Aspects of Infectious Agents and Cancer: Effects of Human Papillomavirus 16 and *Escherichia coli* on the Innate Immune Defense of Human Keratinocytes

By

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Dedication

To my parents for their love and support.

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List of Abbreviations

28S	Large Ribosomal RNA Subunit
18S	Small Ribosomal RNA Subunit
A_{260}	UV Density at 260nm
ANOVA	Analysis of Variance Statistical Method
APC	Antigen Presenting Cell
CARD	Caspase Recruiting Domain
cDNA	Complementary DNA
CpG	Cytosine/Guanine Rich Sequence
Ct	Threshold Cycle
DNA	Deoxyribose Nucleic Acid
DCs	Dendritic Cells
DI	De-ionized Water
DMSO	Dimethylsulfoxide
dNTPs	Nucleotide Bases
dsDNA	Double Stranded DNA
dsRNA	Double Stranded RNA
DMEM	Dulbeccos Modified Eagle Medium
E1	Early gene 1 of HPV
E2	Early gene 2 of HPV
E4	Early gene 4 of HPV
E5	Early gene 5 of HPV
E6	Early (6) oncogene of HPV
E7	Early (7) oncogene of HPV
E1^E4	Early gene 1 Early gene 4 mRNA splice
ECM	Extracellular Matrix
E. coli	*Escherichia coli*
Ex vivo	Experiment performed on living tissue outside an organism
FasL	Fas-Fas Ligand
FCS	Fetal Calf Serum
FFPE	Formalin-Fixed, Paraffin Embedded Tissue
GAS	Interferon Gamma Activated Sequences
GTPase	Enzyme that hydrolyzes GTP
H_2O_2	Hydrogen peroxide
HeLa	Henrietta Lacks-Cervical cancer cell line
HKG	Housekeeping Gene
HPV	Human Papillomavirus
HPRT-1	Hypoxanthine-guanine phosphpribosyltransferase-1
HR	High-Risk

IFN	Interferon
IFNAR-1/2	Interferon Alpha/Beta Receptor
IFNGR-1/2	Interferon Gamma Receptor
IFN-α	Interferon-Alpha
IFN-β	Interferon-Beta
IFN-ε	Interferon-Epsilon
IFN-γ	Interferon-Gamma
IFN-|	Interferon-Kappa
IFN-λ	Interferon-Lambda
IFN-λR1	Interferon-Lambda Receptor 1
IFN-ω	Interferon-Omega
Ii	Invariant Chain
ILs	Interleukins
IL-10	Interleukin-10
IL-10R2	Interleukin-10 Receptor 2
IRF-1	Interferon Regulatory Factor-1
ISGs	Interferon-stimulated Genes
In vitro	Experiment in controlled cell culture environment
IRF	Interferon Regulatory Factor
ISGF	Interferon Stimulated Gene Factor
ISRE	Interferon Stimulated Regulatory Element
JAK	Janus Kinase
L1	HPV Late Gene 1
L2	HPV Late Gene 2
LB	Luria Bertani
LC	Langerhans Cell
LPS	Lippopolysaccharide
L.reuteri	*Lactobacilli reuteri*
mRNA	Messenger RNA
MCP-1	Monocyte Chemoattractant Protein-1
MHC	Major Histocompatability Complex
MIP-3α	Macophage Inflammation Protein-3 alpha
MOA5	Melanoma Differentiation Associated Gene 5
MRS	de Man, Rogosa and Sharpe
MxA	Interferon-Induced GTPase
MyD88	Myeloid Differentiation Primary Response Gene 88
NF H_2O	Nuclease-free water
NF-| B	Nuclear Factor of Kappa B
NIKS	Near-diploid Immortalized Keratinocytes
NKs	Natural Killer Cells

OAS	2'-5' Oligoadenylate Synthetase
OD_{600}	Optical Density at 600nm
$p16^{INK4a}$	Cell Cycle Regulator, marker for High Risk HPV Infection
p16	Cyclin Dependant Kinase Inhibitor
p53	Tumor Protein 53
PAMPs	Pathogen-Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pH	Power of Hydrogen
PKR	Protein Kinase R
pRb	Retinoblastoma Protein
PRR	Pattern Recognition Receptor
pUC19	Plasmid cloning vector University of California 19
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RBP-1	Retinoblastoma Binding Protein-1
REST	Relative Expression Software Tool
RGD	Recognition sequence for integrins binding to matrix proteins
RIG-1	Retinoic Acid Inducible Gene-1
RNA	Ribonucleic Acid
R-PE	R-Phycoerythrin
RT	Reverse Transcription
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel
SN	Supernatent
SOC	Super optimal broth
SOB	Synthetic oil based medium
ssDNA	Single Stranded Deoxyribonucleic Acid
ssRNA	Single Stranded R Ribonucleic Acid
STAT	Signal Transducer and Activator of Transcription
STI	Sexually Transmitted Infection
TB	Transformation Buffer
Th1	T-Helper 1
TLR	Toll-Like Receptors
Tyk-2	Tyrosine Kinase 2
XPGC	Xenoderma Pigmentosum Group G

Abstract

Persistent human papillomavirus (HPV) is the causative agent of cervical cancer and bacterial vaginosis (BV) caused by *Escherichia coli* (*E. coli*) has been implicated as a contributing factor. The innate immune system is our body's first defense against these invading pathogens. Using a novel 3-D organotypic tissue culture model to recreate the viral life cycle of HPV16 we examined mRNA expression of crucial innate immune molecules such as integrins, toll-like receptors (TLRs), and interferons (IFNs). Our model was comprised of normal immortalized keratinocytes (NIKS) and NIKS containing full-length HPV16 (NIKS+HPV16). These models were also exposed to *E. coli*, which simulated a BV infection. Models were characterized by examining morphology via hematoxylin and eosin (H + E) staining, keratin expression, DNA synthesis, and viral gene and protein expression. Extraction methodologies were optimized prior to continuing experiments. Extracted RNA was reverse transcribed into cDNA and mRNA was detected for $_{\alpha}$ and $_{\beta}$ integrins, toll-like receptors 3, 4, and 9, and their downstream molecules interferons –β, -γ, and –λ. Integrin expression was the highest in the control NIKS samples. Expression of α5 integrin was significantly increased in NIKS+*E. coli* compared to NIKS, which implicates its role in facilitating bacterial infections. Expression of α5 significantly decreased in HPV16 positive samples that could be associated with the increase in proliferation found in HPV infected tissues. Expression of TLRs showed similar trends to previously published data, indicating decreases in all TLRs in HPV16 positive samples. An increase in TLR3 was found in samples containing *E. coli*, which implicates the role of TLR3 in facilitating *E. coli* infections. IFN-β was found to increase in the presence of *E. coli* and HPV16 and this could be associated with the expression of E7, an event that has been implicated in increasing IFN-β production (previously unpublished results). Expression of this interferon was truly unique as it was

upregulated in samples exposed to *E. coli,* while no change was seen in NIKS and NIKS+HPV16 samples. Implications of IFN-β have yet to be elucidated and further investigation into their role in established carcinoma cell lines as well as *ex vivo* tissues would be ideal. The use of a novel tissue culture model in assessing the status of innate immune molecules provides a new vehicle of investigation. Further investigation into both the adaptive and innate immune response could lead to better treatment options for those affected by persistent and non-persistent HPV infections.

1.0 Introduction

1.1 Cervical Cancer

Cervical cancer affects many women worldwide, and it is the third most common cancer in women. Nearly 80% of new cases of the disease occur in low or middle-income countries (Hoory et al., 2008), (**Figure 1**). The decreased incidence in developed countries is associated with readily available screening and education. Cervical cancer is directly related to infection with human papillomavirus (HPV), which is a sexually transmitted infection (STI) that can affect both males and females. Over 100 subtypes of this virus exist and are relative factors in many anogenital cancers as well as head and neck cancers (zur Hausen, 2006).

In the early 1900's Peyton Rous was the first to elucidate the association of viruses and formation of sarcomas (Jarvier et al., 2008). This idea was further illustrated by Harald zur Hausen who in the 1970's determined that HPVs could cause cancer and were the causative agents in cervical cancer (zur Hausen et al., 1974). Ultimate vindication for this finding was awarded in 2008 when zur Hausen received the Nobel Prize in Medicine/Physiology.

Many types of HPV exist, some of which are low risk types (HPV6 and 11) forming benign lesions or papillomas, in contrast to the high risk (HPV16, 18, 31, and 33) that are commonly associated with anogenital and oropharyngeal cancers and specifically 90% of cervical cancers (zur Hausen, 1991).

Most women are able to clear urogenital HPV infections, but, in those who are not, the virus may become persistent and lead to the formation of cervical lesions. These lesions can then progress further to cervical cancer. In those who do progress, there is an increased prevalence of subtype HPV16. The prevalence of this subtype has reached 70% making it of upmost importance to study (WHO, 2009).

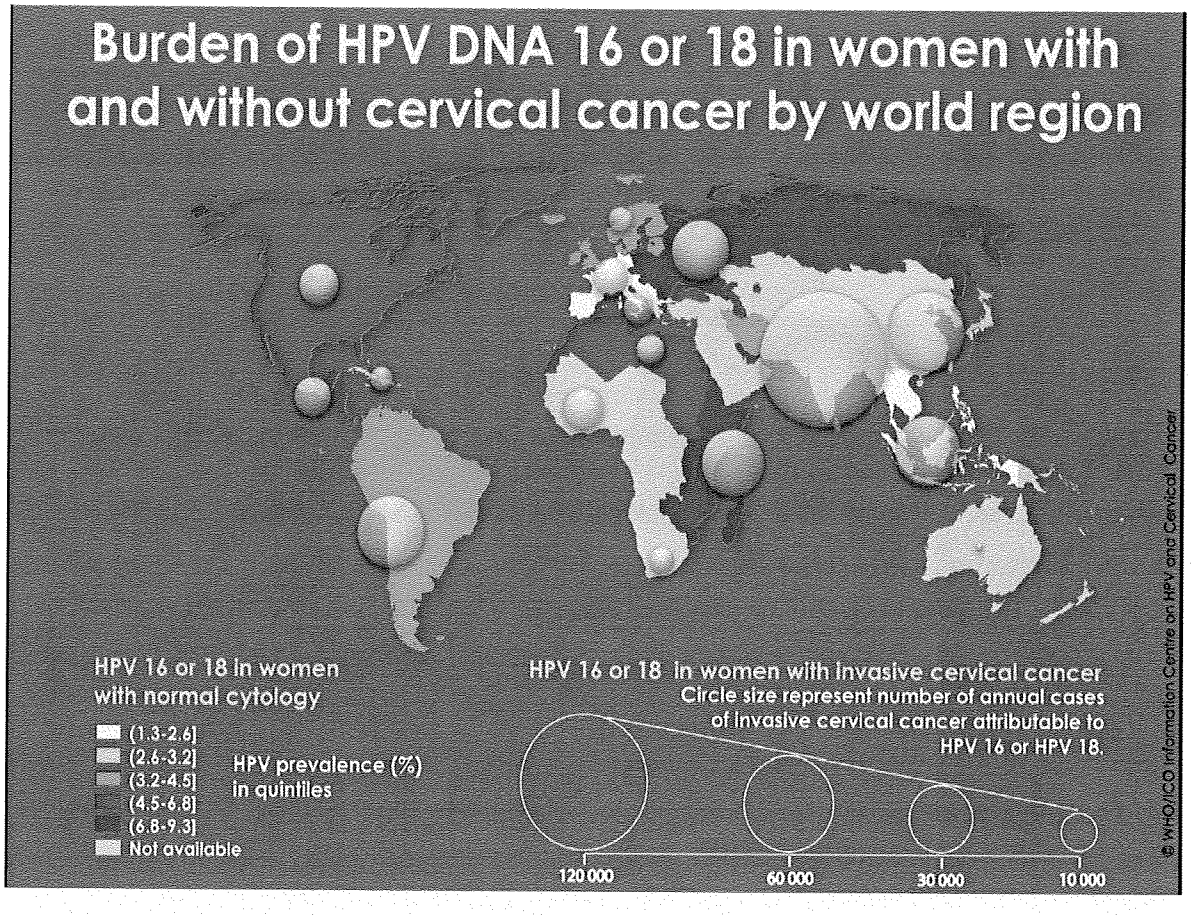

Figure 1. Burden of HPV16 or 18 in women with and without cervical cancer. (WHO, 2009)

1.2 Human Papillomavirus

Papillomaviruses have icosahedral symmetry and are non-enveloped with seventy-two capsomeres surrounding the genome. A major and minor capsid protein comprises the outer protein coat of the virus and approximately 8000 base pairs (8kbp) comprise the closed, double stranded- circular HPV genome. Coding information for HPV exists on one strand and it is believed that transcription occurs in a clockwise direction from a single promoter region (P97) (Beutner and Tyring, 1997). Three major regions comprise the HPV genome. The early region (E1-7, excluding E3) consists of genes responsible for inhibition of replication, transcription, plasmid replication, and transformation. In particular two early genes E6 and E7 are thought to be the two most important factors in determining the malignant phenotype of HPV-positive cervical cancer cells (von Knebel Doeberitz et al., 1992). The control region contains the regulatory elements for transcription and replication. The late region of HPV codes for the major (L1) and minor (L2) capsid proteins. A third late gene product E1^E4 is encoded by spliced messenger ribonucleic acids (mRNAs). This fusion of the E1 and E4 open reading frames is an important indicator of the productive stage of the viral life cycle (Nakahara et al., 2005) (**Figure 2**).

1.3 Viral Life Cycle

The life cycle of the virus relies heavily on the differentiation program of keratinocytes, the host cell of HPV. The non-productive stage of the viral life cycle begins in the basal epithelial layer. The virus penetrates through micro wounds in the cervical epithelium and establishes itself as a low copy number episome. One possible means of HPV entering the cell is mediated via clathrin-mediated endocytosis (Spoden et al., 2008), although others have suggested integrin α6 as a possible mechanism (Evander et al., 1997).

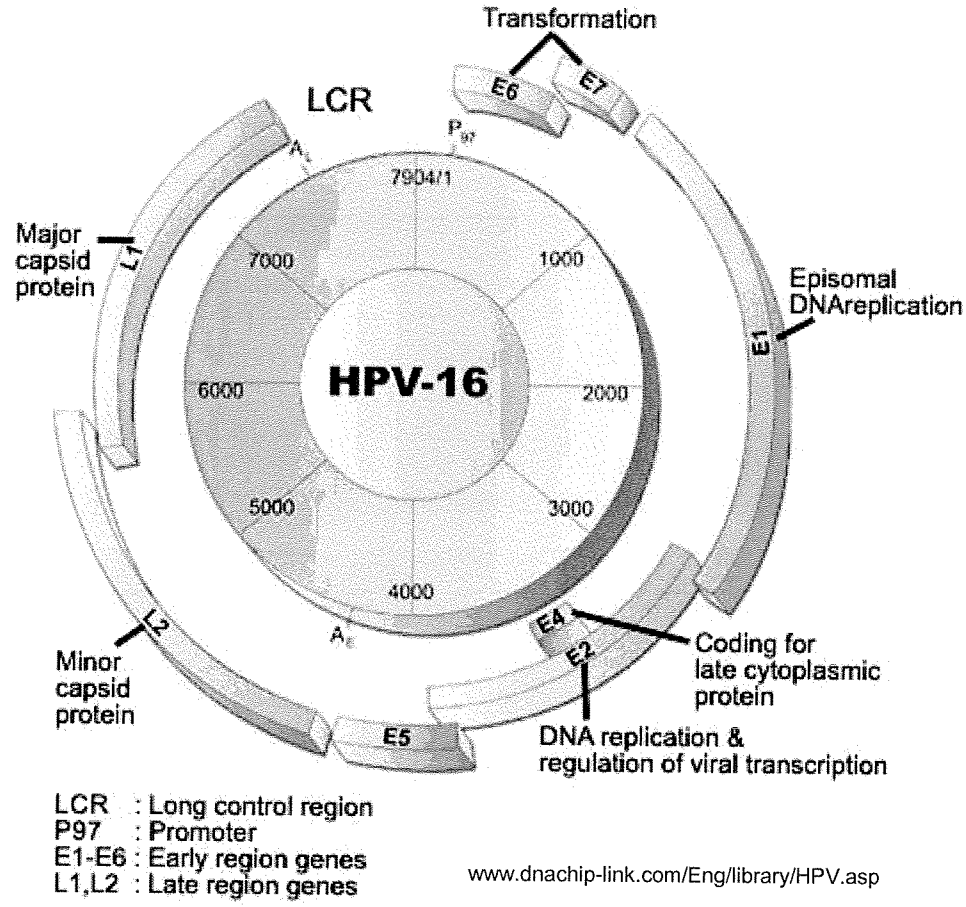

Figure 2. HPV -16 virus structure.

The virus synthesizes deoxyribonucleic acid (DNA) via a bidirectional theta mode at an average of once per cycle utilizing the host cell's replication mechanisms (Auborn, et al., 1994; Flores and Lambert, 1997; Gilbert and Cohen, 1987; and Yang and Botchan, 1990). In contrast, the productive stage of the viral life cycle occurs in the suprabasal portion of the epithelium. Here DNA exists at a high copy number and switches to a rolling circle mode of DNA replication (Flores and Lambert, 1997). In this layer, L1 and L2 proteins are synthesized and encapsulate the virons which are subsequently shed from the suprabasal epithelium and released as viral progeny (Howely, 1996) (**Figure 3**).

Some key features exist in the HPV viral life cycle. The virus relies on the host DNA replication machinery as the virus itself does not encode any DNA polymerases. E1 and E2 are responsible for the recruitment of host factors such as DNA polymerase α (Chow and Broker, 1994). E7 has been postulated as an important factor in causing suprabasal cells to become permissive to DNA synthesis (Flores et al., 2000). E6 and E7 jointly play a role in the deregulation of the normal host cellular machinery via deregulation of the tumor suppressor genes tumor protein 53 (p53) and retinoblastoma protein (pRb) respectively (**Figure 4**). As a result of this deregulation levels of cell cycle regulators such as $p16^{INK4a}$ become increased. $p16^{INK4a}$, in particular has been proposed as a biomarker for HR HPV infection (Riethdorf et al., 2004).

1.4 Innate Immune System

The innate immune system is a rapid, first response to viral infection. As opposed to the adaptive immune system, which employs antigen-specific effector cells, the innate response is non-specific (Medzhitov et al., 2002). Through the recruitment of leukocytes such as natural killer

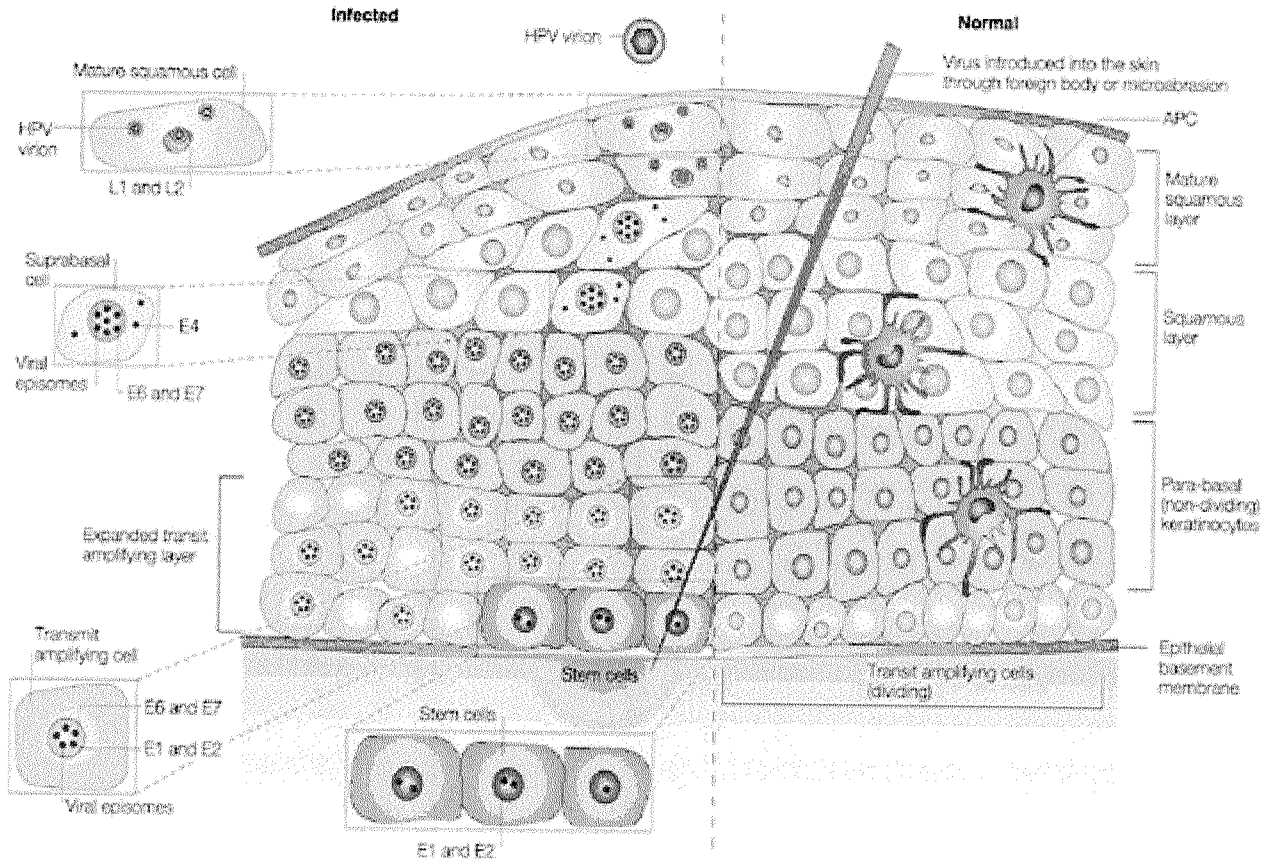

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Figure 3. The location in squamous epithelium of the main stages of the papillomavirus life cycle. (Frazer, 2004.)

Cervical stratified squamous epithelial cell architecture and the expression of human papillomavirus proteins after infection. After introduction of HPV into stem cells in the basal layer of the epithelium, expression of viral non-structural proteins occurs. The dividing-cell population expands vertically and epithelial cell differentiation is delayed. Viral proteins are expressed sequentially with differentiation as shown and mature virions are produced only in the most superficial layers of the epithelium. Intraepithelial antigen-presenting cells are depleted in the HPV-infected epithelium.

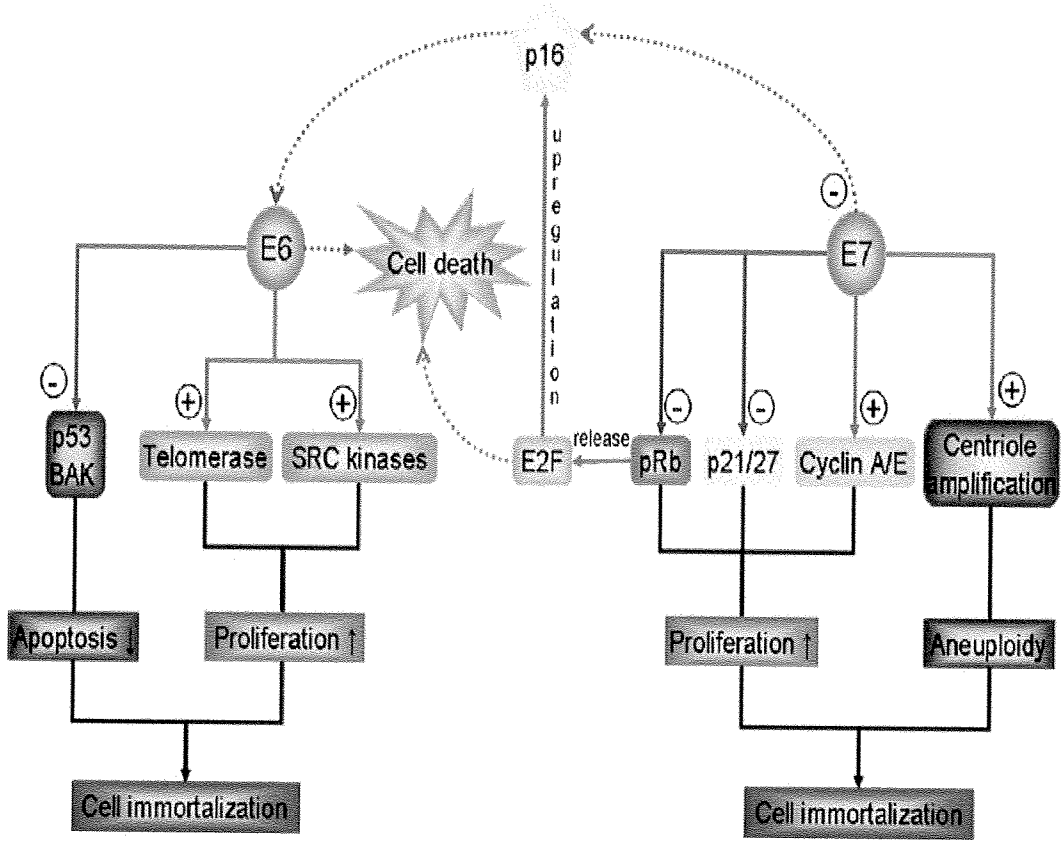

Figure 4. Cellular interactions of E6 and E7 oncoproteins (Boulet et al., 2007)
E6 and E7 upregulate (green) and downregulate (red) to contribute to increased proliferation leading to cellular immortalization.

cells (NKs), mast cells, eosinophils and basophils; and the phagocytic cells such as macrophages, neutrophils, and dendritic cells (DCs), our bodies can identify and eliminate pathogens that may cause infection (Murphy et al., 2008). These phagocytic cells become activated by means of surface receptors that bind common constituents of bacterial and viral surfaces. Once activated these cells secrete cytokines as well as chemokines that perform vital functions within the immune system (Biron, et al., 1999)

1.5 Interferons

Interferons were first discovered in 1957 by Lindenmann and Isaacs and belong to the family of cytokines. Interferons are substances that protect cells from viral infection. These IFNs were found to be secreted in response to stimuli after experiments demonstrating interference effects caused by heat-inactivated influenza virus on the growth of the live virus (Isaacs and Lindenmann, 1957). Part of the vertebrate cytokine family, 3 types of IFNs exist; I, II, and III with the last, being only discovered less than a decade ago. Interferons play significant roles in antiviral (Isaacs and Lindenmann, 1957), antitumor (Takaoka et al., 2003), and immunostimulatory mechanisms (Boehm et al., 1997). Human Type I IFNs consist of IFN-alpha (IFN-α) (many subtypes), IFN beta (IFN-β), IFN-epsilon (IFN-ε), IFN-kappa (IFN-κ), and IFN-omega (IFN-ω). These IFNs can be produced by all nucleated cells and are located on chromosome 9. Type II IFN consists solely of IFN-gamma (IFN-γ). IFN-γ is mainly produced by lymphocytes and NKs. This interferon plays a major role in the upregulation of the major histocompatibility complex I (MHC I) and serves as a link between the innate and adaptive immune systems. Lastly, Type III IFN-lambda (IFN-λ) is the newest and subsequently lesser established IFN.

1.6 Interferon Lambda

IFN-λ, discovered in 2003 (Kotenko et al., 2003; Sheppard et al., 2003), is found on chromosome 19 and exists in three different isotypes; IFN-lambda 1 (IFN-λ1), IFN-lambda 2 (IFN-λ 2), and IFN-lambda 3 (IFN-λ 3), which are also designated as interleukins (ILs) -28a, -28b, and -29 respectively. This group of IFNs demonstrates similar biological effects to that of the IL-10 superfamily as well as types I and II IFNs. Activation of the receptors by the complementary ligands leads to the phosphorylation of Signal Transducer and Activator of Transcription (STAT) -1, - 2, and - 3. Most interestingly though, type III IFNs, signal through heterodimeric class II cytokine receptors. The IL-10 receptor 2 (IL-10R2) is expressed in most cell types where as IFN-lambda receptor 1 (IFN-λR1) is restricted to expression in epithelial cells. This different pattern in receptor expression would therefore limit IFN-λ expression to the epithelial cells. The limitation of IFN-λ to epithelial cells correlates with tissues that are susceptible to a high number of infections compared to type I IFNs which function systemically. IFN-λ may therefor, play a more prominent role in the regulation of innate immunity (Gad et al., 2009). This cell type also serves as the primary site of replication for viruses, HPV in particular. Although IFN-λ is structurally closely related to IL-10 it resembles IFN type I through its functions. Both type I IFN, and type III IFN are induced by viral infections (Ank et al., 2008), both are capable of stimulating expression of interferon stimulated genes (ISGs) (Kotenko et al., 2003), and both perform antiviral actions in culture as well as antitumor functions (Lasfar et al., 2006; Sato et al., 2006).

1.7 Interferon Induction and Signaling Pathways

1.7.1 Extracytoplasmic Induction

The extracytoplasmic induction pathway is activated by stimulation with microbial agents. These microbes contain exclusive molecules known as pathogen associated molecular patterns (PAMPs). These PAMPs are recognized by the pattern recognition receptors (PRRs) on toll-like receptor (TLR) cellular membranes (**Table 1**). TLRs 3, 4, 8 and 9 act as signaling receptors for extracellular double-stranded ribonucelic acid (dsRNA) (Alexopoulou et al., 2001), lipopolysaccharide (LPS) (Hoshino et al., 1999) , G-rich oligonucelotides (Heil et al., 2004; Diebold et al., 2004; Lund et al., 2004), and CpG DNA (Hemmi et al., 2000) respectively. TLR3 recognizes ssRNA and double-stranded DNA (dsDNA) viruses as well dsRNA viruses because the two former produce double-stranded RNA (dsRNA) during replication (Kumar et al., 2009).

1.7.2 Cytoplasmic Induction

Alternatively, interferons can be induced in the cytoplasm involving caspase recruitment domains (CARDs) and the recognition of intracellular double stranded RNA viruses (Yoneyama et al., 2004). The two constituents of CARD are the retinoic acid inducible gene-1 (RIG-I) and the melanoma differentiation associated gene 5 (MDA5) (Kawai et al., 2005). These two proteins contain RNA helicase domains that interact directly with and process dsRNA. This makes the CARD domains available for interaction with downstream adaptors that are anchored within mitochondria. It is through this signaling that CARD proteins are responsible for eliciting activation of transcription factors responsible for IFN gene expression. In regards to Type I IFN induction, the CARD of RIG-I transduce signals that ultimately lead to the activation of IRF-3 and NF-┤ B (Kawai et al., 2005).

1.8 Signaling Pathways

Following the induction of IFNs, intracellular signaling cascades result in the induction of the genes responsible for the anti-viral activities within the cell (**Figure 5**). Type I IFNs such as β

and κ, associate with the IFN-α/β receptor (IFNAR). This receptor has 2 subunits, IFNAR-1 and IFNAR-2 (Uze et al., 1990), whereas IFN-γ signals via another receptor, IFN- γ receptor 1

Table 1. Toll-like receptor (TLR) Recognition

TLR1	Peptidoglycan and lipoprotein (in concert with TLR2)
TLR2	Gram positive bacteria
TLR3	dsRNA
TLR4	Lipopolysaccharide on Gram negative bacteria
TLR5	Flagellin
TLR6	Lipopolysaccharide (with TLR2)
TLR7	ssRNA in endosomes
TLR8	G-rich oligonucelotides
TLR9	CpG motifs

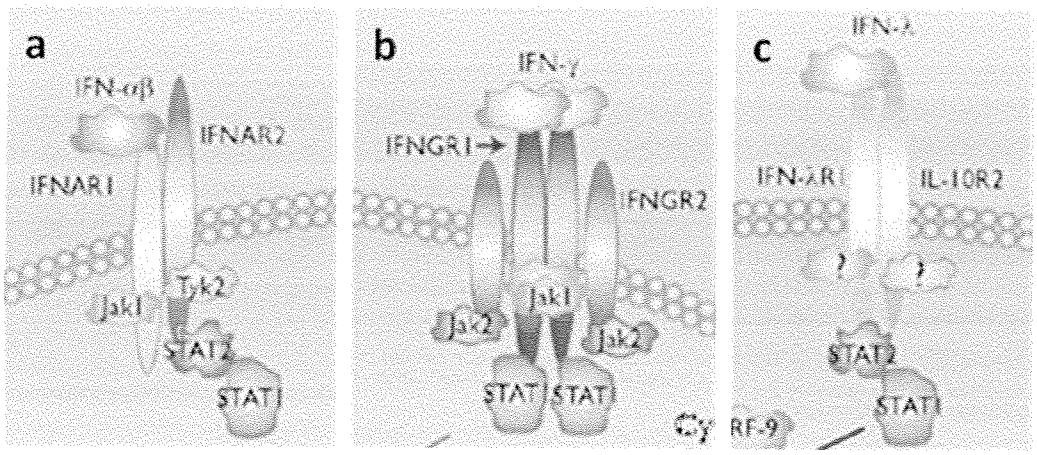

Figure 5. Comparison of signaling pathways activated by IFN-αβ, IFN-γ and the newly described IFN-λ (Vilcek 2003).
a) Type 1 interferon-α and –β binding to cell surface receptors IFNAR1 and 2. b) Type 2 interferon-γ binding to cell surface receptors IFNGR1 and 2. c) Type 3 interferon-λ binding to cell surface receptors IFN- λ1 and IL-10R2.

(IFNGR1) and IFN- γ receptor 2 (IFNGR2) (Bach et al., 1997). Type III IFN signals through heterodimeric receptors, IL-10R2 and IFN-λR1. Activation of these receptor complexes results in the initiation of the JAK-STAT pathway. In Type I and III signaling, STAT molecules become phosphorylated allowing the formation of trimeric interferon stimulated genes(ISGs) which bind to IFN stimulated response elements (ISREs) of downstream genes. Alternatively when STATs become phosphorylated in Type II signaling, the activation complex differs resulting in the downstream binding to IFN-γ activated sequences (GAS) (Decker, et al., 1991).

1.9 Interferon Stimulated Genes

In HPV infection, many ISGs become down regulated, such as IFN-inducible proteins like protein kinase R (PKR) (Dey, et al., 2005), and 2'-5' oligoadenylate synthetase (OAS) (Nees et al., 2001). OAS recognizes dsRNA and is significantly down regulated upon exposure to E6/E7 viral vectors (Nees et al., 2001). Also, ISGs that play important roles in antiviral activity induction such as p53, pRb, and MyD88 are influenced by the E6 and E7 oncogenes (Nees et al., 2001). IFN induced GTPase Mx is capable of exerting antiviral functions via interference with viral protein transport within the cell (Haller and Kochs, 2002). GTPase MxA acts as a cellular surveillance detecting the major capsid protein of HPV (Haller and Kochs, 2002, and Haller et al., 2007) and is able to interfere with viral protein transport via trapping the viral particles and sorting them to locations where they become unavailable for viral generation (Haller et al., 2007). MxA is also capable of inhibiting tumor motility (Mushinski et al., 2009)

1.10 Integrins

Integrins are glycosylated, heterodimeric, transmembrane adhesion receptors. Integrins are composed of non-covalently bound alpha and beta subunits (**Figure 6**). These molecules bind

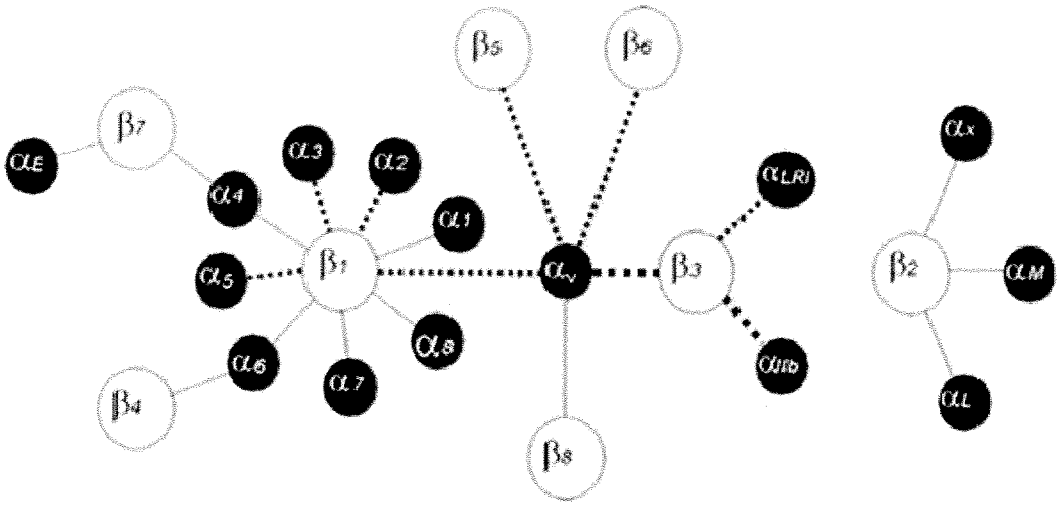

Figure 6. Integrin-α and –β subunits. (Beer and Schwaiger, 2008).
Integrin subunits and the subunit's that they heterodimerize with. Subunits connected by dotted lines bind to ligand via arginine-glycine-aspartate sequence.

to components of the extracellular matrix (ECM), such as, collagen, laminin, and fibronectin, while others are counter-receptors of the immunoglobulin-like super family (Van der Flier and Sonnenberg 2001). Integrins are found in several tissue types including epithelial and fibroblast cell types where these molecules modulate many pertinent cell activities; adhesion, cell motility, proliferation, differentiation. These molecules also play a role in immune defense as well as tumor progression. The relationship between integrin and cancer development is so strong that integrins are considered as therapeutic agents in certain cancers (Tucker, 2006).

1.11 Human Papillomavirus Evasion Strategies

HPV has developed strategies to modulate immune responses, although none of the HPV genes primarily function in regulation of the immune system. The virus maintains a low profile by replicating in undifferentiated cells and forming viral particles in differentiated epithelial cells, thereby reducing its exposure to the immune system. The virus has a non-lytic nature and therefore does not elicit any pro-inflammatory signals, limiting the number of antigens processed and presented to the adaptive immune system (Kupper and Fuhlbrigge, 2004). Lastly there is no blood-born phase of the viral life cycle so minimal amounts of replicating virus are exposed to the humoral immune system (Kanodia et al., 2007). Rather, to eliminate the virus, the cell-mediated immune response is usually necessary (Frazer, 1998). HPV has evolved to exploit the redundancy in the genetic code to control expression levels of its gene products (Smith, 1996) resulting in preferred codon usage. The virus has developed sequence similarity to human proteins. The E7 protein has been shown to mimic the xeroderma pigmentosum group G complementing protein (XPGC) as well as the retinoblastoma binding protein I (RBP-I) which inhibits excision repair (O'Donovan and Wood, 1993; Scherly et al., 1993), and deregulation of the cell cycle respectively (Lai et al., 1999). E5 and E7 have been implicated in disruption of

antigen presentation (Kanodia et al., 2007). E5 via degradation of the invariant chain (Ii) alters the expression of major histocompatability complex-II (MHC II) (Zhang et al., 2003), and E7 represses the promoter for MHC I (Georgopoulos et al., 2000). As discussed briefly before, HPV is able to interfere with the induction of interferons (IFNs). In regards to type I IFNs, E6 impairs Janus kinase-signal transducer and activator of transcription (Jak-STAT) activation by binding to tyrosine kinase-2 (Tyk-2), and binding to IFN-regulatory factor-3 (IRF-3) inhibiting the expression of IFN-α and -β respectively (Li, et al., 1999 and Ronco et al., 1998). E7 can inhibit formation of IFN stimulated gene factor-3 (ISGF-3), IRF-1 disabling IFN-α and IFN-β induction (Barnard et al., 2000, and Park et al., 2000). Nuclear factor of kappa B (NF-| B) induced gene expression can also be affected by E7 (Perea et al., 2000). HPV is also able to inhibit cytokines and chemo-attractants such as monocyte chemoattractant protein-1 (MCP-1) which is important for clearance of viral infections by attracting memory T cells, NK cells, and monocytes (Baggiolini et al., 1995). MCP-1 is suppressed in HPV infected tissues (Rosl et al., 1994) by E6 and E7 proteins either alone or in tandem (Kleine-Lowinski et al., 2003). HPV is able to skew cytokine profiles which can lead to an incorrect immune response. HPV is able to inhibit cytotoxic lymphocyte response by inhibiting T-Helper 1 (Th1) response (Kanodia et al., 2007). HPV infection has effects on adherence molecules which affects such molecules as E-cadherin. E-cadherin is down-regulated by the E6 protein, which is important for adhesion of Langerhans cells (LCs) which are reduced at sites of HPV infection (Lehtinen et al., 1993). Again, in regards to LCs, in cells expressing both E6 and E7 we see a decreased expression of macrophage inflammatory protein-3alpha (MIP-3α) which is an important agent for LC precursors (Dieu-Nosjean et al., 2000). This in association with decreased expression of surface adhesion

molecules for attraction of antigen presenting cells (APCs) could be a mechanism for viral persistence (Kanodia et al., 2007).

Lastly, HPV has mechanisms that prevent apoptosis via the extrinsic and intrinsic induction pathways both converging at caspase-3 induction. E5 via suppression of the Fas-Fas ligand (FasL) affects the extrinsic pathway (Kabsch and Alonso, 2002) and E6, via suppression of p53 affects the intrinsic pathway (Werness et al., 1990).

1.12 Urogenital bacterial Infections

Urogenital bacterial infections affect millions of people worldwide. Some infections are asymptomatic whereas others can result in adverse health problems. In terms of female urogenital health, approximately 1 billion women worldwide are affected by bacterial infections per year (Reid, G., 2008). In regards to normal flora, the urogenital tract consists almost entirely of species of *lactobacilli* (Pfau and Sacks 1977) that play a probiotic role. Various strains of *lactobacilli* are being used as treatment for recurrent bacterial vaginosis infections (Reid 1999; Reid et al., 2001; Reid et al., 2003a; Reid et al., 2003b; Liu et al., 2007; Saunders et al., 2007; Reid 2008; Martinez et al., 2008; Eschenbach et al., 1989), which are most commonly caused by the pathogenic bacterium *Escherichia coli (E.coli). Lactobacilli* trigger a variety of cell responses *in vitro* that are believed to play an important role in the bacterium's probiotic capabilities. These bacteria increase mucus production, increase expression of cell-cell adhesion molecules, capable of inducing innate immune response (Reid et al., 2001), produce H_2O_2, and are resistant to spermicide. Cumulatively these mechanisms form an attack against pathogenic invaders. Reduction in numbers of *lactobacilli* facilitates colonization of the urogenital tract with pathogenic bacteria such as *E.coli* resulting in a bacterial vaginosis infection. Recently, some researchers have speculated that there is an association between bacterial vaginosis and the

development of cervical cancers (Castle et al., 2001; Discacciati et al., 2006; Kharsany et al., 1993; Mikamo et al., 1999; Platz-Christensen et al., 1994).

2.0 Rationale

HPVs have an exclusive tropism for skin and mucosal tissues targeting epithelial cells or keratinocytes more specifically. Keratinocyte organotypic raft cultures are three-dimensional structures that mimic "real" skin. This model is capable of developing a fully stratified epithelium and thus replicates the physiological conditions of the cervix more so than monolayer cultures. More importantly, this model allows for the recreation of the viral life cycle within the laboratory. HPV16 is most commonly associated with cervical cancer and therefore is of upmost importance to examine in this study. By using a cell line stably transfected with full length HPV16 the viral life cycle can be illustrated nicely. An increase in knowledge of the activities of important immune molecules during the viral life cycle is crucial in understanding immune response during HPV infection. Through the examination of key surface receptor molecules such as TLRs and integrins, and their downstream effectors, the interferons, the immune status of HPV infected tissues can be better understood. Our model allows for a unique infection process whereby an infection with pathogenic bacteria can also be applied. The presence of pathogenic bacteria such as *Escherichia coli* in the female genital tract is relevant as this microbe is frequently reported in bacterial vaginosis infections. Therefore, an infection process that includes a pathogen such as *Escherichia coli* demonstrates a highly relevant model. Using this model under the above stated conditions, immune molecules have never been examined in regards to HPV to our knowledge, nor has the viral life cycle. This unique approach to illustrating the viral life cycle and studying immune molecules in HPV infection represents intriguing experimental research.

2.1 Hypothesis

We expect to find the viral life cycle present in the 3-D organotypic raft cultures as found in previous studies (Flores et al., 1999). We expect to see differing gene expression profiles of the IFNs, TLRs, and integrins in the tissues infected with HPV16 as the viral proteins are capable of interfering with cellular signaling networks. We believe that post infection with pathogenic bacteria these profiles will again change. These changes would be a result of the altered antiviral states induced upon viral as well as bacterial infection.

2.2 Research Aims

1) To characterize 3-D organotypic raft cultures morphologically grown from normal immortalized keratinocytes (NIKS) in 4 model systems: 1) NIKS alone, 2) NIKS stably transfected with HPV16, 3) NIKS transiently infected with pathogenic bacteria and 4) NIKS stably transfected with HPV16 and transiently infected with pathogenic bacteria.

2) To illustrate the viral life cycle in a 3-D organotypic raft culture based on models 2 and 4 and compare with NIKS alone (model 1) and NIKS transiently infected with pathogenic bacteria (model 3).

3) To characterize the status of important innate immune molecules such as integrins (αv,-3,-5,-6, and β1, -3,-4,-6) toll-like receptors (TLRs) 3, 4, and 9, as interferon (IFN) –β, -γ, and -λ in all 4 models.

3.0 Methods

3.1 Cell Lines

3.1.1 Monolayer Cultures

J2 cells (ATCC, CCL-92) were grown in medium consisting of 90% Dulbeccos Modified Eagle Medium powder (DMEM) (Sigma, D5648-10X1L), 10% FCS, 2% Sodium Bicarbonate (Gibco, 25080-094), 1% Antibiotic/Antimycotic (Gibco, 15240), and 1% L-glutamine. Cells were passed at 70% confluence. J2 were inactivated in preparation for addition of NIKS+HPV16 by inactivation with 100μL of 0.2mg/mL mitomycin-c for 2 hours at 5%CO_2 and 37°C. Cells were then washed 2X with J2 medium and 2X with 1X PBS and NIKS+HPV16 cells were seeded on top. Aliquots of J2 were frozen in 1mL of freezing medium containing 90% FCS and 10% DMSO and stored at -80°C for up to 1 month and then transferred to liquid nitrogen.

Fibroblasts (passage 3, a gift from Paul Lambert) were maintained in medium containing 90% DMEM (Sigma, D5796) 10% FCS, and 1% Antibiotic/Antimycotic. Fibroblasts were passed at 70% confluence, frozen in medium containing 90% DMSO and 10% FCS and stored at -80 °C for one month before they were transferred to liquid nitrogen.

Normal immortalized human foreskin keratinocytes (NIKS) (Allen-Hoffmann et al., 2000) a gift from Paul Lambert (University of Wisconsin, Madison, Wisconsin), were grown in the presence or absence of Human Papillomavirus type 16 (HPV16) (Flores et al., 1999) in monolayer and 3D cultures (Lambert et al., 2005). To ensure the virus was not incorporated into the host genome monolayer NIKS were maintained on J2 feeder cells (ATCC, CCL-92) (Allen-Hoffman and Rheinwald, 1994) in NIKS medium (following STRATATECH®, Madison, Wisconsin, USA, protocol). Cells were washed with 1X Phosphate Buffered Saline (PBS) (Sigma, D8537), and J2 cells were removed from culture via incubation with EDTA/PBS. Cells were then washed with

1X PBS and trypsinized (Gibco, 25300) at 70% confluence and seeded on mitomycin (0.2mg/mL) (Roche Molecular Biochemicals, 107409) inactivated J2 feeder cells to maintain culture. Aliquots of cells were frozen in 1mL of freezing medium containing 90% fetal calf serum (FCS) (PAA, A15-701) and 10% dimethylsulfoxide (DMSO) (Sigma, 34869), frozen in styropor at -80°C for at least 24 h and then transferred to liquid nitrogen.

3.1.2 Three-Dimensional Cultures

Fibroblasts were trypsinized (Gibco, 25300) and counted. The number of cells needed (10^6) was calculated and resuspended in 2mL of FCS (sufficient 10 rafts). A volume 10mL of gel was prepared at 4°C in sterile conditions on ice with stirring as follows: Bovine Collagen I (Cultrex 3442-050-01, 5mg/mL) was added to 0.1% acetic acid to a final concentration of 2mg/mL along with 1X Hanks Balanced Salt Solution (Sigma H4385). NaOH (5M) was used to neutralize the solution until a pinkish-red colour (~pH 7.0) was achieved. Fibroblasts mixed with the FCS were added slowly to the gel solution ensuring no bubbles were formed. One mL of gel culture containing 10^6 cells was placed into wells again ensuring no bubbles were formed. Cultures were incubated at 5%CO_2 and 37 °C for 2 hours until the gel solidified and appeared opaque. 1mL of fibroblast medium was added to each well and incubated over night at 5%CO_2 and 37 °C when fibroblasts reverted to an elongated shape. NIKS were trypsinized counted, and $5x10^6$ cells were resuspended in 1mL of fresh NIKS medium (following STRATATECH®, Madison, Wisconsin, USA). The fibroblast medium was removed from each well and 100µL of NIKS cells for a final count of 10^5 cells per well was added. Following 2 hours of incubation at 5%CO_2 and 37 °C and, an additional 1mL of NIKS medium was added. Cultures were then incubated for 24 hours at 5%CO_2 and 37 °C. Medium was aspirated and the gel was removed using sterile tweezers. The gel was placed on top of a 0.4um Millicell Cell Culture Insert (Millipore, PICMORG50) with the

NIKS side facing up. One mL one-hundred microlitres of FAD medium (NIKS medium with epidermal growth factors omitted and replaced with 19μL of 2.5M $CaCl_2$) was added to each well slowly to ensure even dispersion around and under the membrane. Rafts were maintained in 5%CO_2 and 37°C, changing the medium every 2 days with 1.1mL of FAD medium. Rafts were grown for 14 days to facilitate a stratified epithelium. 5-bromo-2-deoxyuridine (BrdU) is the nucleoside analogue of thymidine and is used for the detection of proliferating cells. Prior to harvesting rafts, BrdU was added to cultures for 8 hours using 1.1ul of 10mM BrdU. The BrdU becomes incorporated into newly synthesized DNA substituting the thymidine during DNA synthesis and can be detected via immunohistochemistry.

3.2 Harvesting for RNA and Protein

3.2.1 Monolayer

Cells were trypsinized following methods previously described and cells were washed in 1X PBS and centrifuged at 85 g for 5 minutes. PBS was aspirated and cells were stored at -80°C until use.

3.2.2 Three-Dimensional Cultures

Medium was aspirated and rafts were washed by filling plates with cold, sterile, 1X PBS, rafts were placed in a cryovial and snap-frozen in liquid nitrogen (~20 seconds) then stored at -80 °C.

3.3 Harvesting Rafts for Morphological Assessment

Medium was aspirated and rafts were washed by filling plates with cold, sterile, 1X PBS; 4% buffered formaldehyde solution was added for fixation (24 h). Rafts were embedded in paraffin and cut (Shi et al., 2005). Sections of 5μm were used for immunohistochemical analysis. One slide per sample was also prepared for hematoxylin and eosin staining to examine epithelial structure.

3.4 Bacterial Infections

Expression of integrins αv,-3,-5,-6, and β1, -3,-4,-6 was examined in normal monolayer NIKS, 3D NIKS, HPV-infected NIKS, and HPV-infected 3D NIKS in the presence or absence of *E. coli*. *E. coli* (ATCC catalogue #25922; Vermeulen, et al., 2008), was kindly provided by Dr. K Leung, Lakehead University, Thunder Bay, ON.

3.4.1 Drop-plating for Verification of Bacterial Numbers

E. coli culture was grown at 37°C for 16h in a shaking incubator at 150rpm.

One mL of *E. coli* culture was transferred to 20mL of fresh Luria-Burtani (LB) medium, and allowed to grow for an additional 1 hr. Each bacterial culture was centrifuged at 3500 x g for 20 min at 4°C, and pellets were washed 3 times with 10mL of cold, sterile, phosphate buffered saline (PBS), pH 7.4. The final pellet was re-suspended in PBS to achieve optical density at 600nm (OD_{600}) of 0.39-0.44. Serial dilutions of *E.coli* were made to calculate the colony forming units per mL (CFU/mL). Drop-plating was performed using 60μL (6 drops of 10 μL) each of 10^{-5} and 10^{-6} dilutions. *E.coli* was plated to LBA and stored in 37°C for 24 hours. The number CFU/mL was calculated using the following equation: CFU/mL = # colonies/volume plated (μL) x 1/dilution factor x 10^3

i.e. 75 colonies/60μL x $1/10^{-6}$ x 10^3 = 1.25 x 10^9 CFU/mL

Bacterial counts were then plotted against the OD to create a line of best fit which was then used to calculate the CFU/mL in future experiments.

3.4.2 Monolayer Bacterial Infections

E. coli culture was grown in LB at 37°C for 16h in a shaking incubator at 150rpm. 3 million NIKS cells at 3 million were seeded into 3 flasks, one for the bacterium, a control, and a cell count control. Flasks were incubated in 5% CO_2 and 37°C overnight to allow cells to adhere.

One mL of *E. coli* culture was transferred to 20mL of fresh LB medium, and allowed to grow for an additional 1 hr.

Bacterial cultures were centrifuged at 3500 x g for 20 min at 4°C, and pellets were washed 3 times with 10mL of cold, sterile, PBS, pH 7.4. The final pellet was re-suspended in PBS to achieve OD_{600} of 0.39-0.44. Medium from flasks was removed, and the cells were rinsed 2 times with 4mL of sterile PBS. The appropriate volume of bacterial suspension calculated for the desired multiplicity of infection (MOI) was added to the treatment flask. A volume of PBS equal to the volume of suspended bacteria in the treated flask was added to the control flask. Serum and antibiotic/antimycotic free medium was added to each flask to achieve a final volume of 15mL. Flasks were placed in a 5%CO_2, 37°C incubator for 4hr. Images of the cells, the culture medium as well as pH were taken. MOI was confirmed in all the experiments by counting NIKS in control flasks and serial dilutions of drop plates.

3.4.3 Flow Cytometry

Medium from the bacteria infected cells was removed and saved, and the cells were subsequently washed 2 times with 2mL of cold sterile PBS. Adherent cells were trypsinized and neutralized with NIKS medium. Suspended cells were centrifuged along with PBS wash supernatants at 500 x g for 6 minutes at 4°C and washed two times with PBS. Cells that were not infected with bacteria were washed with PBS two times and all supernatants saved. Cells were trypsinized and washed as previously described and counted using the Vi-CELL™ XR (Beckman Coulter, Mississauga, ON, CA). For immunostaining, cell pellets were re-suspended in 1.5mL of PBS with 0.1% bovine serum albumin (BSA, Sigma, A1470-100G), and 100µL aliquots ($2x10^5$ cells) were transferred to flow cytometry tubes. Antibodies (**Table 2**) were used at 1:50 dilutions and tubes were vortexed, covered, and incubated in the dark at 4°C for 1.5 hours. Following

incubation, cells were washed 3 times with 1mL of PBS as previously described. Cells incubated with unconjugated primary antibodies were resuspended in 100μL of PBS and secondary antibody conjugated to R-Phycoerythrin (R-PE) (BD Pharmingen 552603) at a dilution of 1:50 and incubated under previous conditions. Post-incubation, cells were resuspended in 700μL of PBS and analyzed on a Becton Dickinson FACS Caliber.

3.4.4 3D Culture Bacterial Infections

E. coli culture was grown at 37°C for 16h in a shaking incubator at 150rpm. One mL of *E. coli* culture was transferred to 20mL of fresh LB medium, and allowed to grow for an additional 1 hr. Each bacterial culture was centrifuged at 3500 x g for 20 min at 4°C, and pellets were washed 3 times with 10mL of cold, sterile, PBS, pH 7.4. For each bacterium the final pellet was re-suspended in PBS to achieve optical density at 600nm (OD_{600}) of 0.39-0.44. Rafts were grown for 14 days and infected with the bacteria on the final day of growing. The amount of bacteria needed per raft was calculated based on 10^6 cells and an MOI of 1:50. Bacteria were added directly on top of rafts and diluted further to 2.2mL with FAD medium void of antibiotic/antimycotic as well as FCS. For control rafts the volume of bacteria added was replaced with cold sterile 1X PBS. Rafts were incubated at 5%CO_2 , 37°C incubator for 4hr after which were washed with 1X PBS 3 times and subsequently snap frozen in liquid nitrogen then stored at -80°C until needed.

3.4.5 Assessment of Infection Method

Monolayer bacterial infections using the NIKS cell line as well as the NIKS+HPV16 cell line were preformed to assess the effectiveness of the infection method. Testing of the method also determined if there were any differences between the 2 cell lines and the 2 cell lines in the presence of *Escherichia coli.* Bacterial infections were carried out as described above along with

assessment of surface integrin expression, cellular morphology, pH and medium consistency was also investigated. Bacterial infections were carried out for a 4 hour time period, with control parameters assessed at 0, 2 and 4 hours. At these time points medium conditions (**Appendix, Figure 21**) and power of hydrogen (pH) (**Appendix, Figure 22**) were recorded. In cell lines infected with *E.* coli the medium became cloudy at the time of initial infection and remained in this state throughout the 4 hour time point. Medium within the control flask remained clear and translucent throughout the experiment. Images were captured of cell distribution in the flasks at the same time period at 10X, 20X, and 40X magnification to assess the amount of cell debris, apoptotic cells (assessed only by morphology), and to confirm that controls remained uninfected. pH also remained constant throughout the 8h time span. As expected in the flasks containing *E.coli* (NIKS and NIKS+HPV16) there was a large amount of debris which was visible at the 2 hour time point which increased at the 4 hour time point (**Appendix, Figures 23, 24, and 25**). Surface integrin expression was analyzed using Phycoerythrin (PE) conjugated antibodies at concentrations of 1:50.

Table 2. Antibodies used in flow cytometry.

Specificity	Clone	Isotype	Format	Source	Catalogue Number
β_1	MAR4	Mouse IgG1, |	PE conjugated	BD Pharmingen	555443
β_3	VI-PL2	Mouse IgG1, |	PE conjugated	BD Pharmingen	555754
β_4	439-9B	Rat IgG2b, |	PE conjugated	BD Pharmingen	555720
β_6	442.5C4	Mouse IgG	Unconjugated	Calbiochem	407317
α_v	13C2	Mouse IgG1	PE conjugated	Chemicon	CBL490P
α_3	C3 II.1	Mouse IgG1, |	PE conjugated	BD Pharmingen	556025
α_5	IIA1	Mouse IgG1, |	PE conjugated	BD Pharmingen	555617
α_6	GoH3	Rat IgG2a, |	PE conjugated	BD Pharmingen	555736
I.C	DD13	Rat IgG2a	PE conjugated	Chemicon	CBL605P
I.C	MOPC-31C	Mouse IgG1, |	unconjugated	BD Pharmingen	550878
I.C	Polyclonal	Mouse IgG (H+L)	PE conjugated	eBiosciences	12-4012-87

I.C – isotype control
PE-Phycoerythrin

3.5 Protein Analysis

3.5.1 Immunohistochemistry

Using paraffin embedded 3D raft cultures, protein expression analysis was performed via immunohistochemistry. Sections were de-paraffinized through a series of 3 X 10 min washes with 100% xylene (v/v) and 100% ethanol (v/v). Slides were allowed to air-dry and subsequently submerged in 0.01M citrate buffer. Antigens were retrieved via Pascal pressure cooker (Dako, S2800) set at 126°C and 20psi. Slides were allowed to cool and rinsed 2 times with distilled water followed by 1 X PBS. Primary antibodies were applied at concentrations varying from 1:100μL to 1:1000μL in background reducing dilution reagent (Dako, S3022). Primary antibodies were applied overnight at 4°C. The following day slides were washed in a series of 3 X 10 min washes with 1X PBS. Secondary antibodies conjugated to an Alexa-fluorophore were diluted in the same background reducing dilution agent (Dako, S3022) at concentrations of 1:800μL. Sections were incubated in the dark at RT for 30 minutes followed by three X 10 minute washes with 1X PBS. Sections were then mounted with mounting medium containing DAPI (Vectashield, H-1000). Negative controls were performed where the primary antibody was replaced with volumes of diluent.

3.5.2 Western Blot

Protein was harvested from NIKS cell lines and rafts using an in house 4% lysis buffer (0.25M Sucrose, 02M NaCl, 10mM Tris pH 8.0, 2mM $MgCl_2$, 1mM $CaCl_2$, 1% Triton X-100) and stored in 1 X sodium dodecyl sulphate (SDS). Samples were run on a 12% SDS polyacrylamide gel (SDS-PAGE) for 2 hours at 100 volts. Gels were transferred to a polyvinylidene fluoride membrane (PVDF) (Thermo-Scientific, 88518) at 100 volts for 1 hour on ice. Membranes were incubated in 8% blocking solution (5% milk powder, 0.1% TBST) at room temperature for 1

hour and then washed in 0.1% TBST. Primary antibodies were added in concentrations ranging from 1:200 to 1:1000 diluted in 5% milk (8% milk powder, 0.1% TBST), and incubated overnight at 4°C. Horseradish peroxidase secondary antibodies were added at a concentration of 1:2000 diluted in 8% milk solution (8% milk powder, 0.1% TBST), and incubated at RT for 1 h. Blots were visualized using the Western Lightening™ Plus-ECL Enhanced Chemislumenescence Substrate (PerkinElmer NEL104001EA). Membranes were exposed for varying times (1-20 minutes) using the Biospectrum® 410 Imaging System (UVP 97-0342-01).

For examination of late protein 2, primary monoclonal mouse E7 antibody was kindly provided by Werner Zwerschke (Institute for Biomedical Aging Research of the Austrian Academy of Science in Innsbruck, Austria) and was incubated for 2 hours at room temperature with an HRP conjugated Donkey anti-mouse secondary. Bands were detected using an enhanced chemilumincescent solution (Perkin-Elmer, NEL104001EA).

3.6 Gene Transcription Analyses

3.6.1 RNA Extraction

RNA was extracted from 3-D organotypic rafts frozen at -80°C using two kits in order to achieve optimal quantity of RNA. The RNAqueous®-4PCR kit (Ambion, 1914) was used following the method described for extraction from frozen tissue and cell pellets using 150µL lysis/binding solution and EtOH as well as 5µL DNase I, DNase inactivation treatment. Extraction of RNA from 3-D organotypic rafts was also performed using the PicoPure™ RNA Isolation Kit (Arcturus KIT0202) following the method described for RNA extraction from cell pellets using 100µL extraction buffer and EtOH followed by DNase inactivation using RNase-Free DNase Set (Qiagen, 79254) and DNase I (Qiagen, 1010395).

3.6.2 Nucleic Acid Quantification and Integrity

Nucleic acid quantification and integrity was performed using the Bio-Rad Experion™ Automated Electrophoresis System following manufacturer's instructions. Quality of the samples was determined using electropherograms as well as gel images. Those samples which displayed distinct 18S and 28S ribosomal peaks on electropherograms as well as strong bands on the gel images were indicative of high quality RNA. Ratio values were not used as indicators as these numbers did not always correlate with strong samples. This method was also used to assess the efficiency of the RNA extraction protocol for raft cultures.

3.6.3 Reverse Transcription

RNA isolated from tissue pellets and raft cultures was reverse transcribed to complementary DNA (cDNA) using the High-Capacity cDNA Archive Kit (Applied Biosystems, 4322171). A 1:1 ratio of sample to master-mix was performed. Reverse transcription was performed following the manufacturer's instructions using random hexamer primers. Reactions were carried out in 30-70μL volumes depending on the obtained RNA concentration.

3.6.4 Quantitative Real-Time Polymerase Chain Reaction

Reactions were performed according to the qPCR protocol defined in the TaqMan® PreAmp Master Mix Kit. cDNA volumes of 500ng were added to each reaction and ran in triplicate volumes of 25μL in 96-optical well plates (Applied Biosystems, 4306737). Standard qPCR conditions followed (50°C for 5 minutes, 95°C for 10 minutes and then cycled at 95°C for 16 seconds and 60°C for 1 minute for 40 cycles) for expression assays for hypoxanthine phosphoribosyltransferase 1 (HPRT1) housekeeping gene (HKG), IFN-β, IFN-λ, IFN-γ, INTαv, INTα3, INTα5, INTα6, INTβ1, INTβ3, INTβ4, INTβ6, as well as toll-like receptors (TLRs) 3, 4 and 9. Positive controls were included on plates and negative controls consisted of nuclease free water (NF-H_2O). cDNA was omitted which monitored for contamination or non-specific primer

binding. Ct values were normalized to HPRT1 and auto Ct, baseline settings, and a threshold of 0.20 was used for relative quantification of target genes.

3.6.5 Statistical *Analysis*

Samples were run in duplicates where each sample type contained three rafts. Rafts were pooled due to the large volume of RNA required. Relative values were calculated using the 1 over delta Ct value method. Gene expression analysis was performed using the Analysis of variance test (ANOVA) where there were more than 2 groups. NIKS rafts were deemed the control sample and therefore the Dunnetts post-hoc test was chosen to compare all other samples to the control. For all statistical tests, p-values ≤ 0.05 indicated statistical difference in expression.

4.0 Results

4.1 Sample Characterization

4.1.1 Histological Staining

Initial morphology of the rafts was determined using hematoxylin and eosin (H + E) to stain the nuclei and cytoplasm respectively (**Figure 7**). In rafts produced with normal NIKS (model 1) a well-structured epithelium with well defined cell borders was evident. The basal portion consists of only one layer of nucleated cells (dark blue-purple staining). The suprabasal layer consists of a few layers of differentiated cells where the nucleated cells are sparsely distributed. In the rafts produced using NIKS+HPV16 (model 2), dense staining of the epithelium indicating a higher number of cells than in NIKS (model 1) is evident. The basal layer is thicker with tightly packed dark nuclear staining. The suprabasal layer contains more nuclear staining compared to that of model 1, which represents evidence of hyperplasia. Morphology was also assessed in models 3 and 4, no gross morphological differences were observed in comparison to the H + E staining of rafts void of bacteria.

4.1.2 Keratin Staining

Keratins are a family of fibrous structural proteins. These intermediate filaments are important to epithelial structure and can be used as epithelial subtype markers. Keratin 5 (K5) is a type II cytokeratin specifically expressed in the basal layer of the epithelium corresponding to proliferating cells. Alternatively, keratin 10 (K10), a type I cytokeratin, is a marker for the suprabasal portion of the epithelium. Via Immunohistochemistry, keratin 5 expression was assessed using a polyclonal rabbit primary antibody conjugated to an Alexa Fluor® 488 labeled anti rabbit secondary (green).

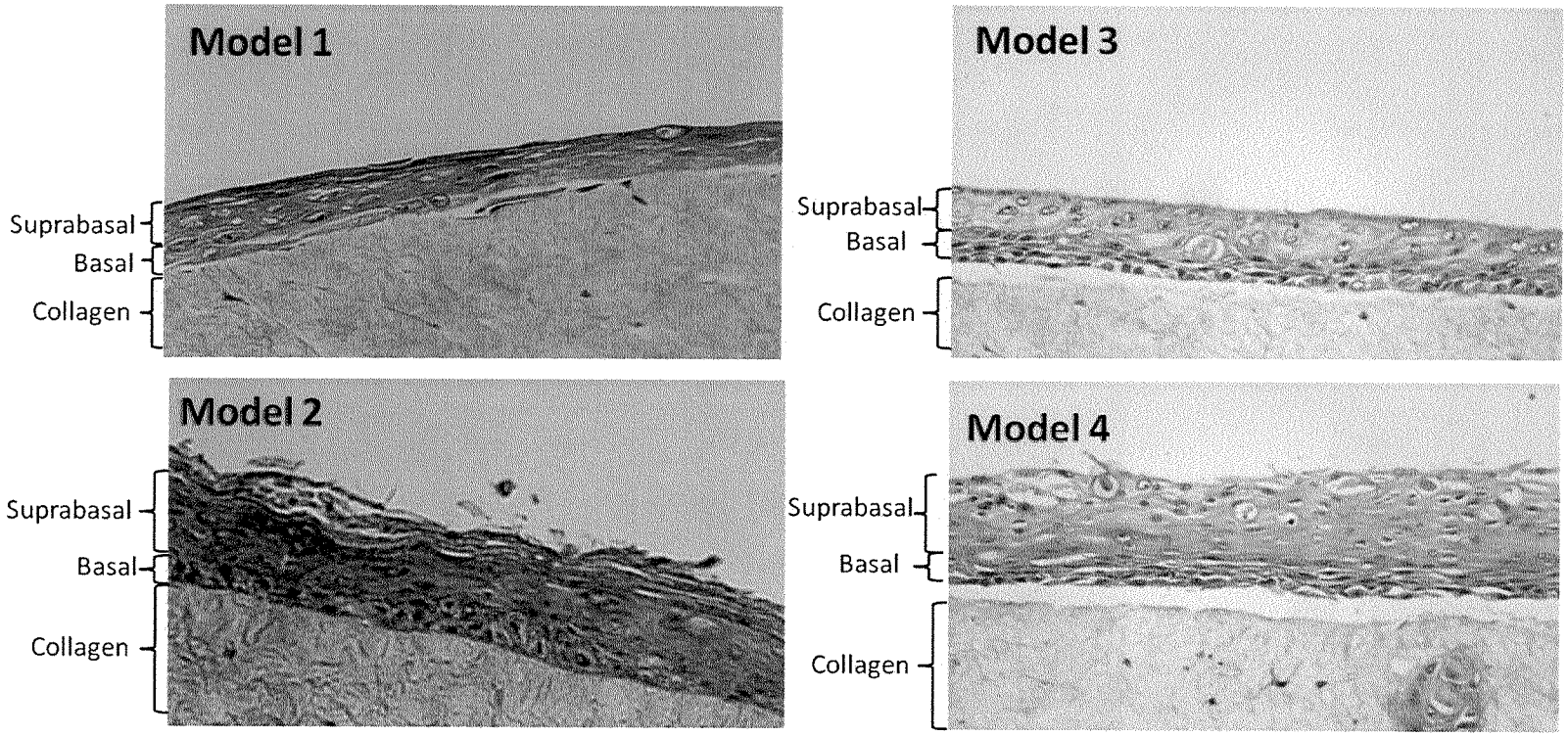

Figure 7. Haematoxilyn and Eosin Staining of 3-D Organotypic Rafts. Rafts were stained with H&E to distinguish the nuclei and cytoplasm respectively. The epithelial portion of the rafts containing HPV16 (model 2) contains more nuclei compared to normal NIKS rafts (model 1) and cell borders are not well defined.

Normally, K5 is found in the proliferating part of the epithelium only as can be seen in the NIKS (model 1). In contrast, in NIKS+HPV16 (model 2) both the basal and the suprabasal epithelium show K5 staining (**Figure 8**). K10 staining in both types of rafts is solely expressed in the suprabasal layer. Staining of K10 is indicated by a monoclonal mouse primary antibody conjugated to an Alexa Fluor® 594 labeled anti-mouse secondary antibody (red). A triple overlay image illustrates areas of overlapping K5 and K10 staining (yellow/orange). Here sole staining of K5 in the basal layer is evident. This overlay also incorporates DAPI staining to identify the nuclei. As a comparison, keratin staining of NIKS (model 1) and NIKS+HPV16 (model 2) in the presence of *Escherichia coli* (*E.coli*) (models 3&4 respectively) were also analyzed following the same method. In the models exposed to bacteria no differences in keratin expression were observed compared to rafts unexposed to bacteria (data not shown).

4.1.3 Assessment of DNA Synthesis

Cells positive for BrdU were then isolated using a donkey anti-mouse bromodeoxiuridine primary antibody, which was then detected using an Alexa Fluor® 594 conjugated anti-mouse secondary antibody (**Figure 9**). In the basal portion of the NIKS+HPV16 (model 2) we see very strong fluorescence in several of the basal layer cells as well as weaker staining in many cells of the suprabasal epithelium where the viral DNA is being amplified.

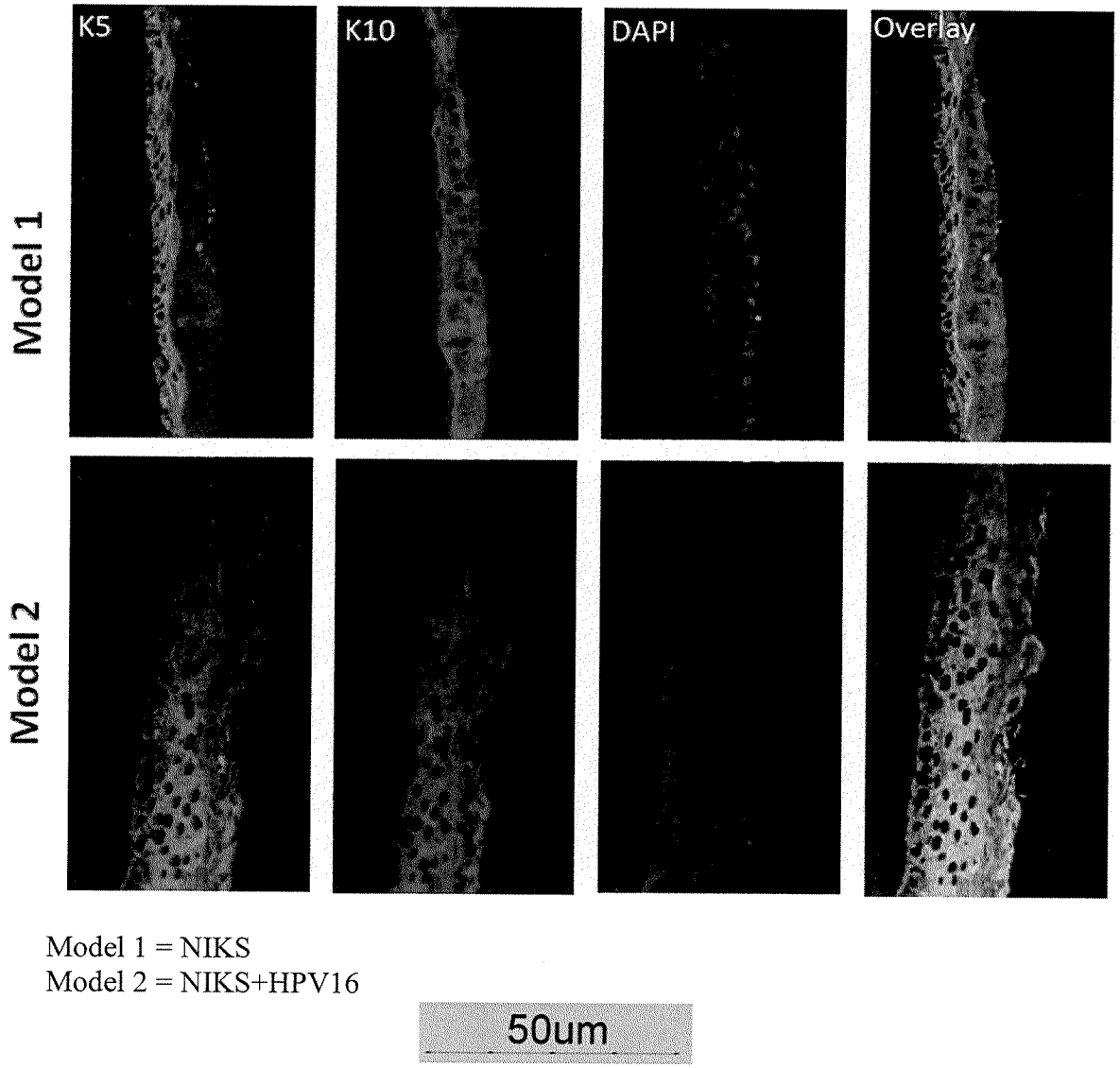

Figure 8. Keratin Staining of 3-D Organotypic Rafts. Rafts were stained with K5 (green) and K10 (red) cytokeratin antibodies. Nuclei were detected using mounting medium containing DAPI (blue). The triple overlay image reveals the overlapping staining.

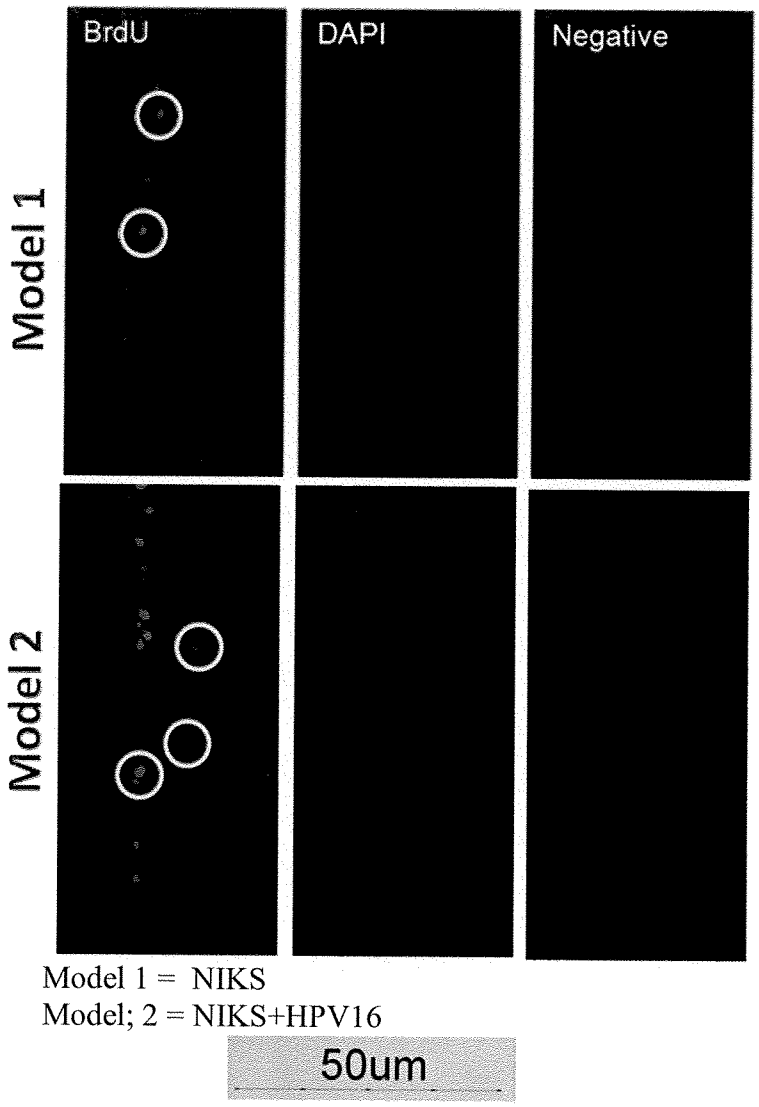

Figure 9. Identification of DNA Synthesis in 3-D Organotypic Rafts. Prior to harvesting 3-D organotypic rafts BrdU was added to cultures for 8 hours using 1.1ul of 10mM BrdU. Incorporated BrdU (red) is found in the basal cells of the NIKS (model 1) but throughout the epithelium of NIKS+HPV16 (model 2).

4.1.4 Viral Gene Expression

E6 and E7 play a joint role in deregulating cell cycle control, contributing to the loss of a normal phenotype and allowing an undue accumulation of cells. Their role in the viral life cycle is critical to the development of a malignant phenotype. qPCR was performed using RNA extracted from NIKS (model 1) and NIKS+HPV16 (model 2) and assessed for relative E6 and E7 expression (**Figure 10**). Results indicate that NIKS+HPV16 (model 2) are positive for both E6 and E7 oncoproteins. E7 has a higher relative expression in comparison to E6 in NIKS+HPV16 (model 2). The isolation of both E6 and E7 in the NIKS+HPV16 (model 2) confirms the presence of HPV16 in these tissues.

4.1.5 Viral Protein Isolation

4.1.5.1 Western Blot for E7

In HPV16 infected tissues, E7 acts as the primary transforming protein. Along with E6, E7 contributes to the disruption of the cellular cycle promoting uncontrolled cellular growth resulting in genomic instability and immortalization. A western blot was performed to confirm the presence of the E7 protein and thus the presence of the virus (**Figure 11**). A Positive band was observed in the lane containing NIKS+HPV16 (model 2), as well as the positive control NIKS+E7 (data not shown) at approximately 17kDa. Actin blots were also positive (42kDa).

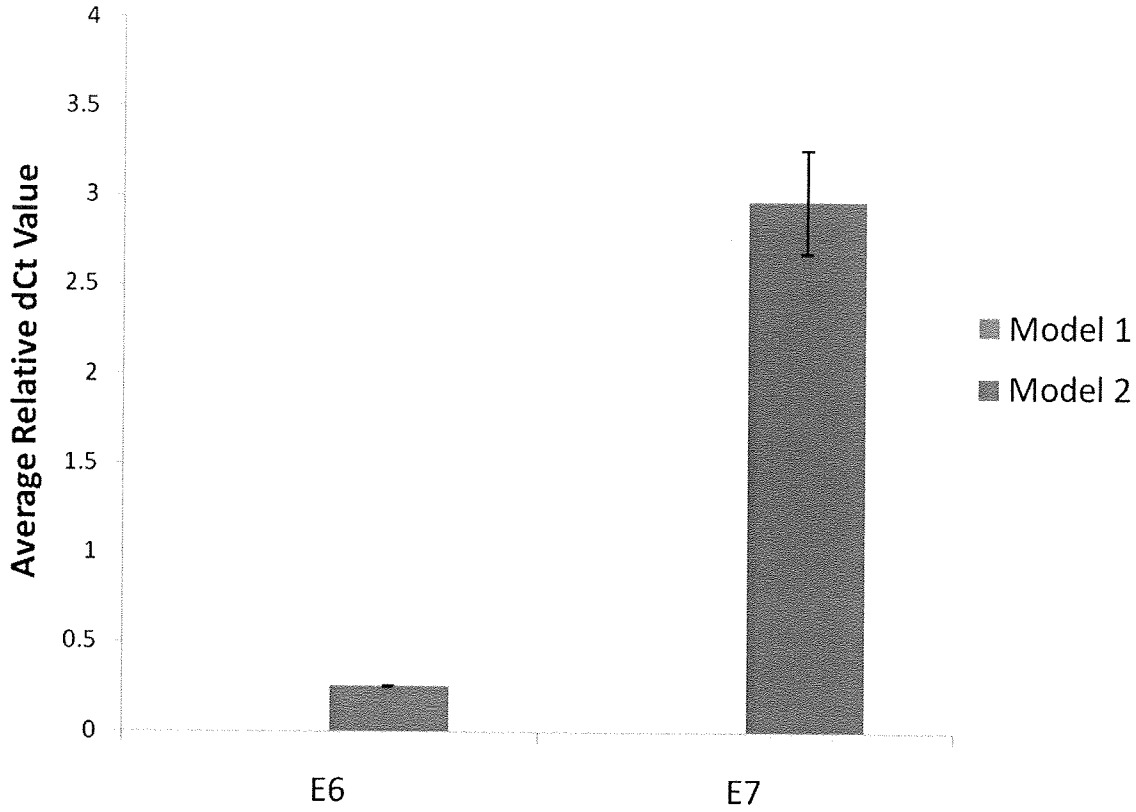

Figure 10. HPV gene expression in rafts using qPCR. NIKS and NIKS+HPV16 (n=2) were analyzed for HPV E6 and E7 mRNA levels via qPCR. Values represent an average dCt-qPCR value ± SD Presence and absence of HPV genes E6 and E7 were assessed in NIKS (model 1) and NIKS+HPV16 (model 2). "*" indicates $p \leq 0.05$. cDNA inputs were verified using the Experion™ where " ▲ " corresponds to zero values.

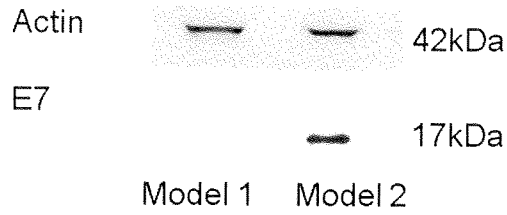

Figure 11. E7 Protein Expression in NIKS and NIKS+HPV16 3-D Organotypic Rafts. E7 expression in 3-D organotypic rafts measured using 15ug of extracted protein and run on 12% gel Gels were transferred for 1 hour at 100V to a PVDF membrane and incubated with a monoclonal E7 primary. Bands were visualized using an enhanced chemiluminescence solution (Perkin-Elmer, NEL104001EA) using a UVP imager. Model 1 corresponds to NIKS and model 2 corresponds to NIKS+HPV16

4.1.5.2 Identification of L2

The HPV16 late protein, L2, is a major component of the viral capsid, which encapsulates viral particles. These particles are indicative of a completed viral life cycle and are subsequently shed from the suprabasal epithelium where they are found. Expression of the HPV16 L2 protein is limited to tissues infected with the HPV virus making it a prominent marker for not only HPV infection, but also the viral life cycle. In the normal NIKS rafts (model 1, **Figure 12**) L2 is not present. In contrast, in the NIKS+HPV16 (model 2), we see positive staining of the suprabasal epithelium indicating presence of L2 using a primary monoclonal mouse L2 antibody (kindly provided by Martin Müller, German Cancer Research Center, Heidelberg, Germany) detected using an Alexafluor® 488 labelled anti-mouse secondary. Presence of L2 is strictly limited to the differentiating portion of the epithelium. This confirms that the viral life cycle has occurred and was completed in these tissues resulting in positive L2 staining.

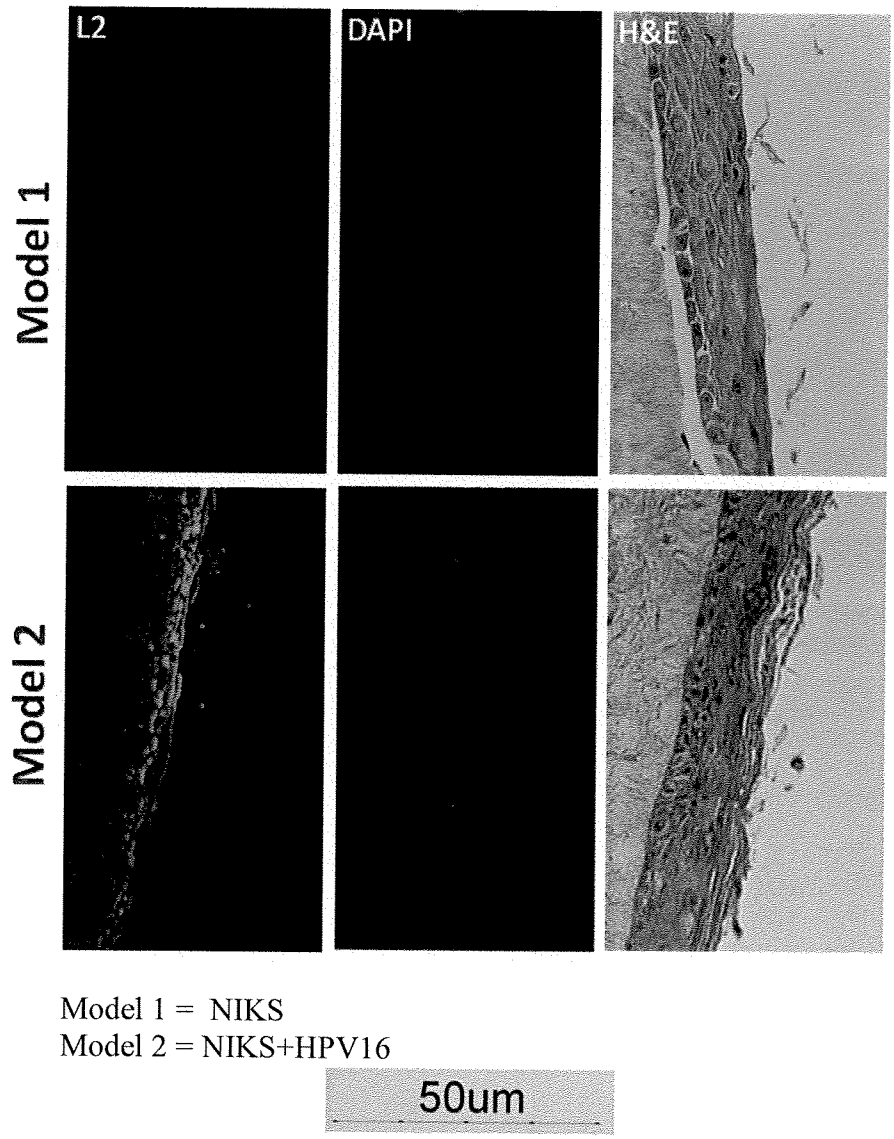

Figure 12. L2 Staining of 3-D Organotypic Rafts. L2 staining (green) is found in the NIKS+HPV16 (model 2) only. The staining is localized in the suprabasal portion of the epithelium and corresponds to the completed viral life cycle.

4.2 Innate Immune Response during the Viral Life Cycle and Bacterial Infection

4.2.1 Integrin Gene Expression

All 8 integrins examined were found in the 4 models (**Figure 13**). Integrin expression was higher than that of the housekeeping gene. To illustrate relative integrin expression 5 Cts were subtracted from the HKG and relative calculations were performed for all 8 integrins using these values. Gene expression by sample type follows a similar trend throughout the 8 integrins examined. NIKS (model 1) and NIKS+*E.coli* (model 3) express integrins at higher levels than NIKS+HPV16 (model 2) and NIKS+HPV16+*E.coli* (model 4) and NIKS (model 1) expresses integrin the highest consistently except in regards to α5. α5 is increased in NIKS+*E.coli* (model 3) versus NIKS (model 1) ($p=0.007$). α5 is also increased in NIKS+HPV16+*E.coli* (model 4) compared to NIKS+HPV16 (model 2), although this increase is not significant. α5 increase in the presence of *E.coli* in our model possibly relates to the role of this integrin in facilitating bacterial entry into the cell.

4.2.2 TLR Gene Expression

Like for the integrins, relative gene expression of 3 TLRs in 3-D organotypic raft models was measured via qRT-PCR (**Figure 14**). TLR 3 gene expression was found in all 4 models. Expression of TLR3 was varied throughout the sample groups but was found to be the highest expressed of the three TLRs examined. TLR3 was found to be significantly different from NIKS rafts (model 1) in NIKS+*HPV16* (model 2) ($p=0.001$) where expression was the lowest of all 4 models. Interestingly, NIKS+HPV16+*E.coli* (model 4) expressed TLR3 higher than NIKS+HPV16 rafts (model 1) but remained lower than that of NIKS (model 1). TLR3 expression is increased in NIKS+*E.coli* (model 3) and NIKS+HPV16+*E.coli* (model 4). These results elucidate to the role of TLR3 involvement in *E.coli* infections.

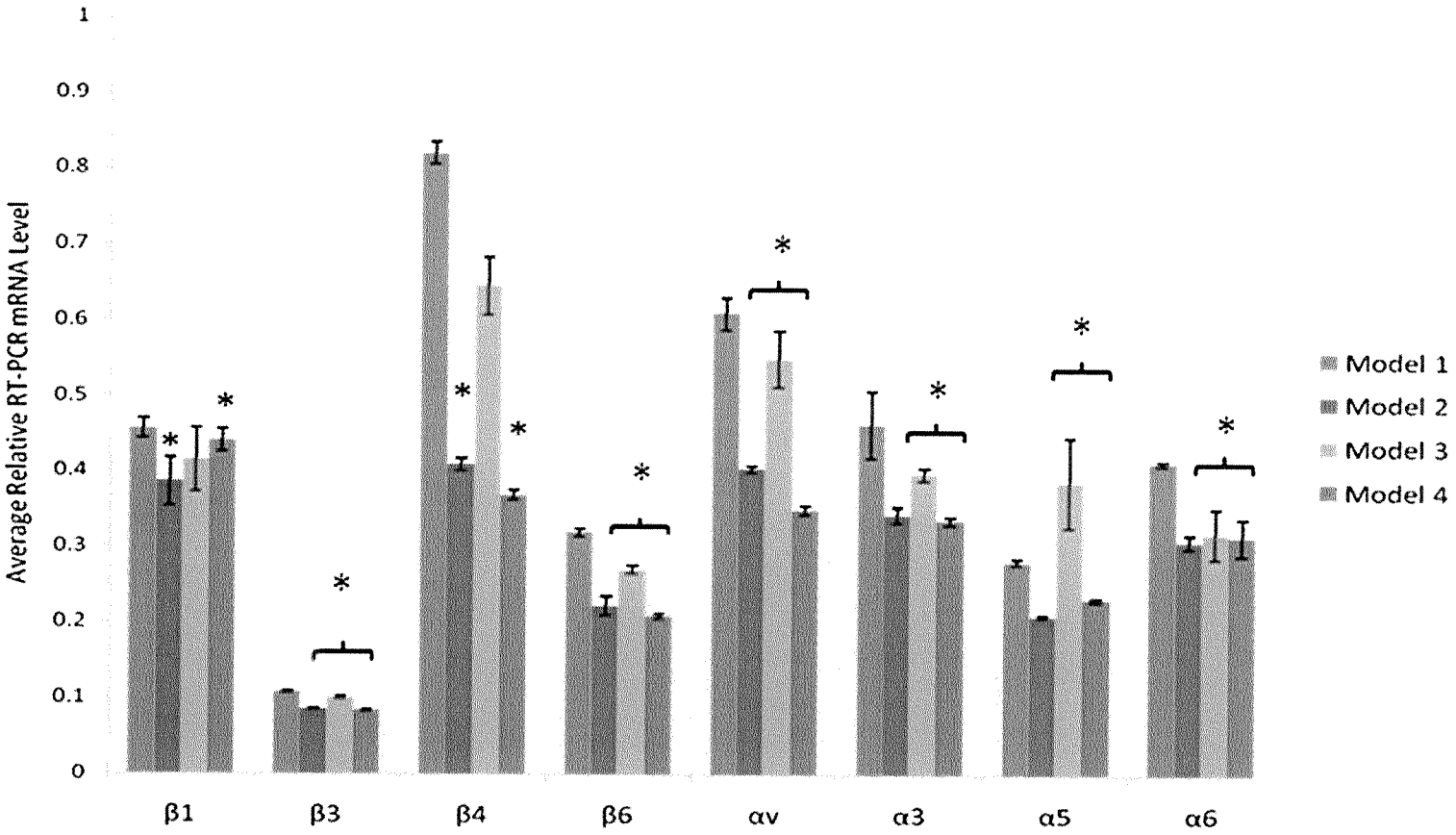

Figure 13. Average Relative dCt Integrin Expression in 3-D Organotypic Raft Models. NIKS (model 1), NIKS+HPV16 (model 2), NIKS+*E.coli* (model 3) and NIKS+HPV16+*E.coli* rafts (model 4) were analyzed for integrin mRNA levels via qPCR where n=2 experiments consisting of 3 samples each. Values represent an average RT-PCR Ct value ± SD of two experiments. "*" indicates $p \leq 0.05$ as compared to NIKS (model 1). cDNA inputs were verified using the Experion™.

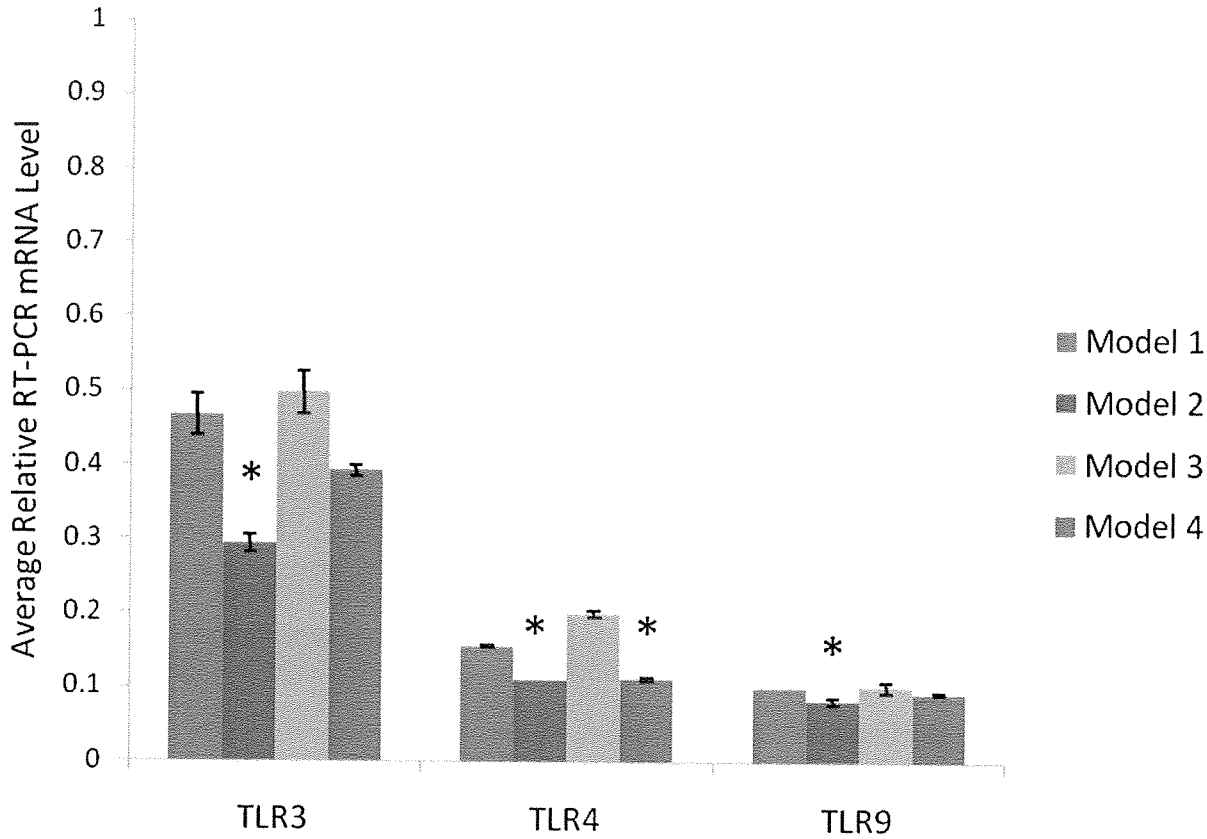

Figure 14. Average Relative dCt TLR Expression in 3-D Organotypic Rafts. NIKS (model 1), NIKS+*E.coli* (model 3), NIKS+HPV16 (model 2) and NIKS+HPV16+*E.coli* (model 4) rafts were analyzed for TLRs 3, 4, and 9 mRNA levels via qPCR where n=2 experiments consisting of 3 samples each. Values represent an average relative delta RT-PCR Ct value ± SD of two experiments. "*" indicates $p \leq 0.05$ as compared to NIKS (model 1). cDNA inputs were verified using the Experion™.

TLR4 was found expressed in all four samples but at lower levels than TLR3. TLR4 was slightly increased in NIKS+*E. coli* and NIKS16+*E. coli* samples but NIKS16 and NIKS16+*E. coli* were decreased and statistically significant when compared to NIKS (p=0.001 and p=0.002 respectively).

TLR9 expression was similar to the other TLRs in that it too was found in all 4 samples but at lower expression levels than TLR3 but similar levels as TLR4. TLR9 follows a similar trend to TLR4 in that NIKS (model 1) express TLR9 at the highest levels and NIKS+HPV16 (model 2) expresses TLR9 less (p=0.002). *E.coli* does not appear to play a role in the expression of TLR9 in the control samples compared to the other TLRs. We see a minute increase in the expression of TLR9 in NIKS+HPV16+*E.coli* (model 4) but this increase is not significant. This pattern is similar to that seen in TLR3 althoguh not to the same extent though. The absence of any effects of *E.coli* on TLR9 expression demonstrates that HPV plays a predominant role in expression of this receptor.

4.2.3 IFN Gene Expression

Relative gene expression of 3 interferons in 3-D organotypic raft models were again measured via qRT-PCR (**Figure 15**): IFN-β, -γ and -λ. Type I, IFN-β was present in all 4 models. Expression remained similar in both NIKS (model 1) and NIKS+*E.coli* (model 3) samples. In NIKS+HPV16 (model 2) and NIKS+HPV16+*E.coli* (model 4) expression of IFN-β was significantly increased (p= 0.049 and 0.001 respectively) compared to NIKS (model 1). This expression pattern demonstrates that *E.coli* does not play a role in IFN-β expression but that HPV16 does.

Type II, IFN-γ, was found at very low levels in NIKS+*E.coli* (model 3) and NIKS+HPV16 (model 2) samples but absent in the other two models. This IFN was expressed similarly in the

two models. IFN-γ expression in these samples was found a maximum of two times and at very high Ct levels in both cases. Inconsistencies in IFN-γ expression could be related lower expression of this gene in general compared to other sample types. Type III, IFN-λ, was found in all 4 models. NIKS+*E.coli* (model 3) and NIKS+HPV16+*E.coli* (model 4) showed increased levels of IFN-λ ($p=0.000$ and $p=0.000$ respectively) compared to NIKS (model 1). The increase of IFN-λ in samples containing *E.coli* indicates the influence of the pathogen on type III IFN. The increased expression of IFN-λ could be related to increases in TLR expression as IFNs are their down-stream molecules. In NIKS (model 1) and NIKS+HPV16 (model 2) expression was similar demonstrating that HPV16 would not be an important factor dictating IFN-λ expression.

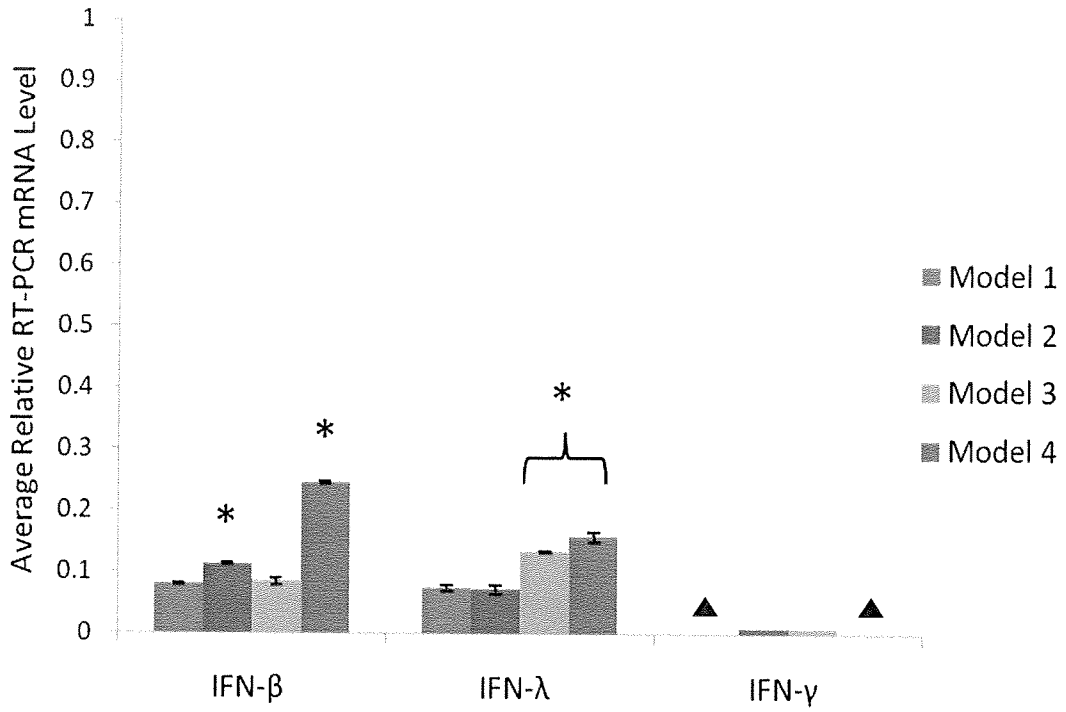

Figure 15. Average Relative dCt Interferon Expression in 3-D Organotypic Rafts. NIKS (model 1), NIKS+*E.coli* (model 3), NIKS+HPV16 (model 2) and NIKS+HPV16+*E.coli* (model 4) were analyzed for IFN-β, and -λ mRNA levels via qPCR where n=2 experiments consisting of 3 samples each. Values represent an average relative delta RT-PCR Ct value ± SD of two experiments. "*" indicates $p \leq 0.05$ as compared to NIKS (model 1). cDNA inputs were verified using the Experion™ where " ▲ " corresponds to zero values.

5.0 Discussion

Although organotypic cultures have been used in cell culture since the 1960s, these models have not been applied to studies surrounding HPV until the last decade. The development of a 3-D organotypic model has launched HPV research into an exciting new realm as it enables the mimicry of the viral life cycle *in vitro* (Flores et al., 1999). In this work, we illustrated the use of this organotypic model as the vehicle for demonstrating an active viral life cycle in the presence or absence of *E.coli*, a common pathogen found in the female genital tract. We were able to characterize the cellular differentiation program, confirm the presence of important viral genes, and proteins, and examine crucial innate immune molecules in the presence of both the virus and pathogenic bacteria during the viral life cycle. Our results exhibit an innate immune profile in regards to the 3-D organotypic model, including some molecules that have not been previously characterized in regards to HPV infection.

5.1 Sample Characterization

5.1.1 Morphological and Keratinocyte Expression

Our H+E staining results were consistent with results obtained by Flores et al., (1999), Allen Hoffmann et al., (2000) and Zehbe et al., (2009). Raft cultures obtained from NIKS (model 1) demonstrate thin stratified epithelium comparable to normal skin as previously described (Allen-Hoffmann et al., 2000) where the basal and suprabasal cells are easily distinguished. This model also produced normal keratinocyte differentiation as per previous observations (Flores et al., 1999; Allen-Hoffmann et al., 2000; Zehbe et al., 2009). In NIKS (model 1), the strict localization of K5 in the basal portion of the epithelium is a potent indicator of normal differentiation (Lersch and Fuchs, 1988). Rafts obtained from NIKS+HPV16 (model 2) demonstrated the expected hyperplastic differentiation pattern. It has been suggested that the hyperplasia exhibited in these

tissues is also due to the presence of E6, whereas previous results have shown that E6 was able to induce epidermal hyperplasia in transgenic mice (Nguyen et al., 2006; Song et al., 1999) as well as inhibiting terminal differentiation and stratification in primary human keratinocytes induced by calcium and serum in monolayer cultures (Alfandari et al., 1999; Sherman and Schlegel, 1996). Abnormal keratinocyte differentiation patterns were present as previously observed (Zehbe et al., 2009) as K5 was found throughout the epithelium. Abnormal differentiation is implicated in the progression of carcinogenesis (Zehbe et al., 2009), but individual contributions of viral proteins are easily identifiable, although E6 and E7 are considered the main contributors (Flores, et al., 2000; Halbert et al., 1992; Hudson, et al., 1990; McCance et al., 1988; Woodworth et al., 1992).

5.1.2 Cellular Proliferation

Proliferation is restricted to the basal cells in normal epithelial tissues. In tissues infected with HPV16, the proliferating cells are able to expand through the barriers and proliferate in the suprabasal portion. This pattern is reminiscent of that found in HPV positive epithelia (Stoler et al., 1989; Stoler et al., 1992). The E7 protein has been implicated to cause this transformation from cycling cells in the basal portion only to the suprabasal portion of the epithelium (Cheng et al., 1995; Crish et al., 2000).

5.1.3 Viral Molecule Assessment

We have shown in 3-D organotypic raft cultures using TaqMan chemistry, amplification of the E6 and E7 early genes of HPV16. Our results indicate a higher level of HPV16 E7 mRNA compared to that of HPV16 E6 mRNA. Differences in the levels of the two oncogenic proteins have been reported previously (DeFilipis et al. 2003; Smothkin et al. 1989). Possible mechanisms for the differences in the expression of these two genes could be related to cis

elements in the 3' end of the HPV ORF, which affect E6 and E7 levels (Münger et al., 1989). The expression of the E2 protein of HPV16 has also been reported to play a role in the expression/repression of these two genes (DeFilipis et al. 2003). As reported by others, the presence of both oncogenes is necessary for immortalization and transformation of tissues to the malignant state (Hawley-Nelson et al., 1989; Münger et al., 1989; Romanczuk et al., 1991; and Münger and Howley, 2002).

E7 has important transformation properties and is necessary for the productive stage of the viral life cycle (Phelps et al., 1988; Flores et al., 2000). E7 creates a favourable environment for HPV-16 DNA synthesis by inducing the host DNA replication machinery. The positive identification of E7 via western blot demonstrates that HPV DNA synthesis can occur and the viral life cycle can continue in our model. This also demonstrates that the translation of E7 mRNA to E7 protein is successful. Rigorous efforts were made to isolate the E6 protein without any success. This could relate to the lower expression of the E6 gene and therefore lower protein expression. Alternatively, the E6 protein could be affected by post-translational modifications such as phosphorylation by molecules such as protein kinase A, hindering its detection (Kühne et al., 2000).

The final stage of the viral life cycle includes the production of viral particles, which are subsequently released into the environment when the upper layer of the epithelium is shed (Howely 1996). These late proteins 1 and 2 (L1 and L2 respectively) are responsible for assembly of the virus into particles and therefore a marker for the completed viral life cycle (Gönermann et al., 2002). Because our organotypic model is able to demonstrate the productive stage of the virus we were able to confirm the presence of L2 in the suprabasal epithelium via immunofluorescence. The reason that we did not apply this technique to demonstrate L1 is due to

the fact that no suitable antibodies were available for paraffin-embedded tissue. The use of L2 as a possible therapeutic target has been recently proposed (Hitzeroth et al., 2009) as opposed to the majority of HPV vaccines, which target L1 (Koutsky et al., 2002). As L2 was observed to be present throughout our model, a therapeutic drug that incorporates the use of both L1 and L2 capsid proteins may be beneficial.

Combined with the evidence of hyperplastic epithelial development caused by E6, abnormal keratinocyte differentiation, altered proliferation pattern, the presence of E6 and E7 mRNA, E7 protein, and L2 protein in the suprabasal portion of the epithelium, we successfully re-created the viral life cycle in our organotypic model.

5.2 Innate Immune Molecule Expression

The innate immune system requires invasion of foreign microbes to become activated. The recognition of these microbes occurs on the cell surface, as well as internally via the endosome. Activation of these molecules is important for the regulation of the mammalian immune system and to prevent infection. Here we have shown that expression of key innate immune molecules can be assessed in a 3-D organotypic culture where an active viral life cycle is occurring. This is the first study to our knowledge to characterize these molecules in this manner.

5.2.1 Integrin Gene Expression

The integrins play important roles in apoptosis, migration, angiogenesis and proliferation (Bentz and Yurochko 2008, Unfried et al., 2007; Yoon et al., 2001). Alterations in such events have been shown to be related to the development of cervical cancer (Gruber et al., 2005; Hazelbag et al., 2007; Hughes et al., 1994). It is also known that Gram-negative pathogenic bacteria interact with integrins and epithelial cells (Roger et al., 1999). Our results indicate a common trend expressed in 7 of the 8 integrins examined where NIKS (model 1) express integrins the highest.

Expression of these integrins in the absence of the microbes could produce an environment conducive to facilitating viral and/or bacterial infection. Upon infection by these microbes, integrins are down-regulated halting the induction of the immune response and allows advancement of the cell cycle and allows the microbes to persist.

The increase in α5 integrin in the NIKS+*E.coli* (model 3) is thought to be a mechanism by which pathogens such as *E. coli* infect epithelial cells (Frankel et al., 1996; Gu et al., 2008) through heterodimerization with β1 integrin. Although only α6 has been implicated as a possible means by which HPV enters the cell, implications for α5 playing a role have yet to be determined. A slight increase was observed in α5 expression in the NIKS+HPV16+*E.coli* (model 4) model; further supporting the idea that *E.coli* stimulates an increase in α5 to aid in infection and questioning its role in viral infection. High levels of α5 have also been implicated in causing a decrease in proliferation and loss of the transformed phenotype in HT29 colon carcinoma cells (Varner et al., 1995). This could show that the decrease of α5 in the HPV16 positive tissues (models 2 and 4) creates an environment conducive to increased proliferation, which is what is exhibited in HPV16 infections.

Downregulation of β1 and β4 integrins have also been previously shown to have effects on epithelial proliferation and differentiation. Decreases in β4 have been found to be related to increases in proliferation of epithelial cells (Oldak et al., 2006; Sashiyama et al., 2002). Interestingly this decrease in β4 has been found to be related to high expression of E2 (Oldak et al., 2006). The expression of β4 has also been found to change through the stages of cervical carcinogenesis where it is expressed highly in CIN III and invasive tumors but lower in CIN I and II (Aplin et al., 1996; Carico et al., 1993). The localization of this integrin has has been found to differ in normal tissue and lesions compared to epithelial neoplasias where in the former

β4 was found in the basal/spinous layers and the later in the suprabasal layers (Williams et al., 1995). Down regulation of β4 is now being thought to be important in inducing differentiation and enabling the virus to being the productive stage of the viral life cycle which is necessary for its survival (Oldak et al., 2006).

β1 integrin heterodimerized with other integrins plays important roles in cellular adhesion and wound healing (Watt 2002). Down regulation of β1 integrin would cause these dimers to not be made and thus adhesion of cells to the basal membrane would be lost forcing these cells into differentiation. In turn this would lead to an increase in the thickness of the suprabasal epithelial tissues.

Integrin expression has demonstrated altered expression patterns in several different cell lines harbouring different viral serotypes (Gavrilovskaya et al., 2002 and Halasz et al., 2008). These observations would explain differences in integrin expression in our cell line and model compared to established carcinogenic cell lines such as HeLa. In our model, integrins become decreased during the viral life cycle which appears to contribute to increased proliferation and promotion of increase differentiation in the epithelium.

5.2.2 TLR Gene Expression

TLR 3 is an endosomal TLR similar to TLR9 with the exception that its ligand is double stranded RNA (dsRNA) and bacterial and viral CpG motifs. The decrease in TLR3 in NIKS+HPV16 (model 2) compares to previous results (Zehbe, unpublished, 2008). A decrease in TLR3 could be a mechanism by which HPV infection is facilitated. Interestingly, TLR3 increased in NIKS+HPV16+*E.coli* (model 4) compared to NIKS+HPV16 (model 2). The increased expression of TLR3 in concert with the HPV16+*E.coli* (model 4) infection elucidates the role of TLR3 in *E.coli* infection. This increase is also related to the increased numbers of

necrotic cells as a result of cell death induced by bacterial infections. This causes large amounts of RNA to become exposed and thus recognized by TLR3 (Lai 2009). IRF-3 mediates TLR3/TLR4-specific antiviral gene program (Doyle et al., 2002) and E6 inhibits IRF-3 (Li et al., 1999, and Ronco et al., 1998) halting the induction of this antiviral response. The correlation between our finding that TLR3 is decreased in HPV16 infection and that of previous results validates our findings.

TLR9 is an endosomal receptor, which recognizes bacterial as well as viral CpG motifs (Hemmi et al., 2000). Expression of TLR9 in the NIKS+HPV16 (model 2) was significantly different from that of normal NIKS (model 1) demonstrating that HPV16 does play a role in down-regulating TLR9 expression. This was previously observed by other groups (Hasan et al., 2007 and Daud et al., 2010) where decreases in TLR9 were also found in cervical cancer-derived cell lines and endocervical specimens respectively, as well as previous data using this model (Zehbe, unpublished, 2008). These results illustrate that HPV16 has built in mechanisms for viral evasion via down-regulation of endosomal receptor molecules (Daud et al., 2010) most likely through the E6 and E7 oncoproteins (Hasan et al., 2007). The downregulation of TLR9 by HPV16 has also been suggested as a type specific mechanism by which this particular subtype is able to evade detection (aDud et al., 2010). As a result of this down-regulation of TLR9, persistent infection could possibly ensue which is necessary for the onset of cervical carcinogenesis (Yu et al., 2010). Again, similar to TLR3 our findings are supported by previous research further validating our results.

TLR4 is a receptor expressed on the surface of immune cells recognizing LPS of Gram-negative bacteria. Expression of TLR4 in our model mimics the observation that TLR4 was decreased in expression during progression of cervical neoplasia (Yu et al. 2010). This decrease in expression

has been postulated to be a result of persistent HPV infection (Yu et al. 2010). TLR4 also plays a role in the detection of viral proteins. As TLR4 is decreased in HPV infection, viral proteins will not be recognized by the innate immune system and therefore the virus is able to persist (Bowie and Unterholzner, 2008).

As previously discussed, TLR4 in combination with TLR3 plays a role in the antiviral gene program mediated by IRF3 (Doyle et al., 2002) leading to the induction of INF-α/β. There was no difference in the expression of TLR4 observed in the models that have been infected with *E. coli* (models 3+4) compared to NIKS+HPV16 (model 2). Because the receptor ligand for this TLR is expressed on the surface of Gram-negative bacteria such as *E. coli*, some sort of difference in the expression of TLR4 would be expected. These unexpected results in TLR4 expression could be related to the levels of TLR9 expression. In contrast, an increase in the expression of TLR4 in the NIKS+*E. coli* (model 3) was seen. It has been shown previously by others that TLR4 mRNA expression in patients with BV have a 60 fold increase in this gene compared to those without BV (Sonnex 2010, and Zariffard et al., 2005).

Our results show that dampened TLR expression may be a type specific mechanism by which HPV16 interferes with immune response contributing to viral persistence which correlates with findings by Daud et al., (2010).

5.2.3 Interferon Gene Expression

Expression of interferons in HPV16 positive tissues has been previously reported as being varied, and this depends on the type of cervical tissue (Pao et al., 1995), virus, or cell line (Scott et al., 2002) used. Our results illustrate an increase in the expression of IFN-β in NIKS+HPV16 (model 2) samples and greater expression of this gene in those samples infected with *E.coli* (model 3+4). Previous results (De Carlo, unpublished) using the same cell line containing viral

transcripts of the E6 or E7 genes have demonstrated an increase in IFN-β expression in the presence of E7 compared to the control. Because our model contains full-length HPV16, and thus E7, the presence of E7 corresponds to this increase in IFN-β expression that could contribute to a varied expression for this gene as previously reported (Park et al., 2000, and Ronco 1998). Also, increases in IFN-β are related to levels of TLR3 expression where higher levels of TLR3 lead to increases of this IFN (Doyle et al. 2002). IFN-β production is also related to decreased levels of E6. Recall that E6 is able to inhibit IRF-3 which is important in the upregulation IFN-β. In our model we had low levels of E6 which demonstrates the IRF-3 binding may not have been inhibited allowing for increased IFN-β production. IFN-β may also not be the most potent IFN in regards to eliciting an immune response in HPV infected tissues.

Type II IFN, IFN-γ, has previously been reported as both increased (Lee et al., 2001) and decreased (Nees et al., 2001) in cervical tissues compared to in normal tissues (El-Sherif et al., 2001). A possible reason for the impairment of IFN-γ response in HPV-infected cells is due to the expression of HPV16 E7 (Perea et al., 2000). The impairment of IFN-γ could be attributed to the inhibition of IRF-1 and NF-kappaB function by E7. IFN-γ was expressed at very low levels which could be due to the high expression level of E7 in our models.

As previously mentioned, type III IFN-λ has not been examined in regards to HPV infection and therefore not in regards to a combination infection of HPV16 and *E.coli*. IFN-λ has been shown to be upregulated in the presence of DNA viruses (Brand et al., 2005) and several RNA viruses (Coccia et al., 2004, Kotenko et al., 2003, Mihm et al., 2004, Sheppard et al., 2003, Spann et al., 2004). It appears that *E.coli* stimulates expression of IFN-λ as the levels in NIKS (model 1) and NIKS+HPV16 (model 2) remain similar. Induction of this type III IFN is suggested to occur through the MyD88-independent pathway regulated by TLR3 (Zhang et al, 2007), correlating

with our results for IFN-λ and TLR3. Comparing NIKS+HPV16 (model 2) to NIKS+HPV16+*E.coli* (model 4), there is a significant difference in the expression of TLR3. The increase in TLR3 expression in the presence of HPV16 and *E.coli* could be the mechanism by which IFN-λ increases in those samples. Previous studies have shown that the treatment of herpes simplex virus (HSV) with IFN-λ blocked viral replication in the vaginal mucosa, preventing the disease (Ank et al. 2006). Because both HSV and HPV, are DNA viruses, it is reasonable to suggest that IFN-λ could be a possible therapeutic agent in both cases. Although Type I and Type III IFNs are distantly related, because they utilize different receptors they would not have similar expression patterns. Expression patterns of these genes would not be the same as their receptors are expressed in different cell types. Although there is an increase in IFN-λ, ISGs such as OAS and PKR stimulated by its production may still be inhibited by HPV infection but these need to be further examined in our model.

6.0 Conclusions and Future Work

Worldwide, the prevalence of cervical cancer is still high. This makes the need for biologically relevant models, such as the 3-D organic raft cultures used in this investigation, even more necessary. These models allow for the investigation of innate immune molecules, our body's primary defense against pathological invaders during an active viral life cycle. In order to decrease HPV infection incidence we need to better understand what is occurring in vivo at the time of infection. Most importantly, the status of the immune system in collaboration with or without pathogenic and non-pathogenic microbes needs to be further investigated. Because these microbes are commonly present in the female genital flora, understanding HPV infection under these conditions should be of increasing importance. This would help characterize the ideal infectious environment for HPV and aid in developing highly specific preventative medicine.

The availability of a therapeutic vaccine targeting L1 capsid proteins thereby preventing HPV infection will most probably help reducing cervical cancer. However this prophylactic step does not help women that are already infected with HPV. Investigations into other immune molecules as alternatives to currently available therapeutics could provoke more potent treatment options.

Our results complement or confirm previously obtained results in tissue culture as well as *ex vivo* samples adding validity to this study using 3-D cell culture models. This indicates the effectiveness of a 3-D organotypic culture model for investigations of immune molecules during an active viral life cycle.

Examination of viral proteins such as E2 which play important roles in the regulation of not only E6 and E7 but also proliferation and differentiation would most certainly add to this study and possibly reveal the mechanism by which integrin expression is regulated.

Further examination of signaling pathways leading to IFN induction would be most beneficial. Because the viral proteins are able to inhibit key members of these pathways it would be pertinent to examine whether this can be mimicked in our model. Also, because of the different effects of variants of HPV16 genes it would be fitting to examine immune signaling pathways and the binding affinities of various components with HPV genes to assess immune regulation.

The role of IFN-λ has only briefly been touched upon in regards to HPV infection. We examined IFN-λ1 (IL-29) in our study. Two other forms IFN-λ2 (IL-28a) and IFN-λ3 (IL-28b) of this IFN exist and have not yet been examined along with HPV. Also, examination of IFN-λ in *ex vivo* samples would elucidate the status of IFN-λ in cervical disease. Lastly, examination of IFN-λ in cervical cancer derived cell lines would further illustrate the role of λ as we have only examined this IFN in regards to initial infections.

Application of a *Lactobacilli* bacterium to the model would mimic "normal" physiologic flora and possibly further validate the model.

Lastly, and possibly the most important next step would be the examination of cell lines containing different variants of HPV16, as it is known that certain variants are more potent elicitors of cervical cancer.

This study has provided the first steps in the applications of a 3-D cell organotypic cell culture model in the study of the innate immune system during an active viral life cycle of a cancer-producing virus.

7.0 References

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8.0 Appendix A

8.1 RNA Extraction Optimization

We show that the Arcturus kit by far provides the highest RNA yield both by sample type and combined samples. Through visual inspection of the Experion™ gel images the bands are much more defined and there is less background in the lanes using the Arcturus PicoPure™ RNA Isolation Kit (KIT0202) compared to samples extracted using the Ambion® RNAqueous®-4 PCR kit (1914) (**Figure 17**). In the electropherograms we notice two things: differences in peak ratio and noise (**Figure 18**). The amount of RNA present can be determined by examining the three peaks where the first illustrates the amount of ladder and the next two the 18S and 28S ribosomal subunit peaks respectively. The ratio of the last two peaks compared to the first determines the amount of RNA in relation to the amount of ladder. In **Figure 18a**, we see that the 18S/28S peaks are similar in height to the ladder indicating a lower yield. Also we notice the noise in between the peaks that is also an indicator of the lack of integrity of the RNA. In **Figure 18b**, we see that the 18S/28S ribosomal subunit peaks are much higher in relation to the ladder. This indicates that there is a much higher quantity of RNA present. Also the minimal noise in between the peaks illustrates high quality RNA. When the amount of RNA yield is compared between the two kits we found that the Arcturus PicoPure™ RNA Isolation Kit provided a much higher sample yield (5 times) (**Figure 19**) compared to the Ambion® RNAqueous®-4 PCR kit. Nearly 70% of the samples extracted using the Arcturus kit produced a yield greater than 500ng/ul, which we determined an excellent RNA yield, compared to only 12% using the Ambion kit (**Figure 20**).

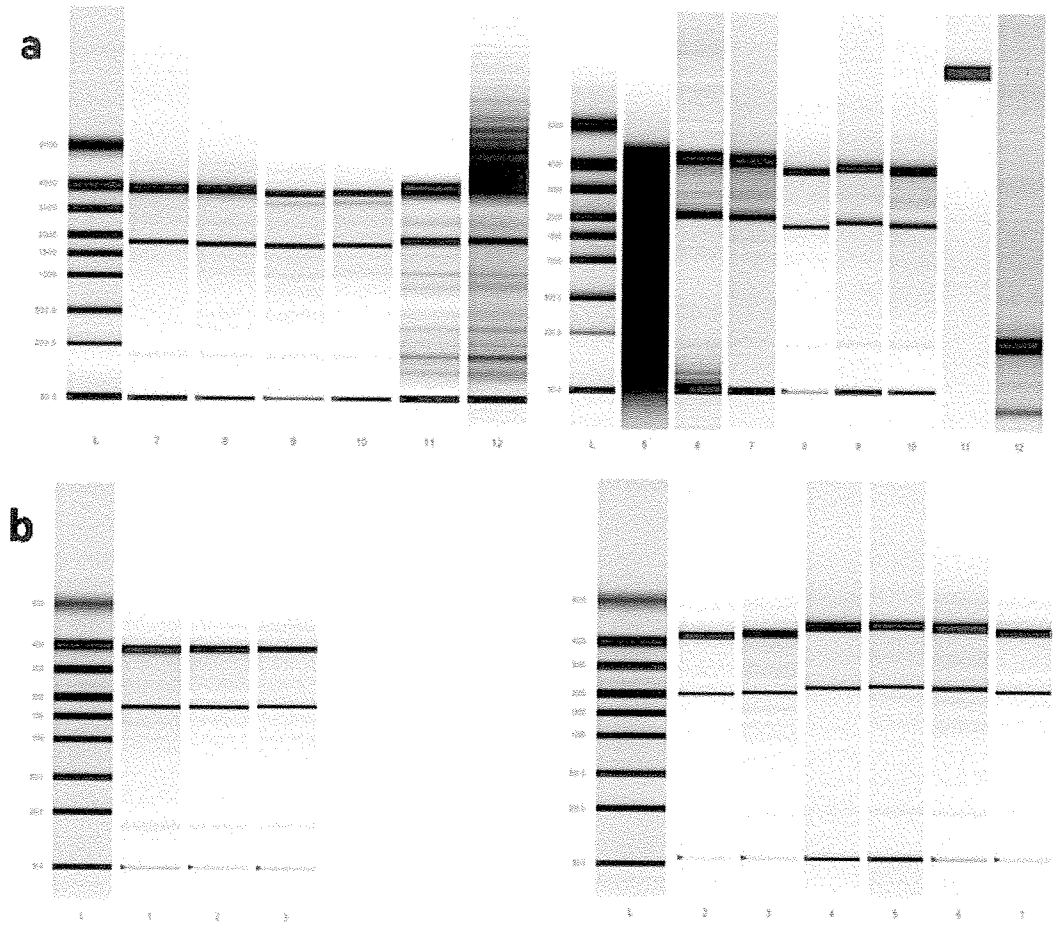

Figure 16. Electrophoresis Gel Images Comparing RNA Extraction Kits.
Experion™ gel images of ribosomal subunits for rafts. **a)** Representative gel images of RNA extracted using the Ambion® RNAqueous®-4 PCR kit (#1914) and standard sensitivity Experion™ chips. **b)** Representative gel images of RNA extracted using the Arcturus PicoPure™ RNA Isolation Kit (#KIT0202) and Experion™ RNA Std Sens chips (700-7153). The top band represents the 28S ribosomal subunit, the middle band represents the 18S subunit and the bottom band represents the ladder. One μL of extracted RNA from NIKS rafts were run in each lane.

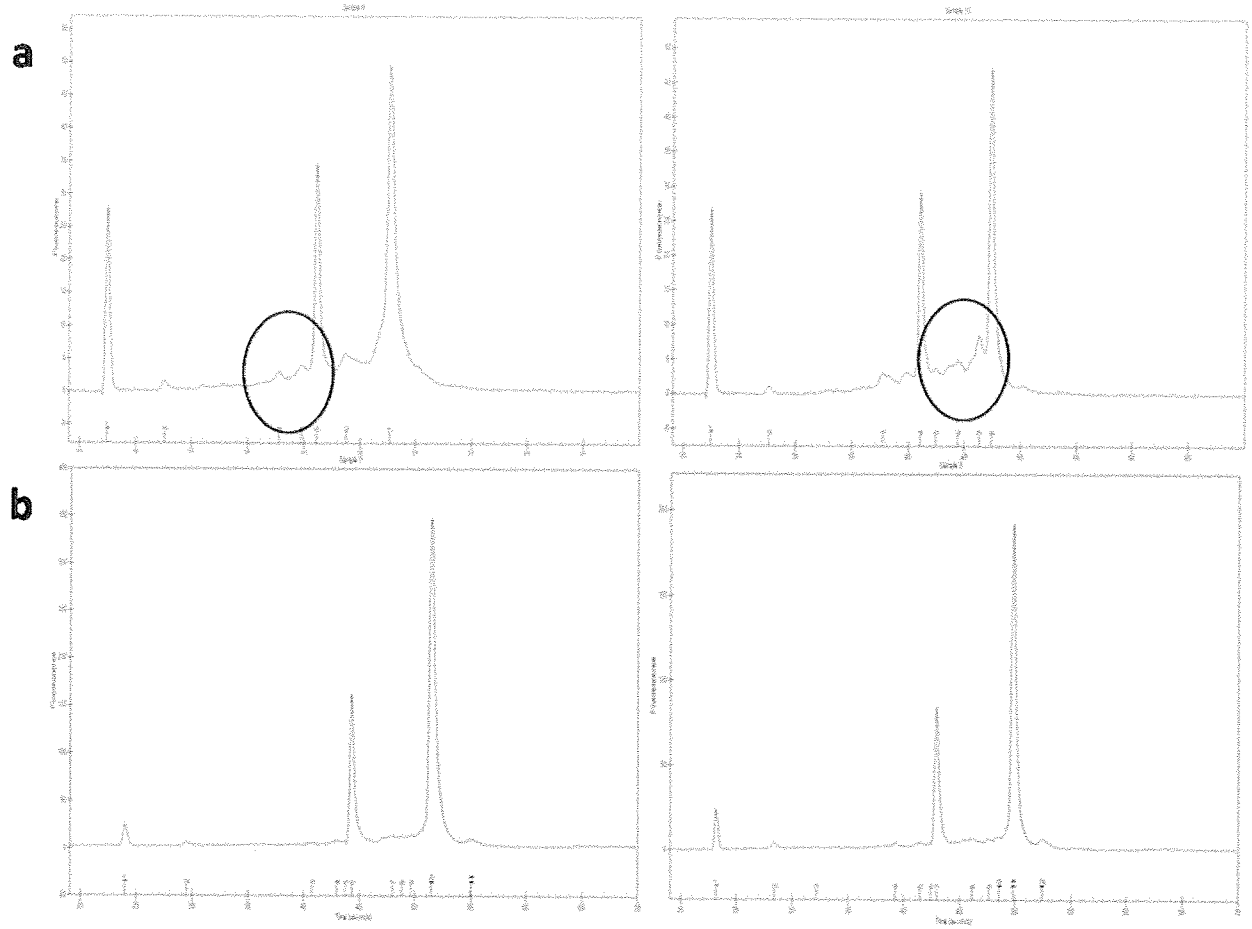

Figure 17. Representative Electropherogram Images Comparing RNA Extraction Kits. Experion™ electropherogram images illustrating the 18S and 28S ribosomal subunit peaks. The first peak is representative of the ladder, the second of 18S and the third 28S. The height of the 18/28S peaks illustrates the amount of RNA in relation to the amount of ladder. Peaks were measured by emitted fluorescence. The noise in between the peaks (indicated by the circle) illustrates the integrity of the extracted RNA. **a)** Representative images of those samples extracted using the Ambion® RNAqueous®-4 PCR kit (#1914) and **b)** representative images of those samples extracted using Arcturus PicoPure™ RNA Isolation Kit (#KIT0202) where samples are run using Experion™ RNA Std Sens chips (700-7153).

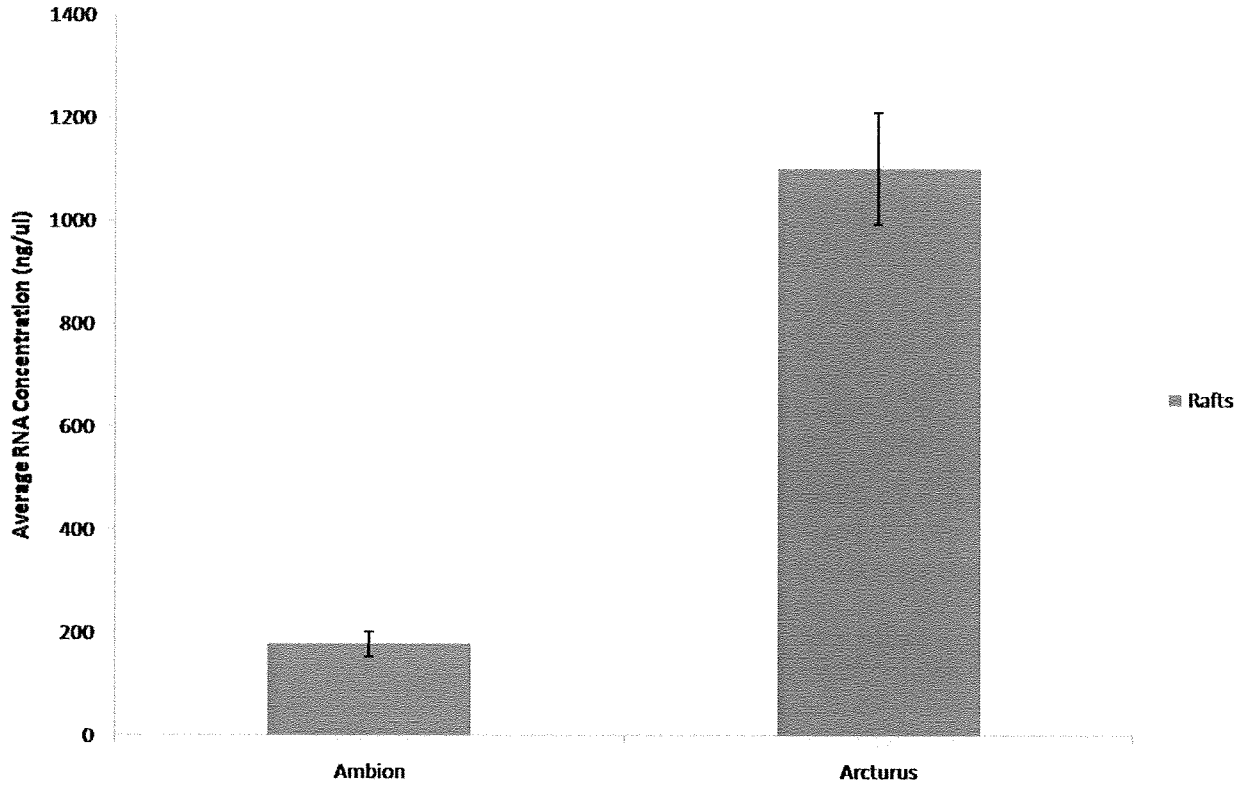

Figure 18. The Average RNA Yield By Kit. RNA was extracted from 3-D organotypic rafts using the Ambion Kit (n=14) and the Arcturus Kit (n=16). Graph illustrates the mean sample yield from each kit in nanograms per microlitre.

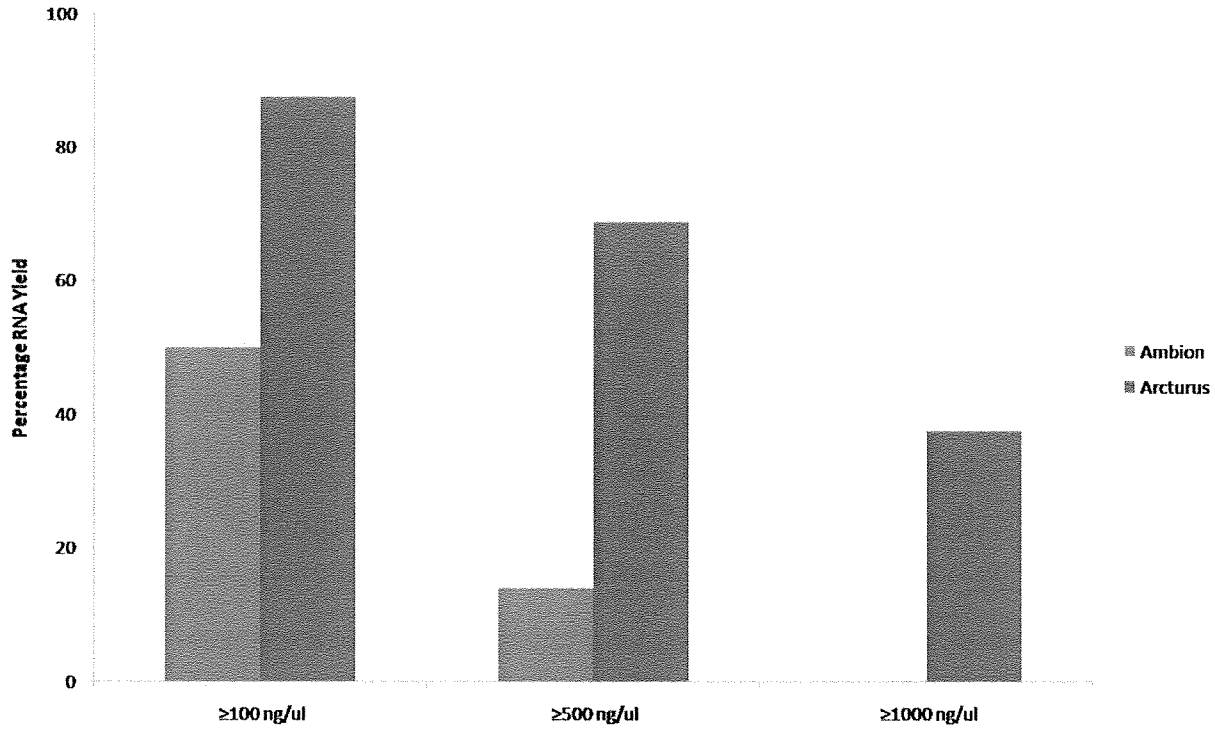

Figure 19. The Percent of Samples Yielding an Acceptable Quantity of RNA. RNA was extracted from 3-D organotypic rafts using the Ambion Kit (n=14) and the Arcturus Kit (n=16).

Graph illustrates percentage of samples within three ranges of yield where " ▲ " corresponds to zero values.

9.0 Appendix B

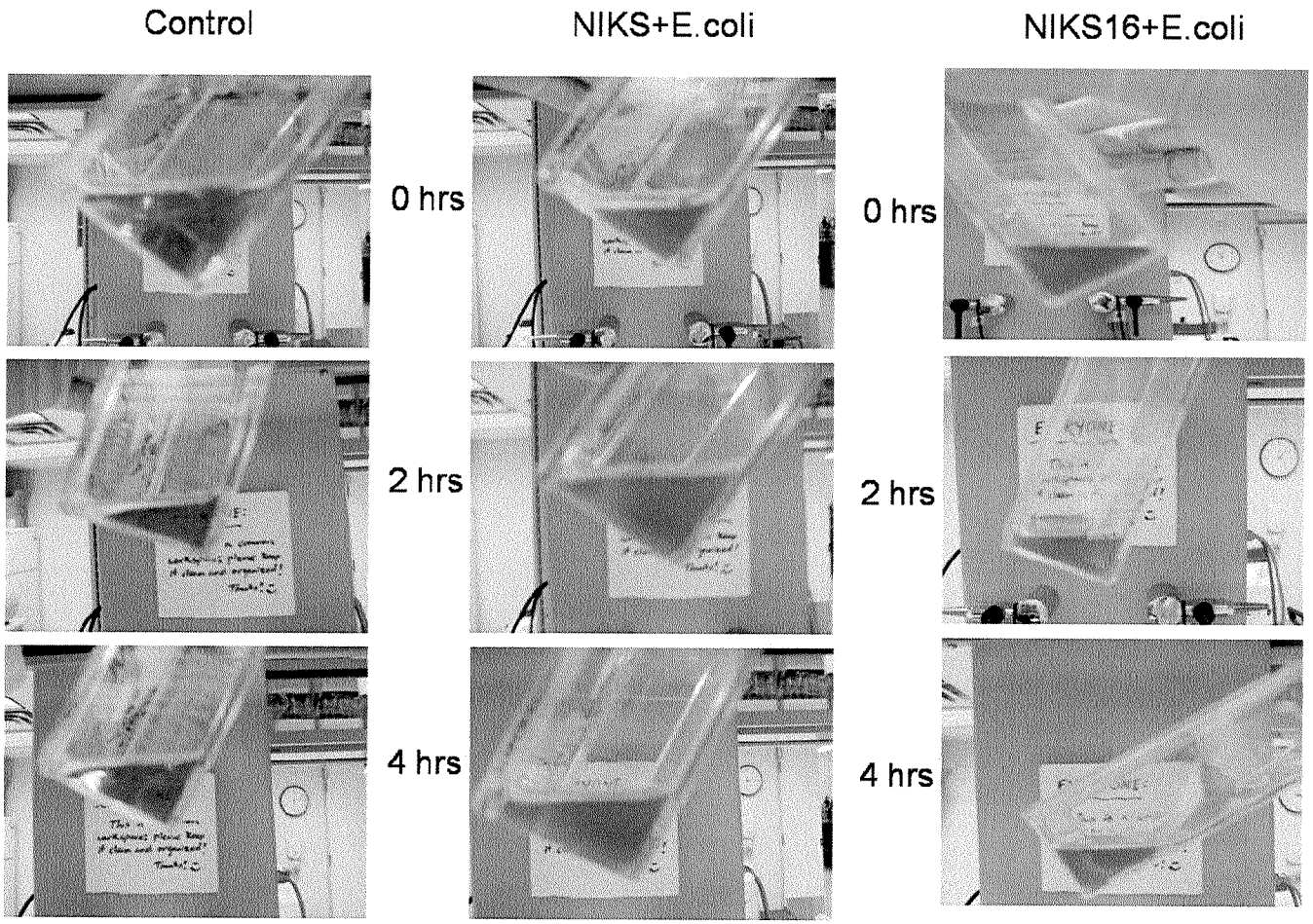

Figure 20. Bacterial Infection Control Measures. Medium clarity and colour were monitored throughout the 4 hour infection. Medium became opaque immediately following bacterial infections and remained translucent in control flasks.

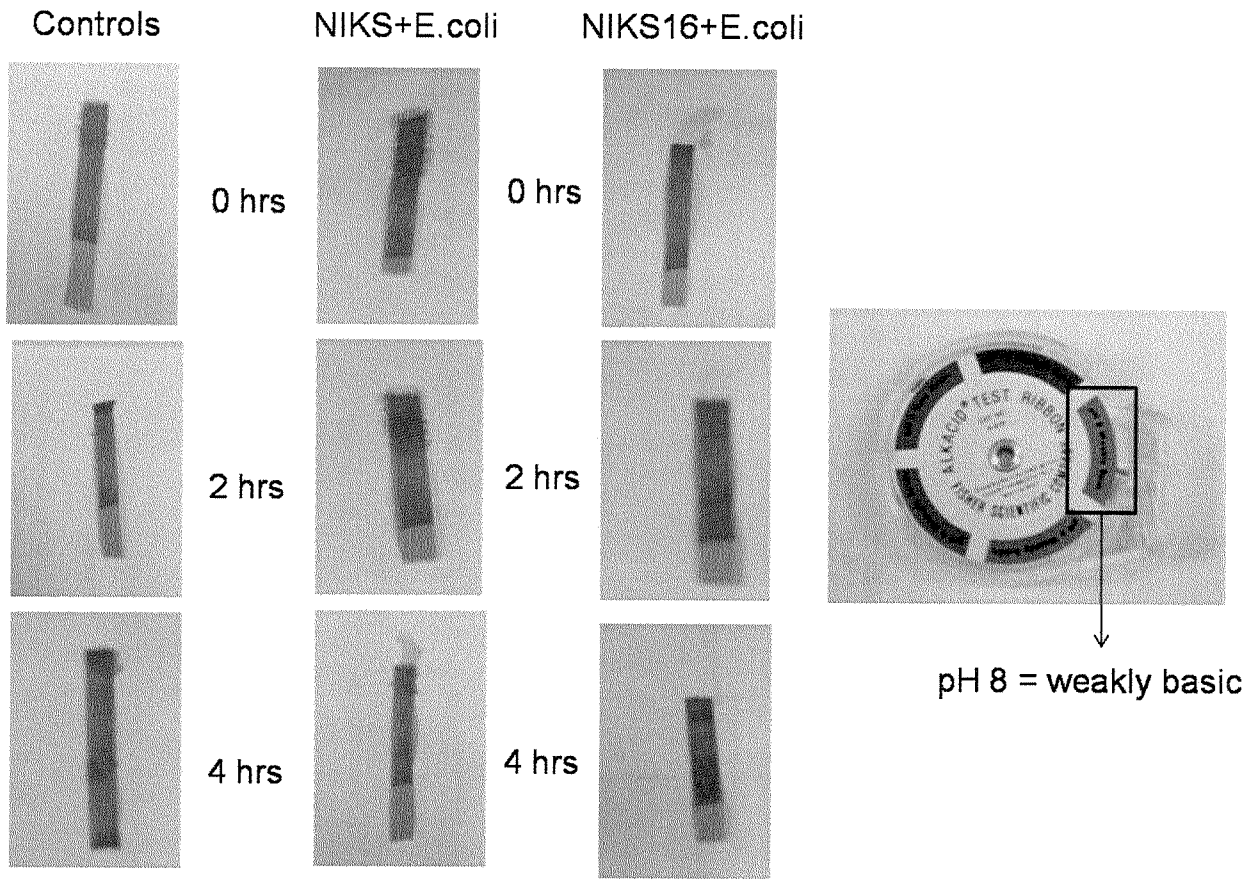

Figure 21. pH Measurement during Bacterial Infections. pH was monitored throughout the 4 hour infection. pH remained constant throughout the infection period in both the control and cells infected with *E.coli*.

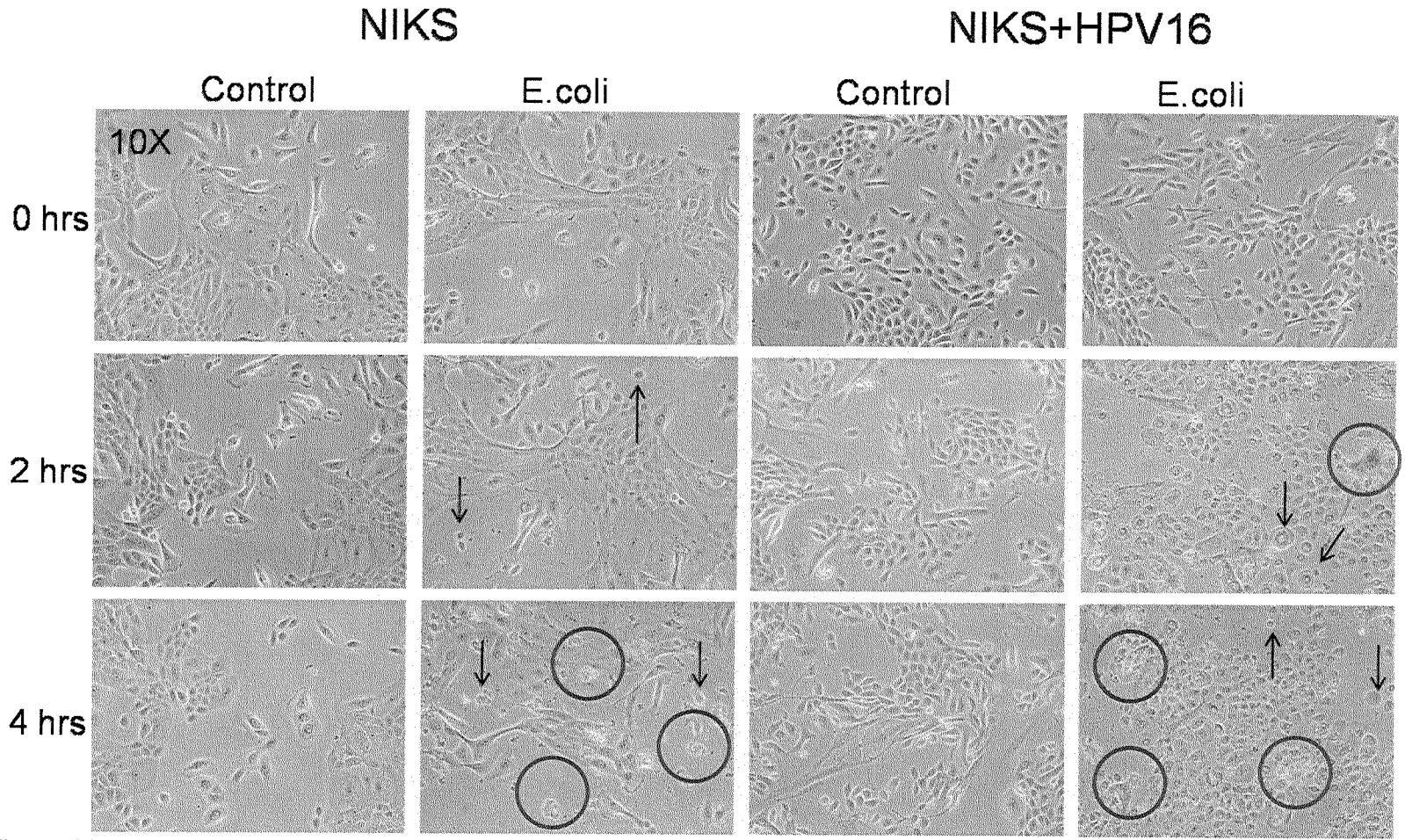

Figure 22. NIKS and NIKS+HPV16 Cultured with *E.coli* (10X). Cells were infected at an MOI of 1:50. Cellular debris is increased in those cells infected with *E.coli* (red circles). Control cells remained consistent throughout the infection period. Apoptotic cells are identified with arrows.

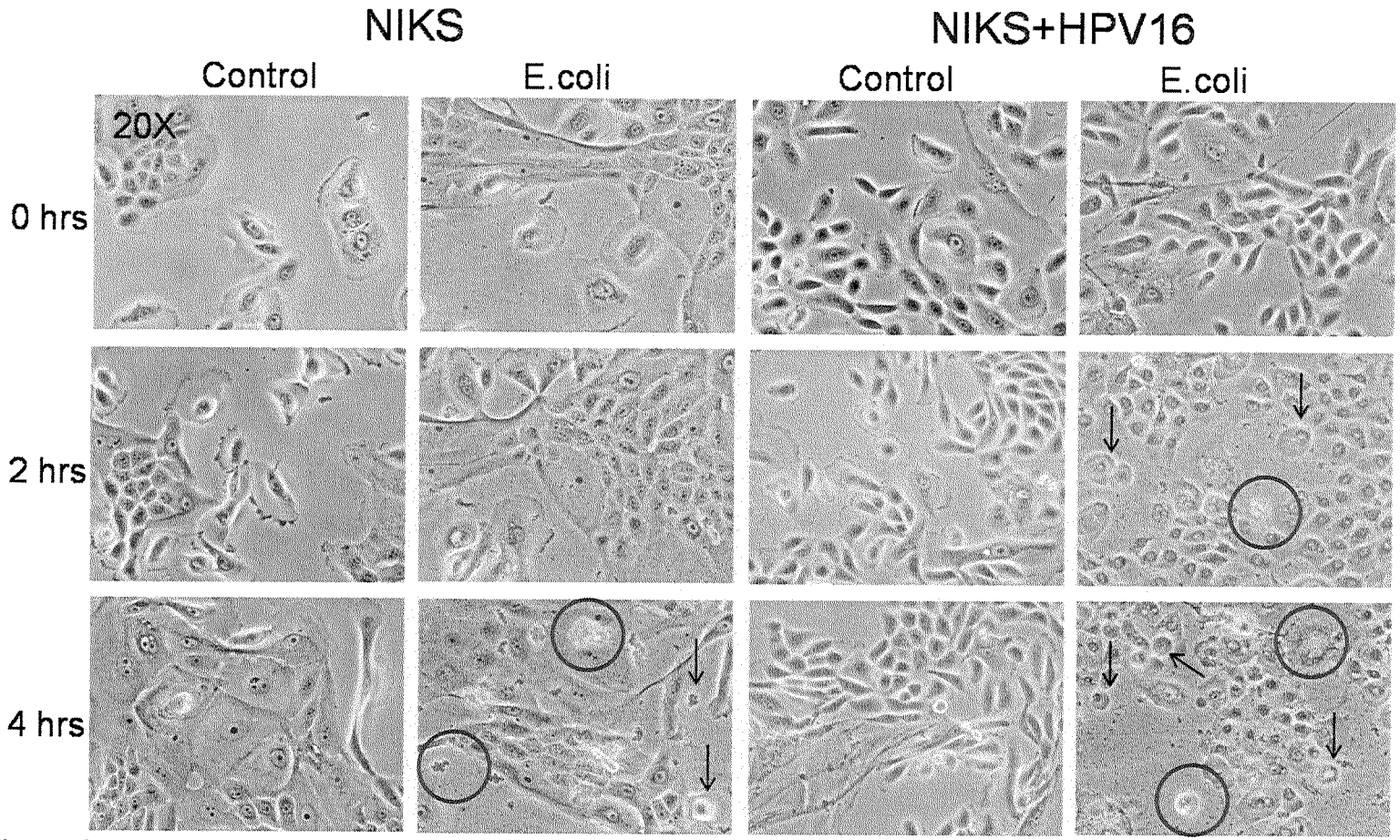

Figure 23. NIKS and NIKS+HPV16 Cultured with *E.coli* (20X). Cells were infected at an MOI of 1:50. Cellular debris is increased in those cells infected with *E.coli* (red circles). Control cells remained consistent throughout the infection period. Apoptotic cells are identified with arrows.

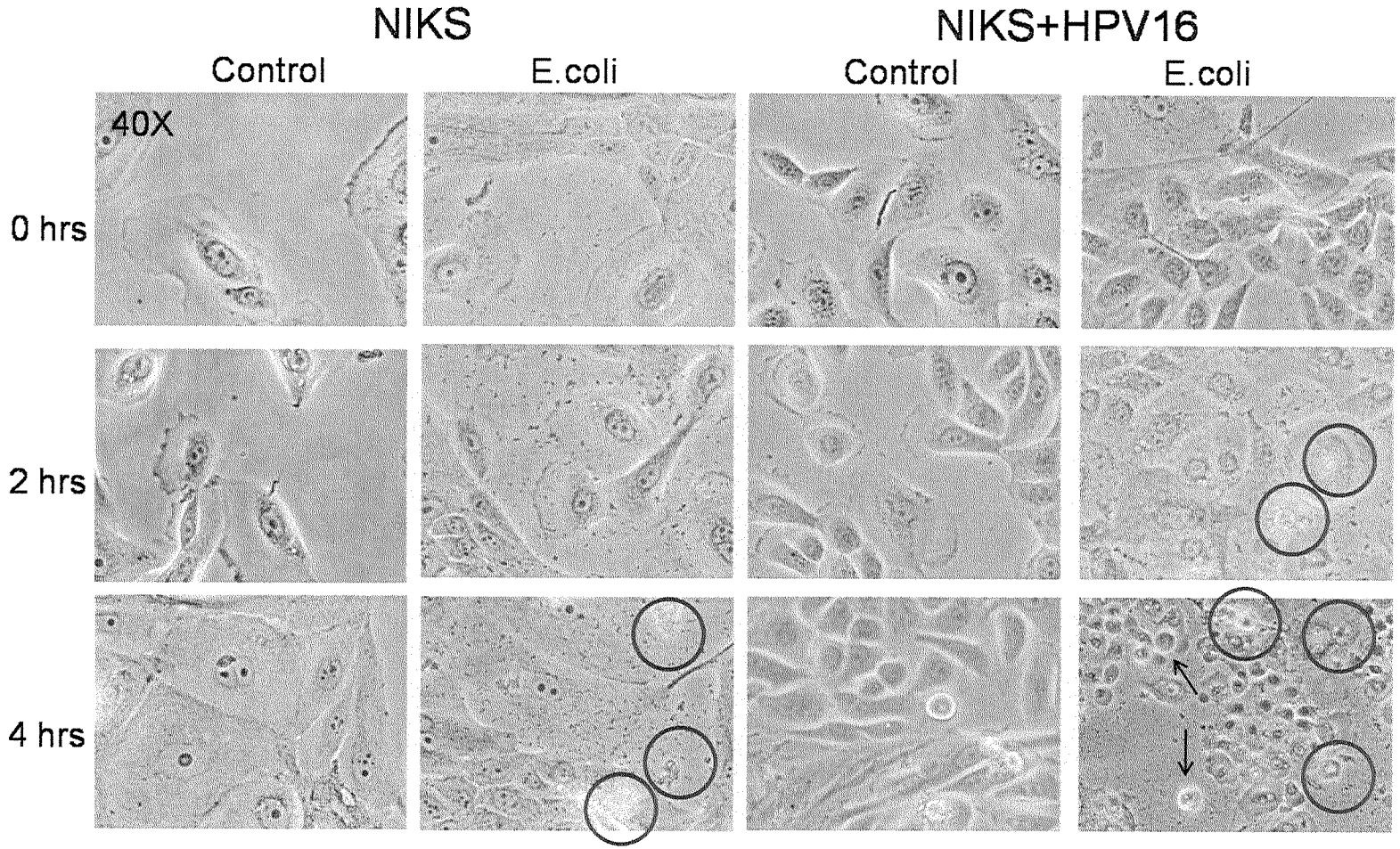

Figure 24. NIKS and NIKS+HPV16 Cultured with *E.coli* (40X). Cells were infected at an MOI of 1:50. Cellular debris is increased in those cells infected with *E.coli* (red circles). Control cells remained consistent throughout the infection period. Apoptotic cells are identified with arrows.