day 16 in repeated experiments (Ch. 4. Figs. 1-3 and data not shown; Wang et al. 2009. Figs. 4, S2, S3). Beyond this age, we observed a dramatic reduction in superficial live cells positive for viral DNA amplification or for the L1 antigen. The frequency of BrdU positive cells also reduced (data not shown, but see Ch. 4. Figs. 1-3, Ch. 5. Figure; Wang et al. 2009. Figs. 4, S2 & S3, Chow et al. 2009. Figure). Comparatively, rafts containing HPV-31b genomes also achieved maximal late transcript expression by day 12 that significantly diminished by day 16 (Ozbun and Meyers 1999). In both labs, a single wave of virus production occurred. The mechanism(s) underlying this apparent spontaneous regression or limitation to sustainable production is not understood.

Summary

In summary, we have developed a simple and reproducible method to recapitulate a highly productive program of HPV infection in organotypic cultures of PHKs. It is no longer necessary for the HPVs under investigation to possess the immortalization
functions or to use pre-immortalized epithelial cell lines. This method is ideal for genetic and molecular analyses of all HPV mutants. Indeed, we have successfully examined an immortalization-defective E6 mutant, not previously possible. It will be interesting to test whether low-risk HPV types can also be studied in this system. In the course of our experiments, we have gained novel insights into viral gene expression, regulation of viral DNA amplification and virion morphogenesis. We envision that the viruses so produced will be most useful in studying the infection process and in testing potential agents to prevent HPV infection or to interfere with the infection program.

My multiplexed fluorescent detections established novel temporal and spatial trends relative to differentiation and cell cycle (within a specific cell and its microenvironment) appertaining to host-virus interactions. Though time-consuming and laborious (in situ: 2-4 days; microscopy and image processing: >1-2 wks), normalizing 2-3 parallel fluorescent detections to viral DNA content establishes trends that remained consistent throughout experiments.
CHAPTER 7

FUTURE DIRECTIONS AND PERSPECTIVES

The S phase induced in suprabasal keratinocytes by the full-length HPV-18 genome is veridical to previous studies expressing E7 only or E6-E7. Surprisingly, though HPV DNA typically accrues in the mid- to upper-spinous strata in situ, analogous to increases in cycling cells, I provide the first definitive temporal and spatial evidence that this critical step for HPV progeny formation is distinct from cellular S phase. While earlier publications had hinted this might be the case, that it was accompanied by a simultaneous cessation of HR E7 activity was inconceivable. For the first time, I demonstrate an inversion of E7 expression in cells with amplified HPV DNA. This loss of canonical early expression is underscored by the parallel stabilization of E1^E4 protein and eventual formation of infectious progeny virions. Two important conclusions are drawn from my in situ data: 1.) The stochastic induction of S phase by E7 (i.e. BrdU, cyclin A, etc.) does not represent the simultaneous timing nor the exclusivity of productively infected nuclei. 2.) In stark contrast, the uniformity of intense viral DNA that amplified throughout upper-spinous cells emphasizes HPV’s dependence on differentiation. In other words, the prevention of additional rounds of HPV induced cellular replication, followed by the initiation of HPV to replicate, are both direct consequences intrinsic to all terminally differentiating keratinocytes.

This begins to explain commonly observed delays in epidermal differentiation, as detected by cytokeratin 10 (CK10) and involucrin (IVL), in raft cultures of HR-HPV E7
immortalized cells (Collins et al. 2005). Since cell lines require HR \( E6-E7 \) expression to sustain neoplastic progression, limiting the potential for differentiation may prevent the inactivation of either the viral oncogenes or oncoproteins. This is emphasized in HPV-18 \( E6-E7 \) or \( E7 \) alone expressing PHK raft cultures that do not delay normal differentiation (Cheng et al. 1995; Chien et al. 2002). Another report found IVL, but not CK10, coincided with Late gene expression upon inducing an epithelial cell line containing extrachromosomal HPV DNA to differentiate (Ruesch et al. 1998). In the day 8 and day 10 cotransfected HPV-18 raft cultures, when the number of \( E7 \) induced S phase nuclei were maximum, there was no apparent delay in CK10 expression (Fig. 1). Epithelial differentiation was also maintained even in the presence of viral DNA amplification through days 12 and 14 (Fig. 1). At every harvest, both CK10 was expressed in all cells outside of the basal stratum, regardless of cell cycle state. These results emphasize the disparity between utilizing PHKs versus immortalized epithelial cell lines to recreate an \textit{in situ} environment to follow HPV pathogenesis.

Of note, in some of the upper-spinous cells strata with high viral DNA, there was a reduction or thinning of detectable CK10 (Fig. 1, day 12). This may be explained by the earlier observation that \( E1^*E4 \) protein binds to keratin filaments and reduces their ability to be detected (Doorbar et al. 1986; Davy et al. 2002, 2005, 2006; Knight et al. 2004). This phenomenon was attributed to the various monolayer cell cultures expressing a lighter-weight CK network that failed to support extensive \( E1^*E4 \) protein binding (Wang et al. 2004). Indeed, HPV-31b \( E1^*E4 \) protein does not collapse CK networks in a keratinocyte cell line harboring viral genomic plasmids (Pray and Laimins 1995). Further, extensive networks of fibrous \( E1^*E4 \) proteins were always detected in
Fig. 1. HPV-18 does not delay squamous epithelial differentiation. Thin sections from days 8, 10, 12 and 14 rafts were triple fluorescently labeled for the keratinocyte differentiation marker cytokeratin 10 (CK10) (Alexa Fluor 488, green) as well as HPV-18 DNA (Cy3, red) and BrdU (Alexa Fluor 647, Purple). Total DNA was stained with DAPI (blue).

superficial strata by day 12 in the HPV-18 raft cultures (Ch 5. Figure; Chow et al. Figure). IVL is readily detected in comparable regions throughout all time points, suggesting HPV likely does not affect the differentiation program (Fig. 2).

All cellular antigens detected in HPV-18 raft cultures can be found in control PHK epidermis at some point, though often less exaggerated and occasionally in different strata. Importantly, though HPV-18 rafts were more dysplastic, the number of S phase cells and the degree of differentiation of highly productive regions by day 14 began to appear similar to untransfected PHK raft cultures harvested on day 10 (Figs. 1,2).

This relationship is also apparent in the novel data depicting the initial appearance of amplified viral DNA from differentiated G2-arrested epithelial cells. The dramatic
Fig. 2. HPV-18 does not delay differentiation dependent Involucrin expression. Thin sections from days 8, 10, 12 and 14 rafts were triple fluorescently labeled for the keratinocyte differentiation marker involucrin (IVL) (Alexa Fluor 488, green) as well as HPV-18 DNA (Cy3, red) and BrdU (Alexa Fluor 647, Purple). Total DNA was stained with DAPI (blue).

accumulation of cyclin B in stochastic cells of day 10 HPV-18 raft cultures can resolve momentarily to delineate the boundary between cycling and productively infected keratinocytes on day 12. Interestingly, comparison of various day 10 HPV E6-E7 and E7 only raft cultures revealed significantly fewer differentiated cells stabilizing less intense cyclin B then equally aged HPV-18 full replicon rafts though both displayed a similar stochastic distribution (data not shown). While this pre-mitotic population of differentiated HPV-18 cells increased from day 8 to day 10 and steadily decreased with successive harvests until day 14, they typically remained directly proportional to S phase cells (as did E6-E7 and E7 only rafts) (Ch.4 Fig. 3.; Wang et al. 2009 Fig. S3). This suggests that every cell in which E7 inactivated the restriction point progressed through S
phase free of cellular interference and into G2. The successive reductions in the G2 population suggests that, independent of cell-mediated immunity and the humoral immune response, immortalization-free raft cultures intrinsically represses E7 induced cycling disproportionately to those cells amplifying viral DNA, making cyclin B also an inaccurate predictor of the productive infection.

Conversely, keratinocytes naturally increase proliferation to migrate and rapidly establish or reestablish the epidermis at sites of trauma. The proportionality of cycling to non-cycling epithelial cells may be more representative of this cellular state during HPV-induced hyperproliferation. Since the exposed artificial dermis of the raft culture system partially recapitulates the wound-healing environment, how is HPV pathogenesis divergent from actively proliferating and differentiating keratinocytes?

To gain snapshots of proliferating epithelia as through differentiation, day 2, day 4, day 6, day 8 and day 10 PHKs only raft cultures were probed for BrdU, CK10 and p21\textsuperscript{CIP1} (Fig. 3). For E7 raft cultures, p21\textsuperscript{CIP1} protein is stabilized in an inactive complex with Cyclin E throughout spinous cells, indicative of G1 arrest (Noya et al. 2001). Further, p21\textsuperscript{CIP1} accumulates in normal keratinocytes to arrest proliferation and commence the differentiation program (Di Cunto et al. 1998; Harvat et al. 1998). The day 2 epidermis was only 1-2 cell layers deep, with each cell being BrdU positive, contained some p21\textsuperscript{CIP1}, with little detectable CK10. As the cell layers increased at successive harvests, so did detection of CK10. BrdU positive cells receded by day 6 and day 8 to the basal strata, while p21\textsuperscript{CIP1} protein signals were greatly reduced in the spinous cells. By day 10 there were only a few BrdU positive basal cells and sporadic p21\textsuperscript{CIP1} spinous nuclei, while CK10 filled the spinous strata.
The p21\textsuperscript{CIP1} protein is stabilized in normal PHKs differentiation. Thin sections from days 2, 4, 6, 8 and 10 rafts were triple fluorescently labeled for the keratinocyte differentiation marker cytokeratin 10 (CK10) (Alexa Fluor 488, green), the G1/S arrest protein p21\textsuperscript{CIP1} (Cy3, red) and BrdU (Alexa Fluor 647, Purple). Total DNA was stained with DAPI (blue).

Though with proportionally far more cells, the mechanisms governing PHKs as they establish the epidermis may not be that distinct from those influencing HPV pathogenesis. This becomes more evident in day 8 and day 10 HPV-18 raft cultures that stabilize p21\textsuperscript{CIP1} protein amidst proliferating epithelial cells, which contain very little viral DNA (Data not shown, but see Fig. 5). As this is proposed to be a defensive response to unscheduled E7-induced S phase to repress the virus, it was surprising to observe a transition from elevated p21\textsuperscript{CIP1} protein to amplified HPV-18 DNA. While \textit{CDKN1A} constitutively transcribes p21\textsuperscript{CIP1} mRNA in differentiating epithelia, the protein is perpetually degraded. The p21\textsuperscript{CIP1} protein is stabilized in an inactive complex at G1 with cyclin E-Cdk2. In either PHK only or HPV-18 raft cultures, the p21\textsuperscript{CIP1} protein level diminished in superficial CK10 positive epithelial cells prior to the appearance of squame
or viral DNA amplification. This suggests that CK10 does not simply represent a singular differentiated state, but encompasses the entire differentiation process.

To follow the transition of proliferating epithelia through differentiation, the same day 2, day 4, day 6, day 8 and day 10 PHKs raft cultures were probed with CK14, CK1, PCNA and gut-enriched Krüppel-like factor (GKLF or KLF4). Once imaged the same slides were antigen retrieved, reprobed for Filaggrin (Flg), Loricrin (Lor) and BrdU, then the same region was reimaged for the new detections (Fig. 4) It was immediately apparent that while the CK10 binding partner, CK1, represented a population of exclusive spinous cells, it also represented two additional differentiation states. CK14 is specific to the basal strata, underscored by colocalizing with BrdU and PCNA nuclei, thus overlap with CK1 should represent parabasal cells transitioning into the spinous strata. Both Flg and Lor are critical components that require enzymatic processing to establish the epithelial barrier. GKLF is a zinc-finger transcription factor that is necessary and sufficient to establish a functional epidermal permeability barrier (Morasso et al. 1999). Flg, Lor, GKLF and CK1 all colocalize in the most superficial epithelial cells as early as day 2 and day 4. As more of the CK14 strata differentiates, the CK1/GKLF population extends from differentiated cells undergoing cornification, to the fully differentiated strata and finally to cells transitioning from the parabasal into the spinous strata (Fig. 4). Of importance, activation of CDKN1A transcription is among the GKLF transcriptional targets that inhibits proliferation to promote differentiation and correlates to the p21CIP1 protein detected (Figs. 3,4) (Yonish-Rouach et al. 1991; Garrett-Sinha et al. 1996; Shields et al. 1996; Zhang et al. 2000; Chen et al. 2001; Sancho et al. 2003; Weiss 2003; Xia et al. 2004; Foster et al. 2005; Rowland and Peeper 2006). The lack of GKLF and p21CIP1
Normal PHK differentiation. Thin sections from days 2, 4, 6, 8 and 10 rafts were quadruple fluorescently labeled cytokeratin 14 (CK14) (Alexa Fluor 594, red), cytokeratin 1 (CK1) (Alexa Fluor 488, green), PCNA (Alexa Fluor 555, yellow) and gut-enriched Krüppel-like factor (GKLF) (Alexa Fluor 647, brown). Once imaged, slides were antigen retrieved and triple fluorescently labeled Filaggrin (Flg) (Alexa Fluor 488, orange) Loricrin (Lor) (Alexa Fluor 555, turquoise) and BrdU (Alexa Fluor 647, purple). Total DNA was stained with DAPI (blue).
protein in the terminal differentiating strata of HPV-18 raft cultures coincided with the appearance of amplified viral DNA on day 10 (Fig. 5A-C).

Future work can determine the molecular mechanisms surrounding this transition from early to late HPV productive programs. The observation that p21CIP1 protein foreshadows viral DNA amplification is both novel and unprecedented yet it makes sense and is testable. In response to DNA damage, the p53/p21CIP1 pathway slows or arrests cells at cell cycle checkpoints (Kachnic et al., 1999; Taylor and Stark, 2001; Gudkov and Komarova, 2003; Kohn and Pommier, 2005). Specifically, p21CIP1 mediates p53-dependent G1 growth arrest by inhibiting cyclin/cdk and PCNA to block cell cycle progression and DNA replication (Mandal et al. 1998; Smits et al. 2000; Abbas et al. 2007; Moldovan et al. 2007). The time the upregulation of the p53/p21CIP1 pathway provides the cell has been implicated to permit either repair of damaged DNA to prevent deleterious mutations accumulating, or depending on the nature and severity of the damage, to enter apoptosis (Fujimori et al., 1996, Weinert, 1998; Taylor and Stark, 2001; Han et al., 2002; Melo and Toczyski, 2002; Zhivotovsky and Kroemer, 2004; Furuta et al. 2006).

As mentioned earlier, HPV E7 expression in PHK raft cultures stabilizes inactive p21CIP1/cyclin E/Cdk2 complexes to arrest spinous cells at G1 in a p53-independent manner (Jian et al. 1998; 1999). Since the restriction point is bypassed in HPV infections by E7, this implies that p21CIP1 protein may more precisely indicate those cells on the cusp of S phase. In the absence of DNA damage, p21CIP1 protein pauses the cell in late G1, post-restriction point, until E2F-dependent gene expression accumulates sufficiently to promote the cell into S phase. In normal epithelia, suprabasal cells have ceased cycling
Fig. 5. HPV-18 amplified in terminally differentiating epithelia. Thin sections from day 10 HPV-18 raft cultures were triple or quadruple fluorescently labeled. A. For the basal keratin CK14 (Alexa Fluor 594, Red), differentiated keratins CK10 (Alexa Fluor 488, green). Imaged, antigen retrieved and reprobed for Flg (Alexa Fluor 488, Orange) and Lor (Cy3, Turquoise). Then following denaturation, HPV-18 DNA (Alexa Fluor 647, Purple). B. Triple fluorescent labeling for HPV-18 DNA (Cy3, Red), p21CIP1 (Alexa Fluor 488, Green) and BrdU (Alexa Fluor 647, Purple). C. Quadruple fluorescent labeling for CK14 (Alexa Fluor 594, Red), CK10 (Alexa Fluor 488, Green), HPV-18 DNA (Alexa Fluor 647, Purple) and GKLF (Cy3, Brown). Total DNA was stained with DAPI (blue).
at a G1 like state, as demonstrated by the stabilization of p130 pocket protein (Ch. 4. Fig. 4H; Wang et al. 2009. Fig. 5H). Yet the E7-independent stabilization of p21\textsuperscript{CIP1} protein in the differentiated strata at early PHK only raft culture harvests may constitute an arrested transit amplifying population following rapid expansion from a wounded/healing state. While p21\textsuperscript{CIP1} prevents these cells from excessively cycling, it may also indicate their proliferative potential. Further, subjugation of HPV to E7 induced p21\textsuperscript{CIP1} repression would maintain low levels of viral genomes by repressing the requisite S phase machinery. Thus inactivation of p21\textsuperscript{CIP1} through inducible shRNA in HPV-18 rafts at earlier harvests may demonstrate the state of the differentiated epithelia sufficient to support robust HPV DNA amplification. It is unlikely that this would increase the population of S phase cells since the majority of nuclei are likely similar to \textit{E6-E7} expressing raft cultures, >4N with only a stochastic relicensing population. The elegance of such an epithelial model for a viral infection is that, intrinsic to the differentiation dependent p21\textsuperscript{CIP1} cell cycle arrest, terminal differentiating dependent repression of \textit{CDKNIA} would serve as a signal for HPV to replicate and encapsidate preparatory to cornification. Additional detections of specific stages within differentiating epithelia would elucidate whether the alleviation of p21\textsuperscript{CIP1} permits viral DNA amplification or the environment that represses p21\textsuperscript{CIP1} arrest is required for the viral productive state. These should be specific to the spinous to granular transition as well as processes that establish the cornified envelope from the granular strata.
CHAPTER 8
MATERIALS AND METHODS

Plasmid construction, recovery of PHKs, DNA transfection into primary human keratinocytes, the preparation, development and harvesting of organotypic epithelial raft cultures, retrovirus transduction, PCR and Southern blot analyses for HPV-18 DNA excision and amplification, the fixation, sectioning and immunohistological analyses of raft culture tissues, transmission electron microscopy of raft culture ultrathin sections and HPV-18 virion recovery and titer determination and infectivity assays were performed by Dr. Hsu-Kun Wang (Wang et al. 2009).

Construction of HPV-18 E6*I parental plasmid

In the HPV-18 E6*I mutant, the intron E6 coding sequence (nt234-nt415) was deleted. I designed a four primer PCR mutagenesis to construct the pNeo-LoxP HPV-18 E6*I parental plasmid: two partially overlapping sense and antisense strand primers (A&B) recreate the E6*I splice, an upstream sense strand primer C spanning the Asc I site (nt7572) and a downstream antisense strand primer D spanning the Blp I site (nt823). First, a PCR product encompassing HPV-18 E6 was generated using primers C and D with HPV-18 genomic template. The 1142 bp PCR product was digested with the Xho I/Hind III sites and inserted into pBlueScript II (pBS), sequenced and utilized for subsequent as well as future mutational analyses.

To produce HPV-18 E6*I, pBS-18E6 template was used to PCR amplify a fragment from either side of the E6*I intron. Primers B and C generated the 541 bp
110

E6*I del 1 product and primers A and D generated the 432 bp E6*I del 2 product, which were both agarose gel purified. The two fragments were pooled as template and PCR amplified using primers C and D to generate the 952 bp Xho I-Asc I-HPV-18E6*I-Blp I-HindIII fragment. The E6*I fragment was cloned into pBS as before to produce pBS-18E6*I and verified by sequencing. The 931bp Asc I/Blp I 18E6*I fragment was repeatedly double digested alongside pNeo-LoxP-HPV-18, gel purified and ligated.

Unfortunately, I was unable to produce pNeo-LoxP-HPV-18E6*I. Hsu-Kung Wang offered his assistance and successfully inserted the Asc I/Blp I HPV-18E6*I fragment into the HPV-18 parental vector to generate his published HPV-18 E6*I parental vector (Wang et al. 2009).

Cell Culture

CaSki, HeLa and SiHa cells maintained in DMEM supplemented with 10% fetal bovine serum (FBS).

Oligonucleotide probe 3′-end labeling

HPV-16 16E6, 16E6*I or E7 mRNA. The 3′-half of either E6 ~40-mer probe targeted the same 20 bp upstream of the splice donor site (nt226-nt207) (Table 2). Probe specificity was designed into the 5′-half of either mRNA probe by extending into the

<table>
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<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>A</td>
<td>5′-ACTTACAGAGCGTGGCTGCAGGCCAGAAACC-3′</td>
<td>(nt204-nt233*nt416-nt441)</td>
</tr>
<tr>
<td>B</td>
<td>5′-CCGCGAGGCACCTCGTAAACTTCAATTCTTGTC-3′</td>
<td>(nt425-nt416*nt233-nt208)</td>
</tr>
<tr>
<td>C</td>
<td>5′-TAATGCTGAGGACAAATGCGGCGGCCTCTTGG-3′</td>
<td>(Xho I-Asc I (nt7572))</td>
</tr>
<tr>
<td>D</td>
<td>5′-ACGAGCTTGCTGAGCTTTTACTACTAGCTCAATTCTGGC-3′</td>
<td>(Hind III-Blp I (nt823))</td>
</tr>
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Table 1. PCR primer sequences to clone HPV-18 E6*I.

intron (nt248-nt227-nt226) to detect full-length HPV-16 E6 or across the resulting spliced junction (nt430-nt409^nt226) to detect HPV-16 E6*I. Additional antisense (nt580-nt539) or sense (nt539-nt580) 40-mer probes were designed to detect HPV-16 E7 message or to function as an annealing control.

A PerkinElmer 3'end-labeling kit was originally used to tail oligonucleotides specific for either the HPV-16 E6, E6*I, E6*II or E7 messages with a dATP/ biotin-16-dUTP mix at a ratio of 1:10, allowing for 3-5 biotinylated nucleotides per tail. While these labeled probes work well in detecting abundant mRNA messages, it is difficult to discern between lower expressed mRNAs and background. To improve detection of the lower expressed unspliced E6 message, primers were designed to PCR the 183 bp E6*I intron (nt226-nt409) and used as template for to biotin nick translate probe. The Roche Biotin-Nick Translation mix of biotin-16-dUTP to dTTP ensures every 20\textsuperscript{th}-25\textsuperscript{th} nucleotide of the newly synthesized DNA is modified. Now, instead of a single probe with 4-5 biotins per tail, we have a population of probes spanning a 183 bp region each containing 4-5 biotin labeled nucleotides.

Biotin nick translated FISH probe synthesis

Gel-purified DNA templates were nick-translated using the protocol outlined in the biotin: nick translation kit (NEN, Boston, Mass). Products (5–10\% of reaction) were

<table>
<thead>
<tr>
<th>HPV-16 oligonucleotide probes for biotin 3’-end-labeling</th>
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<tbody>
<tr>
<td><strong>E6</strong></td>
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<tr>
<td></td>
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<tr>
<td><strong>E6*I</strong></td>
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<td><strong>E7</strong></td>
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<tr>
<td><strong>E7 (sense)</strong></td>
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Table 2. Complimentary sequences used in RNA-TSA-FISH.
confirmed by agarose gel electrophoresis to be 50–500 bp in length. Labeling reactions were precipitated with 0.3 M sodium acetate/70% EtOH in the presence of 40 μg of cot-1 DNA, 60 μg of salmon sperm DNA and 20 μg of yeast t-RNA. The cot-1 DNA, yeast tRNA and salmon sperm DNA help to reduce background. The biotinylated HPV-18 probe precipitate was resuspended in 100 μl Hybrisol VII (Oncor, Galthersburg, MD) and stored at -20°C. Prior to use, probes were denatured at 74°C for 10 minutes, quick spun and placed on ice.

Tyramide signal amplified fluorescent *in situ* hybridization (TSA-FISH)

Raft sections were fixed with 4% paraformaldehyde and then treated with 3% hydrogen peroxide to quench endogenous peroxidases. Slides were treated with RNase A (100 μg/ml RNase A (Sigma)/2X SSC) for 1 hour at 37°C, washed (1X PBS), ethanol dehydrated and dried. For detection of RNA, slides were incubated with 10 μl of either biotinylated-oligonucleotide (1:10) or appropriate biotinylated HPV-18 probe (1:1).

Cover-slips were sealed over samples and the slides incubated overnight in a 37°C humid chamber. To detect DNA, dried slides were denatured (70% formamide, 2X SSC, pH 7, 72°C for exactly 2 minutes), ethanol dehydrated, dried and incubate with probe as with RNA.

After overnight incubation at 37°C, slides were washed 3x (50% formamide/2X...
SSC for DNA or 20% formamide/2X SSC for RNA) at 37°C for 10 minutes each, followed by a 1X SSC wash for 30 minutes at 37°C. At the following washes are preformed at r.t.: 1X SSC, 15 minutes (1x); 3% H2O2/1X SSC, 15 minutes (1x); 4X SSC/0.1% Triton X-100, 2 minutes (3x); 4X SSC, 2 minutes (1x).

Biotinylated probes were indirectly detected by incubating slides with streptavidin-HRP (SA-HRP) (1:100) in 4X SSC at 37°C for 30 minutes. Slides were washed 3x for 5 minutes in 4X SSC/0.1% Triton X-100 and TSA labeled using a fluorophore conjugated tyramide (1:100) (Alexa Fluor 488, Alexa Fluor 647 (Invitrogen) or Cy3 (PerkinElmer, Wellesley, MA)) per the manufacturer’s protocol. Slides were finally stained in 4’,6-diamidino-2-phenylindole (DAPI), mounted in Antifade with coverslips and sealed with clear nail polish.

Immunofluorescence (IF)

Antibodies used for IF were as follows: mouse anti-BrdU (Calbiochem, San Diego, CA) 1:50; mouse anti-cyclin B1 (Novocastra, Newcastle-upon-Tyne, UK) 1:25; mouse anti-cyclin A (Novocastra) 1:25; Mouse anti-p53 D07 (Novocastra) 1:50; PCNA (DakoCytomation) 1:250; mouse anti-Rb2 (BD Biosciences) 1:50; HRP-conjugated rabbit anti-mouse IgG (Invitrogen) 1:50; concentrated MultiLink biotinylated goat anti-IgG to either mouse or rabbit (BioGenex) 1:20.

Briefly, if required, slides were first subjected to antigen retrieval (0.01 M sodium citrate, pH 6.0) at 95°C for twenty minutes. After cooling to r.t., slides were rinsed in PBS and incubated with primary antibody either for 1 hour at r.t. or 4°C overnight. After washing, slides were incubated with the appropriate fluorescent, HRP or biotinylated (followed by SA-HRP) secondary antibody for one hour at r.t. Indirect labeling of HRP
by TSA was performed as described above for FISH. Slides were DAPI treated and sealed also as before.

Multiplexed RNA-DNA-FISH or DNA-FISH and IF

IF detection of antigens coupled to DNA-FISH was performed as previously published (Van Tine et al. 2005). Briefly, RNA is always detected first, followed by detection of DNA or antigens. IF was typically performed prior to DNA-FISH, with the exception of BrdU IF. To multiplex TSA reactions, prior to each additional round of detection, HRP labeling was quenched in 3% H₂O₂/PBS for 15 minutes at r.t. Samples were subsequently subjected to DNA-denaturation for FISH, antigen retrieval or immediate incubation with a primary or secondary antibody for IF. Distinctly conjugated tyramides were used for each sequential labeling reaction. TSA was always coupled to detections prior to denaturing conditions, whereas fluorescently conjugated secondary antibodies could be used after (i.e. BrdU). Slides could be quenched in 3% H₂O₂/PBS and reprobed as before, or DAPI-stained, mounted in Antifade and sealed with coverslips as before.

Image acquisition and processing

Images were acquired using the 10x, 20x or 60x objective lens on an Olympus AX70 fluorescence microscope equipped with Speicher filters (Chroma, Rockingham, VT) and a Carl Zeiss Axiocam HR digital camera. For each distinct label (Alexa Fluor 350, Alexa Fluor 488, Alexa Fluor 555, Alexa Fluor 565, Alexa Fluor 647, Cy3, Cy 5, FITC, Texas Red and DAPI) AxioVision software captured multi-layered color images (.ZVI). The dynamic range for each layer was preserved for processing in Adobe Photoshop CS2 (Adobe Systems, Mountain View, CA) by converting each original
fluorescent RGB detection into an individual TIFF file. Each fluor (i.e. DAPI, FITC, Cy3 and Cy5) was combined into a single, multi-layered image in which each detection can be independently adjusted.

For example, FITC fluorescence is not exclusively green, but a bluish-green with a hint of red (i.e. R050, G255, B100). Upon robust labeling, each fluorescing RGB component increases until saturation and the perceivable signal is a bright greenish/white. Thus the intensities of each fluor permits some semi-quantification when comparing regions. These relationships are typically lost upon pseudocoloring. Consider my four channel images: Cy5 was typically colored yellow by copying the red channel of the image and pasting it into the green channel. If each fluorescent detection was included in a common image, adjusting either green or red alone would disrupt the yellow labeling. An additional concern is that this pseudocoloring method eliminates the dynamic range of the RGB components. I have found that these relationships can be preserved by instead merging two copies of the same fluorescence: one with the original RGB profile and the other (in the case for yellow) swaps the red and green channels. In this way, areas of higher or lower efficiencies of labeling can more easily be distinguished and certain types of background can be resolved.
CHAPTER 9

REFERENCES


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CHAPTER 10
APPENDIX
IRB Approval
DATE:

MEMORANDUM

TO: Aaron Duffy  
Student Investigator

FROM: Sheila Moore, CIP  
Director, UAB OIRB

RE: Request for Determination—Human Subjects Research  
Work done on IRB Protocol #X981228006 – Mechanisms of Human Papillomavirus DNA Replication (Louise T. Chow, Principal Investigator)

An IRB Member has reviewed your description of your work conducted in conjunction Dr. Louise Chow on her protocol referenced above.

The reviewer noted that a description of the work should have been submitted prior to the start of the research in compliance with IRB policies and procedures. It was noted that the work would have been determined not to be subject to FDA regulations and would have been considered Not Human Subjects Research.

Note that any future changes to the above referenced projects should be resubmitted to the Office of the IRB for determination.

SM/hw