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A Novel & Efficient Method to Produce High Titers of Infectious HPV in Organotypic Cultures of Primary Human Keratinocytes

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A NOVEL & EFFICIENT METHOD TO PRODUCE HIGH TITERS OF INFECTIOUS HPV IN ORGANOTYPIC CULTURES OF PRIMARY HUMAN KERATINOCYTES

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A DISSERTATION

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A NOVEL & EFFICIENT METHOD TO PRODUCE HIGH TITERS OF INFECTIOUS HPV IN ORGANOTYPIC CULTURES OF PRIMARY HUMAN KERATINOCYTES

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BIOCCHEMISTRY AND MOLECULAR GENETICS

ABSTRACT

Infections by the high-risk human papillomavirus type 18 (HPV-18) or type 16 (HPV-16) can lead to cancers of the anogenital tract. Because the propagation of HPVs is restricted to differentiated human squamous epithelia, experimental studies of the productive program are conducted in organotypic cultures. The current methods are of low efficiency and time consuming and have relied on immortalized epithelial cell lines that do not support efficient viral DNA amplification or virion production, compromising HPV genetic analyses and obscuring normal virus-host cell interactions. I used Cre-loxP site-specific recombination to develop a relatively simple, fast and highly efficient system to produce HPV-18 genomic plasmids in primary human keratinocytes (PHKs). The transfected primary human keratinocytes can be quickly developed into organotypic raft cultures, obviating the need for immortalized cells or the immortalization function of the high-risk HPV types. Upon keratinocyte differentiation and early viral gene expression, the HPV-18 replicons amplified to high copy numbers in raft cultures. The major capsid protein (L1) expression was widely detected in superficial cells and in cornified layers on and after day 14 or 16. Electron microscopy examination revealed HPV-18 particles were packed in paracryatalling arrays in shrunken nuclei with condensed chromatins. These

virions infected PHKs efficiently and were passaged in fresh PHK raft cultures, a feat not achieved until now. Thus, this system should be a useful tool to study HPV mutants or low-risk HPV genotypes. I further verified the utility of this system in HPV genetic analyses. Indeed, I was able to analyze the phenotypes of the HPV-18 E6*I and E6-null mutant replicons which had not been studied previously in raft cultures due to their inability to immortalize PHKs or failure to be maintained in transfected PHKs as plasmids. The mutant genomes were highly deficient in L1 production but were complemented *in trans* by a retrovirus expressing wild type HPV-18 E6. In support of translating basic scientific capabilities into clinical outcomes, this system should also be highly informative for testing agents that can prevent or treat human papillomavirus infections.

Keywords: organotypic raft cultures of primary human keratinocytes, Cre-loxP site specific recombination, HPV-18 infection cycle, viral DNA amplification, HPV-18 E6*I and E6-null mutants, genetic complementation in *trans*

DEDICATION

To my dearest wife, Eun-Young, our sweet daughter, Michelle, my parents, sister, brother, my parents-, sisters- and brothers-in-law and all of my good friends

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TABLE OF CONTENTS

Page

TABLE OF CONTENTS (Continued)

Page

LIST OF TABLES

Table *Page*

INTRODUCTION

ROBUST PRODUCTION AND PASSAGING OF INFECTIOUS HPV IN SQUAMOUS EPITHELIUM OF PRIMARY HUMAN KERATINOCYTES SUPPLEMENTAL RESEARCH DATA (WANG, DUFFY, BROKER, AND CHOW)

SUMMARY

LIST OF FIGURES

Figure *Page*

INTRODUCTION

ROBUST PRODUCTION AND PASSAGING OF INFECTIOUS HPV IN SQUAMOUS EPITHELIUM OF PRIMARY HUMAN KERATINOCYTES

LIST OF FIGURES (Continued)

Figure *Page*

LIST OF ABBREVIATIONS

Ad	adenovirus
BrdU	bromodeoxyuridine
BS	binding site
CKII	casein kinase II
CRPV	cottontail rabbit papillomavirus
DAPI	4′,6-diamidino-2-phenylindole
E6AP	E6-associated protein
FISH	fluorescence *in situ* hybridization
H&E	hematoxylin and eosin
HR	high risk
HPV	human papillomavirus
HSPG	heparin sulfate proteoglycan
IF	immunofluorescence
IHC	immunohistochemistry
LCR	long control region
LR	low risk
MCS	multiple cloning sites
MOI	multiplicity of infection
MPF	mitosis promoting factor
ORF	open reading frame

Ori	viral origin of replication
PCNA	proliferating cell nuclear antigen
PHK	primary human keratinocyte
polyA	poly-adenylation
pRB	retinoblastoma susceptibility protein
SA-HRP	streptavidin-conjugated horseradish peroxidase
TAg	large T antigen
TPA	12-O-tetradecanoyl phorbol-13-acetate
TEM	transmission electron microscopy
TSA	tyramide signal amplification
URR	upstream regulatory region
VLP	virus like particles
wt	wild-type

CHAPTER 1

INTRODUCTION

Human Papillomaviruses

The papillomaviruses are small tumor DNA viruses with a non-enveloped protein capsid that harbors a double-stranded circular genome. Formerly they were classified in the family of papovaviruses (**pa**pilloma/**po**lyoma/**va**cuolating) but now comprise their own family. This is a result of better genomic and molecular characterization that reveals that polyoma and SV40 viruses are not particularly related to the papillomaviruses. PVs can cause hyperproliferative lesions called warts, papillomas or condylomata (according to the anatomic location) in many vertebrates from amphibians to humans. Cottontail rabbit papillomavirus (CRPV) was the first papillomavirus isolated and characterized by Shope in 1933 (Shope and Hurst 1933). Many of the principles of viral oncogenesis and the role of cofactors in neoplastic progression were derived from studies of CRPV over the next 30 years. Bovine papillomaviruses also emerged as important model systems for deriving the principles of viral infections and host neoplastic responses. Human papillomaviruses (HPVs) were first isolated and molecular cloned in the laboratories of Harald zur Hausen and Gerard Orth (Gissmann and Hausen 1976; Orth et al. 1977; Durst et al. 1983)

HPVs only infect human epithelial tissues, either cutaneous skin or mucosal lining. zur Hausen's lab identified that certain HPV types are highly associated with cervical

cancer. Over the years, about 120 genotypes of HPVs have been cloned from patient specimens and sequenced (de Villiers et al. 2004). Among the mucosotropic HPVs, infections by the high-risk (HR) genotypes, such as HPV-16 or HPV-18 and closely related types, can lead to cervical, penile, anal and a subset of head & neck / oral cancers. In contrast, infections by the low-risk (LR) genotypes, such as HPV-6 and HPV-11, rarely progress to cancer but do cause the preponderance of genital warts and laryngeal papillomas. Cervical cancer accounts for 6% of all malignancies in women. Each year, at least 470,000 new cases of cervical cancer are diagnosed worldwide and about 230,000 women die from invasive cervical cancer (Parkin et al. 2001). The Nobel Prize in Physiology or Medicine in 2008 was awarded to Dr. Harald zur Hausen in recognition of his discovery that human papillomaviruses cause cervical cancer and his contributions to the characterization of their interactions with the infected cells.

HPV Genome Organization and Protein Functions

Electron microscopy of negatively stained papillomavirions reveals non-enveloped particles with a size of ~55 nm in diameter (Orth 1999; Lowy 2001). Each virus has a circular double-strand DNA genome 7.4 to 8.2 kb in length. The PV genome is comprised of three main regions (Figure 1): a non-coding upstream regulatory region (URR), an early gene region, and a late gene region. The early and late regions are both followed by a poly-adenylation (polyA) site. Early regions contain E6, E7, E1, E2, E4 (which overlaps the E2 open reading frame) and E5 genes, plus a very short open reading frame called E8 which overlaps the E1 open reading frame (ORF). There is no E3 gene. The purpose of the early viral proteins is to modulate the activity of numerous host genes

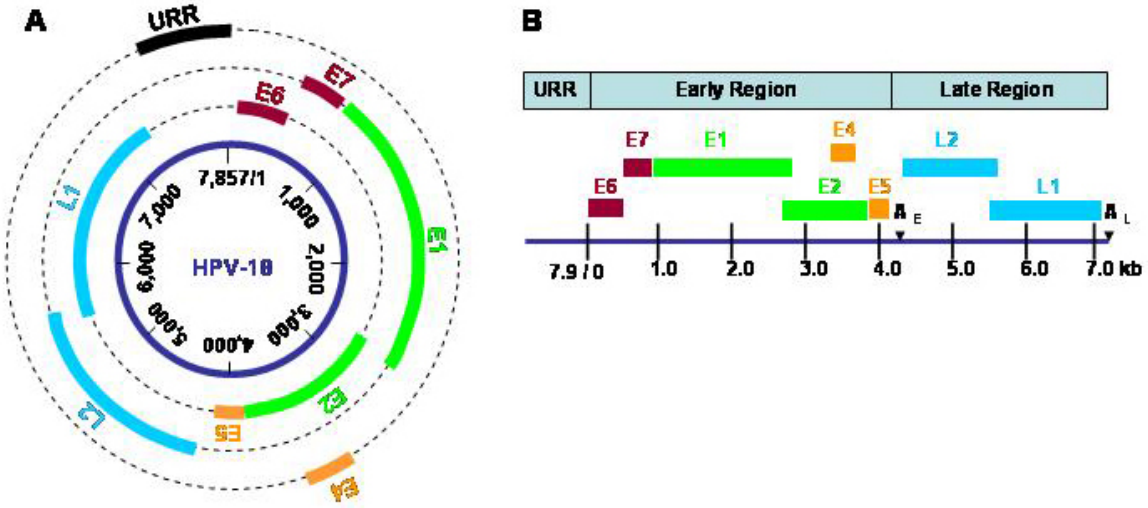

Figure 1. HPV-18 genome organization.
A, the circular genome. **B**, linear view. All genes are transcribed from the same DNA strand. The early and late gene blocks are each followed by a polyadenylation site.

or proteins and to support and regulate viral transcription and DNA replication. Late region genes L1 and L2 encode the major and minor capsid proteins, respectively. All the proteins are encoded by the same DNA strand, and the primary transcripts are derived from multiple promoters and then undergo complex patterns of mRNA splicing and alternative polyadenylation to generate a large number of mRNA species. While the basic transcription maps are similar, the various papillomavirus types can have different promoter locations and slightly different splicing patterns.

Upstream Regulatory Region (URR)

The URR is also called long control region (LCR) or non-coding region. It is shorter than 1 kb in length and is located immediately upstream of the early genes and downstream of the late genes in the circular genome. The URR contains the origin of DNA replication, one or more promoters, the late RNA polyA site and numerous transcriptional enhancer or repressor protein binding sites that are responsive to cellular transcription factors (reviewed by Bonnez 2002). In addition, the URR also contains several binding sites for viral proteins, for example, a cluster of E1 and E2 binding sites (E1/E2BS) for the E1 and E2 protein to bind, coordinate the assembly of pre-replication complexes and initiate HPV DNA replication. Binding of the E2 protein can also regulate the viral promoter responsible for the expression of the viral early genes (reviewed by Chow and Broker 1994). The sequence organization, function, and regulation of both the promoter elements and the replication origin in the URR are highly conserved and have been heavily studied in many papillomavirus types. Generally, the up-regulation of viral RNA transcription and DNA replication depends on the differentiation of the squamous

epithelium, but wounding and healing can also transiently stimulate these viral activities in the basal keratinocytes.

E6, E7and E5 Proteins

The structures of E6 and E7 proteins are relatively conserved among all papillomaviruses. These proteins contain two (E6) or one (E7) conserved Cys-X-X-Cys structural motifs, which form coordination complexes with zinc necessary for their proper folding and function. Remarkably, the PV E7 protein has key homologies and similar functions to adenovirus E1A (AdE1A) and SV40/PY large T antigen (TAg), while the E6 protein has sequence homologies to AdE1B 55 K protein and SV40/PY large T-antigen (reviewed by Chow and Broker 1997). The HR HPV E6 protein can bind the important host cell tumor suppressor protein p53 and, in cooperation with adaptor proteins, promote its ubiquitination and destabilize it. A highly conserved region of HPV E7, AdE1A and SV40/PY TAg proteins can bind with the pRB (retinoblastoma susceptibility protein) tumor suppressor and other related regulatory proteins. Together, targeting p53 and pRB plays key roles in the immortalization and transformation of cells (Dyson et al. 1989; Werness et al. 1990). The similarity of molecular mechanisms of transformation displayed by the small DNA tumor viruses was one of the important unifying concepts in virology and emphasized the crucial activities of p53 and pRB in controlling cellular responses to DNA damage, unscheduled DNA replication and cell cycling. Inappropriate and repeated expression of E6 and E7 of HR HPVs in basal or stem cells plays a major role in initiating as well as sustaining viral carcinogenesis (Jones and Munger 1996). In contrast to the HR E6 and E7 protein, the LR E6 and E7 proteins bind relatively weakly

to p53 and the pRB, do not promote their ubiquitination and degradation, and have weak or no immortalization activity (Munger et al. 1989; Gage et al. 1990; Werness et al. 1990).

The phosphorylation state of the retinoblastoma protein is regulated throughout the cell cycle. In G0/G1 phase, pRB is hypophosphorylated and binds to and inhibits the E2F family of cellular transcription factors, which controls genes necessary for S phase entry and cell cycle progression (Chellappan et al. 1991). Therefore, pRB binding with E2F acts as a negative regulator of the cell cycle. In late G1/S transition, pRB is phosphorylated by cyclin D/cdk4 or cdk6 complexes and is unable to bind to E2F; and de-repressed E2F activates transcription. However, the HR HPV E7, AdE1A and SV40TAg proteins can each bind to the hypophosphorylated form of pRB, causing the dissociation from E2F and thereby bypassing the requirement for cyclin D/cdk4 for S phase entry (Table 1) (reviewed by Chow and Broker 1997). To have full activity, the E7 protein must be phosphorylated by casein kinase II (CKII) (Munger et al. 1992; Chien et al. 2000; Genovese et al. 2008). The difference in binding affinity to pRB between HR and LR HPV E7 proteins, that may also play a crucial role in the immortalization function, has been attributed to a single amino acid change (eg. Asp 21 in HPV-16 E7 vs. Gly 22 in HPV-6 E7) (Heck et al. 1992). However, HR HPV E7 alone immortalizes PHKs very inefficiently. The synergistic activity of HR HPV E6 is necessary.

In addition to the effects on pRB, the E7 proteins of both HR and LR HPV genotypes can destabilize the pRB related p130 protein (Zhang et al. 2006). Our lab recently showed that p130 maintains the homeostasis of differentiated keratinocytes and that its destabilization by the high-risk or low-risk HPV E7 protein then allows S phase reentry by the post-mitotic differentiated keratinocytes and that E7 phosphorylation by

pRB and p130 Regulation of Replication Genes

G Phase	*Transition*	*S Phase*
Basal Keratinocytes		
HDAC : pRB : E2F2/3 Not bound to DNA, genes are inactive	cyclin D/cdk4 : pRB~P degraded.	HAT : E2F2/3 bound to S-phase genes, genes are activated
G0 and Differentiated Keratinocytes		
HDAC : p130 : E2F4 Bound to DNA, genes are inactive	HPV E7 : p130 : E2F4 degraded. E2F1/2/3 induced	HAT : E2F2/3 bound to S-phase genes, genes are activated

Table 1. E7 abrogates cell cycle regulation by pRB and p130.

CKII is critical for this activity (Munger et al. 1992; Chien et al. 2000; Genovese et al. 2008).

The HR HPV E6 protein alone is insufficient to immortalize PHKs; it needs to act in concert with the HR HPV E7 for efficient immortalization (Kiyono et al. 1998; Liu 1999). HR HPV E6 can activate the catalytic component of the telomerase hTert (Klingelhutz et al. 1996). In addition, HR HPV E6 binds to p53 and accelerates its degradation through the ubiquitination pathway (Scheffner et al. 1990). p53 is induced after DNA damage or unscheduled DNA replication. p53 is a transcription factor and induces p21cip genes leading to G1 arrest, and also transactivates DNA repair genes. If the damage is excessive, p53 induces apoptotic genes, leading to cell death. Therefore, the E6-induced p53 loss can allow unscheduled DNA replication to proceed and has been proposed to delay apoptosis to enable viral reproduction; as a consequence E6 inactivation of p53, cycling cells can accumulate mutations and DNA damage (Wu and Levine 1994). p53 destabilization is mediated by the E6/ E6-associated protein (E6AP) complex (Huibregtse et al. 1991), which acts as a E3 ubiquitin-protein ligase (Scheffner et al. 1993). The carboxyl terminus of HR HPV E6 protein also binds to and degrade may PDZ domain-containing host proteins that regulate cell polarity and proliferation ((Thomas et al. 2008). Collectively, all these activities are crucial to viral carcinogenesis.

Due to the alternative splicing of a substantial fraction of the primary transcripts that include the E6 gene, most of the translation products are carboxyl terminus truncated E6 peptides, called E6* (Schneider-Gadicke et al. 1988). These E6* peptides exert a dominant negative effect on the full-length E6 protein (reviewed by Thomas et al. 1999). Accordingly, not all of the p53 is degraded in HPV-induced cervical tissues (reviewed by

Mantovani and Banks 1999). Only the HR HPVs undergo intragenic and intergenic splicing and express E6* peptides. For example, HPV-16 has four E6* peptides (E6*I-IV) whereas HPV-18 has three. In cervical cancer cells, the E6* mRNAs are the most abundant species among E6 mRNAs (Schneider-Gadicke and Schwarz 1986; Smotkin and Wettstein 1986). E6* and full-length E6 proteins could have a dynamic balance and thereby appear to fine tune the levels of E6-mediated activities during HPV infection and cell cycle regulation. The role of E6 protein in the productive program of papillomaviruses is not known because such mutants are incapable of destabilizing p53, cannot immortalize PHKs, and the mutant genome is quickly lost in transfected cells.

The E5 protein of the bovine papillomavirus type 1 (BPV-1) is an oncoprotein because of its transformation ability in rodent cells (Groff and Lancaster 1985; DiMaio et al. 1986; Rabson et al. 1986; Schiller et al. 1986). E5 protein sequences are rather different among animal and human papillomaviruses, but all appear to be hydrophobic proteins that insert into membranes. Briefly, BPV-1 E5 interacts with and activates platelet-derived growth factor (PDGF) receptor. The up-regulated signal transduction of this receptor tyrosine kinase (RTK) then leads to cell transformation (DiMaio et al. 2000). For both BPV-1 E5 and HPV E5, their activity is mediated by an interaction with 16kDa vacuolar H^+-ATPase in the endoplasmic reticulum, resulting in alkalinization of the early endosomes. Thus, the degradation of RTKs is reduced whereas their recycling to the cell surface is increased (Disbrow et al. 2005) and refs therein). However, the specific RTKs in the human keratinocytes that are up-regulated by HPV E5 proteins have not been identified (Fehrmann et al. 2003; Genther et al. 2003).

As HR HPV-infected lesions progress toward high grade neoplasias and carcinomas, the episomal viral DNA usually integrates (more or less randomly) into a host cell chromosome. It follows a period of chromosome instability which may includes viral genome amplification, translocation to multiple other host chromosome sites, and strong selection for both particular HPV genomic deletions and silencing of most viral genomes imposed by DNA methylation. In the surviving clonal cells that comprise the tumor, only the E6 and E7 genes along with at least the amino-terminal portion of the E1 protein are consistently highly expressed, whereas other early genes including E2 and E5 are deleted, disrupted, or silenced after integration (Van Tine et al. 2004) and refs therein). Therefore, E5 only participates in the viral persistence and productive program in benign lesions but does not play an essential role in late stage of HPV-mediated carcinogenesis and tumor maintenance.

E1, E2 and E4 Proteins

Viral DNA replication requires the origin sequence, the viral origin recognition protein E2, and the replicative helicase E1. All other enzymes, replication proteins, and substrates are supplied by the host (reviewed by Chow and Broker 2006). The E1 protein is a DNA-dependent ATPase and a DNA helicase (Seo et al. 1993; Yang et al. 1993) which functions as a replication initiation protein and binds to an array of motifs in the viral origin of replication (ori). E2 protein has two domains which are conserved among HPVs: a DNA binding and dimerization domain in the carboxyl terminal region and a trans-activating domain located in the amino terminal region. The two domains are separated by an internal hinge region which is not well conserved among HPV types

except for very short motifs (Giri and Yaniv 1988; McBride et al. 1989). E2 protein binds the consensus sequence, $ACCN_6GGT$, as a dimer (Haugen et al. 1988; Hirochika et al. 1988; Spalholz et al. 1988; McBride et al. 1991). In addition to its role in recruiting the E1 to the origin, E2 can down-regulate the E6 promoter by binding to adjacent E2BSs in the ori (Androphy et al. 1987; Haugen et al. 1988; Hirochika et al. 1988; Spalholz et al. 1988; Li et al. 1989). Mutations in the E1 or E2 gene increases HR HPV immortalization efficiency (Romanczuk and Howley 1992).

The E4 ORF precisely overlaps the hinge region of the E2 ORF in a different reading frame and is translated from a spliced E1^E4 mRNA which derives 4 to 7 codons from the beginning of the E1 ORF (Nasseri et al. 1987). Although the E4 ORF is located in the early region, the protein is expressed at the highest levels in the upper, terminally differentiating strata and in cells with abundant viral DNA and the L1 protein (Breitburd et al. 1987; Crum et al. 1990; Palefsky et al. 1991; Brown et al. 1994). This is a consequence of the inclusion of the E1^E4 coding region in the late transcripts upstream of the L2 or L1 ORF (reviewed by Chow and Broker, 2007a). Indeed, it is the most abundant HPV protein in HPV-18 infected tissue (Chow et al. 2009). The E4 protein undergoes proteolytic cleavage to form amino terminal truncated peptides. They can form hetero- as well as homo-dimers. E4 is not essential for viral carcinogenesis nor is it for DNA replication (Hermonat and Howley 1987; Neary et al. 1987). The role of E4 protein in viral DNA amplification in squamous epithelium is not understood, as reports have not been consistent among different investigators (Fehrmann et al. 2003; Genther et al. 2003; Nakahara et al. 2005; Wilson et al. 2005; Fang et al. 2006). The common feature of the E4 protein family is that they bind cytokeratin fibers (Roberts et al. 1994). HPV-16 E4

over-expression in undifferentiated keratinocytes has been shown to induce the collapse of the cytokeratin network (Doorbar et al. 1991; Roberts et al. 1993) with the suggestion that this property may aid virion release from desquamated cell envelopes.

L1 and L2 Proteins

The HPV capsid is comprised of the late L1 and L2 proteins. L1 is the major capsid protein because 83% of the capsid is L1. HPV late gene expression occurs after vegetative viral DNA amplification. DNA packaging and virion assembly and maturation all occur in the terminally differentiating uppermost and cornified layers of the epithelium. Indeed, late gene expression are usually lacking in high–grade lesions for lack of terminal differentiation. Moreover, the HPV genome is often found integrated into host chromosomes of carcinomas-in-situ and invasive cancers, associated with deletion and/or or silencing of the late genes (described earlier).

The minor capsid protein L2 plays an import role in HPV entry into host cells. L1 alone or L1 plus L2 together can spontaneously assemble to virus like particles (VLPs) (Kirnbauer et al. 1992). The current HPV vaccines are based on VLPs composed of exclusively of the L1 protein. They provide only type-specific protection, with a small amount of cross-protection to closely related genotypes. For example, Cervarix contains the L1 VLPs from HPV-16 and -18 and Gardasil contains the VLPs from HPV types -16, -18, -6 and -11. None of them provides absolute protection against infections with other types of HPV. Therefore, the development of a vaccine with wider protection is necessary. HPV-16 L2 residues 17-36 are highly conserved among HPVs so this region

could be a candidate for developing antibodies to recognize for a wider-protection, L2 peptide-based vaccine (Kawana et al. 2003).

HPV Life Cycle

The reproductive program and life cycle of papillomaviruses are very different from other virus families. Infection requires wounding of the epithelium down to the basal strata and this is followed by possibly three modes of viral replication. 1. Establishment replication during which incoming virus maintains a steady state copy number (to approximately 20-50 copies / cell) during the healing phase of the wounded epithelium. 2. A persistent mode during which the low copy number is maintained in synchrony with host cell cycling, chromosome replication, and cell division (Gilbert and Cohen 1987). The two modes occur in the basal and parabasal layers of epithelium or, in the case of bovine papillomaviruses capable of forming sarcoids, in the dermal fibroblasts that BPV can also infect. Maintaining low copies in the basal cells of epithelium is one strategy for HPV to avoid the immune surveillance. 3. A vegetative mode, during which the viral DNA amplifies to very high copy numbers in a subset of differentiated cells of proliferative warty lesions. Viral DNA can quickly expand, achieving hundreds to thousands of DNA copies per cell, and then is packaged into progeny capsids in the superficial strata (reviewed byHowley 1996)

To infect the basal cells exposed upon wounding, the capsids first bind to heparin sulfate proteoglycan (HSPG) receptors (Joyce et al. 1999). Binding leads to a conformational change of the viral particle and unmasking of critical peptide motifs in L2, which then bind to a yet-to-be-identified secondary receptor (Yang et al. 2003). Through

endocytosis, the virions are delivered to endosomes for acidification and uncoating. L2 protein then guides the uncoated genome to the nucleus where enhancer proteins associated with wound healing are able to activate the transcription of the early viral genes. The early proteins then promote transient viral DNA replication to establish the infection and also cause a sustained cell proliferation to expand the infected cell population. Persistent infections can be maintained for years with little or no clinical symptoms of infection. Occasionally, the papillomavirus can activate, typically as a result of weakened immune surveillance or as a consequence of wounding of the latently infected tissue.

In normal squamous epithelium, spinous cells withdraw from the cell cycle to undergo squamous differentiation. In contrast, in HPV infected epithelium, as the keratinocytes differentiate, expression of the E7 and E6 early genes can recondition the non-cycling cells to reenter unscheduled S-phase, leading to the duplication of the host chromosomes. The viral DNA replication can then begin to enter the amplification and productive stage of the life cycle in the differentiation of the epithelial cells (Figure 2). L1 and L2 capsid proteins are expressed in superficial cells, in which virion assembly takes place. Finally, the nuclear membrane breaks down and mature virions are released within cornified envelopes as they slough off. The virions may re-infect the basal cells through wound healing and begin a new life cycle (reviewed by Chow and Broker 2007a). Of all possible experimental cell culture systems, this highly orchestrated differentiation dependent productive program can only be achieved in organotypic cultures of epithelial keratinocytes (reviewed by Chow and Broker 2007b).

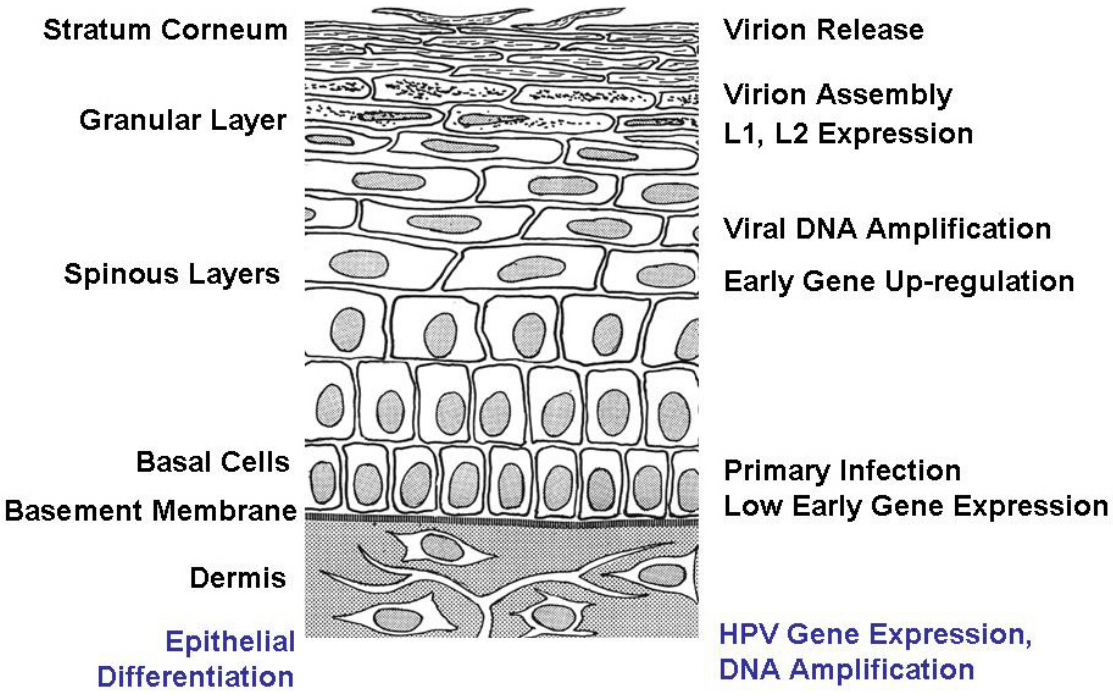

Figure 2. HPV productive program during squamous differentiation.
Adapted from Chow, L.T. and Broker, T.R. 1997. Small DNA Tumor Viruses. in *Viral Pathogenesis* (ed. N. Nathanson et al.), pp. 274. Lippincott-Raven Publishers, Philadelphia.

Organotypic PHK (Raft) Cultures

Papillomaviruses cannot propagate in regular, monolayer tissue cultures because the viral productive program completely depends on the progressive stages of differentiation in a squamous epithelium. An *in vitro* tissue culture system called epithelial or organotypic raft culture has become an invaluable tool for HPV researchers. The system was developed in late 1970s as a means to grow cutaneous epithelia for autologous skin grafting of patients with burns and other major wounds (Bell et al. 1983; Asselineau and Prunieras 1984; Kopan et al. 1987). The basic attribute is that the epithelial cells are placed on a dermal equivalent and grown at the liquid medium:air interface, just as real skin tissues have an air-exposed "dry" side as well as a "wet" internal side.

The system was first suggested as applicable to HPV research by Broker and Botchan (1986) and was first applied to modeling HPV-caused dysplasia in 1988 (McCance et al. 1988). Briefly, keratinocytes from neonatal foreskins, which are most conveniently and reproducibly available, are seeded onto a dermal equivalent consisting of a rat tail collagen bed containing J2 3T3 fibroblast feeder cells, and this is grown submerged in tissue culture medium. Once the PHK monolayer growth is almost confluent on the dermal equivalent, the whole assembly is raised to the medium-air interface for 10 or more days, and the cells stratify and differentiate into a full thickness terminally differentiated squamous epithelium (Wilson et al. 1992) (Figure 3). The raft culture medium contains growth factors, serum and a moderate concentration of calcium. Via capillary action due to evaporation from the tissue surface, nutrients are drawn upward through the dermal equivalent, calcium gradients are established through the

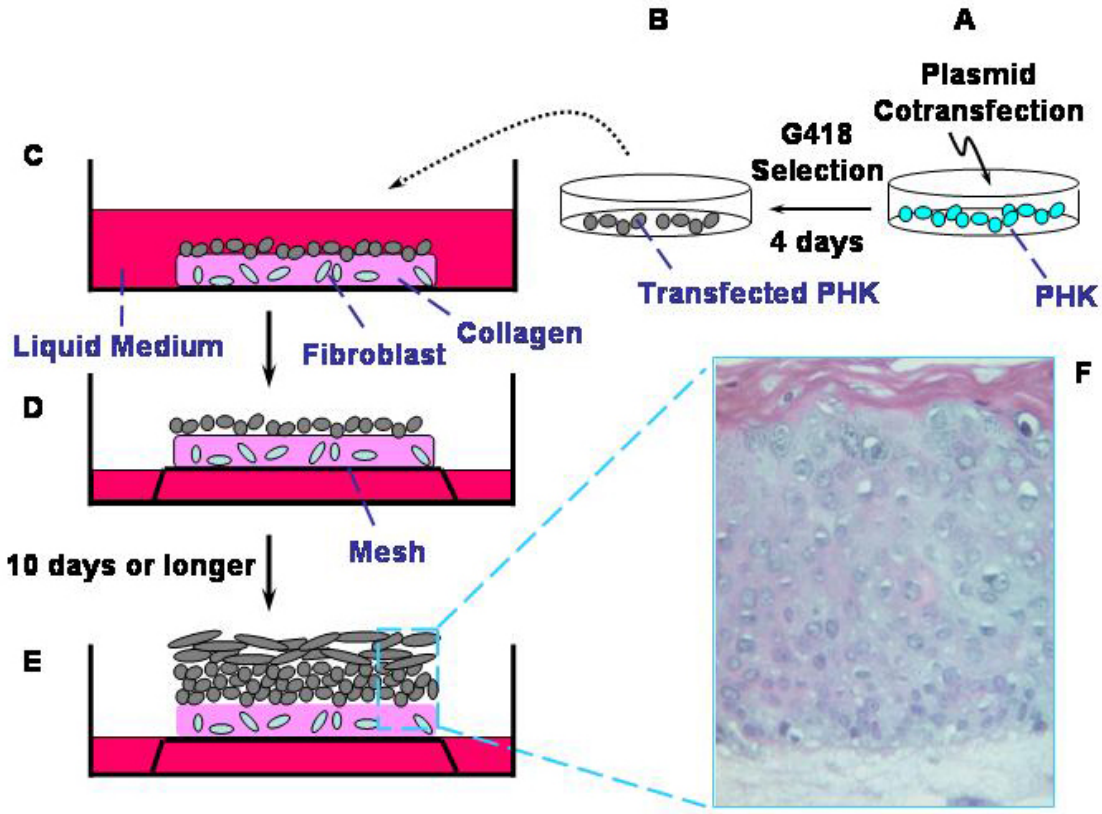

Figure 3. Organotypic raft cultures.
A, cotransfection of plasmids or infection with retrovirus into PHKs in submerged cultures. **B**, select for transfected cells by drug resistance. **C**, transfer cells to collagen bed and culture in K-SFM for 1 to 2 days. **D**, lift the assembly to air:medium interface. **E,** culture for 10 or more days whereupon cells differentiate into squamous epithelium. **F**, sectioning of formalin fixed and paraffin embedded tissue for histology and in situ analyses, or harvest fresh cultures for biochemical analyses.

developing squamous epithelium as the water continues to evaporate, and the conditions necessary for differentiation of PHKs are induced. Unexpectedly, using mouse J2 3T3 fibroblast as feeder cells for human epithelium raft cultures promotes better differentiation than using primary human foreskin fibroblasts (Wilson et al. 1992). In raft cultures, the expression of the differentiation markers (eg. involucrin or cytokeratins) is achieved (Wilson et al. 1992).

Using a variation of this raft culture system, Dollard et al. (Dollard et al. 1992) explanted small pieces of an experimental condyloma onto a dermal equivalent and culturing at the medium-air interface. The basal/parabasal cells grew out from the tissue, went through a phase of very high HPV gene expression comparable to that occurring during wound healing, and then stratified and differentiated on the dermal equivalent. HPV virions were detected by transmission electron microscopy in the most differentiated layers of the outgrowth. This experiment provided the proof-of-principle that such culture conditions are capable of supporting the viral productive program. The experimental condyloma was obtained by infecting chips of foreskin with an HPV-11 isolate and allowing it to develop into warty cysts under the renal capsule of an athymic mouse over a period of several months. This HPV-11 isolate was originally present in a pooled extracts from patient condylomata. It was successfully propagated in the foreskin xenografts in the athymic mice (Kreider et al. 1987). Meyers et al. (Meyers et al. 1992) used a cell line (CIN-612) which contained extrachromosomal HPV-31b for raft cultures and treated the CIN-612 by 12-O-tetradecanoyl phorbol-13-acetate (TPA) to induce differentiation. Virions were detected in the system. However, neither system is amenable

for HPV genetic analyses that would require introduction of mutant viral DNA into the keratinocytes prior to raft culturing.

Raft cultures usually completely stratify and differentiate between day eight to ten. While the basal cells divide to supply daughter cells that ascend and differentiate, the superficial cells undergo programmed cell death to form to the cornified layers (stratum corneum). After prolong culturing, the basal cells exhaust their proliferative potential; thus, cornified layers become prominent while live epithelium becomes very thin. In contrast, native foreskin tissue can be constantly replaced with new epithelial cells, probably from stem cells, so it can last a human lifetime). For the HPV-infected lesion, the transformation from low grade or mild dysplasia to high grade dysplasias and further on to carcinoma-in-situ is always a long term development. In place of a "long lived" raft culture system, raft cultures can be established with keratinocyctes immortalized by HPVs or with cervical carcinoma cell lines. The epithelium developed resembles moderate dysplasia or carcinomas (Steenbergen et al. 1998; Genovese et al. 2008)

Additional Systems for HPV Virion Production

There is no convenient source of HPV viral particles to study the wild type HPV life cycle in host cells, notably because most ano-genital lesions do not produce high titers of virions. A good tissue culture system for propagating HPVs in the laboratory did not exist when I began my research project. Only foreskin xenografts in nude or SCID mice could be used to produce wild type HPVs from virions initially harvested from rare patient specimens that contained ample HPV particles, as described above (Kreider et al. 1987). In an alternative approach, Sterling et al. (Sterling et al. 1990) transplanted a

human cervical cell line (W12) which carries extrachromosomal HPV-16 DNA (Stanley et al. 1989) onto nude mice skin. A small amount of HPVs could be detected in the differentiated W12 cells. These systems are expensive, not easily manipulated, and cannot be used for HPV genetic analyses.

Using a totally different approach, cloned and religated HPV DNA can be transfected into 293T or 293TT cells without undergoing replication and directly packaged into pseudovirions in cells expressing L1 and L2 proteins from cotransfected vectors. “High titers” of pseudovirions were generated (Figure 4) (Buck et al. 2004; Buck and Thompson 2007). However, there has not been any report to demonstrate that these particles can productively infect PHKs in raft cultures, nor was there any report on using this system to examine HPV mutants. In a collaboration with Buck and Schiller, our preliminary data showed that the pseudovirions that express a GFP reporter gene has low infectivity on PHKs. Nevertheless, they have been widely used for research on viral capsid structures, neutralization antibodies, and infection processes.

Limitations on HPV Genetic Studies

The current protocol to study HPV genetics in the context of full length viral plasmid and the squamous epithelium has many limitations. First, the viral genome is excised from the recombinant plasmid. The linear or recircularized viral DNA is then cotransfected into PHKs with a drug-resistance marker plasmid to select for transfected cells (Frattini et al. 1996). Because of the very low transfection efficiency of non-supercoiled DNA and because PHKs senesce after a few passages, only cells immortalized by the transfected HR HPV DNA can be recovered. However,

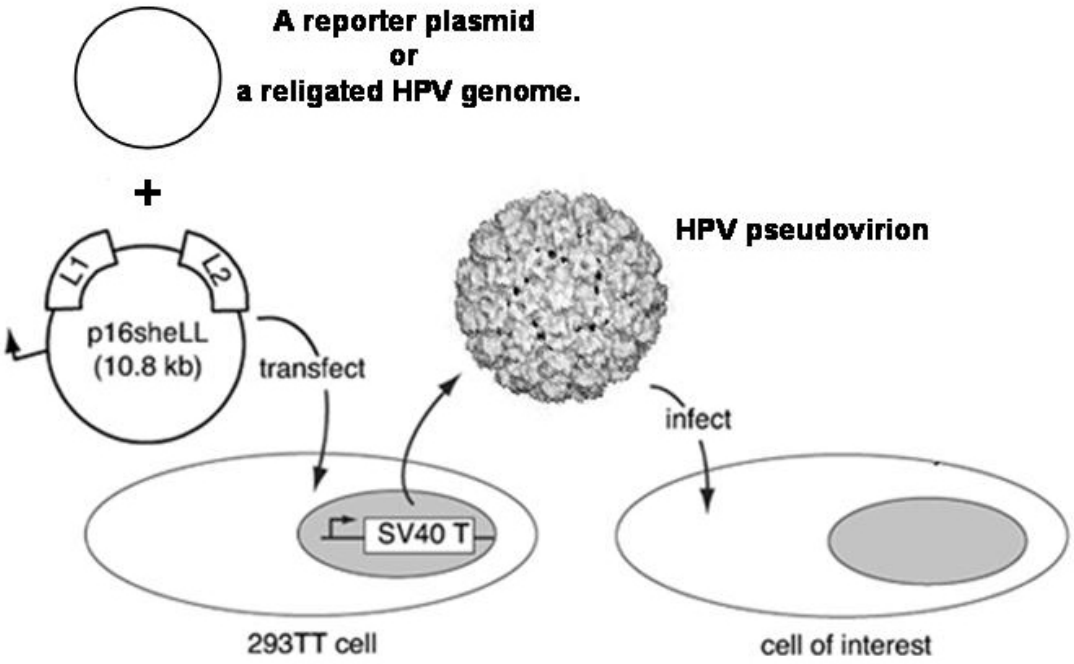

Figure 4. Production of pseudovirions.
Adapted from Buck et al. Current Protocols in Cell Biology, 26: 1.1-1.19, 2007.

immortalized cells often contain integrated DNAs, and they are completely unsuitable to study the viral life cycle. Only very few colonies still carried extrachromosomal HPV DNA and were useful for the studies of viral life cycle in raft cultures. Even in the presence of a PKC inducer to effect differentiation, these immortalized cells have not supported efficient viral DNA amplification or virion production. Consequently, the roles of the E4 and E5 genes in the productive program were examined by this system not resolved. Furthermore, this system cannot be used to study E6 or E7 mutants as they are incapable of immortalizing PHKs. As a result, the E6 function in the viral life cycle has not been elucidated. Selected immortalized epithelial cell lines have also been used (reviewed by Lambert et al. 2005). However they have a similar deficiency in being unable to support a highly productive program. Clearly, there is a need for a new strategy to generate HPV genomic plasmids in PHKs for analyses in raft cultures without extensive passaging or colony expansion.

Cre-LoxP Recombination

The Cre-loxP recombination system was discovered in bacteriophage P1. It mediates site-specific DNA recombination. Cre is a 343 amino acid protein and promotes recombination between two loxP sites. LoxP consists of a 34-bp DNA sequence containing two 13-bp inverted repeats and an asymmetric 8-bp spacer region (Sternberg and Hamilton 1981). Following the action of the Cre recombinase, the DNA sequence flanked by direct or inverted repetitions of loxP sequences is excised or inverted, respectively (Hoess et al. 1986; Stricklett et al. 1999). The Cre-loxP recombination system has been widely applied in gene knockout studies in transgenic mice (Sauer and

Henderson 1988; Liu et al. 2000). We considered that Cre-mediated *in vivo* recombination had the potential to generate at high efficiency HPV genomic plasmids in PHKs. Indeed I succeeded in developing such a system and the details are elaborated in Chapter 2, which is reprinted from the publication in **Genes & Development**, January, 2009.

Lee et al. (Lee et al. 2004) previously used the Cre-loxP strategy to construct a chimeric adenovirus in which the HPV-16 genome was flanked by direct repeats of loxP sites. Cervical or airway epithelia were then coinfected with another adenovirus expressing the Cre protein to generate the HPV-16 replicons in doubly infected cells. However, these authors ultimately relied on epithelial cells immortalized by the incoming HPV-16, and the system did not produce abundant HPV virions. Furthermore, it is quite complicated to construct such chimeric adenoviruses. Adenoviruses are highly infectious in humans, so the chimeric virus is potentially dangerous. Moreover, adenoviruses are extremely pathogenic in PHK raft cultures and target many of the same tumor suppressor proteins for inactivation (Balague et al. 2001). Thus, the results of HPV genetic analyses could be confounded.

Questions Addressed in These Projects

The purpose for this study has been to develop an efficient system to investigate the details of the HPV life cycle and the interactions between viruses and host cells associated with benign infection. We constructed an HPV-18 genome flanked by loxP recombination sites in a kanamycin/neomycin-resistant plasmid, pNeo-loxP HPV-18. My working hypothesis is that, when cotransfected into PHKs with an nlsCre-expression vector, an HPV genomic plasmid (harboring a 34-bp loxP site) will be excised efficiently

from the parental plasmid via Cre-mediated recombination. Acute selection with the neomycin analog G418 would then eliminate all untransfected PHKs. Raft cultures could be established within one week of transfection without any selection for viral E6 or E7 functions. In the absence of immune surveillance, the HPV replicon should then efficiently amplify in differentiated strata in raft cultures and virions should assemble in the superficial cells, as in foreskin xenografts in nude mice, recapitulating the entire sequence of viral-host interactions, viral gene expression, DNA amplification and packaging, and virion maturation and shedding (Figure 5). To construct the system, the following objectives needed to be addressed step by step:

1. Construction of a parental plasmid, pNeo-loxP HPV-18.

The vector that we use for the system should be able to amplify in bacteria and also be selectable in PHKs. To increase the efficiency of transfection, a small size of parental plasmid is desirable. pEGFP-N1 is a candidate for both purposes and it is commercially available. The kanamycin resistance gene can be selected with G418 in mammalian cells, and its size is small (~4.7kb). In order to reduce the vector size more, pEGFP-N1 was modified: A fragment of pEGFP-N1, the pCMV-MCS-EGFP sequences, was removed and replaced with its multiple cloning site (MCS), called pNeo.

Before inserting the fragment loxP flanked HPV genome into pNeo, the site at which the HPV genome is linearized has to be preselected. After the genome is re-circularized by Cre recombinase, leaving behind a single 34-bp loxP at the insertion site, the function of HPV replicon ideally should not be disturbed by the extra loxP insertion. After characterizing the resulting raft cultures after a reiterative process of using different

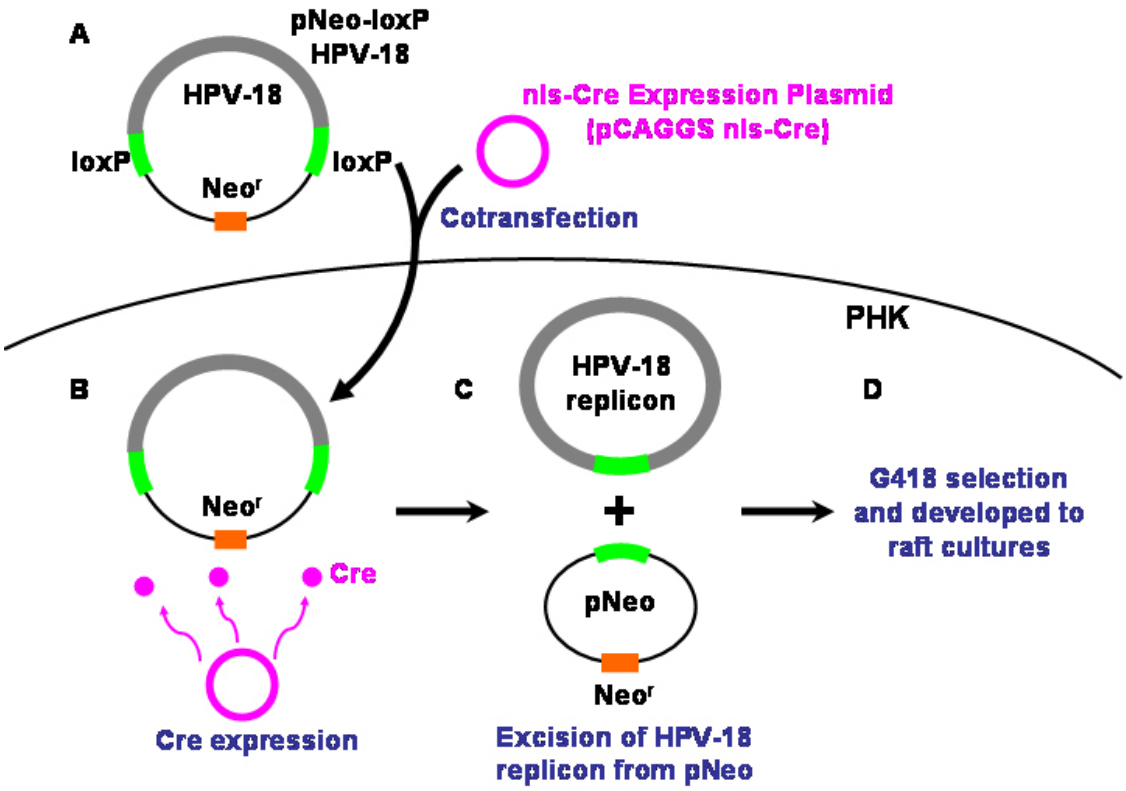

Figure 5. A simple and efficient method to Generate HPV-18 replicons in PHKs.
A, pNeo-loxP HPV-18 and pCAGGS nls-Cre were cotransfected into PHKs. **B**, Cre recombinase was transiently expressed from pCAGGS nls-Cre in PHKs. **C**, HPV-18 replicon was excised by Cre recombinase, generating HPV-18 replicon and pNeo vector each with one loxP site. **D**, after G418 selection, the PHKs were grown as raft cultures.

parental HPV plasmids and the different Cre expression system (see below), I found that opening HPV-18 between nt 7473 and nt 7474 is benign. This site is located downstream of the late polyA site in the upstream regulatory region in a region not known to have binding sites for viral or host factors critical for viral gene expression or DNA amplification. Before the HPV-18 genome was linearized, I inserted the fragment of 5'-loxP-Xho I-Xma I-loxP-3' into nt 7473-7474. Therefore, after linearization with Xma I, the genome became 5'-Xma I-loxP-HPV18 7474-7857/1-7473-loxP-Xho I-3'. Finally, the fragment was inserted between the Xho I and Xma I sites in the MCS of pNeo to generate pNeo-loxP HPV-18 (Figure 6A, B).

2. Co-transfection of pNeo-loxP HPV-18 and Cre expression plamid into PHKs.

After testing several Cre expression constructs, including a retroviral vector with a Cre-ER (Tam) fusion protein inducible by tamoxifen (Hayashi and McMahon 2002), I found that the Cre expression plasmid, pCAGGS nls-Cre (Hardouin and Nagy 2000), has the best function. It can transiently express Cre recombinase in PHKs and the plasmid is then lost after the PHKs divide. I also optimized the DNA ratio between pNeo-loxP HPV-18 and pCAGGS nls-Cre as well as the concentration of G418 needed to select the transfected cells in 4 days. After PHKs were selected, the evidence for and efficiency of Cre excision were examined by PCR amplification with appropriate primer sets and the selected PHKs were ready to develop into raft cultures.

3. Development of G418-resistant PHKs into raft cultures to assess HPV productivity.

The tissue histology, capacity for and patterns of bromodeoxyuridine (BrdU)

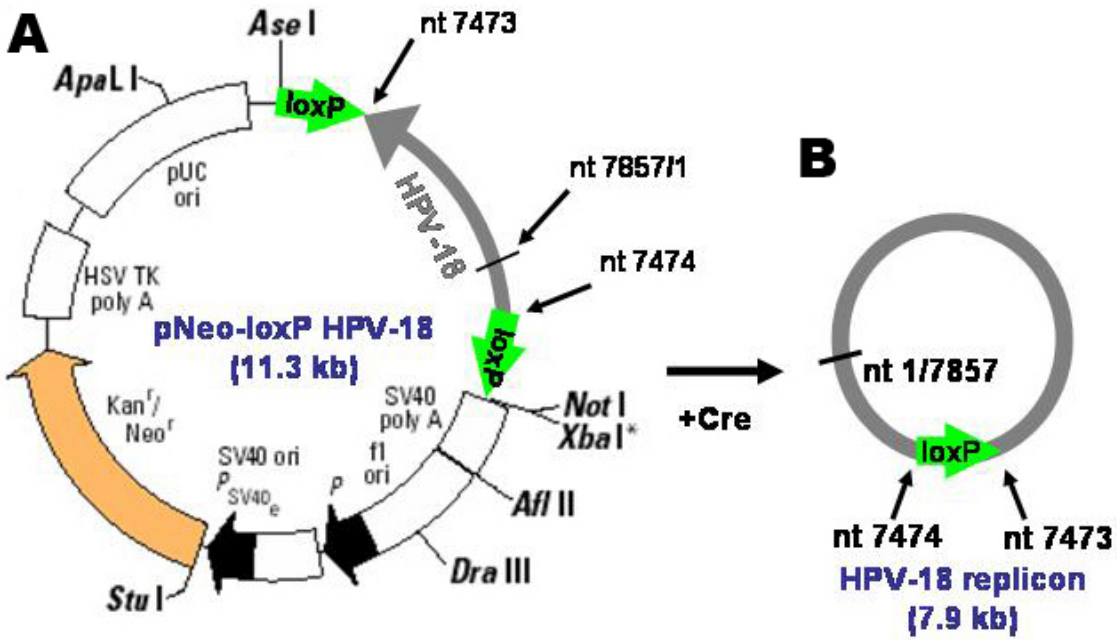

Figure. 6. Construction and excision of pNeo-loxP HPV-18 plasmid.
A, pNeo-loxP HPV-18: the CMV-EGFP in pEGFP-N1 was replaced with 5'-loxP-HPV 18-loxP-3' fragment. **B,** After excision recombination by nls-Cre, HPV-18 replicon generated carries a loxP fragment between nt 7473 and 7474.

incorporation, HPV DNA amplification, and major late protein (L1) expression were all systematically investigated. Additionally, the time course of HPV-18 containing raft cultures is helpful to understand the changes of HPV expression and viral-host interactions as a function of time and keratinocyte differentation. Viral particle assembly was visualized by transmission electronic microscopy of fixed, embedded, and thin sectioned specimens at a time point when L1 expression was high.

4. Isolation of progeny HPV virions and infectivity assay in PHKs.

HPV-containing PHK raft cultures were harvested at the time point when L1 expression was high, and HPV virions were purified from the epithelial layer of raft cultures. The titers of HPV virions were determined by real-time quantitative PCR and then used for PHK infection to test for infectivity. The ability to passage the virions was determined in submerged PHKs and in fresh PHK raft cultures.

More Questions Could Be Answered After The System Was Developed

*1. Characterization of an HPV-18 E6*I mutant.*

To demonstrate the utility of this system, a mutation in the HPV-18 E6 gene, E6*I was created. Unable to immortalize PHKs in collaboration with E7, this and other similar E6 mutants have not been studied and the role of E6 in the viral life cycle remains unknown. The E6*I peptide cannot degrade the target proteins of the wild type E6 protein. I decided to test my system using this mutant. The parental plasmid pNeo-loxP HPV-18 E6*I was constructed and also was co-transfected with the Cre expression plasmid to generate the HPV-18 E6*I mutant genome in PHKs. This HPV-18 E6*I mutant genome

was assessed in PHK raft cultures for its ability to amplify viral DNA and to produce progeny virions. It is defective in both activities. Importantly, successful *trans*-complementation of this mutant genome with a retrovirus which delivers HPV-18 URR-E6 rules out the possibility that the defective phenotypes are a consequence of unintended mutation in the genome. The successful *trans*-complementation also provided the necessary proof-of-principle that mutagenesis of E6 could be accomplished and the E6 functions elucidated.

2. Drug discovery and vaccine development.

HPV virions generated from the system can be used for tests initially conducted with pseudovirions using immortalized cells. Our results should be more relevant to HPV infection in patients than the existing system. For example, I tested the ability of carrageenan and the L2 antibody to inhibit HPV infection of PHKs.

Carrageenan is a family of linear sulphated polysaccharides extracted from red sea weeds. They are widely used in the food and other industries as thickening and stabilizing agents. Buck et al. (Buck et al. 2006) showed that carrageenan acts primarily by preventing the binding of HPV pseudovirions to immortalized human HaCaT ells. We believe that there is a need to use genuine virions and PHKs for validation.

The development of the next generation of broadly cross-reactive HPV vaccines based on an L2 peptide relies on pseudovirions containing a reporter plasmid (Gambhira et al. 2007). L2 is much less antigenic than VLPs assembled from L1 alone. An L2 peptide spanning residues 17-36 induces antibodies is cross-reactive with multiple HPV pseudovirions as well as with genuine cottontail rabbit papillomavirus (Pastrana et al.

2005). However, because the infectivity of pseudovirions is poor in PHKs, studies were performed using cell lines such 293TT or HaCaT. The neutralization results could be different from infection of PHKs with genuine HPV virions.

Thus, I initiated experiment in which genuine HPV-18 virions were used infect PHKs in the presence or absence of carrageenan and the L2 antibody. RT-PCR or RT nested-PCR to detect the HPV-18 E1^E4 transcripts was used to establish the effectiveness of inhibition or neutralization. In the future, quantitative RT-PCR will be conducted to determine the degree of inhibition at certain concentrations of carrageenan or at certain fold dilution of the antibodies.

CHAPTER 2

ROBUST PRODUCTION AND PASSAGING OF INFECTIOUS HPV IN SQUAMOUS EPITHELIUM OF PRIMARY HUMAN KERATINOCYTES

by

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THOMAS R. BROKER, AND LOUISE T. CHOW

Genes and Development 23:181-194

Abstract

Utilizing Cre-loxP mediated recombination, we established a highly efficient and reproducible system that generates autonomous HPV-18 genomes in primary human keratinocytes (PHKs), the organotypic raft cultures of which recapitulated a robust productive program. While E7 promoted S-phase reentry in numerous suprabasal differentiated cells, HPV DNA unexpectedly amplified following a prolonged G2-arrest in mid and upper spinous cells. As viral DNA levels intensified, E7 activity diminished and then extinguished. These cells then exit the cell cycle to undergo virion morphogenesis. High titers of progeny virus generated an indistinguishable productive infection in naïve PHK raft cultures as before, never before achieved until now. An immortalization-defective HPV-18 E6 mutant genome was also characterized for the first time. Numerous cells accumulated p53 protein, without inducing apoptosis, but the productive program was severely curtailed. Complementation of mutant genomes by E6-expressing retrovirus restored proper degradation of p53 as well as viral DNA amplification and L1 production. This system will be invaluable for HPV genetic dissection and serves as a faithful *ex vivo* model for investigating infections and interventions.

Introduction

HPVs are non-enveloped DNA viruses with a circular genome of ~7.9 kbp that replicates as multi-copy nuclear plasmids. Various HPV genotypes infect mucosal or cutaneous squamous epithelia, where they may cause benign hyperproliferation. A small fraction of infections by the mucosotropic high-risk (HR) HPVs (e.g. HPV-16, -18 and related genotypes) can undergo neoplastic progression to high grade intraepithelial lesions and cancers, specifically cervical, penile, anal, and oropharyngeal carcinomas (for a review, see zur Hausen 2002). In contrast, infections by low-risk (LR) types (e.g. HPV-6 and -11) rarely promote carcinogenesis. Wounding of epithelium is necessary to establish infections in basal keratinocytes, but viral DNA amplification only occurs in mid and upper spinous cells undergoing terminal squamous differentiation (Stoler and Broker, 1986). Major and minor capsid proteins, L1 and L2, express in the superficial strata of the epithelia where progeny virions assemble and eventually shed as cornified envelopes desquamate (reviewed by Chow and Broker 2007). HPV DNA replication depends on cellular replication machinery and substrates to complement the viral origin-binding protein E2 and the replicative helicase E1 (reviewed by Chow and Broker 2006). Because spinous cells have withdrawn from the cell cycle, HPV E7 protein destabilizes p130 pocket protein, thereby disrupting the homeostasis of the squamous epithelium and promoting S phase reentry (Zhang et al. 2006; Genovese et al. 2008 and refs therein). HPV E6 protein blocks p53 transactivating functions (Thomas and Chiang, 2005), though its significance in productive infections remains unknown; E6 alone is neither necessary nor sufficient to induce S phase reentry in spinous cells (Cheng et al., 1995). In addition, HR HPV E7 and E6 can destabilize pRB and p53, respectively (for reviews, see Münger

and Howley 2002; zur Hausen 2002). Thus their inappropriate over-expression in cycling basal cells can initiate neoplastic progression and, *in vitro*, immortalize PHKs.

Organotypic cultures of PHKs or selected epithelial cell lines have been a core format for HPV investigations. Briefly, keratinocytes stratify and differentiate into a squamous epithelium in 10 or more days when cultured on a dermal equivalent, consisting of collagen with embedded fibroblasts, and held at the medium:air interface. This "raft" culture system can support the viral productive program (Dollard et al. 1992; Meyers et al. 1992). Nonetheless, current methods for HPV genetic analyses in such cultures have severe limitations. Typically, linear or recircularized viral DNA, excised from a recombinant plasmid, is cotransfected into PHKs along with another plasmid encoding a selectable marker (for reviews, see McLaughlin-Drubin and Meyers 2005; Wilson and Laimins 2005). Since transfection of non-supercoiled DNA is inefficient and PHKs senesce after only a few passages, selection and expansion of drug-resistant colonies depends on HR HPV oncogenes to immortalize PHKs. One alternative has been to use certain immortalized epithelial cell lines as recipient cells (Fang et al. 2006b; reviewed by Lambert et al. 2005). In either case, only a small number of spinous cells in raft cultures of immortalized cells support viral DNA amplification and progeny virus production. Critically, there is no report that these low yields of virus can initiate a *de novo* productive infection in PHK raft cultures. Rather, infectious virus is demonstrated by detecting spliced early viral transcripts. Cell lines are typically used in these assays for their elevated susceptibility to infection over PHKs (Ozbun, 2002). This inefficient productive program could explain the inconsistent results for E5 and E4 mutants regarding their roles in viral DNA amplification. Moreover, immortalization-defective E6

mutants cannot be studied as the mutant plasmids were rapidly lost in transfected cells (Thomas et al. 1999; Oh et al. 2004). Thus, there is a critical need for an efficient strategy to obviate the need for immortalized cells and to conduct analyses in raft cultures of PHKs.

In this study, we have developed just such a system. It is highly efficient, yet simple and reproducible. Importantly, high titers of progeny virions elicited, for the first time, equally productive infections in fresh PHK raft cultures. The productive program revealed novel virus-host interactions. The significance of this new methodology is highlighted by the successful characterization of an immortalization-defective E6 mutant in PHK raft cultures. This new system would be invaluable for definitive genetic analyses of HPVs and for investigating infection processes and validating potential antiviral agents for intervention.

Results

Excision and amplification of the HPV-18 genomic plasmids in PHKs

Upon PHKs transfection with pNeo-loxP HPV-18 parental vector alone or together with pCAGGS-nlsCre, greater than 30% of the cells typically survived the acute G418-selection. Cre-mediated recombination should generate a circular 7.9 kb HPV-18 genome and a pNeo vector, each now with a single loxP site (Figure 1A). This 34 bp loxP sequence is placed In the upstream regulatory region (URR) between nts 7473 and 7474 of HPV-18, a site downstream of the late poly-A to which no host or viral transcription or other regulatory factors are known to bind. For simplicity, excised HPV-18 DNA is hereafter referred to as wild type since because, by all criteria, the patterns of its early and

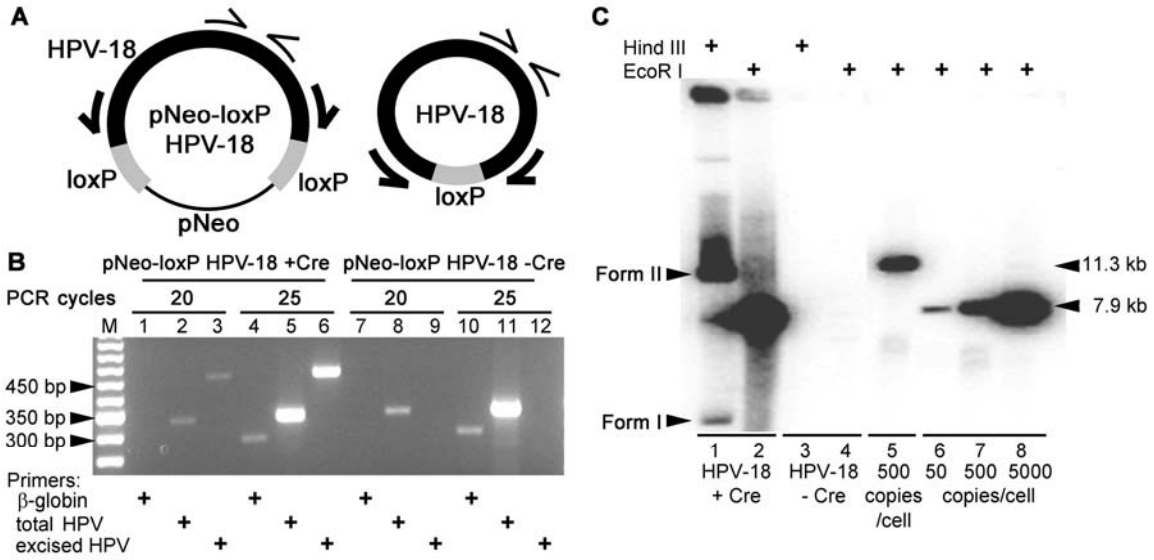
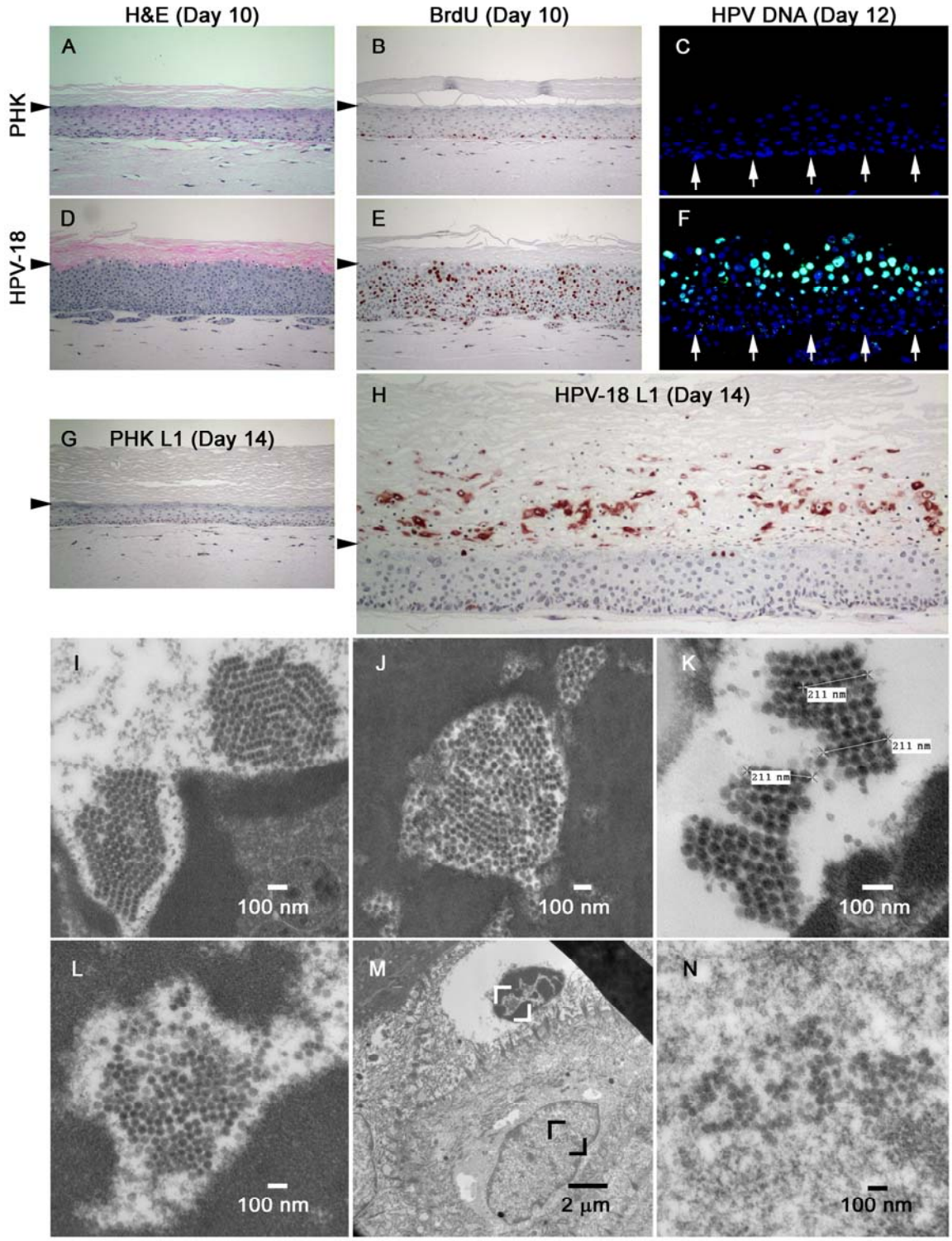


Figure 1. HPV DNA excision and amplification

(A) Schematic diagrams depicting the parental pNeo-loxP HPV-18 and Cre-excised HPV-18 plasmids and PCR primers (thin and thick arrows) to detect total and excised HPV DNA. (B) Ethidium bromide-stained agarose gel of PCR amplification products after 20 and 25 cycles, revealing total HPV DNA (332 bp), excised HPV DNA (450 bp), or β-globin gene (275 bp) from submerged PHK cultures after transfection with pNeo-loxP HPV-18 plasmid with (lanes 1-6) or without (lanes 7-12) the nls-Cre expression plasmid. M, DNA length markers. (C) Southern Blot hybridization for HPV genomic plasmid in day-12 raft cultures. Total DNA from raft cultures of PHKs transfected with pNeo-loxP HPV-18 in the presence (lanes 1, 2) or absence (lanes 3, 4) of the nls-Cre expression plasmid. Length and copy number standards for EcoR I-linearized parental plasmid (lane 5) or HPV-18 genomic DNA (lanes 6-8).

late gene expression are identical to genuine HPVs in productively infected patient specimens (reviewed by Doorbar, 2006). Most important, the virions generated were infectious in naïve PHK raft cultures, recapitulating the same productive program.

Total DNA isolated from G418-resistant PHKs was analyzed using PCR and primer sets specific for total HPV-18 DNA, excised viral DNA (Figure 1A), or β-globin gene as a positive control. In the absence of nls-Cre, only the total HPV DNA band was observed (Figure 1B lanes 7-12). In the presence of nls-Cre, the similar intensities of the total and the excised HPV-18 bands demonstrated efficient excision recombination (Figure 1B lanes 1 - 6). The excision- specific DNA band was confirmed by direct DNA sequence (data not shown). G418-resistant PHKs were also immediately developed into raft cultures. Total DNA from 12-day raft cultures was subjected to Southern blot analysis following digestion with a no-cut (Hind III) or one-cut (EcoR I) restriction enzyme (Figure 1C). The 11.3 kb parental plasmid was below detection in the presence or absence of nls-Cre (compare lanes 2-4 with 5). In contrast, in the presence of nls-Cre, an HPV-18 plasmid of 7.9 kb length amplified to approximately 2,000-5,000 copies per diploid chromosome equivalent in different experiments (compare lanes 1, 2 to 6-8; and data not shown). These results demonstrate that excision from the parental plasmid is prerequisite to HPV-18 DNA amplification in the squamous epithelium. The productivity per cell is an approximation. Although many of the cells have gone through S phase and could very well be tetraploid, viral DNA amplified only in a fraction of the cells (see Figure 2). On the other hand, the amount of host DNA remaining in the residual nuclei in the stratum corneum (see Figure 2) is not known.


H&E (Day 10)
BrdU (Day 10)
HPV DNA (Day 12)
PHK
HPV-18
A
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I
J
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211 nm
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Figure 2. Productive HPV-18-containing PHK raft cultures and visualization of HPV virions by TEM. (A-H), 4 μm sections of control (A-C and G) or HPV-18 containing PHK raft cultures (D-F and H) analyzed. Day-10 culture sections were stained with hematoxylin and eosin (A and D) or probed for BrdU incorporation (reddish brown, B and E) after a 12 hr exposure immediately prior to harvest. (C and F), DNA-FISH to reveal HPV-18 DNA (green) in a day-12 culture sections. Arrows point to the basal stratum. (G and H), immunohistochemistry to detect the major capsid protein L1 (reddish brown) in a day-14 culture sections. Horizontal arrowheads point to the boundary between the upper cornified layer and live epithelium below. Under the epithelium is the collagen matrix. (I-N), transmission electron microscopy of ultra thin sections of day-14 cultures to visualize virions. (I and J), paracrystalline arrays of mature virions in degenerated nuclei with condensed chromatins. (K), 3 measurements, each across 5 virions in a closely packed ordered array, gave the same value of 211 nm, or 42.2 nm/virion diameter. (M), two adjacent cells shown in low magnification. (L), the enlarged area (white brackets) in the upper cornified envelope with a degenerated nucleus. (N) the enlarged area (black brackets) in the lower, living cells with a normal nucleus.

Recapitulation of the HPV-18 productive program in raft cultures

H&E staining showed that day-10 HPV-18 plasmid containing raft cultures were mildly hyperproliferative when compared to untransfected PHK raft cultures (Figure 2A and D). When probed with an antibody to BrdU, positive cells were distributed stochastically in all cell strata of the HPV-18 raft cultures (Figure 2E), similar to patient specimens and raft cultures expressing E7 gene from a pro-retrovirus (Cheng et al., 1995; Genovese et al., 2008 and refs therein). In contrast, only basal keratinocytes in normal PHK raft cultures were BrdU-positive (Figure 2B). DNA-FISH of day-12 cultures revealed intense HPV-18 DNA nuclear signals in a high percentage of mid- to upper-spinous cells, with occasional punctate signals observed in basal and parabasal cells (Figure 2F). This abrupt increase in viral DNA is identical to the pattern consistently observed in naturally occurring HPV lesions (Stoler and Broker 1986). By day 14, abundant L1 antigen was readily detected in numerous cornified envelopes and in some superficial live cells across the entire length of raft cultures, verifying a highly productive program (Figure 2H). Cultures of control PHKs or PHKs transfected with pNeo-loxP HPV-18 alone remained negative for L1 and viral DNA signals (Figures 2C, 2G, S1C and data not shown). Multiple independent time course experiments showed that low L1 signals typically appeared on day 10 in a small number of superficial cells. Signals dramatically increased in the stratum corneum on day 12, and then leveled off by days 14 or 16, after which virtually all signals were restricted to cornified envelopes (Figure S1). This shut off in late gene expression in superficial live cells coincided with diminished host DNA replication (see Figure 4A and data not shown) and thinning of the raft

cultures (Figure S1). Raft cultures of immortalized cells with HPV-31b genomes also had reduced late mRNA levels after day 14 (Ozbun and Meyers 1997).

Visualization of HPV-18 virions in organotypic cultures

Examination of day-14 raft culture sections by transmission electron microscopy (TEM) revealed numerous viral particles in the cornified envelopes, each with a degenerated, shrunken nucleus containing electron-opaque, presumably highly condensed chromatins (Figures 2I, J). The persistence of residual nuclei in cornified envelopes (termed parakeratosis) is one the hallmarks of productive HPV infections. Viral particles were electron dense, icosahedral in shape and often formed paracrystalline arrays. Measurements with an internal scale of the TEM approximated the diameter of virions to be 42.2 nm (Figure 2K), consistent with previous reports that used similar TEM techniques (Dollard et al. 1992 and refs therein). Purified HPV virions are often referred to be 55 nm in diameter. The smaller size observed in tissue sections might be attributable to extensive dehydration procedures during sample preparation or to positive staining as opposed to the negative staining of purified virions.

We also observed what appeared to be slightly larger empty or immature particles, as they were of lower electron density or less distinct in overall shape and capsomere patterning. Paracrystalline arrays of mature virions were invariably disrupted by the inclusion of such immature or empty particles (Figure 2I, J, L). Interestingly, live superficial cells rarely contained highly ordered arrays of mature virions. For instance, the more superficial of two adjacent cells in Figure 2M had a degenerated nucleus, in which paracrystalline arrays of mature virions had begun to form (Figure 2L) while, a

live cell immediately below, with a normal-appearing nucleus, contained probably "immature" virions that were not yet organized into large ordered arrays (Figure 2N).

Productive infections of PHK raft cultures by HPV-18 virus

The titers of two independent virus preparations were determined by quantitative real time PCR to be 3.2 x 10^8 and 4.9 x 10^8/raft culture, each initiated from 2 to 2.5 x 10^5 PHKs. Virus infectivity was examined in submerged PHK cultures by detecting a spliced E6-E7-E1^E4 cDNA fragment using RT-PCR. The 521 bp cDNA was easily visualized in ethidium bromide stained agarose gels at an MOI of 42 and as low as 5 in other experiments (our unpublished results). The band intensity increased with the multiplicity of infection (MOI) and was absent in uninfected PHKs (MOI=0) (Figure 3A, left). The identity of the cDNA was verified by direct DNA sequencing (data not shown). RT-nested PCR detected the same cDNA fragment at an MOI of 2 (Figure 3A, right). In contrast, β-actin cDNA was detected in all samples (Figure 3B). As a further test, infected PHKs were developed into raft cultures and then harvested on day 14. At MOI of 800 or higher, cultures contained widespread L1 antigen-positive cornified envelopes (Figure 3C, left panel and data not shown). Unexpectedly, at MOI's of 400 and lower, the cultures had little or no L1 antigen (Figure 3C, middle panel and data not shown).

Interestingly, regardless of L1 productivity, all infected cultures were mildly hyperplastic relative to uninfected ones (Figure 3C, right panel), indicative of successful infection and early viral gene expression. To verify this interpretation, sections were probed for viral DNA and PCNA, a DNA polymerase δ processivity factor induced in suprabasal cells by HPV E7 (Cheng et al. 1995). In control PHK cultures, PCNA was

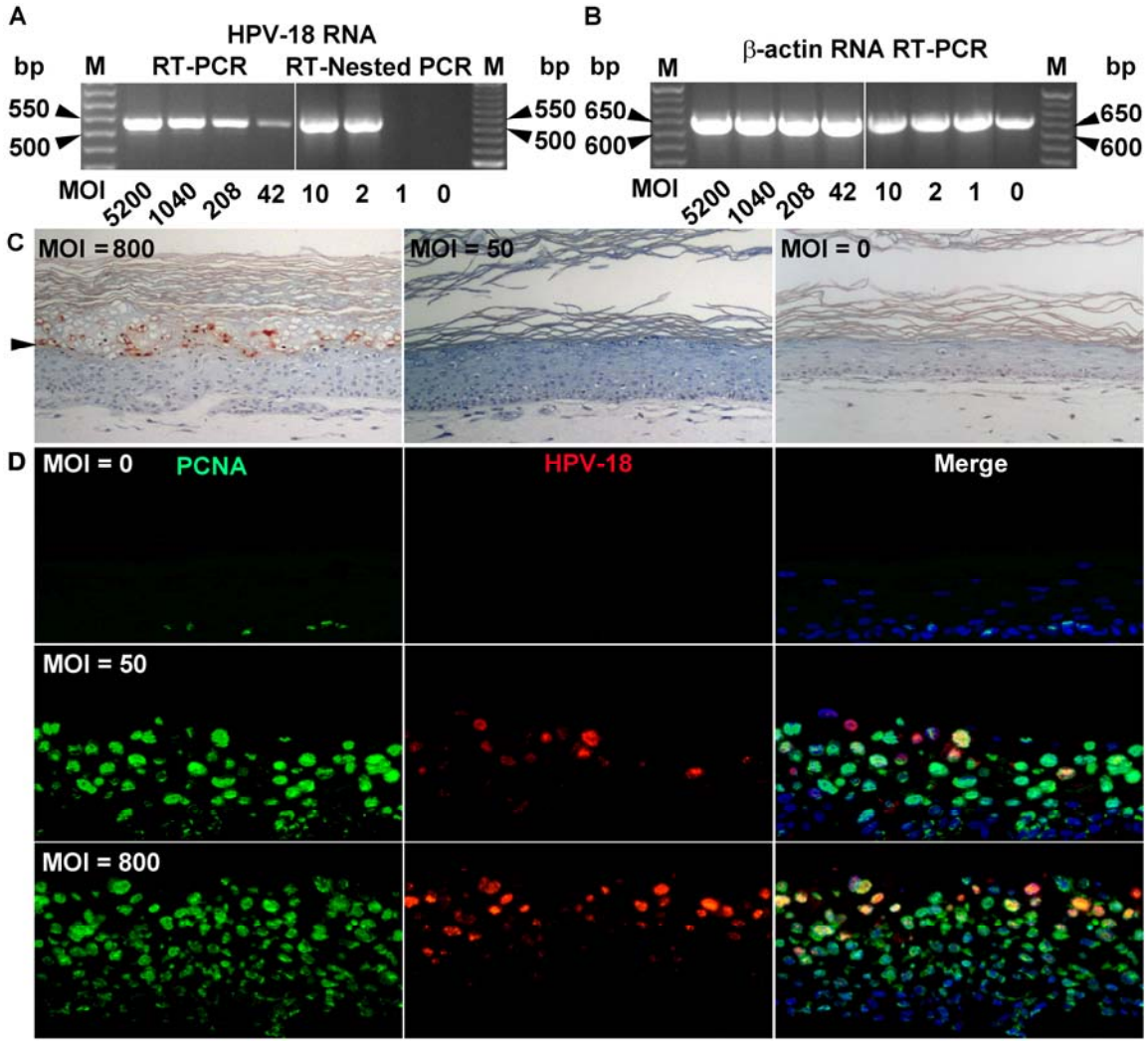

Figure 3. Infectivity assays of HPV-18 virions in PHKs. HPV virions were titered by quantitative real time PCR

(A) HPV cDNA detection in submerged PHK cultures after infection at the indicated MOI. A 521 bp cDNA fragment of a spliced early viral mRNA was detected by ethidium bromide staining after RT-PCR (left panel) or RT-nested PCR (right panel). (B) A 642 bp long β-actin cDNA served as an internal control. M, 50 bp ladder. (C) Immunohistochemical detection of the HPV major capsid protein L1 (reddish brown) in 14-day raft cultures of PHKs infected with HPV virions. MOI of 800 or higher (left panel); MOI of 50 up to 400 (middle panel). An uninfected culture is shown in the right panel. Arrowheads point to the boundary between the upper cornified strata and live epithelium below. (D) Four μm sections from the raft cultures above were probed for PCNA (Alexa Fluor 488, green) and for viral DNA (Cy3, red). Cellular DNA was revealed by DAPI (blue).

only detected in basal cells (Figure 3D). In contrast, in all infected cultures, down to an MOI of 50, the lowest MOI tested, most differentiated cells were positive for PCNA, indicative of successful infection in the majority if not all the cells. Further, occasional pockets of superficial cells in these nonproductive cultures had low levels of amplified viral DNA (Figure 3D and data not shown). Accordingly, we suggest that HPV DNA must amplify to a high copy number for efficient late protein expression, contrary to a previous report (Spink and Laimins 2005). Rarely we detected faint L1 antigen in cells not also having high viral DNA. Interestingly, intense viral DNA signals detected at productive (high) MOIs were restricted to the differentiated strata, as in cultures initiated by DNA cotransfection. This latter observation indicates either that a majority of virions failed to enter cells under our conditions or that the preponderance of internalized viral DNA was lost.

Temporal order of host DNA replication and HPV-18 DNA amplification

Intriguingly, in productive raft cultures initiated by DNA transfection, 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 (Figure 2E and F). 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 pulse-labeled with BrdU for the final 12 hours and harvested on days 8, 10, 12 and 14 and analyzed as before (Figure 4A). A large fraction of nuclei on days 8 and 10 were intensely positive for BrdU. There was 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. These inverse patterns of host DNA replication and viral DNA amplification were reproducible in multiple experiments, with strong viral DNA signals appearing on day 12 +/- 2. The fraction of spinous cells with high viral DNA significantly declined in older cultures as did spinous cells in S phase (data not shown). 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 Figure 5). Thus, BrdU signals represent host DNA replication. As HPV DNA only amounts for about 1% of host DNA by mass even at 5000 copies/per cell, the amount of BrdU incorporated into HPV DNA is clearly not sufficient to generate strong signals.

Punctate dots or streaks of viral DNA signals were observed in the stratum corneum, consistent with DNA packaged in virions as observed in Figure 3. Indeed, some of the L1 signals colocalized with viral DNA (Figure 4B). One of the factors attributed to the incomplete colocalization of these two signals is the difficulty in detecting packaged

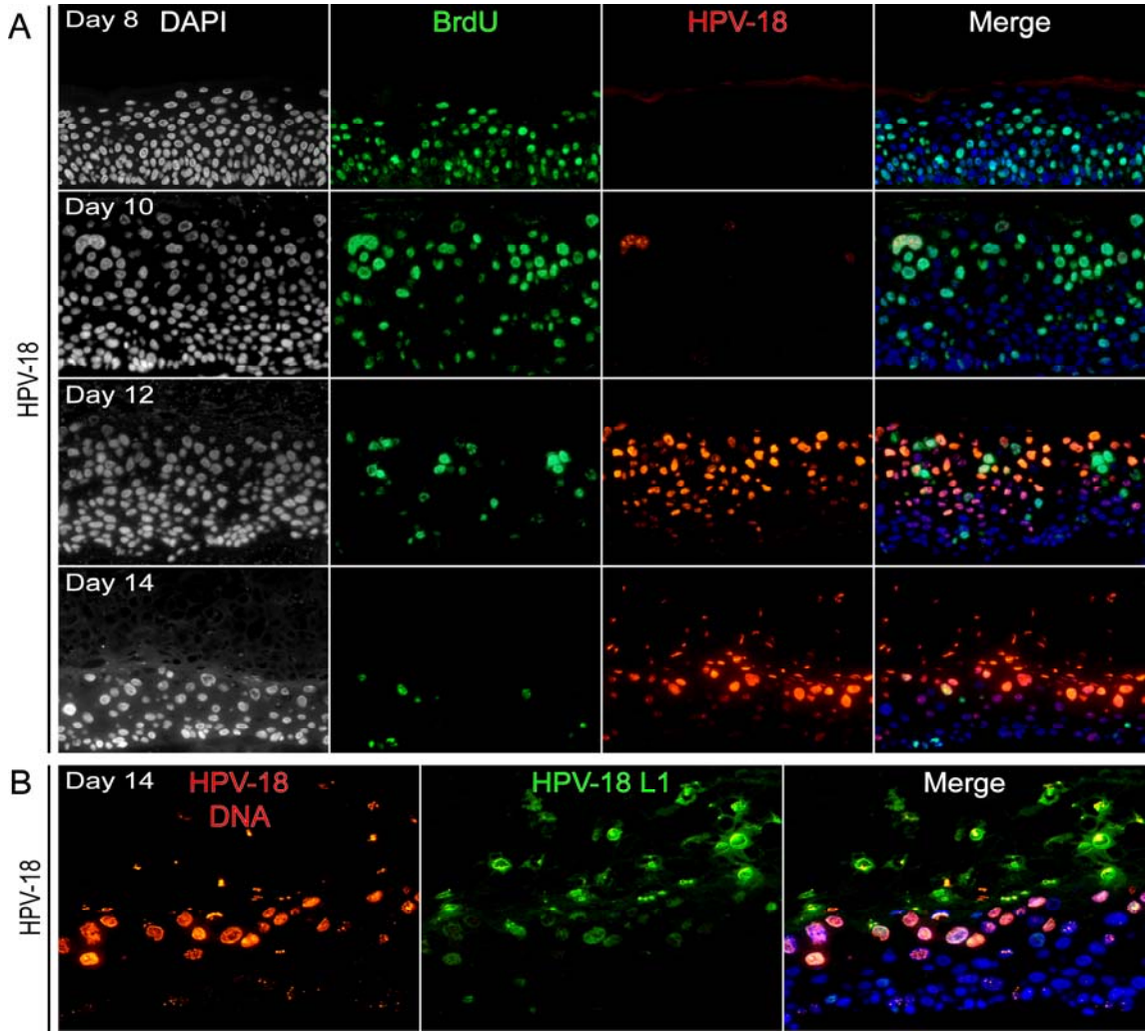

Figure 4. (A) HPV-18 DNA amplification in differentiated keratinocytes lags behind cellular DNA replication. HPV-18 containing PHK raft cultures were harvested on days 8, 10, 12 and 14, each following a 12-h incubation with BrdU. Sections were probed for HPV-18 DNA (red) and incorporated BrdU (green). Cellular DNA was revealed by DAPI (blue). For better visualization of tissue morphology, DAPI staining is also presented in black/white images (left column). (B) Detection of viral DNA (red) and the major capsid protein L1 (green) in a 14 day old culture.

viral DNA and possibly a significant loss of virions during DNA denaturation for *in situ* hybridation. The latter is evident when one compares the reduction in DNA signals in the cornified strata relative to those in the live cells. Similarly, the L1 signals revealed in this double IF image was much reduced relative to those detected by immunohistochemistry without the denaturation step (Figures 2H and S1).

HPV-18 genome amplification occurs in G2-arrested cells

To verify and elaborate upon the above conclusions, we probed the raft cultures for the S phase cyclin A, amplified viral DNA, and BrdU incorporation. Control PHK raft cultures demonstrated a few basal cells positive for both cyclin A and BrdU (Figure 5A). Cyclin A was induced in many suprabasal cells in HPV-18 raft cultures in early times, but the fraction of cyclin A positive cells progressively decreased by days 12 and 14 (Figures 5D and S2), as expected from the reduction in BrdU-positive cells as the cultures aged (Figure 4A). At all time points, many nuclei positive for cyclin A were also positive for BrdU, indicative of cells well into S phase. Cells positive for nuclear cyclin A, but negative for BrdU, could represent very early S phase prior to extensive cellular DNA replication. Another small subset of cells was positive for BrdU but contained cytoplasmic cyclin A or no cyclin A. These cells may have been at the S/G2 transition or in G2. Most importantly, the great majority of cells with amplified HPV DNA were distinct from cells positive for both cyclin A and BrdU. These observations demonstrate that viral DNA amplification did not occur concurrently with host DNA replication in S phase.

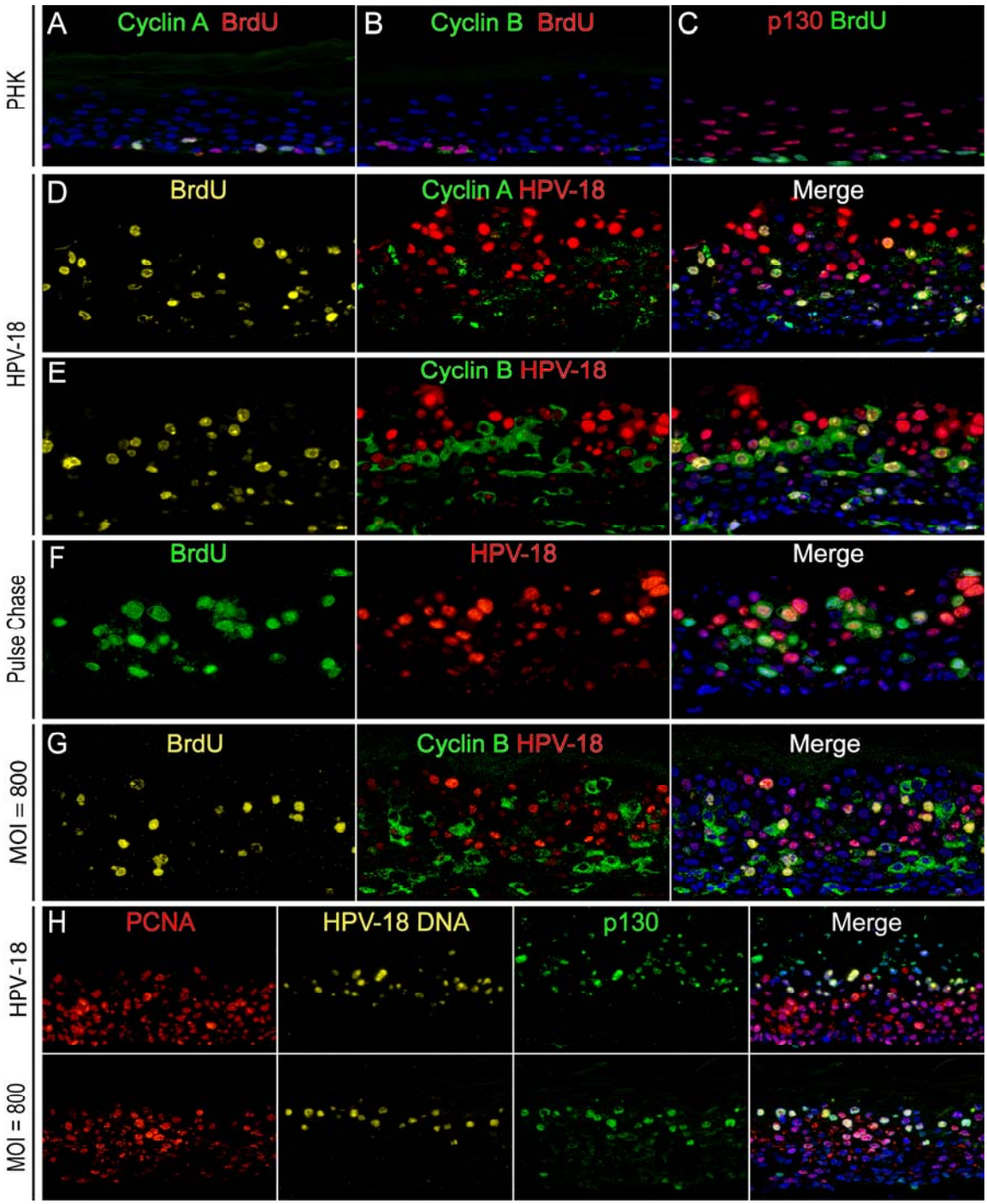
PHK
A Cyclin A BrdU
B Cyclin B BrdU
C p130 BrdU
HPV-18
D BrdU
Cyclin A HPV-18
Merge
E
Cyclin B HPV-18
Pulse Chase
F BrdU
HPV-18
Merge
MOI = 800
G BrdU
Cyclin B HPV-18
Merge
HPV-18
H PCNA
HPV-18 DNA
p130
Merge
MOI = 800

Figure 5. HPV-18 plasmid DNA amplification as it relates to the cell cycle. Panels A-C, day-10 raft cultures of normal PHK raft cultures. Panels D-F, day-12 raft cultures initiated from PHKs transfected with HPV-18 DNA. Sections were analyzed for cyclin A (green in Panels A, D) or cyclin B (green, Panels B, E), HPV-DNA FISH (red, Panels D-F) and for BrdU (red in Panels A, B; green in Panel C or yellow Alexa Fluor 647 in panels 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). Cellular DNA was revealed by DAPI (blue) in all Panels.

If not in S phase, did HPV DNA amplify in G2? We next probed for the mitosis promoting factor (MPF) component cyclin B1, amplified viral DNA, and BrdU-incorporation. Nuclear import of the cyclin B1/cdk1 complex is essential for the conclusion of G2 and initiation of mitosis. In control PHK raft cultures, weak cyclin B1 signals were detected in occasional basal cells positive for BrdU (Figure 5B). In day-8 HPV-18 raft cultures, cytoplasmic cyclin B1 was detected in a small fraction of spinous cells, and there was a modest increase in day-10 cultures. The signals were typically observed in intense BrdU-positive cells (Figure S3). On day 12, there was a more dramatic increase in the signal strength and in the number of cytoplasmic cyclin B1-positive spinous cells (Figures 5E and S3). This increase coincided with the reduction in cyclin A- and BrdU-positive cells. 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 cells. Critically, it was in the nuclei of many of the cyclin B1-positive mid spinous cells that low to moderate HPV DNA signals first appeared (Figure 5E and S3).

To investigate further the relationship between BrdU-positive cells and those with amplified viral DNA, we probed raft cultures that had been pulsed with BrdU for 6 h on day 10 and harvested on day 12. There was an increased population of cells in which intense viral DNA colocalized with strong BrdU signals (Figure 5F). Collectively, these observations demonstrate that HPV DNA amplification initiated after cellular DNA replication in cells that are subsequently arrested in G2.

When these experiments were conducted on day-14 raft cultures of PHKs productively infected with HPV-18 virions at MOI of 800, the same relative distributions

of cyclin B1, amplified viral DNA, and BrdU were observed (Figure 5G). These results verify that the delayed viral DNA amplification relative to S phase and the loss of cyclin B1 subsequent to viral DNA amplification are not an artifact of raft cultures containing transfected HPV-18 DNA.

Cessation of HPV-18 E7 activity in cells with abundant viral DNA

Intriguingly, spinous cells with high HPV DNA were negative for cyclin B1 signals in cultures of any age (Figures 5E, G and S3). We considered a number of possible explanations: (1) Cyclin B1 was degraded as cells transitioned through mitosis. This is unlikely because the enlarged nuclei in these cells are consistent with a greater than 2n DNA content (Chien et al. 2002). (2) Cyclin B was degraded to allow cellular DNA re-replication. This is also unlikely as cells with high viral DNA were negative for either nuclear cyclin A or intense BrdU (Figures 5D and S2). (3) Cells exit the cell cycle following viral DNA amplification due to the loss of E7 activity, resulting in cyclin B1 disappearance. Evidence is consistent with this last interpretation, as described below.

Our lab 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 reentry by spinous cells (Genovese et al. 2008). Thus, the loss of p130 is an excellent 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 (Figure 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 (Figure 5H, top row). 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 reenter 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 seen in cells with high viral DNA in PHK raft cultures productively infected with HPV-18 virus (Figure 5H, bottom row).

An essential role of E6 in viral DNA amplification and virion production

To demonstrate that our experimental system indeed obviates a dependence on HPV E6 oncoprotein for long-term viral plasmid maintenance and PHK immortalization while permitting analyses in PHK raft cultures, we prepared an HPV-18 mutant in which the E6 gene (which spans nts 105-579) was deleted of the E6*I intron coding sequence (nts 234-415). The great majority of HR HPV E6 transcripts are alternatively spliced intragenically or intergenically, abrogating the ability to encode the E6 protein. The predominant HPV-18 E6*I mRNA contains a frameshift that terminates translation shortly after the splice. The resulting E6*I peptide 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 and other E6-targeted host proteins (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 E6*I mutant genome or the wild type HPV-18 genome over a time course extending to 22 days. The wild type HPV-18 cultures were highly productive as before (Figure S1B) whereas the mutant HPV-18 cultures were virtually negative for L1 (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, we probed for PCNA by IHC. A great majority of the superbasal cells were positive. Relative to cultures containing the wild type genome, a few pockets in the epithelium were negative for PCNA (Figure 7, left panels). 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 have previously demonstrated 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

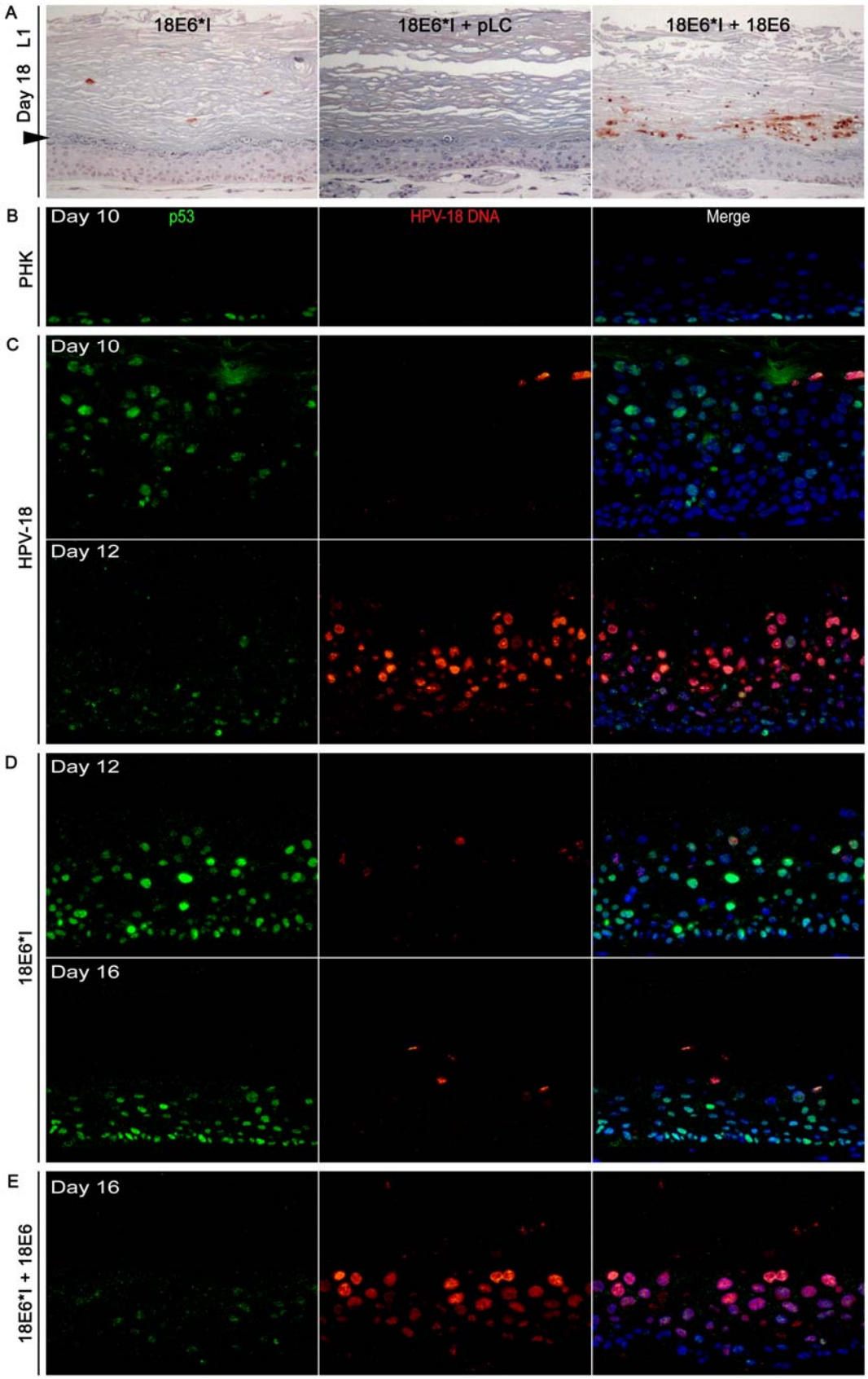
A
Day 18 L1
18E6*I
18E6*I + pLC
18E6*I + 18E6
B
PHK
Day 10
p53
HPV-18 DNA
Merge
C
HPV-18
Day 10
Day 12
D
18E6*I
Day 12
Day 16
E
18E6*I + 18E6
Day 16

Figure 6. 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; and (E) day-16 HPV-18 E6*I containing PHKs *trans*-complemented with pLJ HPV-18 URR-E6. Cellular DNA was stained with DAPI (blue).

provide yet another marker of 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 normal localization for E6 and E6* peptides.

In untransfected PHK cultures, p53 was detected in a few BrdU-positive and -negative basal cells but not in suprabasal cells (Figure 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 day 10. 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 (Figure 6C and data not shown). 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 (Figures 6D). This pattern did not change out to 22 days. 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 over 3 weeks. To verify the absence of apoptosis, we probed 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 (Figure 7, right panels). In contrast, the antibody yielded wild spread signals in drug-treated tissues with apoptotic histology (E.-Y. Kho, H.-K. Wang, T.R, Broker and L.T. Chow, unpublished observation).

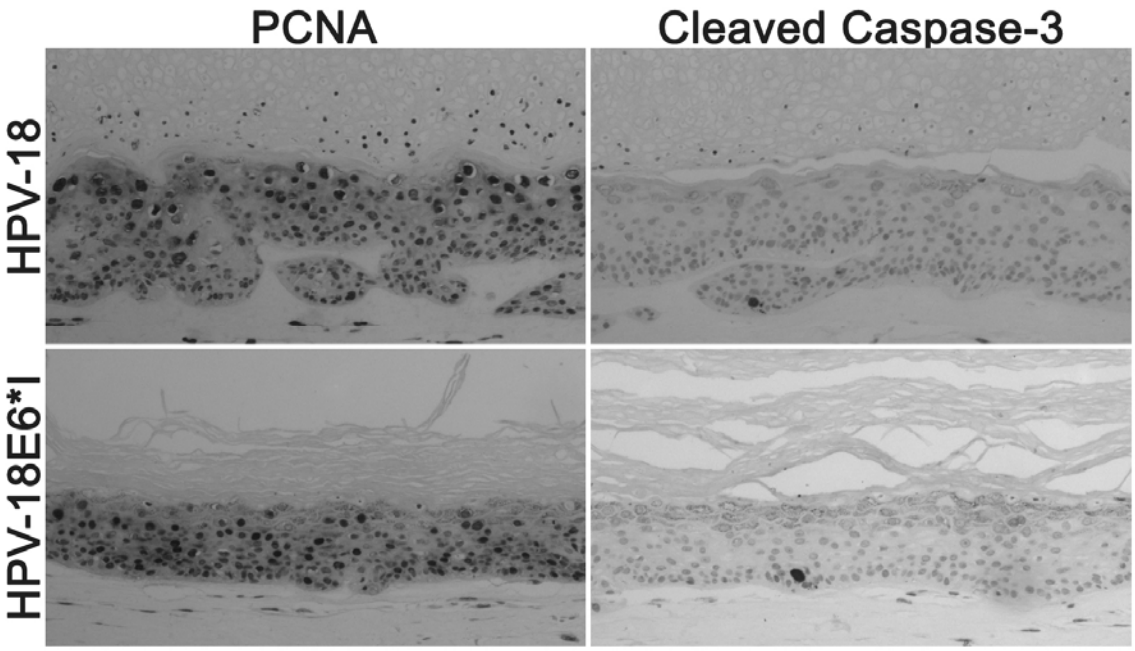

Figure 7. Wide spread induction of PCNA and virtual absence of cleaved caspase 3 in raft cultures harboring the wild type or E6*I mutant plasmid. IHC was conducted to detect PCNA (left column) or the cleaved caspase 3 (right column) in day-16 raft cultures of PHK transfected with the wild type HPV-18 (top row) or the HPV-18 E6*I mutant (bottom row).

To ascertain that the defective phenotypes were not attributable to unintended mutations in E6*I genomes, we infected the E6*I DNA-containing PHKs with a retrovirus expressing HPV-18 E6 or E6-E7 controlled by the contiguous viral URR, which harbors transcription enhancers, the E6 promoter, and the HPV origin of replication. With either retrovirus**,** about 30% of the cornified envelopes across the day-18 and older rafts were abundantly positive for L1 (Figure 6A, right panel, and data not shown). Empty vector-only retrovirus had no effect on L1 synthesis (Figure 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 (Figure 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. E6 t*rans*-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.

Discussion

By using the Cre-loxP recombination *in vivo*, we have developed an efficient strategy to generate HPV genomic plasmids in PHKs suitable for organotypic raft cultures without extensive passages. The cultures support an unprecedented productive program which yields high titers of infectious HPV virus (Figures 1-4) with little or no effects on squamous differentiation (our unpublished observations). Critically, the ability to examine an immortalization-defective E6 mutant phenotype (Figure 6), which could not be investigated in PHK raft cultures until now, clearly demonstrates that our approach obviates the need for immortalized host cells. In so doing, we have overcome the inefficient amplification of the wild type viral DNA in immortalized cells, a limitation on HPV genetic analyses.

Lee at al. (2004) similarly used Cre-loxP recombination to generate an HPV-16 genomic plasmid in PHKs from chimeric adenoviruses, but the immortalized cells had a very low yield of virus. Construction of such a virus is time-consuming. Given that adenoviruses themselves are highly infectious to humans, the chimeric virus requires greater care in handling. In contrast, our method is simple, expedient, efficient, and reproducible. The critical factors are a high efficiency of cotransfection of supercoiled plasmids into PHKs, a rapid and effective acute drug selection for cells harboring the parental plasmid which expresses the drug-resistance gene, and a highly efficient excision of HPV genomes from the parental plasmids by nls-Cre recombinase *in vivo* (Figure 1). Relative to the wild type genome, the HPV-18 plasmid generated has a 34 bp loxP insertion downstream of the late poly A site. We believe this insertion exerts no adverse effects on the viral productive program. A mildly hyperproliferative tissue morphology,

differentiation-dependent viral DNA amplification, and patterns of E7 activity and L1 protein synthesis (Figures 2, 4, 5 and S1) all recapitulate those observed in productively infected patient tissues (Stoler and Broker 1986; Doorbar, 2006). Moreover, the exuberant viral DNA amplification and robust virus production (Figures 1-3) resemble those observed in HPV-11 infected foreskin xenografts in nude mice deficient in immune surveillance (Stoler et al. 1990).

Virion production and maturation

Taking into account the significant fraction of spinous cells with amplified viral DNA, the high numbers of amplified viral genomes per cell, and the numerous L1-positive cornified envelopes (Figures 1-5), the titers of virus produced in our PHK rafts are orders of magnitude higher than viruses recovered from immortalized cells (see Frattini et al., 1997). A highly productive infection of naive PHKs in raft cultures by a human papillomavirus has finally been achieved (Figure 3). Our TEM studies of tissue sections have revealed that, in degenerated nuclei of cornified envelopes, HPV particles undergo a maturation which alters the particle surface to enable the tightly packed paracrystalline arrays (Figure 2I-2N), similar to those observed in highly productive canine papillomas (Campo, 2002). Notably, disulfide bond formation between neighboring L1 capsomeres is required for virion maturation (Buck et al. 2005b and refs therein) and apparently this process does not take place in the reducing environment of living cells, further linking the viral productive program to the successive stages of epithelial differentiation.

HPV-16 pseudovirions must undergo a maturation process *in vitro* to gain stability (Buck et al. 2005b). High titers of HPV pseudovirions are assembled in 293T or 293TT cells from L1/L2 expressed from vectors and co-transfected, recircularized HPV DNA (Buck et al. 2005a; Pyeon et al. 2005). However, no productive infection in PHK raft cultures has been reported despite efficient infection of immortalized or transformed cells. Given the wide use of HPV pseudovirions in diverse studies, a side-by-side comparison between pseudovirions against our genuine HPV virions will be particularly relevant in examining infection processes and outcomes.

Viral DNA amplification in G2 arrested cells

Most intriguing is our finding that, in the great majority of the cells, HPV DNA amplification did not occur in cells in S phase. Rather, it follows host DNA replication. Significant colocalization of cellular DNA replication and amplified viral DNA occurs only upon a BrdU pulse followed by a long chase (Figures 4, 5, S2 and S3). Furthermore, viral DNA first appears in cells strongly positive for cytoplasmic cyclin B1. Collectively, these observations strongly suggest that viral DNA amplification initiated in G2-arrested cells. For small DNA tumor viruses, the assumption has been that, after inducing an S phase milieu by one of the viral "oncoproteins" (HPV E7, adenovirus E1A and SV40/polyomavirus T-antigen), viral DNA then amplifies concurrently with host DNA replication in S phase. Nevertheless, the possibility that the two events might not exactly coincide has been suggested for HPV (Swindle et al. 1999). Others have observed that sporadic HPV DNA signals were separated spatially from S phase markers (reviewed by Davy and Doorbar 2007). However, in the absence of simultaneous detections for viral

DNA, BrdU incorporation, and markers of cell cycle status in time course studies, as presented here, no definitive interpretations had been offered.

Why would HPV DNA amplification lag behind host DNA replication? The simplest and most straightforward explanation is that HPV is not able to hijack host replication machinery while it is engaged in replicating host DNA. A virus-induced G2 arrest would then create an uncontested window of opportunity for a highly efficient and rapid viral genomic amplification. A number of DNA and RNA viruses are known to arrest cells in G2 phase for reasons not well understood (reviewed by Davy and Doorbar 2007). Perhaps viral replication in G2-arrested cells is not an uncommon occurrence, just not widely appreciated.

G2 arrest of proliferating cell lines caused by ectopic over-expression of the cytoplasmic HPV E1^E4 protein is well documented (Davy and Doorbar, 2007). However, mutational analyses of the E4 gene have not resolved its role in viral DNA amplification (Nakahara et al., 2005; Wilson et al., 2005; Fang et al., 2006a). In our productive raft cultures, the E1^E4 protein did not colocalize with cyclin B1 and thus unlikely to be responsible for G2 arrest (A. Duffy, H-K. Wang, T. Broker, and L. Chow, unpublished observations). The experimental system reported here is ideal for investigating its possible roles in the viral infection cycle. In contrast, in PHK raft cultures expressing HPV-18 E6 and E7 genes, there is an elevated transcription of cdk1, cyclin B1, other G2/M genes, and Wee1, a negative regulator of cyclin B1/cdk1 activation at G2/M (Garner-Hamrick et al. 2004). Indeed, our recent results in PHK raft cultures demonstrate that E7 alone causes cytoplasmic accumulation of cytoplasmic

cyclin B1 in BrdU-positive cells (N. Banerjee, T. Broker, and L. Chow, unpublished observation).

A critical function of E6 in viral DNA amplification

We show that the full-length E6 protein is critical for viral DNA amplification and hence capsid protein synthesis (Fig. 6). The defect of the E6*I mutant cannot be attributed to a loss of the plasmid, as E7-induced PCNA was detected in the great majority of the superbasal cells (Figure 7). This interpretation is supported by the very high levels of p53 protein, which is induced in response to E7, in a great majority of cells (Figure 6). The elevated p53 protein level may have trigger check point control to inhibit cellular DNA replication. Also, in wild type and E6*I raft cultures, viral DNA signals did not colocalize with p53 signals, suggesting that the high levels of p53 protein also inhibited viral DNA amplification. This interpretation would agree with transient HPV DNA replication assays in which ectopic p53 protein inhibits HPV DNA replication (Lepik et al. 1998). The ability to *trans*-complement the E6 deletion mutation in the context of an autonomous viral plasmid (Figure 6) will facilitate future analyses of site-specific E6 mutations to verify the role of p53 and to identify additional E6 functions relevant to the viral productive program.

Comparative results between wild type and E6*I mutant genome-containing raft cultures have several additional implications: (1) the function of E6 is not to counter E7-induced apoptosis from elevated levels of p53. In cervical carcinoma derived HeLa cells, expression of E7 in the absence of E6 leads to apoptosis (DeFilippis et al. 2003). Over-expression of HPV-18 E6*I in CaSki cells, another cervical carcinoma cell line,

antagonized E6 function and triggers p53-dependent apoptosis (Pim and Banks 1999). In addition, HPV-31 E6 and E7 have been reported to activate caspase 3, important for HPV-31 DNA amplification in raft cultures of immortalized cells (Moody et al., 2007). We did not observe apoptotic cells by histology in raft cultures with the wild type or the HPV E6*I mutant. Furthermore, we hardly ever detect cleaved caspase 3 in either raft cultures (Figure 7). (2) The patterns of p53 detection suggest that E6 is expressed in both basal and in suprabasal cells in raft cultures. This could account for an increase in p53-positive cells in the basal stratum of E6*I containing raft cultures, resulting in a less hyperproliferative morphology than in the wild type HPV-18 containing cultures. Also revealing is the observation that, in cultures containing the wild type HPV, the increased p53 signals in spinous cells relative to the basal cells would suggest an increase in spliced E6* transcripts during squamous differentiation, reducing but not abolishing the synthesis of the E6 protein. It is possible that, because of the anti-recombinational properties of p53 (Bertrand et al. 2004), the low levels of p53 remaining in the spinous cells could limit recombination of HPV DNA into oligomers that cannot be packaged into HPV capsids. An increase in E6* spliced RNAs could bolster translation of downstream early ORFs from polycistronic mRNA to support viral DNA amplification (Hubert and Laimins, 2002; Tang et al., 2006). The absence of p53 in cell with high HPV DNA is consistent with the loss of E7 activity in these cells.

A switch from early to late phase

The temporal and spatial patterns of cyclin A and cyclin B, BrdU incorporation, viral DNA amplification, destabilization and subsequent reappearance of p130 pocket

protein (Figures 5 and S2, S3), and the synthesis of the L1 protein in superficial cells following viral DNA synthesis (Figures 2 and S1) in both DNA-transfected and HPV virus-infected PHK raft cultures all point to a transition from a state of viral DNA amplification to late protein synthesis for virion morphogenesis. What then inactivates E7 as viral DNA amplification progresses? The viral E6 promoter might be down-regulated by increasing levels of viral E2 protein expressed from the increasing viral DNA templates (Chow and Broker 2007 and refs therein). Alternatively, elevated E2 protein has been reported to directly bind and inactivate E7 (Gammoh et al. 2006).

We propose the following scenario for the sequence of events in the productive phase. The E6 protein is expressed in the lower strata to increase the parabasal, transit amplifying cell population. Elevated E7 expression, attributable to increased E6* mRNA in suprabasal differentiated cells, leads to p130 destabilization and S phase reentry. Following host chromosome duplication, spinous cells are arrested in a prolonged G2 phase, during which viral DNA amplification occurs, a process which appears to require E6 to degrade most of the p53 induced by E7. High levels of viral DNA amplification are accompanied by a reduction in and eventual loss of E7 activity, leading to a switch to late protein synthesis. The late events are characterized by a dramatic increase in E1^E4 protein (Doorbar, 2006; Duffy et al., unpublished observation) and the expression of capsid proteins in superficial cells (Figures 2, 4, and S1) for virion morphogenesis. The assembled virions then mature in the cornified envelopes. Viral DNA amplification in the live tissue is then attenuated after the productive phase. The cause of this shut-down remains to be investigated.

In summary, we have developed a simple and reproducible method to recapitulate a complete and highly productive infection cycle in organotypic cultures of PHKs in which viral genomic plasmids were generated by *in vivo* recombination. A detailed molecular portrait of virus-host interactions were revealed and further verified using raft cultures infected with the progeny virus. Our system is ideal for genetic and molecular analyses of mutants of high-risk as well as low-risk HPV types in PHK raft cultures. The recapitulation of the full infection cycle will finally enable realistic *ex vivo* evaluation of potential agents to prevent HPV infection or reproduction.

Materials and Methods

Plasmids

The nls-Cre expression plasmid pCAGGS-nls Cre was a gift (Hardouin and Nagy 2000). All PCR primers and the construction of pNeo-loxP HPV-18 and pNeo-loxP HPV-18 E6*I plasmids are provided in **Table 1** and **Materials and Methods** in **Supplemental Research Data**. For both plasmids, the 34 bp loxP sites flank the linear HPV-18 sequence upstream of nt 7474 and downstream of nt 7473. The vector carries the Neomycin-resistance marker gene selectable in bacteria and in mammalian cells. In the HPV-18 E6*I mutant, the intron coding sequence (nts 234-415) in the predominant E6*I mRNA was deleted. For *trans*-complementation experiments, the empty vector-only retrovirus pLC and pLJ HPV-18 URR-E6 or URR-E6/E7 retroviruses were used. Each expresses the Neomycin-resistance gene (Cheng et al. 1995; Chien et al., 2002). All plasmids were purified by banding in CsCl-ethidium bromide equilibrium density gradients.

DNA transfection and organotypic raft cultures of primary human keratinocytes

PHKs recovered from neonatal foreskins collected from UAB Hospital Well Baby Nursery (Birmingham, AL) with IRB approval were grown in keratinocyte serum-free medium (K-SFM) (Invitrogen, Carlsbad, CA). $2X10^5$ PHKs at the second passage were seeded onto 6-well plates, incubated overnight in K-SFM and then cotransfected with 1 μg of pCAGGS-nlsCre and 5 μg of pNeo-loxP HPV-18 or pNeo-loxP HPV-18 E6*I plasmids using FuGENE 6 (Roche, Indianapolis, IN). After selection with 100 μg/ml of G418 (Invitrogen) for 4 days, the cells were cultured in fresh K-SFM for an additional 1 or 2 days. Each raft culture was then initiated from 2 - 2.5 x 10^5 PHKs (Banerjee et al., 2005 and refs therein). As specified in each experiment, the cultures were grown for different numbers of days after being raised to the medium:air interface. To mark cells in S phase, bromodeoxyuridine (BrdU) was added to the medium prior to harvest as indicated in **Figure Legends**. The cultures were fixed in 10% buffered formalin, and embedded in paraffin. For *trans*-complementation, the G418-selected PHKs were transduced with various retroviruses. Raft cultures were initiated with or without a re-selection using 300 μg/ml G418 for 2 days. BrdU labeling and tissue processing were similarly performed.

PCR and Southern blot analyses for HPV-18 DNA excision and amplification

Fifty ng of total DNA from submerged PHKs were examined by PCR amplification for Cre-mediated HPV DNA excision. The primer sets G, H and I (**Table I, Supplemental Research Data**) detected total HPV-18 DNA, excised HPV-18 DNA, or the control β-globin gene, respectively, after electrophoresis in a 2% agarose gel and

staining with ethidium bromide. For Southern blot hybridization, 15 μg of total epithelial DNA from day-12 raft cultures were digested overnight with Hind III (no cut in the parental plasmid or the excised HPV DNA) or with EcoR I (one cut in each). Copy number and length standards were prepared by mixing the parental plasmid or pGEM2-HPV-18 with 15 μg of cellular DNA from control PHK raft cultures to yield 50, 500 or 5000 copies of the HPV genome/diploid cell. The mixture was then digested with EcoR I which linearizes the parental plasmid or excises the 7.9 kb HPV-18 DNA from the pGEM2 vector. DNA samples were electrophoretically separated in 0.8% neutral agarose gels, and Southern-blotted membranes were hybridized to [α-^{32}P]-labeled, genomic HPV-18 DNA probes. Blots were documented using a Storm 820 PhosphorImager (GE Healthcare, Piscataway, NJ).

Histology and in situ analyses

Four μm sections of raft cultures were deparaffinized and stained with hematoxylin and eosin (H&E) for histology or by immunohistochemistry (IHC) to detect PCNA induction, BrdU, and cleaved caspase 3, and the L1 capsid antigen. DNA-fluorescence *in situ* hybridization (DNA-FISH) and indirect immunofluorescence (IF) coupled with tyramide signal enhancement were conducted as previously described, with minor modifications (Van Tine et al. 2005). Additional details are provided in the **Supplemental Research Data**.

Transmission electron microscopy of raft culture sections

Day-14 raft cultures were fixed with 2.5% glutaraldehyde in phosphate-buffered saline, followed by 2% osmium tetroxide and embedded in Spurr resin (Electron Microscopy Sciences, Hatfield, PA). One hundred nm sections were cut and stained with 1% uranyl acetate in 50% ethanol, followed with Reynold's lead citrate (EMS). Grids were scanned at 60 KV in a FEI T12 Spirit electron microscope (Advanced Microscopy Techniques, Danvers, MA). Images were electronically captured and measured using an AMT XR 60B digital camera and its software.

HPV-18 virion recovery and titer determination

HPV-18 virions were recovered from day-14 or day-16 epithelia as described (Favre 1975). To titer the virus, aliquots of the virus stocks were digested with DNase I (Invitrogen), which was then inactivated by heating for 5 min at 100ºC. Packaged viral DNA was then purified by digestion with Proteinase K and phenol/chloroform extractions. Serial dilutions of viral DNA were analyzed by real time quantitative PCR using SYBR® GreenER™ qPCR SuperMix (Invitrogen) and primers J and K (**Table I, Supplemental Research Data)**. As standards, purified pNeo-LoxP HPV-18 plasmid DNA was serially diluted to approximately 40 to $4x10^{8}$ copies/well. Forty cycle PCR amplification reactions in triplicates were performed in 384-well plates using the ABI 7900HT. Data were processed with SDS2.1 software (Applied Biosystems, Foster City, CA).

HPV-18 infectivity assays

Approximately $1x10^5$ PHKs were inoculated with various amounts of virus stock, corresponding to an MOI of 5200, 1040, 208, 42, 10, 2, 1, or 0 in 1 ml of K-SFM and incubated overnight. The medium was changed and the cells were cultured for 4 more days. Total RNA was then extracted by TRIzol (Invitrogen). Reverse transcription was conducted in a 50 μl reaction on 10 μg of RNA. One μl of RT reaction was then subjected to 30 cycles of PCR or nested PCR amplification (30 cycles each) in a 35 μl reaction mixture to generate a cDNA fragment of the spliced HPV-18 E6-E7-E1^E4 ,RNA or the β-actin mRNA, as described (Meyers et al. 2002). Fifteen μl of each reaction was resolved by electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining. PHKs were also infected with various MOI in K-SFM overnight and developed into raft cultures, fixed on day 14 and processed as described.

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SUPPLEMENTAL RESEARCH DATA (WANG, DUFFY, BROKER, AND CHOW)

Table I. PCR Primer Sequences

For constructing pNeo:

Primer A 5′-TTAGTGAACCGTCAGATCCATTAATGCTAGCGCTA-3′ (Ase I site)

Primer B 5′-CTTGCTCACCATGGTGGCGGCGGCCGCACCGGTGG-3′ (Not I site)

*For constructing pNeo-loxP HPV-18 E6*I*

Primer C 5′-ACTTACAGAG^GTGCCTGCGGTGCCAGAAACCG-3′ (nts 204-233^416-441, splice site)

Primer D 5′-CCGCAGGCAC^CTCTGTAAGTTCCAATACTGTCTTGC-3′ (nts 425-416^233-208, splice site)

Primer E 5′-TACGCTCGAGGAACAATTGGCGCGCCTCTTTG-3′ (Xho I, nt 7572 Asc I sites)

Primer F 5′-TACGAAGCTTGCTGAGCTTTCTACTACTAGCTCAATTCTGGC-3′ (Hind III, nt 823 Blp I sites)

For detecting excision of HPV-18 DNA and for β-globin gene

Primer pair G for detecting total HPV-18 DNA:

5′-GTTAGATGATGCAACGACCACG-3′ and 5′-TGTCTGCATCTTCCTCTTCCTC-3′

Primer pair H for detecting excised HPV-18 DNA:

5′-TGCGTGTACGTGCCAGGAAGTAAT-3′ and 5′-GTTTGCAATAGTGCCAGCGTACTG-3′

Primer pair I for detecting the human β-globin gene as internal control:

5′-CTGACTCTCTCTGCCTATTGGTCT-3′ and 5′-CCATAGACTCACCCTGAAGTTCTC-3′.

For real time quantitative PCR to quantify HPV-18 virions

Primer J 5′-AAGCTCAGCAGACGACCTTC-3′

Primer K 5′-ACCTTCTGGATCAGCCATTG-3′.

Materials and Methods

Plasmid construction

pNeo-loxP HPV-18 plasmids were constructed as follows: The multiple cloning sites (MCS) of pEGFP-N1 (BD Biosciences, San Jose, CA) were PCR-amplified using primers A and B (**Table I**) that carry Ase I and Not I sites, respectively. The Ase I- and Not I-digested PCR product was used to replace the CMV promoter-EGFP fragment between the Ase I and Not I sites of pEGFP-N1, generating pNeo. Using PCR, a double-stranded synthetic DNA fragment containing loxP - Xho I - Xma I - loxP (5′-ATAACTTCGTATAGCATACATTATACGAAGTTATCTCGAGCCCGGG ATAACTTCGTATAGCATACATTATACGAAGTTAT-3′) (loxP sites underlined) was inserted between nts 7473 and 7474 of HPV-18 which is cloned at the EcoR I site (nt 2440) in pGEM-2 (Promega, Madison, WI), generating pGEM-2 loxP HPV-18. The Xma

I-EcoR I fragment (Xma I-loxP-7474-7857/1-2440) and the EcoR I-Xho I fragment (2441-7473-loxP-Xho I) from pGEM-2 loxP HPV-18 were then inserted into the MCS of pNeo at the Xma I and Xho I sites to create pNeo-loxP HPV-18.

PCR mutagenesis was used to construct the pNeo-loxP HPV-18 E6*I parental plasmid by utilizing four primers: two partially overlapping sense and antisense strand primers (C & D) that recreate the E6*I splice, an upstream sense strand primer E spanning the Asc I site (nt 7572), and a downstream antisense strand primer F spanning the Blp I site (nt 823). The two overlapping fragments from PCR amplification with primer pairs D/E and C/F were pooled and re-amplified with primers E/F to generate the 931bp Asc I - Blp I fragment spanning the HPV-18 E6*I ORF. The pNeo-loxP HPV-18 E6*I was then prepared as the wild type plasmid except that, after the fragment Xma I-EcoR I fragment was inserted into the MCS of pNeo, the Asc I - Blp I fragment containing E6*I was used to replace the wild type genomic counterpart. pNeo-loxP HPV-18 and pNeo-loxP HPV-18 E6*I parental plasmids were verified by sequencing.

Immunohistochemistry

The slides were probed with antibody against BrdU (at 1:50; Invitrogen, Carlsbad, CA), the L1 major capsid protein (at 1:100, DakoCytomation, Carpinteria, CA), PCNA (at 1:300, Upstate, Temecula, CA) and cleaved caspase-3 (at 1:500, NEB, Beverly, MA) and reacted with Concentrated Detection System (BioGenex, San Ramon, CA) and DAB kit (Innovex Biosciences, Richmond, CA). The sections were counterstained with hematoxylin and mounted with Permount. Images were acquired using a 10x objective lens and visible light with an Olympus AX70 fluorescence microscope (Center Valley,

PA) and a Carl Zeiss Axiocam HR digital camera (Thornwood, NY). Adobe Photoshop CS2 (Adobe Systems, Mountain View, CA) was used to process the images.

DNA-FISH and Indirect IF for Antigen.

Biotinylated DNA probes were synthesized by nick translation (Roche, Indianapolis, IN) of the 8 kb HPV-18 genome. The biotinylated HPV-18 probes were precipitated, 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. After RNase treatment and DNA denaturation, slides were incubated with 10 µl of biotinylated HPV-18 probe stock, cover-slipped, sealed and incubated overnight in a 37°C humid chamber. After washing, biotinylated DNA probes were detected using streptavidin-conjugated horseradish peroxidase (SA-HRP) and tyramide signal amplification (TSA) per the manufacturer's instructions for either TSA Alexa Fluor 488 (Invitrogen) or TSA Cy3 (PerkinElmer, Wellesley, MA). Slides were then stained in 4′,6-diamidino-2-phenylindole (DAPI), mounted in Antifade, and sealed with coverslips.

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. IF detection of antigens coupled to DNA-FISH was performed as previously published (Van Tine et al. 2005). Briefly, slides were first subjected, if required, to antigen retrieval (0.01 M sodium citrate, pH 6.0) at 95°C

for twenty minutes. After cooling to room temperature (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, the slides were then incubated with the appropriate HRP-conjugated or biotinylated (followed by SA-HRP) secondary antibody for one hour at r.t. and detected by the specified fluorescent TSA kit. TSA labeling was quenched by washing the slides in 3% H_2O_2/PBS for 15 minutes at r.t. Slides were then prepared and probed for HPV-18 DNA by DNA-FISH. Detection of BrdU incorporation following either DNA-FISH alone or IF/DNA-FISH multiplexing was achieved by incubating the slides with mouse anti-BrdU for 1 hour at r.t. or overnight at 4°C. The slides were then incubated for 45 minutes at r.t. with goat anti-mouse secondary antibody conjugated to either Alexa Fluor 488 or Alexa Fluor 565 (Invitrogen). Thereafter, slides were DAPI-stained, mounted in Antifade and sealed with coverslips as before. Images were acquired using a 20x or 60x objective lens with an Olympus AX70 fluorescence microscope equipped with Speicher filters (Chroma, Rockingham, VT) and a Carl Zeiss Axiocam HR digital camera. Processing and assembly of images were the same as above. Four color images were captured with Alexa Fluor 488, Cy3 and DAPI in color and Alexa Fluor 565 black and white, then pseudo-colored yellow using AxioVision 3.1 software.

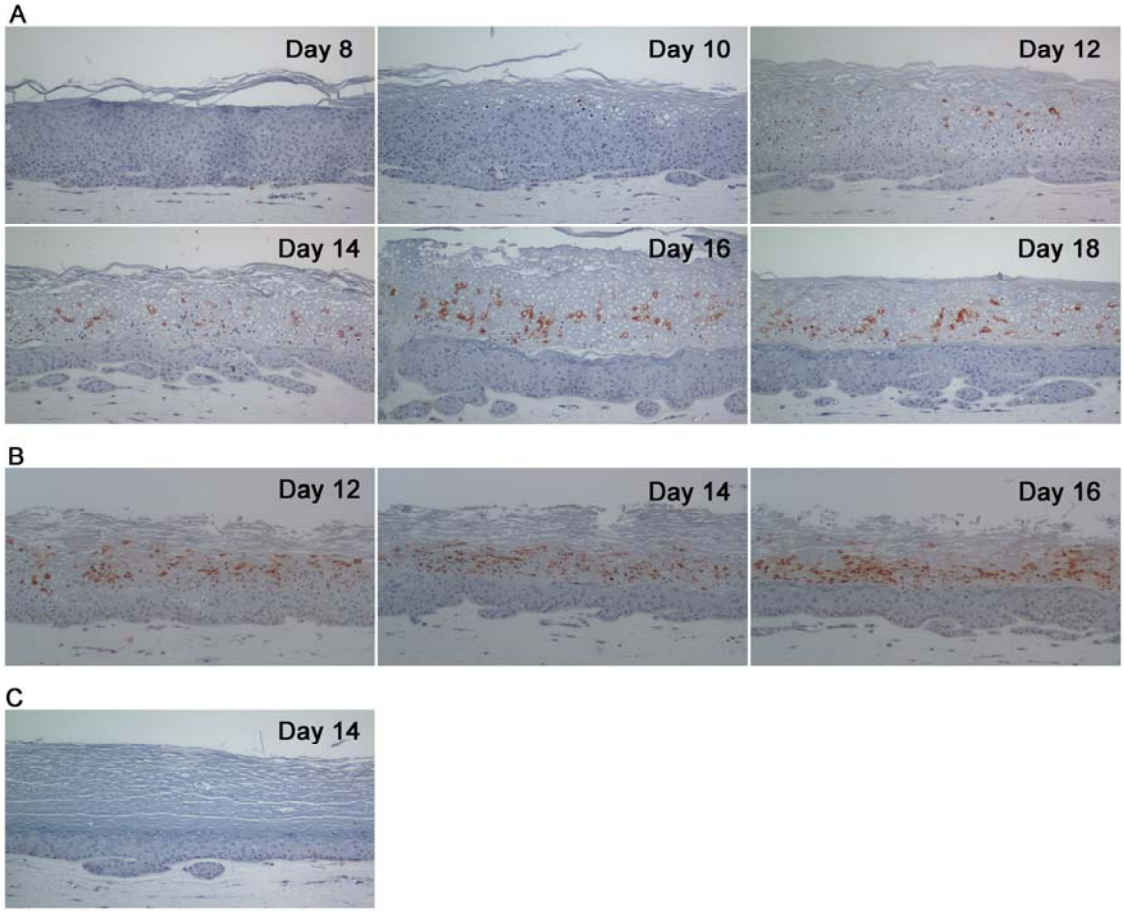

Figure S1. Time course of HPV-18 major capsid protein (L1) detection in raft cultures of PHKs

(A, B) Raft cultures of PHKs cotransfected with pNeo-loxP HPV-18 and nls-Cre expressing plasmid. The cultures were harvested on different days as indicated. Five time course experiments were conducted with similar results. One complete time course set of time course was presented in (A). The set in (B) is the wild type HPV-18 control raft cultures in parallel with the HPV-18 E6* mutant genome presented in Figure 6. (C) Day-14 raft culture of PHKs transfected with pNeo-loxP HPV-18 alone.

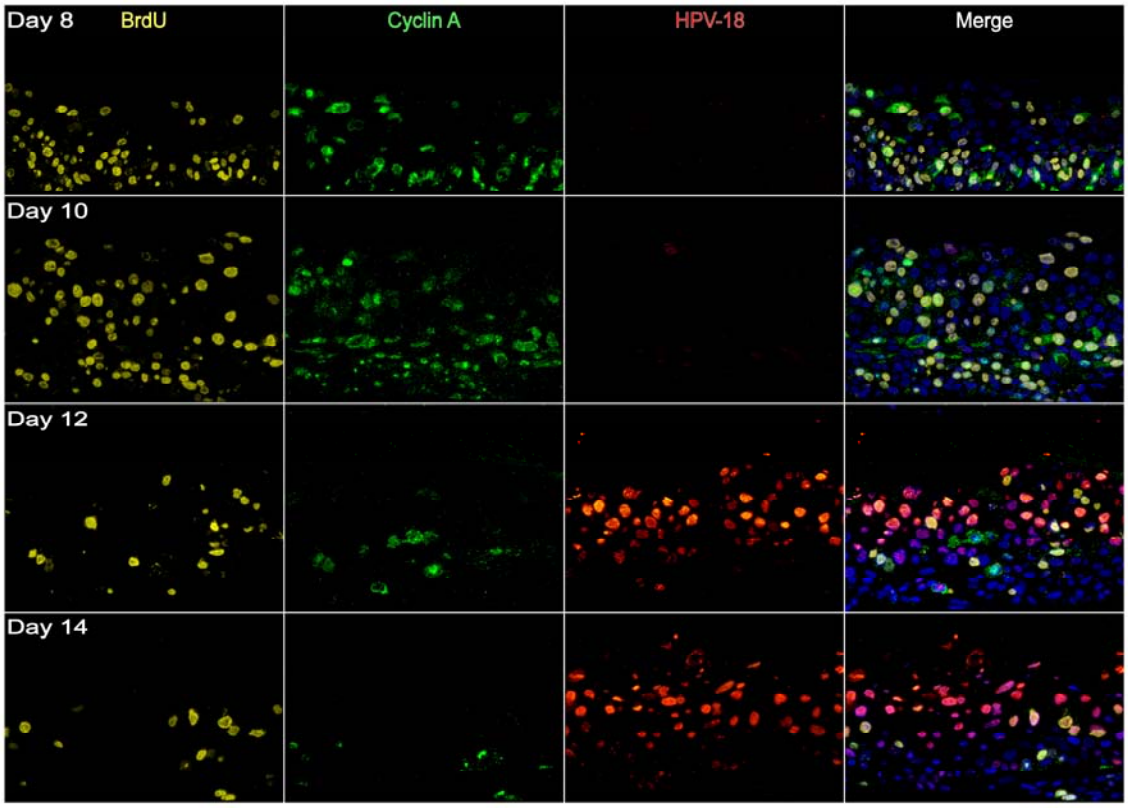

Figure S2. HPV-18 DNA amplification not in S phase, as revealed by the time course to detect viral DNA detection, BrdU incorporation, and cyclin A. Thin sections from the same day 8, 10, 12 and 14 raft cultures as shown in Figure 4 were subjected to sequential triple fluorescence detection of cyclin A (Alexa Fluor 488, green), HPV-18 DNA (Cy3, red), and BrdU (Alexa Fluor 647, yellow). Cellular DNA was stained with DAPI (blue).

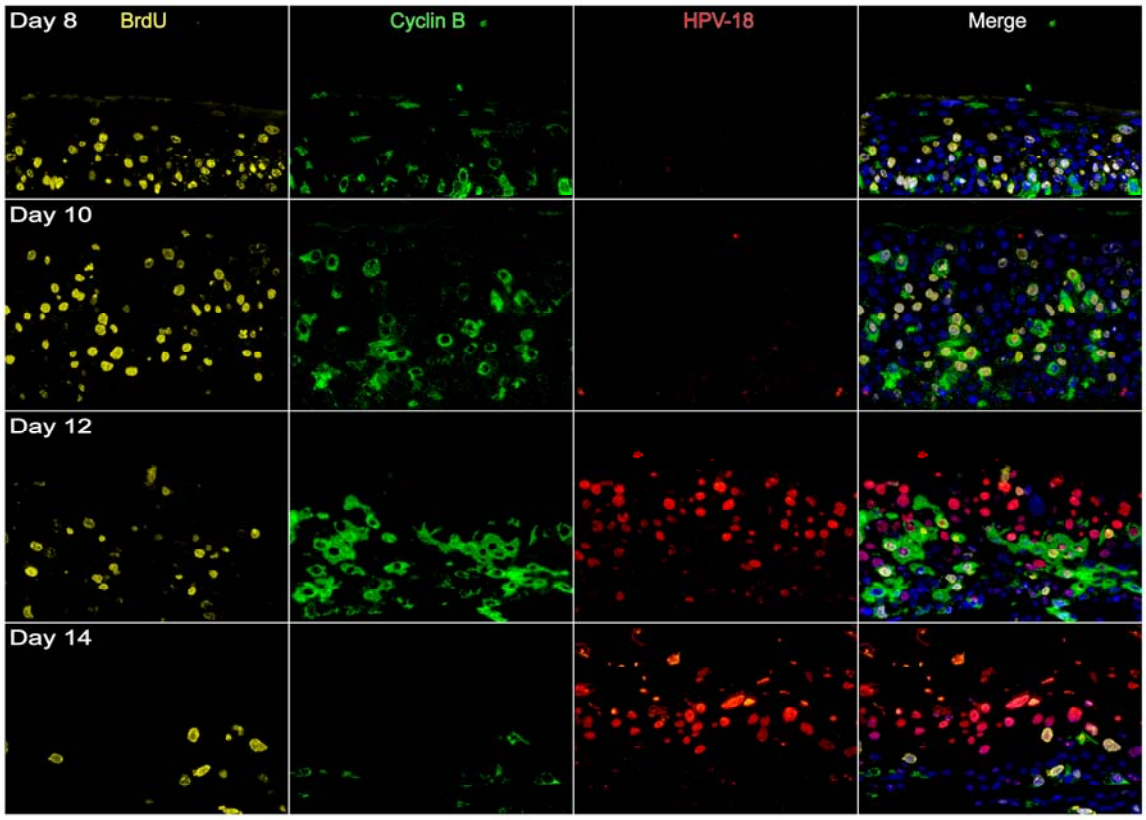

Figure S3. HPV-18 DNA amplification in the G2 phase, as revealed by the time course to detect viral DNA detection, BrdU incorporation, and cyclin B1. Thin sections from the same day 8, 10, 12 and 14 raft cultures as those shown in Figure 4 were subjected to sequential triple fluorescence detection of cyclin B (Alexa Fluor 488, green) HPV-18 DNA (Cy3, red), and BrdU (Alexa Fluor 647, yellow). Cellular DNA was stained with DAPI (blue).

CHAPTER 3

HPV-18 E6-NULL MUTANT GENOME AS A TOOL FOR FUTURE IDENTIFICATION OF E6-TARGET HOST PROTEINS CRITICAL FOR VIRAL DNA AMPLIFICATION

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Abstract

We recently developed a system in which Cre-loxP recombination efficiently liberates HPV whole genomic plasmid from a parental plasmid transfected into primary human keratinocytes (PHKs). The organotypic cultures of these PHKs support a robust productive life cycle. The system is particularly suitable to study HPV mutants in the context of full thickness squamous epithelia. The function of E6, which target many host proteins for degradation, is not understood in the productive program. Using the Cre-loxP systmer, we have shown that the immortalization-defective HPV-18 E6*I mutant genome was unable to support efficient viral DNA amplification nor progeny virus production. This mutant genome was complementation in *trans* by a retrovirus expressing the wild-type E6 gene from its natural, differentiation-dependent upstream regulatory region (HPV-18 URR E6). However, expression of the major late capsid protein L1 was seen in only 30% of the superficial keratinocytes. Here we optimized the *trans*-complementation system. We constructed pBabePuro HPV-18 URR-E6, a puromycin-resistant retroviral vector carrying with HPV-18 URR-E6. The PHKs were first infected with either an empty retrovirus or a virus delivering HPV-18 URR-E6 prior to transfection with an nls-Cre expressing plasmid and pNeo-loxP HPV-18 E6-null, a parental plasmid carrying HPV-18 mutant lacking the E6 initiation codon. The E6-null mutant genome was defective but was *trans*-complemented in virtually all the cells by the HPV-18 URR-E6. This optimized system should be applicable to systematic analysis of mutations in the E6 gene to identify the critical host proteins targeted by E6 during the productive life cycle.

Results and Discussion

Human papillomaviruses (HPVs) are non-enveloped DNA viruses with ~7.9 kb double stranded circular genome. About 120 genotypes of HPVs have been characterized (de Villiers et al. 2004). Among the mucosotropic HPVs, the normally benign infections by the high-risk (HR) types such as HPV-16 or HPV-18 can occasionally progress to cancers, such as cervical, penile, anal and a subset of oral cancers. On the other hand, infections by the low-risk (LR) genotypes such as HPV-6 and -11 very rarely progress to cancer. The productive phase of HPV takes place in differentiated strata of squamous epithelium. Organotypic cultures of immortalized epithelial cells have been used to recapitulate this phase of the viral life cycle (for reviews, see Lambert et al. 2005; Wilson and Laimins 2005). However genetic analyses of HPV to determine viral protein functions in this system have met with only limited success because immortalized cells have not supported efficient viral DNA amplification or virion production. In particular, the function of the E6 protein cannot be studied because most E6 mutants are unable to immortalize primary human keratinocytes (PHKs) or are not stably maintained in the transfected keratinocytes. The HR HPV-16 E6 protein alone has been studied in great detail. It accelerates the degradation of p53, E6TP1, hAda3, E6BP1, and many PDZ domain-containing proteins. With the exception of p53, the roles that the other proteins may have on viral reproduction are simply not known.

Utilizing Cre-loxP mediated recombination, we have recently established a highly efficient and reproducible system which efficiently generates autonomous HPV-18 genomic plasmids in primary human keratinocytes. Within a week, the transfected PHKs can be used to develop organotypic raft cultures that recapitulates a robust productive

program. The high yield of virus particles generated were passaged in naïve PHK raft cultures for the first time. Using this system, we have also shown that the HPV-18 E6*I mutant genome defective in immortalization is unable to amplify viral DNA efficiently in PHK raft cultures, while it induces high levels of p53 protein in numerous cells for prolonged period of times. These phenotypes were partially corrected when PHKs harboring the mutant genome is subsequently *trans*-complemented with a retrovirus expressing the wild type HPV-18 E6 gene alone or the E6 and E7 genes together under the control of the HPV-18 differentiation-dependent upstream regulatory region (i.e., URR-E6 or URR-E6E7) (Wang et al. 2009). These results are consistent with previous reports that p53 can inhibit HPV DNA replication (Lepik et al. 1998; Massimi et al. 1999). *Trans*-complementation of an E6 mutant genome should serve as a convenient assay to screen a panel of E6 mis-sense mutations with defined properties to identify the target host proteins, the degradation of which is critical for efficient viral DNA amplification.

A comparison of the kinetics and amount of L1 expression between *trans*-complemented mutant and wt HPV-18 revealed that the complemented mutant is delayed in L1 expression (day 18 vs. day 12 or less on wt). Furthermore, although p53 was completely abolished by E6 expressed in *trans*, L1 expression was detected in 30% of the superficial cells *versus* virtually 100% for cultures harboring the wild type viral genome. We suggest that the difference can be attributed one or more of the following factors. 1. Retrovirus infection was carried out about five days after PHKs are cotransfected with HPV-containing plasmid and nlsCre expressing plasmid DNA. Thus, the slower kinetics of complementation would have contributed to several days of delay in L1 expression

versus wild-type HPV-18 cultures. 2. E7 expression in the absence of E6 stabilizes p53 protein (Demers et al. 1994; Jian et al. 1998; Eichten et al. 2002) which inhibits viral DNA replication. Thus, the HPV-18 E6*I mutant genome might not be able to replicate for several days and becomes diluted or lost when PHKs divide before the trans-complementation takes place. 3. The intragenic E6 splice has been reported to be important for efficient E1 protein expression (Hubert and Laimins 2002; Lace et al. 2008). The E6*I genome might not be able to express sufficient E1 protein to support efficient viral DNA amplification. 4. The E6*I peptide may act as a dominant negative toward the full-length E6 protein. It is possible that, in some of the cells, the E6 levels expressed from the retrovirus may not be high enough to cause degradation of host proteins other than p53 to allow efficient viral DNA amplification.

Although the complementation was not observed in all regions of the raft cultures, these experiments nevertheless provided the proof of principle that it is possible to screen the ability of various E6 mutations to rescue an E6 mutant genome. However, to determine the phenotype of mis-sense E6 mutations unambiguously, more efficient *trans*-complementation by the wild type E6 would have to be achieved. In this work, we improved the test system to eliminate the above potential complications which may have limited the efficiency of *trans*-complementation. This is achieved by modifying both the mutant genomic plasmid and the retrovirus with which to deliver the E6 gene.

An improved test system to assess E6 functions.

pNeo-loxP HPV-18 E6-null mutant genome was constructed as described for pNeo-loxP HPV-18 (Wang et al. 2009). The mutant genome encodes no E6 peptide of

any length (E6-null) while preserving all the intragenic mRNA splice sites. The T106A mutation abolishes the AUG initiation codon, which is the only AUG in the E6 ORF. Using PCR, the fragment spanning HPV-18 URR-E6 (EcoRI-nts 6929-7857/1-581-SalI) was inserted into multiple cloning site in the pBabe Puro vector which carries the puromycin-resistance gene (Morgenstern and Land 1990). The strategy is to infect PHKs with a retrovirus prior to generating the HPV-18 genomic plasmid in PHKs. It is anticipated that L1 protein production by the E6 mutant rescued by wild type E6 should not be delayed relative to PHKs harboring the wild type HPV-18 plasmids (Figure 1). Indeed, this was the case. Furthermore, a similar percentage of L1 positive superficial cells was observed. The results as described in details below.

HPV-18 E6-null mutant genome is defective in PHK raft cultures.

PHKs were transfected with nls Cre expression plasmid and the pNeo-loxP HPV-18 wild type or the HPV-18 E6-null mutant as described (Wang et al. 2009). After G418 selection to eliminate untransfected cells, raft cultures were harvested on day 12 or day 16. Twelve hours before harvest, BrdU was added to each raft culture to labeling cells in S phase. The cultures were fixed with formalin, embedded in paraffin, and cut into 4 μm sections for *in situ* assays, as described (Wang et al. 2009). H&E staining of the formalin-fixed tissue sections showed that the raft cultures containing wt HPV-18 were mildly dysplastic (Figures 2A and 3A, left panels) relative to the normal PHK raft cultures (Figure 2C, left panel). When probed with BrdU, positive cells were distributed stochastically in all cell strata of the HPV-18 containing raft cultures (Figures 2A and 3A.

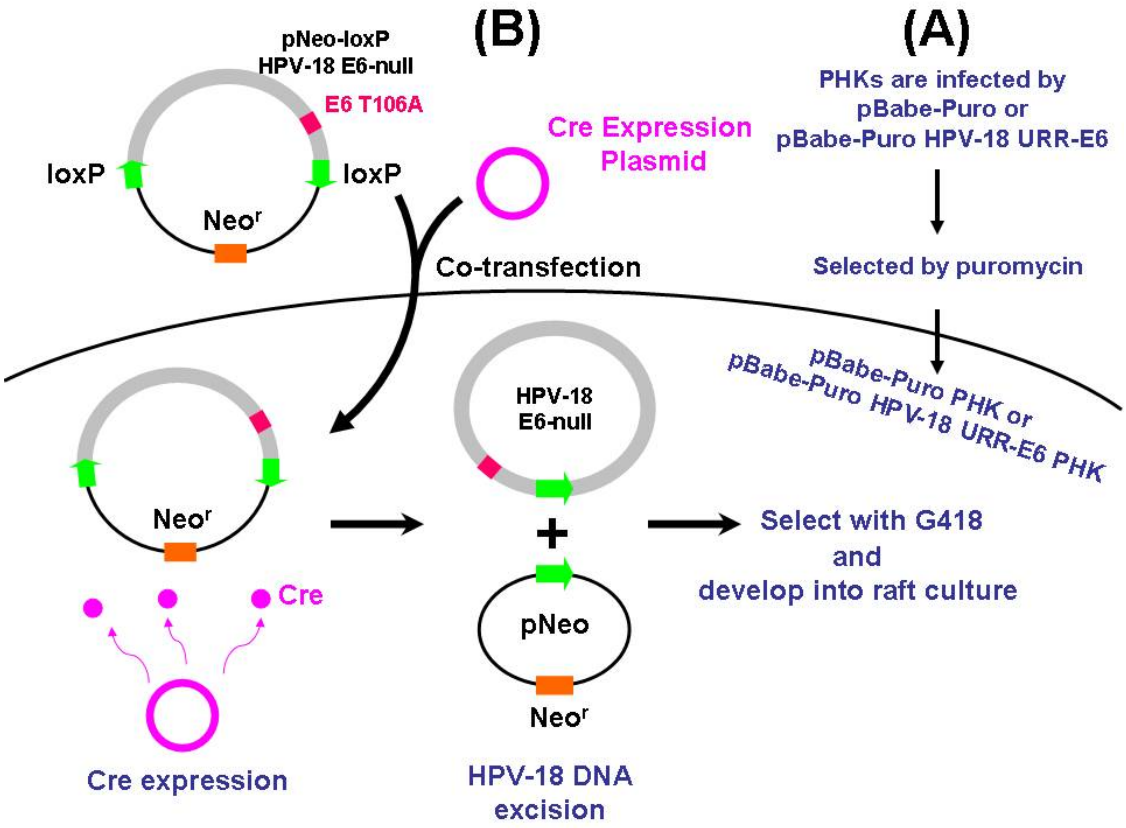

Figure 1. An improved *trans*-complementation for the HPV-18 E6-null mutant. **A**, prepare the retrovirus (empty or carrying HPV-18 URR-E6) infected PHKs first. **B**, then apply the same procedures as Wang et al., 2009 to co-transfect the pNeo-loxP HPV-18 E6-null and nls-Cre plasmids into the PHKs to generate HPV-18 E6-null.

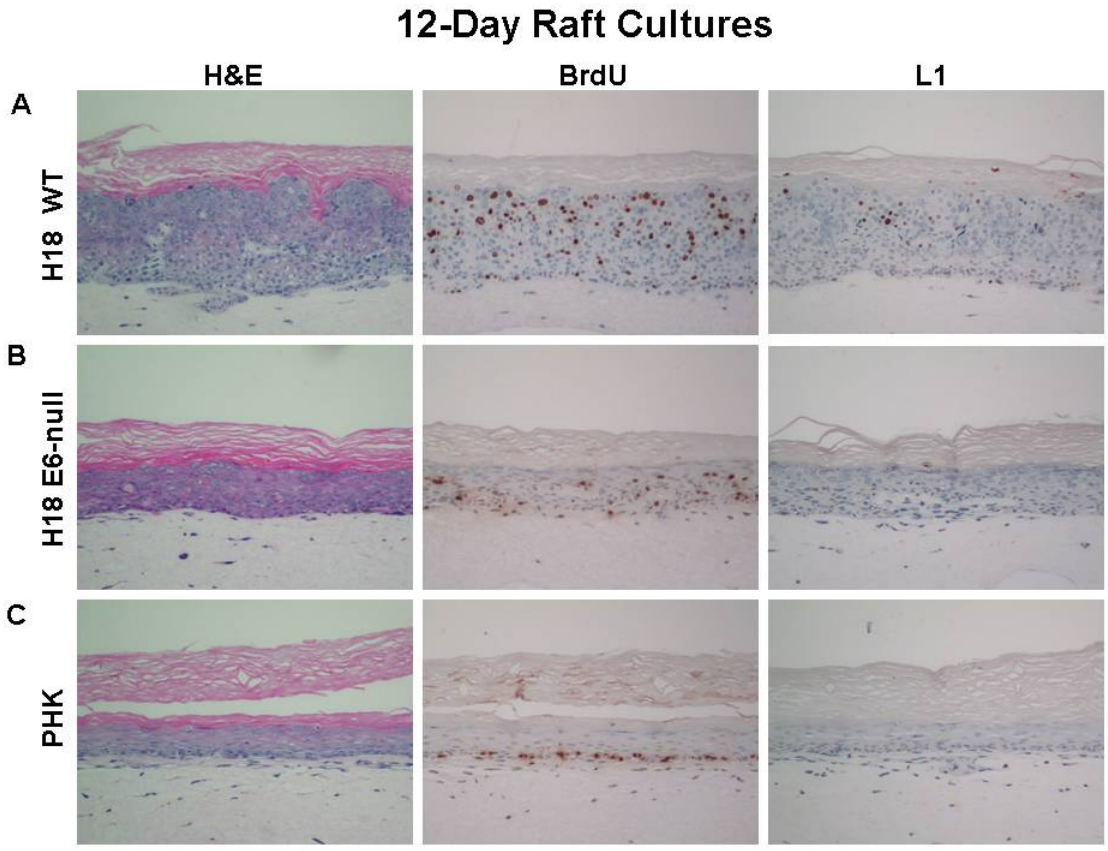

Figure 2. Histology and *in situ* analyses of day-12 PHK raft cultures.
A, wild-type HPV-18. **B,** HPV-18 E6-null. **C,** normal PHK. Left panels, H&E staining. Middle panels, probed for BrdU. Right panels, probed for major capsid protein (L1).

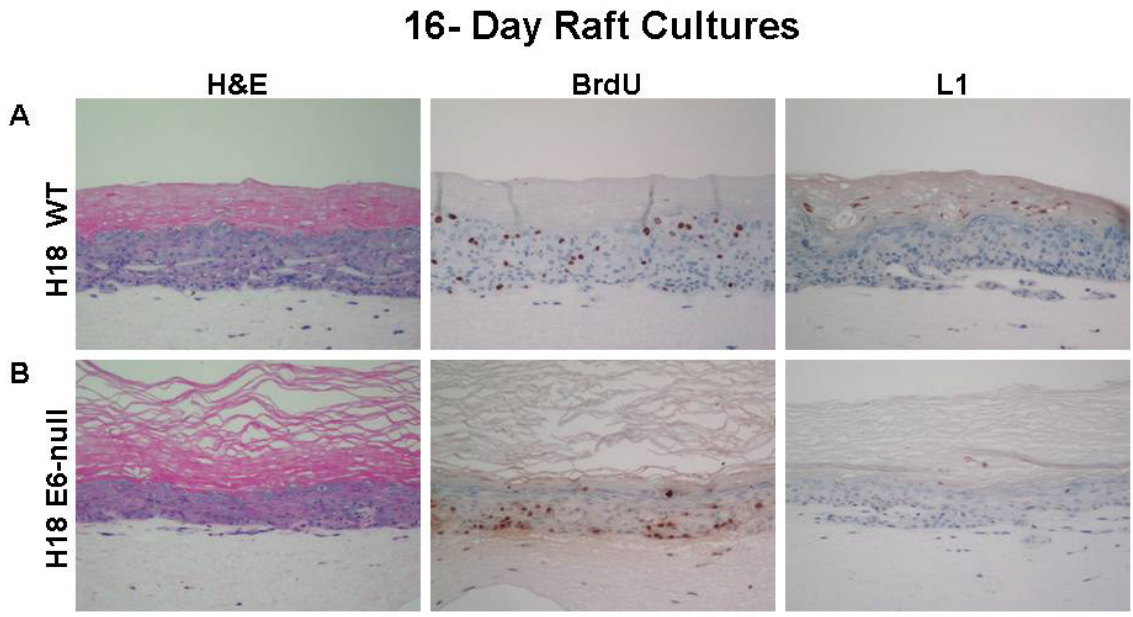

Figure 3. Histology and *in situ* analyses of day-16 PHK raft cultures.
A, wild-type HPV-18. **B,** HPV-18 E6-null. Left panels, H&E staining. Middle panels, probed for BrdU. Right panels, probed for L1.

middle panels), whereas only basal cells were positive for BrdU in the control PHK raft cultures (Figure 2C, middle panel). Raft cultures containing the HPV-18 E6 null mutant genome had histological appearances in between the above two raft cultures (Figures 2B and 3B, left panels). Conspicuously, BrdU-positive suprabasal cells were significantly reduced relative to cultures containing the wild type HPV-18 (Figures 2B and 3B, middle panels).

In wt HPV-18 raft cultures, the expression of the major capsid protein was low on the 12th day, while abundant L1 antigen was readily detected on the 16th day in cornified envelopes, verifying a highly productive program, as in our previous report (Figures 2A, 3A, right panels). No signal was detected in the control PHK cultures (Figure 2C, right panel). The E6-null mutant has little or no L1 signal on days 12, 16, day 20 (Figures 2B, 3B, right panels and data not shown). These results are in complete agreement with our previous observation made with the E6*I mutant genome.

HPV-18 URR-E6 retrovirus efficiently rescues the E6-null mutant genome.

Retroviruses were produced in amphotropic producer cell line, GP + envAm12 (Markowitz et al. 1988), upon selected for 3 days with 1 μg/ml puromycin. PHKs with first infected with a vector-only retrovirus or the retrovirus carrying HPV-18 URR-E6 and selected for 2 days with 1.5 μg/ml puromycin. The surviving cells were then cotransfect with an nls-Cre expressing plasmid and the parental plasmid which carries the HPV-18 E6-null mutant genome. G148 selected PHKs were developed into raft cultures. As controls, PHKs were infected with the empty retrovirus pBabe Puro or with pBabe Puro HPV-18 URR-E6 without DNA transfection. Prior transduction with empty pBabe

Puro had no effect on the phenotypes of the HPV-18 E6-null containing raft cultures (Figure 4). In contrast, prior transduction with the HPV-18 URR-E6 retrovirus successfully rescued the mutant (Figure 5). The histology, the pattern of BrdU incorporation, and the kinetics, and the frequency of L1-positive cornified envelopes were virtually indistinguishable from those detected in raft cultures harboring the wild type HPV-18 genome. As a further control, PHKs transduced with either the pBabe Puro retrovirus or HPV-18 URR-E6 retrovirus had phenotypes identical to that of the normal PHK raft cultures (compare Figures 6A, B to 2C). These results demonstrate that E6 itself has no effect on S phase reentry or dysplasia, concordant with our earlier report (Cheng et al. 1995).

In summary, we have developed an effective *trans*-complementation system with which to screen a panel of HPV-18 mis-sense mutations for their ability to support the productive life cycle. From the well characterized properties of the homologous HPV-16 E6 mutations, it is then possible to identify with little or no ambiguity, among the numerous E6 targets, host proteins the degradation of which is critical for high levels of viral DNA amplification and virion production.

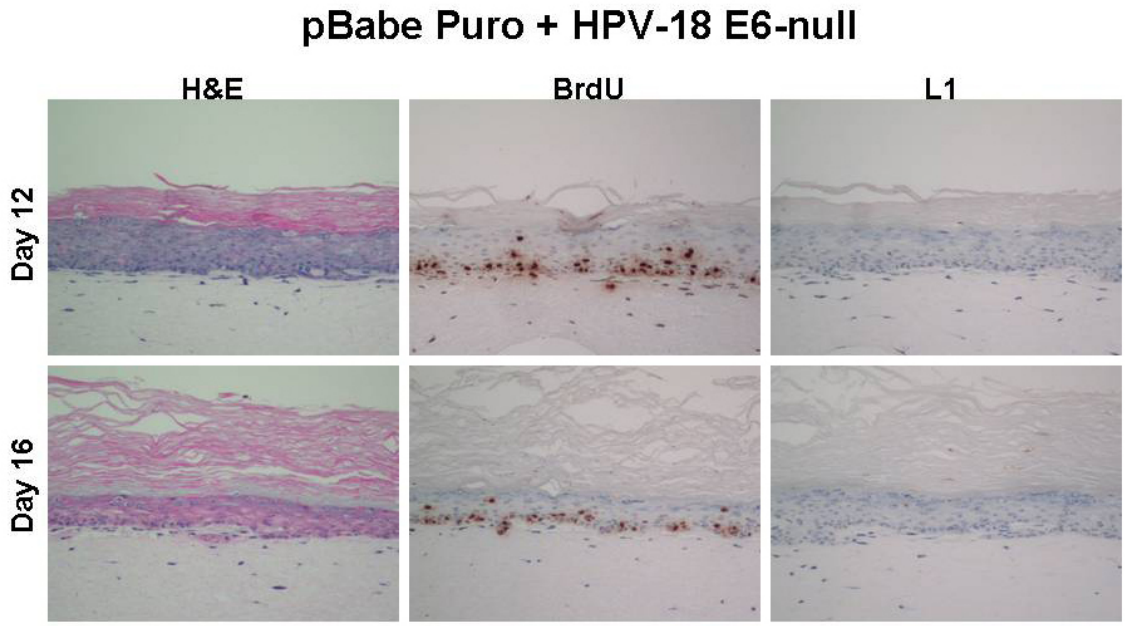

Figure 4. Histology and *in situ* analyses of HPV-18 E6-null containing PHK raft cultures *trans*-complemented by empty retrovirus.
Left panels, H&E staining. Middle panels, probed for BrdU. Right panels, probed for L1.

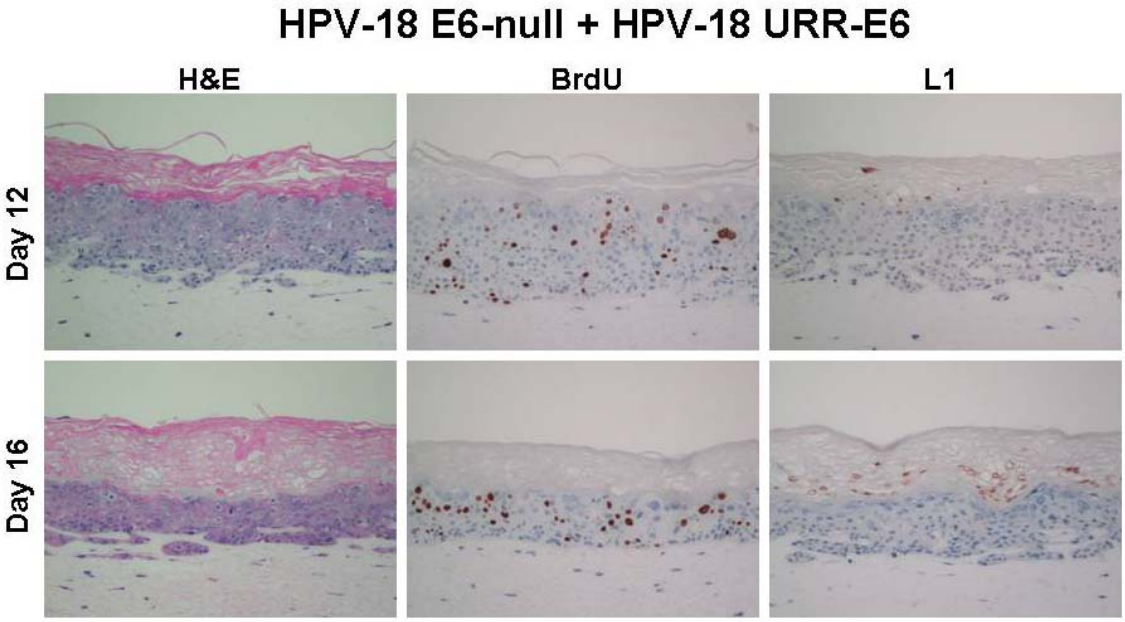

Figure 5. Histology and *in situ* analyses of HPV-18 E6-null containing PHK raft cultures *trans*-complemented by HPV-18 URR driven E6.
Left panels, H&E staining. Middle panels, probed for BrdU. Right panels, probed for L1.

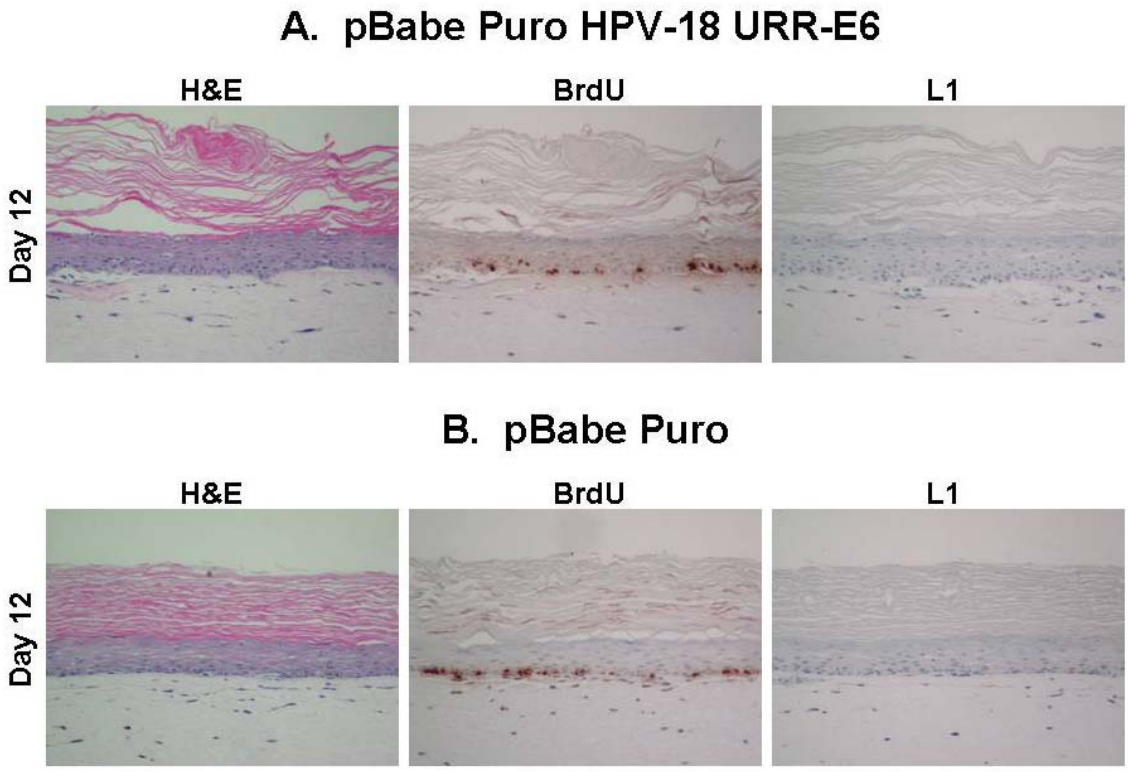

Figure 6. Histology and *in situ* analyses of negative control PHK raft cultures.
A, a pBabe Puro HPV-18 URR-E6 infected PHK raft culture. **B,** a pBabe Puro (an empty retrovirus) infected PHK raft culture. Left panels, H&E staining. Middle panels, probed for BrdU. Right panels, probed for L1.

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CHAPTER 4

SUMMARY

A. Overview of the organotypic system for recapitulating the HPV-18 infection cycle.

The long sought goal of highly efficient production of infectious HPV and passaging the virus in an experimental cell or tissue culture system has been achieved. We constructed a parental plasmid which carries the complete HPV type 18 genome flanked by loxP sites in a commercial vector, which encodes a drug resistance gene selectable in both bacteria and mammalian cells. Upon co-transfection of the negatively supercoiled parental plasmid along with an nlsCre expression plasmid into primary human keratinocytes, nlsCre-mediated recombination liberated an HPV genomic plasmid. Untransfected cells are eliminated by a brief drug selection. Within a week post-transfection, PHKs are used to establish organotypic squamous epithelial raft cultures, recapitulating each stage of the reproductive program from early gene expression, E7-mediated induction of host chromosome replication, vegetative amplification of the viral genome to very high copy numbers, late gene expression, virion assembly, and particle maturation in desquamated keratinocytes. For the first time, the progeny virions infected fresh PHKs and were passaged through a complete infection cycle, again with high yields. Compared with previously described systems which produce very low yields of virus particles and which are incapable of passaging, our Cre-loxP system is simple,

reproducible, less expensive, far less time-consuming and much closer to the tissue environment encountered in natural infections.

The establishment of this novel methodology is a major advance in the field for the following reasons. (a) It is highly informative in revealing virus – host interactions over the course of the entire infection cycle (further elaborated in section **B** below). (b) It circumvents the need for the immortalization functions unique to the high-risk HPV types. Thus it should, in principle, be applicable to other HPV types, including the low-risk HPV genotypes. (c) It is ideal for genetic and functional analyses of the various HPV genes, especially for E6 mutants not previously possible, or E4 or E5 mutants for which functions in the infection cycle have not been resolved. The analyses of E6 mutants are further aided by the *trans*-complementation system which I also developed and optimized. (d) It can be used for antiviral drug discovery and comprehensive pharmacologic characterization *ex vivo* (to be elaborated in Section **C**).

B. Novel virus-host interactions revealed by the productive program in PHK raft cultures.

With the PHK raft culture system, many new insights into the viral life cycle have been discovered. In particular, HPV DNA amplification strictly follows replication of the host chromosomes and occurs during G2 arrest. This G2 arrest is not perpetrated by the E1^E4 protein, as had been published by other labs. Recent work by Dr. N. Sanjib Banerjee of our lab showed that G2 arrest is a consequence of the viral E7 protein (personal communication), which interacts with p130, a member of the family of retinoblastoma proteins that maintains homeostasis of non-cycling differentiated cells

(Genovese et al., 2008). The studies of E6*I and E6-null mutants reveal for the first time that the full-length E6 protein is required for viral DNA amplification and L1 expression. The infection assay unexpectedly reveals that only a high MOI can progress to a productive infection culminating to progeny virus production. At low MOI, the cells are infected but viral DNA fails to amplify and there is no L1 production. This interesting observation will be discussed in Section **C**. The results from the infection experiments and from the E6 mutant both demonstrate convincingly that L1 expression is dependent on viral DNA amplification, contrary to a previous report (Terhune et al. 2001)

C. Possible Role and Consequence of Viral DNA Methylation.

In our infection assays, productive infection is achieved only at very high MOI (800 particles/cell or higher). We suggest that this might be explained by methylation of viral DNA. Methylation of papillomavirus DNA was discovered 20 years ago but the details are unclear. DNA methylation may regulate viral activity through the introduction of methyl groups to cytosine residues at CpG dinucleotides which can lead to transcriptional silencing and also inhibition of DNA replication (reviewed by Zheng and Baker 2006). This could be a host defensive response to foreign DNA. Furthermore, the E2 protein binding sites that comprise the replication origin of all papillomaviruses are ACCGNNNNCGGT, and often AACCGNNNNCGGTT, where N is usually A or T for mucosal tropic HPV types. Note that this sequence has two CpG motifs on each DNA strand (upper/lower; Watson/Crick; sense/antisense). Our lab has previously proposed that the E2BS could be methylated to modulate viral DNA replication (Chiang et al. 1992). Indeed, methylation at the ori prevents E2 protein binding (Kim et al. 2003).

Since E2 is critical to recruit the E1 replicative DNA helicase to the ori, a methylated ori would inhibit viral DNA replication. On the other hand, E2 binding to the ori, which overlaps the E6 promoter, would inhibit transcription (Demeret et al. 1994; Dong et al. 1994; Thomas and Chiang 2005). Thus, a methylated ori would allow early transcription to occur if methylation is not overly extensive to prevent binding of critical host transcription factors at other regulatory sequences.

With this understanding, one hypothesis for the need of high MOI for productive infection is that the URR region (or the coding regions as well) of packaged viral DNA is relatively heavily but perhaps stochastically methylated in the URR. Most of the viral DNA are templates for transcription while, only at high MOI, are some rare genomes able to serve as templates for viral DNA replication. However, at low MOI, viral early gene expression can take place but there are few unmethylated origins to allow replication complexes to assemble and amplification to occur. An alternative hypothesis is that host DNA methyltransferease acts as a defensive mechanism toward the incoming viral DNA. Only at high MOI are the methylation enzymes overwhelmed and the virus can then progress to a productive phase.

It is not known at this time whether URR is methylated *in vivo* or in raft cultures and if so, whether this can occur in basal strata, lower or upper spinous strata. We are collaborating with Dr. Magnus von Knebel-Doeberitz, Department of Applied Tumour Biology, University of Heidelberg, Germany, to test the hypothesis by assessing the degree and site specific localization of DNA methylation in the URR of DNA packaged in mature virions. They will also analyze the level of DNA methylation on viral DNA

extracted from tissue sections using laser confocal microdissection of productive and nonproductive raft cultures.

D. Comparison of Authentic HPV-18 Virions and Pseudovirions

HPV pseudovirions can be simply packed and produced through 293TT monolayer (Buck et al. 2004). It obviates the need for epithelia raft cultures. The authors always claim that that there is no difference from wt HPVs, especially on capsid structure (Pyeon et al. 2005). Conversely, we believe that HPV maturation needs to rely on complete keratinocyte differentiation culminating in the transition from a reducing state (preventing disulfide bond formation) characteristic of metabolically active cells to a dying, oxidizing state where L1 protein cross-linking can occur (Buck et al. 2005). Producing HPV as close to naturally in organotypic raft cultures is necessary for such maturation. For example, in HPV containing PHK raft cultures, paracrystalline arrays can be seen forming from mature HPV virions in cornified envelopes whereas immature virions in live nuclei lack of such ordered array (Wang et al. 2009). This maturation process imparts stability attributable to the formation of disulfide bonds between neighboring L1 capsomers (Buck et al. 2005). That appears to be why the pseudovirions have substantially different properties from wt virions produced in rafts and have poor infectivity in the natural host cell. In the future, a side-by-side comparison between our genuine virions and pseudovirions need to be conducted to compare and contrast the infectivity. The experiments described in section **E** have indeed revealed differences between authentic virion and pseudovirions.

E. Potential Impact on Medicine and Public Health

This system can be used to characterize potential inhibitors of HPV infection currently being conducted. I tested the abilities of the sea weed extract carrageenan and of an antibody to the minor capsid protein L2 to inhibit HPV infection of PHKs.

a. Carrageenan

Carrageenans are large galactose-linked sulfated polysaccharides which can be extracted from red seaweeds such as *Chondrus, Gigartina,* and *Eucheuma.* These compounds are very stable and bacteria cannot generally take them up or metabolize them. Carrageenans confer higher viscosity on liquids and are widely used in foods and in pharmaceutical products such as cosmetics and sexual lubricants. Carrageenan has been reported to be potent inhibitors viruses including herpes simplex virus, human cytomegalovirus, and human immunodeficiency virus, etc (Baba et al. 1988). Recently, Buck et al. reported that ι-carrageenan at 100ng/ml was also a potent inhibitor of infection by HPV pseudovirions that harbors a reporter gene in cell lines or in mouse genital tract after brushing (Buck et al. 2006; Roberts et al. 2007). Here, we used genuine HPV-18 virions harvested from PHK raft cultures to test the inhibitory effect of ι-carrageenan on PHKs.

First, we found that carrageenan had little or no cytotoxicity on normal PHKs at 0.1 to 1600μg/ml overnight. Even at the highest concentration tested, 1600μg/ml, the growth of PHKs was only slightly retarded (data not shown). Second, we test its ability to inhibit infection of PHKs by our authentic HPV virions. The MOIs of HPV-18 used to test the effects of carrageenan in suberged PHKs were 0, 5, 50 and 500. Carrageenan and

HPV virions were mixed together in various ratios (Table 1) for 10 minutes and then added to the PHKs for incubation overnight. Then the culture medium was changed to fresh K-SFM and the submerged cultures were grown for ~4 days to 90% confluence. To determine whether infection took place, RT-PCR or RT-nested PCR was conducted to detect the 521 bp long spliced E6-E7-E1^E4 cDNA fragment using agarose gel electrophoresis followed by ethidium staining. Our conclusion is that carageenan can inhibit HPV infection and the degree of inhibition is dependent both the MOI and the concentration of carageenan. At MOI of 5 with or without carrageenan treatment, there was no expected band of 521 bp cDNA fragment band on RT-PCR (data not shown) similar to the uninfected cultures (Figure 1 lane 1). However, RT-nested PCR detected the spliced cDNA without carrageenan or when carrageenan was present at 0.5 μg/ml or lower (lanes 2 to 4). At 2.5 μg/ml, carrageenan completely abolished infection (lanes 5 and 6). At the MOI of 50 and 500 in the absence of carrageenan, the cDNA was easily detected by RT-PCR (lanes 7 and 12). At MOI of 50 and 1 μg/ml and 5 μg/ml or higher of carrageenan , infection was partially or completely inhibited based on RT-nested PCR (lane 8, 9-11). At MOI of 500, 10 μg/ml or higher of carrageenan abolished , there was any cDNA band detectable by RT-PCR; however, aberrant products were detected upon RT-nested PCR (lanes 13, 14). The identity of these aberrant bands remains to be determined. RT-PCR of β-actin showed that all RT reactions were successful (Figure 1 bottom). These preliminary observations indicate that 5 μg/ml of carrageenan completely inhibited nonproductive HPV-18 infection at MOI of 50, whereas at 10 μg/ml, carrageenan might disrupt the productive infection at MOI of 500. Therefore, carrageenan significantly reduces HPV-18 infection of PHKs.

HPV MOI	Carrageenan Concentration (μg/ml)				
0	0				
5	0	0.1	0.5	2.5	12.5
50	0	1	5	25	125
500	0	10	50	250	1250

Table 1. Multiplex tests of carrageenan inhibition of HPV-18 infection in PHKs.

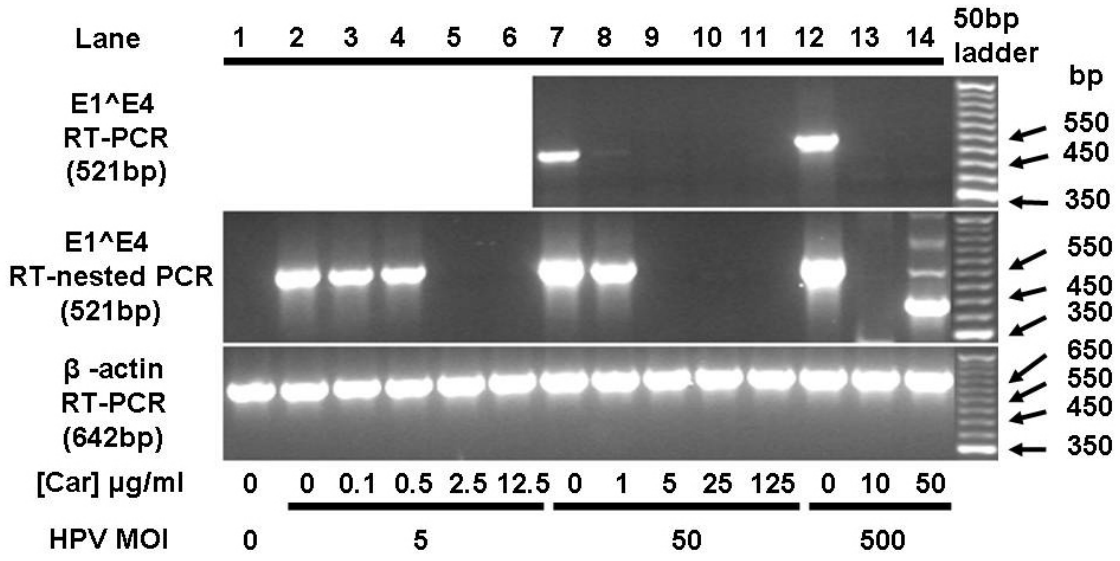

Figure 1. Carrageenan inhibition of HPV-18 infection of PHKs.
Total RNA was isolated from submerged PHKs four days post infection. Successful infection was detected by RT- or RT-Nested PCR of a cDNA fragment of spliced HPV-18 E6-E7-E1^E4 RNA or the β-actin mRNA, as described (Wang et al. 2009 and refs therein).

We also tested the ability of carrageenan to inhibit of infection of PHKs by HPV-18 virions in raft cultures. Submerged PHK cultures seeded on collagen beds were grown for about 18 hr and then exposed overnight to HPV-18 virions (MOI=1000) that had been pre-mixed for 10 minutes with carrageenan (0, 200, 400, 800 or 1600 μg/ml). Then the culture medium was changed to a 1:1 mixture of K-SFM and normal raft medium. On day 4, the collagen-cell culture assembly was lifted to medium-air interface, grown as raft cultures, and harvested 16 days later. The expression levels of the DNA polymerase δ processivity factor PCNA induced in suprabasal cells by HPV E7 and the viral major capsid protein L1 were examined (Cheng et al. 1995). Normal PHK raft culture only has PCNA signals in basal layer (Figure 2A). In PHK raft cultures infected with virions not exposed to carrageenan, PCNA induction was highly induced in virtually all the cells and L1 expression was easy to detect in the cornified layers (Figures 2B and C). At the lowest carrageenan concentrations used (200 μg/ml), PCNA induction became more sporadic than in untreated HPV-18 infected PHK rafts, and no L1 signal was detected (Figures 2D and E). When virions were preincubated with carrageenan at 400 μg/ml or higher, the tissue morphology was very similar to normal, uninfected PHK rafts (Figures 2F to I) and suprabasal cells positive for PCNA was greatly reduced. These results indicate that high concentrations of carrageenan significantly inhabited infection.

For comparison, Buck et al. showed that 100% inhibition of infection on HeLa cells with pseudovirions when carageenan is present at 100ng/ml (Buck et al. 2006).

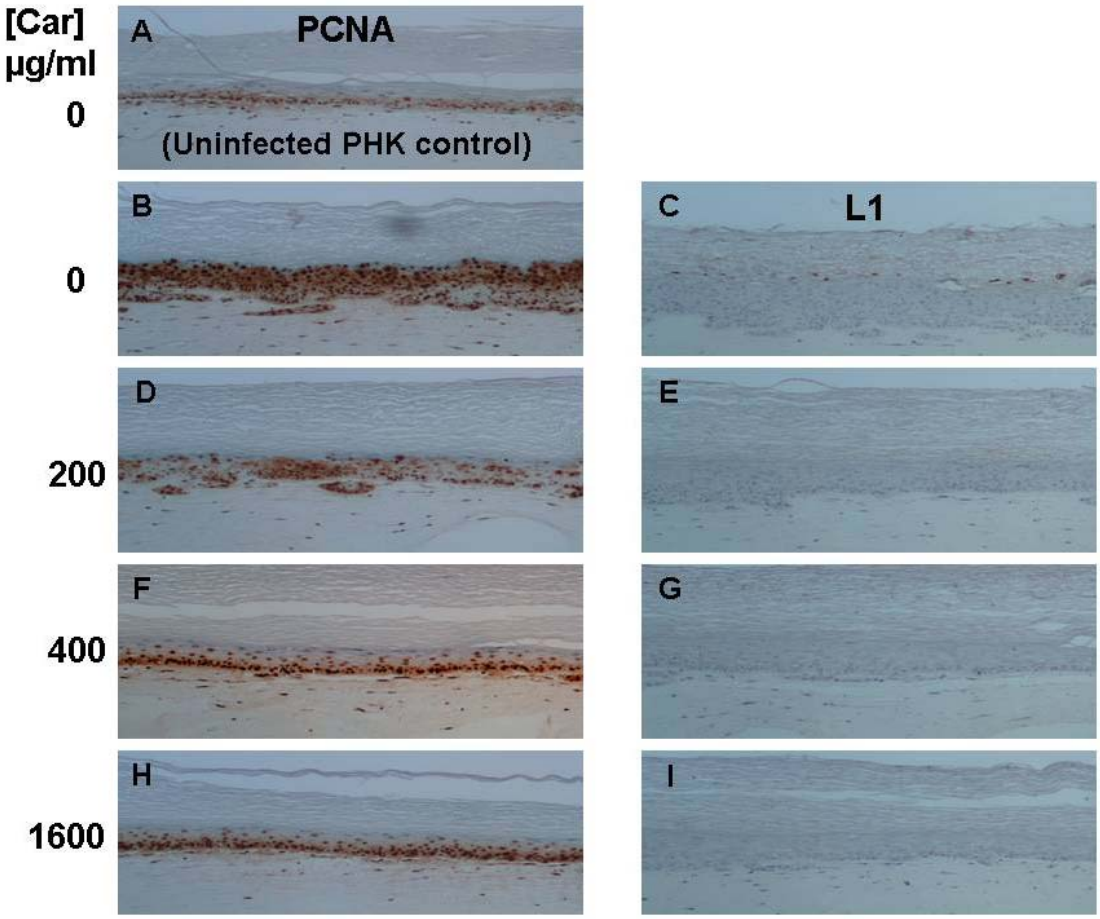

Figure 2. Histology of carrageenan treated HPV-18 infected PHK raft cultures.
A, normal untreated PHK raft culture. **B to I,** HPV-18 virions (MOI=1000) and carrageenan (0 to 1600 µg/ml) were added together to PHKs on collagen bed overnight. Raft cultures were harvested on day 16 for analyses.

However, our data showed that at high MOI necessary for productive infection by our authentic HPV-18 virions, carrageenan at 1600 μg/ml did not completely abolish infection. Even at a MOI of 50 which leads to a nonproductive infection in PHK raft cultures (Wang et al. 2009), it takes 5 μg/ml of carrageenon to abolish infection. Thus, we can conclude that our authentic HPV virions are much more infectious in PHKs when compared to pseudovirions on epithelial cell lines.

In the future, we will conduct quantitative RT-PCR to determine the degree of inhibition achieved at different concentration and various MOI. Moreover, we would also like to perform a time course to assess the duration of incubation required to exert the inhibitory activity (eg. 05, 2, 4, or 8 hours of incubation of the PHKs with the mixture of virions and carrageenan). We also would like to determine whether carrageenan can (a) block HPV infectivity if added various lengths of time after exposure of the cells to virions, and (b) whether pre-treatment of cells for various lengths of time and over a range of concentrations can block subsequent infection with the virion. The outcome of these experiments will determine whether carrageenan can be developed into a useful preventive measure. If, for instance, pretreatment of cells with carageenan is not effective. Rather it takes a long pre-incubation between virus and carageenan to prevent infection. In that case, carrageenan would not be a useful agent to prevent infection.

b. Testing of antibodies to the L2 minor capsid protein.

We have established a collaboration with Dr. Richard Roden, Department of Pathology, The Johns Hopkins University. His lab has extensively characterized cross-reactive L2 antibodies and is in the process of developing an L2 peptide-based pan-HPV

vaccine (Gambhira et al. 2007). L2 protein is much less antigenic than HPV L1 VLPs. However, the L2 peptide spanning amino acid residues 17-36 is conserved among various PV types and induces antibodies that are cross-reactive with multiple HPV pseudovirions as well as with genuine cottontail rabbit papillomavirus (Pastrana et al. 2005). Furthermore, the neutralization studies were conducted in HaCaT or 293TT cells. Since pseudovirions efficiently infect these immortalized or transformed epithelial cell lines but poorly infect PHKs, the natural host of HPVs, these neutralization results could be substantially different from infection of PHKs with genuine HPV virions. Thus, it is critical to repeat the neutralization tests with my authentic HPV-18 virions.

Using the E6-E7-E1^E4 cDNA production revealed by RT-PCR as a readout in HPV-18 infected PHKs, my preliminary results showed that the anti-L2 RG-1 antibody indeed reduced the cDNA production (for details, see Table 2) (Figure 3A) compared with the negative control, C-9 antibody (Figure 3B). Yet, the inhibition appeared to be much less efficient than the published results. This difference of course raises concerns about both the pseudovirion system and the L2 peptide as an antigen for vaccine development.

F. Future Applications

There are numerous experiments that my lab mates or I can pursue by taking advantage of the Cre-loxP system. First, the system should be extended to other HPV genotypes to prove its general applicability. Although initial cursory attempts by members of the lab have not been successful, the main reason might be attributable to cryptic mutations in the prototype genomic clones. Indeed, some HPV-16 isolates fail to amplify in raft cultures of immortalized cells (McLaughlin-Drubin et al. 2004). The

A

Ab dilu / HPV MOI	1:10	1:50	1:250	1:1250	No RG-1
0	X	X	X	X	X
5	X	X	X	X	X
50	X	X	X	X	X
500	X	X	X	X	X

B

Ab dilu / HPV MOI	1:10	1:50	1:250	1:1250
5	X	X	X	
50	X	X	X	X

Table 2. Multiplex tests of L2 antibodies neutralization of HPV-18 infection in PHKs.
A, neutralizing L2 antibody, RG-1; **B**, non-neutralizing L2 antibody, C-9.

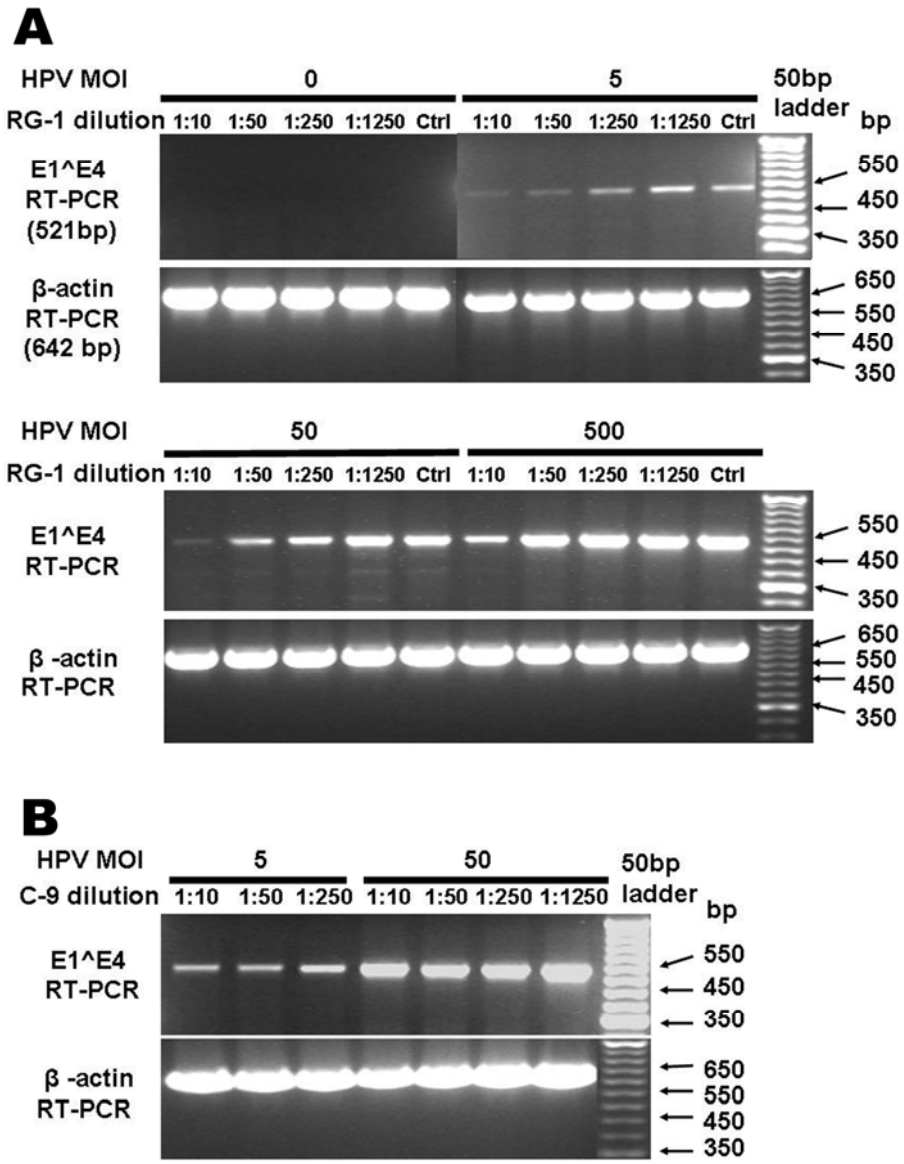

Figure 3. Antibody neutralization of HPV-18 infection of PHKs.
A, Neutralizing L2 antibody, RG-1. **B,** non-neutralizing L2 antibody, C-9. Virions and antibodies at various dilutions were added together to PHKs and incubated overnight. Total RNA was isolated from submerged PHKs four days post infection. Successful infection was detected by RT- or RT-Nested PCR of a cDNA fragment of spliced HPV-18 E6-E7-E1^E4 RNA or the β-actin mRNA, as described (Wang et al. 2009 and refs therein).

recombinant HPV genomic DNAs were all isolated more than two decades ago and have been repeatedly amplified in *E. coli* in different laboratories. There is no assurance that detrimental mutations have not occurred. It is best to reclone the viral genomes from benign lesions before attempting to extend the replicon system to additional HPV types.

The functional dissections of HPV E6 and E5 genes are being conducted in the lab. I will continue to use the authentic virions to study the potential of the L2 antigenic epitope (residues 17-36) as a pan-HPV vaccine. I will (a) use higher quality RG-1 antibody for the experiment, (b) use real-time PCR to obtain quantitative results of RG-1 antibody neutralization and (c) perform a time course of viral entry into PHKs to determine the best experimental schedule for RG-1 antibody neutralization or, more specifically, the precise stage of infection at which the antibody is acting, given that RG-1 epitope is exposed only upon cleavage by furin after the virion has undergone a conformational change upon binding to the extracellular matrix, essential for the next stage of engagement with cellular receptors for the virus. In addition, I also plan to understand why the L1 VLPs function so effectively as a prophylactic vaccines. These experiments involve the determination of the kinetics of VLP antibody to neutralize infection by the authentic HPV-18 virions in both submerged PHKs and in PHK raft cultures.

CHAPTER 5

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CHAPTER 6

APPENDIX

IRB Approval

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Review Board for Human Use

DATE:

MEMORANDUM

TO: **Hsu-Kun Wang**
Student Investigator

FROM: Sheila Moore, CIP
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.

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