THE INTERACTIONS OF KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS WITH HUMAN TOLL-LIKE RECEPTORS

AND

HUMAN PAPILLOMAVIRUS AND PDZ-DOMAIN CONTAINING PROTEINS

by

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Contents Listing

List of Abbreviations

AIDS - Autoimmune deficiency syndrome

B2M - β2-microglobulin

cDNA - complementary DNA

DC - Dendritic cell

DLG1 - Discs Large 1

DNA - Deoxyribonucleic acid

dsDNA – Double stranded DNA

EBV - Epstein Barr virus

EBM2 - Epithelial basal media-2

GAPDH - Glyceraldehyde-3-phosphate dehydrogenase

GFP - Green Fluorescent Protein

GIPC1 - GAIP interacting protein C-terminus

GK - Guanylate kinase

GOPC - Golgi-associated PDZ and coiled-coil motif containing protein

HCMV - Human cytomegalovirus

HEK - Human endothelial kidney

HFK - Human foreskin keratinocyte

HHV8 - Human herpesvirus 8

HPRT - Hypoxanthine-guanine phosphoribosyltransferase

HPV - Human papillomavirus

HSV - Herpes simplex virus

HTERT - human telomerase reverse transcriptase

HTLV1 - Human T-lymphotrophic virus

HUVEC - Human umbilical vein endothelial cells

ICP0 - Infected cell polypeptide 0

IFN - Interferon

INADL – InAd-like protein

IRF - Interferon response factor

IU - Infectious unit

KCP - KSHV complement control protein

KS - Kaposi's Sarcoma

KSHV - Kaposi's sarcoma-associated herpesvirus

LANA - Latency-associated nuclear antigen

LPS - Lipopolysaccharide

LRR - Leucine-rich repeat

MAGI - membrane-associated guanylate kinase with inverted domains

MAGUK - membrane associated guanylate kinase

MCD - multi-centric Castleman's disease

MCMV - Murine cytomegalovirus

MHC - Multi-histocompatabilility complex

MHV-68 - murine gammaherpesvirus 68

MLV RT - Moloney murine leukemia virus reverse transcriptase

MOI - Multiplicity of infection

MPDZ - multi-PDZ domain protein 1

NFκB - Nuclear factor kappa B

ORF - Open reading frame

PAMP - Pathogen-associated molecular pattern

PARD3 - Par-3 partitioning defective 3 homologue

PATJ - Pals-associated tight junction protein

PBM - PDZ-binding Motif

PBMC - Peripheral blood mononuclear cell

PCR - Polymerase chain reaction

PDZ domain - PSD/Dlg/ZO1 domain

PEL - Primary effusion lymphoma

PRR - PAMP recognition receptor

PTPN - Protein tyrosine phosphatase

qPCR - Quantitative PCR

RFP - Red fluorescent protein

RNA - Ribonucleic acid

RSV - respiratory syncytia virus

RTA – Replication and transcription activator

RTPCR - Reverse transcription PCR

SAP97 - synapse-associated protein 97

SDS-PAGE - Sodiumdodecylsulphate-polyacrylamide gel electrophoresis

shRNA - Sshort hairpin RNA

siRNA - Silencing RNA

sqPCR - Semi-quantitative PCR

ssDNA - Single stranded DNA

TAX1BP3 - Tax-1 binding protein 3

TJ - Tight junction

TLR - Toll-like receptor

vIRF - virally encoded IRF

VZV - Varicella-Zoster virus

THE INTERACTIONS OF KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS WITH HUMAN TOLL-LIKE RECEPTORS

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Abstract

Approximately 15-20% of human cancers are associated with viral agents (Parkin, 2006). The human tumour virus Kaposi's sarcoma associated herpesvirus (KSHV) is one such virus. It is the aetiological agent of the AIDS-defining malignancy Kaposi's sarcoma (KS) and primary effusion lymphoma (PEL), and is linked to the development of multi-centric Castleman's disease (MCD). As a herpesvirus, KSHV resides in a latent state within infected host cells, evading various innate and adaptive immune mechanisms. The widely expressed and highly conserved toll-like receptor (TLR) family plays a major role in binding of pathogen-associated molecular patterns (PAMPs), thus identifying pathogens and leading to the stimulation of relevant host immune responses. Members of the *Herpesviridae* family are known to act upon, interact with and/or be detected by certain TLRs. Based on this precedence, we have investigated the interaction of KSHV on human TLRs in the human epithelial kidney cell line, 293. We evaluated the initial effect of ectopic TLR expression on KSHV infection level and the effect of KSHV infection on ectopic TLR transcript levels. Using flow cytometry, we found that KSHV infection is sensitive to the expression of TLRs 2, 3, 4 and 5, presumably via KSHV PAMP recognition, and is not sensitive to the ectopic expression of TLRs 1, 6, 7, 8 and 9. Semi-quantitative PCR revealed that TLR 7 mRNA levels are decreased by KSHV infection. We suggest that this result indicates that KSHV utilises some mechanism to decrease mRNA levels in order to evade potential antiviral responses triggered by the receptor.

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Interactions between Kaposi's Sarcoma-Associated Herpesvirus and Human Toll-like Receptors

1. Introduction

1.1 Kaposi's sarcoma-associated herpesvirus (KSHV) and related malignancies

KSHV, also known as human herpesvirus 8 (HHV8) (Chang *et al.* 1994), belongs to the lymphotrophic gamma (γ) herpesvirus subfamily. As a herpesvirus, KSHV consists of a large, double-stranded DNA (dsDNA) genome enclosed by an icosahedral capsid and has a life cycle that includes both lytic and latent stages. During latent infection, a restricted set of genes is expressed to ensure the virus' survival within a host cell, whilst reducing detection by the host immune system. It is this characteristic, similar to other human tumour viruses, that is thought to contribute to the oncogenic nature of the virus (de Oliveira, 2007). KSHV is transmitted via saliva, and is the aetiological agent of Kaposi's sarcoma (KS) and primary effusion lymphoma (PEL) (Bouvard *et al.* 2009). It is also strongly associated with the rare, lymphoproliferative malignancy multi-centric Castleman's disease (MCD) (Soulier *et al.* 1995). KS is a malignant transformation of endothelial cells seen in immunosuppressed patients, middle-aged men of Mediterranean and Jewish descent, and in 60% of untreated AIDS patients (Parkin, 2006). The disease manifests as a tumour of lymphatic endothelial type cells, appearing as KSHV protein-containing, and highly vascularised, lesions on the skin, mouth, gastro-intestinal tract and respiratory tract (Antman and Chang, 2000; Colman and Blackbourn, 2008*).* With the AIDS pandemic, KS incidence has increased in sub-equatorial African countries and is the most frequently detected tumour in AIDS patients (Damania, 2007). Unlike KS, only half of MCD cases are associated with KSHV; MCD is a follicular hyperplasia of lymphoid tissue and, in its more aggressive form, has been described as a disease resulting from B-cell hyperproliferation (Menezes *et al.* 2007). Similarly, PEL is a B-cell lymphoma arising in immunodeficient patients,

characterised by lymphomatous effusions. The associated cancers reflect KSHV tropism, and both MCD and PEL have a poor prognosis, and are yet to have a successful treatment formalised (Bower, 2010). Thus, KSHV provides an attractive target in the search for cancer therapies, and the role of KSHV in the induction and development of malignancies is an area of intense research. Not only will this, but knowledge of viral mechanisms of proliferation, persistence, immune modulation and oncogenesis benefits the fields of virology, oncology and cell biology. Understanding viral oncogenesis and immune modulation can be extrapolated to other viruses, and tumour virus interactions with cell machinery highlights important cellular oncoproteins and tumour suppressors.

As a herpesvirus, KSHV has evolved to establish persistent infection in the host and therefore exist in equilibrium with the host immune defences. KSHV alone is not enough to transform endothelial cells, but is a major contributor once the equilibrium is disturbed. For example, in immunodeficient situations immune control of the virus is insufficient, allowing reactivation of the virus and disease manifestation (de Oliveira, 2007). Thus, the initial detection and production of an immune response against the virus is an important factor in viral suppression and dissemination.

1.2 KSHV Evasion of the Immune System

As previously mentioned, KSHV, like other herpesviruses, is able to evade and subvert host cell machinery in order to promote viral survival and growth. In order to establish a chronic infection, it is essential to avoid host detection and set up a latent state. Consequently, more than 22 of the 86 genes encoded by the KSHV genome are thought to act upon human adaptive and innate immune mechanisms, in order to facilitate viral persistence (see Rezaee *et al.* 2006) (Figure 1). KSHV immunomodulatory proteins are known to act upon the host interferon response, complement and cytokine secretion in order to dampen initial responses against the virus and prevent recruitment of immune cells and further immune responses (see Arresté and Blackbourn

2009). For example, the virally encoded vIRFs and ORF45 proteins inhibit the host interferon response (Zhu *et al.* 2002), and the KSHV ORF4-encoded complement control protein (KCP) inhibits complement-mediated lysis of infected cells (Spiller *et al.* 2003). KSHV proteins also act upon adaptive host immune responses. Examples include the viral E3 ubiquitin ligases MIR1 and MIR2, which target MHC1, enhancing its endocytosis and thus inhibiting viral antigen presentation (Coscoy *et al.* 2001). Furthermore, by encoding chemokines that bind Th1 receptors but do not incur intracellular signalling, KSHV prevents Th1 chemotaxis to the infected area and skews the ensuing cell-mediated immune response towards a Th2 effector cell focus, shifting the immune response to an antibody-mediated response (Stine *et al.* 2000). There are more immune modulatory functions of KSHV than can be comprehensively covered here, and together immune evasion proteins advance viral persistence. These same viral mechanisms contribute to the deregulated cellular proliferation and apoptotic resistance that leads to the oncogenic transformation of a host cell. Evasion of the host immune response allows KSHV to set up a persistent state and thus expression of genes that promote cell existence. Such viral proteins include latency-associated nuclear antigen (LANA), which increases infected cell life span by interaction with hTERT (Verma *et al.* 2004), v-cyclin which mimics cellular D-cyclin (Chang *et al.* 1996) and virally encoded miRNAs (Cai *et al.* 2005), all of which contribute to latency and extended cell lifespan (see Mesri *et al.* 2010). Such evasion of immune surveillance and promotion of cellular proliferation can contribute to mutational accumulation and development of the characteristic hallmarks of cancer (Hanahan and Weinberg, 2000).

As such a large proportion of the KSHV genome is dedicated to immune evasion, it is logical that TLRs, as one class of innate sentinels of the immune response, are targets of interaction and manipulation by KSHV. Activation of TLRs may initiate antiviral responses, but these pathways may also aid viral replication and propagation. Activation of the transcription factor NFκB leads to the transcription of pro-inflammatory cytokines such as interleukin-6 and -8, which induce an

antiviral state in neighbouring cells, and an immune response aimed at eliminating virus infection (Takeuchi and Akira, 2009). At the same time, NFκB inhibits the activation of viral lytic promoters and proinflammatory cytokine production may aid KSHV by recruiting cells for further infection (Brown *et al.* 2003). KSHV is implicated in the activation of pDCs via TLR9 (West *et al.* 2011), thus promoting an inflammatory state and bringing KSHV tropic cells into close contact for infection. It is important for KSHV to avoid the initial detection and signalling cascades that would cause an antiviral state, but to balance this with allowing the activity of NFκB to suppress lytic replication that would be damaging to the establishment of long-term infection.

1.3 Toll-like receptors (TLRs)

Elimination or control of pathogen replication requires interaction between the innate and adaptive arms of the immune response. TLRs occupy the central position in this system, acting as the first response to recognise pathogen-associated molecular patterns (PAMPs). These innate receptors detect pathogen-associated ligands and alert the appropriate immune cells and subsequent responses. Currently, 10 TLRs are known in humans, designated TLR 1 to TLR 10 (Takeuchi and Akira, 2010), which form homo- or hetero- dimeric receptors at the cell surface or endosomally (Table 1). The TLR family of receptors are type 1 transmembrane glycoproteins with an immunoglobulin-like extracellular domain and a Toll/interleukin-1 receptor (TIR) intracellular domain, through which downstream signalling cascades are initiated (Bowie and O'Neill 2000) (Figure 2). The conserved extracellular domain in all TLRs contains between 21 and 25 leucine-rich repeats (LRRs), which recognise and bind cognate PAMPs (Akira *et al.* 2006). Once activated, signalling cascades operate via either the MyD88-dependent or the MyD88 independent pathways to activate the transcription factor NFκB, which directs the transcription of proinflammatory cytokines (see Akira and Takeda, 2004) (Figure 2). Various viral components, such as surface glycoproteins and nucleic acids, are recognised by TLRs, typically leading to a

type 1 interferon response, and the induction of an antiviral state in infected and surrounding cells (Akira and Takeda, 2004). The type 1 interferon response is a prominent host virus control mechanism, and it is already known that KSHV encodes several genes to counteract the innate interferon system. For example, cellular interferon response factor 7 (IRF-7) is targeted for caspase-mediated degradation by virally-encoded RTA (Arresté *et al.* 2009, Yu *et al.* 2005), and viral protein LANA is able to block interferon activation via cellular IRF-3 (Cloutier *et al.* 2010). KSHV encodes four interferon regulatory factor (IRF) homologues (vIRF1, vIRF2, and vIRF3), which are able to downregulate the production of interferons by acting dominantly over cellular IRFs on regulatory factors and elements (Fuld *et al.* 2006, Bisson *et al.* 2009). Thus, we hypothesise that KSHV is likely to encode proteins to counteract the interferon response induced by TLRs, which would otherwise be detrimental to establishment of KSHV infection.

TLRs are known to be central to viral detection, and are found on cells central to the initial detection of pathogens and influencing the resulting direction of the immune response. These cells include plasmacytoid dendritic cells (pDCs), which produce large amounts of interferon in response to viral infection (Krug *et al.* 2004). There is specific evidence for TLR-mediated detection of the family *Herpesviridae* by human TLRs (Table 1). For example, TLR 2 is activated by human cytomegalovirus (HCMV) stimulation of peripheral blood mononuclear cells (PBMC) (Compton *et al.* 2003), and has been shown to be activated by EBV in human monocytes (Gaudreault *et al.* 2007). The endosomal receptor TLR 3 detects the viral dsRNA of herpesviruses, including the non-coding Epstein-Barr virus (EBV) transcripts and dsRNA intermediates produced during KSHV replication (West and Damania, 2008). Similarly, TLR 9 is activated by DNA sequences containing unmethylated deoxy-CpGs, motifs abundant in HSV-1, HSV-2 and murine cytomegalovirus (MCMV) (Hochrein *et al.* 2004, Krug *et al.* 2004). Further complexity is evident through cooperation between different TLRs in viral detection is likely; TLR 2 and 9 have both been shown in mouse models to be required together for HSV-2

detection (Sorensen *et al.* 2008), TLR 3 and 9 important in resistance to MCMV (Tabeta *et al.* 2004), and TLR 7 and 9 are highly expressed on pDCs and are both involved in the detection of EBV ds and ss RNA by these cells *in vitro* (Quan *et al.* 2010).

The importance of TLRs in immune surveillance is reflected in the number and variety of viral evasion strategies employed to evade them. More specifically, downregulation of TLRs has already been seen in other herpesviruses. For example, TLR 9 expression is downregulated by EBV at both an mRNA and a protein level (van Gent *et al.* 2010), and EBV LMP-1 protein acts upon the TLR 9 promoter to downregulate expression (Fathallah *et al.* 2010). EBV also negatively affects TLR 2 transcription and protein functionality (Fathallah *et al.* 2010) and HSV-1 downregulates TLR 2 mediates inflammatory responses (van Lint *et al.* 2010). Converesly, KSHV is known to upregulate TLR 3 pathway components in human monocytes, leading to transcription of proinflammatory cytokine genes through NFκB activation (West and Damania, 2008). Thus viruses are able to both positively and negatively modulate TLR function at both the mRNA and protein level. It is highly likely that KSHV utilises mechanisms to evade immune detection by TLRs and their anti-viral consequences, and the study of such mechanisms will provide and exciting insight into the establishment and control of infection by the host.

With such precedence seen in the *Herpesviridae* family, and some suggestion of KSHV-TLR interactions, it is reasonable to hypothesise that KSHV will influence TLR levels, acting either at a transcriptional, translational and/or post-transcriptional level. We hypothesise that one or more of the human TLRs is responsible for detecting KSHV infection and providing some resistance to infection, and that KSHV targets relevant TLRs by negatively regulating mRNA levels. Having obtained stable cell lines expressing each of the nine human TLRs individually, and in order to gauge the effect of KSHV infection on TLR mRNA levels, we aim to determine whether KSHV infection is sensitive to the expression of individual TLRs, and to identify any changes in individual TLR gene transcription by quantifying their mRNA levels.

1.4 Hypothesis, Aims and Objectives

We hypothesise that one or more human TLR is responsible for detecting KSHV infection and providing some resistance. Therefore, KSHV may target relevant TLRs by negatively regulating their mRNA levels. We aim to determine whether KSHV infection is sensitive to the expression of individual TLRs, and to determine whether KSHV infection affects the mRNA levels of particular TLRs. We used cell lines expressing individual human TLRs, subject them to infection by KSHV and analysed by flow cytometry and reverse transcription (RT) PCR, and RT semi-quantitative PCR (sqPCR). Throughout the experiment a non-transfected control cell line was treated in parallel. In the PCR experiments, infected and uninfected cells were compared.

2. Methods

Unless otherwise stated, materials and chemicals were obtained from commercial sources.

2.1 Cells and Cell culture

Human embryonic kidney (HEK) 293 cell lines ectopically expressing individual human TLRs (TLRs 3, 4, 5, 7, 8 and 9 as homodimers and TLRs 1/2, 2/CD14 and 2/6 heterodimers) were obtained from collaborators (Dr M. Ressing, Department of Medical Microbiology, University Medical Center Utrecht). The expression vectors conferred antibiotic resistance (van Gent *et al.* 2010). These cell lines were maintained alongside non-transfected HEK-293 control cells taken from an already growing source within the Blackbourn lab. Cells were grown within flasks containing media comprising Dulbecco's modified eagle media (DMEM, Gibco) supplemented with foetal bovine serum (FBS, Gibco, 10% v/v), L-glutamine (Sigma, 2mM), penicillin (Sigma, 200mM) and streptomycin (Sigma, 67mM). This supplemented DMEM cell culture media is referred to as complete DMEM throughout. Cells were cultured by incubation at 37°C and 5% CO_2, in a humidified environment. Human TLR-expressing cell lines were cultured in media containing selective antibiotics as recommended (Table 2). Non-transfected HEK-293 control cells were unable to grow in antibiotic-containing media, giving reassurance that the transfected cells were selected for. Cells underwent at least three passages in selective medium before use in experiments.

Table 1: Selective antibiotics used to isolate and culture TLR-expressing cell lines

Antibiotic	Final concentration (in complete DMEM)	Selected HEK-293 cell lines expressing ectopic TLR
Blasticidin	10 μg/ml	3, 5, 6, 7 and 8
G418	700 μg/ml	9
Puromycin	10 μg/ml	2 and 4

2.2 KSHV infection of cells and flow cytometry

The rKSHV.219 strain used in these studies expresses green fluorescent protein (GFP) from the EF-1α promoter during latent infection and red fluorescent protein (RFP) from the PAN

promoter during lytic infection (Vieira and O'Hearn, 2004). Virus was prepared as described previously (Viera and O'Hearn, 2004), and infectious units (IU) of rKSHV.219 determined by titre of virus in cell-free medium on HEK-293 cells, counting GFP-positive cells 2 days post-infection. Virus was resuspended in epithelial basal media-2 (EMB2, Gibco).

For infection experiments, HEK-293 cells were isolated from main stocks and seeded at $2x10^5$ cells per ml in a 96 well plate, and incubated overnight in the appropriate media before stimulation with purified rKSHV.219 at multiplicities of infection (MOIs) of 10, 5, 1, and 0.1. MOI is a measure of IUs per cell, and allows comparability between different experiments. The plate of virus plus cells was then centrifuged (450 x *g*, 30 min, room temperature) and incubated for an hour. Virus was then removed, the cells washed with media and incubated for 48 hours. Cells were removed by trypsinisation, fixed in 1% paraformaldehyde (PFA) and transferred to cytometry tubes for quantification of GFP expression, as a measure of the extent of rKSHV.219 infection. A Coulter EPICS XL-MCL flow cytometer was used to analyse GFP levels (Beckman Coulter, UK) and the FlowJo programme (Treestar Inc.) used to analyse the resulting data, gating first on live cells, and then on GFP-expressing cells. Different gates were used for each cell line, due to the differences in morphology, but the same gate was used within a cell line, applied to the different MOIs.

For RNA analysis, cells plated at $2x10^5$ cells per ml in a 6 well plate were infected the next day with purified rKSHV.219 at an MOI of 5, using the same method as described for the 96 well plates. Corresponding wells were plated with cells in parallel and treated with EMB2 medium rather than virus for analysis of non-infected cells as a control. Latent infection was confirmed by observation of GFP fluorescence under a fluorescent microscope. Cells were harvested by trypsinisation, pelleted and snap-frozen in liquid nitrogen and stored at -80°C for further use.

2.3 RNA isolation, cDNA transcription and PCR

From each TLR-expressing cell line and a HEK-293 control cell line, approximately $5x10^6$ cells were isolated, pelleted and snap frozen. The total cell RNA was extracted from the thawed pellet using an RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions, and quantified by spectrophotometry.

RNA was treated with DNase (Promega) as recommended by the manufacturer, in order to remove any genomic DNA that could be amplified by PCR and give false positive results. Two hundred and fifty nanograms of DNase-treated RNA was used for complementary DNA (cDNA) transcription using random hexodeoxynucleotide primers (100mM, Invitrogen) and the Moloney murine leukemia virus reverse transcriptase (MLV RT, Promega) in the presence of RNase inhibitor (Promega) and nucleotides (10mM, Promega). MLV RT synthesises a complementary strand of DNA from a template in the presence of random primers. Before addition of enzyme, the reaction was heated to 65°C for 5 min and chilled on ice quickly, in order to denature any RNA secondary structure that would prevent efficient primer annealing. Polymerase and RNase inhibitor was then added and the reaction incubated at 37°C for 50 min to allow sufficient primer elongation. The enzyme was inactivated by heating to 70°C for 15 min. To confirm the absence of genomic DNA in the later stages of the experiment, parallel reactions were performed without the reverse transcriptase enzyme.

Resulting cDNA was used as a template for amplification in a PCR using GoTaq polymerase (Invitrogen), 2μl of cDNA from the first strand reaction and specific primers (Table 3) to amplify TLR genes, in seperate reaction volumes (50μl). Amplification of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a positive control for each cell line, alongside a reaction incorporating DNA extracted from a HEK-293 cell line where GAPDH is present in the genome. DNA fragments of expected length were visualized by 1% agarose gel electrophoresis and ethidium bromide staining, using a bromophenol blue loading

buffer dye and tris-acetate-EDTA (TAE) electrophoresis buffer, loading 10μl of sample against 1μg of 100bp DNA ladder (Invitrogen).

Table 2: Primer sequences for the detection of TLR expression

mRNA target	Forward 5'-3'	Reverse 5'-3'	Amplified Fragment size (kbp)
TLR 1/2	AAAAGAAGACCCTGAGGGCC	TCTGAAGTCCAGCTGACCCT	340
TLR 2/CD14	AACCCTAGGGGAAACATCTCT	GGAATATGCAGCCTCCGGAT	549
TLR 3	AAATTGGGCAAGAACTCACAGG	GTGTTTCCAGAGCCGTGCTAA	320
TLR 4	TACAAAATCCCCGACAACCTC	AGCCACCAGCTTCTGTAAACT	264
TLR 5	TGCATTAAGGGGACTAAGCCTC	AAAAGGGAGAACTTTAGGGACT	351
TLR 6/2	TTGACAGTTTTGAGACTTTCCC	TGAACCTCTGGTGAGTTCTG	516
TLR 7	TCCAGTGTCTAAAGAACCTGG	TGGTATATATACCACACATCCC	532
TLR 8	TAATAGGCTCAAGCACATCCC	TCCCAGTAAAACAAATGGTGAG	621
TLR 9	GTGCCCCACTTCTCCATG	GGCACAGTCATGATGTTGTTG	260/212
GAPDH (a)	CCCACTCCTCCACCTTTGAC	CCTCTTGTGCCTTTGCTGGG	178
GAPDH (b)	GAGCCACATCGCTCAGACAC	GCTTCCGTTCTCAGCCTTG	220

GAPDH primers were used to amplify the housekeeping genes, and act as a standard in sqPCR. Where the PCR reaction was paused at 20, 25 and 33 cycles and 10ul aliquots removed from each reaction and stored at 4°C. All samples were analysed together by agarose gel electrophoresis. TLRs 1/2, 2/CD16 and 6/2 work as heterodimers involving one of each of the named receptor. The first number is the one that will be used to refer to the receptor as a whole within our experiments. Annealing temperature of 55°C was used for all primer pairs.

2.4 Semi-quantitative PCR (sqPCR)

Unless stated otherwise, cDNA from infected and uninfected cell samples were treated in exactly the same way. KSHV infected cell samples from the infection step were subject to mRNA extraction and cDNA transcription carried out as described above. cDNA was specifically amplified with relevant TLR primers and primers for GAPDH, by polymerase chain reaction (PCR). During the reaction, 10μl aliquots from these samples were taken at 20, 25 and 35 cycles, by stopping the machine at the relevant number of cycles and transferring aliquots for storage at 4°C. All samples were visualised together on a 1% agarose gel with ethidium bromide staining, as described above. Relative brightness of the amplified bands was compared visually between

infected and non-infected cDNA from the same cell line, using GAPDH brightness as a loading volume control.

3. Results

In order to investigate whether ectopic expression of individual TLRs affected the efficacy of rKSHV.219 infection, we infected HEK-293 cells expressing individual human TLRs with this virus at a range of MOIs. The extent of infection was quantified by measuring GFP expression by flow cytometry, using a non-transfected HEK-293 control for comparison. This strategy revealed whether rKSHV.219 infection was sensitive to the expression of any TLR, and the results translate to wild type virus. Extraction of mRNA and subsequent analysis of infected and uninfected samples from each cell line confirmed TLR expression and allowed a comparison of mRNA levels between infected and uninfected cells. Thus we could suggest whether KSHV infection affects the levels of TLR mRNA within this cell system.

3.1 HEK-293 cell lines express the transfected human TLRs

To confirm TLR expression, each cell line was subjected to RNA analysis via RT-PCR. TLR mRNA transcribed to cDNA and amplified by PCR, using specific primers to amplify TLR cDNA. In parallel, untransfected control cells were subject to RT-PCR in order to test whether the HEK-293 cell line naturally expresses any TLR. Visualisation of amplified fragments on an agarose gel showed that each cell line expressed the expected individual TLRs (Figure 3). Bands were seen at the expected size for each TLR and control cDNA prepared in the absence of reverse transcriptase showed no bands of amplified TLR cDNA (Figure 3). The absence of TLR DNA amplification in samples where no reverse transcriptase was present suggests that there was no contaminating cDNA, and that bands corresponded to the presence of TLR mRNA in the cultured cells. The absence of bands in control HEK-293 cell samples indicate that the cells do not express any TLRs at a level detectable by ethidium bromide (Figure 3). Individual amplified fragment amounts vary, for example TLR 3 appears to have amplified to a greater amount than TLR 2, and the band for TLR 9 is very faint (Figure 3, top and bottom panels). However, PCR

amplification is sensitive to conditions and cycles, and these differences are attributable to the variations in primer pair length and GC content as well as gel loading. This information was enough to give us confidence that the provided cell lines were expressing the relevant receptors and could be subject to further experiments, alongside the current control. It is important to note that the control untransfected HEK-293 cell line did not show expression of any tested TLR (Figure 3), and thus we can confidently attribute differences in KSHV infection efficiency to ectopic TLR expression.

3.2 Cell lines expressing human TLRs are infected by KSHV at different efficiencies

Using flow cytometry, we investigated whether the ectopic expression of a human TLR in a HEK-293 cell line was responsible for detecting rKSHV.219 infection and whether such expression affected infection efficiency. This model cell system mimics cellular expression of TLRs and their effect on initial infection of wild type KSHV. Infection of cell lines expressing each of the human TLRs 1-9 separately, at a range of MOIs, allowed the determination of whether KSHV infection is sensitive to TLR expression. Infection was quantified by flow cytometry, detecting the GFP expression induced by rKSHV.219 infection. Initial observations under a fluorescence microscope showed successful infection and latent expression of the virus, seen as green fluorescence emission (Figure 4). Expression of GFP rather than RFP indicates a latent infection (Viera and O'Hearn, 2004). Infection of the different cell lines is visibly comparable at the same MOI, but more accurately measured by flow cytometry. All cell lines showed GFP expression upon infection, although the proportion of GFP-expressing cells appears to vary. Figure 4 shows that cell lines expressing TLRs 4 and 9 appear to be less infected than the other cell lines at comparable MOIs. Cell lines expressing TLR 4 and 9 show GFP expression in approximately 30-40% of cells at MOI 5, whereas the TLR 2, 6 and 5 expressing cells show approximately 50-60% of cells infected, and TLR 1 and 3 expressing cells appear to have the highest infection, with approximately 70-80% of cells expressing GFP at the same MOI.

Cell densities vary, due to the difference in growth rates between cell lines (Figure 4). The higher MOIs, 10 and 5, show visibly higher levels of infection as higher GFP density in these images, compared to lower MOIs, 1 and 0.1 (Figure 4). In the case of TLR 1, 2, 4, 7, 8 and 9 expressing cells, infection at an MOI of 0.1 showed no visible GFP expression (Figure 4), and is reinforced by flow cytometry data (Figure 5, panel A). As was expected, varying MOIs produced different extents of infection; a higher MOI gave a higher density of GFP-expressing cells (Figure 4). Morphology of the cell lines differed subtly compared to control, which was observable via the light microscope (Figure 4) or when analysing by flow cytometry. TLR 7 and 8 expressing cells were less adherent and more rounded in shape compared to the control HEK-293 cells, which have a more spikey appearance (Figure 4). Differences in morphology led to the need for different gating on live cells during the flow cytometry, although the gates were maintained within a cell line for quantification of GFP at different MOIs.

Flow cytometry data shows infection of cells at MOIs 0.1 and 1 gave less consistent results than higher MOIs (5 and 10) (Figure 5 panel A). Inconsistency was seen both between individual experiments and within each data set; values at these MOIs are extremely variable. It is likely that this variability is due to the small amounts of virus involved at the low MOIs. With smaller volumes, any discrepancy in volume has a bigger impact on amount of virus (Figure 4 panel A), and thus creates a large amount of deviation between values. The differences between the cell lines and control were not analysed in depth at low levels of infection, and data gained from MOIs of 5 and 10 form the focus of conclusions drawn.

Efficiency of infection of HEK-293 at MOIs of 5 and 10 appeared to be unaffected by the ectopic expression of TLRs 1, 6, 7, 8 and 9 compared to control (Figure 5 panel B). These data suggest that KSHV infection is not sensitive to the expression of these receptors.

The flow cytometry data indicates that KSHV infection is sensitive to the expression of TLRs 2, 3, 4, and 5, with TLRs 2 and 4 having the most effect (Figure 5 panel C). HEK-293 cell lines

expressing these receptors show lower infection efficiency than control cells, these data are consistent at the MOIs of 5 and 10 (Figure 5 panel C). Both TLR 2 and 4 have lower infection efficiencies than control cells by a factor of approximately 50% whereas KSHV is less sensitive to the expression of TLRs 3 and 5, with infection efficiency of 25% less than the average control cell infection efficiency (Figure 5, panel C).

3.3 Levels of cellular TLR mRNA levels can be affected by KSHV infection

RT sqPCR analysis was applied to cells expressing TLRs 3, 4, 5 and 7, comparing infected and uninfected samples of the same passage. Samples were taken from the paused PCR at 20, 25 and 33 cycles and all were visualised by agarose gel electrophoresis. In the samples analysed, amplified bands of DNA taken at 33 cycles were all at a comparable brightness, as expected, due to the reaction reaching a plateau as reagents become limiting. Samples taken at the earlier cycles of 20 and 25 are predicted to be within the logarithmic expansion phase, and thus should give an indication of any difference in sample cDNA template levels when compared to each other, assuming that housekeeping gene (GAPDH) amplification is equivalent. In most cases, faint or no bands were visible at 20 cycles, and thus most of the comparison occurs at 25 cycles.

When analysed by sqPCR, both infected and uninfected cell lines expressing TLR 3 showed unvarying levels of TLR 3 mRNA at 33 cycles, the only stage at which template amplification was detected. Indicating that TLR 3 mRNA levels are not affected by KSHV infection (Figure 6). Bands were barely visible at 20 cycles, and not useful to draw conclusions from, and the brightness of bands compared between infected and non-infected cells was seen to be almost equal at 25 cycles and 33 cycles.

sqPCR of the cell line expressing TLR 4 does not appear to show a difference in the amount of TLR template between infected and non-infected cell samples (Figure 7). Amplified cDNA from uninfected cells show a marginally brighter band than the equivalent from the infected cell

samples, but the GAPDH brightness decreases, suggesting that KSHV infection does not affect TLR 4 mRNA.

From RT sqPCR analysis, TLR 5 mRNA levels appear to be unchanged in TLR 5 expressing 293 cells before and after infection (Figure 8). Levels of both TLR 5 and GAPDH at 33 cycles are comparable, but are not at 25 cycles. Because GAPDH levels are not comparable, the difference between TLR 5 fragment brightness between infected and uninfected samples is slight and would be better analysed by a more quantitative method.

The most obvious change in mRNA levels between KSHV positive and negative samples was seen in the analysis of TLR 7. Amplified cDNA from TLR 7 expressing cells is absent in infected samples (Figure 9). Analysis by sqPCR showed no detectable expression of TLR 7 following KSHV infection. No bands are visible at either 25 or 35 cycles, compared to equivalently treated, uninfected cells expressing TLR 7 (Figure 9). The difference seen between infected and uninfected samples is considerable and merits further investigation.

4. Discussion

TLR detection is the 'first-line' of defence in immunity, and thus the receptor family are an important group in terms of viral evasion; avoiding initial detection by TLRs promotes virus infection and dissemination. Thus, one can assume that KSHV employs mechanisms to interact with and modulate any of the TLRs that affect its ability to infect cells and set up persistence. These experiments give an overview of the changes that occur in TLR mRNA when infected with recombinant KSHV, and form a solid basis for further verification and investigation.

The receptors TLR 1, 6, 8 and 9 have been implicated in herpesvirus detection. TLR 1/2 heterodimers expressed on the cell surface of pDCs are activated by HCMV glycoproteins (Boehme *et al.* 2006). TLR 8 has been implicated in KSHV reactivation from latency (West *et al.* 2011) and TLR 9 signalling is activated by KSHV infection of pDCs, despite mRNA levels being unaffected by KSHV infection (Lagos *et al.* 2008). However, we saw no effect of the expression of these TLRs on HEK-293 cells on KSHV infection efficiency. It is likely that the interactions and effects of KSHV and TLRs is cell-specific and that these differences are attributable to the variance in cell type.

TLR 2 is prominent in the detection of hydrophobic PAMPs, and is expressed on DCs, monocytes and macrophages (Table 1), primary responders to KSHV infection (Krug *et al.* 2004). There are examples of herpesviruses stimulating proinflammatory cytokine production via stimulation of TLR 2 (Table 1). Notably, HCMV activates inflammatory response via TLR2/CD14 heterodimer on human monocytes (Compton *et al.* 2003), and EBV induces monocyte chemotactic protein-1 (MCP1) production via stimulation of TLR 2 on primary monocytes (Gaudreault *et al.* 2007). Our data shows that ectopic TLR 2 expression by HEK-293 cells reduces susceptibility to KSHV infection. The mechanism of this is unknown, and whether KSHV affects TLR 2 mRNA levels is also unknown. It is likely that the receptor recognises viral

glycoproteins, as it does the glycoproteins of HCMV (Boehme *et al.* 2006). As TLR 2 is expressed on the surface of the host cell, detection of viral glycoproteins is likely to occur whilst virus is still outside of the cell, during initial infection. The epitope detected by TLR 2, and which stage of infection detection occurs is something that can be deduced structurally or investigated using site-direct mutagenesis. In this system the TLR 2 gene has been transduced alongside CD14, allowing the two to act together as a heterodimer. Transfection of TLR 2 with other known molecular partners, such as CD36 and RP105 (Kumar *et al.* 2009), would highlight the specificity of TLR 2-KSHV interactions. On this basis, the effect of TLR 2 expression on KSHV infection warrants further investigation.

Dendritic cells and B cells are known to express TLR 3, which detects pathogen-associated double-stranded and single stranded RNA (Alexopoulou *et al.* 2001) (Table 1). KSHV sets up a latent infection in B cells (Dupin *et al.* 1999), indicating that TLR 3 may be especially relevant to viral detection. Our evidence suggests that TLR 3 expression causes cells to be slightly resistant to infection by KSHV; expression of TLR 3 is not affected by KSHV infection but KSHV infection is sensitive to ectopic expression of TLR 3. It is possible that encounter of ligand and TLR occurs following endocytic entry of the virus into the cell, not a conventional method of viral entry, but one seen in some cell types, including B cells (Akula *et al.* 2003). To become activated by the virus, TLR 3 may detect packaged RNA within the KSHV virion, exposed to the receptor during fusion-mediated entry of the virus within the endosome (West and Damania, 2008). With respect to other herpesviruses, activation of TLR 3 is detrimental to HSV-1 latent infection of neurones (Zhou *et al.* 2009). However, KSHV infection has been shown to upregulate TLR 3 at a transcriptional level in monocytes, a cell type that the virus is able to infect (West and Damania, 2008). It has been suggested that TRIF, a signalling protein downstream of activated TLR 3 and 4 signalling, is degraded by KSHV (Ahmed *et al.* 2011). By acting to prevent intracellular signalling, KSHV prevents an antiviral inflammatory response that could be caused

by TLR 3 upregulation. The sensitivity to TLR 3 expression that we see may be overcome once the virus has infected host cells. The position of TLR 3 in our model system may not resemble the *in vivo* situation, and experiments in naturally TLR 3 expressing cell lines could yield contrasting results.

TLR 4 is involved in viral glycoprotein and lipopolysacharide (LPS) detection and is expressed endosomally by Monocytes/macrophages, DCs, mast cells and intestinal epithelial cells (Table 1). Our results disagree with previous data that show that infection of lymphatic endothelial cells with KSHV rapidly suppresses TLR 4 mRNA and protein levels (Lagos *et al.* 2008), and also with data that suggests that lack of TLR 4 expression renders cells more susceptible to KSHV infection (Lagos *et al.* 2008). These differences are attributable to the differences in the model systems used and altogether suggest a role for TLR 4 in innate immunity against KSHV, which needs to be characterised fully. It is feasible that TLR 4 recognises herpesvirus glycoproteins, as it does the glycoprotein F of respiratory syncytia virus (RSV) (Kurt-Jones *et al.* 2000). Like TLR 3, it is likely that KSHV has mechanisms to overcome the antiviral response once a cell is infected.

TLR 5 is known to detect bacterial flagellin (Hayashi *et al.* 2001), and thus it is unclear why its expression would affect KSHV infection. However, most TLRs have a range of ligands (Akira and Takeda, 2004), and we are open to the possibility that there are undiscovered ligands for all of the TLRs. In this case, TLR 5 may well interact with lipids present in the virion envelope. The structure of TLR 5 may have regions of homology with the other cell-surface expressed TLRs, and could loosely interact with a common viral epitope to become activated. Therefore, expression of TLR 5 renders KSHV sensitive to cell infection, due to an unspecific interaction that causes the virus the difficulty in entering a target cell that we see in our flow cytometry data. This is speculation, and elucidation of the mechanism behind this result requires further exploration.

Like TLR 3, TLR 7 recognises viral ssRNA (Heil *et al.* 2004). KSHV has been suggested to activate pDCs via TLR 7 and TLR 9, leading to interferon production (West *et al.* 2011). TLR 7 is also known to interact with the herpesviruses Varicella-Zoster virus (VZV) (Martin *et al.* 2007) and EBV RNA and DNA is known to stimulate pDCs to produce interferon, via interaction with TLRs 7 and 9 (Quan *et al.* 2010). Here we have shown a large decrease in TLR 7 mRNA in KSHV infected cells, compared to uninfected, despite no observable KSHV sensitivity to TLR 7 expression seen in flow cytometry experiments. Reduction of TLR 7 at an mRNA level presumably decreases the amount of protein and aids KSHV evasion of innate immune activation. The lack of sensitivity to infection of TLR 7 expressing cells may be due to KSHV reducing TLR 7 protein levels relatively quickly after infection, in order that a TLR 7–activated anti-viral response is not triggered by KSHV. The mechanism of TLR 7 reduction may has arisen as a mechanism by of KSHV to overcome TLR 7 activated signalling that was originally detrimental to the life cycle of KSHV. KSHV has co-evolved with the host to evade this particular mechanism of immune defence. It may also be due to the difference in receptor localisation in our model. *In vivo* TLR 7 is expressed endosomally, and thus KSHV would only come into contact with the receptor post-infection. During viral infection, viral ssRNA may reach the endosome via receptor-mediated uptake of viral particles or by fusion of virus and cell membranes. Exposure of viral nucleic acids, such as RNAs packaged within the mature virion, may occur within the endosome during primary infection (Bechtel *et al.* 2005). Such RNAs ensure quick transcription of viral genes after entry, but could endanger the virus by binding to TLR 7, leading to an immediate and strong induction of type 1 IFN (West and Damania, 2008). Therefore, it is sensible that the virus has evolved mechanisms of evading an anti-viral interferon response via downregulation of a receptor that would begin the signalling cascade. The speed of TLR 7 suppression following KSHV infection in these experiments suggests that a viral structural protein is involved in the downregulation mechanism. Both HSV-1 and -2 utilise a similar

mechanisms, where the VP16 tegument protein acts to transactivate genes favourable for viral immediate early gene transcription (Campbell *et al.* 1984). As herpesviruses, many of the mechanisms of immune evasion are comparable. KSHV protein RTA is present in infectious virions, and is known to target cellular proteins for downregulation (Bechtel *et al.* 2005b, Yu *et al.* 2005). It is possible that the transactivation properties of RTA allow downregulation of TLR 7 at a transcriptional level, thus reducing mRNA levels relatively soon after entry into the host cell.

Type one interferons are important in the control of viral infections, including KSHV. Counter-action of TLR-induced interferon responses are required for KSHV survival *in vivo*. Furthermore, interaction with TLRs allows KSHV to capitalise on the host innate immune response; without such a response to KSHV infection, the virus could prove fatal for the host. There is evidence that pDCs play a beneficial role in herpesvirus infection (Krug *et al.* 2004). Thus, activation of the host innate immune response against KSHV not only restrains viral replication and dissemination, but provides selective pressure on the virus to enter a latent state. Consequently, the virus is able to evade the adaptive immune responses that are activated by a successful innate immune response. Virus and host are in a constant, dynamic equilibrium between infection and immunity.

This model cell system emulates TLR expression and KSHV interaction with the receptors at the cell surface, as well as with TLR gene expression. TLRs 1, 2, 4, 5 and 6 are expressed on the cell surface in the *in vivo* situation (Table 1), as they are in our model system. However, TLRs are expressed *in vivo* on a variety of cell types, including monocytes, epithelial cells and dendritic cells (Table 1) and the HEK-293 cells do not normally express TLRs. *In vivo* expression localisation of TLRs is not necessarily represented in this experiment. For example, TLRs 3, 7, 8 and 9 are expressed endosomally (Table 1), but it is likely that they are cell-surface expressed in our model system. Thus localisation of TLRs in the model may not exactly mimic an *in vivo* situation, and

virus interactions may also therefore differ. It would be useful to confirm localisation of the receptors by immunofluorescence in future investigations using this model.

Selection-containing medium killed control HEK-293 cells, indicating that cell survival in selection media is due to the expression of the correct TLR gene transduced into cells alongside antibiotic resistance. RT-PCR also confirmed the expression of TLRs in the relevant cell lines, and that control cells did not express TLRs. On this basis, we can assume that changes in KSHV infection levels in the TLR-expressing cell lines seen by flow cytometry compared to control cell lines are due to receptor expression.

sqPCR identified novel TLRs whose expression appears to be affected by KSHV infection. This can now be further investigated using quantitative PCR methods, which would provide a more sensitive identification method over ethidium bromide, and allow precise quantification and comparison of differences in template levels between infected, uninfected and control samples.

4.1 Future Directions

We have investigated the effect of ectopic TLR expression on KSHV infection and formed a basis for which more detailed analyses in KSHV-tropic cells, such as HUVECs, can be done. Given that B cells express a majority of TLRs (Bourke *et al.* 2003) and KSHV latency occurs in B cells (Dupin *et al.* 1999), the experiments should be confirmed in these cells to closer resemble *in vivo* infection. The expression of these TLRs *in vivo* may change the effect that they have in KSHV infection. TLRs are expressed on a range of cell types, and often more than one on a single cell type (Table 1). A further direction could be taken in investigating the mechanism behind the sensitivity of KSHV infection to the ectopic expression of TLR 2, 3, 4 and 5 expressing cell lines. Given that expression of TLRs has been seen on KSHV tropic cells, as well as immune surveillance cells, such experiments would be analogous to clinical situations.

Receptors of innate immunity act in concert to enhance a response to viral infection (Sorensen *et al.* 2008). Therefore, it would be of interest to investigate the effect of the expression of TLRs in

combination on KSHV infection. For example, investigating combinations of TLRs that act as heterodimers with other TLRs and endogenous cellular receptors, such as TLR 2/RP105, TLR 2/CD36, TLR4/RP105 and TLR 4/CD14 heterodimers. Different combinations of receptors can affect ligand interactions (Kang *et al.* 2009), which may be relevant for viral recognition, manipulation and downregulation. Similarly, concerted expression of TLRs may have a greater, lesser or no effect on KSHV infection efficiency. TLR 2 and 9 are required together for detection of HSV-2 in mouse models (Sorensen *et al.* 2008). Based on our data, it would be of interest to investigate the effect of TLR 7 in concert with TLR3, which is similarly situated in the cell and detects ssRNA (Table 1). Nucleic acid detecting TLRs may overlap in ligand binding specificity, and thus the expression of more than one may counteract KHSV evasion techniques, such as mRNA degradation or transcriptional downregulation.

Degradation of mRNA is a recognized method of viral evasion from immune detection. HSV-1 induces ICP0 expression, a cellular protein which destabilises mRNA (Kummer *et al.* 2008). KSHV encodes a host-shutoff and exonuclease protein (Sokoloski *et al.* 2009), and HSV encodes a similar protein, both of which have exonuclease activity (Glausinger and Ganem, 2004, Korom *et al.* 2008). EBV BGLF-5 protein is known to promote mRNA degradation (Rowe *et al.* 2007), and is a homolog of KSHV host-shutoff and exonuclease protein. Given the similarities between the two proteins, it is possible that the TLR 7 mRNA decrease seen in our experiments is orchestrated by KSHV host-shutoff and exonuclease protein, and this is an interesting avenue for the exploration of KSHV interactions with TLR-induced pathways. However, mRNA levels can also be affected at a transcriptional level, as previously mentioned, and it would be of interest to investigate this also. For example, gene transcription may be inhibited by the viral RTA transactivator (Bechtel *et al.* 2005).

We have analysed the effect of ectopic TLR expression on KSHV infection, and the effect of KSHV infection upon TLR expression levels to find that TLRs that are affected by KSHV

infection do not necessarily effect KSHV infection. Notably, TLR 7 expression does not affect KSHV infection efficiency, but KSHV infection downregulates TLR 7 mRNA levels. This information provides a solid foundation for future work into the interactions of a prominent herpesvirus with human innate immune receptors, and may provide relevant information for therapeutic targetting.

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HUMAN PAPILLOMAVIRUS AND PDZ-DOMAIN CONTAINING PROTEINS

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Abstract

Human papillomavirus (HPV) is responsible for 5.2% of the world's cancer burden, and is associated with cervical, ano-genital, and head and neck cancers. Interaction of high-risk E6 proteins with cellular PDZ-domain containing proteins such DLG1 INADL, SCRIB and PTPN13, has been shown to contribute to invasive and metastatic properties of advanced carcinomas. Investigation into PDZ proteins not only presents an insight into the mechanism and consequences of host PDZ protein gene expression changes that contribute to the progression of cancer, but also highlights important cellular oncoproteins and tumour suppressors. Here, we investigate the impact of HPV16 and HPV18 infection, in a keratinocyte model of HPV replication, on the expression of DLG1, INADL, PTPN13 and SCRIB. Gene expression array and qPCR analysis showed increased expression of DLG1, INADL and PTPN13, but not SCRIB, in the HPV genome containing cells. However, the upregulation of gene expression was lost upon extended passage of these cells. Such HPV-induced changes in expression of DLG1, INADL and PTPN13 are suggested to be a cellular response to HPV replication, one that might be negated by the subsequent ubiquitin-mediated degradation of the protein, directed by E6.

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1. Introduction

1.1 Cancer and Epithelia

Cancer is a disease of cells, resulting from the accumulation of mutations leading to deregulation of cell proliferation and cell migration (Hanahan and Weinberg, 2000). Around 90% of human cancers are derived from epithelial cells (Tanos and Rodriguez-Boulan, 2008); the cells which form the monolayers or multi-layered tissues at interfaces between the organism and the outside world. Epithelial tissues are involved in complex functions necessary for an organism's homeostatic control, such as cellular trafficking, migration and interactions, for which epithelial polarity is essential (see Etienne-Manneville, 2008). The polarity of tissue, and thus the polarity of the individual cells, is determined and regulated by a number of interacting protein complexes forming scaffolds and trafficking systems (Figure 1). As such, alteration and deregulation of epithelial polarity molecules leads to abnormal cellular trafficking, signalling and migration, and contributes to the development of carcinomas and progression to an invasive phenotype, which are hallmarks of epithelial-derived cancers (Figure 2) (Tanos and Rodriguez-Boulan, 2008). Epithelial cancers are also characterized by epithelial-mesenchymal transition (EMT) (Huber *et al.* 2005) where cell phenotype is altered; cell to cell junctions become disrupted so that neighbouring cells begin to dissociate from each other, no longer making tight junctions (TJs) and forming the interface that characterises their function. With such an important role, control of cell polarity is a major issue in the treatment of cancer patients, and the targeting of such pathways therapeutically represents a wide area of potential research.

Maintenance of epithelial polarity and cell-cell junctions involves a large and diverse number of proteins, many of which contain multiple PDZ-domains. The PDZ (PSD/Dlg/ZO1) domain is an evolutionarily conserved domain of approximately 90 amino acids. It forms a hydrophobic pocket that interacts with specific sequences within or at the C terminus of target proteins (Songyang *et al.* 1997). The presence of multi-PDZ domains within a protein allows it to serve a structural role and act as a scaffold for the organisation of large protein complexes at a specific sub-cellular location. PDZ-domain containing proteins are generally involved in cell polarity, although they have associated roles in signalling and may yet have roles that are not elucidated (Giallourakis *et al.* 2006). Thus there is significant oncogenic potential if these proteins are deregulated at a transcriptional, translational or post-translational level.

1.2 Human papillomavirus and oncogenesis

Human papillomavirus (HPV) is necessary for the transformation of mucosal epithelium in the development of cervical and ano-gential cancer (Walboomers *et al.* 1999). There are 106 types of HPV which infect humans, divided into the non-oncogenic, 'low-risk' types, and the oncogenic, 'high-risk' types (Table 1). Oncogenesis is a rare side effect of infection by high-risk types of HPV; persistent HPV infection leads to increased proliferation of epithelia and decreased response to apoptotic signals, leading to the accumulation of mutations that favour cancerous growth. High-risk types include HPV16 and HPV18, which are responsible for 5.2% of the world's cancer burden (Parkin, 2006), and express the principal HPV oncoproteins E6 and E7. These oncoproteins act in synchrony to utilise host transcriptional and cellular proteins to induce the host-cell proliferation. The consequence E6 and E7 expression is genomic instability, disrupted apoptotic regulation and immortalisation required in transformation (see McLaughlin-Drubin and Munger 2009). The expression of E6 and E7 oncoproteins is retained in cancers (Doorbar, 2005).

HPV is a small, double-stranded DNA-containing virus that infects basal epithelia, and replicates in synchrony with the cells (Figure 3). As cells migrate upwards they would normally differentiate and thus exit the cell cycle, but HPV pushes the cell back into the cycle, mainly via inactivation of viral E7 protein with cellular pRb (Dyson *et al.* 1989, Du and Pogoriler, 2006). The viral genome is multiplied, so that there are hundreds of copies per cell, by hijacking cell machinery and creating an environment conducive to virion production (Doorbar, 2005). This over-proliferation activates the cellular p53 protein, whose actions are countered by viral E6 protein (Scheffner *et al.* 1990, McLaughlin-Drubin and Munger 2009).

1.3 PDZ Proteins

PDZ domain-containing polarity proteins are common cellular targets for inactivation by oncoproteins encoded by the tumour viruses HPV, human T-lymphotrophic virus (HTLV-1) and adenovirus type 9, which are influential in cervical cancer, adult T-cell leukaemia and mammary cancers in experimental animals, respectively. These oncogenic viruses promote tumour growth via interactions with cellular polarity proteins, resulting in deregulation of cell polarity and integrity; interactions with PDZ proteins contribute to virus-mediated tumourigenesis. (see Javier, 2008).

It is the E6 protein of high-risk HPV types that is known to interact with cellular PDZ-domain containing proteins. The E6 protein of high-risk, but not low-risk, HPV types contains a class I PDZ-binding motif (PBM) at its C-terminus (Figure 4). The PBM is required for E6-mediated transformation of cultured rodent cells (Kiyono *et al.* 1997) and immortalised keratinocytes (Watson *et al.* 2002). The absence of PBM in low-risk HPV implies that interactions between E6 and cellular PDZ-domains are significant in viral transformation of epithelia; expression of high-risk E6 protein correlates with metastatic skin cancer formation in transgenic animals (Song *et al.* 1999), and is known to cause EMT of human keratinocytes (Watson *et al.* 2003). The ability of E6 and E7 together to

increase tumour size in the cervix is dependant upon the binding ability of E6 to its PDZ-domain containing proteins (Nguyen *et al.* 2003). Thus, E6-mediated disruption of PDZ proteins, which regulate host-cell polarity and adhesion, may contribute to metastatic properties of HPV-driven tumours.

Viral disruption of cell polarity aims to benefit viral life cycle by aiding proliferation of basal cells, and interaction of E6 PBM with a subset of cellular PDZ proteins appears to be important for HPV life cycle and genome maintenance (see Pim and Banks, 2010), with cancerous effect in the host cells. High-risk HPV E6 binds to a specific domain within a target protein depending upon the viral PBM sequence, a non-canonical upstream amino acid sequence, and on the sequence of the PDZ domain sequence of the target protein (Thomas *et al.* 2008). Despite the presence of many PDZ domains in the PDZ-containing targets of HPV E6, only one PDZ domain is thought to be targeted (Thomas *et al.* 2001, 2002). The interactions appear to be highly specific. As well as this specificity, E6 proteins from different high-risk types of HPV appear to have different affinities and specificities for PDZ-domain containing target proteins, depending on the E6 and on the localisation and modification of the PDZ protein (Thomas *et al.* 2001, Massimi *et al.* 2006). For example HPV18 has a higher affinity for DLG1 and the MAGI-1 proteins than HPV16, which has higher affinity for SCRIB protein (Pim *et al.* 2009, Thomas *et al.* 2005, Kranjec and Banks, 2010). This difference in affinity can be switched by altering the PBM motif in the HPV16 and HPV18 E6 proteins (Thomas *et al.* 2001). It is yet to be seen whether the affinity for different PDZ proteins correlates with ability to cause invasive cancers.

E6 forms the E6-associated ubiquitin ligase complex. Via this ligase activity and PBM, it is thought that high-risk HPV E6 protein couples PDZ-domain containing proteins to ubiquitin, and targets the proteins for proteosomal degradation. HPV18 E6 appears to induce the degradation of certain pools of DLG1 in cell culture, potentially due to the

different localization and/or phosphorylation state of the protein (Massimi *et al.* 2004, 2006).

To date, fourteen PDZ domain-containing proteins that interact with high-risk HPV E6 is have been identified (Table 2). These proteins have varying roles in cell polarity, tissue integrity, scaffold formation, signalling and intracellular trafficking (Table 2). Although most of E6 PDZ-domain targets appear to be degraded via their interaction with E6, it is not clear what the consequences of interaction are for PTPN13 and TAX1BP3, and INADL is degraded in the absence of E6 PBM (Storrs and Silverstein, 2007).

DLG1, INADL, PTPN13 and SCRIB are all human PDZ-domain containing proteins that are involved in the maintenance of cell junctions and cell polarity and have been implicated in cancer progression separately from HPV influence.

1.3.1 DLG1

DLG1 was identified as a tumour suppressor protein in *Drosophilia*, where null mutations lead to loss of apical-basal polarity and to neoplastic growth (Woods *et al.* 1996, Woods and Bryant, 1991). The protein belongs to the membrane-associated guanylate kinase (MAGUK) family (Lue *et al.* 1994, Müller *et al.* 1995), which localise to cell–cell contact sites and are required for junction formation and the establishment of cell polarity (Humbert *et al.* 2003, Laprise *et al.* 2004). Consistent with this, DLG1 is made up of several interaction modules, including an SH3 domain (Figure 5), which are involved in protein-protein interactions (Gonzalez-Mariscal *et al.* 2000), allowing DLG1 to act as a molecular scaffold complex, organising target proteins into signalling complexes and localising them correctly (Kim and Sheng, 2004). As is part of the scribble complex with Lgl and SCRIB proteins, DLG1 is involved in tight junction formation and mitotic spindle formation, as well as cell polarity and attachment (Albertson and Doe, 2003). However, as DLG1 is expressed in epithelial tissue across the body as well as neurons, the protein is likely to have context-dependent functions (Cavatorta *et al.* 2008, Frese *et*

al. 2006). DLG1 was the first identified cellular PDZ protein target of high-risk E6 PBM (Kiyono *et al.* 1997, Lee *et al.* 1997). High-risk E6 interacts with the PDZ1 and 2 domains of DLG1 and recruits ubiquitin ligase, leading to the proteosomal degradation of DLG1 (Gardiol *et al.* 1999). However, there is a nuclear subset of phosphorylated DLG1 protein (Massimi *et al.* 2004 and 2006), and thus HPV E6-mediated degradation does not completely eliminate DLG1 in epithelia.

1.3.2 SCRIB

As well as acting in the scribble complex, SCRIB protein prevents apoptosis and has been designated a tumour suppressor (Liu *et al.* 2007). The protein has been shown to regulate cell migration in a context dependant fashion (Humbert *et al.* 2006). Loss of SCRIB function presents a similar phenotype to DLG1 mutations, reduced levels of both DLG1 and SCRIB in carcinomas is associated with increased dysplasia and invasive phenotype (Gardiol *et al.* 2006, Martin *et al.* 2004). Both proteins are lost at the later stages of many other epithelial cancers (Fuja *et al.* 2004; Navarro *et al.* 2005), indicating that the complex they form, as well as the other roles they perform within a cell, is a significant obstacle to metastatic cancer formation. HPV16 E6 levels are increased by SCRIB in a PDZ-dependent fashion (Nicolaides *et al.* 2011), suggesting that although the PDZ protein may play a role detrimental to viral promotion of proliferation, the protein may affect viral stability, and that E6 selectivity in targeting the PDZ protein is important to viral life persistence.

1.3.3 INADL

Like DLG1 and SCRIB, INADL, is associated with cellular tight junctions (TJ). The protein regulates the formation and localization of TJs, as well as regulating the directional migration of epithelial cells and organising signalling and effector molecules on a scaffold (Shin *et al.* 2007, Chen *et al.* 2009). Interactions of INADL protein with adenovirus 9 E4 ORF oncoprotein causes mislocalisation of the cellular TJ complexes

and correlates with viral oncogenic ability (Latorre *et al.* 2005). As viruses have evolved strategies targeting similar polarity proteins in oncogenesis, INADL is likely to be a relevant protein affected during HPV-driven oncogenesis.

1.3.4 PTPN13

PTPN13 is a fas-associated, non-receptor phosphatase that contains five PDZ domains, as well as a phosphatase domain and a cell membrane localisation domain (Figure 5). PTPN13 protein is increased in aggressive breast cancers (Glondu-Lassis *et al.* 2010), and certain genetic polymorphisms are known to increase the risk of squamous cell carcinomas in the head and neck (Niu *et al.* 2009), a cancer that HPV is increasingly becoming associated with (Shillitoe, 2009). PTPN13 dephosphorylates fas protein and interacts with src kinase, suggesting a role for PTPN13 in fas-mediated apoptotic signalling. Unlike the other proteins described here, this is the only one not yet associated with a cell-to-cell junctions. However, it has been linked to cancer progression in the absence of viral agents, and interacts with high-risk HPV E6 proteins, and is therefore of interest to HPV-associated carcinomas.

1.4 Hypothesis, Aims and Objectives

The HPV infectious cycle is closely aligned with the differentiation programme of keratinocytes. Investigation into the virus-host interactions involved in this alignment are supported by a cell-based model of HPV replication. This model is based upon primary keratinocytes stably transfected with episomal forms of high-risk HPV DNA. The viral DNA is stably replicated as extrachromosomal plasmids within the keratinocytes and these cells are representative of infected basal epithelial cells (Frattini *et al.* 1997). Affymetrix gene expression arrays, performed on primary foreskin keratinocytes stably transfected with HPV16 and HPV18 genomes, showed upregulation of mRNAs coding for a subset of the E6 PDZ-domain containing proteins. The most significantly

upregulated PDZ-domain containing proteins were DLG1, INADL and PTPN13, whilst SCRIB showed only moderate upregulation of expression (Table 3).

On the basis of this data, we hypothesize that replication of the oncogenic viral DNA in keratinocytes induces a host defence mechanism that includes the transcriptional upregulation of selective PDZ-domain containing proteins. Downstream inactivation of these targets by E6 mediated proteosomal degradation may be a key mechanism of the virus to allow virus replication.

The first aim of the study is to verify the results of the microarray analysis by quantitative PCR (qPCR) and the second to determine if this is an early or late response to viral replication in primary keratinocytes. The third aim of this study is to investigate the effect of HPV-PDZ interactions on cell morphology, by examining whether knockdown of DLG1 using shRNA technology will rescue any invasive phenotype.

2. Methods

Unless otherwise stated, materials and chemicals were obtained from commercial sources.

2.1 Cells and Cell Culture

Primary human foreskin keratinocytes (HFKs) isolated from neonate foreskins were cultured from frozen stocks on irradiated 3T3 J2 fibroblast feeder cells in complete E Media (CEM). CEM consists of Dulbecco's modified eagle media (DMEM, 60% v/v, Gibco), Ham's F12 (32% v/v, Gibco), PenStrep (2% v/v, Gibco), hydrocortisone (0.1% v/v, Sigma), foetal bovine serum (FBS, 10% v/v, Gibco), mouse epidermal growth factor (EGF, 0.5 % v/v, BD), L-glutamine (2% v/v, Gibco), cholera toxin A (0.1% v/v, ICN Biomedical), insulin (0.2% v/v, Sigma), transferrin (0.2% v/v, Sigma), tri-iodo–lL-thyronine T3 (4 nM, Sigma) and adenine (36 μM, Sigma).

Cell lines containing the HPV16K (Kanda *et al.* 1987, Roden *et al.* 1996) or HPV18 (Wilson et al. 2007) episomal genome were cultured alongside untransfected cells derived from the same donor. The HPV16 and HPV18 replicating cell lines from this particular donor were generated in Dr Roberts' laboratory by Drs Constandinou-Williams and Roberts. The untransfected cells serve as a control and were treated in parallel to the transfected cells. At each passage, aliquots of $5x10^5$ cells were pelleted by centrifugation, washed with phosphate buffered saline (PBS) and stored at -80°C for RNA extraction

3T3 J2 cells were cultured in DMEM supplemented with L-glutamine (2% v/v, Gibco) and foetal bovine serum (FBS, 10% v/v, Gibco). This will be referred to as J2 medium. Cells were cultured and harvested for irradiation as required. 3T3-J2 cells were irradiated with 40 grays and plated at a density of $2x10^6$ cells per 10 cm^3 dish overnight for preparation as feeder cells. HFK cells were seeded onto 3T3-J2 cells at a density of $2x10^5$ cells.

Lung carcinoma-derived H1299 cells were cultured in DMEM supplemented with foetal calf serum (FCS, 10% v/v) and L-glutamine, (2% v/v, Gibco) which will be referred to as H1299 medium.

HFK models of HPV life cycle are initially established by cotransfection of early passage (p3) primary human foreskin keratinocytes with recircularised HPV16 or HPV18 genomes and a plasmid encoding the gene for neomycin resistance (Wilson *et al.* 2007). After transfection the cells are seeded onto feeder layers of irradiated J2 3T3 fibroblasts and cells expressing the resistance marker are selected using the antibiotic G418 for 8 days during which time the cell are often passaged again at least once or twice (e.g. p3/p2, p3/p3); after this time the antibiotic is removed from the media. Clones that emerge from these antibiotic free cultures are eventually pooled once they reach the size of a 10 pence coin, and then expanded by further growth on J2 3T3 feeder layers. At this point, the actions of HPV E6 and E7 early proteins are the driving force for the extended growth of these cells. Microarray analysis was performed on RNA extracted from the cell lines at this stage of expansion (p3/p4). Establishment and maintenance of HPV episomes was confirmed by Southern blot analysis of total DNA (Wilson *et al.* 2007).

2.2 Transfection of shRNA and protein analysis

H1299 cells were harvested and seeded at a density of $2x10^5$ cells in a 6 well plate, for incubation overnight in the appropriate media before transfection of cells at 90% confluency. For transfection, cells were treated with Lipofectamine 2000 (Invitrogen) and 5 μg of DNA, premixed at room temperature for 20 min. Parallel to this, cells were transfected with green fluorescent protein (GFP) as a control. Cells, DNA and Lipofectamine were incubated for three hours and quenched with warmed H1299 medium (37°C). Initial observations under a fluorescence microscope before cells were harvested (at 12, 24, 48 and 72hr) showed successful transfection, seen as green fluorescence emission. At the appropriate timepoint (12, 24, 48 or 72 hours), cells were harvested for protein quantification and analysis by western blot. To harvest, cells were

washed with ice-cold saline and lysed using urea-tris-βmercaptoethanol (UTB) lysis buffer (9 M urea, 250 mM tris, 50 mM NaCl, 150 mM βmercaptoethanol) before sonication for 30 secs. Lysed cells were centrifuged (450 x *g,* 20 min) and the supernatant transferred for protein quantification. Protein concentration was quantified by Bradford assay (BioRad) and 20 or 30 μg of protein was resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Once SDS-PAGE was complete, protein was transferred to PVDF membrane (15 V, overnight). The presence of protein on the membrane was confirmed by Ponceau-S staining. Membranes were incubated in 2% milk in phosphate-buffered saline (PBS), before separate halves were probed with an anti-rabbit DLG1 polyclonal (NAG) antibody (Roberts *et al.* 2007) or mouse monoclonal antibody specific to GAPDH, for at least 2 hours. Membranes were then washed in PBS and probed with appropriate secondary antibodies conjugated to horseradish peroxidase (HRP). All antibodies were diluted in 2% milk-PBS. The HRP reaction was carried out using enhanced chemiluminescence (ECL) reagents (GE Healthcare) and visualized by exposure to X-ray film. Resulting images were scanned in and subject to densitometry using the ImageJ program (http://rsbweb.nih.gov/ij/), standardising each lane to the intensity of GAPDH to account for loading, and then all samples to GFP transfections to assess the extent of DLG1 knockdown.

Combinations of four shRNA plasmids were tested to identify the optimum combination for DLG1 knockdown (Table 4). Having identified the 5 most efficient knockdown combinations, using the standard incubation time of 24 hours, the combinations were subject to a time titrations, where transfected cells were lysed at 12, 24, 36 or 48 hours. These samples were then treated as above; subject to SDS-PAGE and western blotting. In all experiments, GFP transfected cells served as a control for successful transfection and for showing a normal level of DLG1 protein.

2.3 RNA isolation and cDNA synthesis

Approximately $5x10^6$ cells were isolated from each HFK culture; one transfected with HPV16, one with HPV18 and one untransfected. Messenger RNA (mRNA) was extracted using the Qiaquick RNA extraction kit (Qiagen), according to the manufacturer's instructions. A DNase treatment step was included. The RNA concentration of each sample was measured (ratio A260/280), on a NanoDrop ND-1000 Spectrophotometer (Labtech International). The same mRNA used for the gene expression arrays was used to examine the effect of HPV early after transfection. This mRNA was extracted from early passage HFKs (p3) for untransfected control, and from the established HPV16 and HPV18 transfected cells once they had been expanded in culture (p3/p4). Alongside this, later passage samples, taken at p3/p8 from the same donor cells grown at a different time, were analysed.

Complementary DNA (cDNA) synthesis was performed using 250 ng of DNase-treated RNA, random hexodeoxynucleotide primers and the Moloney murine leukemia virus (MLV) reverse transcriptase (MLV RT, Promega) in the presence of RNase inhibitor and nucleotides (10 mM) (Promega). The RNA, nucleotides and primers were heated to 65°C for 5 min and chilled on ice, in order to denature any proteins present in the mixture before addition of the polymerase and RNase inhibitor. The reaction (25 μl) was incubated at 37°C for 50 min followed by inactivation of the MLV RT by heating to 70°C for 15 min. Resulting cDNA was purified using a Qiaquick kit (Qiagen) according to the manufacturer's instructions and quantified using a NanoDrop ND-1000 Spectrophotometer (ratio A260/280).

2.4 Primer design, optimization and qPCR

Polymerase chain reaction (PCR) primers were designed using primer 3 software (www.primer3.com). Human genomic sequences were obtained from the UCSC genome browser (www.ucsc.com). Basic local alignment search tool (BLAST) searches (http://www.ncbi.nlm.nih.gov/BLAST/) were performed in all cases to ensure that primers were

not complementary to other regions of the genome and bound specifically to the target sequences. The specific primer sequences are listed in Table 5. Initially a temperature gradient was set up to find the optimum temperature for each primer pair. Each 25 μl cDNA synthesis reaction contained; 5 μl cDNA, 12.5 μl hotstart Taq mastermix (Thermo Scientific) and 10 pmol of forward and reverse primers. DNA fragments of expected length were visualized by 2% agarose gel electrophoresis and ethidium bromide staining.

qPCR reactions were set up on ice and performed in 96-well reaction plates (Applied Biosystems). Each 25 μl reaction consisted of 40 ng of cDNA, 12.5 μl of Sybr green mastermix (Applied Biosystems) and 10 pmol of forward and reverse primers. All samples were run in triplicate in the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Samples were subjected to a denaturation step at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec and an extension step at 72°C for 1 min.

Primers specific to a housekeeping gene were also run on each qPCR plate, to account for loading variability. In order to ensure no variability in housekeeping gene levels, three were examined, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β2-microglobulin (B2M) and hypoxanthine-guanine phosphoribosyltransferase-2 (HPRT2). Dissociation curves were performed to ensure specific amplification of the correct template.

3. Results

This study has used a primary HFK based model of the HPV life cycle to examine the effect of HPV16 and HPV18 genome expression on the cellular mRNA level of E6 targeted PDZ-domain containing proteins. This cell-based model represents a powerful system in which to study the function of E6-PDZ domain protein interactions. With this in mind, the study investigates the mechanisms of silencing specific PDZ domain proteins.

3.1 Genome wide expression analysis.

Affymetrix gene expression arrays performed in the school of Cancer Sciences by Professor Ciaran Woodman, Dr Sally Roberts and Dr Sarah Leonard and colleagues were used to identify PDZ-domain containing proteins which are deregulated following transfection of primary HFKs with episomal forms of HPV16 and HPV18 genomes. These arrays identified the three PDZ-domain containing proteins DLG1, INADL and PTPN13 that were significantly upregulated at the mRNA level in both HPV16 and HPV18 transfected HFKs when compared to untransfected HFKs, (Table 3). Another PDZ-domain containing protein, GOPC was upregulated on the microarray, but was considered absent on the array and therefore not included in the validation. Other PDZ-domain containing proteins had either little or no significant changes in expression (Table 3). DLG1, INADL and PTPN13 were therefore chosen for further validation using qPCR. As a control, SCRIB was included in the qPCR analysis, because array data showed it was not significantly deregulated following transfection with either HPV16 (1.57 fold) or HPV18 (2.01 fold).

3.2 Optimisation of DLG1, INADL, PTPN13 and SCRIB primers

Primers designed to specifically amplify SCRIB, DLG1, INADL and PTPN13 were optimised on cDNA from untransfected HFKs, in order to find the most efficient annealing temperature for each primer pair. SCRIB, DLG1, INADL and PTPN13 primers annealed at a range of

temperatures, however each target sequence amplified at 60°C (Figure 6). Therefore, all primers could be utilised for qPCR in subsequent experiments, applying an annealing temperature of 60°C to all samples.

3.3 Housekeeping gene analysis

Initially, GAPDH served as a housekeeping gene in qPCR reactions to account for experimental variability. However, its expression was consistently variable between cDNA samples (Figure 7, panel A); there are about three cycles between transfected samples and the untransfected control. The significance of the variability was not statistically tested. However, it was obvious by comparison that B2M and HPRT2 were less variable than GAPDH between cDNA samples; they differ only by half a cycle at most (Figure 7, panels B and C). The difference in expression between each of the samples for B2M and HPRT2 is slight, but because the HPRT2 expression is the least variable, it is this control that is used in further qPCR analyses.

3.4 Establishment of HPV16 and HPV18 genomes in primary cells is associated with an upregulation of DLG1, INADL and PTPN13 gene expression

qPCR analysis was used to validate the changes in gene expression of the PDZ-domain containing proteins observed in the microarray study; the same donor primary keratinocytes were used in the qPCR analysis. qPCR analysis showed upregulation of DLG1, INADL and PTPN13 in low passage HFKs replicating episomal HPV16 or HPV18 genomes (Figure 8, panel A). As a general trend, transfection with HPV16 caused a higher-fold upregulation of DLG1, INADL and PTPN13 than transfection with HPV18, although PTPN13 shows the least difference between HPV16 and HPV18-induced increases in gene expression (Figure 8 panel A). DLG1 showed the highest relative upregulation; expression increased 23 and 7-fold following transfection of HPV16 and HPV18, respectively (Figure 8, panel A). Expression of INADL increases 14-fold upon HPV16 genome transfection, and 5-fold upon HPV18 genome transfection (Figure 8,

panel A). However, expression of SCRIB gene shows little, if any, change in level upon transfection of either HPV16 or HPV18 as expected (Figure 8, panel A).

In contrast to samples extracted from the HPV DNA containing cell lines at early passage, cDNA from the higher passage cell lines showed little change in DLG1, INADL, PTPN13 or SCRIB expression in HPV16 and HPV18 genome transfected cells, compared to the untransfected control (Figure 8 panel B). Unlike the low passage samples, there is no general trend between cells containing HPV16 or HPV18. DLG1 and PTPN13 expression shows no change upon HPV genome expression and, if anything, HPV appears to downregulate SCRIB and INADL to a small extent (Figure 8 panel B), however the statistical significance of the changes in gene expression was not tested.

3.5 Silencing RNA optimisation

Transfection of combinations of four plasmids encoding shRNA to DLG1 into H1299 cells showed that, after 24 h transient expression, a combination of all four and a combination of plasmids 1 and 3 knocked down DLG1 expression most effectively, lowering levels of the protein to approximately 30% of the control, GFP-transfected cells (Figure 9). Plasmid 4 also achieved a significant decrease in DLG1 levels, lowering protein levels to 45% of control (Figure 9). Knockdown is visible by eye and confirmed by densitometry of western blot analysis (Figure 9).

On testing to find the optimum length of shRNA incubation time that lead to the lowest level of DLG1 protein, it was found that generally 12 hours of transient expression of the shRNA provided the best decrease of DLG1 levels in all combinations tested (Figure 10). By 72 hours, it appears that the transient effects of shRNA have markedly reduced, and levels of DLG1 return to around the level of control in most cases (Figure 10). Use of plasmids 1 and 4 individually led to a large decrease in DLG1 protein, compared to normal levels, most notably following a 12 hour incubation where protein levels are taken down to approximately 55% and 16% of control

DLG1 levels, respectively (Figure 10). Of the combinations, transfecting all four shRNAs seems to be the most efficient for DLG1 knockdown. A combination of all four plasmids led to lower DLG1 levels at all timepoints, and thus seems to be the most consistent (Figure 10). A 12 hour incubation is the most efficient length of time, leading to a reduction of DLG1 protein levels to 35% of control (Figure 10). Unlike the previous transfection, a combination of plasmids 1 and 3 does not appear to be very effective in this time optimisation (Figure 9 and 10).

4. Discussion

HPV16 and HPV18 episomal genome expression in early passage neonate HFKs upregulates transcription of PDZ-domain containing proteins DLG1, INADL, PTPN13, but has no affect on the transcription of SCRIB. The qPCR results verify results from the gene expression microarray analysis, showing a larger increase in expression of DLG1, INADL and PTPN13 when cells were transfected with HPV16, compared to HPV18. These findings are novel and have shown for the first time an effect of HPV replication on the gene expression of a subset of PDZ-domain containing proteins.

The increase in DLG1, INADL and PTPN13 gene expression may be a cellular response to HPV infection; one that requires the virus to negate the consequence of the upregulation of these genes through the action of E6 targeting DLG1, INADL and PTPN13 proteins for proteosomal degradation. Such a response is similar to the activation of p53 upon viral replication mediated by the actions of E7 on the pRb pathways, which are subsequently countered by E6-induced degradation of the p53 protein. Perhaps, however, DLG1, INADL and PTPN13 proteins are necessary for the establishment of HPV genomes in primary keratinocytes, and so the increase in expression of these genes is a viral response to counteract detrimental affects of E6. If increased levels of DLG1, INADL and PTPN13 proteins are required for viral life cycle, then degradation of PDZ-domain containing proteins by E6 may be inhibited in cells; it has already been shown that E6 mediated degradation of PDZ substrates is negatively regulated by protein kinase A phosphorylation of the E6 PBM (Kuhne *et al.* 2000). This is an example of a posttranslational modification that would allow differential degradation of the PDZ substrates during the HPV life cycle; degradation of DLG1, INADL and PTPN13 may not be favourable at the early stages of the virus life cycle, and hence expression of these genes is increased.

Although several cellular PDZ-proteins have been identified as interacting with high-risk E6 proteins (Table 2), it is yet to be seen whether these proteins are genuine targets of E6-mediated proteosomal degradation. Indeed, both DLG1 and SCRIB are lost in the later stages of non-HPV related carcinomas (Fuja *et al.* 2004; Navarro *et al.* 2005), and the loss of these proteins may be a consequence of cancer development in these tissues, rather than as a result of HPV infection. However, phenotypic similarities between HPV-associated cancers and loss of polarity proteins strongly suggest a link between HPV interaction with PDZ-proteins and subsequent oncogenesis. For example, silencing RNA (siRNA) depletion of PTPN13 in tonsillar epithelia presents the same loss of polarity phenotype as expression of E6 within these cells, whilst overexpression of PTPN13 reverses the effects (Javier and Rice, 2011). The phenotypic similarity between HPV infected and PTPN13 depleted cells indicates an important role for PTPN13 protein in epithelial polarity, a characteristic abnormality in HPV-induced epithelial tumours. The HPV16 and HPV18 PBMs are the C-terminal motifs of ETQL and ETQV respectively, which are required for the virus' promotion of EMT, immortalisation of human tonsil keratinocytes, induction of anchorage-independent growth and inhibition apoptosis in epithelia (Watson *et al.* 2003, James *et al.* 2006, Spanos *et al.* 2008), all of which are phenotypic consequences attributable to the depletion of PDZ proteins. Because the E6 PBM is the only protein-protein interaction unique to high-risk HPV types, and the protein is known to target proteins for ubiquitin-mediated degradation (Thomas *et al.* 2008), it is likely that decrease of cellular PDZ proteins is due to HPV E6 PBM-mediated proteosomal degradation. Furthermore the E6 PBM is crucial to tumour size and frequency, mediated by E7 and E6 cooperation in the cervix (Shai *et al.* 2007), which implies a cooperative role for the E7 oncoprotein in PDZ deregulation. High-risk HPV E6 downregulation of PDZ proteins may allow growth of cells regardless of anti-growth signals that are likely to be triggered from neighbouring cells in response to over-proliferation, allowing the virus to proliferate with the cell. But, if this deregulation is detrimental to the life of the cell it is

also detrimental to the life of the virus. For this reason, HPV may utilise mechanisms to limit such damage to the host, including the upregulation of DLG1, INADL and PTPN13 gene expression, in order to prevent cellular protein levels from dropping below a critical level. Evidence suggests that E6 targets specific pools of PDZ proteins (Massimi *et al.* 2004). It is possible that E6-resistant PDZ protein pools are required to function for the benefit the HPV life cycle, and that increased gene expression enables the protein level of these pools to be increased, or at least maintain the required level for the viral persistence. This may be a reason behind the high level of increased DLG1 gene expression, compared to the increase in levels of INADL and PTPN13 gene expression. DLG1 is known to exist in nuclear and cytoplasmic-localised pools, and it may be that a specific pool is required for viral propagation. Thus, it is more important to the viral life cycle to retain the expression, and a high level of gene expression increase is required to compensate for protein degradation in these pools.

It has been suggested that stabilisation of HPV genome replication is mediated by E6 interactions with PDZ proteins (Lee and Laimins, 2004; Nicolaides *et al.* 2011); such stabilisation may be a reason behind the viral requirement to maintain certain PDZ-protein presence in the cell.

The increase in DLG1 gene expression upon HPV genome transfection is the highest of the PDZ-proteins investigated, and the reason for this is unclear. As part of the scribble complex, it may be that DLG1 upregulation is a cellular response to compensate for SCRIB protein downregulation also, seeing as SCRIB itself is not upregulated in response to depletion.

The dependency of increased gene expression and/or decreased PDZ protein levels upon HPV E6 and the influence of other viral proteins can be investigated by expression of viral proteins singly, and in combination, with consequent gene and protein level analysis, using the HFK model. Analysis of individual viral oncoprotein expression would isolate the roles each factor played in the changes we see in PDZ-domain containing protein gene expression levels. Analysis

of protein levels would further elucidate the relationship between changes in gene expression and protein levels.

In both expression analysis and qPCR data, HPV16 increases gene expression levels of DLG1, INADL and PTPN13 more than HPV18. In the case of DLG1, this difference is largest (Figure 9, panel A), this may be linked to the ability of the virus to mediate protein degradation. Including different high-risk E6 types in HFKS and subjecting them to the expression analysis seen in this study would indicate whether E6 affinity for PDZ domain proteins affects the transformation ability of high-risk viruses, thus highlighting how dependant the carcinogenic activity of HPV16 and HPV18, as well as the life cycle of the virus, is upon the ability to bind a PDZ-protein preferentially over another.

In contrast to early passage samples, expression of DLG1, INADL, PTPN13 and SCRIB in late passage (p3/p8) samples did not show change in expression levels upon HPV genome transfection (Figure 9 panel B). Further analysis of DLG1, INADL, PTPN13 and SCRIB at passages in between p3/p4 and p3/p8, as well as later passages if possible, would reveal the kinetics of their regulation. This variability could be a reflection of cell culture conditions at time of harvest; as DLG1, SCRIB and INADL are involved in TJ and adherens complexes that are sensitive to cell-cell interactions, their levels may be affected by confluency of cells in culture. Alternatively, the variability could reflect the progression of HPV infection through to immortalisation. At early passages, the keratinocytes resemble 'normal' infected basal cells, where E6 and E7 are driving cell proliferation and extend the life span of infected cells. Upon extended cell culture, the virus promotes immortalization of the cells and it can be said that this reflects a persistent HPV infection within the host. Keratinocyte immortalization may be driven by downregulation of PDZ proteins both at the transcriptional and translational levels, thus explaining the return of DLG1, INADL and PTPN13 gene expression to a normal level at later passages.

To study the effect of individual PDZ proteins, their depletion can be achieved by silencing gene expression and then compared to the phenotypic effects of HPV infection. To achieve this, an optimum set of shRNA combinations and conditions for silencing of DLG1 has been found, that will allow protein analysis of effects of the PDZ protein knockout, as has been demonstrated for PTPN13 (Javier and Rice, 2011). Now that the conditions for DLG1 silencing have been established, theses shRNAs can be used to investigate the effects of silencing DLG1 in human keratinocytes, and the resulting phenotype compared with E6 expressing cells. This principle of shRNA knockdown can be applied to the PDZ proteins INADL and PTPN13 in a keratinocyte system for similar investigations.

4.1 Future Directions

To investigate the hypothesis that HPV proteins work synergistically to decrease protein levels and/or increase gene expression of certain PDZ proteins, a comparison of the levels of DLG1 INADL, PTPN13 and SCRIB proteins in HFKs transfected with HPV16 and HPV18 genomes with levels of gene expression could be carried out. If protein kinase A phosphorylates E6 to negatively regulate PBM interaction with PDZ-proteins, then no change in protein or transcript levels will be seen, so analysis of the phosphorylation state of E6 concurrent with analysis of PDZ-domain containing proteins could indicate how phosphorylation corresponds to the expression and translational levels of cellular PDZ proteins. Phosphorylation is a transient state, and is likely to vary at different stages of the cell cycle, and thus studying samples at each passage would be beneficial.

As the viral mechanism of gene upregulation is unknown, an analysis of the expression of each viral protein on PDZ-domain protein gene expression would highlight whether E7, for example, is involved. It would then also be of interest to investigate the effect of co-expressing E7 and E6 on the regulation of the PDZ protein transcription.

The findings of this study suggest that downregulation of the gene expression of these PDZ-proteins might be involved in HPV induced immortalisation of keratinocytes. A further marker of cervical disease is integration of the viral genome into the host cell genome. The HPV genome is known to integrate into the genome of the keratinocyte cell lines used following extended passage of cells. Therefore, this cell model can be used to analyse the changes in expression of these cellular PDZ proteins before and after viral DNA integration. DNA analysis of cells would show whether integration of the HPV genome has an influence upon PDZ protein gene expression. Furthermore, use of different primary keratinocyte donors would both extend and strengthen the investigation.

Moreover, PDZ protein expression profiles from clinical samples of cervical carcinomas at different stages would add layers of complexity over i*n vitro* samples. Such studies would allow translation of our *in vitro* results to a disease phenotype, as well as highlighting other factors that may interact where the whole system is present, for example *in vivo* immune cells and growth factors. The HFK model of differentiation is a reliable model of HPV life cycle. Comparisons have been drawn between HPV-mediated oncogenesis of cervical and oropharyngeal mucosa (Shillitoe, 2009). Thus, it would be interesting to look at gene expression of PDZ proteins in head and neck cancer cell lines.

This study shows the changes in DLG1, INADL, PTPN13 and SCRIB gene expression in a HFK model upon transfection with high-risk types of HPV. DLG1, INADL and PTPN13 gene expression is increased in cells replicating episomal HPV genomes, more so by HPV16 than by HPV18. The results are novel and create possibilities for further analysis. Study of the mechanism and consequences gene expression changes will perhaps provide insight into viral mechanisms of persistence and of oncogenesis. Research directed at understanding such virally-induced oncogenesis and viral interactions with cell machinery highlights important cellular

oncoproteins and tumour suppressors, aids our knowledge of molecular events associated with human cancer and ultimately informs potential clinical and therapeutic outcomes.

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, P., Banks, L. and Roberts, S. (2002). Changes in expression of the human homologue of the *Drosophila* tumour suppressor protein in high-grade premalignant cervical neoplasias. *Carcinogensis*. **23**, 1791-1796

Watson, R., Thomas, M., Banks, L. and Roberts, S. (2003). Activity of the human papillomavirus E6 PDZ-binding motif correlates with an enhanced morphological transformation of immortalised keratinocytes. *Journal of Cell Science*. **116**, 4925-4934

Wilson, R., Ryan, G., Knight, G., Laimins, L. and Roberts, S. (2007). The full-length E1^E4 protein of human papillomavirus type 18 modulates differentiation-dependent viral DNA amplification and late gene expression. *Virology*. **362**, 453-460

Woods, D., Hough, C., Peel, D., Callaini, G and Bryant, P. (1996). Dig protein is required for junction structure, cell polarity, and proliferation control in *Drosophila* epithelia. *The Journal of Cell Biology*. **134**, 1469-1482

Woods, D. and Bryant, P. (2007). The Discs-large tumor suppressor gene of *Drosophilia* encodes a guanylate kinase homolog localized at septate junctions. *Cell*. **66**, 451-464

Tables and Figures

Table 3: Human Toll-like Receptors

TLR	Dimer	Cellular Position	Cell type	Ligand(s)	Herpesviruses known to have an affect/be affected	References
TLR 1	Heterodimer with TLR2	Cell surface	Monocyte/macrophages, neutrophil	Triacylated lipoproteins Peptidoglycan	HCMV (envelope proteins)	Boehme *et al.* 2006
TLR 2	Heterodimer with CD14, CD36, RP105	Cell surface	Mast cell monocyte/macrophage DC	Hydrophobic PAMPs e.g. lipopeptides Diacylated lipoproteins	HCMV (envelope proteins) EBV HSV-1	Boehme *et al.* 2006 Juckem *et al.* 2008 Gaudreault *et al.* 2007 Compton *et al.* 2003
TLR 3	Homodimer	Endosomal	Conventional DC, B cell	dsRNA ssRNA	HSV EBV	Alexopoulou *et al.* 2001 Zhou *et al.* 2009 Iwakiri *et al.* 2009
TLR 4	Heterodimers with MD2, CD14, LBP, RP105	Cell surface	Monocytes/macrophage DC, mast cell, intestinal epithelial cell	Lipopolysaccharide Viral glycoproteins	KSHV	Burzyn *et al.* 2004 Lagos *et al.* 2008
TLR 5	Homodimer	Cell surface	Monocyte/macrophage a subset of DC, intestinal epithelial cell	Bacterial flagellin	-	Hayashi *et al.* 2001
TLR 2/6	TLR2/6 heterodimer	Cell surface	Monocyte/macrophage mast cell, B cell	Bacterial diacylated lipoproteins	-	Takeuchi *et al.* 1999 Kang *et al.* 2009
TLR 7	Homodimer	Endosomal	Monocyte/macrophage, pDC, B cell	ssRNA	VZV KSHV	Heil *et al.* 2004 Mancuso *et al.* 2009
TLR 8	Homodimer	Endosomal	Monocyte/macrophage mast cell, pDC	Viral ssRNA	KSHV	Gregory *et al.* 2009
TLR 9	Homodimer	Endosomal	Monocyte/macrophage B cell, pDC	Unmethylated DNA with CpG motif	HSV-1 MCMV MHV-67	Krug *et al.* 2004

TLR 10 did not form part of our analysis and therefore is not featured here.
HSV - herpes simplex virus, HCMV- human cytomegalovirus, EBV- Epstein-Barr virus, KSHV - Kaposi's sarcoma-associated herpesvirus, MHV-68 - murine gammaherpesvirus 68, VZV - Varicella-Zoster virus. pDC - plasmacytoid dendritic cell

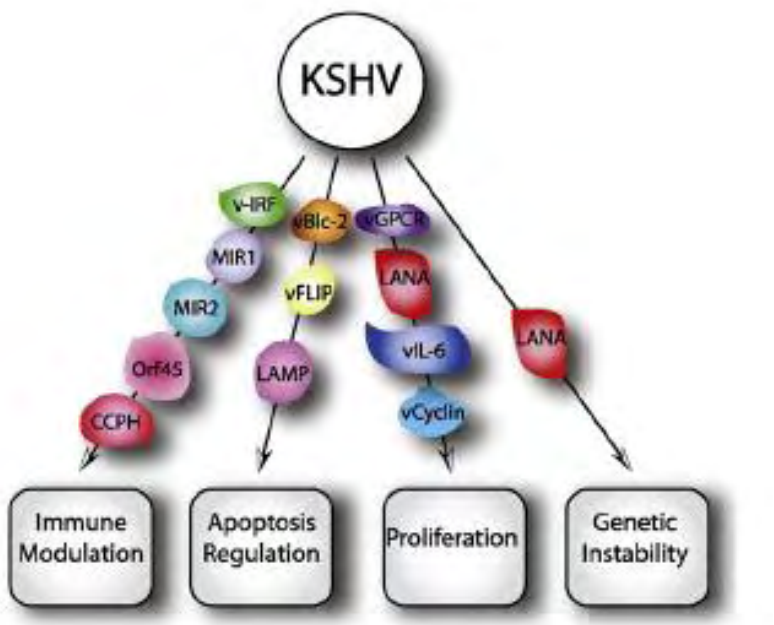


Figure 1: Virally-encoded Factors contributing to Host Cell Transformation
Depiction from McLaughlin-Munger *et al.* 2008 showing the factors involved in the major biological activities that contribute to KSHV-mediated transformation.

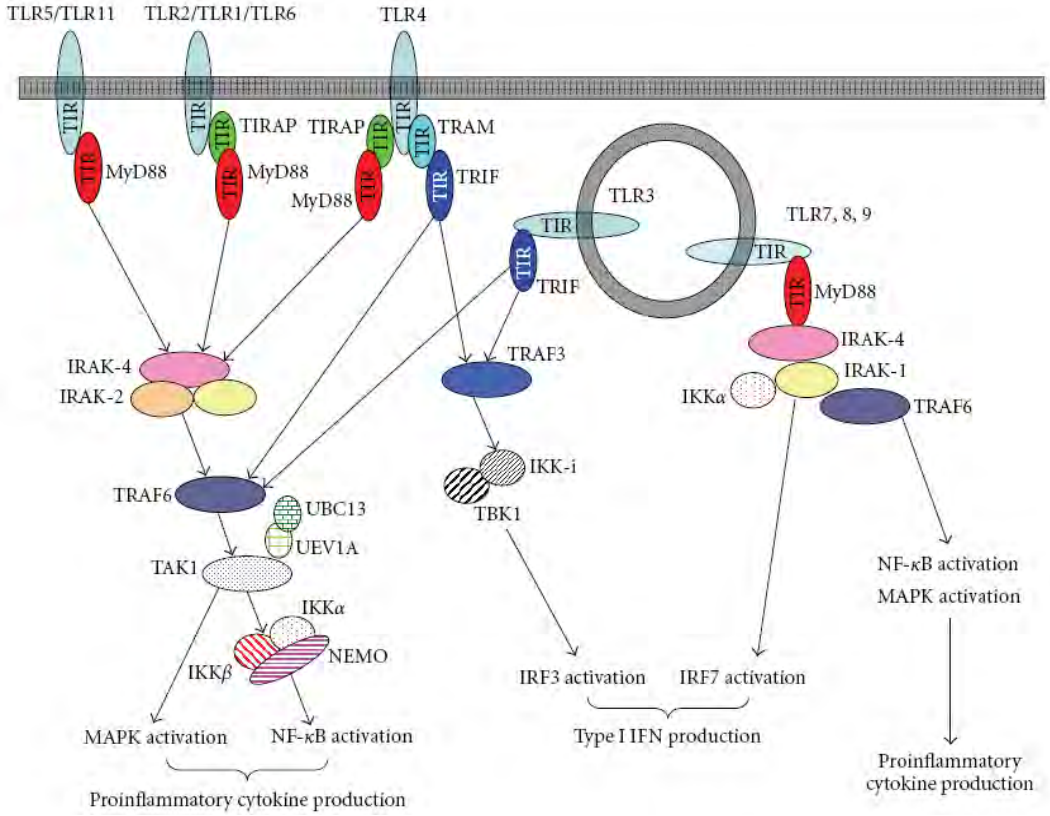


Figure 2: TLR Signalling Pathways
Cytoplasmic components of the TLR-activated signalling cascade overlap to initiate the transcription of genes required to recruit factors that drive the innate immune response. (from Carty and Bowie 2010). Upon stimulation by ligand, activated TLRs, except for TLR3, recruit MyD88. This adaptor protein binds the IRAK-4/IRAK-2/TRAF6 complex which self-ubiquitinyates and activates a signalling cascade that results in the phosphorylation of TAK1. TAK1 activates the IKK complex, leading to the degradation of IκBa, allowing NFκB to translocate into the nucleus and transcribe genes, including those coding for pro-inflammatory cytokines. At the same time, TAK1 is able to activate the MAPK cascade, leading to activation of the transcription factor AP-1, which also induces pro-inflammatory cytokine expression.
TLR 3 bypasses MyD88 and recruits TRIF, an adaptor that interacts with the protein complex composed of TRADD, FADD and RIP-1. This complex triggers the activation of NFκB and AP-1. Activated TRIF binds TRAF3, leading to the activation of TBK1 and IKK-I, which phosphorylate IRF3, allowing its homodimerisation nuclear translocation. Along with NFκB and AP-1, IRF3 cooperates to induce type I IFN expression.

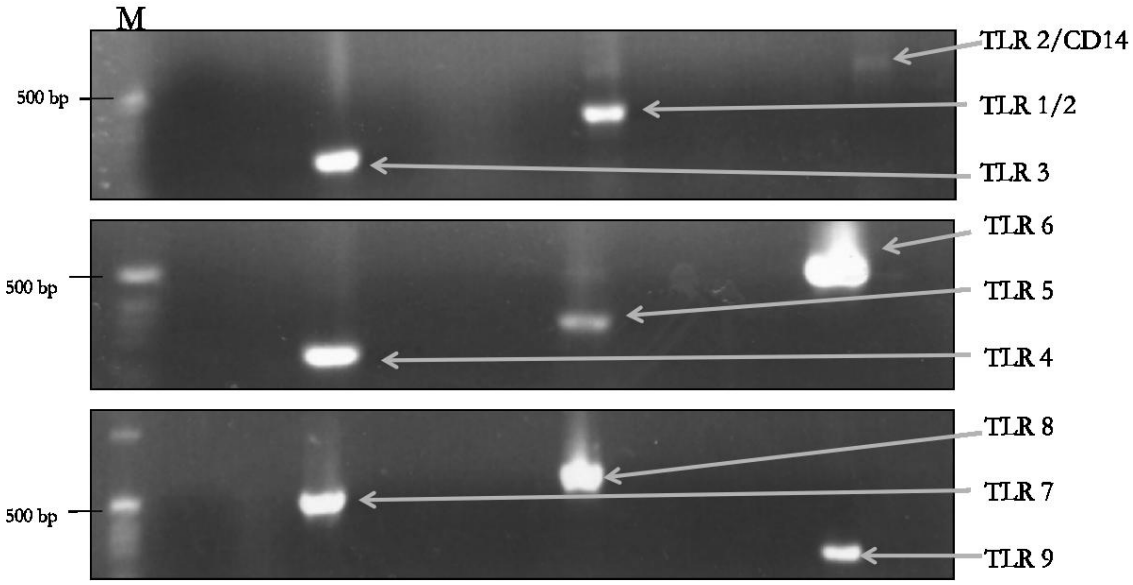


Figure 3: Expression of TLRs in TLR-transfected HEK-293 Cells
RT PCR of mRNA extracted from uninfected HEK-293 cells expressing individual TLRs. Bands visualized on an agarose gel by ethidium bromide, to confirm the expression of TLRs in the correct cell lines, and the absence of TLR expression in control cell lines. Samples without the presence of reverse transcriptase enzyme show no bands, confirming that there is no DNA contamination in samples, and thus amplified fragments are derived from mRNA of expressed TLRs.

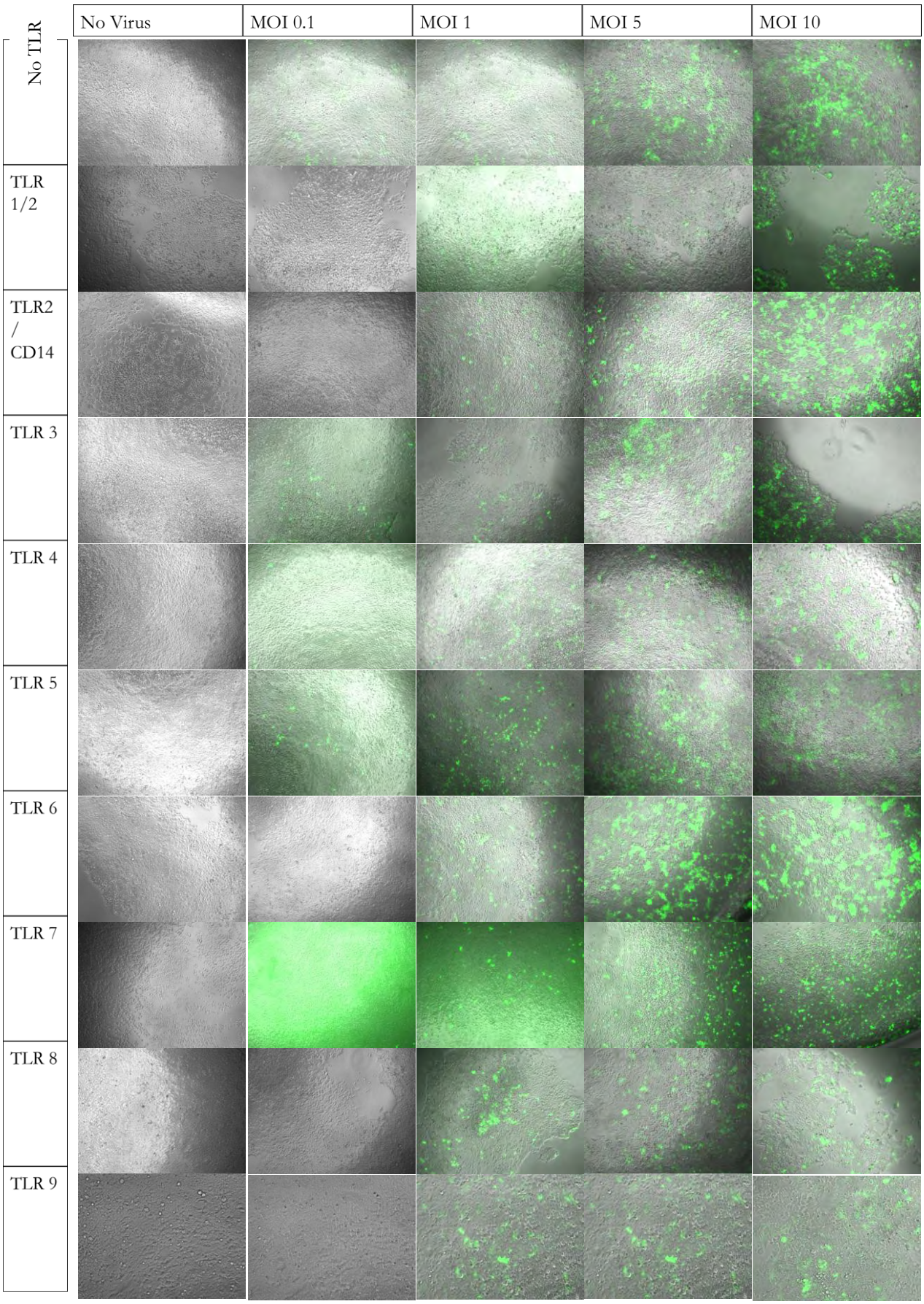
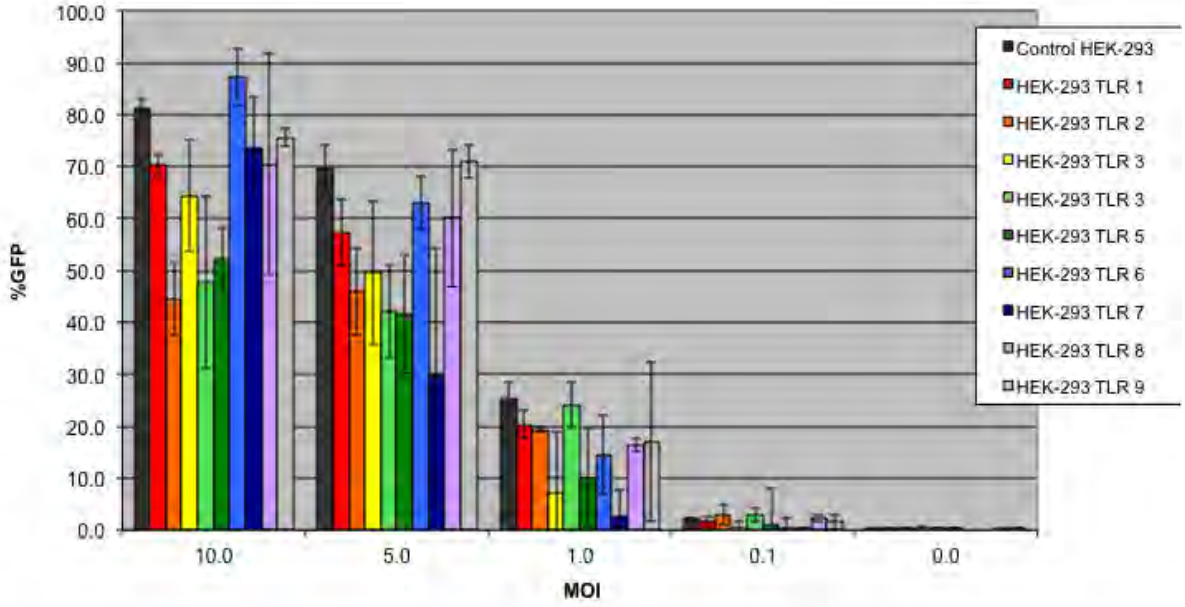
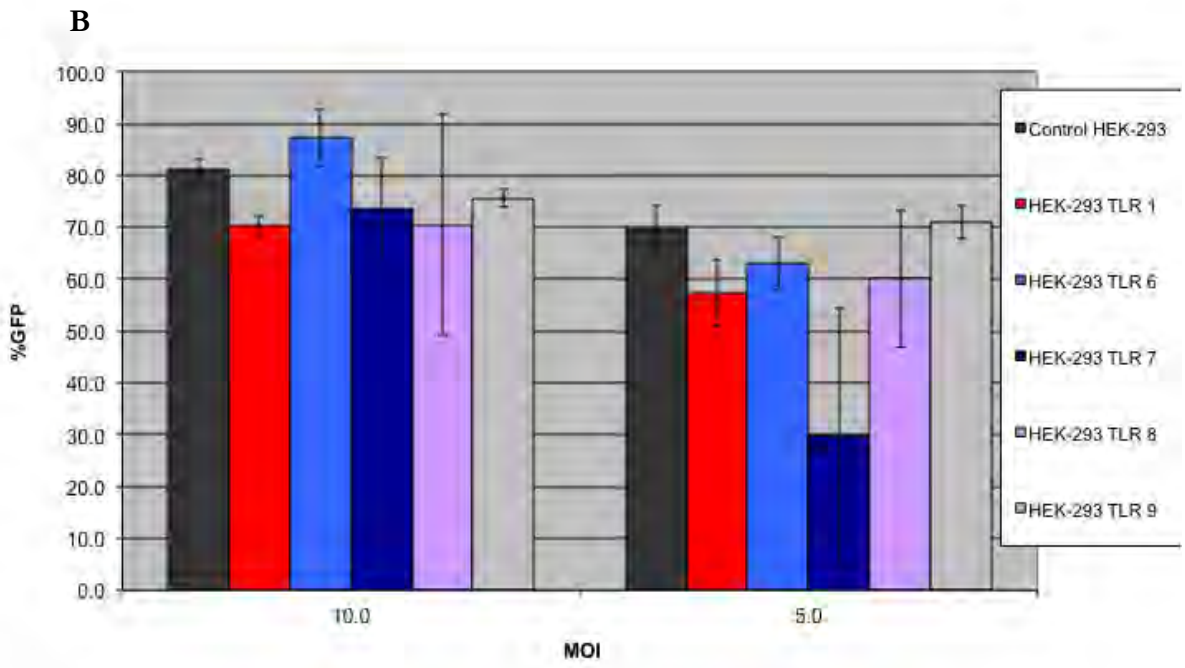

No Virus
MOI 0.1
MOI 1
MOI 5
MOI 10
No TLR
TLR 1/2
TLR2 / CD14
TLR 3
TLR 4
TLR 5
TLR 6
TLR 7
TLR 8
TLR 9

Figure 4: Fluorescence microscopy of infected cells at a range of MOIs
Cells were plated at $2x10^5$ cells, and infected the following day with rKSHV.219 with increasing MOIs. 48 hours post-infection, cells were visualised by fluorescence microscope and images captured using ImagePro.

A


100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
%GFP
10.0
5.0
1.0
0.1
0.0
MOI
Control HEK-293
HEK-293 TLR 1
HEK-293 TLR 2
HEK-293 TLR 3
HEK-293 TLR 3
HEK-293 TLR 5
HEK-293 TLR 6
HEK-293 TLR 7
HEK-293 TLR 8
HEK-293 TLR 9

B


100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
%GFP
10.0
5.0
MOI
Control HEK-293
HEK-293 TLR 1
HEK-293 TLR 6
HEK-293 TLR 7
HEK-293 TLR 8
HEK-293 TLR 9

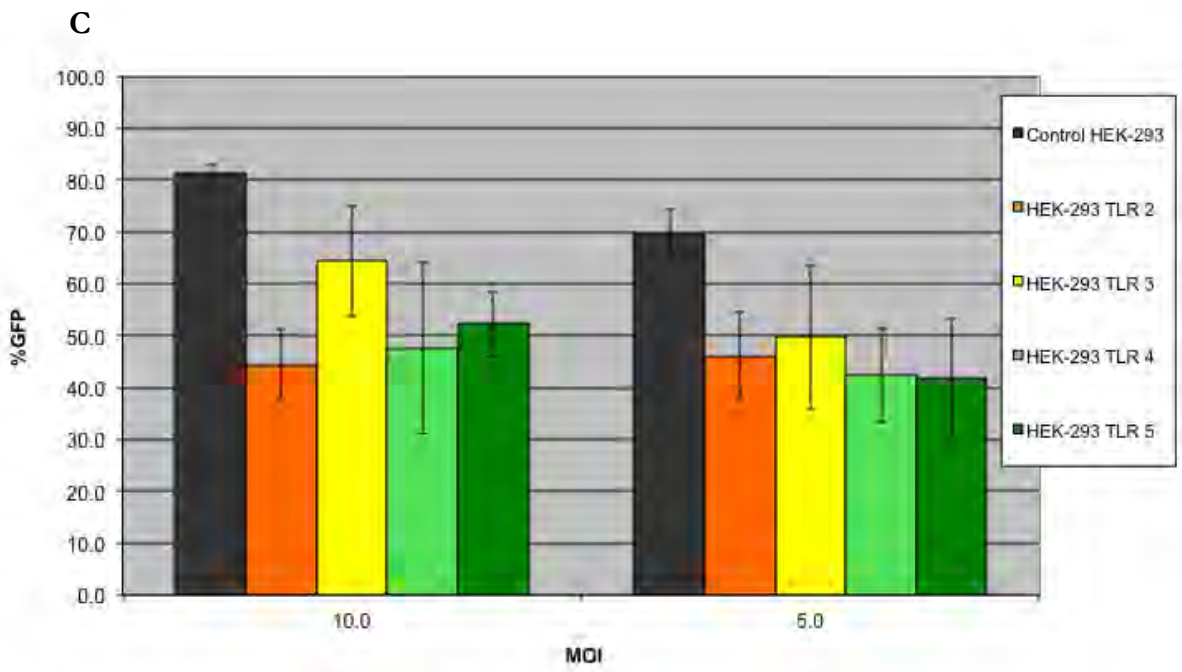


Figure 5: Quantification of KSHV infection of TLR-expressing cell lines at different MOIs
Infection efficiency is measured as the percentage of live cells expressing GFP. (A) The infection efficiency of all TLR-transfected cell lines compared to control HEK-293 cells, at five MOI values. At MOIs of less than 5, such small amounts of virus are used that infection efficiency values are varied between experiments, and data is not consistent.
(B) The infection efficiency of cell lines transfected with TLRs 1, 6, 7, 8 and 9 compared to control HEK-293 cells, at two MOI values. These are the cell lines that showed no difference in sensitivity to infection when compared to control cells.
(C) The infection efficiency of cell lines transfected with TLRs 2, 3, 4 and 5 compared to control HEK-293 cells, at two MOI values. These are the cell lines that showed less sensitivity to infection compared to control cells.
Data shown is the mean of at least three different experiments, error bars show standard deviation.

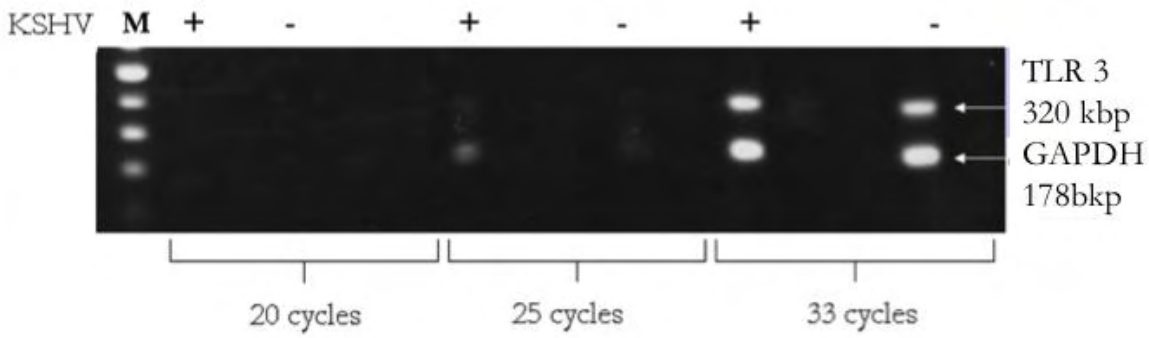


Figure 6: Comparative sq RT-PCR of TLR 3 in cell lines infected and uninfected with KSHV.
Infected and uninfected cells expressing ectopic TLR 3 were subject to mRNA extraction and cDNA synthesis. This was then subject to PCR. 10ul aliquots were taken from the PCR reaction paused at 20, 25 and 33 cycles and run on an agarose gel for ethidium bomide detection. Images are taken under UV light. The brightest marker is the 500bp band, from a 100bp ladder (Invitrogen).

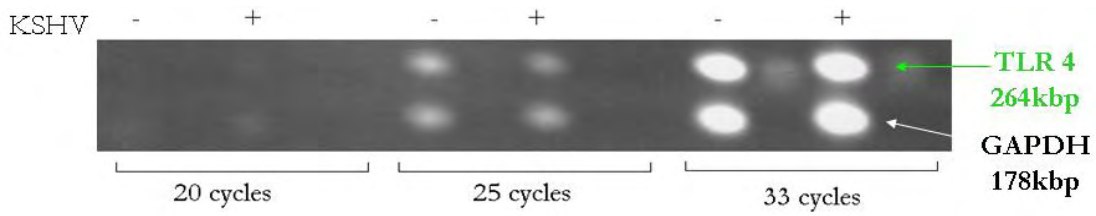


Figure 7: Comparative sq RT-PCR of TLR 4 in cell lines infected and uninfected with KSHV
Infected and uninfected cells expressing TLR 4 ectopically were subject to mRNA extraction and cDNA synthesis. This was then subject to PCR. 10ul aliquots were taken from the PCR reaction paused at 20, 25 and 33 cycles and altogether run on an agarose gel for ethidium bomide detection. Images are taken under UV light.

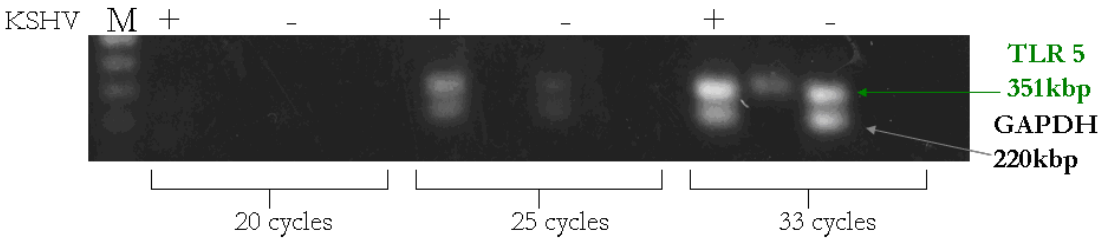


Figure 8: Comparative semi-quantitative RT-PCR of TLR 5 in cell lines infected and uninfected with KSHV
Infected and uninfected cells expressing TLR 5 ectopically were subject to mRNA extraction and cDNA synthesis. See Figure 6. The brightest marker is the 500bp band, from a 100bp ladder (Invitrogen).

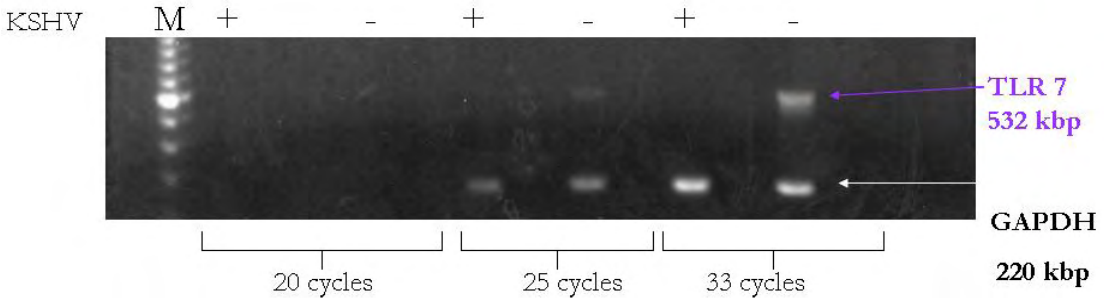


Figure 9: Comparative semi-quantitative RT-PCR of TLR 7 in cell lines infected and uninfected with KSHV
Infected and uninfected cells expressing ectopic TLR 7 were subject to mRNA extraction and cDNA synthesis. See Figure 6. The brightest marker is the 500bp band, from a 100bp ladder (Invitrogen).

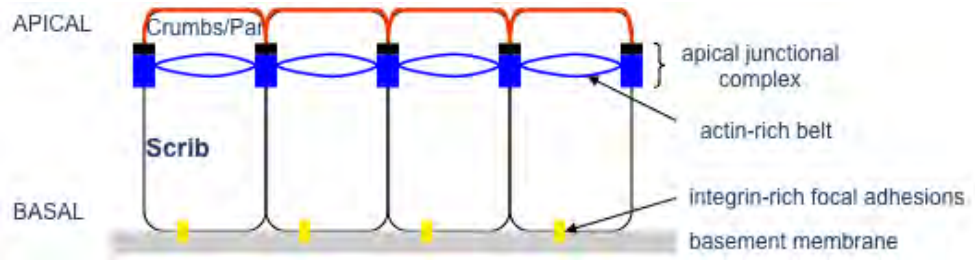


Figure 3: Epithelial cell polarity
Cell polarity is defined by the presence of tight junctions (TJs) and adherens junctions, which define the segregation of the cell membrane into apical and basolateral domains. Theses domains are both biochemically and functionally distinct. Three groups of proteins act to create and maintain apical-basal polarity: the scribble complex (Scribble/Dlg1/Lgl), the par complex (Par3/Par6/aPKC) and the crumbs complex (crumbs/PATJ/PALS). Polarity proteins are at the centre of various context-dependent signalling pathways.

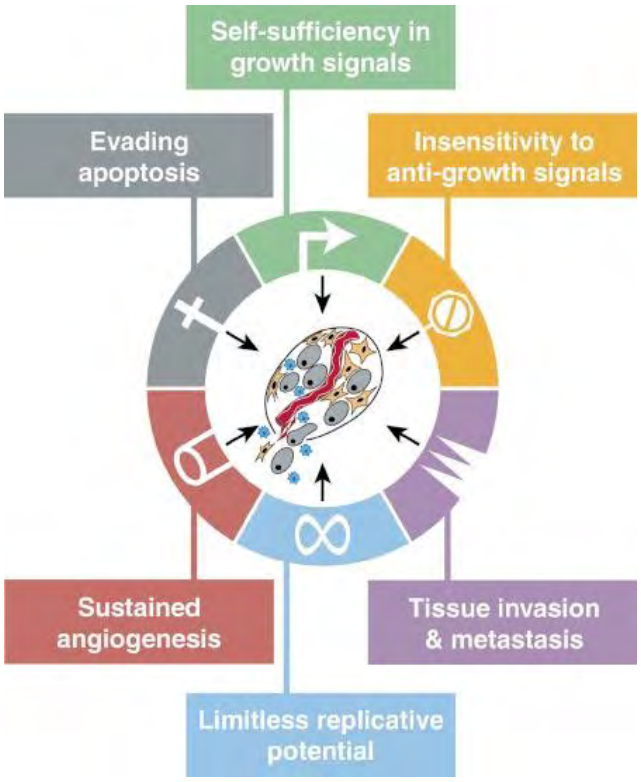


Figure 4: Hallmarks of cancer
Functional capabilities acquired by cancers during their development, via different mechanistic routes (Hanahan and Weinberg, 2000).

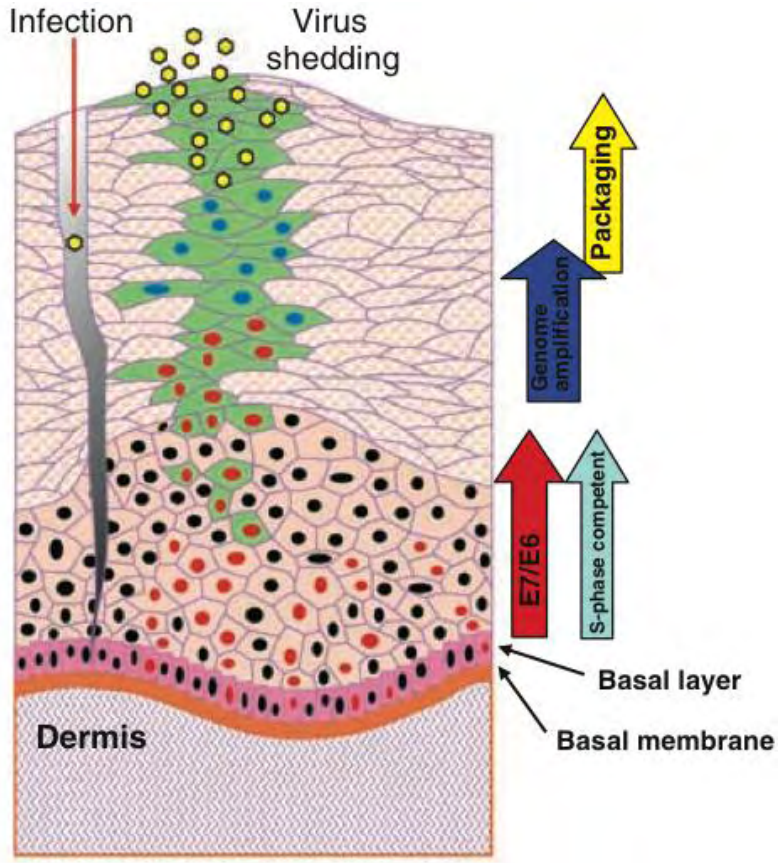
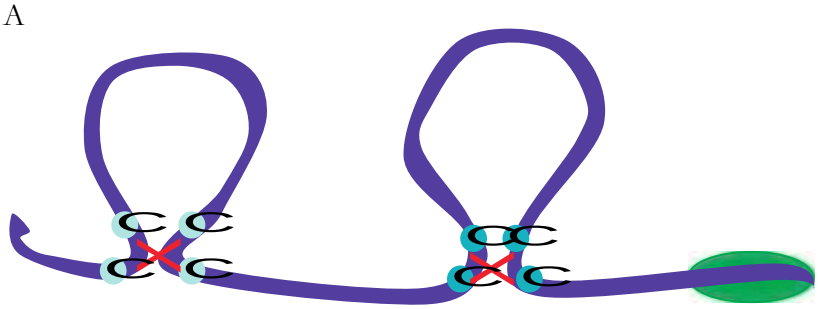


Figure 5: HPV maturation in epithelia
The virus penetrates tissue via microabrasions and infects basal cells. Expression of E 6 and E7 induces cells to enter S phase and replicate, allowing concurrent replication of the viral genome. Amplification of viral genome and migration of cell eventually leads to synthesis and shedding of new viral particles. Adapted from Thomas *et al.* 2008.

A



B

Risk category	Type	PBM
High-risk E6	HPV16	CMSCC-------RSSRTRRETQL
	HPV33	CAACW-------RS--RRRETAL
	HPV31	CIVCW---------RRPRTETQV
	HPV35	CMSCW---------KPTRRETEV
	HPV58	CAVCW---------RPRRRQTQV
	HPV52	CSECW----------RPRPVTQV
	HPV18	CHSCCNRARQE--RLQRRRETQV
	HPV45	CNTCCDQARQE--RLRRRRETQV
	HPV39	CRRCWTTKRED-RRLT-RRETQV
	HPV59	CRGCRTRARHLRQQRQARSETLV
	HPV56	CLGCW------RQTSREPRESTV
	HPV66	CLQCW------RHTSRQATESTV
Low-risk E6	HPV6	CLHCWTTCMED---------MLP
	HPV11	CLHCWTTCMED---------LLP

Figure 6: Structure of HPV E6 protein
Schematic diagram of the structure of HPV E6 from Thomas *et al.* 2008. **A**) The protein consists of two zinc fingers, stabilised by intramolecular salt bridges between cysteine residues (highlighted in blue) and a highly conserved C-terminus region (highlighted in green). **B**) High-risk HPV E6 oncoproteins contain a PDZ binding motif, whereas low-risk HPV E6 does not. (Area shaded in green). HPV = Human papillomavirus.

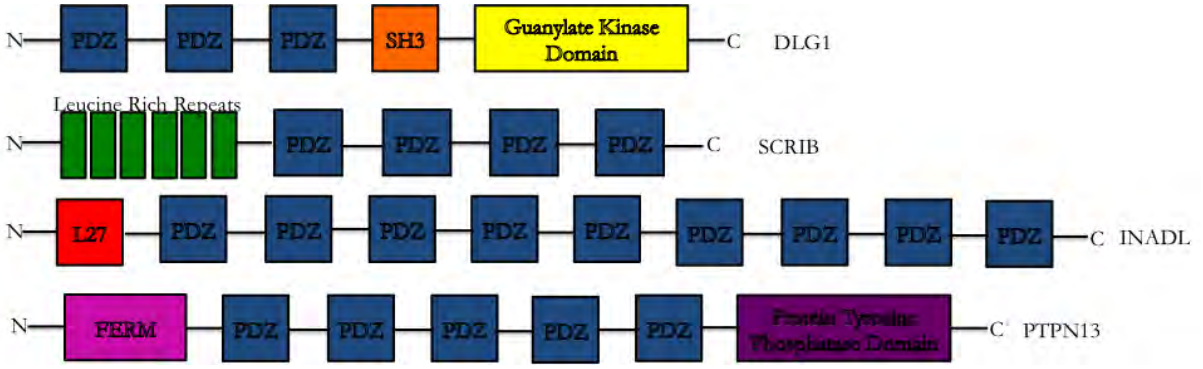
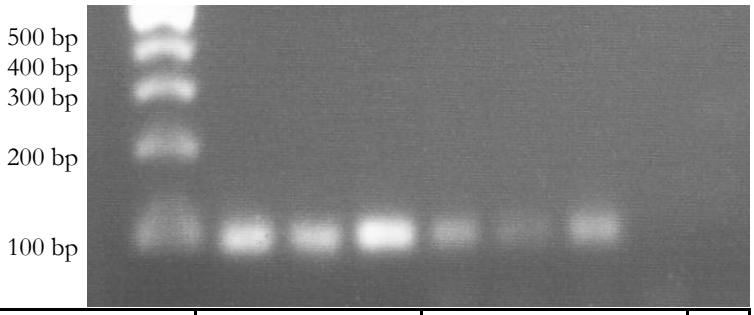


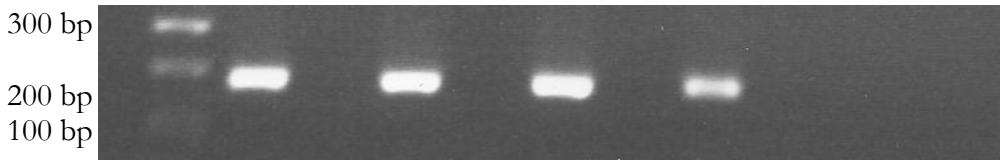
Figure 7: Domain structure of PDZ-domain containing proteins.
Each protein contains multiple PDZ domains, as well as other domains. The L27, Leucine rich repeats and SH3 domains are involved in protein-protein interactions. The FERM domain of INADL binds plasma membrane and elements of the cytoskeleton. The GK domain of DLG1 is not functional. Different modules and catalytic domains allow the proteins to bring proteins together in a scaffold, and in the correct orientation for relevant catalysis.

A



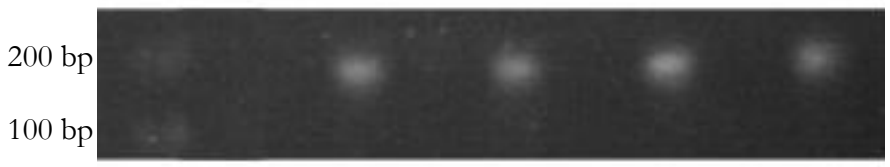
Amplified Fragment	DLG1			INADL			DLG
Annealing Temp/ Control	55	57	60	55	57	60	H_2O

B



Amplified Fragment	PTPN13									
Template	cDNA	H_2O	cDNA	H_2O	cDNA	H_2O	cDNA	H_2O	cDNA	H_2O
Annealing Temp	53		55		57		59		62	

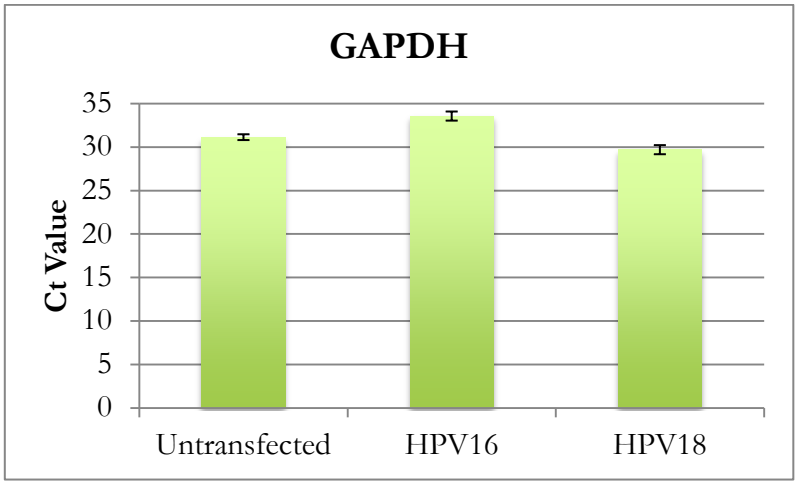
C



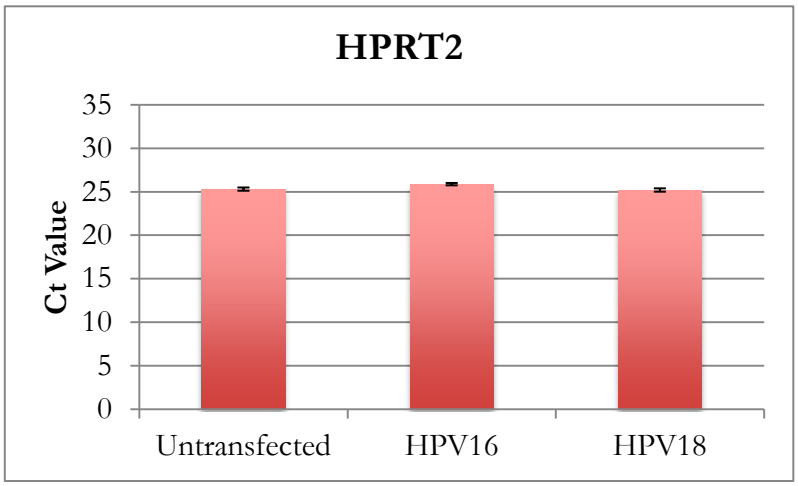
Amplified Fragment	SCRIB							
Template	H_2O	cDNA	H_2O	cDNA	H_2O	cDNA	H_2O	cDNA
Annealing Temp	55		57		59		61	

Figure 8: Optimisation of DLG1, INADL, PTPN13 and SCRIB specific primers
cDNA was translated from DNase treated mRNA extracted from untransfected HFKs. At least three temperatures per primer pair were analysed, and a water control was included. Aliquots from each reaction were then run on a 2% agarose gel for ethidium bromide detection. A 100bp ladder (Invitrogen) was run alongside as a marker to indicate that the correct fragment was amplified. A) DLG1 and INADL B)PTPN13 C)SCRIB

A



B



C

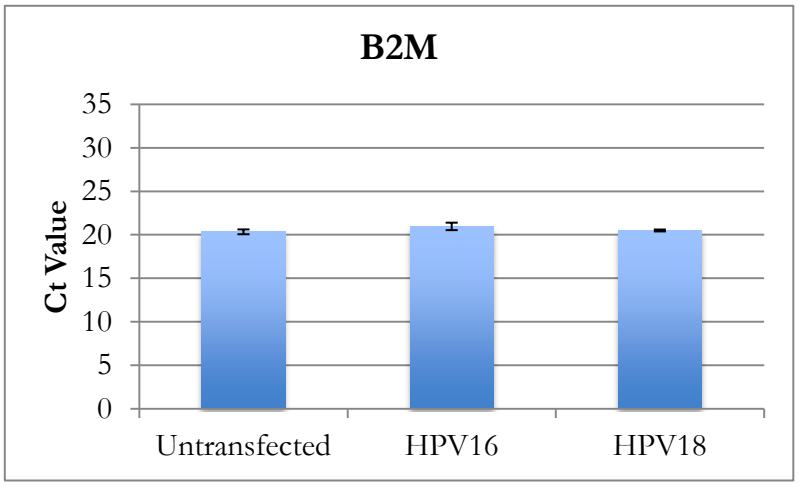
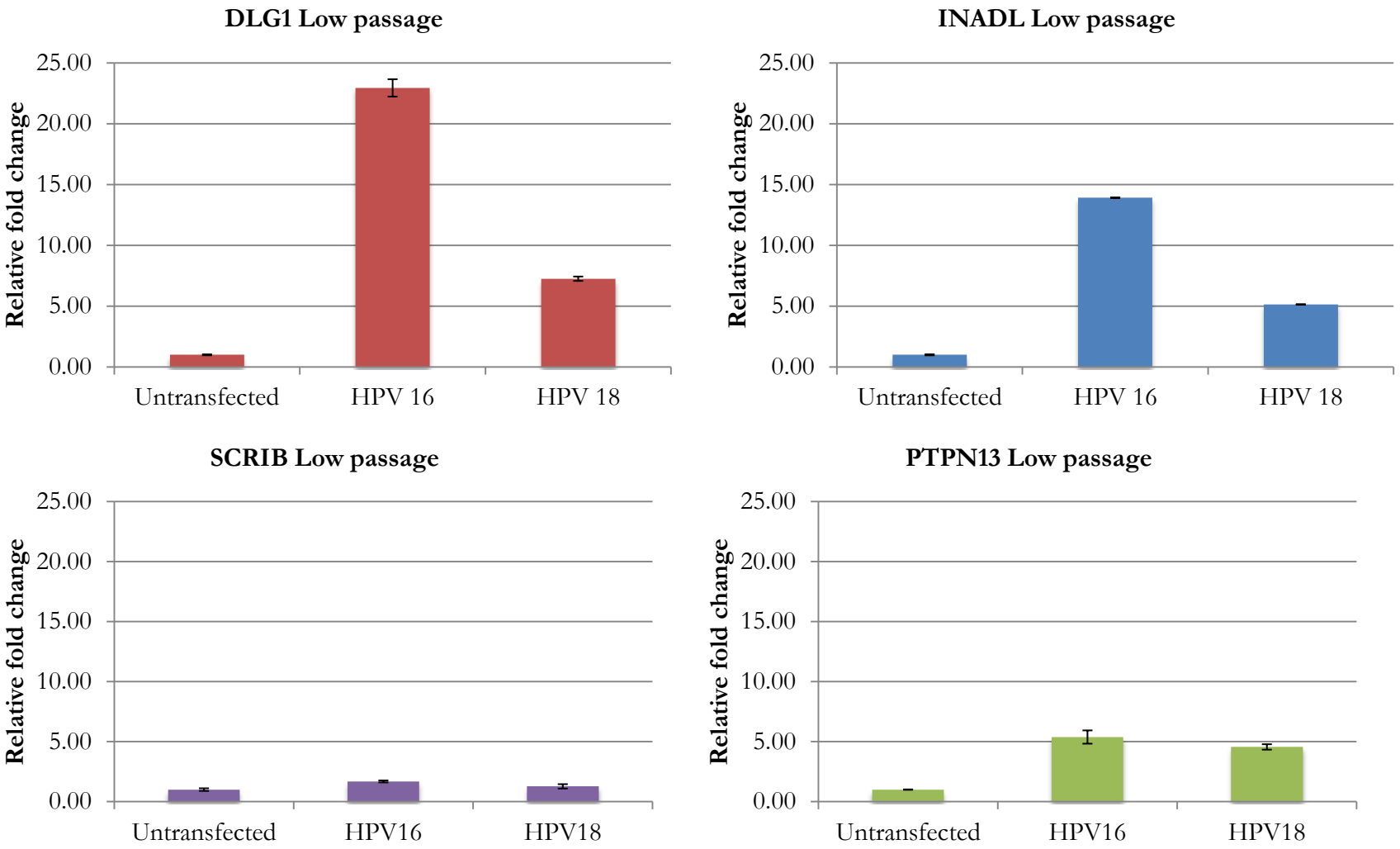


Figure 9: qPCR: housekeeping gene comparison
GAPDH, HPRT2 and B2M expression was analysed in untransfected HFKs and HPV16 and 18 transfected HFKs. Ct values are plotted to show the variation between each of the samples. Assays were performed in triplicate.

A


DLG1 Low passage
Relative fold change
25.00
20.00
15.00
10.00
5.00
0.00
Untransfected
HPV 16
HPV 18
INADL Low passage
Relative fold change
25.00
20.00
15.00
10.00
5.00
0.00
Untransfected
HPV 16
HPV 18
SCRIB Low passage
Relative fold change
25.00
20.00
15.00
10.00
5.00
0.00
Untransfected
HPV16
HPV18
PTPN13 Low passage
Relative fold change
25.00
20.00
15.00
10.00
5.00
0.00
Untransfected
HPV16
HPV18

B

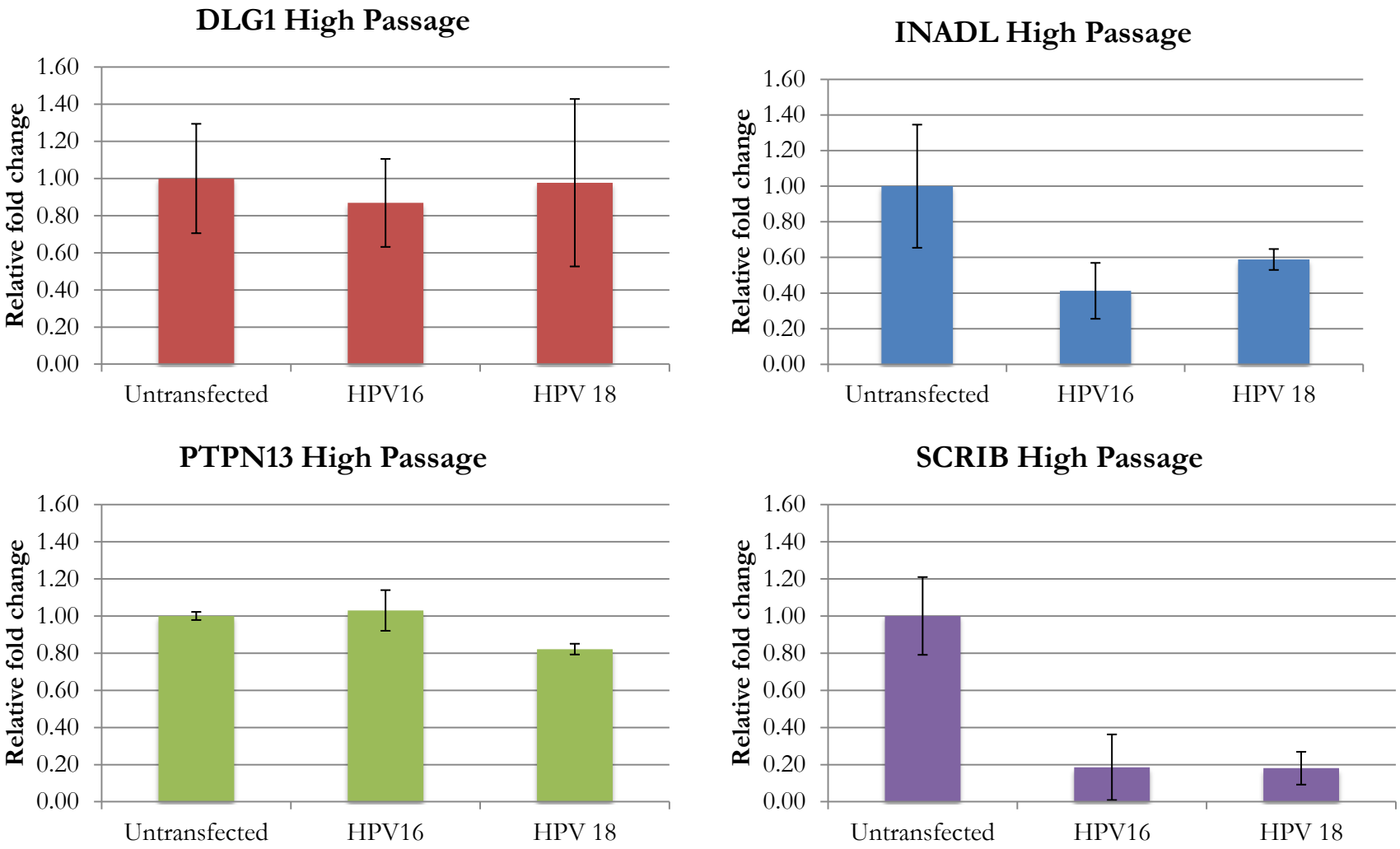
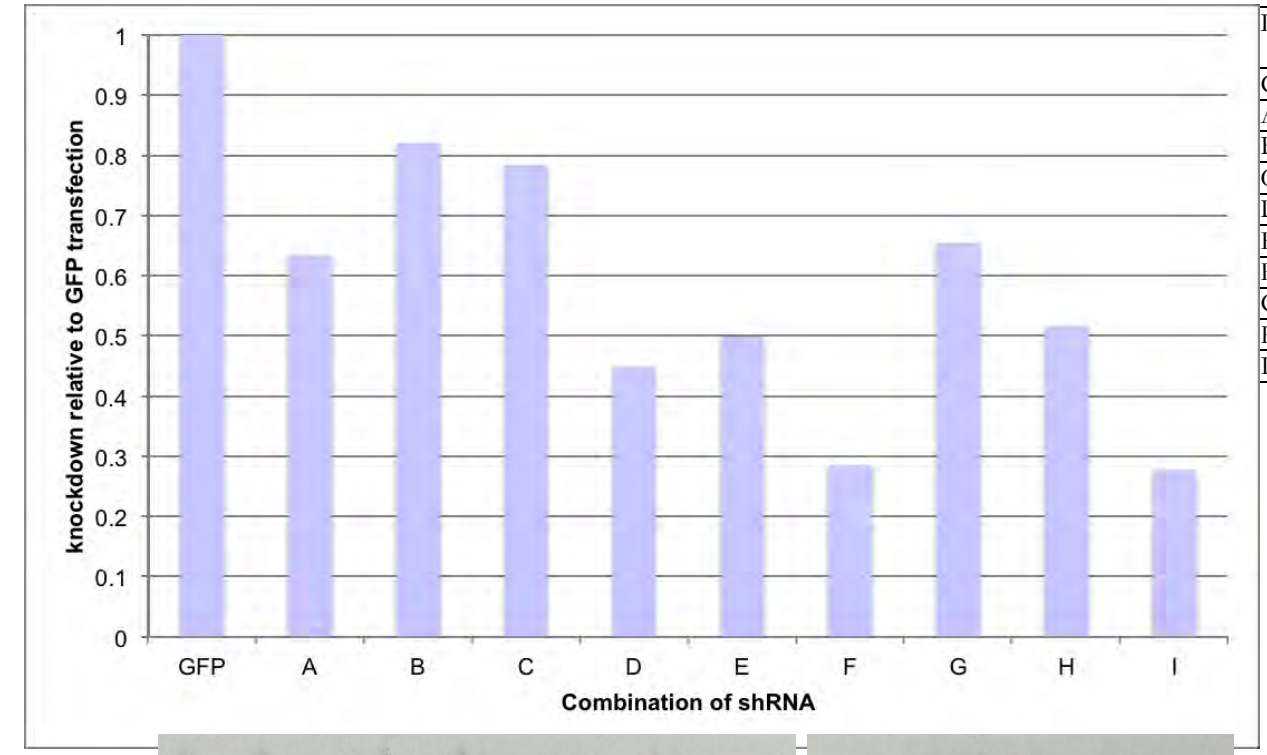

DLG1 High Passage
Relative fold change
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Untransfected
HPV16
HPV 18
INADL High Passage
Relative fold change
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Untransfected
HPV16
HPV 18
PTPN13 High Passage
Relative fold change
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Untransfected
HPV16
HPV 18
SCRIB High Passage
Relative fold change
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Untransfected
HPV16
HPV 18

Figure 10: qPCR analysis of PDZ-domain containing proteins in HPV-transfected HFKs
A) Analysis of cDNA from low passage samples, using mRNA from gene expression array. HPV 16 and 18 transfected HFKs at p3/p4 were normalised to untransfected HFKs (p3). B) Analysis of cDNA from high passage samples. HPV16 and 18 transfected HFs at p3/p8 were normalised to untransfected HFKs (p3). All assays were carried out in triplicates and results presented as $2^{-\Delta\Delta_{Ct}}$ values. Error bars show standard deviation of three experimental repeats. HPRT2 served as the housekeeping gene.



Lane Name	shRNA combinations
GFP	EGFP
A	1
B	2
C	3
D	4
E	1 + 2
F	1 + 3
G	1 + 4
H	1, 2 + 3
I	1, 2, 3 + 4

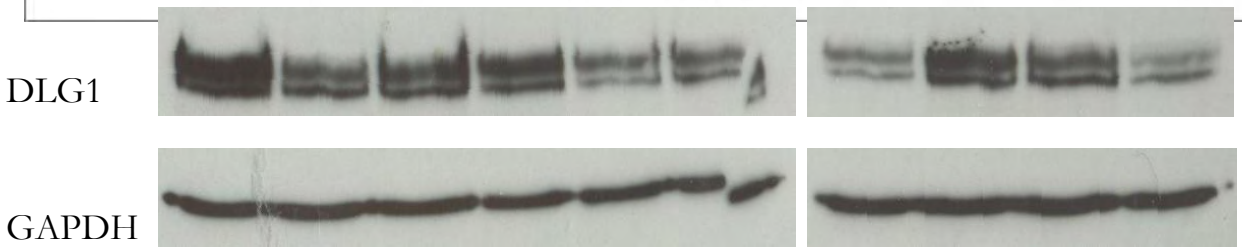
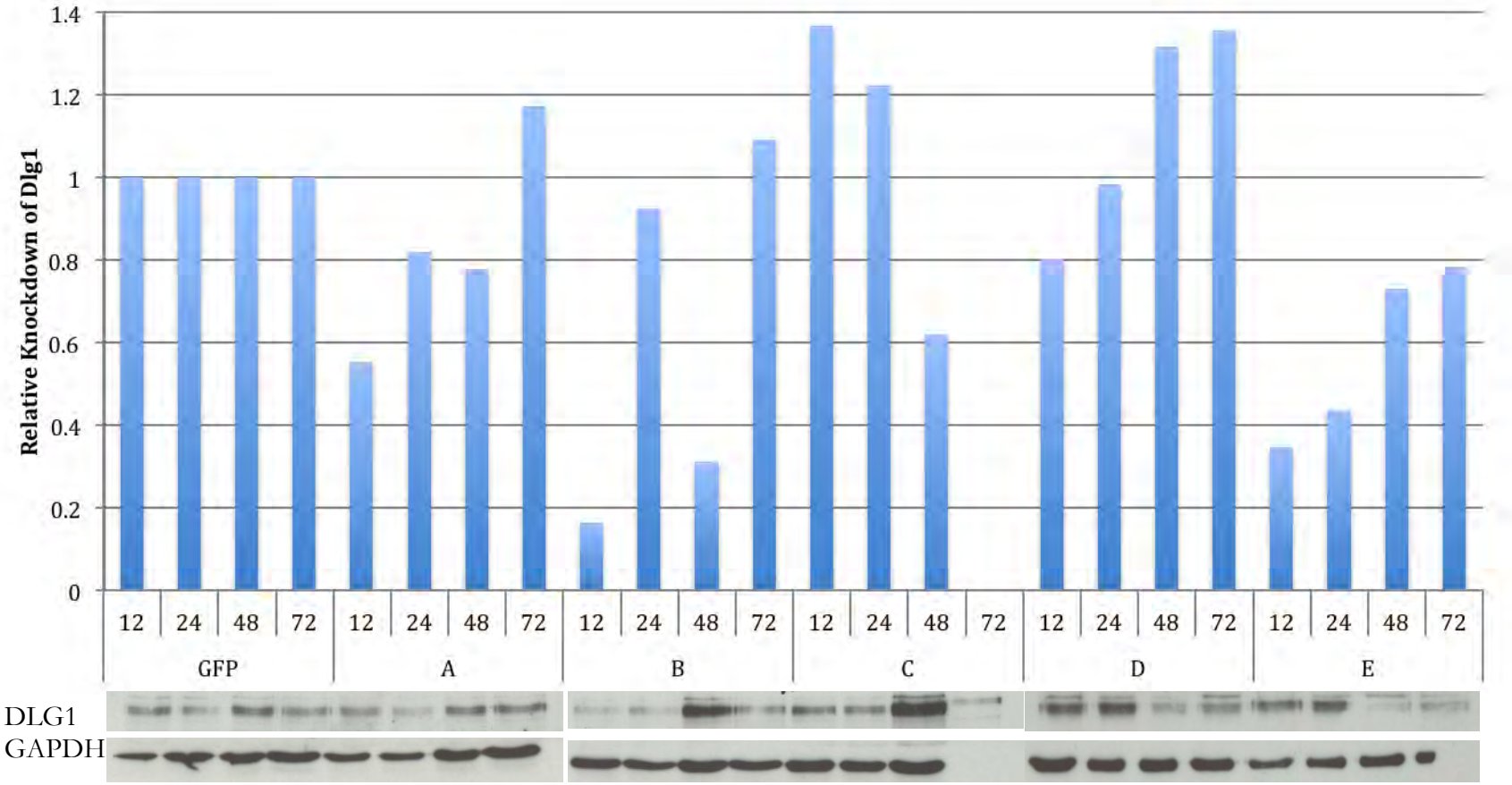


Figure 11: Silencing of DLG1 expression in H1299 cells
ShRNA combinations were transfected into H1299 cells and incubated for 24 hours, before cells were harvested and protein extracted for Western blot analysis. The blot was exposed to film for 1 minute before fixing. Dlg1 and GAPDH band intensity was analysed by Image J software, comparing each lane to the loading control (GAPDH) and then normalising to GFP (Lane 1) intensity.


Relative Knockdown of Dlg1
1.4
1.2
1
0.8
0.6
0.4
0.2
0
12 24 48 72 12 24 48 72 12 24 48 72 12 24 48 72 12 24 48 72 12 24 48 72
GFP
A
B
C
D
E
DLG1
GAPDH
shRNA Combinations

Lane Name	shRNA combination
GFP	EGFP
A	1
B	4
C	1 + 3
D	1 + 4
E	1, 2, 3 + 4

Figure 12: Time titration of selected shRNA combinations to knockdown of DLG1 in H1299 cells
ShRNA combinations were transfected into H1299 cells and incubated for 12, 24, 48 or 72 hours, before cells were harvested and protein extracted for Western blot analysis. These blots were then exposed to film for 1 minute before fixing. DLG1 and GAPDH band intensity was analysed using the Image J program, comparing each lane to the loading control (GAPDH) and then normalising to GFP (Lane 1) intensity. Each lane of the blot below the graph corresponds to bar chart data, and the table indicates the content of each combination of shRNAs applied to cells.

Table 4: HPV types

HPV type	Site of Infection	Associated lesion	Oncogenic Risk
1, 4	Cutaneous skin	Hand warts, verrucae	Benign
6, 11	Anogenital epithelia and laryngeal mucosa	Genital warts, laryngeal papillomas	Low
16, 18, 33, 45	Anogenital epithelia and oropharyngeal mucosa	CIN, anogenital cancers including cervical carcinoma, oropharyngeal cancer	High
5, 8, 38	Cutaneous skin	Non-melanoma skin cancer	High

CIN = cerival intraepithelial neoplasia

Taken from Roberts**, S**. and Young, L. (2008) HPV and disease. *In*: Oxford Oncology Library - Vaccines for the Prevention of Cervical Cancer (eds. P. Stern and H. Kitchener). Oxford University Press, pp 25-33 [ISBN 978-019-954345-8].

Table 5: PDZ domain-containing targets of papillomavirus oncoproteins

Protein	Function	Effect of E6 on target	Reference
DLG1	Cell polarity and integrity	Ubiquitination and proteosomal degradation	Gardiol *et al.* 1999 Kiyono *et al.* 1997
SCRIB	Cell polarity and integrity Apoptotic signalling	Ubiquitination and proteosomal degradation	Nakagawa and Hulbregtse, 2000
MAGI-1, -2, -3	Cell polarity PTEN membrane localisation	Ubiquitination and proteosomal degradation	Glaunsinger *et al.* 2000 Thomas *et al.* 2002
MUPZ	Signalling complex scaffold	Ubiquitination and proteosomal degradation	Lee *et al.* 2000
INADL	TJ formation and localisation	Ubiquitination and proteosomal degradation E6* directed degradation	Latorre *et al.* 2005 Storrs and Silverstein, 2007
DLG4	Signalling complex scaffold	Ubiquitination and proteosomal degradation	Handa *et al* 2007
PTPN3	Protein tyrosine kinase	Ubiquitination and proteosomal degradation	Jing *et al.* 2007 Topffer *et al.* 2007
PTPN13	Non-receptor protein tyrosine phosphatase Apoptotic signalling	Unknown	Spanos *et al.* 2008
TAX1BP3	Downregulation of PDZ interactions	Unknown	Hampson *et al.* 2004
GIPC1	TGFβ signalling	Ubiquitination and proteosomal degradation	Favre-Bonvin *et al.* 2005
GOPC	Intracellular trafficking	Ubiquitination and proteosomal degradation	Jeong *et al.* 2007
PARD3	Cell polarity	Ubiquitination and proteosomal degradation Relocalisation	Tomalc *et al.* 2008

Those highlighted are those focussed on in this report.
DLG1- discs large 1, MAGI- membrane-associated guanylate kinase with inverted domains, MPDZ - multi-PDZ domain protein 1, DLG4 - post-synaptic density protein 95, PTPN - protein tyrosine phosphatase, TAX1BP3 - tax-1 binding protein 3, GIPC1 - Golgi-associated PDZ and coiled-coil motif containing protein, GOPC - GAIP interacting protein C-terminus, PARD3 - par-3 partitioning defective 3 homologue.
E6* is a truncated form of E6, without the PBM motif.

Table 6: Gene expression array data

Probe Set ID	Gene Symbol	Chromosomal Location	fold change HPV 16	fold change HPV 18	Detection of transcript in HFK	Detection of transcript with HPV16	Detection of transcript with HPV18
230229_at	DLG1	3q29	11.67	7.50	Present	Present	Present
214705_at	INADL	1p31.3	4.47	2.58	Present	Present	Present
243792_x_at	PTPN13	4q21.3	4.22	4.40	Present	Present	Present
212556_at	SCRIB	8q24.3	1.57	2.01	Present	Present	Present
213306_at	MPDZ	9p24-p22	-1.79	1.27	Absent	Absent	Absent
206144_at	MAGI1	3p14.1	2.82	3.63	Absent	Present	Present
207702_s_at	MAGI2	7q21	-1.23	-2.08	Absent	Absent	Absent
226770_at	MAGI3	1p12-p11.2	2.13	1.57	Present	Present	Present
204592_at	DLG4	17p13.1	1.18	1.00	Present	Absent	Absent
203997_at	PTPN3	9q31	1.24	1.26	Present	Present	Present
209154_at	TAX1BP3	17p13	-1.75	-1.78	Present	Present	Present
207525_s_at	GIPC1	19p13.1	1.23	1.17	Present	Present	Present
236862_at	GOPC	6q21	22.07	16.94	Absent	Absent	
221527_s_at	PARD3	10p11.21	1.32	1.23	Present	Present	Present

All proteins are PDZ-domain proteins. Gene expression of transfected HFKs at early passage (p3/p4) compared to untransfected HFKs (p3). From Woodman, Leonard, Roberts *et al.*, unpublished data.

Table 7: shRNA constructs against DLG1

Tube ID	Nucleotide Sequence (5'-3')	Designated number
TI353825	TCCTTGATGTGTCTGGAAATGCCATAAAG	1
TI353826	GCAGCACATAAGGATGGCAAACTTCAGAT	2
TI353827	GTAACTTCTAATGCCAGCGATAGTGAAAG	3
TI353828	CAGAGCAACTCTTTCAGGCTTTAATAGA	4

Every 29mer shRNA vector is cloned in pRS plasmid under U6 promoter for mammalian cell expression. Source: Origene Technologies Inc. Plasmids were transfected into H1299 cells using Lipofectamine in different combinations, referred to throughout experiments using the designated number.

Table 8: Primers used in qPCR

mRNA amplification target	Forward Primer (5'-3')	Reverse Primer (5'-3')
DLG1	GCCCATTCCTAGCGCATGAACTCC	GCCCATTCCTAGCGCATGAACTCC
PTPN13	ATGAAACCTGTGCTAGGGATTATA	CGACCTCCCAGGCTCAAGTAG
INADL/PATJ	AGATCGGAGTTTTGCAGAGGT	TCGAAGCAATGTTCTTGACCCA
SCRIB	CCTGCCAGCTCCAGCACCAC	CGGCTGGGGTGGGGCAGTTA