Interactions of the Human Papillomavirus E6 protein and their role in the persistence of viral episomes

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Statement of declaration

I, Lietta Nicolaides, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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" Πάντα στον νου σου νάχεις την Ιθάκη.
Το φθάσιμον εκεί είν' ο προορισμός σου.
Αλλά μη βιάζεις το ταξίδι διόλου.
Καλλίτερα χρόνια πολλά να διαρκέσει·
και γέρος πια ν' αράξεις στο νησί,
πλούσιος με όσα κέρδισες στον δρόμο,
μη προσδοκώντας πλούτη να σε δώσει η Ιθάκη."

~ Ιθάκη (1911)
Κ.Π. Καβάφης

(from "Ithaka"by C.P.Cavafy)

Abstract

The E6 protein from high-risk Human Papillomaviruses (HPVs) has previously been shown to be necessary for the persistence of viral episomes in cells, however, the mechanism for this remains unclear. High-risk E6 proteins have many activities including the ability to degrade p53 and the ability to bind to and degrade PDZ proteins. In this study I aimed to further elucidate the role of E6 in the persistence of viral episomes.

I used two HPV16 mutant genomes with mutations in the E6 open-reading frame; one that is unable to degrade p53 (16E6p53m), and one that lacks the PDZ-binding motif (16E6PDZ). I found that both are unable to persist episomally in cells thereby implicating these two activities of E6 in HPV episomal persistence.

Upon closer investigation of the two mutant genomes, I found that the 16E6p53m genome does not replicate as efficiently as the wild-type genome. This result suggests a function for p53-degradation in genome replication, and consequently in genome persistence. Furthermore, by carrying out a more detailed analysis of the relationship between E6 and the PDZ protein hScrib, I showed that the wild-type E6 protein is stabilised by virtue of the PDZ-binding motif, present on its C-terminus. On the other hand, the mutant E6 protein that lacks the PDZ-binding motif (E6PDZ) is more susceptible to proteasomal degradation. These findings provide evidence for a previously unknown outcome of the E6-PDZ protein interaction, in stabilising wild-type E6 protein. In addition to the implications of this stabilisation in the persistence of viral episomes, it is also significant when considering the activities and properties of E6 that contribute to the development of neoplasia.

Finally, I have also found that wild-type HPV16 genomes cannot persist in cells that constitutively express E6 protein, suggesting that the correct regulation of E6 expression is crucial.

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List of abbreviations

APC	adenomatous polyposis coli
APS	ammonium persulphate
β-gal	beta-galactosidase
BPV	bovine papillomavirus
cDNA	complementary DNA
CIN	cervical intraepithelial neoplasia
CMV	cytomegalovirus
CRPV	cottontail rabbit papillomavirus
dH_2O	distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleic triphosphate
DPBS	Dulbecco's PBS
DTT	dithiothreitol
E6AP	E6-associated protein
E6p53m	E6 protein that is unable to degrade p53
E6PDZ	E6 protein with a deleted PDZ-binding motif
E6TP1	E6-targeted protein 1
E6WT	wild-type E6 protein
EBNA1	EBV nuclear antigen 1
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EV	epidermodysplasia verruciformis
Exo III	Exonuclease III
FBS	fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
H2B	histone H2B
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HFK	human foreskin keratinocyte

HMEC	human mammary epithelial cell
HPV	human papillomavirus
HRP	horseradish peroxidise
HSIL	high-grade squamous intraepithelial lesion
HTLV-1	Human T-lymphotropic virus Type 1
IFN	interferon
IgG	immunoglobulin G
IRF	interferon regulatory factor
kb	kilo base
kDa	kilo Dalton
KSHV	Kaposi's sarcoma-associated herpesvirus
LANA	latency-associated nuclear antigen
LB	Luria Bertani
LCR	long-control region
LSIL	low-grade squamous intraepithelial lesion
MCM	minichromosome maintenance
mg	milligram
µg	microgram
ml	millilitre
µl	microlitre
mRNA	messenger RNA
ng	nanogram
NIKS	normal immortalised human keratinocytes
NLS	nuclear localisation signal
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
Par3	partitioning-defective 3
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDZ	structural domain named after PSD95, DlgA and ZO-1 proteins
Pen/strep	penicilin/streptomycin solution
poly (A)	polyadenylation
Puma	p53 up-regulated modulator of apoptosis

PVDF	polyvinylidene fluoride
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
ROPV	rabbit oral papillomavirus
RRP	recurrent respiratory papillomatosis
RT-qPCR	reverse transcription quantitative polymerase chain reaction
SDS	sodium dodecyl sulphate
SF2/ASF	splicing factor 2/alternative splicing factor
SR	serine-arginine-rich
SV40	Simian virus 40
TA	transit amplifying
TBP	TATA box binding protein
TIP60	tat-interacting protein 60 kDa
TopBP1	topoisomerase II-binding protein 1
Topo II	topoisomerase II
v/v	volume/volume
VLP	virus-like particle
w/v	weight/volume

Chapter 1: Introduction

1.1. Introduction to papillomaviruses

Papillomaviruses (PVs) are small, non-enveloped viruses and consist of a circular, double-stranded DNA molecule, within an icosahedral protein capsid. PVs are epitheliotropic viruses, infecting both mucosal and cutaneous epithelial tissues, and their life-cycle is tightly linked to the differentiation of their host cells (Favre *et al.*, 1997). The viruses infect the basal layer of the epithelium and production of mature virions at the end of the viral life-cycle is confined to the upper, most differentiated layers of the tissue (reviewed in Doorbar, 2006). Many PVs have been shown to cause a range of diseases, from benign proliferative skin or genital warts to cancer. Human PV types (HPVs) that cause benign disease are classified as low-risk viruses, whereas the types that can cause cancer, such as HPV types 16 and 18, are classified as high-risk types (Favre *et al.*, 1997; Laimins, 1993; Walboomers *et al.*, 1999).

The first PV to be identified in animals was the cottontail rabbit PV (CRPV), which was shown to cause cutaneous lesions that sometimes progressed to malignancy (Shope & Hurst, 1933). PVs are now known to infect a variety of different animal species including ungulates, cetaceans, birds (Bernard *et al.*, 2010; de Villiers *et al.*, 2004) and more recently, reptiles (Herbst *et al.*, 2009) and have been shown to be very species-specific, suggesting that they have been co-evolving with their host species through time (Bernard *et al.*, 2006). More recently the first PV to naturally infect laboratory mice was identified and was shown to be transmissible to both nude and immunocompetent laboratory mice (Ingle *et al.*, 2010). This discovery has opened new doors and has provided an important tool for the future of PV research.

1.2. Classification of papillomaviruses

PVs were initially classified under the family of *Papoviridae*, together with polyomaviruses, based on their similarities in structure (non-enveloped capsids) and circular, double-stranded DNA genome. However, sequencing of the PV genome revealed many differences between PVs and polyomaviruses, such as

the size and organisation of their genomes (de Villiers *et al.*, 2004). Another difference is that PV transcription is unidirectional that is, all transcripts are expressed from one strand of DNA (Amtmann & Sauer, 1982; Danos *et al.*, 1983). This led to PVs being designated as a separate family, the *Papillomaviridae*.

To date, over 180 PV types have been identified (Bernard *et al.*, 2010). Sequencing of PV genomes has led to a phylogenetic analysis based on the homology of the L1 open reading frame (ORF) that encodes the major capsid protein and is the most conserved PV ORF. PVs are broadly categorised in genera, and types within the same genus share more than 60% identity in their L1 DNA. Genera can be sub-categorised into species, types, subtypes and variants. Types within the same species have 71% - 89% identity to each other (de Villiers *et al.*, 2004).

1.3. Human papillomaviruses (HPVs)

Using the classification system described above, HPVs are found in five out of twenty-nine genera (Apha, Beta, Gamma, Mu and Nu) with the animal and bird PVs comprising the other twenty-four genera. The genus with the greatest medical importance is Alpha, which includes the HPVs associated with genital cancers (the high-risk types) such as HPV16 and HPV18. This genus also includes the low-risk HPV types causing genital warts, for example HPV11, as well as those causing cutaneous, non-genital warts, such as HPV2 (Bernard *et al.*, 2010).

Members of the Beta genus, such as HPV5 and 8, infect cutaneous skin. They are typically associated with mild skin lesions, but can spread widely in patients suffering from a rare hereditary skin condition called Epidermodysplasia Verruciformis (EV). In these patients, infections by Beta PVs are associated with the development of non-melanoma skin cancer (Harwood & Proby, 2002; Pfister, 2003). The Gamma, Mu and Nu PVs generally infect cutaneous sites and cause skin lesions such as verrucas (de Villiers *et al.*, 2004).

1.4. HPVs and disease

Detailed studies of different types of HPVs have found a correlation between the ability of HPVs to immortalise cells in culture, and the degree of disease caused. For example, the high-risk types HPV16, 18, 31 and 33 are able to immortalise primary epithelial cells whereas the low-risk types HPV1a, 5, 6b and 11 are not (Pecoraro *et al.*, 1989; Schlegel *et al.*, 1988; Woodworth *et al.*, 1989). Moreover, the HPV E6 and E7 proteins have been shown to be able to immortalise cells independently (Band *et al.*, 1991; Halbert *et al.*, 1991), although their immortalisation efficiency is increased when both are expressed together in cells (Halbert *et al.*, 1991).

1.4.1. Low-risk and cutaneous HPVs

Low-risk HPVs are infrequently linked to cancer, but can cause a wide-range of benign diseases. Anogenital warts are prevalent amongst the sexually active population and are most commonly caused by HPV6 and 11 (Greer *et al.*, 1995). These two low-risk types are also the cause of most benign HPV-related oral lesions (Praetorius, 1997). Cutaneous warts are caused by a large number of HPV types, including HPV1 (Egawa *et al.*, 1993) and HPV2, which is also able to infect mucosal epithelia (Chan *et al.*, 1997; de Villiers *et al.*, 2004).

Although benign to the majority of the population, low-risk and cutaneous HPVs are sometimes associated with rare but severe diseases such as EV and recurrent respiratory papillomatosis (RRP). As mentioned above, EV patients have a higher susceptibility to infections by HPVs from the Beta genus, and develop skin lesions that may progress to cancer (Harwood & Proby, 2002; Pfister, 2003). RRP is typically caused by the Alpha-PVs HPV6 and HPV11 (Gissmann *et al.*, 1982; Mounts *et al.*, 1982) and patients develop papillomas in their respiratory tracts (particularly the larynx), which lead to obstruction of the airway. It is thought that PVs persist in these patients in a latent state, and their re-activation causes the recurrent disease (Steinberg *et al.*, 1983).

1.4.2. High-risk HPVs

The best-studied malignancy caused by HPVs is cervical cancer and it is now believed that over 99% of cervical cancers are caused by HPVs. HPV16 and 18 are thought to account for over 60% of these, with other HPV types, including 31, 33, 39, 45, and 58, accounting for the rest (Bosch *et al.*, 1995; Clifford *et al.*, 2003; Munoz *et al.*, 2003; Walboomers *et al.*, 1999). Cervical cancer is the second most common cancer in women worldwide, with approximately 500,000 newly diagnosed cases and 250,000 deaths every year (World Health Organisation). Most cervical cancers are squamous cell carcinomas, with the rest being adenocarcinomas and a small number being small cell neuroendocrine tumours. HPV16 is more commonly associated with squamous cell carcinomas whereas HPV18 is more commonly associated with adenocarcinomas (Clifford *et al.*, 2003).

The majority of cervical cancers are thought to occur in the transformation zone of the cervix, which is an area of metaplastic change, from columnar cells to a stratified squamous epithelium (Burghardt & Ostor, 1983; Sun *et al.*, 1992). The higher susceptibility of the transformation zone may be due to better access of the virus to the basal layer of the epithelium, as well as reduced immune surveillance compared to other sites of the cervix (Giannini *et al.*, 2002).

Genital HPV infection is a sexually transmitted infection and the highest prevalence is seen in women in their late teens or early 20s. However, the incidence of cervical cancer is highest in older women. This delay between infection and onset of disease suggests that cervical cancer may arise after persistent infection. In such persistent infections, disease is thought to ensue due to the prolonged expression of the viral oncoproteins (Schiffman & Kjaer, 2003).

High-risk HPVs can also cause pre-cancerous cervical disease, which can precede the development of cancer. These pre-cancerous lesions are histologically classified based on their severity, with cervical intraepithelial neoplasia (CIN) - 1 exhibiting the lowest form of dysplasia, and CIN-3 exhibiting severe dysplasia (Woodman *et al.*, 2007). In addition to accounting for almost all cases of cervical cancer, HPVs are thought to cause a variety of other

anogenital cancers such as anal, vulvar and penile cancers, as well as head and neck cancers, such as those of the pharynx, larynx and tonsils (zur Hausen, 2009).

1.5. The HPV16 genome

The HPV16 genome is a 7.9 kb circular, double-stranded DNA genome (Fig. 1.1) and is divided into three regions by two polyadenylation (poly (A)) sites; the early region, the late region, and a non-coding region called long-control region (LCR) (Zheng & Baker, 2006). The E1, E2, E4, E5, E6 and E7 non-structural proteins are the early proteins and are expressed from the early promoter (p97), which lies within the LCR. The L1 and L2 capsid proteins are the late proteins and are thought to mainly be expressed from the late, differentiation-dependent promoter (p670). This promoter lies within the E7 ORF and can also be used to express E1, E2, E4 and E5, depending on the differentiation stage of the epithelium (Grassmann *et al.*, 1996; Hummel *et al.*, 1992; Smotkin & Wettstein, 1986).

The LCR of HPV16, which lies upstream of the E6 ORF also contains regulatory elements involved in viral transcription. These *cis* elements include binding sites for various cellular transcription factors, including Sp1, AP-1, Oct-1, and YY1, which can both positively and negatively regulate transcription. It has further been suggested that regulatory sequences in the LCR may play a role in the tissue- or cell-specificity of HPVs (Chan *et al.*, 1990; Gloss & Bernard, 1990; Lace *et al.*, 2009; Marshall *et al.*, 1989; Morris *et al.*, 1993, Offord *et al*., 1993). For example, the HPV promoter has been shown to be active in keratinocytes but not in fibroblasts (Bernard *et al.*, 1989) and this correlates with higher levels of the AP-1 transcription factor in keratinocytes compared to early passage fibroblasts (Offord *et al.*, 1993).

Moreover, the LCR contains binding sites for the viral protein E2 (Androphy *et al.*, 1987), and the HPV16 LCR contains four such E2-binding sites. Binding of E2 has been shown to regulate viral gene transcription (Bernard *et al.*, 1989; Bouvard *et al.*, 1994; Phelps & Howley, 1987; Romanczuk *et al.*, 1990; Spalholz *et al.*, 1985; Spalholz *et al.*, 1987). E2 has been reported to have both positive

and negative regulatory effects on transcription and recent studies have suggested that this differential transcriptional regulation may be determined by the amounts of E2 viral protein present (Steger & Corbach, 1997), or by the physical state and conformation of the viral genome in the cells, (Bechtold *et al.*, 2003; Schmidt *et al.*, 2005). Moreover, the LCR contains the viral origin of replication (Chiang *et al.*, 1992a), and a binding site for the viral replication protein E1, a DNA helicase that is recruited to the viral origin of replication by E2 (Desaintes & Demeret, 1996), to initiate viral replication.

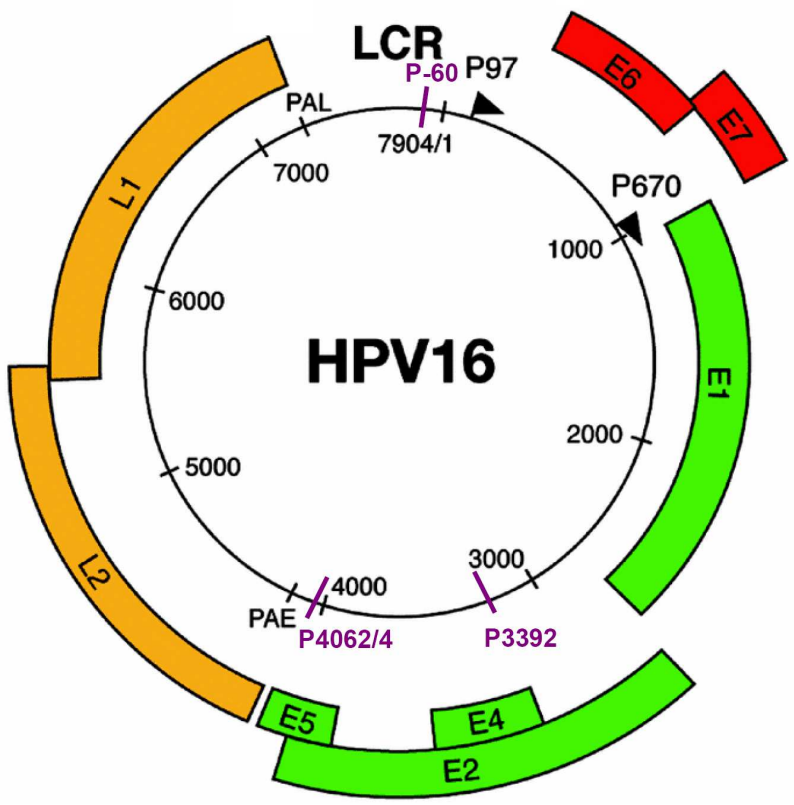


Figure 1.1: The HPV16 genome

The HPV16 genome is a 7.9 kb, circular, double-stranded DNA genome and consists of 8 main ORFs. These encode for 6 non-structural, early proteins, E6, E7 (red), E1, E2, E4 and E5 (green) and 2 structural, late proteins, L1 and L2 (yellow). The 2 main viral promoters p97 and p670 are shown, as are the early and late poly (A) sites (PAE and PAL respectively). The early proteins are expressed from both promoters, whereas the late proteins are thought to be expressed from the late, differentiation-dependent promoter (p670). Three recently identified promoters (p3392 at the 5' of the E4 ORF, p4062/4 at the end of the E5 ORF and p-60 in the LCR), which were found to be active in differentiating cells, are shown in purple (Milligan *et al.*, 2007). The LCR is a non-coding region and contains *cis*-acting elements involved in viral replication and transcription, including binding sites for transcription factors and for the E1 and E2 proteins, as well as the viral origin of replication. (The figure has been modified from Doorbar, 2006).

1.6. HPV16 transcription

The study of different HPV16 mRNA species in cells that harbour HPV episomes has been carried out mostly in W12 cells (Doorbar *et al.*, 1990; Milligan *et al.*, 2007). This cell line is a cervical keratinocyte line isolated from a low-grade cervical lesion and has been shown to contain predominantly episomal forms of the HPV16 genome (Stanley *et al.*, 1989). As such it presents a better candidate for the study of viral transcripts than the cervical cancer cell lines, such as CaSki and SiHa, as these lines contain only integrated copies of the viral genomes and do not support the viral life-cycle.

HPV16 transcripts are divided into early and late transcripts and are polyadenylated by an early and two late poly (A) sites respectively (Milligan *et al.*, 2007). The early promoter, p97, is regulated by the binding of E2 and cellular transcription factors to their binding sites in the LCR (Zheng & Baker, 2006). Regulation of expression from the late promoter, p670, is less well understood but studies suggest that it may be activated by differentiation signals within the cell and its activation appears to be independent of viral genome amplification (Spink & Laimins, 2005). Transcription from the early promoter appears constant throughout the life-cycle of the virus, whereas transcription from the late promoter, p670, is greatly enhanced upon differentiation (Grassmann *et al.*, 1996; Hummel *et al.*, 1992).

HPV16 transcripts have also been identified that initiate at positions other than the abovementioned early and late promoters (p97 and p670). This suggests the existence of additional promoters on the HPV genome (see Fig. 1.1). One such promoter was mapped to the 5' of the E4 ORF (p3392), another one at the end of the E5 ORF (p4062/4) and a third in the LCR (p-60). These were described in W12 cells (Milligan *et al.*, 2007). A promoter in the E4 ORF has been previously described in HPV31 genomes, as have promoters in the LCR (Ozbun & Meyers, 1997; Ozbun & Meyers, 1999), suggesting that these may be conserved across different HPV types.

Almost all HPV transcripts are polycistronic and gene expression is regulated by alternative splicing, which generates different mRNA products (Zheng & Baker, 2006). One example is the alternative splicing of the E6/E7 polycistronic or

bicistronic transcripts. High-risk E6 ORFs contain an intron sequence, and several splice sites and the excision of the intron has been shown to generate alternatively spliced E6 products (Zheng & Baker, 2006). In addition to mRNAs containing the full-length E6 sequence, at least 2 spliced species have been described, E6*I and E6*II, both of which have the same N-terminal sequences as full length E6, but lack the intron sequence and have different C-terminal truncations (Doorbar *et al.*, 1990; Schneider-Gadicke & Schwarz, 1986; Smotkin *et al.*, 1989). It has been suggested that mRNAs carrying the spliced forms of E6 (E6*) are more abundant in cervical cancer lines than ones carrying the full-length E6 form (Smotkin *et al.*, 1989; Zheng & Baker, 2006). Although this splicing would prevent the production of full-length E6 protein, it appears that the spliced transcripts favour the expression of E7 protein. (Smotkin *et al.*, 1989; Tang *et al.*, 2006a; Zheng *et al.*, 2004). Earlier studies have also identified a protein product thought to be expressed from the spliced E6* species (Schneider-Gadicke *et al.*, 1988). More recent studies have attempted to elucidate the activities of the E6* protein compared to the full-length E6 protein (Pim *et al.*, 2009; Storrs & Silverstein, 2007) but its precise function and role in the viral life-cycle remains unclear.

Little is known about the regulation of E6 splicing in cells. Interestingly, a recent study suggested that in the presence of epidermal growth factor (EGF), full-length E6 is expressed, whereas the absence of EGF favours the expression of E6* and E7 (Rosenberger *et al.*, 2010). This may suggest a mechanism for the regulation of this splicing process in the differentiated epithelium, where growth factors are depleted.

Another example of splicing of HPV transcripts, is the generation of the E8^E2 protein, which is derived from 2 viral ORFs. E8 is an ORF found within the E1 ORF and is spliced with the C-terminus of E2, to generate E8^E2 protein. E8^E2 mRNA species have been identified in a variety of HPVs (they are sometimes called E2C), including HPV16 (Doorbar *et al.*, 1990; Sherman *et al.*, 1992), HPV31 (Stubenrauch *et al.*, 2000) and the low-risk type HPV11 (Rotenberg *et al.*, 1989). HPV16 and 31 E8^E2 proteins are thought to repress viral gene transcription and viral replication (Lace *et al.*, 2008; Stubenrauch *et al.*, 2000). Moreover, the HPV31 E8^E2 protein was suggested to inhibit

episomal persistence of HPV31 genomes (Stubenrauch *et al.*, 2000). In contrast HPV16 E8^E2 did not appear to have an inhibitory effect on the persistence of HPV16 genomes (Lace *et al.*, 2008).

Alternative splicing is also essential for the expression of late viral proteins from late viral transcripts. Splicing of late transcripts takes place in the differentiated layers of the epithelium. The levels of splicing factors, such as SR (serine-arginine-rich) proteins, in the differentiated layers of uninfected epithelia are decreased (compared to the levels in undifferentiated cells) (McPhillips *et al.*, 2004; Mole *et al.*, 2009a). Recent studies have shown that the viral E2 protein up-regulates the expression of different SR proteins, one of which being SF2/ASF (splicing factor 2/alternative splicing factor) (McPhillips *et al.*, 2004; Mole *et al.*, 2009a; Mole *et al.*, 2009b). Furthermore, the expression of these proteins increases upon differentiation of HPV-positive cells, suggesting that E2 may play a role in altering the cellular environment in differentiated cells, to facilitate late viral gene expression (McPhillips *et al.*, 2004; Mole *et al.*, 2009a). Interestingly, there also appears to be a correlation between the expression of SF2/ASF and the severity of cervical disease, suggesting a potential use of this protein as a biomarker for disease (Mole *et al.*, 2009a).

1.7. HPV16 viral life-cycle

Most work on HPVs has been carried out using the high-risk types and in particular HPV16, due to their clinical importance. From this work, a general pattern of the HPV life-cycle has been elucidated (Fig. 1.2). HPVs are exclusively epitheliotropic viruses, and their life-cycle is dependent on the differentiation of the epithelium for its completion. As such, the levels and pattern of viral gene expression are tightly regulated and change during the differentiation of the epithelium. Different stages of the life-cycle are often categorised into early and late events. Early events take place in the basal and parabasal layers of the epithelium and comprise the viral entry into cells and the establishment of the viral episomes in the nucleus. Late events occur in the upper, differentiated layers of the epithelium and comprise the amplification of the viral genome, the production of the capsid proteins, and the assembly and release of new virions (reviewed in Doorbar, 2005).

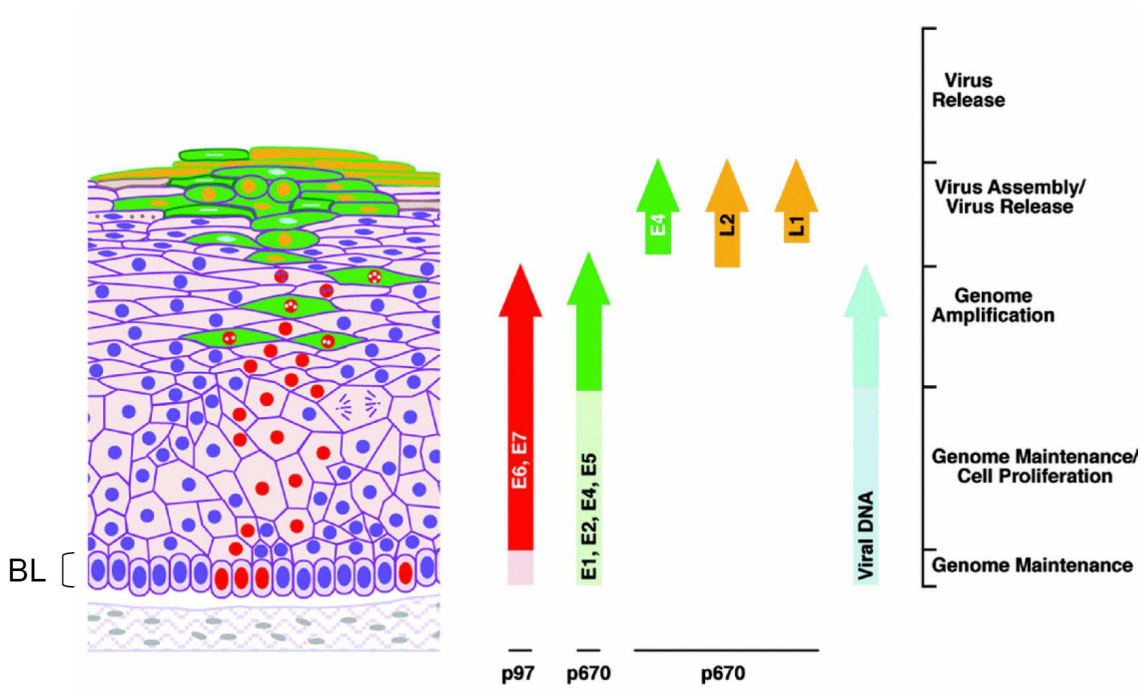


Figure 1.2: The HPV life-cycle

Papillomaviruses infect the epithelium by gaining entry to the cells in the basal layer (BL), where they establish themselves as low copy number extrachromosomal episomes in the nucleus of the cell. Early events in the life-cycle require expression of the E1 and E2 replication proteins, and E6 and E7 oncoproteins by the early promoter, p97. When infected cells exit the basal layer, they remain in cycle through the activities of E6 and E7. In the middle and upper layers, the cells differentiate, and expression from the late promoter p670 is activated. In these layers, E1, E2, E4 and E5 expression increases, leading to viral genome amplification. Following amplification, L1 and L2 are expressed, the viral DNA is packaged, and new infectious virions are shed from the surface of the epithelium. (The figure has been modified from Doorbar, 2006).

1.7.1. Site of infection – basal layer of the epithelium

The site of infection of HPVs is the basal layer of the epithelium. As mentioned earlier, neoplasia usually arises in the transformation zone of the cervix, where the squamous epithelium tapers down towards the columnar cells. This metaplastic change is thought to afford the virus easy access to the basal cells to initiate its life-cycle, and also has reduced immune surveillance compared to other cervical sites (Giannini *et al.*, 2002).

Basal cells are the only cells in the epithelium that are capable of division and are thought to be comprised of epithelial stem cells and transit amplifying (TA) cells. Epithelial stem cells are considered to be undifferentiated cells with enhanced proliferative capacity. These cells give rise to TA cells but can also self-renew and are thought to persist throughout the lifetime of the epithelium. TA cells on the other hand have a finite life-span and continue to differentiate (Potten & Loeffler, 1990). It has been suggested that for an infection to be persistent, an epithelial stem cell must be infected (Egawa, 2003; Schmitt *et al.*, 1996), although this has not been proven to date.

A model for how PVs gain entry to epithelial cells has recently been proposed by Schiller *et al*., and implicates both L1 and L2 capsid proteins in the process (Schiller *et al*., 2010). This model suggests that L1 first binds to heparan sulfate proteoglycans on the basement membrane, once this has been exposed by a micro-lesion in the tissue (Johnson *et al.*, 2009; Joyce *et al.*, 1999). This interaction brings about a conformational change, which subsequently exposes sites on the L2 capsid protein to being cleaved by enzymes, one of which being furin (Richards *et al.*, 2006). This is then thought to expose an L1 site that can bind to a cell-surface receptor, mediating the attachment of the virion to keratinocytes. The cell-surface receptor involved in this process has not yet been identified, but studies have implicated alpha-6 integrin (Evander *et al.*, 1997) in this process. Following attachment to the cell-surface receptor, PVs enter cells by endocytosis. However, which pathway is involved in this remains unclear as studies have implicated both clathrin- and caveolae-mediated pathways. These discrepancies may reflect differences between the various HPV types (Bousarghin *et al.*, 2003).

1.7.2. Early events

1.7.2.1. Establishment and maintenance of HPV episomes

Following infection, HPV genomes establish themselves as episomes in the nucleus of the infected basal cell. It is generally believed that for establishment to take place, the viral genome first undergoes a transient replication phase, whereby its copy numbers are amplified. Following the initial establishment phase, genomes are thought to replicate along with the cellular DNA and divide equally into the two daughter cells during cell division, thus maintaining a more or less constant copy number in the cells of the basal layer (reviewed in Kadaja *et al*., 2009).

1.7.2.2. Early proteins

Viral transcripts have been detected in the basal cells of epithelia (Stoler & Broker, 1986), although which viral proteins are actually expressed in these cells remains unclear. It is widely believed that the viral replication proteins E1 and E2 are expressed in basal cells from the early promoter, p97, and both of these proteins are necessary for the persistence of HPV16 viral episomes (Ken Raj, unpublished data). The role of these proteins in replication is well established. E2 binds to regions near the viral origin of replication and is able to recruit the E1 viral helicase, which in turn recruits the cellular replication proteins that are necessary for viral replication (reviewed in Kadaja *et al*., 2009). In addition to its role in viral replication, E2 has also been shown to have an important role during cell division by anchoring the viral episomes to mitotic chromosomes or the mitotic spindle, thus ensuring their correct segregation and localisation into the nucleus of daughter cells (Feeney & Parish, 2009). Episomal replication and segregation will be discussed in more detail later. Interestingly, several HPVs, as well as BPV1, were also found to be able to replicate in yeast, a process that was shown to be independent of both E1- and E2-expression (Angeletti *et al.*, 2002).

It is not clear which other viral proteins are expressed in the basal layer of the epithelium and this is partly due to the low levels of protein expressed, as well as to the lack of sensitive detection methods. However, studies in monolayer cell cultures have suggested that both the E6 and the E7 proteins are

necessary for the episomal persistence of HPV31 genomes in primary cells (Thomas *et al.*, 1999), and that E6 but not E7 is necessary for persistence of HPV16 episomes in the immortalised cell line, NIKS (Flores *et al.,* 2000; Laurson *et al*., 2010; and Ken Raj, unpublished data).These studies imply that both of these proteins are expressed in the basal layer, as the monolayer cell culture system is considered to be a good model for the study of the early events of the life-cycle (see section 1.8).

1.7.2.3. Cell proliferation

In the uninfected epithelium basal cells are eventually pushed to the suprabasal layers and exit the cell cycle. This would be detrimental to the virus as the virus relies on the cell's replication machinery in order to replicate its own genome. In an HPV infected epithelium, the normal differentiation program of the tissue is delayed, and cells in the suprabasal layers are pushed to cycle. This is thought to be caused by the combined activities of the E6 and E7 proteins, which push cells into S-phase (Cheng *et al.*, 1995; Dollard *et al.*, 1992). The activities of E6 and E7 that are thought to be responsible for driving suprabasal cell proliferation have been extensively studied and the most well characterised ones are the degradation of tumour suppressors p53 and pRb respectively (Boyer *et al.*, 1996; Scheffner *et al.*, 1990). Moreover, high-risk E6 is able to bind to and degrade PDZ proteins, such as hScrib and hDlg, via a PDZ-binding motif on its C-terminus. This motif has been linked to the development of epidermal hyperproliferation in transgenic mice (Nguyen *et al.*, 2003) and raft cultures (Lee & Laimins, 2004).

Both E6 and E7 have several other activities which can contribute to driving cells to proliferate and these will be discussed later.

1.7.3. Late events

In productive HPV infections, the suprabasal cells do eventually enter terminal differentiation and this is required for the induction of the late promoter, p670, and consequently the expression of the late proteins. It is not yet clear what causes the switch from early to late promoter, but it is believed to occur via changes in cell signalling.

1.7.3.1. Genome amplification

As the infected cell approches the upper layers of the epithelium, it becomes necessary for the viral genome to be amplified in preparation for packaging into the new virions. The switch from early to late promoter leads to an increase in the expression of the proteins necessary for genome amplification, E1, E2 (for replication) (Klumpp & Laimins, 1999), and E4 and E5 (Hummel *et al.*, 1992).

The roles of E4 and E5 in genome amplification are not very clear. E5 has been shown to contribute to the maintenance of a replication competent environment in the upper epithelial layers, and to facilitate genome amplification and transcription from the late promoter (Fehrmann *et al.*, 2003). E4 on the other hand has been shown to arrest cells in the G2 phase (Davy *et al.*, 2002). The current model suggests that amplificational replication takes place in cells that express E4 as well as E7. These cells are thought to be in a pseudo-S phase state, where cellular replication proteins are available for viral DNA replication but the cells themselves are not dividing (Davy & Doorbar, 2007)

1.7.3.2. Virus assembly and release

At the end of the viral life-cycle the two capsid proteins, L1 and L2, are expressed and localise to the nucleus (Day *et al.*, 1998; Doorbar & Gallimore, 1987; Florin *et al.*, 2002). This is followed by the encapsidation of the newly replicated viral DNA, and studies have suggested that this process may be enhanced by E2 (Day *et al.*, 1998; Zhao *et al.*, 2000). As HPVs are not lytic viruses the release of progeny virions relies on the natural shedding of dead skin cells (Bryan & Brown, 2001). Viral release from cells may be facilitated by the E4 protein which has been shown to disrupt the keratin network of the cells thereby making the cells more fragile (Doorbar *et al.*, 1991; Wang *et al.*, 2004).

1.7.4. Abortive infections

Abortive infections arise in situations where the viral life-cycle is not completed and if left untreated, these infections can develop into cancer. It is thought that abortive infections occur at specific sites where productive PV infections are not supported. An example of this is CRPV infections, which can cause productive

infections in the virus's natural host (the cottontail rabbit), but cause abortive infections when inoculated in another host, the domestic rabbit. Similarly cutaneous HPVs, which usually cause benign skin lesions, occasionally cause cancers when they infect a mucosal site. It has been considered that the transformation zone of the cervix may be a sub-optimal site for the completion of the life-cycle of high-risk HPV types, thereby making it more prone to the development of neoplasia (reviewed in Doorbar, 2006).

CIN-1 lesions closely resemble productive infections, as the life-cycle of the virus, although delayed, is eventually completed. In CIN-2 and CIN-3 lesions however, the tissue shows higher degree of deregulation and CIN-3 lesions closely resemble abortive infections. It has been found that the severity of a lesion is reflected in the pattern of viral protein expression. As lesions become less productive, there is less expression of the late viral proteins (Middleton *et al.*, 2003). Of particular importance is the deregulation of the expression of the two viral oncoproteins, E6 and E7, seen in abortive infections. This leads to cells in the upper-most layers of the epithelium (which would have normally exited the cell cycle during a productive infection) being pushed to cycle (Middleton *et al.*, 2003). Aberrant expression of E6 and E7 can lead to genomic instability. One way in which deregulation of E6 and E7 expression is achieved is by the integration of the viral genome into the cellular chromosomes, which is frequently observed in cancers (Durst *et al.*, 1985).

1.8. Papillomavirus models

The dependence of PVs on the stratified epithelium for the completion of their life-cycle has made the study of the life-cycle difficult. It is generally accepted that monolayer cell cultures of keratinocytes are good models for the study of the events that occur in the basal layer of the epithelium. HPV DNA (isolated from plantar warts) was first shown to replicate and persist episomally in cultured epidermal keratinocytes in 1982 (LaPorta & Taichman, 1982). Since then, many studies have used monolayer cell cultures as systems to study the early events in the viral life-cycle such as episomal persistence. Different cell isolates have been used, including primary foreskin keratinocytes (Thomas *et al.*, 1999) as well as immortalised cell lines, such as NIKS (Flores *et al.*, 1999).

However, monolayer cell cultures do not provide good models for the study of the late events in the viral life-cycle, such as genome amplification and virion production. Clinical samples are sometimes used to study HPV infections but these are difficult to obtain and may not contain a productive infection, as these samples are often isolated from patients with high-grade disease. Another hindrance to the study of PVs is the species specificity which makes several animal models unsuitable for the study of the human viruses. In addition to this, until very recently no PV that naturally infects laboratory mice had been isolated, making the most common laboratory animal model unavailable for studies. The recent isolation of such PV (Ingle *et al.*, 2010) promises to provide an important tool for PV research, with more reagents and more expertise being available for the mouse than any other laboratory animal model.

The study of the HPV life-cycle has been aided by the development of the organotypic raft culture system, a three-dimensional system in which keratinocytes are allowed to differentiate in culture to form a complete stratified epithelium, supported by a collagen dermal equivalent. These rafts support the viral life-cycle and have been used in studies aimed at characterising the different stages of the life-cycle (Flores *et al.*, 1999) as well as identifying factors that may affect it (Flores *et al.*, 2000; Lee & Laimins, 2004). Other methods that have been used to induce the differentiation of keratinocytes are the culturing of cells in medium with high calcium concentration (Hennings *et al.*, 1980) or in semi-solid medium such as methylcellulose (Ruesch *et al.*, 1998).

Raft cultures can also be used for the production of mature infectious viral particles (McLaughlin-Drubin *et al.*, 2004). Alternatively virions can be made by co-transfecting viral genomes with L1- and L2-expressing plasmids in monolayer cultures of 293T cells (Buck *et al.*, 2005). Production of infectious viruses to be used for infectivity studies, is still in early stages. Hence most studies employ other means for introducing viral genomes into cells, such as tranfections.

1.9. The E7 protein

The HPV E7 protein is a small protein (11 kDa for HPV16) and is one of the oncoproteins expressed by HPVs. It has been shown to be able to immortalise keratinocytes (Halbert *et al.*, 1991), and when expressed together with E6, can also transform them (Munger *et al.*, 1989a). Furthermore, HPV16 E7 was found to be able to induce hyperplasia when expressed on its own in the skin of transgenic mice (Herber *et al.*, 1996). Many studies have shown that E7 can interact directly with several cellular proteins and its biological effects are thought to be conferred by its protein-protein interactions. Interestingly, the HPV E7 protein shares many similarities with the SV40 large T antigen and the adenovirus E1A protein (DeCaprio *et al.*, 1988; Massimi *et al.*, 1996; Whyte *et al.*, 1988).

In a normal epithelium, the basal cells are the only cells capable of undergoing cell division. HPVs are dependent on the cellular replication machinery in order to replicate their DNA. Therefore, in an infected epithelium, it is essential that the suprabasal cells are kept in cycle, in order to support viral replication. A major role of E7 is to delay the terminal differentiation of the suprabasal cells, thereby keeping them in cycle (reviewed in McLaughlin-Drubin & Munger, 2009). The main activities of E7 are outlined below.

1.9.1. Association with retinoblastoma proteins and effects on cell cycle regulators

The best characterised activity of E7 is its ability to bind to the retinoblastoma family of proteins. E7 binds to pRb and pRb-related proteins p107 and p130 and targets them for proteasomal degradation (Boyer *et al.*, 1996; Davies *et al.*, 1993; Dyson *et al.*, 1989; Jones & Munger, 1997; Munger *et al.*, 1989b). Interactions with pRb appear to be a common feature of tumour virus proteins, as the SV40 large T antigen and the adenovirus E1A protein also bind to it (DeCaprio *et al.*, 1988; Whyte *et al.*, 1988). E7 preferentially degrades the hypophosphorylated form of pRb (Boyer *et al.*, 1996) and this is the form that interacts with the E2F transcription factor (Chellappan *et al.*, 1991.) Binding of hypophosphorylated pRb to E2F prevents the transactivation activities of E2F and when pRb gets phosphorylated, E2F is free to activate the transcription of

genes that promote entry into S-phase (reviewed in Donjerkovic & Scott, 2000). Targets of E2F include cyclins A and E, the proliferating cell nuclear antigen (PCNA), minichromosome maintainance proteins (MCMs) and DNA polymerase α (Cheng *et al.*, 1995; Chien *et al.*, 2000; Dyson, 1998; Leone *et al.*, 1998). By interacting with pRb, E7 interrupts the pRb-E2F binding, thus causing the release of free E2F. As a result, E7 is able to promote S-phase progression in suprabasal cells, which would have otherwise exited the cell cycle. This was demonstrated in the analysis of wild-type and E7-deficient HPV16 raft cultures which demonstrated a clear need for E7 in the induction of PCNA expression and DNA synthesis in suprabasal cells (Flores *et al.*, 2000).

The E7 proteins of low-risk viruses interact with pRb with a much lower efficiency than the E7 proteins of high-risk viruses and this correlates with their lack of transforming ability (Gage *et al.*, 1990; Heck *et al.*, 1992). Despite this, low-risk HPVs can also cause hyperproliferative lesions, suggesting that they too have a mechanism for causing aberrant entry into S-phase. It has been shown that whereas the E7 protein from the high-risk type HPV16 can degrade pRb as well as the related p107 and p130 proteins, the E7 protein from the low-risk type HPV6 can only degrade p130. Interestingly, in the uninfected epithelium, p130 is more abundant in differentiating cells, as opposed to pRb and p107, which are more abundant in undifferentiated cells (Zhang *et al.*, 2006). These observations suggest a mechanism by which both the high- and low-risk HPVs can promote the replication of their genomes in the undifferentiated epithelium.

Furthermore, both high- and low-risk E7 proteins have been shown to augment the activities of the cdk2/cyclin A and cdk2/cyclin E complexes, by directly binding to them (Arroyo *et al.*, 1993; He *et al.*, 2003; Nguyen & Munger, 2008; Tommasino *et al.*, 1993). The cdk2/cyclin E complex controls entry into S-phase whereas the cdk2/cyclin A complex controls progression through S-phase, and the G2/M transition (reviewed in Sherr, 1993). E7 also affects the activities of these complexes indirectly, by abrogating the activities of cyclin-dependent kinase inhibitors (CKIs), $p21^{CIP1}$ and $p27^{KIP1}$ (Funk *et al.*, 1997; Jones *et al.*, 1997; Zerfass-Thome *et al.*, 1996).

1.9.2. Association with other cellular binding partners

In addition to the above, E7 interacts with a variety of other cellular proteins, many of which have effects on cellular gene transcription. One of these interactions is with histone deacetylase 1 (HDAC-1) (Brehm *et al.*, 1999), which results in increase of E2F2-mediated transcription in differentiating keratinocytes (Longworth *et al.*, 2005). E7 also binds to AP-1 transcription factors, such as c-Jun, and up-regulates their transcriptional activities (Antinore *et al.*, 1996). Furthermore, E7 binds to the TATA box binding protein (TBP), another activity that it shares with the adenovirus E1A protein (Massimi *et al.*, 1996). This interaction was shown to inhibit TBP's ability to bind to DNA (Maldonado *et al.*, 2002), suggesting the inhibition of TBP's transcriptional activity, and has also been shown to correlate with E7's transforming ability (Massimi *et al.*, 1997).

1.9.3. Role in episomal persistence

In addition to its role in maintaining a replication-competent environment in the suprabasal layers of the epithelium, E7 has also been implicated in the persistence of viral episomes in the basal layer. This was suggested for HPV11 as well as HPV31, using monolayer cultures of primary cells (Oh *et al.*, 2004; Thomas *et al.*, 1999). Interestingly, studies using HPV16 showed that this is not true when an immortalised keratinocyte cell line (NIKS) is used instead (Flores *et al.*, 2000; Laurson *et al.*, 2010). This suggests that E7 may not have a direct role in episomal persistence, but its effect may instead be a consequence of its role in cellular immortalisation.

1.10. The E6 protein

The HPV E6 proteins are small proteins and for HPV16 approximately 18 kDa and 151/158 amino acids long (Foster *et al.*, 1994). E6 proteins contain two zinc-finger motifs which have been suggested to play a role in protein stability and localisation (Kanda *et al.*, 1991) (Fig.1.3). No enzymatic activity of E6 is known and, as with E7, most of its activities are thought to occur through protein-protein interactions, although E6 has also been shown to have DNA-binding abilities (Ristriani *et al.*, 2000). Recent studies have demonstrated that

E6 can dimerise or oligomerise *in vivo*, however, whether or not the monomeric and oligomeric forms have different functions remains unclear (Garcia-Alai *et al.*, 2007; Zanier *et al.*, 2010).

Studies of the E6 protein have been hindered due to the low levels of protein thought to be expressed, as well as the lack of sensitive antibodies. However, E6 is still one of the best studied HPV proteins and many of its activities have been well characterised. Similarly many cellular binding partners of E6 have been identified and these include proteins that E6 targets for proteasomal degradation. These interactions implicate E6 in a variety of cellular pathways the consequences of which include blocking of apoptosis, evasion of immune surveillance and chromosomal instability. In fact, HPV16 E6 has been shown to be able to induce cancers when expressed alone in the skin of transgenic mice (Song *et al.*, 1999) and interestingly, its PDZ-binding motif was found to be necessary for this (Nguyen *et al.*, 2003).

Not much is known about the regulation of the levels of E6 in HPV-infected cells. The levels of high-risk E6 proteins are regulated by the proteasome, however results vary with regards to low-risk E6 proteins (Kehmeier *et al.*, 2002; Stewart *et al.*, 2004). Moreover, a recent study has shown that HPV16 and 18 E6 proteins are stabilised by their interactions with the E6-associated protein (E6AP) (Tomaic *et al.*, 2009b). The main activities of E6 are outlined below.

Figure 1.3: Sequence of the 158 amino acid product of the HPV16 E6 ORF

The E6 protein of HPV16 is 151/158 amino acids long, depending on which of the two ATG codons is used in translation. The two methionines that could potentially represent the first amino acid of E6 are shown in red and are marked as amino acids 1 and 8. The 2 zinc-finger motifs are shown in the boxes. The PDZ-binding motif at the C-terminus is shown in blue.

1.10.1. Binding to E6AP

E6 proteins from both high- and low-risk HPV types have been shown to bind to E6AP, which is a cellular E3 ubiquitin ligase (Brimer *et al.*, 2007; Huibregtse *et al.*, 1991). Through its interaction with E6AP, E6 is able to direct the degradation of many of its cellular binding partners, for example p53.

More recently, it has been shown that E6-E6AP binding promotes the ubiquitination and proteasomal degradation of E6AP (Kao *et al.*, 2000). As the catalytic activity of E6AP was found to be necessary for this, it has been suggested that through its interaction with E6, E6AP ubiquitinates itself and targets itself for degradation. Contributing further to the understanding of the interaction between E6 and E6AP, a recent study found that E6AP is able to stabilise E6, in a manner independent of the former's catalytic activity (Tomaic *et al.*, 2009b). Taken together, these results demonstrate the complex relationship between E6 and its binding partners, the outcome of which is probably influenced by many factors, including signalling, differential protein localisation in the cell and differences in the levels of each protein at various stages of the viral life-cycle and disease progression. Many cellular proteins have been shown to be destabilised by the presence of E6, however, the Tomaic et al. paper is the first report of a protein that alters the stability of E6 itself. This opens new doors for studying possible effects of other cellular proteins on E6.

1.10.2. Association with p53

The best characterised activity of E6 is its ability to bind to p53 (Werness *et al.*, 1990). p53 is a transcription factor as well as one of the key signal transducers during times of cellular stress. It is usually present at low levels, and kept in an inactive state. It is activated when cellular stress is detected, and initiates a cascade of events leading to cell cycle arrest, DNA repair and even apoptosis, if the damage is too large to repair (reviewed in Gottlieb & Oren, 1996). Interaction with p53 appears to be a common feature amongst proteins from different DNA tumour viruses, as both the SV40 large T antigen and the adenovirus E1B 55 kDa protein have also been shown to bind to this protein (Lane & Crawford, 1979; Sarnow *et al.*, 1982). Interestingly though, whereas the

SV40 large T antigen and the adenovirus E1B 55 kDa protein stabilise p53, the high-risk HPV E6 proteins degrade it (Oren *et al.*, 1981; Scheffner *et al.*, 1990; van den Heuvel *et al.*, 1993).

Due to p53's essential functions in preventing replication of damaged DNA, it is not surprising that over 50% of cancers have mutations in the p53 gene (Vogelstein *et al.*, 2000). p53 is also activated when cells inappropriately enter the cell cycle. As mentioned earlier, one of the functions of E7 is to push suprabasal cells to divide. As this is not a "natural" occurrence, p53 is activated in order to prevent it, and it has been shown that cells that express E7 alone have higher levels of p53 than control cells (Demers *et al.*, 1994; Laurson *et al.*, 2010). HPVs therefore need to circumvent this check-point and they do so by the E6-mediated degradation of p53. As such, most HPV-induced cancers actually harbour wild-type p53 genes (Crook *et al.*, 1991; Scheffner *et al.*, 1991).

In HPV-negative cells the levels of p53 are kept tightly regulated and the protein is degraded by the ubiquitin-proteasome, and via interaction with the cellular E3 ubiquitin ligase Mdm-2 (Haupt *et al.*, 1997; Honda *et al.*, 1997). Interestingly, in HPV-positive cells the degradation of p53 by E6 is mediated though the binding of E6 to p53 and E6AP (Huibregtse *et al.*, 1991; Scheffner *et al.*, 1993). E6AP does not target p53 in the absence of a high-risk E6 protein (Huibregtse *et al.*, 1991). By binding to both E6AP and p53, E6 alters the target specificity of E6AP, and causes the proteasomal degradation of p53 by a new pathway. Although the role of E6AP in E6-mediated p53-degradation by the ubiquitin-proteasome pathway seems well established, more recent reports suggest that E6AP-independent (Massimi *et al.*, 2008) and even ubiquitin-independent (Camus *et al.*, 2007), pathways may also be involved.

E6 proteins from high- and low–risk HPVs bind to p53, however, only high-risk types degrade p53 (Foster *et al.*, 1994; Scheffner *et al.*, 1990). The difference is thought to lie in the specific regions of p53 that interact with E6 and it had been suggested that E6 proteins from high- and low-risk types can bind to the C-terminus of p53, while only high-risk types can bind to its core DNA-binding region (Li & Coffino, 1996). A recent study showed that upon expression of low-

risk HPV11 E6 protein, p53 is predominantly present in the cytoplasm (Sun *et al.*, 2008), suggesting that even without inducing the degradation of p53, low-risk viruses may still have an effect on its function.

High-risk E6 proteins also employ other approaches to inhibit the activities of p53, which do not involve its degradation. For example, they interact with histone acetyltransferases CBP/p300 and hADA3, the latter of which they also degrade, (Kumar *et al.*, 2002; Patel *et al.*, 1999) which are p53 co-activators.

Several studies have used mutants of the E6 protein in an attempt to identify the precise region of E6 necessary for the degradation of p53. One such study found that most N-terminal mutations of E6, as well as mutations in either of the two zinc-fingers, inhibit the degradation of p53, whereas mutations in the C-terminus of E6 do not. This study also showed that wild-type E6 and E6 mutants that retained the ability to degrade p53, showed abrogation of actinomycin D-induced growth arrest. On the other hand, E6 mutants that were unable to degrade p53 responded to actinomycin-D (low levels of which cause DNA breaks) by inducing growth arrest (Foster *et al.*, 1994). This highlights the importance of p53-degradation by E6 in overcoming cellular stress. Interestingly, the truncated form of E6, E6*, has been shown to bind to full-length E6 and inhibit E6-mediated degradation of p53 (Pim *et al.*, 1997). The same authors also reported that exogenous expression of E6* has been shown to inhibit proliferation of HPV-positive cells (Pim *et al.*, 1997). These findings suggest a dominant negative role for E6* over full-length E6 with rergards to p53-degradation and cell proliferation.

1.10.3. Inhibition of apoptosis

A consequence of E6-mediated p53-degradation is bypass of apoptotic signalling and subsequent death of HPV-infected cells. E6 proteins from low-risk types are unable to degrade p53, however, results from a recent study suggest that low-risk E6 proteins may bypass the p53-induced apoptotic response by degrading the acetyltransferase TIP60 (Tat-interacting protein 60 kDa) (Jha *et al.*, 2010). TIP60 has been shown to be necessary for the p53-dependent activation of the proapoptotic factor Puma (p53 up-regulated

modulator of apoptosis) (Tang *et al.*, 2006b) and Jha et al. reported that in the presence of high- or low-risk E6 in cells Puma expression was not induced (Jha *et al.*, 2010).

E6 is also able to bypass apoptosis by interfering with both the intrinsic and the extrinsic apoptotis pathways. The extrinsic apoptotic pathway is activated by viral infections, and E6 has been shown to inhibit this signalling cascade by interacting with and degrading key components of the pathway, such as the Fas-associated death domain (FADD) and procaspase 8 (Filippova *et al.*, 2004; Garnett *et al.*, 2006). The intrinsic apoptotic pathway senses signals from within the cell, such as from DNA damage and oxidative stress. Activation of the intrinsic pathway results in the activation of pro-apoptotic proteins and subsequent release of mitochondrial proteins, such as cytochrome *c* and apoptosis-inducing factor (AIF) into the cytosol (reviewed in Elmore, 2007). E6 proteins from high-risk, low-risk and cutaneous HPVs have been shown to induce the proteasomal degradation of Bak, the protein that forms the mitochondrial pores during apoptosis (Jackson *et al.*, 2000; Thomas & Banks, 1998; Thomas & Banks, 1999). As a result of this, there is no disruption of the mitochondria, no release of mitochondrial proteins and no activation of the effector caspases. By targeting both the intrinsic and extrinsic apoptotic pathways, E6 can ensure that the infected cell circumvents any apoptotic signals it may receive.

1.10.4. Disruption of cell-cell adhesion and polarity

The stratified epithelium is made up of many layers of cells, each at a different stage of differentiation. Therefore, it is important that the cellular organisation of the epithelium is very tightly regulated to ensure that the correct signals for proliferation and differentiation reach the correct cells. This involves the establishment of correct contacts between neighbouring cells, as well as between cells and the extracellular matrix (ECM) and also, the regulation of apico-basal polarity of epithelial cells (Bilder, 2004). For example only basal cells are attached to the extracellular matrix of the basement membrane and receive proliferation signals. Once a cell exits the basal layer, it stops receiving proliferation signals, and starts receiving signals for differentiation. As discussed

earlier, HPVs have many ways in which to keep suprabasal cells in cycle so that the viral DNA is able to keep replicating. Some of these involve the disruption of cell adhesion and polarity by E6.

1.10.4.1. The PDZ proteins – hScrib, hDlg and MAGI

The PDZ-domain containing proteins (PDZ proteins) are a large group of cellular proteins that contain one or more of the structural PDZ domains (named after PSD95, DlgA and ZO-1 proteins) involved in protein-protein binding. Some PDZ proteins have been shown to interact with viral oncoproteins, such as the E6 protein of high-risk HPVs, via the PDZ-binding motifs of the latter. Certain PDZ proteins have been implicated in the regulation of epithelial polarity or are scaffold proteins that are involved in the organisation of multi-protein membrane structures (Thomas *et al.*, 2008). These include hScrib, hDlg and MAGI-1, -2 and -3, which are the best characterised PDZ proteins with respect to their interaction with E6 and have been shown to be targeted for proteasomal degradation, by way of this interaction (Gardiol *et al.*, 1999; Glaunsinger *et al.*, 2000; Kiyono *et al.*, 1997; Lee *et al.*, 1997; Nakagawa & Huibregtse, 2000; Thomas *et al.*, 2002).

hScrib and hDlg are human homologues of the well studied *Drosophila melanogaster* genes *scribble* and *discs large* respectively (Dow *et al.*, 2003; Lue *et al.*, 1994). They are members of the Scribble polarity complex, which localises to the basolateral membrane and is implicated in the formation of adherens junctions (Bilder & Perrimon, 2000; Firestein & Rongo, 2001; Woods *et al.*, 1996). As such, they are involved in keeping the ordered structure of the epithelium as well as in signal transduction (Humbert *et al.*, 2003). Importantly, loss of cell polarity is a common feature in human cancers (Gardiol *et al.*, 2006; Javier, 2008) and it is thus not surprising that these polarity proteins have been shown to interact with proteins from various oncogenic viruses such as the HPV E6 protein, the Human T-lymphotropic virus Type 1 (HTLV-1) Tax protein and the Adenovirus 9 E4ORF1 protein (Glaunsinger *et al.*, 2000; Lee *et al.*, 1997).

In addition to their role in polarity, hScrib and hDlg (and their *Drosophila* counterparts) are important in controlling cell proliferation and have been

classified as tumour suppressors (Bilder *et al.*, 2000; Dow *et al.*, 2003; Woods & Bryant, 1989). Mutations of these proteins in both *Drosophila* and mammals have been shown to cause aberrant growth and loss of tissue morphology and differentiation (Bilder *et al.*, 2000; Woods & Bryant, 1989; Zhan *et al.*, 2008). Suggested ways in which loss of polarity proteins could affect growth include deregulation of signalling pathways and loss of contact inhibition (Bilder *et al.*, 2000).

hScrib and hDlg are also implicated as tumour suppressors in studies that have shown them to bind to the adenomatous polyposis coli (APC) protein, via the PDZ-domains of the former and the PDZ-binding motif of the latter (Matsumine *et al.*, 1996; Takizawa *et al.*, 2006). APC is a tumour suppressor that regulates cell cycle progression (Baeg *et al.*, 1995) and is part of the Wnt pathway, where its role is to keep the levels of free β-catenin low in the absence of Wnt signals (reviewed in Logan & Nusse, 2004). Binding of both hScrib and hDlg to APC has been shown to contribute to the negative regulation of the cell-cycle (Ishidate *et al.*, 2000; Nagasaka *et al.*, 2006). Disruption of this interaction by E6 (Kiyono *et al.*, 1997; Takizawa *et al.*, 2006) would be expected to result in deregulated growth.

The recruitment of both hScrib and hDlg to sites of cell-cell contact appears to be dependent on E-cadherin (Navarro *et al.*, 2005; Reuver & Garner, 1998). Non-membrane forms of hDlg have also been identified, including nuclear and cytoplasmic forms (Garcia-Mata *et al.*, 2007; McLaughlin *et al.*, 2002; Roberts *et al.*, 2007). These seem to be at least in part regulated by cellular differentiation, suggesting different functions of hDlg at different stages of differentiation or even a role for hDlg in the differentiation process (Roberts *et al.*, 2007; Watson *et al.*, 2002). Moreover, different phosphorylated forms of hDlg have been identified (Mantovani *et al.*, 2001; Massimi *et al.*, 2006) some of which exhibit differential cellular localisation. For example, hDlg that is phosphorylated following osmotic stress localises to sites of cell-cell contact (Massimi *et al.*, 2006). Interestingly, phosphorylated forms of hDlg were found to be more susceptible to degradation by HPV E6 proteins (Massimi *et al.*, 2006; Narayan *et al.*, 2009b). Moreover, hDlg was shown to be a substrate for phosphorylation by CDKs 1 and 2 (Narayan *et al.*, 2009a) and its localisation appears to change

during the cell-cycle with one study showing it to be predominantly on the membrane during G1, and on the mitotic spindle in mitosis (Narayan *et al.*, 2009a). Together these studies suggest the existence of distinct cellular pools of hDlg, with different modifications and functions and hint at the complexity of this multifunctional protein.

Loss of polarity has been linked to the development of a more invasive phenotype (Humbert *et al.*, 2003) and this is supported by observations that aggressive cancers harbour lower levels of hDlg (Watson *et al.*, 2002). Studies using mutants of *Drosophila* Scribble or hScrib have shown that its deregulation alone is not enough to cause an invasive phenotype, and additional mutations in oncogenes, such as Ras or Notch, are thought to be necessary for this (Brumby & Richardson, 2003; Dow *et al.*, 2008). One way in which hScrib has been shown to regulate cell migration is by blocking the Ras-activated MAPK signalling pathway. Loss of hScrib in conjunction with activation of Ras, activates this pathway and promotes cell invasion (Dow *et al.*, 2008).

Another group of PDZ proteins that have been shown to interact with E6 are the MAGIs (Glaunsinger *et al.*, 2000; Thomas *et al.*, 2002). E6 targets two pools of MAGI, a nuclear and a membrane pool, suggesting that both may be detrimental for the viral life-cycle (Kranjec & Banks, 2010). Not much is known about the nuclear pool of MAGI, however the membrane pool is involved in tight junction formation (Ide *et al.*, 1999; Thomas *et al.*, 2008). Tight junctions regulate important signalling pathways that are involved in both cell proliferation and differentiation (Matter & Balda, 2003). Loss of MAGI in HPV-infected cells may thus deregulate these pathways.

Membrane bound MAGI plays a role in the localisation of the tumour suppressor protein PTEN, by interacting with it via the PDZ domains of the former and the PDZ-binding motif on the latter (Kotelevets *et al.*, 2005; Wu *et al.*, 2000b; Wu *et al.*, 2000c). Interestingly, PTEN is also stabilised via its interaction with MAGI (Valiente *et al.*, 2005; Wu *et al.*, 2000b). PTEN down-regulates signalling pathways that promote cell growth (Marte & Downward, 1997; Stambolic *et al.*, 1998) and PTEN mutations have been shown to lead to a decrease in apoptosis or an increase in cell growth (Stambolic *et al.*, 1998), both of which can result in

tumorigenesis. Therefore, the disruption of the interaction between MAGI and PTEN is another way in which E6 can promote malignancy.

In addition to the above, other PDZ proteins have also been shown to bind to (and sometimes be degraded by) high-risk E6. These include PATJ (Storrs & Silverstein, 2007), which is involved in tight junction formation, and MUPP-1 (Lee *et al.*, 2000), which localises to tight junctions and is involved in signalling. Other targets include TIP-1 (Hampson *et al.*, 2004), TIP-2 (Favre-Bonvin *et al.*, 2005), PTPN3 (Jing *et al.*, 2007), PTPN13 (Spanos *et al.*, 2008), which are involved in signalling pathways, and CAL (Jeong *et al.*, 2007), which is involved in trafficking of membrane proteins.

1.10.4.2. Degradation of PDZ-proteins by E6

The high-risk E6 oncoproteins have a class I PDZ-binding motif (X-S/T-X-V/L) at their extreme C-terminus (Kiyono *et al.*, 1997; Lee *et al.*, 1997). Several high-risk HPV E6 proteins have been shown to possess a PDZ-binding motif, and binding to PDZ proteins has been implicated in E6's tumorigenic activities (Nguyen *et al.*, 2003). Furthermore, this C-terminal motif is shared by oncoproteins of other tumour viruses such as the Adenovirus E4 ORF1 protein and the HTLV-1 Tax protein (Lee *et al.*, 1997) as well as by the NS1 proteins of influenza viruses (Obenauer *et al.*, 2006).

The low-risk HPV E6 proteins lack a PDZ-binding motif and consequently are unable to bind to PDZ proteins (Kiyono *et al.*, 1997; Lee *et al.*, 1997; Nakagawa & Huibregtse, 2000). Intriguingly, recent studies have suggested the degradation of PDZ proteins by the E6* splice variant of E6 (which lacks the PDZ-binding motif) in the absence of full-length E6 (Pim *et al.*, 2009; Storrs & Silverstein, 2007). Some controversy exists with respect to whether or not E6* can directly bind to the PDZ proteins, however this may be reflective of the different PDZ proteins that were investigated in each study.

Different studies have also looked at whether E6AP is involved in the degradation of PDZ proteins by E6, and the results vary. One study suggested that the E6-E6AP complex is involved in the degradation of hScrib (Nakagawa

& Huibregtse, 2000) whilst others suggested that the degradation of hDlg, hScrib, MAGI and PATJ may be E6AP-independent (Grm & Banks, 2004; Massimi *et al.*, 2008; Pim *et al.*, 2000; Storrs & Silverstein, 2007). It is probable that different mechanisms may be involved in the E6-mediated degradation of cellular proteins. This is also supported by data showing E6AP-independent degradation of p53 (Massimi *et al.*, 2008). E6 may therefore interact with other ubiquitin ligases, apart from E6AP and a recent report has identified the ubiquitin ligase EDD as a novel cellular binding partner of E6 (Tomaic *et al.*, 2011).

E6 proteins from different high-risk HPV types have been shown to have varying affinities to PDZ proteins. HPV16 E6 preferentially binds to and degrades hScrib, with minimal effect on hDlg, whereas the contrary is true for HPV18. This is due to the single amino acid variation in the PDZ-binding motifs of the two E6 proteins (Thomas *et al.*, 2005). Interestingly MAGI was found to be targeted for degradation efficiently by both HPV16 and HPV18 (Kranjec & Banks, 2010).

Most studies looking at the effect of high-risk E6 proteins on PDZ proteins have been carried out using the E6 proteins from HPV16 or 18. Whether other high-risk E6 proteins have the same effects, needs to be determined. Moreover, most such studies have been carried out *in vitro* or using over-expression systems. Whether endogenous PDZ proteins are targeted for degradation by episomally-expressed E6 remains unclear. In fact, a study using HPV31-transfected keratinocytes, reported that there was no significant difference in the levels of PDZ proteins between un-transfected and transfected cells (Lee & Laimins, 2004). However, the authors did report lower viral copy numbers and a slower growth rate of cells harbouring a PDZ-binding mutant HPV31 genome, compared to cells harbouring the wild-type HPV31 genome (Lee & Laimins, 2004).

Although the potential tumorigenic effects of the interaction between E6 and PDZ proteins have been discussed, the role of this interaction in the viral life-cycle remains unclear. One role however may be in the persistence of viral genomes in the basal layer of the epithelium (Lee & Laimins, 2004).

1.10.4.3. Interaction with focal adhesion molecules

In addition to PDZ proteins, E6 from different PVs have been shown to interact with paxillin and zyxin, which are focal adhesion proteins and are involved in signal transduction (Degenhardt & Silverstein, 2001; Tong & Howley, 1997). Binding of E6 to these proteins results in breakdown of the actin cytoskeleton and consequently the cell structure, and could contribute to cell transformation.

1.10.5. Induction of telomerase activity

Expression of high-risk E6 protein can lead to cell immortalisation (Band *et al.*, 1991) and one of the mechanisms employed is activation of telomerase. Telomerase is a ribonucleoprotein whose function is to extend the telomeric ends of chromosomes. It is inactive in most cells and the continuous shortening of the telomeres with each cell division eventually leads to cell senescence. Telomerase is made up of an RNA template (TERC) and a catalytic subunit (hTERT). hTERT activity can be detected in the majority of immortalised cells and cancers (reviewed in Wai, 2004).

E6 induces telomerase activity in different cells including human keratinocytes and this is independent of E6's ability to degrade p53 (Klingelhutz *et al.*, 1996). Telomerase activity is induced by up-regulation of hTERT transcription (Veldman *et al.*, 2001) as well as by direct interaction of E6 with telomerase and with telomeric DNA sequences (Liu *et al.*, 2009).

It has also been suggested that E6-mediated activation of telomerase may be E6AP-dependent (Liu *et al.*, 2005), however, other studies have suggested that there may also be an E6AP-independent pathway (Sekaric *et al.*, 2008). Moreover, E6 and E6AP have been shown to promote histone acetylation of the hTERT promoter, as well as affect many of its activators and repressors (Howie *et al.*, 2009; James *et al.*, 2006).

1.10.6. Association with other cellular binding partners

In addition to its more well-characterised binding partners mentioned above, E6 also interacts with other cellular proteins. Some of these interactions correlate

with the oncogenic potential of the E6 protein, thus suggesting roles in HPV-mediated cancer progression. One protein that E6 binds to is E6-targeted protein 1 (E6TP1), a GTPase-activating protein that E6 has been shown to degrade by means of E6AP (Gao *et al.*, 1999; Gao *et al.*, 2002). Only high-risk E6 proteins were found to degrade E6TP1 (Gao *et al.*, 1999).

Other interactions of E6 have more defined implications for cancer progression, and these include proteins involved in maintaining chromosome stability. One such protein is minichromosome maintenance 7 (MCM7), which both high- and low-risk E6 proteins have been shown to bind to (Kukimoto *et al.*, 1998) and HPV18 E6 has been suggested to degrade (Kuhne & Banks, 1998). MCM7 has a role in ensuring that cellular DNA only replicates once per cell cycle (reviewed in Chong *et al.*, 1996), thus disruption of this control is thought to contribute to genomic instability. Furthermore, E6 interacts with proteins involved in DNA repair, such as XRCC1 (Iftner *et al.*, 2002), and O(6)-methylguanine-DNA methyl-transferase (MGMT) (Srivenugopal & Ali-Osman, 2002), the latter of which E6 also degrades. These activities of E6 have therefore been suggested to sensitise HPV-positive cells to DNA damage and consequently promote genetic instability.

As mentioned earlier, E6 interferes with the interaction of APC with PDZ proteins (Kiyono *et al.*, 1997; Takizawa *et al.*, 2006), and this may in turn affect signalling via the Wnt pathway. Interestingly, a recent study has shown that E6 expression can also affect the Wnt pathway in a manner that is independent of APC (Lichtig *et al.*, 2010). Furthermore, E6 has been shown to down-regulate the levels of E-cadherin, which can also deregulate the Wnt pathway (Matthews *et al.*, 2003).

Although these activities of E6 have potential implications for cancer development, their roles in the viral life-cycle remain unclear.

1.10.7. Effects on viral transcription

In section 1.10.3 I mentioned the E6-mediated degradation of TIP60 (Jha *et al.*, 2010). TIP60 is involved in cellular transcription, apoptosis and activation of

DNA-damage pathways. (Sun *et al.*, 2005; Sykes *et al.*, 2006; Tang *et al.*, 2006b). Experiments in HeLa cells showed TIP60 to be a repressor of the viral early promoter (measured by the levels of E6 mRNA) (Jha *et al.*, 2010). This led the authors to suggest that by degrading TIP60, E6 may be able to counteract this repression, thus promoting its own expression as well. It is important to note however that the viral promoter is regulated differently depending on the physical state and conformation of the viral DNA (Bechtold *et al.*, 2003; Schmidt *et al.*, 2005). Therefore, these results may be true for cancer cell lines but may not hold true for cell lines in which the HPV DNA is episomal.

1.10.8. E6 localisation

E6 has been shown to have activities in the cytoplasm, such as binding to E6AP, as well as in the nucleus, such as binding to transcriptional regulators. Several studies have looked at the localisation of E6 in cells however their results differ. E6 has been reported to be primarily nuclear, or nuclear and membrane-associated, when expressed in COS cells (Kanda *et al.*, 1991; Sherman & Schlegel, 1996) or insect cells (Daniels *et al.*, 1998; Grossman *et al.*, 1989). In contrast, it has also been shown to be both nuclear and cytoplasmic in cervical lesions (Tosi *et al.*, 1993) and perinuclear in the HPV16-positive cervical cancer line SiHa (Daniels *et al.*, 1998). In yet another study, E6 was reported to be primarily cytoplasmic in the HPV16- or 18- integrated cervical cancer lines HeLa, CaSki and SiHa, where it was shown to co-localise with p53 (Liang *et al.*, 1993), but primarily nuclear in transiently transfected COS cells, where it was again shown to co-localise with p53 (Lechner *et al.*, 1992). More recently, Tao *et al.* identified three nuclear localisation signals (NLSs) on the HPV16 E6 protein, which they authors suggest play an active role in driving the protein to accumulate in the nucleus (Tao *et al.*, 2003). It is not yet clear if the inconsistency between these studies is due to the different cell lines or techniques used.

1.11. Immune response and infection clearance

1.11.1. Regulation of the immune response

The HPV life-cycle has some important features that "protect" the virus from being cleared by the immune system. One such feature is the lack of inflammation following HPV infection. This is because the virus is not lytic, and also because the infected keratinocytes are cells that are already destined to die. Moreover, there is no viraemia, as both the infection and the shedding of the progeny virions takes place away from the blood and lymphatic systems (reviewed in Stanley, 2009).

Moreover, HPVs have been shown to interfere with interferon- (IFN) signalling. E7 has been shown to interfere with the transcriptional activity of the IFN-stimulated gene factor 3 complex (Barnard & McMillan, 1999) and to also bind to IFN regulatory factor-1 (IRF-1) and abrogate its transcriptional activity (Park *et al.*, 2000). E6 has been shown to do the same to IRF-3 (Ronco *et al.*, 1998). Microarray analysis of HPV31-positive cells has indicated a significant down-regulation of IFN-inducible genes, such as Stat-1, compared to HPV-negative cells (Chang & Laimins, 2000) and a similar study using HPV16 suggests that this may be at least in part due to expression of E6 (Nees *et al.*, 2001). Moreover, E6 has been shown to interact with Tyk2 thereby reducing Jak-Stat activation by IFN-α (Li *et al.*, 1999) and E6 and E7 inhibit transcription of toll-like receptor 9 (TLR9) and consequently abrogate its signalling pathway (Hasan *et al.*, 2007).

Furthermore, in HPV-induced lesions there is a decrease in the levels of E-cadherin, compared to normal epithelium, and this correlates with reduced infiltration of antigen-presenting Langerhans cells (Hubert *et al.*, 2005). The depletion of E-cadherin by HPV has been attributed to the activities of both E6 (Matthews *et al.*, 2003) and E7 (Laurson *et al.*, 2010). Interestingly, down-regulation of E-cadherin is a feature that HPVs share with other tumour viruses, such as Epstein-Barr virus (EBV) (Fahraeus *et al.*, 1992), Hepatitis B (Liu *et al.*, 2006) and Hepatitis C (Iso *et al.*, 2005) viruses (HBV and HCV). Importantly, a potential consequence of this is the reduction of the anti-viral immune response.

In addition to E6 and E7, E5 is also involved in immune evasion, as it has been shown to decrease the levels of MHC I molecules on the surface of W12 cells. As a result, antigen presentation is inhibited, and cell recognition by the immune system is reduced (Campo *et al.*, 2010).

1.11.2. Infection clearance

HPV infections are thought to occur very frequently, seen in as much as 80% of some adolescent populations (Brown *et al.*, 2005), yet the incidence of high-grade disease is considerably lower. It is thought that neoplasias of even the highest grade (CIN-3) can naturally regress, however the probability that a lesion progresses to cancer increases with the severity of the neoplasia. It has been suggested that, if not treated, 40% of CIN-3 lesions may eventually develop into cancer (Peto *et al.*, 2004).

Despite the abovementioned methods that HPVs use to evade immune responses, it is widely thought that most HPV infections are naturally cleared. This is believed to occur either via the self-limiting nature of the viral life-cycle, or via the mounting of an immune response (Schiffman & Kjaer, 2003). The role of the immune system in infection clearance is also highlighted by the higher incidence of infection and disease progression in immunocompromised individuals, such as HIV patients and organ transplant patients who are given immune-supressants (Palefsky *et al.*, 2006).

Histological analyses of genital warts have revealed infiltration of both T cells and macrophages into the wart, which undoubtedly contribute to the eventual regression of the wart (Coleman *et al.*, 1994). Furthermore, CD4+ T cell responses to HPV E2 and E6 have been implicated in viral clearance, and these were found to be reduced in patients with high-grade lesions (Welters *et al.*, 2003; Welters *et al.*, 2006). Importantly, the L1 capsid protein has been found to be very immunogenic, allowing the development of two HPV prophylactic vaccines. These make use of L1 virus-like particles (VLPs) to generate anti-L1 antibodies, thereby conferring protection to future HPV infection (Stanley, 2009).

1.12. Persistence

Despite the successful clearance of most HPV infections, in some cases the infection can persist, and there appears to be a correlation between this persistence and the development of high-grade disease or cancer (Remmink *et al.*, 1995; Schiffman & Kjaer, 2003). This is thought to occur due to the prolonged expression of E6 and E7, whose combined activities can lead to the accumulation of mutations and genomic instability.

As mentioned earlier, the combined activities of E6 and E7 cause aberrant cell proliferation and also block several important cell-cycle check-points, all of which can promote the accumulation of mutations in the cell. In addition, the expression of E6 and E7 has been shown to cause abnormal centrosome and mitotic spindle formation in HPV-positive cells thereby promoting genomic instability and aneuploidy (Duensing *et al.*, 2000; Duensing *et al.*, 2001; Heselmeyer *et al.*, 1996).

Persistence of viral DNA in the tissue may be accompanied by a continuing productive infection. Alternatively, viral DNA may persist in the basal layer of the epithelium as a latent infection, without any signs of disease (Abramson *et al.*, 2004; Maglennon *et al.*, 2011). Persistence of viral episomes in the basal layer of the epithelium is necessary whether for lasting and symptomatic infections, or for latent infections. Several studies have identified the need for the expression of specific viral proteins in order for episomes to persist. Both of the replication proteins, E1 and E2 have been found to be necessary (Ken Raj, unpublished data) and, for E1, it has been suggested that its export from the nucleus may also be necessary for this (Fradet-Turcotte *et al.*, 2010). Furthermore, E6 and E7 were found to be necessary for the persistence of both low- and high-risk HPV episomes (Laurson *et al.*, 2010; Oh *et al.*, 2004; Thomas *et al.*, 1999), although it appears that, for E7, this may not be the case in immortalised cells (Flores *et al.*, 2000; Laurson *et al.*, 2010). E4 and E5 were found to be dispensable for episomal persistence (Fehrmann *et al*., 2003; Genther *et al*., 2003; and Ken Raj, unpublished data).

In order for extrachromosomal episomes to persist in dividing cells, they must both replicate and actively segregate into the two daughter cells upon cell division and subsequently localise to the newly formed nuclei.

1.12.1. Replication

Apart from expressing E1 and E2, HPVs rely entirely on the cellular replication machinery to replicate their DNA. The replication for PVs has been proposed to consist of three different phases. In the first phase following infection the PV episomes replicate faster than cellular DNA resulting in an increase of viral genomes per cell (Lusky & Botchan, 1986). This initial amplification phase is often referred to as establishment, and is followed by a maintenance phase, during which the viral genomes remain at a more or less constant number per cell. Both the establishment and maintenance phases are thought to take place in the basal cells. In the upper layers of the epithelium, the cells stop dividing and the viral genomes undergo amplificational replication again, where their copy numbers increase dramatically.

The initial establishment phase is thought to require the presence of two viral proteins, E1 and E2, and the viral origin of replication including the E1- and E2-binding sites (Chiang *et al.*, 1992b; Del Vecchio *et al.*, 1992; Frattini & Laimins, 1994; Remm *et al.*, 1992; Sverdrup & Khan, 1994). E2 binds to its binding sites in the LCR and recruits E1 to its own binding site within the replication origin. Following E1 recruitment, E2 is thought to dissociate from its binding sites (Desaintes & Demeret, 1996). The role of E1 in viral replication is varied. It is a DNA helicase, so is actively involved in the unwinding of DNA (Hughes & Romanos, 1993), and is also involved in the recruitment of members of the cellular DNA replication machinery to the viral origin of replication. These include replication protein A (Han *et al.*, 1999), topoisomerase (Clower *et al.*, 2006) and polymerase α-primase (Park *et al.*, 1994).

The maintenance phase of DNA replication is characterised by a relatively stable HPV copy number per cell following successive rounds of cell division. Some controversy exists in terms of the mechanism employed in this phase, with a study suggesting that BPV1 DNA replicates once in every cell cycle

(Roberts & Weintraub, 1988), and another suggesting a more random replication, with some episomes replicating several times and some not replicating at all (Piirsoo *et al.*, 1996). A recent investigation of HPVs however reported that the mode of replication varies depending on the cell type as well as the HPV type studied (Hoffmann *et al.*, 2006). Interestingly, one study also proposed that the E1 protein may not be required for this phase of viral replication (Kim & Lambert, 2002) whereas another proposed that neither E1 nor E2 may be required (Pittayakhajonwut & Angeletti, 2010). Therefore, there appear to be different mechanisms that regulate the establishment and maintenance phases of DNA replication. In fact, it has also been suggested that p53 expression down-regulates establishment replication but not maintenance replication, further hinting at two different modes of replication (Ilves *et al.*, 2003; Lepik *et al.*, 1998).

The last phase of DNA replication is the differentiation-dependent amplificational replication, which occurs in the upper layers of the epithelium and coincides with an increase in E1 and E2 transcripts, generated from the late promoter (Klumpp & Laimins, 1999). E4 expression is also up-regulated in this phase, and E4 has been shown to be important for this phase of replication (Nakahara *et al.*, 2005; Peh *et al.*, 2004; Wilson *et al.*, 2005; Wilson *et al.*, 2007) although this may not be true for all HPV types (Fang *et al.*, 2006).

There appear to be differences between the control of viral replication in undifferentiated and differentiated cells. It appears that E7 is not required for the establishment and maintenance phases of replication but is required for the amplification phase (Flores *et al.*, 2000) and this is reflective of the need for E7 in the induction of the cellular replication machinery in the suprabasal cells. Moreover, it has been suggested that E6 inhibits viral replication in undifferentiated cells (Grm *et al.*, 2005), but is necessary for DNA amplification in differentiated cells (Wang *et al.*, 2009). Furthermore, HPVs have been shown to activate the ATM (ataxia-telangiectasia mutated) DNA damage response pathway and this is necessary for DNA amplification in differentiated cells but not establishment and maintenance replication (Moody & Laimins, 2009). Therefore, it appears that different mechanisms may be at play to promote viral replication across the various stages of the life-cycle.

Interestingly, the replication of viral DNA is targeted by IFN-signalling and it has recently been shown that, for HPV replication, one mechanism of this is the binding of p56, an interferon-inducible protein, to E1 (Terenzi *et al.*, 2008).

1.12.2. Segregation of episomes

Segregation of viral episomes has been studied in different DNA tumour viruses and common features have been identified. Segregation is thought to be controlled by the tethering of viral episomes to cellular chromosomes before or during mitosis. This is mediated via a DNA-binding viral protein, which binds to the viral episomes, and a DNA-binding cellular protein, which binds to the cellular chromosomes. The viral and cellular proteins interact, thereby tethering the viral episomes to cellular chromosomes (Feeney & Parish, 2009).

E2 proteins from a variety of PVs have been shown to associate with cellular chromosomes during mitosis (Bastien & McBride, 2000; Ilves *et al.*, 1999; Lehman & Botchan, 1998; Oliveira *et al.*, 2006; Skiadopoulos & McBride, 1998) or with the mitotic spindle (Van Tine *et al.*, 2004). This proposes a role for E2 in ensuring proper segregation of viral episomes, and also suggests that PVs may employ different mechanisms for episomal segregation.

Several cellular proteins have been implicated in this process and these include Brd4, a member of the bromodomain protein family (Abbate *et al.*, 2006; Baxter *et al.*, 2005; You *et al.*, 2004; You *et al.*, 2005) and ChlR1 (Parish *et al.*, 2006a), a DNA helicase involved in the cohesion of sister chromatids (Parish *et al.*, 2006c). More recently, the topoisomerase II-binding protein 1, TopBP1, was also suggested as a possible mediator of episomal segregation as it was shown to interact with HPV16 E2 in mitosis (Donaldson *et al.*, 2007). The elucidation of this process has been made more difficult by the fact that these cellular proteins are also known to be involved in other viral processes, such as transcription and replication and therefore care must be taken when trying to dissect these roles. Moreover, studies suggest that the mechanisms for the segregation of viral episomes (McPhillips *et al.*, 2006; Oliveira *et al.*, 2006) may vary between different PVs.

Studies of other tumour viruses have revealed similar mechanisms for episomal persistence. The viral protein involved in tethering of EBV episomes to cellular chromosomes was shown to be EBNA1 (EBV nuclear antigen 1) (Petti *et al.*, 1990), whereas for Kaposi's sarcoma-associated herpesvirus (KSHV), it was found to be LANA (latency-associated nuclear antigen) (Cotter & Robertson, 1999). Moreover, both histone H1 (Marechal *et al.*, 1999) and EBNA binding protein 2 (EBP2) (Wu *et al.*, 2000a) have been proposed as the cellular proteins that mediate the binding of EBV episomes to cellular chromosomes, whereas for KSHV possible candidates include Brd4 (You *et al.*, 2006) and histones H2A and H2B (Barbera *et al.*, 2006).

It is interesting to note the various similarities between these three tumour viruses in that E2, EBNA1 and LANA have all been implicated in viral replication, viral transcription and tethering of viral episomes for segregation.

1.13. Aims of thesis

The general aim of this study was to elucidate the role of the viral E6 protein in the persistence of HPV episomes in cells. The specific objectives are outlined below:

1) To investigate specific activities of E6 that are necessary for the persistence of HPV16 episomes;

2) To determine how these activities of E6 may be regulating viral episomal persistence;

3) To further characterise the interaction of the E6 protein with PDZ proteins.

Chapter 2: Materials and Methods

2.1. Commonly used buffers and reagents

Table 2.1: Buffers and reagents	
Name	**Components**
1x Phosphate buffered saline (PBS)	1% NaCl, 0.025% KCl, 0.14% Na_2HPO_4, 0.025% KH_2PO_4
SDS electrophoresis buffer	25 mM Tris base, 250 mM glycine, 0.1% sodium dodecyl sulfate (SDS); pH 8.3
Luria Broth	1% Bacto-Tryptone, 0.5% Bacto-yeast extract, 1% NaCl
Luria Agar	LB medium plus 1.5% Difco agar
SOC medium	2% Bacto-Tryptone, 0.5% Bacto-yeast extract, 0.06% NaCl, 0.02% KCl, 0.2% $MgCl_2.6H_2O$, 0.25% $MgSO_4.{}_7H2O$, 0.36% Glucose
Transfer buffer	48 mM Tris, 39 mM Glycine, 20% Methanol
RIPA protein extraction buffer	150 mM NaCl, 1% Triton X, 0.5% Sodium Deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0, 0.005 mM EDTA. Protease Inhibitor cocktail was added prior to use (3 µl per 1 ml buffer)
RIPA 6% SDS protein extraction buffer	Same as RIPA Protein Extraction buffer but with 6% SDS instead of 0.1% SDS.
Urea protein extraction buffer	8M urea, 0.1M NaH_2PO_4, 0.01M Tris; pH 7.0
Trypsin-versene	0.8% NaCl, 0.02% KCl, 0.12% Na_2HPO_4, 0.02% KH_2PO_4, 0.01% EDTA, 0.13% trypsin, 0.001% phenol red. pH 7.8
20x SSC	3 M NaCl, 300 mM sodium citrate; pH 7.0
5x SDS gel-loading buffer	250 mM Tris-Cl (pH 6.8), 500 mM dithiothreitol (DTT), 10% SDS, 0.5% bromophenol blue, 50% glycerol
50x Tris acetate EDTA (TAE)	242 g Tris base, 57.1 mL glacial acetic acid, 18.6 g EDTA

2.2. Cell culture methods

2.2.1. Cell lines

2.2.1.1. J2-3T3 mouse fibroblast cells

J2-3T3 cells are immortalised mouse fibroblasts, originally isolated from Swiss mouse embryos (Todaro & Green, 1963). Irradiated J2-3T3 cells were used as a feeder layer for the growth of NIKS cells.

2.2.1.2. Normal Immortalised Keratinocytes (NIKS)

Most of the experiments described in this study were carried out using NIKS cells. NIKS cells are an HPV-negative, spontaneously immortalised keratinocyte cell line, which arose from the serial passage of primary cells isolated from neonatal foreskin and designated BC-1-EP (Allen-Hoffmann *et al.*, 2000).

2.2.1.3. 293T and HT1080 cells

293 cells are human embryonic kidney cells that were transformed using adenovirus type 5 DNA (Graham *et al.*, 1977). The 293T cell line is a variant of the 293 cell line into which the gene for the SV40 T-antigen has been inserted (DuBridge *et al.*, 1987). The HT1080 cell line was derived from a biopsy of a fibrosarcoma (Rasheed *et al.*, 1974).

2.2.2. Media and supplements

The media used for the maintenance of the cell lines as well as for the freezing of cells for long-term storage are described in Table 2.2. The supplements used in cell culture media were prepared as 1000x stocks and filter-sterilised using a 0.2 µm filter unit. The supplements were frozen at -20 ºC.

Table 2.2: Cell culture media and freeze media		
Cell type	**Medium type**	**Medium components**
NIKS	F-medium	375 ml F12-Ham's (PAA; E15-817), 125 ml high glucose DMEM (PAA; E15-843), 5% (v/v) FBS (Hyclone; CH30160.03), 5 ml pen/strep (Sigma; P0781), 24 µg/ml adenine (Sigma; A2786), 8.4 ng/ml cholera toxin (Sigma; C8052), 5 µg/ml insulin (Sigma; I4011) and 0.4 µg/ml hydrocortisone (Sigma; H0888). Epidermal Growth Factor (EGF) was added to the medium immediately prior to use at a concentration of 10 ng/ml (Sigma; E9644)
NIKS	F - freeze medium	80% v/v F-medium, 10% v/v FBS, 10% v/v DMSO (Sigma; D2650)
J2-3T3, 293T, HT1080	10% DMEM	500 ml high glucose DMEM, 10% v/v FBS and 5 ml pen/strep
J2-3T3	3T3 - freeze medium	42% v/v 10% DMEM, 50% v/v FBS and 8% v/v DMSO
293T	293T - freeze medium	40% v/v 10% DMEM, 50% v/v FBS and 10% v/v DMSO

2.2.3. Maintenance of monolayer cells

All cells were cultured at 37 ^{o}C and 5% CO_2.

2.2.3.1. J2-3T3, 293T, HT1080

J2-3T3 cells were cultured in 140 mm plates (NUNC; 168381). 293T and HT1080 cells were cultured in 90 mm plates (NUNC; 150350). All cell lines were split twice a week at a ratio of 1:20 for J2-3T3 and 293T cells and 1:10 for HT1080 cells. To harvest, the cells were washed once with trypsin-versene and then incubated with trypsin-versene for 1 minute at 37 ^{o}C. Medium was then added and an aliquot of the cells was transferred to a new plate.

2.2.3.2. NIKS

The NIKS cells (and NIKS containing HPV or NIKS-LXSN cells) were cultured on a layer of γ–irradiated J2-3T3 cells (feeder cells). Prior to splitting the NIKS cells, J2-3T3 cells were irradiated at a dose of 60 Grays using a Caesium source. Feeder cells were plated on 90 mm plates (1.8×10^6 per plate) in 10 ml F-medium and left to attach for 1-2 hours at which point the NIKS cells were plated over them. The NIKS cells were split 1:5 twice a week. For harvesting, NIKS cells were washed twice with trypsin-versene and then incubated in 3 ml trypsin-versene at 37 ^{o}C for 2 minutes, in order to remove the feeder layer. The keratinocytes were then incubated in 3 ml trypsin-versene at 37 ^{o}C for 5-10 minutes. When the cells detached from the plate, 5 ml F-medium was added and they were centrifuged at 240 x *g* for 4 minutes. The cells were re-suspended in F-medium and an appropriate amount was plated over the feeder layer.

2.2.4. Long-term storage of cells

For long-term storage, a confluent layer of cells was harvested as above, pelleted at 240 x *g* for 4 minutes, re-suspended in 1 ml freeze medium (see Table 2.2) and transferred to a cryogenic vial (NUNC; 366656). The vials were stored at -80 ^{o}C overnight and then transferred to liquid nitrogen.

2.2.5. Transfection of cells

For transient transfections, the cells were plated 1 day prior to the transfection. NIKS cells were harvested as above and plated on 6-well plates (NUNC; 140675) at a density of 5.5×10^5 per well and over a layer of 1×10^5 feeder cells. 293T cells were harvested as above and plated at a density of 6.5×10^5 per well. Transfections of NIKS cells were carried out using the Effectene® Transfection Reagent Kit (QIAGEN; 301425) following the manufacturer's instructions. Cells were transfected with a total of 1 µg of DNA, which was purified using the QIAGEN® Plasmid Midi Kit (QIAGEN; 12143) or the QIAGEN® Plasmid Maxi Kit (QIAGEN; 12162). For experiments in which the transfection efficiency was assessed, the transfection mix was "spiked" with 50 ng of the β-galactosidase- (β-gal) expressing plasmid, pMV10. The transfection mix was left

on the cells for 7 hours. The cells were then washed once and fresh medium was applied. Transfections of 293T and HT1080 cells were carried out in the same way as described in section 2.5.2 for the Phoenix cells.

2.2.6. Treatment with proteasome inhibitor

At 46 hours post-transfection, cells were treated with either 40 μM MG-132 (Sigma; C2211) or DMSO in a total volume of 2 ml of medium for 2 hours. Cells were then harvested for protein analysis as described in section 2.9.1.

2.2.7. Treatment with cycloheximide

At 46 hours post-transfection, cells were treated with either 50 μg/ml cycloheximide (Sigma; C4859) or DMSO in a total volume of 2 ml of medium, for 60 or 120 minutes. Cells were then harvested for protein analysis as described in section 2.9.1. Cells that were not treated with the drug were also harvested to serve as the "0 minutes" time-point.

2.3. Persistence assays

2.3.1. Setting-up the population experiments

For population experiments, NIKS cells were plated on 6-well plates 1 day prior to the transfection and at a density of 5 x 10^5 per well on a layer of 1 x 10^5 feeder cells. The transfection was carried out as described in section 2.2.5. The cells were transfected with 800 ng of re-circularised HPV16 genome (prepared as described in section 2.8.2) and 200 ng pcDNA6 plasmid, which carries a blasticidin-resistance gene). The following day, one quarter of the transfected cells were plated on a 90 mm plate with 1.8 x 10^6 feeder cells and the remaining three quarters was plated on another plate with 1.8 x 10^6 blasticidin-resistant feeder cells. The following day, DNA was extracted from the one quarter of the transfected cells. This was used in a qPCR assay (described in section 2.8.9), to determine the number of HPV copies that entered the cells during transfection and this was used as a measurement of the transfection efficiency. The plate with the three quarters of transfected cells was treated with 8 μg/ml

blasticidin antibiotic (Invitrogen; R210-01). A control plate of un-transfected cells was also given the same treatment with blasticidin and was used to determine the end-point of the antibiotic-treatment. The treatment lasted 3-6 days with a change of medium and antibiotics every 2 days. When all control cells were dead, the surviving transfected cells were trypsinised and plated onto a new 90 mm plate with 1.8 x 10^6 feeder cells. The new plate was labelled “passage 1” (p1).

2.3.2. Passing cells for long-term analysis

After passage 1 the cells were split 1:5 - 1:6 twice a week. When possible, the cells were allowed to reach confluence before splitting them. Each time the cells were passed to new plates, genomic DNA was extracted from the remaining cells using the method described in section 2.8.6. The DNA was always extracted fresh on the day the cells were harvested. The cells were passed in this way for several passages, depending on the experiment.

2.3.3. Setting-up the clonal experiments

The plating and transfection of the NIKS cells for clonal experiments is the same as for the population experiments described in section 2.3.1. In the clonal experiments however, the cells were trypsinised 48 hours post-transfection and counted. An equal number of cells from the different transfections, approximately one quarter of the total number of cells, was then divided into ten 90 mm plates each with 1.8 x 10^6 blasticidin-resistant feeder cells. (i.e. approximately one fortieth of the total number of transfected cells was plated in each plate). The following day, the cells were treated with blasticidin at a concentration of 8 µg/ml. As with the population experiments, this treatment was continued until the control cells were dead (3-6 days) with medium changes every 2 days. At the end of the antibiotic selection, new medium was added to the cells (without blasticidin). One million new feeder cells were also added to replenish any feeder cells lost during the selection. The cells were left to grow with medium changes every 2 days, until individual clones became visible and large enough to pick. This process took 2-3 weeks.

2.3.4. Picking individual clones

When the clones were large enough on the 90 mm plates, they were transferred individually into 6-well plates, one clone per well, with 3 x 10^5 feeder cells. To pick the clones, the medium was aspirated off the plate and the plate was washed twice with trypsin. The clone was then outlined with the aspirator and trypsin was pipetted directly onto the clone. Clones to be picked were chosen based on how isolated they were, so as to avoid mixing two or more clones together. No more than 7 clones were picked from each 90 mm plate so as to avoid the drying of the cells. Figure 2.1 shows a clonal plate that was stained with methylene blue solution (1% methylene blue in 50% methanol and 50% PBS).

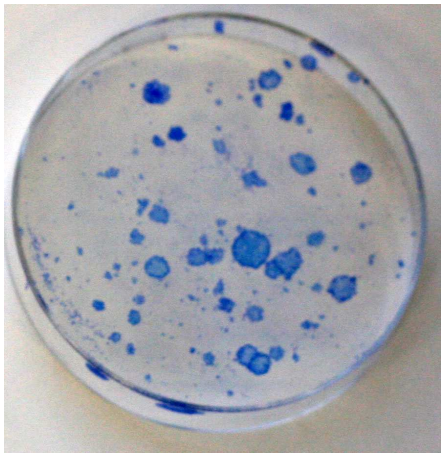


Figure 2.1: Methylene blue staining of plate from clonal experiment

2.3.5. Growing clones and harvesting for analysis

When the clones became confluent in the 6-well plates, they were trypsinised and passed on to a 90 mm plate (one clone per 90 mm plate) with 1.8 x 10^6 feeder cells. The clones were left on the 90 mm plates until they became confluent at which point they were harvested and divided into 2 samples. One sample was frozen (as described in section 2.2.4) while the other sample was used for DNA extraction.

2.4. Transient replication assay

The method for the transient replication assays was modified from a previously described protocol (Taylor & Morgan, 2003). Briefly, NIKS cells (5.5 x 10^5) were seeded onto feeder cells (1 x 10^5) and transfected the following day with equimolar amounts of re-circularised wild-type or mutant HPV16 DNA (1 µg) or pET-28 plasmid carrying the E1^E4 cDNA (700 ng, plus 300 ng of pMV11 plasmid), made by Dr. Pauline McIntosh (NIMR, London) and described in McIntosh *et al*., 2008. Four days post-transfection the cells were trypsinized and re-suspended in 0.5 ml PBS at a concentration of 1.8 x 10^6 cells/ml. Episomal DNA was extracted as previously described (Hoffmann *et al.*, 2006). Following ethanol precipitation, 3 µl of DNA were digested with Dpn I for 3 hours, and then Exonuclease III for 30 minutes, followed by enzyme inactivation at 70 ^oC for 30 minutes. Another 3 µl of DNA were treated in the same way, but without any enzyme, and these represent the undigested samples. Numbers of E4 copies were measured in both Dpn I-digested and undigested samples by qPCR (as described in section 2.8.9), using primers against the E4 ORF of HPV16 (Table 2.8). The former was divided by the latter to give the percentage of replication.

2.5. Retroviral expression system

A retroviral expression system was used for the stable expression of E6 protein or E6 and E7 proteins in NIKS cells, or for the delivery of shRNA constructs. This system utilises Phoenix cells, a 293T-based cell line which is capable of producing the gag-pol and envelope proteins for amphotropic viruses.

2.5.1. Culture of Phoenix cells

The Phoenix cells were kindly provided by Dr. Garry Nolan (Stanford University, Stanford). These cells were cultured in the same way as 293T cells (see section 2.2.3.1). Phoenix cells were plated in 90 mm plates 1 day prior to transfection at a density of 6.5 x 10^6 cells per plate.

2.5.2. Transfection of Phoenix cells and harvesting of retroviruses

Phoenix cells were transfected using polyethylinimine (PEI). The medium was first removed from the cells and 5 ml of high glucose DMEM without any FBS or antibiotics (plain DMEM) was added to the cells. Two mixtures were then prepared: A) 655 µl plain DMEM + 45 µl PEI (1 mg/ml) and B) 15 µg plasmid DNA (pLXSN or pRETROSuper plasmids) topped up to 700 µl with plain DMEM. Mixtures A and B were combined, vortexed and incubated at room temperature for 15-30 minutes. The medium was then removed from the cells, 3.6 ml of plain DMEM was added to the A + B mixture, and this was added to the cells. The transfection mix was left on the cells for 7 hours. The cells were then washed once and the medium was replaced with 10 ml 10% DMEM. The medium was changed again the following day. At 48 hours post-transfection, the medium, which contains retroviruses, was collected and centrifuged at 240 x *g* for 4 minutes. The supernatant was collected and 1 ml aliquots were either used directly to infect NIKS cells or stored at -80 ºC.

2.5.3. Infection of NIKS and NIKS + HPV16 cells

NIKS cells were plated 1 day prior to infection, at a density of 5 x 10^5 per well with 1 x 10^5 feeder cells in 6-well plates. For infection, 1 ml of virus medium was mixed with 3 ml plain F12-Ham's medium (without FBS or antibiotics), with supplements in the concentrations described in Table 2.2 for the F-medium. Polybrene was also added at a final concentration of 10 µg/ml. The existing medium on the cells was replaced with the virus mixture and the cells were incubated at 37 ºC for 7 hours, at which point the cells were washed once and the medium was replaced with F-medium. The following day the cells were passed from the 6-well plates to 90 mm plates with 1.8 x 10^6 antibiotic-resistant feeder cells, and the day after they were treated with neomycin at a

concentration of 300 µg/ml (for the infections with LXSN retroviruses) or puromycin at a concentration of 1 µg/ml (for the infections with shp53 and shScrib retroviruses). This treatment was continued until the control plate (uninfected cells) was completely dead (3-6 days), with a medium change every 2 days.

2.6. Monolayer growth assays

2.6.1. Seeding cells for growth assays

For the growth assays the cells were plated in 6-well plates. Three wells were set up for each cell type and each time-point, in order to obtain triplicate cell counts for each time-point. The feeder cells were first plated in each well and allowed to attach (3×10^5 per well). The keratinocytes were then trypsinised and counted and 1×10^5 cells were plated over the feeder cells. At this point the cells were plated in F-medium without any EGF. The day of plating was denoted as “day 0”. The following day, the cells were counted for time-point “day 1”. The medium was replaced on all the remaining wells with F-medium containing EGF. From that point onwards, the medium was changed every 2 days and the new medium always contained EGF.

2.6.2. Counting cells for growth assays

The cells were harvested for counting on days 1, 3, 4, 5 and 7. For harvesting, each well was first washed with 2 ml of warm PBS, followed by 1 ml of trypsin. To dislodge the feeder cells, 0.5 ml of trypsin was added to each well, and the cells were incubated at 37 ºC for 2 minutes. The trypsin was then aspirated and the well was washed with PBS to ensure the removal of the feeder cells. To remove the keratinocytes, 1 ml of trypsin was added and the cells were incubated at 37 ºC for a further 3–5 minutes. To count the cells, 1 ml of medium was added to the cells and 0.5 ml of the mix was used for counting on a Beckman Coulter Z1 Coulter® Particle Counter.

2.7. Generating p53- and hScrib-knockdown cells

NIKS or NIKS + HPV16 cells were infected with retroviruses carrying shRNA constructs against p53, hScrib or a Scrambled sequence (shScramble) as described in section 2.5.3. Following selection with puromycin, the cells were passed onto new plates. Once the cells had recovered, they were harvested for protein analysis or frozen for future use. Two shRNA constructs were used to knock-down hScrib in NIKS cells. These were kindly provided by Dr. Patrick Humbert. They are labelled shScrib 1 and 2 in this study and refer to shScrib constructs 6 and 7 respectively in Dow et al, 2007 (Dow *et al.*, 2007).

2.8. DNA and RNA techniques

2.8.1. DNA constructs used

Table 2.3: List of plasmids used in this study

Name of plasmid	Use	Reference and/or source
pSPW12	Plasmid used in generating re-circularised HPV16 genomes	Kindly provided by Dr. Margaret Stanley (University of Cambridge, Cambridge)
pSPW12E6p53m and pSPW12E6PDZ	Plasmids used in generating re-circularised mutant HPV16 genomes	Made by site-directed mutagenesis as described in section 2.8.5
pcDNA6	Used for the expression of the blasticidin-resistance gene	(Laurson *et al.*, 2010)
pLXSN, pLXSN-E6WT and pLXSN-E6WT/7	Retrovirus vectors used for the stable expression of HPV16 E6 and E7 proteins	Kindly provided by Dr. Denise Galloway (Fred Hutchinson Cancer Research Center, Seattle) (Halbert *et al.*, 1991)

pLXSN-E6p53m and pLXSN-E6PDZ	Retrovirus vectors used for the stable expression of HPV16 E6SAT and E6PDZ proteins	Made by site-directed mutagenesis as described in section 2.8.5
pMV11-E6WT, pMV11-E6p53m and pMV11-E6PDZ	Used for the transient expression of wild-type and mutant HPV16 E6 proteins	Made by cloning the E6 ORF in the pMV11 plasmid as described in section 2.8.8
pRETROSuper-shScrib 1 and 2 and pRETROSuper-shScramble	Used for knocking-down hScrib protein	Kindly provided by Dr. Patrick Humbert (Peter MacCallum Cancer Centre, Melbourne). The shScrib constructs 1 and 2 correspond to shScrib 6 and 7 respectively (Dow *et al.*, 2007; Dow *et al.*, 2008)
pRETROSuper-shp53	Used for knocking-down p53 protein	(Brummelkamp *et al.*, 2002)
pET-28	Used in transient replication assay. Carries 16E1^E4 cDNA	Made by Dr. Pauline McIntosh (McIntosh *et al*., 2008)
pMV10	Used for the expression of β-gal protein	(Forrester *et al.*, 1992)
pcDNA-HA-hScrib	Used for the transient expression of HA-hScrib protein	Kindly provided by Dr. Lawrence Banks (ICGEB, Trieste) (Thomas *et al.*, 2005)
pcDNA-HA-hScribΔPDZ	Used for the transient expression of HA-hScrib protein that lacks the PDZ domains	Kindly provided by Dr. Lawrence Banks (Thomas *et al.*, 2005)
pGWI-HA-Dlg	Used for the transient expression of HA-Dlg protein	Kindly provided by Dr. Lawrence Banks (Gardiol *et al.*, 1999)

pGWI-HA-11E6	Used for the transient expression of HPV11 E6	Kindly provided by Dr. Lawrence Banks (Glaunsinger *et al.*, 2000)
pcDNA-FLAG-MAGI-1c	Used for the transient expression of FLAG-MAGI-1c protein	Kindly provided by Dr. Lawrence Banks (Glaunsinger *et al.*, 2000)
pCI-EGFP	Used for the expression of GFP	(Tuting *et al.*, 1999)

2.8.2. Preparation of re-circularised DNA

The HPV16 genome used to transfect cells was obtained by digesting the HPV16 DNA out of the pSPW12 plasmid and re-circularising it. The PB, PE and EB buffers as well as the QIAprep spin columns mentioned in this section are all included in the QIAprep® Spin Miniprep Kit (QIAGEN; 27106). The re-circularisation was carried out by first digesting 5 µg of pSPW12 plasmid with 2 µl BamH I enzyme in a total volume of 30 µl for 2.5 hours at 37 ^{o}C. The enzyme was then deactivated by incubating the mix at 85 ^{o}C for 20 minutes. The ligation reaction was carried out in a 16 ^{o}C water-bath overnight and was set up as shown in Table 2.4. To purify and concentrate the re-circularised DNA, the ligation reaction was mixed with 10 ml PB buffer and then put through a QIAprep spin column. The column was then washed once with 750 µl PE solution and the DNA was eluted in 50 µl EB buffer. The quality of the re-circularised DNA was determined by running 3 µl of the DNA on an agarose gel. The re-circularised preps were considered to be of high enough quality if the band representing the HPV16 genome was the strongest band on the gel. Figure 2.2 shows a picture of one such gel.

Table 2.4: Set-up of the ligation reaction to prepare re-circularised HPV16 genomes

Digested DNA	30 µl
Water	1765 µl
10x T4 DNA ligase buffer	200 µl
T4 DNA Ligase (NEB; MO202S)	5 µl

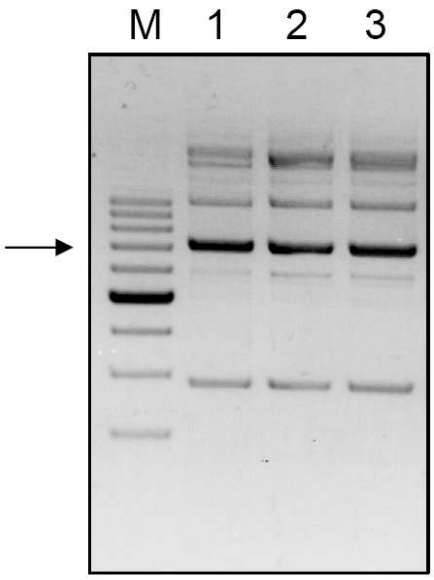


Figure 2.2: Agarose gel showing quality of re-circularised HPV16 genomes
The re-circularised and purified DNA was analysed on a 1% agarose gel to check its quality. The band representing the re-circularised HPV16 genomes is indicated by the arrow. The other bands represent other re-circularised species present in the mix, such as re-circularised pSP plasmid (the smallest band) or multimeric species (the largest bands). The supercoiled marker is indicated (M) and the three lanes (1, 2 and 3) correspond to re-circularised 16WT, 16E6p53m and 16E6PDZ genomes respectively, from one such experiment.

2.8.3. Transformation of *E. coli* with DNA

Plasmid DNA was transformed into competent XL1-Blue cells (grown and made competent by Rachel Chung at the NIMR). Plasmid DNA was mixed with 50 µl of competent cells and incubated on ice for 30 minutes before heat-pulsing at 42 ^{o}C for 90 seconds. The cells were then cooled on ice and 800 µl of SOC medium was added and the cells were incubated at 37 ^{o}C for 45 minutes with constant shaking. Transformed cells were plated on LB agar plates containing ampicillin (100 µg/ml) and incubated overnight at 37 ^{o}C.

2.8.4. Plasmid purification

Three commercial kits were used for plasmid purification, depending on the amount of purified plasmid needed. The QIAprep® Spin Miniprep Kit (QIAGEN; 27106) was used for small-scale preps, from 2–5 ml of bacterial cultures. The QIAGEN® Plasmid Midi Kit (QIAGEN; 12143) and the QIAGEN® Plasmid Maxi Kit (QIAGEN; 12162) were used for larger-scale preps, from 100 ml and 500 ml of bacterial cultures respectively. The purification was carried out according to the manufacturer's instructions. Plasmid DNA was quantified using a Nanodrop ND-1000 Spectrophotometer.

2.8.5. Site-directed mutagenesis

For the purpose of this study, it was necessary to introduce the E6STOP, E6p53m and E6PDZ mutations into several plasmids. This was done by site-directed mutagenesis. The primers used to introduce these mutations are shown in Table 2.5. The mutagenesis reaction was carried out in a total volume of 50 µl, by adding 0.2 mM dNTPs, 1x Pfu Ultra Buffer, 0.2 mM of forward or reverse primer (individual PCR reactions were set up for each of the two primers), 0.15 µg template plasmid and 1 µl Pfu Ultra enzyme (Stratagene; 600380). The conditions of the PCR reaction are outlined in Table 2.6. The template plasmids mutagenised in this way were pSPW12, pLXSN-E6WT and pLXSN-E6WT/7. Following the individual PCR reactions, 25µl of each of the forward and reverse PCR reactions were combined, 1 µl Pfu Ultra enzyme was added and an additional PCR reaction was carried out as shown in Table 2.6 but with 18 repeats of steps 2–4 instead of 4 repeats. Following this, the

template DNA was removed by incubation with 10 U of DpnI (NEB; R0176S) at 37 ^{o}C for 2 hours and 2 µl of this reaction were transformed into XL1-Blue bacteria.

Table 2.5: Primers used for site-directed mutagenesis	
E6STOP mutation	**Forward:** ATGTTTCAGGACCCATAGGAGCGACCCAGAAAG **Reverse:** CTTTCTGGGTCGCTCCTATGGGTCCTGAAACAT
E6p53m mutation	**Forward:** GCAATGTTTCAGGACCCACAGGAGAGCGCCACAAA GTTACCACAGTTATGCACAGAGCTGC **Reverse:** GCAGCTCTGTGCATAACTGTGGTAACTTTGTGGCGCT CTCCTGTGGGTCCTGAAACATTGC
E6PDZ mutation	**Forward:** GCAGATCATCAAGAACACGTAGATAAACCCAGCTGTA ATCATGCATGG **Reverse:** CCATGCATGATTACAGCTGGGTTTATCTACGTGTTCT TGATGATCTGC

Table 2.6: PCR conditions for site-directed mutagenesis
1) 94^{o}C for 30 seconds
2) 95^{o}C for 30 seconds 3) 55^{o}C for 1 minute 4) 68^{o}C for 18 minutes
Repeat steps 2–4 four times

2.8.6. Extraction of total genomic DNA

For the extraction of genomic DNA, cell pellets were first re-suspended in 200 µl PBS with 2 mg/ml RNase A (Sigma; R5500) and incubated at 37 ^{o}C for 5 minutes to remove RNA. DNA was then extracted from cells using the QIAamp® DNA Mini Kit (QIAGEN; 51306) following the instructions for the Blood and

Body Fluid Spin Protocol. Genomic DNA was quantified using a Nanodrop ND-1000 Spectrophotometer.

2.8.7. Agarose gel electrophoresis

To check the presence and quality of plasmid DNA, genomic DNA or PCR products, the DNA was separated on a 1% agarose gel (1% w/v agarose in TAE) containing 0.5 µg/ml Ethidium Bromide.

2.8.8. Cloning of pMV11-E6 plasmids

For the purpose of this study, the wild-type and mutant E6 ORFs were cloned into the pMV11 plasmid, downstream of the cytomegalovirus (CMV) promoter. The pSPW12, pSPW12E6p53m and pSPW12E6PDZ plasmids served as templates for the amplification of the respective E6 ORFs. The primers used to amplify the E6 ORFs are shown in Table 2.7. The forward primer had a BamH I restriction site whilst the reverse primers had an EcoR I restriction site. The E6 ORFs were amplified by PCR, and the PCR products were first purified using the QIAquick® PCR Purification kit (QIAGEN; 28104) and then digested over-night with BamH I and EcoR I restriction enzymes. The pMV11 plasmid was also digested with BamH I and EcoR I restriction enzymes. The digestion mixtures were then separated on an agarose gel and the bands were excised and purified using the QIAquick® Gel Extraction kit (QIAGEN; 28704). For the ligation reaction, the vector and inserts were mixed in a ratio of 1:5 and incubated with T4 DNA ligase and T4 DNA ligase buffer (NEB; MO202S) in a total volume of 10 µl for 6 hours at room temperature and 5 µl of the ligation reaction were then transformed into XL1-Blue bacteria as described in section 2.8.3.

Table 2.7: Primers used for the cloning of wild-type and mutant E6 ORFs into the pMV11 plasmid	
pMV11-E6WT and pMV11-E6p53m	**Forward:** GCTGGGATCCATGCACCAAAAGAGAACTGCAATG **Reverse:** AGGCGAATTCTTACAGCTGGGTTTCTCTACGTGTTC
pMV11-E6PDZ	**Forward:** GCTGGGATCCATGCACCAAAAGAGAACTGCAATG **Reverse:** AGGCGAATTCTTACAGCTGGGTTTATCTACGTGTTC

2.8.9. Quantitative PCR (qPCR)

Quantitative PCR was carried out on genomic DNA, cDNA or plasmid DNA, depending on the assay. When genomic DNA was used as a template, primers detecting part of the E4 ORF were used to detect the presence of HPV16 DNA. In this case GAPDH was used as a control. When cDNA was used as a template, primers that amplify part of the E6 ORF were used to detect the presence of E6 transcripts and primers against β-actin were used as a control. The E6 primers used will only detect full-length E6 transcripts, and will not detect the spliced variants. A list of the primers used in qPCR is shown in Table 2.8. Figure 2.3 shows the location of the E6 qPCR primers on the E6 ORF.

Table 2.8: Primers used for qPCR	
E4 primers	**Forward:** GACTATCCAGCGACCAAGATCAG **Reverse:** CTGAGTCTCTGTGCAACAACTTAGTG
GAPDH primers	**Forward:** CGAGATCCCTCCAAAATCAA **Reverse:** CATGAGTCCTTCCACGATACCAA
E6 primers	**Forward:** AGCGACCCAGAAAGTTACCA **Reverse:** GCATAAATCCCGAAAAGCAA
β-actin primers	**Forward:** TGGGCATGGGTCAGAAGGAT **Reverse:** CGGCCAGAGGCGTACAGGGA

Figure 2.3: Sequence of HPV16 E6 ORF showing location of qPCR primers

The DNA sequence of the E6 ORF of HPV16 (GeneBank accession number: AF125673, region: 83-559). The forward (FOR) and reverse (REV) qPCR primers used to amplify E6 transcripts are indicated, as are the splice donor (SD) and splice acceptor (SA) sites.

2.8.9.1. Reagent cocktails and cycle parameters

For qPCR, the ABsolute™ QPCR SYBR® Green ROX mix was used to amplify and detect the DNA (ABgene; Ab-1163/a). The samples were first plated in 96-well plates and these were read using an ABIPrism 7000 Sequence Detection System (Applied Biosystems). Prior to each qPCR analysis, a fresh master mix was prepared containing 1x SYBRgreen ROX mix and 70 nM of each primer. A separate master mix was prepared for each primer set. To set up the reaction, 75 ng of template (3 µl total) was first pipetted into each well followed by 22 µl of the master mix. Each sample was amplified in triplicate for each primer set (i.e. 6 wells per sample). The cycle parameters for the qPCR are outlined in Table 2.9. The parameters include a dissociation programme at the end of the amplification steps. This was set up due to the fact that SYBRgreen can also bind to unspecific double stranded DNA, such as primer-dimers, and this unspecific binding can interfere with the results. From the dissociation curve obtained, the detection of single or multiple products can be determined.

Table 2.9a: qPCR cycle parameters	
1) 50^{o}C for 2 minutes	x1
2) 95^{o}C for 15 minutes	x1
3) 95^{o}C for 15 seconds	x40
60^{o}C for 1 minutes	
Table 2.9b: Dissociation parameters	
1) 95^{o}C for 15 seconds	x1
2) 60^{o}C for 20 seconds	x1
3) 95^{o}C for 95 seconds	x1

2.8.9.2. Standard Curves

For each set of qPCR primers used in this study, a standard curve was generated in order to establish the sensitivity and efficiency of the primers. To generate each standard curve, a DNA plasmid containing the sequence that is amplified by the primers was chosen as a template. For example, to generate a standard curve for the E4 primers, the pSPW12 plasmid was used as a

template. Serial dilutions of the plasmid were prepared, starting from 60 pg/µl down to 0.6 fg/µl. These dilutions served as the template for the qPCR reaction and 3 µl of the diluted DNA was plated in triplicate wells. To generate the standard curves, the amount of DNA in each dilution sample was first converted to the number of DNA molecules in that reaction. To use the E4 primers as an example, the number of pSPW12 molecules in the dilution samples ranged from 1.5×10^7, 1.5×10^2. The logs of these values were then plotted on a graph, against the Ct value given by the qPCR machine for each sample, and an equation was obtained in the form of y = mx + c. The y-value in these equations is the Ct value that the qPCR machine measures. The ideal standard curve would have a slope of -3.33 as that would represent an increase of 3.33 Cts for every 10-fold dilution of sample. The standard curves generated for each primer set are shown in Table 2.10 and were prepared in collaboration with Dr. Ken Raj.

Table 2.10: Standard curves for primers used for qPCR	
E4 primers	y = -3.39x + 37.29
GAPDH primers	y = -3.473x + 35.932
E6 primers	y = -3.387x + 36.577
β-actin primers	y = -3.387x + 37.204

2.8.9.3. Determination of copy numbers

To determine the number of E4 copies in each sample, the y-value from the equation was replaced by the Ct value and the equation was solved for x. The number of copies is the inverse log of x (i.e. $10^{\wedge x}$). The samples to be analysed by qPCR were always plated in triplicate wells and three Ct values were obtained and the standard deviation determined. When standard deviations were greater than 0.3, the reactions were repeated.

For this study, a "number of HPV copies per cell" was often calculated. To do this, the number of HPV copies per reaction was first calculated (using the E4 primers) as was the number of GAPDH copies per reaction (using the GAPDH primers). The number of GAPDH copies was then divided by 20 to obtain the number of cells in the reaction, as previous work in the laboratory (carried out

by Dr. Ken Raj) had determined that the GAPDH primers recognised 20 copies per cell. The number of HPV copies was then divided by the number of cells to obtain a "number of HPV copies per cell".

2.8.10. Southern blot analysis

Southern blot analysis was carried out on the DNA extracted from the population and clonal persistence assays in order to determine the physical state of the HPV genomes present in the cells. Southern blots were adjusted for brightness and contrast using Photoshop, and a white line is sometimes used to denote a portion of the gel that has been cropped.

2.8.10.1. Restriction enzyme digestion and gel electrophoresis

The cellular DNA used for Southern blotting was extracted as described in section 2.8.6. Genomic DNA (6–10 µg) was left undigested or was digested with Hind III, BamH I or Xba I overnight at 37 ^{o}C. The DNA was then separated on a 1% agarose gel in TAE with 0.5 µg/ml ethidium bromide. The gel was electrophoresed at 100 V.

2.8.10.2. Southern blot transfer

Before transferring, the gel was washed once with distilled water and then soaked in denaturation solution (0.5 N NaOH, 1.5 M NaCl) for 30 minutes. The gel was then rinsed with distilled water and soaked in neutralisation solution (1 M Tris.Cl pH 8, 1.5 M NaCl) for 30 minutes. A capillary transfer stack in 20X SSC was used to transfer the DNA from the gel onto a piece of Millipore Immobilon™-NY+ Transfer Membrane (Millipore; INYC00010). The transfer was carried out overnight at room temperature. The membrane was then washed once in 6x SSC and left to dry completely at room temperature before it was UV cross-linked at 5000 µJ/cm^2.

2.8.10.3. Probe labelling

The probe used in all Southern blots was undigested pSPW12 plasmid. The probe was labelled with ^{32}P-dCTP using the Amersham™ Ready-To-Go™ DNA

Labelling Breads (-CTP) (GE Healthcare; 27-9240-01) following the manufacturer's instructions. Following the labelling reaction, the probe was cleaned by passing it through a DyeEx™ column (QIAGEN; 63204). It was then denatured and applied to the membrane.

2.8.10.4. Pre-hybridisation, hybridisation, washing and exposure

Following cross-linking, the membrane was re-hydrated with distilled water. It was then rolled up and placed in a tube and pre-hybridised in 10 ml hybridisation buffer (5x SSPE, 5x Denhardt's, 100 µg/ml ssDNA, 0.5% SDS) for 1–2 hours at 68 ^{o}C. In the meantime, the probe was prepared (see section 2.8.10.3). Denatured probe was then mixed with another 10 ml of hybridisation buffer. The hybridisation buffer in the tube was discarded, and was replaced with the new 10 ml of buffer containing the denatured probe. The membrane was left overnight in a rotating oven at 68 ^{o}C. The following day, the hybridisation buffer was carefully removed and discarded. The membrane was washed twice for 5 minutes at room temperature with 2x SSC, 0.1% SDS. This was followed by two 15 minute washes at 68 ^{o}C with pre-warmed solution of 0.2x SSC and 0.1% SDS. The membrane was then exposed using a Phosphorimager cassette or X-ray film.

2.8.11. Extraction of total RNA

For extraction of total RNA, cells were harvested and washed once with PBS. Pelleted cells were kept on ice at all times. Total RNA extraction was carried out using the RNeasy® Minikit (QIAGEN; 74104) and QIAshredder™ kit (QIAGEN; 79654) following the manufacturers' protocol. RNA samples were stored at -80 ^{o}C.

2.8.12. Reverse transcription

Prior to carrying out reverse transcription, 4 µg of extracted RNA was cleared of any genomic DNA using the DNA-*free*™ kit (Ambion; AM1906) following the manufacturer's instructions. Clean RNA (5 µl) was then added to a tube containing 6 µl nuclease-free H_2O and 1 µl random primers (Roche; 11034731001) or 1 µl Oligo$(dT)_{20}$ primers (Invitrogen; 18418-020). The mixture

was incubated at 70 ^{o}C for 10 minutes and immediately transferred to ice for 5 minutes. Reverse transcription was carried out using the SuperScript™ II Reverse Transcriptase kit (Invitrogen; 18064-022). The samples were incubated at 42 ^{o}C for 1 hour followed by 70 ^{o}C for 15 minutes. For each RNA sample a "+ reverse transcriptase" (+RT) and a "- reverse transcriptase" (-RT) sample were prepared. The +RT samples included 1 µl of the reverse transcriptase in the reaction whereas the –RT samples included 1 µl of nuclease-free H_2O in the reaction. The latter were used as a control for the absence of genomic DNA from the samples.

2.9. Protein methods

2.9.1. Cell lysis for western blot analysis

Keratinocytes were first washed with PBS and incubated at 37^{o}C for 5 minutes to remove the feeder layer. They were then scraped off the plates using a cell scraper (Corning; 3010) and pelleted. When the RIPA 6% SDS or the Urea protein extraction buffers were used, 1 µl of Benzonase® Nuclease (Sigma; E1014) was first added to each pellet and the pellet was then re-suspended in 120 µl–150 µl of the protein extraction buffer. The lysates were boiled at 95 ^{o}C for 7 minutes and centrifuged at 16100 x *g* for 5 minutes. The supernatant was collected and stored at -80^{o}C. When the RIPA extraction buffer (with no additional SDS) was used, the pellets were re-suspended in 120 µl–150 µl of buffer and incubated on ice for 20 minutes. They were then centrifuged at 16100 x *g* for 10 minutes at 4 ^{o}C and the supernatant was collected and stored at -80^{o}C.

2.9.2. Protein quantification

For protein quantification, the *DC* Protein Assay Kit (Bio-Rad; 500-0111) or the Bio-Rad Protein Assay Kit (Bio-Rad; 500-0006) were used following the manufacturer's instructions. Bovine γ-Globulin standards (Bio-Rad; 500-0208) were used to make a standard curve.

2.9.3. SDS-PAGE

Following protein quantification, equal amounts of protein were prepared in 5x SDS gel-loading buffer, containing DTT (final concentration 100 mM). The samples were mixed, boiled at 95 ^{o}C for 8 minutes and loaded directly on the gel.

2.9.3.1. Preparation of gels

Different percentage gels were prepared depending on the size of the protein to be analysed. Table 2.11 shows the composition of the gels used in this study. The resolving gels were prepared first (10 ml per gel) and left to set for approximately 30 minutes The stacking gels were then prepared and poured over the resolving gel (2 ml per gel). The combs were also put in place and the gels were left to polymerise for 30 minutes.

For some experiments, the 16% Novex® Tricine gels from Invitrogen were also used, following the manufacturer's instructions.

Table 2.11: Composition of 6% , 10% or 15% Tris-glycine SDS-polyacrylamide resolving gels and 5% stacking gel

Resolving Gels (10 ml)	
H_2O	5.3 ml or 4 ml or 2.3 ml
30% acrylamide mix	2 ml or 3.3 ml or 5 ml
1.5M Tris (pH 8.8)	2.5 ml
10% SDS	0.1 ml
10% ammonium persulphate	0.1 ml
TEMED	8 µl or 4 µl or 4 µl
Stacking Gel (2 ml)	
H_2O	1.4 ml
30% acrylamide mix	0.33 ml
1 M Tris (pH 6.8)	0.25 ml
10% SDS	0.02 ml
10% ammonium persulphate	0.02 ml
TEMED	0.002 ml

2.9.3.2. Membrane transfer

All 15% gels were transferred onto 0.2 µm PVDF membranes (Bio-Rad; 162-0176). All other gels were transferred on to 0.45 µm PVDF membranes (Millipore; IPVH00010). Prior to transferring, the membranes were soaked in methanol and then left to soak in transfer buffer for at least 30 minutes. The transfer was carried out using the Bio-Rad Trans-Blot SD Semi-Dry Transfer Cell, at 20 V for 2 hours.

2.9.3.3. Blocking, antibody incubations and washing

Following transfer, the membranes were rinsed twice with dH_2O and then blocked in 5% milk in PBS-0.1% Tween for 1 hour at room temperature or overnight at 4 ^{o}C. In the case of the anti-DYKDDDDK Tag antibody, the membrane was blocked in 5% BSA in PBS-0.1% Tween. The membranes were then incubated with primary antibody for 1–2 hours at room temperature or overnight at 4 ^{o}C. The antibodies were diluted in 5 ml of 5% milk in PBS-0.1% Tween and are outlined in Table 2.12. The membranes were washed in PBS-0.1% Tween with several changes of wash buffer. The amount of washing depended on the antibody. When the E6 antibody was used, the membranes were washed for at least 1.5 hours with a change of wash buffer every 10 minutes. The membranes were then incubated with secondary antibody diluted, as stated in Table 2.13, in 10 ml of 5% milk in PBS-0.1% Tween for 1–2 hours. This was followed by a second round of washing. Table 2.14 outlines the different anti-E6 primary antibodies that were tested in an attempt to reduce the background of the western blots.

Table 2.12: Primary antibodies used for western blotting		
Antibody name	**Dilution**	**Catalogue number**
p53 (DO-1)	1:2000 (2 hours)	Santa Cruz; sc-126
E6 (2E-3F8) - (C-terminus)	1:1000 (overnight)	Euromedex; 2E-3F8
E6 (1E-6F4) - (N-terminus)	1:1000 (overnight)	Euromedex; E6-6F4
Beta-galactosidase (β-gal)	1:2000 (overnight)	Abcam; ab616
HSP-70 (W-27)	1:2500 (1 hour)	Santa Cruz; sc-24
E7 (NM2) E7 (716-325) (used together, as a cocktail)	1:500 each (overnight)	Santa Cruz; sc-65711 and sc-51951
HA	1:1000 (overnight)	Sigma; H6908
Scrib (C-20)	1:2000 (2 hours)	Santa Cruz; sc-11049
DYKDDDDK Tag (FLAG tag)	1:1000 (overnight)	Cell Signalling; 2368 (in 5% BSA)
Histone H2B	1:600 (1 hour)	Upstate; 07-371
HSP-90α/β (H-114)	1:1000 (1 hour)	Santa Cruz; sc-7949
GFP (B-2)	1:750 (overnight)	Santa Cruz; sc-9996

Table 2.13: Secondary antibodies used for western blotting		
Antibody name	**Dilution**	**Catalogue #**
Anti-mouse IgG-HRP (from sheep)	1:2500	GE Healthcare; NA931V
Anti-mouse IgG-HRP (from rabbit)	1:10000	Pierce; 31450
Anti-rabbit IgG-HRP (from donkey)	1:5000	GE Healthcare; NA934V
Anti-goat IgG-HRP (from donkey)	1:5000	Santa Cruz; sc-2020

Table 2.14: Additional anti-E6 primary antibodies tried	
Antibody name	**Catalogue number**
HPV16 E6/18 E6 (C1P5)	Santa Cruz; sc-460
HPV16 E6 (N-17)	Santa Cruz; sc-1584

2.9.3.4. Signal detection

To detect the proteins, 1 of 3 ECL kits were used, depending on the level of sensitivity needed: the Amersham ECL™ Western Blotting Detection kit (GE Healthcare; RPN2106) was used for very abundant proteins, the Immobilon Western Chemiluminescent HRP Kit (Millipore; WBKLS0500) was used for medium sensititivity (such as the detection of E6) and the Amersham™ ECL Advance™ kit (GE Healthcare; RPN2135) was used to detect low levels of protein.

2.9.4. Detecting E6 expressed from the HPV16 genome

2.9.4.1. Transfection of cells

For the detection of E6 expressed from the HPV16 genome, NIKS cells were plated 1 day prior to transfection, as described in section 2.2.5. Thirty-five wells were prepared for each transfection. The 16WT and 16E6PDZ genomes were prepared using the method described in section 2.8.2. and the cells were transfected with 800 ng of re-circularised genome and 200 ng of pCI-EGFP using the method described in section 2.2.5. Master mixes were prepared for the transfection of each genome.

2.9.4.2. Harvesting cells

Forty-eight hours post-transfection the cells were harvested for cell sorting. The medium was first removed from the cells and the cells were washed once in 1x DPBS (bought as a 10x stock from PAA; H15-011). DPBS was added to the cells (2 ml), and the cells were incubated at 37 ^{o}C for 4 minutes in order to allow the feeder cells to detach. The cells were washed once more with DPBS and 1.5 ml of Accutase™ solution (Millipore; SCR005) was added to each well. The cells were then placed at 37 ^{o}C until all the keratinocytes had detached and were in a single-cell suspension. Once the cells were in a single-cell suspension, the enzyme was deactivated by addition of F-medium, and the cells were centrifuged at 240 x *g* for 4 minutes. They were then washed once in buffer (1x DPBS, 0.5% FBS, 5 mM EDTA) and finally re-suspended in buffer to be analysed. Immediately prior to the sorting, the cells were passed through a

40 µm cell strainer (BD Biosciences; 352340) to ensure that only single cells will be analysed.

2.9.4.3. Cell sorting

The cells were sorted on a Becton Dickinson FACS ARIA II Cell Sorter and the sorting was carried out by Graham Preece at the Flow Cytometry Facility at the NIMR. An initial sort using cells that had not been transfected with GFP was carried out in order to set the gate for the forward- and side-scatter as well as the negative gate for FITC. This was followed by a quick sort using the "16WT + pCI-EGFP"-transfected cells in order to set-up the positive gate. To avoid getting many GFP-negative cells in the GFP-positive sort, the negative and positive gates were set so that there was a gap between them. The purity of both the GFP-positive and GFP-negative sorted cells was then checked. The cells were collected in a small volume of F-medium. Once all the "16WT + pCI-EGFP"-transfected cells had been sorted, the same was repeated with the "16E6PDZ + pCI-EGFP"-transfected cells. Once both cell populations had been sorted, the cells were centrifuged at 240 x *g* for 4 minutes, washed once with PBS and the pellets were frozen at -80 ^{o}C. For protein analysis, the cells were lysed in RIPA 6% SDS protein extraction buffer, as described in section 2.9.1. and the lysates were quantified as described in section 2.9.2.

Chapter 3: Investigation of E6 activities necessary for episomal persistence

3.1. Introduction

The E6 oncoprotein of high-risk HPVs has been shown to interact with multiple cellular proteins, such as E6AP (Huibregtse *et al.*, 1991), p53 (Werness *et al.*, 1990) and several PDZ proteins (Kiyono *et al.*, 1997; Lee *et al.*, 1997; Nakagawa & Huibregtse, 2000). Moreover, many of E6's activities have been characterised, including its ability to degrade p53 (Scheffner *et al.*, 1990) and PDZ proteins (Gardiol *et al.*, 1999; Nakagawa & Huibregtse, 2000) as well as its ability to activate human telomerase (Klingelhutz *et al.*, 1996). These activities interfere with important cellular check-points and induce cellular immortalisation and aberrant proliferation, all of which contribute to the accumulation of cellular mutations and the induction of genomic instability. Although these activities of E6 can contribute to the malignant phenotype that sometimes results from a prolonged HPV infection, their role in the viral life-cycle is less clear.

Most HPV infections are cleared, either by the natural physiology of the tissue, whereby cells from the bottom layer continuously move up towards the surface and are sloughed off, or by immune responses mounted by the host against HPV-containing cells (Schiffman & Kjaer, 2003). It is the failure to clear an HPV infection, therefore permitting persistence of viral episomes and expression of viral genes, that is thought to bring about accumulation of mutations, induction of genetic instability and eventual development of cancer (Remmink *et al.*, 1995; Schiffman & Kjaer, 2003).

Ectopically-introduced DNA, such as plasmids, is not known to persist as un-integrated extrachromosomal DNA in cells upon successive cell divisions. Hence it stands to reason that HPV must actively participate in its successful persistence as extrachromosomal DNA in the nucleus of its host cell. Numerous studies have attempted to elucidate the nature and extend of HPV's contribution in episomal persistence in the basal layer of the epithelium. In these studies,

mutant viral genomes were introduced into monolayer cultures of keratinocytes (which are taken to represent the basal layer of the epithelium) and the ability of the mutant episomes to persist in the population of cells several weeks post-transfection was assessed using Southern blotting. These studies have indeed yielded some unexpected results. The E6 and E7 genes, which were only known to possess oncogenic properties, were both found to be necessary for persistence of HPV DNA in primary human foreskin keratinocytes (HFK). That this was reportedly the case for both the high-risk type HPV31 (Thomas *et al.*, 1999) as well as the low-risk type HPV11 (Oh *et al.*, 2004) suggested that it was the non-oncogenic properties of E6 and E7 that were needed for episomal persistence. However, this notion was challenged by observations made by Thomas *et al*. who demonstrated that HPV31 p53-degradation mutant genomes were unable to persist in HFKs (Thomas *et al.*, 1999). Another study supporting this observation was carried out using hybrid HPV genomes, in which the E6 ORF of HPV31 was substituted for that of HPV16 (Park & Androphy, 2002). Furthermore, a third study investigated the role of the PDZ-binding motif in different aspects of the HPV life-cycle, including episomal persistence, using mutant HPV31 genomes and primary HFKs (Lee & Laimins, 2004).

However, several unresolved issues need to be highlighted when considering the conclusions from these studies. In all of these studies, primary HFKs were used in the persistence assay. This point is significant, as mutations within the HPV31 E6 or E7 ORF that disrupt the immortalisation activity of the virus will produce a negative result for viral episome persistence as the primary cells will not survive indefinitely to sustain the persistence of the viral genomes. Indeed Thomas *et al*. pointed out that the HPV31 p53-degradation mutant genomes that reportedly failed to persist, were also incapable of immortalising the recipient primary HFKs (Thomas *et al.*, 1999). In the study by Park *et al*., where hybrid genomes were used, it is important to consider that such a strategy is susceptible to unintentional effects of the E6 ORF substitution, which may impinge on the replication/persistence of the viral episomes. For example, it is not known if this substitution has inadvertently interfered with the splicing pattern of the HPV transcripts, which can in turn affect the quality and quantity of viral proteins in the cells. The fact that HPV16 is associated more frequently with cancer than HPV31 (Clifford *et al.*, 2003), as well as observations that the

HPV16 E6 protein degrades p53 more efficiently than the HPV31 E6 protein (Lee & Laimins, 2004), suggest that the two E6 proteins do in fact have different quantitative effects.

Interestingly, the need for E7 in episomal persistence was challenged by a study that carried out persistence assays in an immortalised cell line, NIKS, instead of in primary HFKs (Flores *et al.*, 2000). NIKS are a spontaneously immortalised keratinocyte cell line (Allen-Hoffmann *et al.*, 2000) which has been shown to support the HPV life-cycle (Flores *et al.*, 1999). In these cells, HPV16 E7 was found to be dispensable for the episomal persistence of HPV genomes (Flores *et al.*, 2000). This suggests that E7 may have been necessary in the previous studies only in so far as to immortalise the cells to allow the persistence of viral episomes, rather than because it is directly required for persistence itself.

As genome persistence is vital for the development of cancer (Remmink *et al.*, 1995; Schiffman & Kjaer, 2003), it is important to take a closer look at the viral requirements that drive this process. Previous work in the laboratory, carried out by Dr. Ken Raj, focused on identifying which viral proteins are necessary for the episomal persistence of HPV16. HPV16 was chosen as it is the most important type from a medical perspective, and no study had looked at how these episomes persist in cells in detail before. NIKS cells were chosen so as to be able to identify proteins that are necessary for persistence alone, without the need for cellular immortalisation interfering with the results. Furthermore, unlike primary cells, NIKS provide an isogenic cell background for all the experiments. Persistence was measured by the number of HPV-positive clones that could be isolated from the transfection of the HPV genomes into the cells, as was determined by quantitative PCR (qPCR). This method differs from previous studies in two respects: firstly, qPCR is more sensitive than Southern blotting and secondly, the cellular cloning method allowed for the analysis of mutations that may have otherwise conferred a growth disadvantage to the cells. These cells would have potentially been lost from the population, giving the false impression that the mutation had a direct effect on episomal persistence.

As part of this work, mutant HPV16 genomes were generated, each with a STOP codon inserted in a different ORF. The results from this study showed that E7 was dispensable for episomal persistence (Laurson *et al.*, 2010), thereby confirming the results from the Flores *et al*. study (Flores *et al.*, 2000), as were E4 and E5 (Ken Raj, unpublished data). On the other hand, E1, E2 and E6 were found to be necessary for episomal persistence (Laurson *et al.*, 2010; and Ken Raj, unpublished data). The need for E1 and E2 was clear, as these are the viral replication proteins. However, the role of E6 in episomal persistence is less apparent.

The aim of the investigation described in this chapter is to determine which activities of the E6 protein are necessary for the persistence of HPV16 episomes in NIKS cells. Two of the best characterised activities of E6, 1) degradation of p53 and 2) binding to PDZ proteins, will be dissected and analysed for their role in episomal persistence. I will assess persistence both quantitatively by qPCR, as well as qualitatively, using Southern blotting, to determine whether the viral genomes persist episomally, are absent or are integrated into the host cell's DNA.

3.2. Results

3.2.1. Three different mutations of the E6 ORF are introduced into the HPV16 genome

To investigate the role of the p53-degradation and PDZ-binding activities of E6 in episomal persistence, I constructed mutants of the HPV16 genome, with different mutations in the E6 ORF. Three different mutant genomes were made; the first has a one base-pair change which introduces a premature STOP codon in the E6 ORF, and was denoted 16E6STOP. The second has a three amino acid substitution mutation, which abolishes E6's ability to degrade p53 (Kiyono *et al.*, 1998; Klingelhutz *et al.*, 1996). This mutant was denoted 16E6p53m. The third has a four amino acid deletion on the extreme C-terminus of E6, which corresponds to the protein's PDZ-binding motif (Kiyono *et al.*, 1997; Lee *et al.*, 1997). This mutant was denoted 16E6PDZ. The mutations are shown in the context of the E6 ORF in Figure 3.1 and outlined in Table 3.1.

The p53-degradation and PDZ-binding mutations have been previously used in the literature and have been well characterised. Table 3.2 lists publications in which these two mutants have been used. Most PDZ-binding mutations of E6 in the literature have been either six amino acid deletions or single amino acid deletions/substitutions. The E6 mutation used in this study is a four amino acid deletion, which corresponds to the exact PDZ-binding motif (Kiyono *et al.*, 1997; Lee *et al.*, 1997). It should also be noted that most of the available data regarding mutations of E6 have been obtained from studies in which E6 was over-expressed in cells. Very little information is available with regards to the activities of these mutant E6 proteins when they are expressed in the context of the whole HPV genome.

Figure 3.1: Sequence of HPV16 E6 ORF highlighting introduced mutations

The DNA sequence of the E6 ORF of HPV16 (GeneBank accession number: AF125673, region: 83-559). The regions that have been mutated for this study are shown in boxes. The splice donor (SD) and splice acceptor (SA) sites are also indicated.

Table 3.1: Outline of the base-pair mutations introduced into the E6 ORF and the resulting amino-acid mutations		
	Base-pair mutation	**Amino-acid mutation**
E6STOP	cag to tag	Introduction of STOP codon: Q to STOP codon
E6p53m	cgacccag to agcgccac	Three amino acid substitution: RPR to SAT
E6PDZ	gaaacccagctgtaa to taaacccagctgtaa	Four amino acid deletion: ETQL to STOP codon

Table 3.2: Summary of the information available in the literature about the E6 mutants used in this study		
	E6p53m (or E6SAT)	**E6PDZ**
Degradation of p53?	No; (Kiyono *et al.*, 1998; Klingelhutz *et al.*, 1996)	Yes; (Kiyono *et al.*, 1998; Klingelhutz *et al.*, 1996)[1]
Binding/degradation of PDZ proteins?	Yes; (Nakagawa & Huibregtse, 2000)	No; (Gardiol *et al.*, 1999; Glaunsinger *et al.*, 2000; Kiyono *et al.*, 1997; Nakagawa & Huibregtse, 2000)[2] (Lee & Laimins, 2004)[3]
Binding to E6AP?	Yes; (Gewin & Galloway, 2001; Nakagawa & Huibregtse, 2000)	N/A
Activation of telomerase?	Yes; (Kiyono *et al.*, 1998) (for human mammary epithelial cells - HMEC) (Gewin & Galloway, 2001; Klingelhutz *et al.*, 1996) (for human foreskin keratinocytes – HFKs)	Yes; (Kiyono *et al.*, 1998) (for human mammary epithelial cells - HMEC)[1] (Klingelhutz *et al.*, 1996) (for human foreskin keratinocytes – HFKs)[1]

[1] The mutant in these studies was an HPV16 PDZ-binding E6 mutant protein with a six amino acid deletion in the C-terminus.

[2] The mutants in these studies were HPV16 or HPV18 PDZ-binding E6 mutant proteins with deletions/substitutions of single amino acids within the PDZ-binding C-terminal motif.

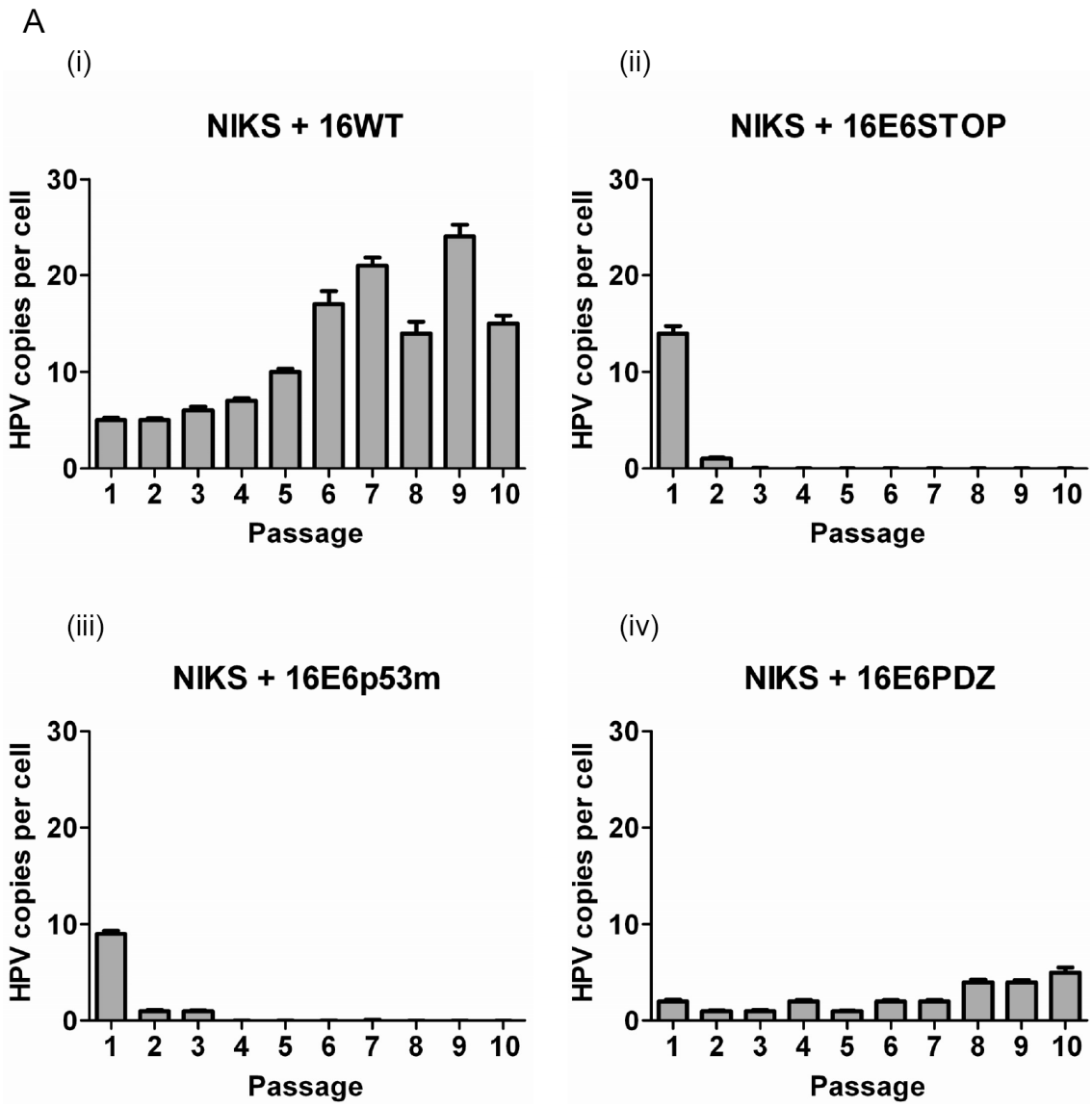
[3] The mutant in this study was an HPV31 PDZ-binding E6 mutant protein with deletion of the last 4 amino acids of the E6 protein.

N/A – No information was available for direct binding of a PDZ-binding mutant E6 to E6AP. However, this interaction may be inferred by the ability of the mutant E6 protein to degrade p53.

3.2.2. The 16WT genomes persist in NIKS cells whereas the 16E6STOP genomes do not

Before addressing the persistence of the 16E6p53m and 16E6PDZ mutant genomes, it was important to confirm the need for E6-expression in HPV16 episomal persistence in NIKS cells. To do this, NIKS cells were transfected with 16WT or 16E6STOP genomes (the latter being unable to make E6 and described in Table 3.1). The two genomes were excised from the pSPW12 plasmid, re-circularised and co-transfected with the pcDNA6 plasmid that carries a blasticidin-resistance gene. Following transfection and antibiotic selection, the cells were cultured for ten passages and DNA was collected at each passage. The DNA was then analysed by qPCR (using primers against the E4 region of the HPV genome and against GAPDH as a control) to measure the number of HPV copies present per cell (Fig. 3.2A i and ii). It is important to note that in these experiments, where a population of cells was analysed, the term “HPV copies per cell” actually refers to the mean number of “HPV copies per cell” for the cells in the population.

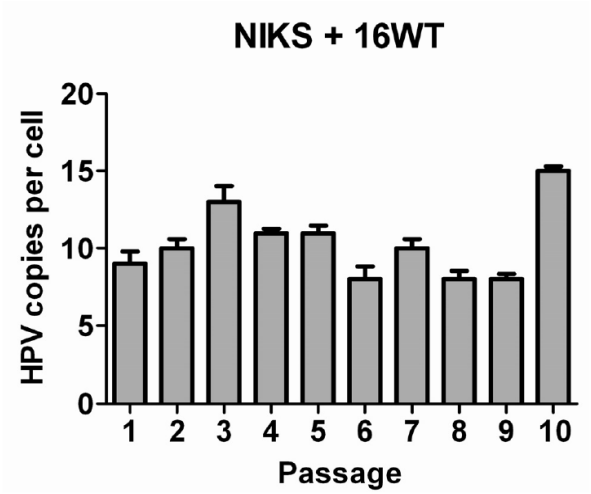
It is clear that the 16WT genomes can persist in NIKS cells, as the number of copies per cell remained constant at first and then even increased at later passages. The increase in copy numbers is not very surprising as cells harbouring the viral genomes may be expected to have a growth advantage. In contrast to 16WT genomes, 16E6STOP genomes did not appear to persist and the number of copies per cell dropped to zero within three passages. These results confirm previous observations (Laurson *et al.*, 2010) and verify that E6 is necessary for viral persistence in this system.



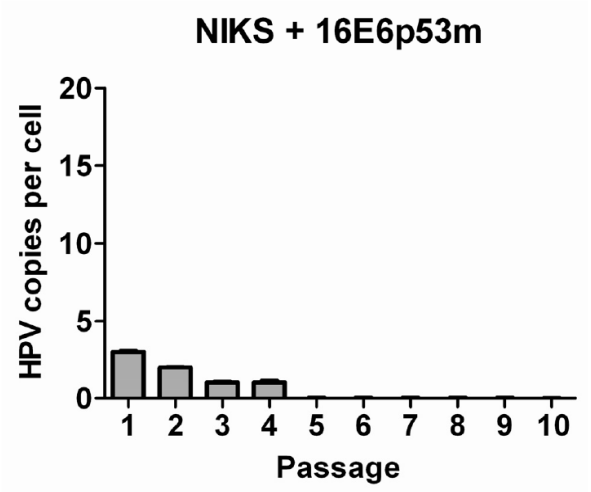
(legend on page 99)

B

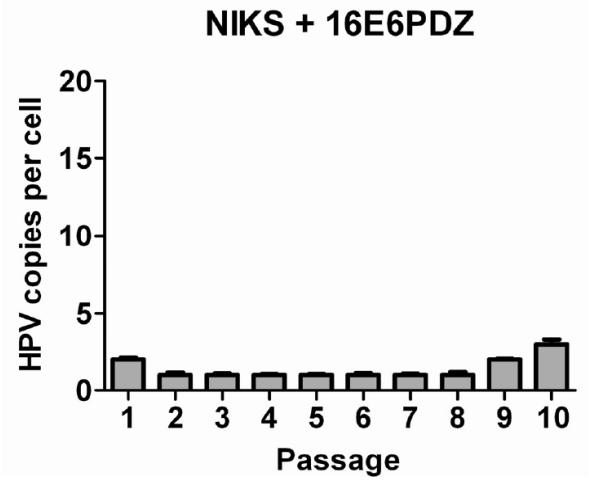
(i)



(ii)



(iii)



(legend on page 99)

Figure 3.2: 16WT genomes persist in NIKS cells whereas 16E6STOP and 16E6p53m genomes do not; 16E6PDZ genomes persist in NIKS cells but at lower numbers than 16WT genomes

In two independent transfection experiments (A and B), NIKS cells were co-transfected with 16WT, 16E6STOP, 16E6p53m or 16E6PDZ genomes, and the pcDNA6 plasmid. Following antibiotic selection, the cells were grown as populations for ten passages, and DNA was extracted at each passage. The DNA was analysed by qPCR to determine the number of HPV copies per cell. The error bars represent the standard deviation of the qPCR triplicates.

3.2.3. E6WT and E6PDZ proteins degrade p53 but E6p53m does not

Although previously characterised in other systems, it was important to confirm that the mutant E6 proteins are indeed defective in their respective activities in the system used in this study, before assessing their role in episomal persistence. Firstly, I wanted to assess the ability of the wild-type and mutant proteins to degrade p53. To do this, I used a retroviral expression system to express wild-type and mutant E6 proteins in NIKS cells. Briefly, I took a pLXSN retroviral vector that has the HPV16 E6 ORF cloned into it (LXSN-E6WT), kindly provided by Dr. Denise Galloway, and used it as a template to make LXSN-E6p53m and LXSN-E6PDZ vectors by site-directed mutagenesis. These vectors, along with the empty LXSN vector, were used to make retroviruses which were then used to infect NIKS cells. Infected cells would express the E6 protein from the retroviral promoter and would also be resistant to neomycin.

After infection and antibiotic selection, the cells were lysed and levels of p53 protein were assessed by western blotting. Figure 3.3 shows one such blot. As expected, the wild-type E6 protein and the E6PDZ mutant protein degrade p53, although it appears that they do so with different efficiencies. In contrast, the E6p53m protein does not degrade p53, and in fact, the levels of p53 appear to increase when E6p53m protein is expressed.

3.2.4. The E6WT and E6p53m proteins degrade hScrib but E6PDZ does not

I next wanted to assess the ability of the wild-type and mutant E6 proteins to degrade the PDZ protein hScrib. hScrib was chosen as a target of E6 because it has been shown to be preferentially degraded by HPV16 E6. Other PDZ proteins such as hDlg on the other hand, were shown to be preferentially degraded by HPV18 E6 (Thomas *et al.*, 2005).

To address this, the levels of hScrib protein were analysed in cell lysates from the stable E6-expressing cells described in the previous section. The levels of hScrib protein in the E6-expressing cells were not lower than in the absence of E6 expression (Fig. 3.4A), which is contrary to is expected based on previous publications.

This result prompted me to have a closer look at the literature. I noticed that the available data regarding the degradation of PDZ proteins by HPV E6, were obtained either from *in vitro* degradation assays, from the analysis of cancer cell lines, or from experiments in which both E6 and the relevant PDZ proteins were over-expressed in cells, from heterologous promoters (Gardiol *et al.*, 1999; Kranjec & Banks, 2010; Nakagawa & Huibregtse, 2000; Thomas *et al.*, 2005). I therefore decided to use a similar assay to look at the degradation of hScrib by the HPV16 E6 protein in this system.

To do so, I sub-cloned the E6WT, E6p53m and E6PDZ ORFs down-stream of the immediate early CMV promoter in the pMV11 plasmid. These vectors were used to co-express the E6 proteins in NIKS cells, together with an HA-hScrib protein expressed from the pcDNA-HA-hScrib plasmid (Thomas *et al.*, 2005), kindly provided by Dr. Lawrence Banks. The cells were harvested 48 hours post-transfection and lysed for protein analysis. The transfection was carried out in triplicate and the levels of HA-hScrib were assessed by western blotting (Fig. 3.4B and C). The levels of HA-hScrib appear to be lower in the presence of E6WT and E6p53m proteins (Fig. 3.4B). However, this was not the case when E6PDZ protein was co-expressed instead (Fig. 3.4C), indicating that the E6PDZ mutant protein is indeed unable to degrade PDZ proteins.

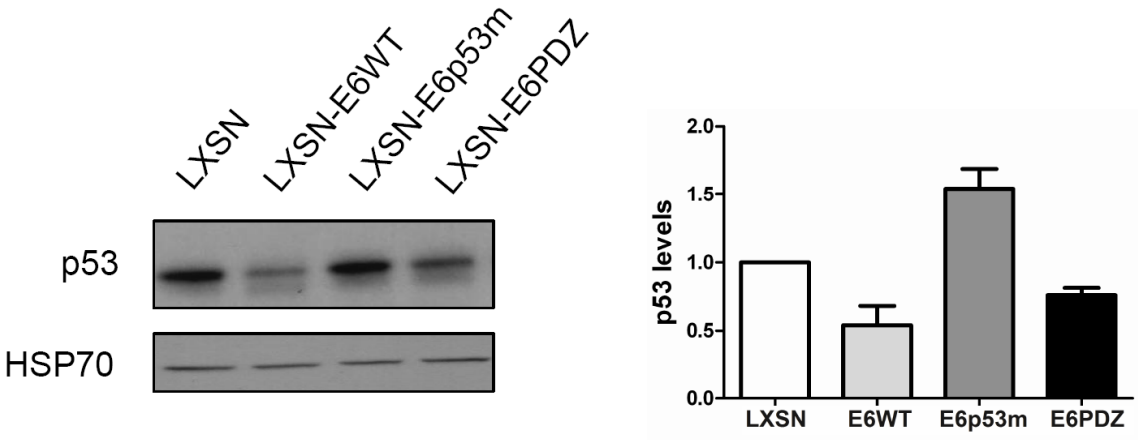


Figure 3.3: Degradation of p53 induced by wild-type and mutant E6 proteins

NIKS cells were infected with retroviruses bearing wild-type or mutant E6 genes. Following antibiotic selection, cell lysates were collected and the levels of p53 in the cells were determined by western blotting. HSP70 was used as a loading control. The levels of p53 protein were measured using ImageJ software and normalised to the loading controls. The bar graph shows the mean levels of p53 protein (in arbitrary units) in cells infected with LXSN-E6WT, LXSN-E6p53m and LXSN-E6PDZ viruses, normalised to the levels of p53 in cells infected with control LXSN viruses for two independent experiments.

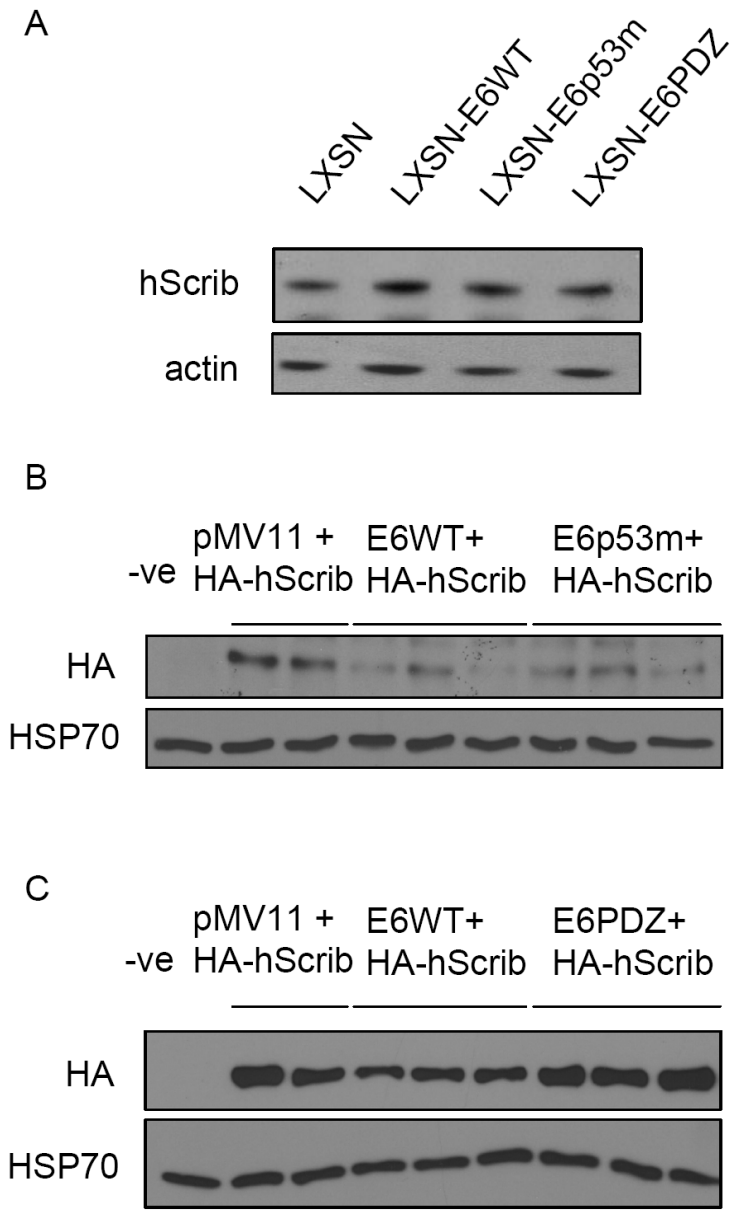


Figure 3.4: Degradation of hScrib induced by wild-type and mutant E6 proteins

A) NIKS cells were infected with retroviruses bearing wild-type or mutant E6 genes. Following antibiotic selection, cell lysates were collected and the levels of hScrib in the cells were determined by western blotting. Actin was used as a loading control. B) NIKS cells were transiently co-transfected with pcDNA-HA-hScrib, and pMV11 control plasmid, pMV11-E6WT or pMV11-E6p53m or C) pMV11-E6PDZ. Whole-cell extracts were prepared 48 h post-transfection. The western blots show the levels of HA-hScrib. HSP70 was used as a loading control.

3.2.5. Wild-type E6 protein is able to degrade endogenous hScrib protein when expressed from stable clonal cell lines

Having demonstrated that the E6PDZ mutant protein is indeed deficient in degrading hScrib when both are expressed exogenously, I also wanted to determine whether the wild-type E6 protein is able to degrade endogenous hScrib protein. From the results presented in Figure 3.4A, using stable LXSN-E6WT cells, it appeared that, at least in these cells, endogenous hScrib protein was not degraded by wild-type E6 protein. Interestingly, a study by Lee and Laimins showed that the levels of endogenous PDZ proteins (in this case hDlg, hScrib and MUPP1) were not significantly different in cells transfected with HPV31 genomes compared to un-transfected cells (Lee & Laimins, 2004).

I therefore hypothesised that the levels of E6 present in the population of LXSN-E6WT cells may not be high enough to cause significant degradation of PDZ proteins. To test this, I isolated individual clones of LXSN-E6WT cells with the intention of obtaining some that express high levels of the E6 protein. Such clones would provide me with the opportunity to test my hypothesis. As a quick means of identifying such high E6-expressing clones, I screened clones of LXSN-E6WT cells for the p53 protein. The rationale for this approach is based on the fact that HPV16 E6 degrades p53 protein (Scheffner *et al.*, 1990), and clones with very low p53 levels are likely to express high levels of E6 protein. I chose two clones that had very low levels of p53 compared to control cells, and western blotting of extracts from these revealed that they were indeed expressing readily detectable level of the E6 protein (Fig. 3.5). Assessment of the levels of endogenous hScrib by western blotting showed that, when E6 was present, the levels of hScrib were clearly decreased (Fig. 3.5). This supports the notion that the inability to detect hScrib reduction in a population of E6-expressing cells was owed to the heterogeneity of E6 expression within the cells in the population.

Interestingly, the difference in the levels of p53 protein was much more pronounced than the difference observed in the levels of hScrib protein, suggesting that more E6 protein may be required to degrade hScrib than to degrade p53. Nonetheless, this experiment confirms that wild-type E6 protein is able to degrade endogenous hScrib protein.

However, the levels of E6 in this experiment are likely to be higher than those expressed during a natural HPV infection. This is because protein expression from the HPV promoter is kept under tight control by the activities of other viral proteins. This control is absent in the LXSN-E6WT cells. Therefore, whether the amount of E6 protein expressed in an HPV infection is sufficient to induce the degradation of endogenous hScrib protein remains to be established.

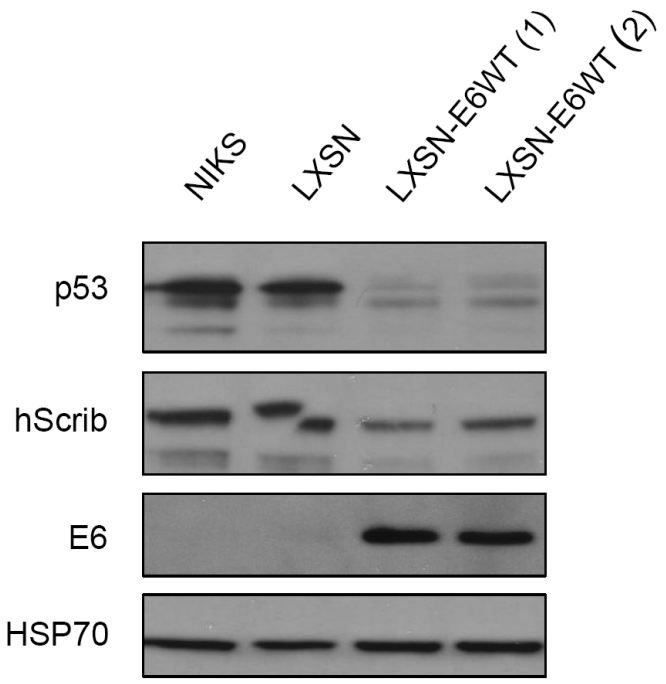


Figure 3.5: E6WT expressed from stable clonal cell lines can degrade endogenous hScrib

NIKS cells were infected with retroviruses bearing the wild-type E6 gene, and the cells were cloned. Cell lysates were collected and the levels of p53, hScrib and E6 in two individual clones were determined by western blotting. HSP70 was used as a loading control.

3.2.6. The wild-type and mutant HPV16 genomes have similar transfection efficiencies in NIKS cells

Having characterised the mutant E6 proteins, I next wanted to determine whether the mutant genomes are able to persist in NIKS cells. It was first important to assess whether the genomes have similar transfection efficiencies in NIKS cells, as major differences could potentially affect the results of the persistence assays. The transfection efficiencies of the 16WT, 16E6p53m and 16E6PDZ genomes were assessed in two different ways.

In the first, the cells were co-transfected with the viral genomes and the pcDNA6 plasmid (which carries a blasticidin-resistance gene) in the same way as they would be for the persistence assays, and DNA was extracted from them 48 hours post-transfection. The DNA was then analysed by qPCR to determine the number of HPV copies per cell. In this assay, the cells were first trypsinised and passed onto new plates 24 hours prior to the DNA extraction, to minimise the chances of having any Effectene-DNA complexes attached on the outside of the cells and interfering with the results. The trypsinisation process of these cells involved one quick wash with trypsin-versene, a 2 minute incubation to remove the feeder cells and a 5-10 minute incubation to remove the NIKS cells, followed by neutralisation of the trypsin and centrifugation of the detached cells. The copy numbers for the transfection of the two mutant genomes were normalised to those for the transfection of the wild-type genome, for three independent experiments (Fig. 3.6A). The transfection efficiencies of the three genomes were found to be comparable.

The second method I used to compare the transfection efficiencies of the three genomes was to co-transfect the genomes with the pMV10 plasmid that expresses β-galactosidase (β-gal). Comparing the protein levels of β-gal in these cells confirmed that the transfection efficiency of the 16WT genome was not higher than that of the mutant genomes, and therefore would not affect the results of the transfection experiments (Fig. 3.6B).

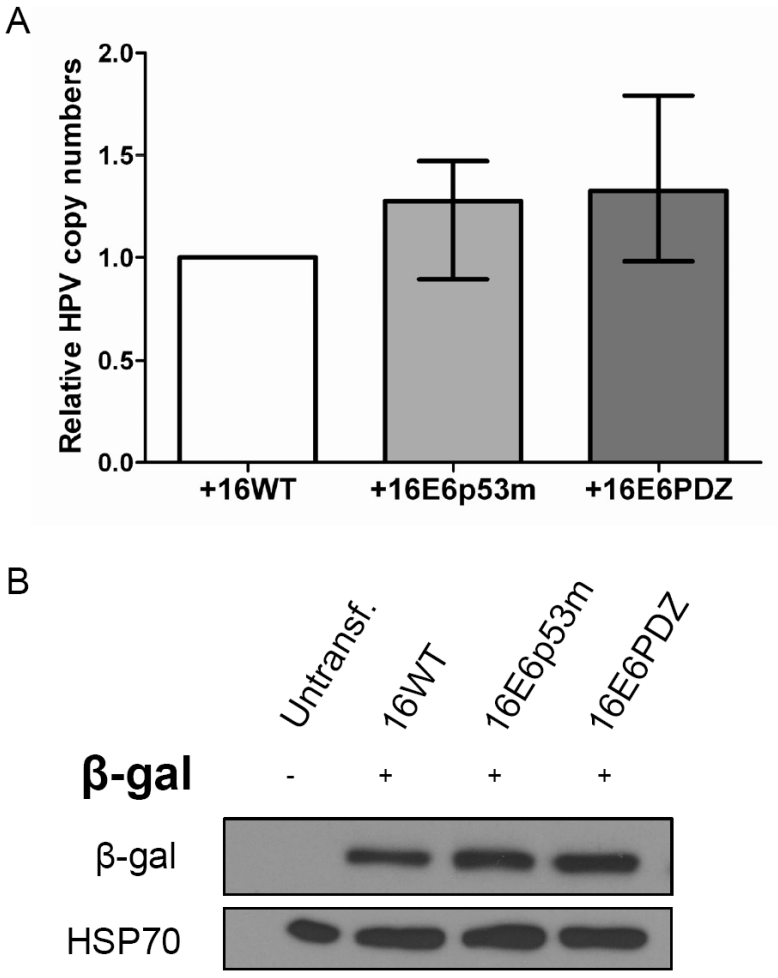


Figure 3.6: There are no major differences in the transfection efficiencies of the wild-type and mutant HPV16 genomes

A) NIKS cells were co-transfected with wild-type or mutant HPV16 genomes, and pcDNA6 plasmid and the HPV copies per cell were measured by qPCR at 48 hours post-transfection. The copy numbers for the transfection of the two mutant genomes were normalised to those for the wild-type genome. The bar graphs show the average of three experiments and the error bars show the range. B) NIKS cells were co-transfected with wild-type or mutant HPV16 genomes and the pMV10 plasmid and lysates were collected at 48 hours post-transfection. The levels of β-gal were determined by western blotting. HSP70 was used as a loading control.

3.2.7. 16E6p53m genomes do not persist in NIKS cell populations

Having confirmed the need for E6 in the persistence of viral DNA in NIKS cells (section 3.2.2), I wanted to assess the ability of the E6 mutant genomes to persist. The first mutant genome to be analysed was 16E6p53m. As described in section 3.2.2. NIKS cells were co-transfected with the viral genomes and the pcDNA6 plasmid and treated with blasticidin. Surviving cells were subsequently cultured for ten passages, with DNA being collected at each passage. The results from two transfection experiments are shown in Figures 3.2A (panels i and iii) and Figure 3.2B (panels i and ii).

In both experiments the 16WT genomes were able to persist, as the number of HPV copies per cell either stayed more or less constant or even increased at later passages. In contrast, the 16E6p53m genomes were unable to persist as the HPV copies per cell dropped to zero within four or five passages. Again this is consistent in both experiments. I thus conclude that the 16E6p53m genomes are unable to persist in NIKS cells.

The persistence profile for the 16WT genomes appears different in the two experiments presented in Figure 3.2. As mentioned earlier, an increase in the copy number is not surprising, as the presence of viral genomes might be expected to give a growth advantage to the cells. This is what seems to be happening in the experiment presented in Figure 3.2A. In the experiment presented in Figure 3.2B however, the copy number stayed more or less constant. This may indicate that an increase in copy number has already occurred, prior to the first analysable time-point for this experiment, after which the copy number was stabilised. The apparent difference in the persistence profiles of the 16WT genome between the two experiments may also be a consequence of the heterogeneity that is expected after the transfection and culturing of a population of cells, which will be discussed further later on. As experimental variations, which may arise from different cell batches or different transfection efficiencies were beyond my control, it was imperative that the persistence of the 16WT genomes was always assayed in parallel to that of any mutant genome, to serve as a positive control.

Importantly however, the 16E6p53m genomes were unable to persist in NIKS cells in either of the two experiments presented above. This result suggests that the region of the E6 protein that was mutated in this genome has a role in viral genome persistence.

3.2.8. 16E6PDZ genomes persist in NIKS cell populations

I next wanted to determine whether the PDZ-binding motif of E6 is necessary for viral genome persistence. To do this I transfected NIKS cells with the 16E6PDZ genomes. This was done as part of the same experiments presented in section 3.2.7 and the results are shown in Figures 3.2A (panels i and iv) and Figure 3.2B (i and iii).

Unlike the 16E6p53m genomes, these mutant genomes appear to persist in NIKS cells. However, the number of HPV copies per cell for the 16E6PDZ genomes was lower than for the 16WT genomes and this was consistent in both experiments. The 16E6PDZ genomes were in fact present at lower copies than the 16WT genomes from passage 1 (p1) and this may be due to a biological effect that has already taken place prior to this first analysable time-point. Moreover, the copy number of the 16E6PDZ genomes seems to decrease initially and then rise again. This is seen in both experiments and is consistent with a possible growth advantage for HPV-positive cells in the population.

From the results presented in this section, I conclude that the 16E6PDZ mutant genomes can persist in NIKS cells but do so at lower levels than the wild-type genomes.

3.2.9. 16E6PDZ genomes do not persist episomally in NIKS cell populations

In the previous section I concluded that the 16E6PDZ genomes can persist in NIKS cells, but appear to do so at lower levels compared to wild-type genomes. Wild-type HPV16 genomes have been shown before to persist episomally in NIKS cells (Lambert *et al.*, 2005; Laurson *et al.*, 2010). I next wanted to investigate whether the 16E6PDZ DNA that was detected in the cells by qPCR is in fact episomal DNA or whether it has integrated into the cellular genome.

To do this, I analysed the DNA collected from the persistence assay described in section 3.2.8 by Southern blotting to look at the physical state of the viral DNA. The copy numbers of the 16WT and 16E6PDZ genomes (as measured by qPCR) were very low in both experiments presented in Figure 3.2. For this assay, I analysed the DNA collected from the experiment shown in Figure 3.2A, as these samples contained more HPV DNA per cell, thus increasing my chances of detecting viral DNA by Southern blotting.

DNA from one passage of the 16WT-transfected cells was digested with Hind III in the first instance, which does not cut the HPV16 genome. In all Southern blots, an HPV-positive episomal NIKS cell line was used as a positive control (Laurson *et al.*, 2010). The positive controls show the three-band pattern expected for episomal DNA. The fastest migrating band represents supercoiled DNA, the middle band represents linear DNA and the slowest migrating band represents open circular DNA. The 16WT genomes appear to have the same band pattern as the positive control, which suggests that these genomes persisted in a primarily episomal form (Fig. 3.7A). However, the bands were difficult to detect due to the low copy number present in these cells. In order to confirm the episomal state of the 16WT DNA in the cells, I repeated the Southern blot, but digested the DNA with BamH I which cuts the HPV DNA once, linearising it. The positive control was also digested with BamH I (Fig. 3.7B). Single bands of the same size were obtained for the positive control and the 16WT sample and represent the linear 8 kb HPV16 genomes. This result indicates again that the 16WT genomes were present primarily in an episomal form in these cells.

To look at the physical state of the 16E6PDZ DNA, a DNA sample from one passage of the 16E6PDZ-transfected cells was again digested with Hind III in the first instance (Fig. 3.7C). In this case, the band obtained, although very difficult to detect, was of a different size to any of the bands of the positive control. This is indicative of integration of the viral genome into cellular DNA. This was confirmed by another Southern blot using samples digested with BamH I (Fig. 3.7D). Again, the band obtained from the digestion of the 16E6PDZ sample, although very faint, was shown to be of a different size to that of the positive control. This result suggests that the BamH I digestion did

not simply linearise the circular 8 kb mutant HPV genome but that an integration event has occurred, which brought the HPV DNA in the proximity of cellular BamH I sites.

From these experiments, I conclude that, although the 16E6PDZ genome does appear to persist in NIKS cells, it does not persist episomally.

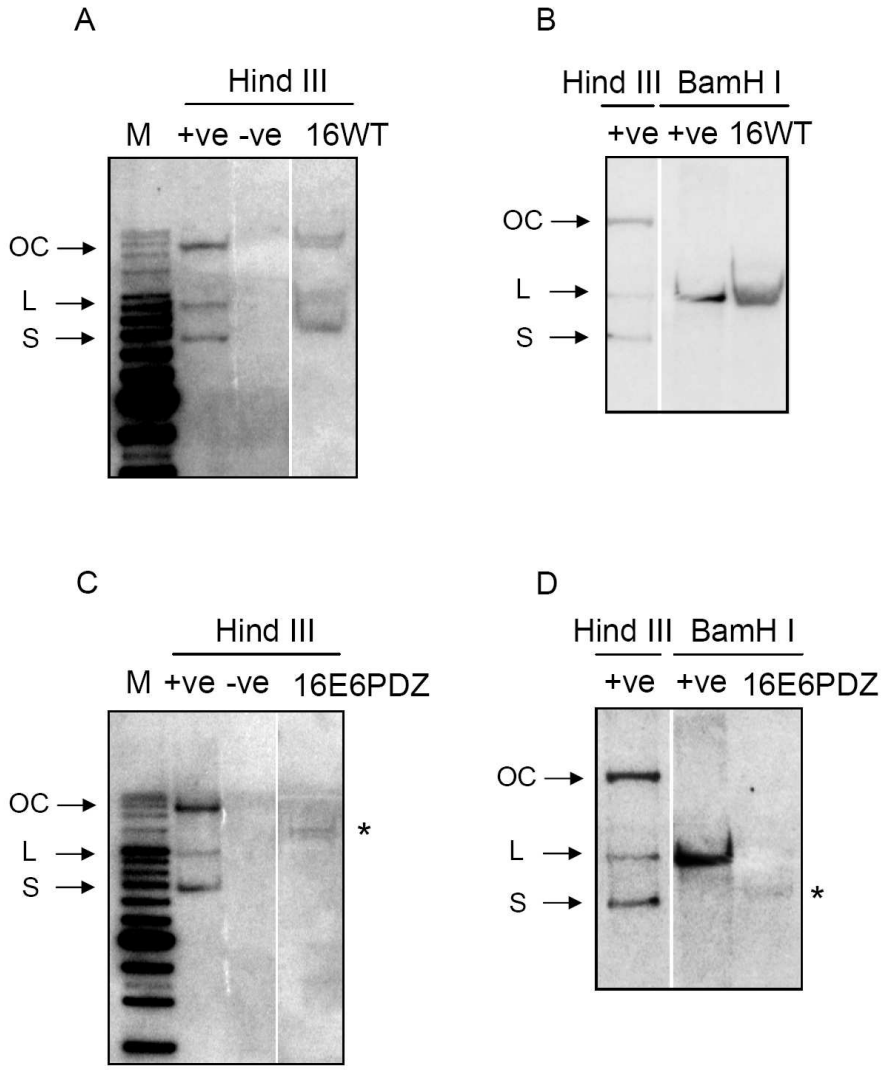


Figure 3.7: 16WT genomes persist episomally in NIKS cells whereas 16E6PDZ genomes do not

DNA extracted from cells from the persistence assay was digested with restriction enzymes which either cut or do not cut the HPV genome (BamH I or Hind III respectively), and subjected to Southern blotting. DNA from cells transfected with 16WT (A and B) or 16E6PDZ (C and D) genomes was analysed. The positive control was DNA from an episomal HPV16-positive NIKS cell line and the negative control was DNA from HPV-negative NIKS cells. The positions of linear (L), open circular (OC) and supercoiled (S) episomal genomes are indicated, as is the marker (M) and the position of likely integrated DNA (*). The middle portion of each gel was cropped and this is indicated by a white line.

3.2.10. 16E6p53m genomes do not persist in NIKS clonal cell lines

In the experiments described in the previous sections, the DNA analysis was carried out on cells that were transfected and grown as a population. An issue that arises in these kinds of experiments is that the results represent an average of the entire population of cells and may be inordinately influenced by a sub-population of cells with growth advantage over the others. Specifically in regards to the population experiments above, it is a concern that 16E6p53m genomes could induce growth retardation to cells that harbour them. This is because, while the mutant E6 protein expressed cannot target p53 for degradation, the E7 protein encoded by this HPV16 genome is wild-type, and would be capable of increasing the p53 level above that of the basal level (Demers *et al.*, 1994; Laurson *et al.*, 2010). In such a scenario, elevation of p21 levels (transcriptional target of p53) (Stoppler *et al.*, 1998), could confer a growth disadvantage to cells that harbour 16E6p53m genomes. In support of this, NIKS cells that express only the E7 protein (in the absence of E6) have been observed to grow slower than NIKS cells that do not express any HPV proteins (Ken Raj, personal communication). Similar concerns apply to the 16E6PDZ genomes as Lee *et al*. observed that cells harbouring HPV31 E6 mutant genomes, incapable of targeting PDZ proteins, grew slower than control cells (Lee & Laimins, 2004). Since abrogation of either of these E6 activities within the whole HPV16 genome could potentially result in growth disadvantage to cells harbouring the mutant genomes, it is important to employ another experimental system that addresses these concerns, to verify the results from the population studies.

Hence I decided to carry out the persistence assay by analysing individual clones of NIKS that were derived from the population of transfected cells. In these experiments, NIKS cells were transfected with HPV16 DNA and pcDNA6, and then plated at very low density, followed by selection with antibiotics. This experimental design allowed single clones to be easily isolated and cultured individually. This eliminates the risk of cells bearing only the pcDNA6 vector and devoid of HPV mutant genomes, or cells that may have spontaneously lost the viral episomes (Stewart *et al.*, 1994), outgrowing those that harbour the mutant viral genomes. Furthermore, some clones would be expected to have a higher number of HPV copies per cell, compared to the average of the population, and

these could be used to overcome the issue of the very low average HPV copy number within the populations that made Southern blotting analysis very difficult.

In the first instance, cells were transfected with either 16WT or 16E6p53m genomes. Individual clones were isolated and DNA was extracted and analysed by qPCR 2-3 weeks post-transfection (Fig. 3.8). As expected, the populations of transfected cells were observed to be heterogeneous, with some clones harbouring much higher numbers of HPV copies per cell than others. The results show that 100% of the 16WT clones and 91% of the 16E6p53m clones were positive for HPV DNA. However, the mean number of HPV copies per cell of the 16WT clones was 239 whereas that of the 16E6p53m clones was 101. The much reduced mean copy number of the 16E6p53m clones (only 42% of that of the 16WT clones), supports my previous conclusions about there being a difference between these two genomes (section 3.2.7). Table 3.3 gives a summary of the results from this experiment. From these, it is clear that both genomes (16WT and 16E6p53m) were able to persist in NIKS (albeit with different efficiencies) up to this point in the analysis (2-3 weeks post-transfection). This observation appears to challenge those from the population analyses, and supports the idea that the clonal assay has a higher resolution than the population assay.

However, the lower average number of HPV copies per cell for the 16E6p53m genomes in the clonal cell lines suggests that the mutant HPV genomes in the 16E6p53m clones may be on the way to being lost and have already dropped in numbers. To test this hypothesis, I took one 16WT and one 16E6p53m clone and cultured them for several additional passages, extracting DNA at each passage. The DNA was then analysed by Southern blotting to determine whether the genomes persist during more long-term culturing, and also to establish the physical state of the HPV DNA in the cells. The viral DNA in both the 16WT (Fig. 3.9A) and the 16E6p53m clone (Fig. 3.9B) appears to be episomal, as the bands were of the same size as the episomal positive control. Moreover, the 16WT genomes persisted episomally throughout the ten passages. In contrast, the 16E6p53m genomes dropped in copy number over

time, which is consistent with my hypothesis that the 16E6p53m genomes may not be able to persist.

To verify that the eventual loss of 16E6p53m genomes, is not specific to the individual NIKS cell clone that was used in the study above, I repeated the experiment from the beginning with a fresh transfection and analysed all the cell clones at a later time-point; at 4-5 weeks post-transfection instead of 2-3 weeks. Figures 3.10A and B show the results from the transfection of the 16WT and 16E6p53m genomes. In this experiment, 70% of the 16WT clones were positive for HPV DNA, compared to the 100% of positive clones in the previous experiment. Strikingly, only 24% of the 16E6p53m clones were positive for HPV DNA, compared to 91% in the previous experiments. More importantly though, the mean number of HPV copies per cell for all the 16E6p53m clones was only 5% of that of all the 16WT clones (39 copies per cell for the 16WT clones and 2 copies per cell for the 16E6p53m clones). Taken together with the results from the population experiments, these results suggest that the 16E6p53m genomes cannot persist in NIKS cells.

3.2.11. 16E6PDZ genomes do not persist in NIKS clonal cell lines

The clonal experiment was also carried out using the 16E6PDZ mutant genomes, with clones being analysed at 4-5 weeks post-transfection and compared to the 16WT clones (Fig. 3.10A and C). Strikingly, only 8% of the analysed 16E6PDZ clones were found to be positive for HPV DNA (2 out of 26 clones) and those 2 positive clones were found to have similar numbers of HPV copies per cell as some of the 16WT clones. This was very interesting and made apparent the lower sensitivity of the population experiments. Table 3.4 gives a summary of the results from this experiment, for the transfection of the 16WT, 16E6p53m and 16E6PDZ genomes.

Having observed that the 16E6PDZ genomes integrated into the cellular DNA in the population experiment, I was keen to determine the physical state of the two HPV-positive 16E6PDZ clones from the experiment described above. To do this, I carried out a Southern blot analyses on DNA from two 16WT clones with different copy numbers, together with DNA from the two 16E6PDZ-positive

clones, as well as an HPV-negative clone from each transfection (as controls) (Fig. 3.11). DNA samples from two 16E6p53m clones were also analysed by Southern blotting but no HPV DNA was detected, probably due to the low number of HPV copies in these cells (as measured by qPCR). For this Southern blot, the DNA was digested with Hind III.

Both HPV-positive 16WT clones analysed by Southern blotting were found to harbour episomal HPV DNA. In contrast, both HPV-positive 16E6PDZ clones were found to harbour integrated HPV DNA as indicated by the HPV DNA bands that were of different sizes compared to the positive control. The higher copy numbers in these clones allowed me to clearly confirm the integration by Southern blotting and these results confirmed those obtained from the population assay (section 3.2.9).

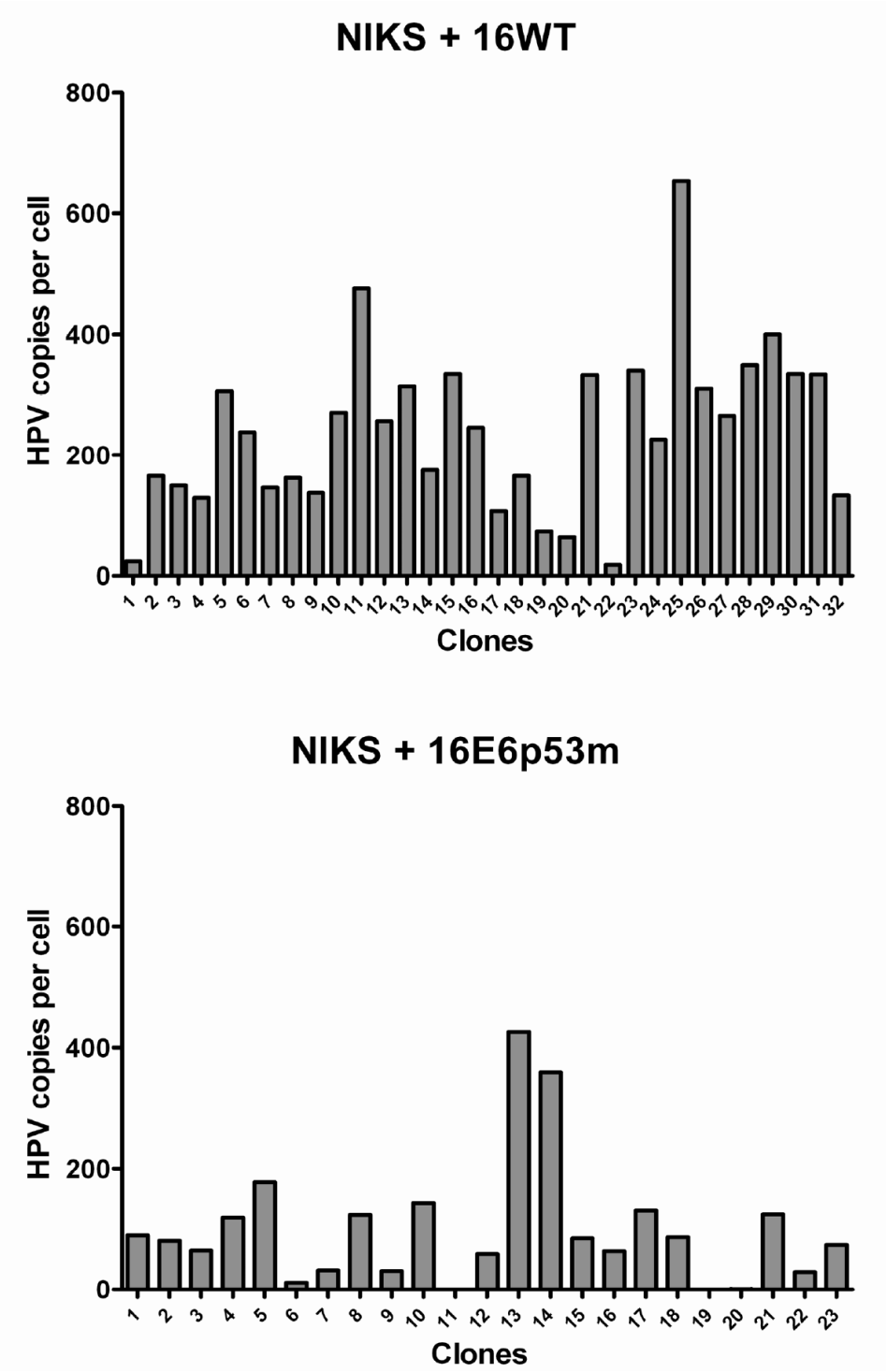


Figure 3.8: 16WT and 16E6p53m genomes persist in clonal cell lines but at different levels

NIKS cells were co-transfected with 16WT or 16E6p53m genomes, and the pcDNA6 plasmid and plated at low density post-transfection. Following antibiotic selection and further growth, clones were isolated and grown independently. DNA was extracted from individual clones 2-3 weeks post-transfection and was analysed by qPCR to determine the number of HPV copies per cell.

Table 3.3: Summary of the data from the clonal experiment (analysed 2-3 weeks post-transfection)

	Percentage of HPV +ve clones	Mean number of HPV copies per cell	
		In all clones	In +ve clones
16WT	100%	239	239
16E6p53m	91%	101	110

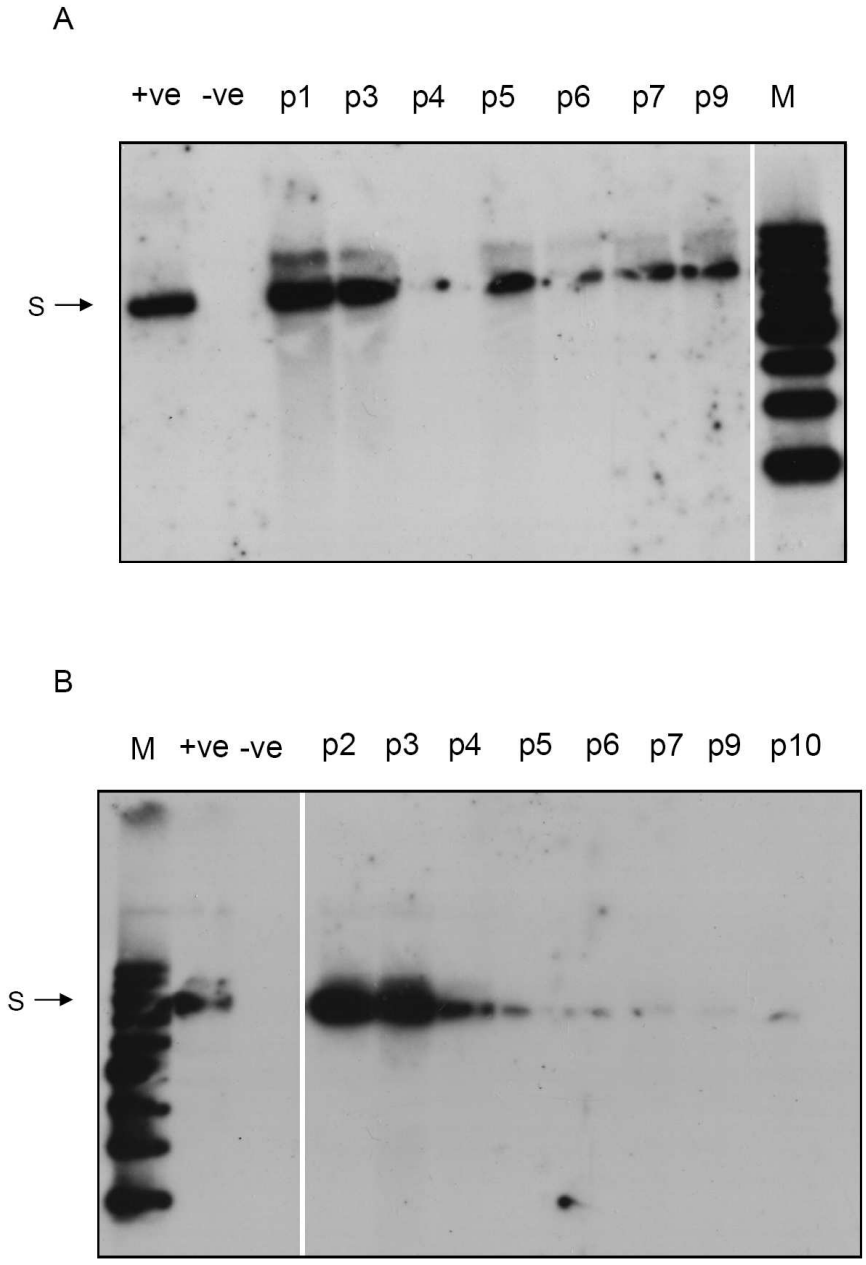
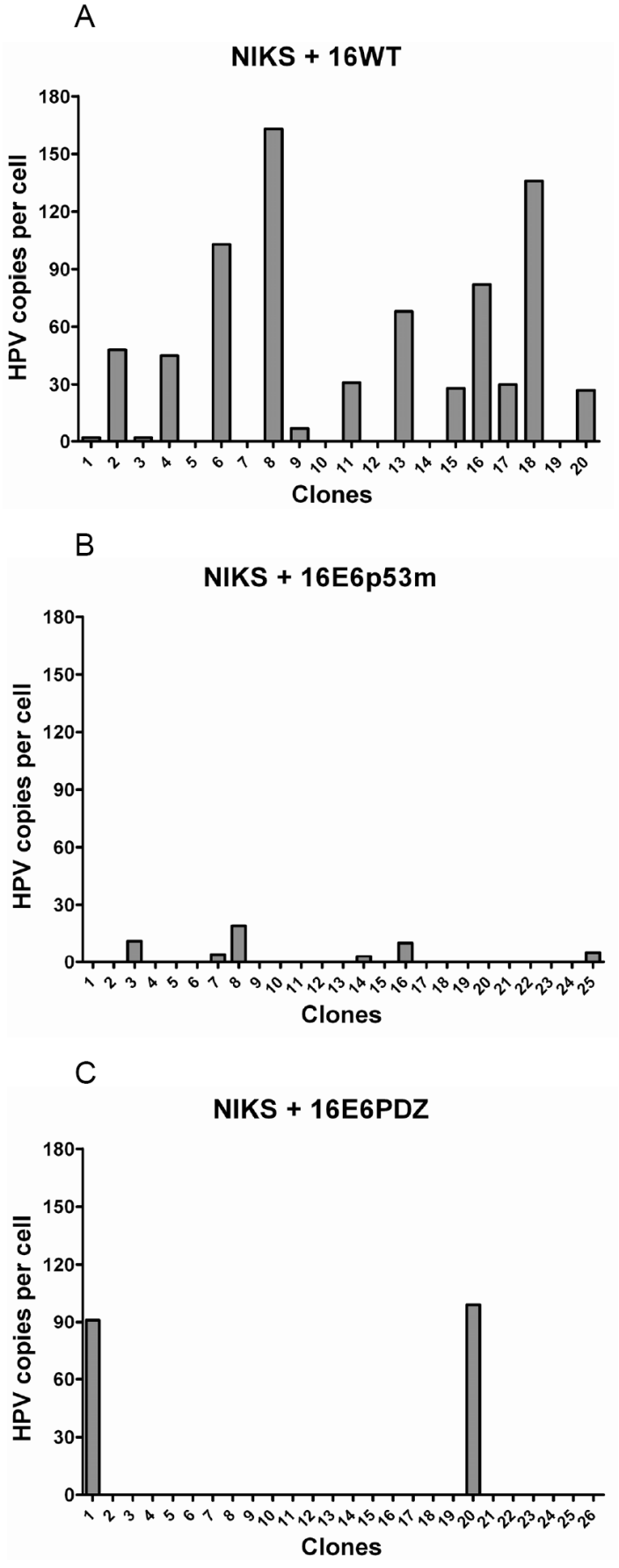


Figure 3.9: 16WT genomes persist episomally in long-term passaging of clonal cell lines, whereas 16E6p53m genomes drop in copy numbers

Undigested DNA from different passages of A) a 16WT and B) a 16E6p53m clone was analysed by Southern blotting. The positive control was DNA from an episomal HPV16-positive NIKS cell line and the negative control was DNA from HPV-negative NIKS cells. The position of supercoiled (S) episomal genomes is indicated, as is the marker (M). The "p" values above each lane indicate the passage number of the cells from which DNA was extracted (e.g. p1 indicates "passage 1"). The white lines denote portions of the gels that have been cropped.



(legend on page 122)

Figure 3.10: 16WT genomes persist in clonal cell lines whereas 16E6p53m and 16E6PDZ genomes do not

NIKS cells were co-transfected with (A) 16WT, (B) 16E6p53m or (C) 16E6PDZ genomes, and the pcDNA6 plasmid and plated at low density post-transfection. Following antibiotic selection and further growth, clones were isolated and grown independently. DNA was extracted from individual clones 4-5 weeks post-transfection and was analysed by qPCR to determine the number of HPV copies per cell.

Table 3.4: Summary of the data from the clonal experiment (analysed 4-5 weeks post-transfection)

	Percentage of HPV +ve clones	Mean number of HPV copies per cell	
		In all clones	In +ve clones
16WT	70%	39	55
16E6p53m	24%	2	9
16E6PDZ	8%	7	95

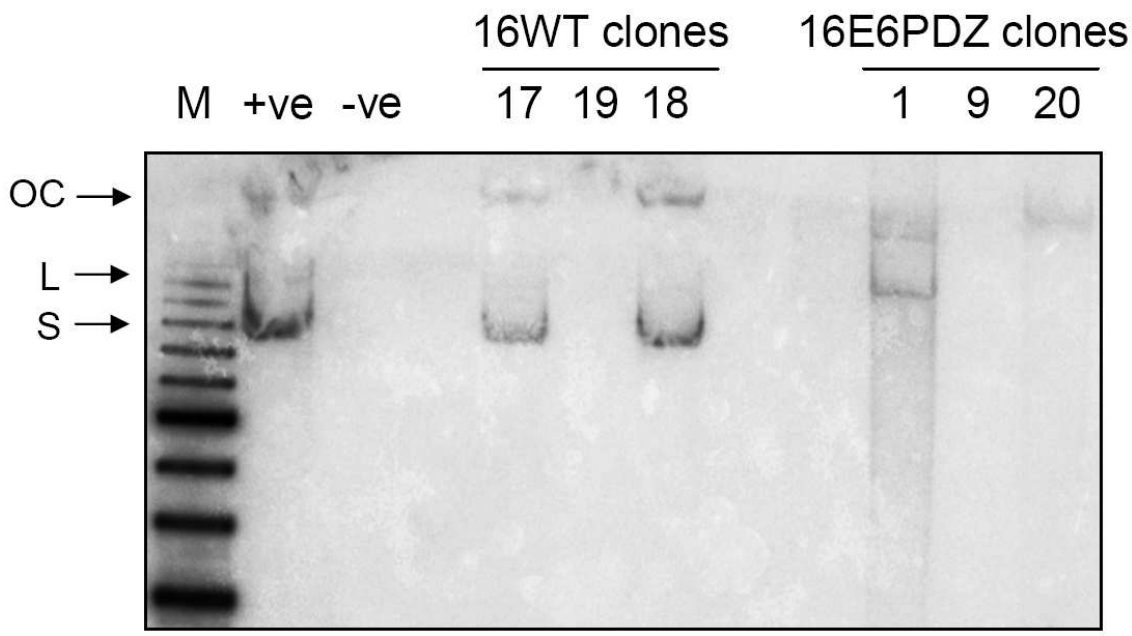


Figure 3.11: 16WT genomes persist episomally in clonal cell lines whereas 16E6PDZ genomes do not

DNA from one HPV-negative and two HPV-positive 16WT and 16E6PDZ clones was digested with Hind III and analysed by Southern blotting to determine the physical state of the HPV DNA. The positive control was DNA from an episomal HPV16-positive NIKS cell line and the negative control was DNA from HPV-negative NIKS cells. The positions of linear (L), open circular (OC) and supercoiled (S) episomal genomes are indicated, as is the marker (M). 16WT clones 17 and 18 and 16E6PDZ clones 1 and 20 were HPV-positive. 16WT clone 19 and 16E6PDZ clone 9 were HPV-negative.

3.2.12. 16WT genomes persist episomally in NIKS clonal cell lines in long-term passaging

In the previous section I observed that the 16WT genomes were episomal in the analysed clones whereas the 16E6PDZ genomes were not. I then wanted to confirm that the integration phenotype that I observed with the 16E6PDZ genomes was specific to these mutant genomes, and not a result of my methodology. To do this, I cultured one of the 16WT clones shown in Figure 3.11 for a further ten passages, extracting DNA at each passage and analysing it by Southern blotting (Fig. 3.12A). The wild-type genomes were observed to be episomal irrespective of the time-point analysed. I thus have no reason to believe that the loss of the episomal phenotype was a result of my methodology, and rather is specific to the 16E6PDZ genomes. To further support my results, I also cultured a 16WT clone from an independent experiment and analysed its DNA by Southern blotting (Fig. 3.12B). Once again, the HPV DNA was found to be episomal and this was consistent over all the analysed passages.

I also wanted to verify the integration of the 16E6PDZ genomes analysed in the clonal experiment. To do this, I cultured one of the clones shown in Figure 3.11 for several additional passages, extracted DNA and analysed it by Southern blotting (Fig. 3.13A). For this Southern blot, DNA was digested with a different enzyme than the one used in the Southern blot in Figure 3.11, to confirm the integration event. Xba I, which does not cut HPV16 DNA, was used for the digestion. The HPV DNA bands in this clone were again of different size to those of the positive control, and in fact appeared as a smear which is indicative of multiple integration events. This was consistent for all the analysed passages, confirming that it truly represents an integration event.

Intriguingly, the HPV DNA in this clone did not appear to persist even as an integrant as the band intensity in the Southern blot decreased over time. This suggests that some growth competition may exist within the clone. This would be possible if the integration event did not occur in the very first cell from which the clone was generated. If this is the case, then the clone would be heterogeneous with some cells harbouring integrated copies of the mutant HPV genome and some being HPV-negative cells, or even harbouring different

integration events. Moreover, it is possible that these cells did not originate from a single clone, but maybe from two merged clones that were growing too close together. This would again introduce heterogeneity and possible growth competition between the cells. It is therefore possible that this specific integration event, or the expression of an E6 protein that cannot degrade PDZ proteins, may confer a growth disadvantage. However, the precise nature of the integration events in these clones, and their effects on the growth of the cells is beyond the scope of this study.

Lastly, to confirm that the integration of the 16E6PDZ genome was not specific to this experiment, a 16E6PDZ clone from an independent experiment was also cultured for several passages and its DNA was analysed by Southern blotting (Fig. 3.13B). The HPV DNA from this clone was also found to be integrated into the cellular DNA, supporting my previous conclusions that the 16E6PDZ genomes cannot persist episomally in NIKS cells.

From these experiments I conclude that neither the 16E6p53m nor the 16E6PDZ genomes can persist episomally in NIKS cells and this was demonstrated by the population as well as the clonal experiments.

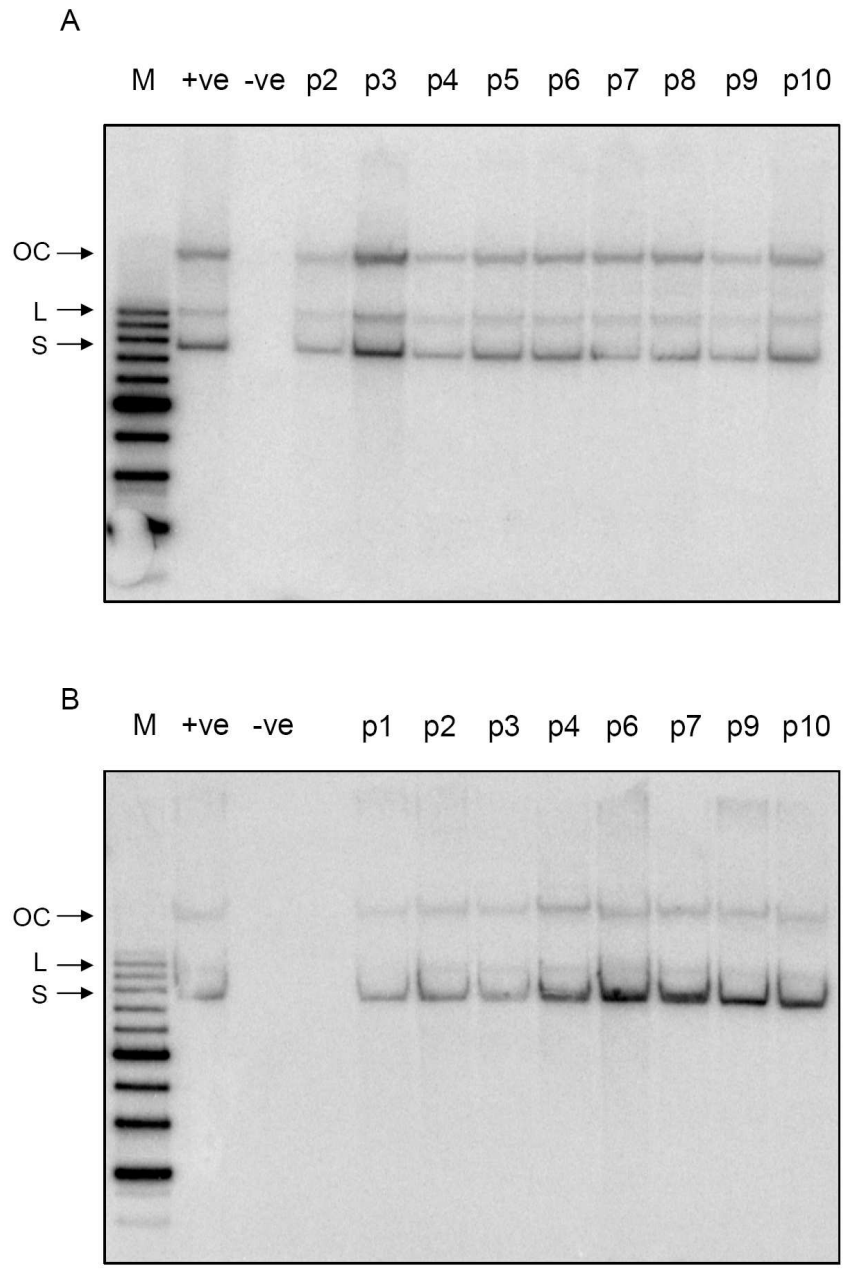


Figure 3.12: 16WT genomes persist episomally in NIKS cells over several passages

Two 16WT clones (A and B) were cultured for several passages. DNA was extracted at each passage, digested with Hind III and analysed by Southern blotting. The positive control was DNA from an episomal HPV16-positive NIKS cell line and the negative control was DNA from HPV-negative NIKS cells. The positions of linear (L), open circular (OC) and supercoiled (S) episomal genomes are indicated, as is the marker (M). The “p” values above each lane indicate the passage number of the cells from which DNA was extracted (e.g. p1 indicates “passage 1”).

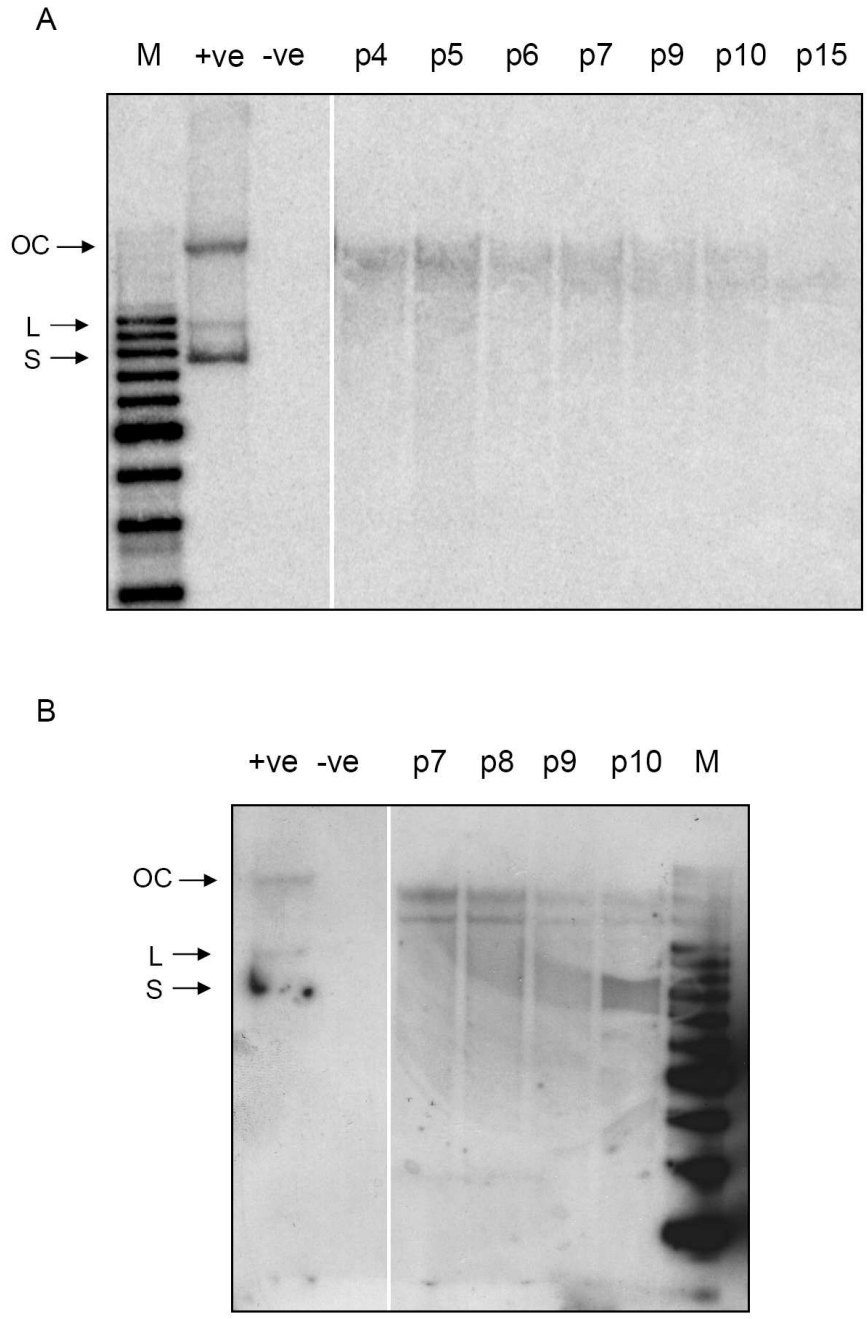


Figure 3.13: 16E6PDZ genomes do not persist episomally in NIKS cells

Two 16E6PDZ clones (A and B) were cultured for several passages. DNA was extracted at each passage, digested with A) Xba I or (B) Hind III and analysed by Southern blotting. The positive control was DNA from an episomal HPV16-positive NIKS cell line and the negative control was DNA from HPV-negative NIKS cells. The positions of linear (L), open circular (OC) and supercoiled (S) episomal genomes are indicated, as is the marker (M). The “p” values above each lane indicate the passage number of the cells from which DNA was extracted (e.g. p1 indicates “passage 1”). The white lines denote portions of the gels that have been cropped.

3.2.13 Cells expressing mutant E6 do not have a growth disadvantage compared to control cells

3.2.13.1. Generation of wild-type and mutant E6/7-expressing cells

In the experiments described above, I addressed the possibility that the cells that carry the E6 mutant genomes may have a growth disadvantage over the HPV-negative cells in the population, and may therefore be lost from the culture. This potential problem was addressed by carrying out clonal experiments, which were aimed at removing the growth competition from the population of cells. However, it is possible that the clones themselves may eventually become heterogeneous, by spontaneous loss of episomes (Stewart *et al.*, 1994), or by un-even segregation of viral genomes. This possibility would undoubtedly affect the confidence in my conclusions. Hence, although clonal analyses can significantly reduce the influence of potential differential cell growth on the outcome of the experiments, it does not eliminate this likelihood entirely.

I therefore wanted to determine whether the expression of the mutant E6 proteins induced a growth disadvantage to cells, compared to control cells that express no viral proteins. To do this, I employed a retroviral expression system to express E6 proteins in NIKS cells. As the activities of E6 and E7 are closely related and often affect each other (for example p53 augmentation by E7 (Demers *et al.*, 1994; Laurson *et al.*, 2010) and p53-degradation by E6 (Scheffner *et al.*, 1990)) I considered it important to co-express E6 and E7 in these experiments.

To do this, I used a pLXSN retroviral vector that has the HPV16 E6 and E7 ORFs cloned into it (LXSN-E6WT/7), kindly provided by Dr. Denise Galloway, and used it as a template to engineer LXSN-E6p53m/7 and LXSN-E6PDZ/7 vectors by site-directed mutagenesis. These vectors, along with the empty LXSN vector, were then used to make retroviruses with which I infected NIKS cells. Infected cells would express the E6 and E7 proteins from the retroviral promoter and would also be resistant to neomycin. Following antibiotic selection and recovery, the cells were lysed and the lysates were analysed by western

blotting which confirmed that the infectants were indeed expressing the HPV16 E6 and E7 proteins (Fig. 3.14).

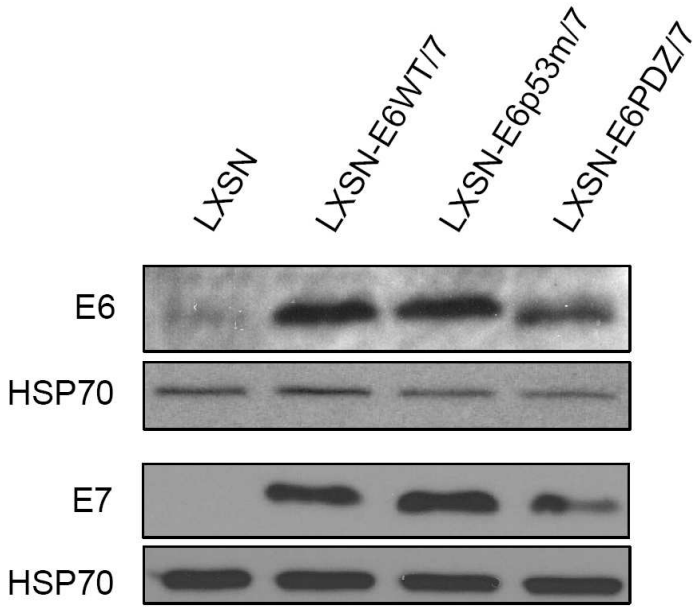


Figure 3.14: Expression of E6 and E7 proteins in LXSN-E6/7 cells

NIKS cells were infected with retroviruses bearing wild-type or mutant E6 genes as well as the E7 gene. Following antibiotic selection, cell lysates were collected and the levels of E6 and E7 proteins were determined by western blotting. HSP70 was used as a loading control.

3.2.13.2. The E6mut/7-expressing cells do not have a growth disadvantage compared to the LXSN cells

To determine whether any of the E6/7-expressing cell populations have a growth disadvantage compared to the LXSN cells, a growth assay was performed. I considered that by performing a growth assay I would be able to observe the consequences of different growth-related variations that may exist between the cells. These include differences in the speed of growth and in the number of cells that are cycling at any given time, as well as potential differences in the levels of cell death. All of these factors would affect the number of cells present in each well and would be reflected in the growth curve.

The cells were counted and plated in 6-well plates, so that three wells could be counted per sample at various time points. In addition to the 1 x 10^5 cells seeded per well, 3 x 10^5 feeder cells were plated as well. This is the feeder density that the cells have always been grown in, and it was considered important to keep this constant, as the speed of growth of the cells may vary at different feeder concentrations. The cells were counted at various time-points over seven days, when they were at both sub-confluent and confluent stages. To ensure that the feeder cells were not counted in this assay, the cells were first washed with trypsin-versene and then incubated at 37 ^{o}C for 2 minutes to allow feeder cells to detach. The cells were then washed with PBS prior to a second incubation in trypsin-versene to remove the keratinocytes for counting.

The mean number of cells per sample was plotted on a graph, with error bars representing the standard deviation of triplicate wells (Fig. 3.15). The growth curves show that neither the LXSN-E6WT/7 cells nor any of the two LXSN-E6mut/7 cell types grew slower than the control LXSN cells at any stage of the assay. This suggests that the expression of the E6p53m or E6PDZ proteins, together with E7, does not confer a growth disadvantage to the cells. I thus have no reason to think it likely that the lack of persistence of these mutant genomes is due to a growth disadvantage of cells harbouring the mutant genomes.

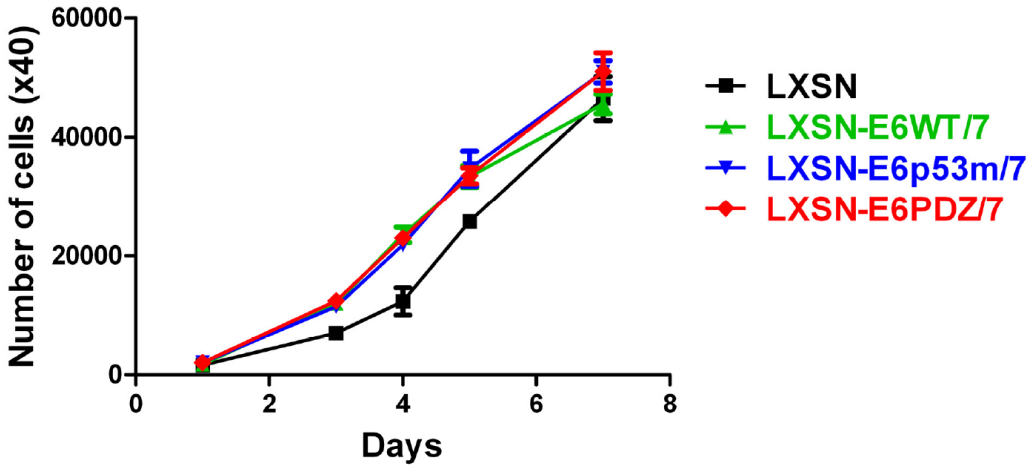


Figure 3.15: Growth assay of E6/7-expressing cells

Equal numbers of cells were plated in 6-well plates, and counted in triplicate at 5 time-points over seven days. The mean number of cells was plotted against the time in days. The error bars represent +/- the standard deviation of the triplicate counts.

3.3. Discussion

In this chapter I presented data that implicate two regions of the HPV16 protein in episomal persistence of HPV16 in monolayer cultures of keratinocytes. Previous studies have attempted to look at how different activities of E6 may affect episomal persistence, by generating E6-mutant genomes (Lee & Laimins, 2004; Park & Androphy, 2002; Thomas *et al.*, 1999). These studies used primary HFKs to study the persistence of either the HPV31 genome (Lee & Laimins, 2004; Thomas *et al.*, 1999) or a hybrid HPV31/16 genome (Park & Androphy, 2002). In all of these studies, persistence was assessed by the detection of HPV DNA by Southern blotting several weeks post-transfection.

Considering that viral persistence in the basal layer of the epithelium is considered to be vital for the development of high-grade HPV-related disease (Remmink *et al.*, 1995; Schiffman & Kjaer, 2003), I decided that a thorough analysis of the requirements for episomal persistence of HPVs would be important. To identify factors necessary solely for episomal persistence, without any interference from the potential need for cellular immortalisation, I decided to study this in NIKS cells instead of primary cells. Furthermore, the HPV16 genome was chosen for this study, as this is the most significant HPV type with respect to cancer development.

The aim of the work presented in this Chapter was to extend the observations that the E6 viral protein was necessary for episomal persistence of HPV16 in NIKS cells (Laurson *et al.*, 2010), by determining which activities of E6 are involved in this function. To this end, two HPV16 mutant genomes were constructed, each harbouring a well-characterised mutation in the E6 ORF. The first was a p53-degradation mutant genome (16E6p53m) and the second was a genome that lacked the PDZ-binding motif of E6 (16E6PDZ).

As part of my studies to characterise these mutations, I showed that the wild-type E6 protein, when expressed at high levels from a stable clonal cell line, is able to degrade endogenous hScrib. More work however is needed in order to determine if this degradation also occurs in natural HPV infections, or if this is

an effect that is brought about only by abnormally high expression level of the E6 protein.

In my assays, the ability of the mutant genomes to persist in NIKS cells was always assessed in comparison to that of the wild-type genome (16WT) from the same experiment. Two methods for looking at persistence were employed, each giving important insight. Firstly, the two genomes were transfected into NIKS cells and the cells were grown as a population for several passages. Analysis of DNA from these experiments showed that 16E6p53m genomes do not persist in NIKS cells whereas 16E6PDZ genomes do persist but not episomally. It therefore seems that the p53-degradation activity of E6 may be necessary for the persistence of viral DNA in cells and the PDZ-binding activity of E6 may be necessary for keeping the episomal state of the genome.

However, these experiments, which resemble the ones carried out by the earlier studies mentioned above (Lee & Laimins, 2004; Park & Androphy, 2002; Thomas *et al.*, 1999), only give a general idea of what is happening in these cells. Therefore, they may be influenced by a small number of atypical phenotypes within the population rather than being representative of most cells. Even more important however is the fact that some cells in the population may carry a growth advantage, which may be unrelated to the ability of the viral episomes in those cells to persist. These cells may consequently outgrow the others, thus giving a false result with regards to viral DNA persistence.

To avoid such potential problems, I assessed the ability of the mutant genomes to persist in individual cell clones instead, which were isolated and grown individually so that they would not be subjected to growth competition by other cells in the population. An added advantage of this system is the insight gained into the degree of heterogeneity that exists within the population of cells. In these clonal experiments, the 16E6p53m genomes were again found to be unable to persist in cells. Interestingly however, the results from the transfection of the 16E6PDZ genomes showed that the phenotype I had observed with the population experiments was not the most common across the whole population. The analysis of the clones indicated that the 16E6PDZ genomes had in fact been lost from the majority of the clones, with a few clones having multi-copy

integrations. It should be noted however, that the primers used in the qPCR analysis of these clones recognise sequences within the E4 ORF. It is therefore possible that other clones may also contain integrated copies of the genome, but are not being detected as positive here because their E4 ORF has been disrupted. Further analyses with primers to other HPV ORFs can be carried out to measure the degree of integration more precisely.

I also considered the possibility that the clones themselves may eventually become heterogeneous and therefore decided to directly address the question of whether or not the expression of mutant E6 proteins, together with the wild-type E7 protein, confers a growth disadvantage to the cells. My results indicate that cells that express a mutant E6 protein and the E7 protein do not have a growth disadvantage compared to control cells, hence conclusively ruling out the possible influence of growth competition described earlier.

Taking the results from this chapter together, I conclude that two distinct regions of the E6 protein are necessary for the persistence of viral episomes in cells. My investigation into episomal persistence went beyond that of previous studies in that I have investigated persistence in the absence of the need for cellular immortalisation. Furthermore, I have looked at the heterogeneity within transfected cells and have taken care to prevent my results from being influenced by different growth rates within the cell population. By doing so, I am able to conclude that these two regions of E6 are directly involved in episomal persistence, and not implicated in it by way of some other function, such as loss of HPV-positive cells from the culture.

The two regions of the E6 protein I have been studying were chosen as their mutations have been shown to disrupt two key activities of the HPV16 E6 proteins, the degradation of p53 and the binding to and degradation of PDZ proteins. Therefore, the data presented in this chapter would suggest that these two activities of E6 are necessary for episomal persistence. However, this has not been conclusively proven as the possibility that the introduced mutations also disrupt other activities of E6 cannot be dismissed. To determine whether degradation of p53 and binding/degradation of PDZ proteins are indeed necessary for persistence, it would be necessary to directly interfere with these

processes. For example, I could determine whether the 16E6p53m genome is able to persist in cells that have constitutively low levels of p53, such as NIKS cells that stably express shRNA against p53. Experiments to this end will be discussed in Chapter 4. Similar assays could be carried out with the 16E6PDZ genomes in NIKS cells in which PDZ proteins have been knocked down. The latter however is less straightforward as it is not currently know which specific PDZ protein, or combination of proteins, may be implicated in HPV episomal persistence.

Intriguingly, my results also indicate that the two mutant genomes I have been studying have two different phenotypes with regards to persistence. The 16E6p53m genomes appear to gradually be lost from the cells, in a similar way as the 16E6STOP genomes. On the other hand, the 16E6PDZ genomes are either completely lost from the cells, or are occasionally integrated at high copy numbers. These observations suggest that the p53-degradation activity and the PDZ-binding activity of E6 may affect episomal persistence by different mechanisms.

Further work is needed to determine the persistence mechanisms that these activities of E6 are involved in. My studies to this end will be the topics of the following chapters.

Chapter 4: Investigating factors that may affect HPV16 persistence

4.1. Introduction

As mentioned in Chapter 1, in order for viral episomes to persist in cells, they need to be able to replicate as well as segregate correctly into the two daughter cells. Both establishment replication and stable maintenance replication are necessary for long-term persistence of viral episomes in basal cells. For correct segregation, the episomes must segregate equally into the daughter cells, and localise to the nucleus of the newly formed cells, in order to be able to undergo replication again. The interruption of any of these processes will result in viral episome loss.

The mechanisms that regulate the segregation of viral episomes have been studied for PVs as well as for some of the other tumour viruses, and common features have been highlighted. It is generally thought that viral episomes bind to viral proteins which in turn attach the episomes to the cellular chromosomes by interacting with a DNA-binding cellular protein (reviewed in Feeney & Parish, 2009). The only PV-encoded protein that has been identified to play a role in this is E2 (Abbate *et al.*, 2006; Bastien & McBride, 2000; Ilves *et al.*, 1999; Lehman & Botchan, 1998; Oliveira *et al.*, 2006; Skiadopoulos & McBride, 1998; Van Tine *et al.*, 2004). Several cellular proteins, such as Brd4 (Abbate *et al.*, 2006; Baxter *et al.*, 2005; You *et al.*, 2004; You *et al.*, 2005), Chlr1 (Parish *et al.*, 2006a) and TopBP1 (Donaldson *et al.*, 2007), have been suggested to mediate the interaction between PV episomes and cellular chromosomes. In addition to the cellular chromosomes, E2 has also been reported to tether viral episomes to the mitotic spindle to enable segregation (Van Tine *et al.*, 2004) and studies suggest that alternative pathways may be used by different PV types (McPhillips *et al.*, 2006; Oliveira *et al.*, 2006). Although it is possible that other viral proteins, in addition to E2, may also play a role in episomal segregation, at this point there is no evidence to suggest a role for E6.

On the other hand, a potential role for E6 in viral DNA replication has been suggested. p53 binds to members of the cellular DNA replication machinery, such as DNA helicases (Sakurai *et al.*, 1994) and replication protein A (RPA), the latter of which p53 has been shown to inhibit (Dutta *et al.*, 1993). In addition, p53 has been found to localise to sites of viral DNA replication along with cellular replication proteins (Wilcock & Lane, 1991).

Moreover, p53 binds to the SV40 large T antigen. This interaction down-regulates the replication of SV40 DNA (Friedman *et al.*, 1990; Wang *et al.*, 1989) and it has been shown that p53 prevents the helicase activity of the large T antigen (Sturzbecher *et al.*, 1988) and also interferes with its ability to bind to DNA polymerase α (Gannon & Lane, 1987).

Importantly, p53 has been implicated in the replication of PVs, having different effects at different stages of replication. p53 inhibits the replication of BPV1 as well as that of the high-risk HPV type 18 and the low-risk HPV type 11. However, this was only found to be true for the amplificational/establishment replication that immediately follows infection (Ilves *et al.*, 2003; Lepik *et al.*, 1998). In contrast, p53 was found to have no effect on the maintenance replication of BPV1 that keeps the viral copies at a stable number (Ilves *et al.*, 2003). These studies provide evidence that establishment replication and maintenance replication are regulated by different cellular and viral factors. However, it should be noted that in both the Lepik *et al*. and the Ilves *et al*. studies, p53 was over-expressed in order for it to have an effect on replication. Seeing as BPV1 and HPV11 do not encode proteins that have p53-degradation activity (unlike HPV16 E6) it is unlikely that physiological levels of p53 would inhibit their DNA replication.

Studies have also shown that p53 interacts with the viral replication protein E2 (Massimi *et al.*, 1999) and that the negative effect of p53 on the replication of HPV16 DNA is dependent on this interaction (Brown *et al.*, 2008). These data however also hint at a difference between the replication of high- and low-risk HPVs. As Brown *et al*. discuss, HPV11 E2 was unable to bind p53 (Parish *et al.*, 2006b), and yet p53 has been shown to inhibit HPV11 replication as well (Lepik

et al., 1998). This suggests that p53 may down-regulate the replication of high- and low-risk HPVs by different pathways.

In this chapter I aim to address possible reasons that may lead to episomal loss. I considered that DNA replication was the most obvious to investigate first, as cellular targets of E6 (p53) have been directly implicated in it. On the other hand, E6 has not been implicated in episomal segregation.

4.2. Results

4.2.1. Replication of wild-type and mutant HPV16 genomes

I first wanted to address the possibility that the failure of the 16E6p53m and 16E6PDZ mutant genomes to persist in NIKS cells was due to their inability to replicate. A previous study has shown that certain mutations in HPV31 E6 protein rendered genomes unable to replicate in transient assays (Thomas *et al.*, 1999). These mutations were in the splice-donor or splice-acceptor sites of E6 and their effect on replication could have been due to one of two things: firstly, it was possible that expression of the E6* splice variant protein was necessary for replication. Secondly, it was also possible that the introduced mutation inadvertently affected the expression of E1 and E2, thereby inhibiting replication. The latter possibility gains credence from the observation that ectopic expression of E1 and E2 in the transfected cells rescued the replication of the mutant genomes (Thomas *et al.*, 1999).

It was therefore important to determine whether the mutations I have introduced into the E6 ORF of HPV16 genomes affect the ability of the genomes to replicate, as this would consequently affect their ability to persist. Previous studies that addressed the persistence of HPV genomes in cells assessed their ability to replicate by carrying out transient replication assays (Lee & Laimins, 2004; Lee *et al.*, 2007; Park & Androphy, 2002; Thomas *et al.*, 1999). In these assays, viral genomes were transfected into cells, and DNA was extracted five days post-transfection. The DNA was digested with Dpn I, which digests the methylated (input) DNA, and Southern blotting was used to detect any Dpn I-resistant DNA, which is DNA that has undergone replication in the transfected cells (Peden *et al.*, 1980). In the abovementioned studies, the transient replication assays were carried out in SCC13 or C33a cells. These were not the same cells in which the persistence assays were carried out in these studies, which were primary human foreskin keratinocytes (HFKs). Although not specifically pointed out in these reports, the SCC13 and C33a cell lines may have been used because they are much more amenable to being transfected than HFKs. The results from these transient replication assays would reveal whether there is something inherently wrong with the mutant genomes, for

example an inadvertent effect on the expression of E1 and E2. However, they may not accurately reflect how efficient the replication of each genome is in the HFKs. For example, viral replication in HFKs may have a requirement for expression of intact E6 protein, which may not be similarly necessary in SCC13 or C33a cells.

For this study it was important to look at the ability of the 16E6p53m and 16E6PDZ mutant genomes to replicate in NIKS cells. As the transfection efficiency of the NIKS cells is relatively low, I did not expect to obtain enough cells that harbour replicated viral DNA, to be able to see the DNA by Southern blotting. Even the use of antibiotics to select for cells that harboured viral DNA would not generate sufficient number of cells for a Southern blot-based transient replication assay in such a short period of time. To bypass this problem, I decided to use a qPCR-based transient replication assay instead, which is more sensitive than Southern blotting, and therefore requires much less starting material. This was modified from a previously described technique (Taylor & Morgan, 2003), in which a probe and primers were designed to amplify an area of the HPV replication origin. In that study, a Dpn I site was introduced into the binding site of the probe, so that non-replicated, methylated DNA would be digested and therefore not amplified in the qPCR reaction. Newly replicated, un-methylated DNA on the other hand, would be resistant to digestion, and would consequently be amplified in the qPCR. Taylor and Morgan highlighted the advantages of their qPCR-based assay over the Southern blotting method by demonstrating its superior sensitivity in detecting small differences in replication (Taylor & Morgan, 2003).

My experimental procedure was different to that described by Taylor and Morgan in that I have been working with the entire HPV16 genome and could utilise Dpn I sites that exist naturally within the viral genome, to distinguish between transfected (input) and newly replicated DNA. A similar qPCR-based assay has also been used previously to determine the replication of BPV1 mutant genomes (Parish *et al.*, 2006a).

In the HPV16 genome, a Dpn I site is present in the E4 ORF and lies within the target sequence that is amplified by the set of primers I have been using for

qPCR. Hence amplification of this region by qPCR in Dpn I-digested and undigested samples would allow the measurement of the magnitude of replication of the viral DNA, based on the principles described above.

To test this principle pSPW12 plasmid (which contains the entire wild-type HPV16 genome in a pSP64 vector and was grown in bacteria) was digested with Dpn I, or left undigested. qPCR was then used to measure the amounts of pSPW12 DNA. As a control, the DNA was also digested with Mbo I, an enzyme that recognises the same restriction site as Dpn I but would only digest un-methylated DNA, and would therefore be expected to leave the pSPW12 DNA intact. Dpn I digestion significantly decreased the levels of E4 detected ($p=0.0013$), whereas the Mbo I digestion did not ($p=0.37$) (Fig. 4.1A). However, there was still a significant amount of DNA amplification detected in the Dpn I-digested sample, indicating that the qPCR assay has a high background. The Taylor and Morgan study suggested that digesting the DNA with Exonuclease III in addition to Dpn I, would help reduce the background (Taylor & Morgan, 2003). To determine whether this would improve the assay, I compared the levels of E4 in samples digested with Dpn I with or without Exonuclease III (Fig. 4.1B). Digestion with Exonuclease III did reduce the background to almost undetectable levels. I therefore decided to use both Dpn I and Exonuclease III digestions for the actual experiments.

An unfortunate consequence of Exonuclease III digestion however, is the complete removal of all digested DNA, which eliminates the possibility of using another viral ORF as template for an internal qPCR control. Such a control would be useful to ensure that the amount of template loaded on the qPCR is comparable between samples. To bypass this problem, I decided to analyse the results from the transient replication assay by measuring the number of E4 copies in the Dpn I-digested and undigested samples and dividing the former by the latter. Apart from the presence of the enzyme, these two samples were treated identically. This would ensure that any variation between the samples, as a consequence of the cell harvesting or DNA extraction process, would not interfere with my results.

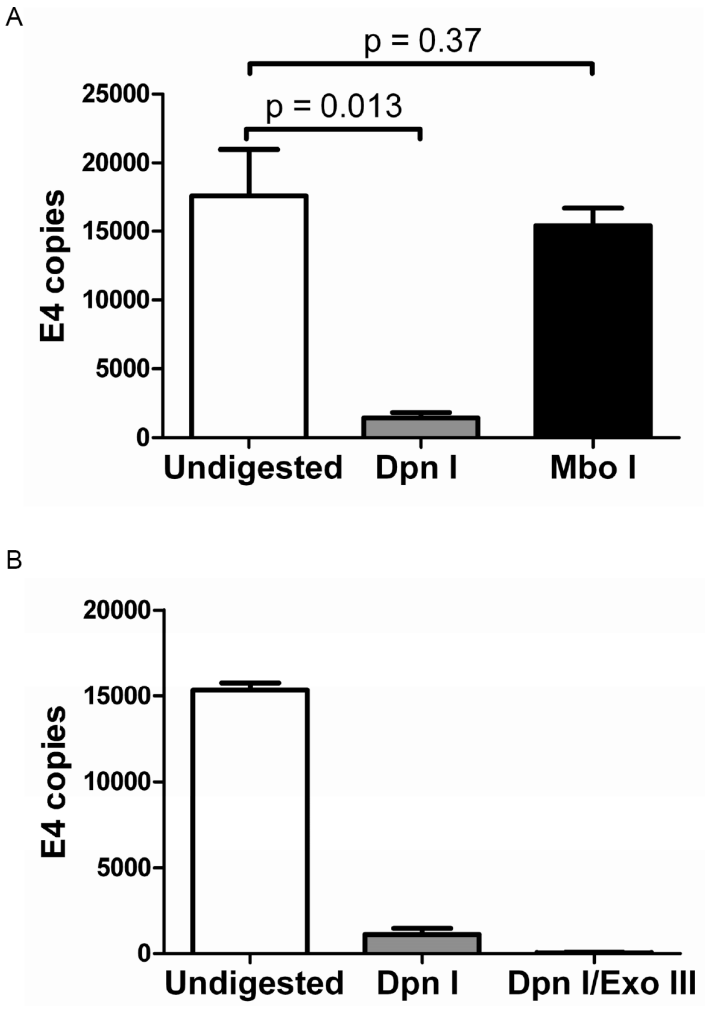


Figure 4.1: Controls for transient replication assay

pSPW12 plasmid was digested with A) Dpn I or Mbo I, or left undigested and B) Dpn I or Dpn I and Exonuclease III (Exo III), or left undigested. The levels of E4 were measured by qPCR. The bar charts show the mean E4 copies and the standard deviation of three replicates.

The controls presented in Figure 4.1 were set up using pure plasmid that was isolated from bacteria. I therefore wanted to ensure that the qPCR-based assay also works in transient transfections. To do this, I carried out another control in which NIKS cells were transfected with either the replication-competent 16WT genomes, or a cloning plasmid carrying the E1^E4 cDNA, but no eukaryotic promoter or origin of replication (E4 plasmid) (McIntosh *et al.*, 2008). Low molecular weight DNA was extracted four days post-transfection, and divided into samples that would remain undigested, or would be digested with Dpn I and Exonuclease III. The number of E4 copies measured in the digested samples was divided by that measured in the undigested samples and multiplied by 100 to give the percentage of replication. The results are presented in Figure 4.2 and show that the replication detected in the cells transfected with the 16WT genomes was significantly higher that that detected in the cells transfected with the replication-deficient E4 plasmid (p=0.048). This indicates that this assay can be used to measure DNA that has replicated above background level, as this is set by the E4 plasmid.

Having tested the principles of this assay, I set out to determine whether the 16E6p53m and 16E6PDZ mutant genomes are replication-competent. To do so, NIKS cells were transfected with either of the two mutant genomes, the 16WT genome or the E4 plasmid. Low-molecular weight DNA was extracted at four days post-transfection and samples were either digested with Dpn I and Exonuclease III or were left undigested. The transfection was carried out in four replicates and Figure 4.3 shows the results.

From these results, I make two important observations. Firstly, the levels of replication detected in the cells transfected with any of the three HPV genomes were significantly higher than the background level detected with the E4 plasmid (16WT: p=0.0001, 16E6p53m: p=0.0004 and 16E6PDZ: p=0.017). This indicates that, in addition to the 16WT genome, both of the mutant genomes are also replication-competent. Secondly, the replication efficiency of the 16E6PDZ genomes was found to not be statistically different (p=0.17) to that of the 16WT genomes. In contrast, the 16E6p53m genomes were found to replicate less efficiently than the 16WT genomes (p=0.0037).

In light of these results, I can conclude that although they are not able to persist in NIKS cells, both the 16E6p53m and 16E6PDZ mutant genomes are nevertheless able to replicate in these cells. However, 16E6p53m genomes replicate less efficiently than 16WT genomes and this might be significant in terms of the inability of these genomes to persist in NIKS cells.

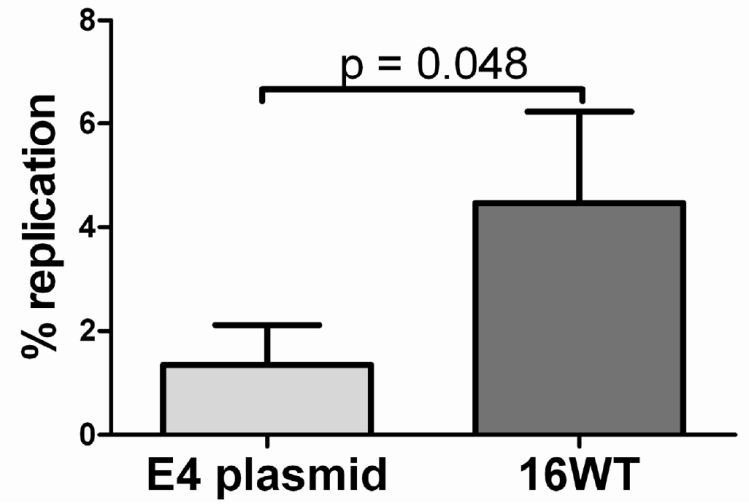


Figure 4.2: Transient replication of 16WT genomes and control plasmid
NIKS cells were transfected with 16WT genomes or a control plasmid (E4 plasmid) that has the E4 ORF without any eukaryotic promoter or origin of replication. Episomal DNA was extracted at four days post-transfection and the number of E4 copies was measured by qPCR in undigested and digested samples. The bar chart shows the mean percentage replication and the standard deviation of three replicates.

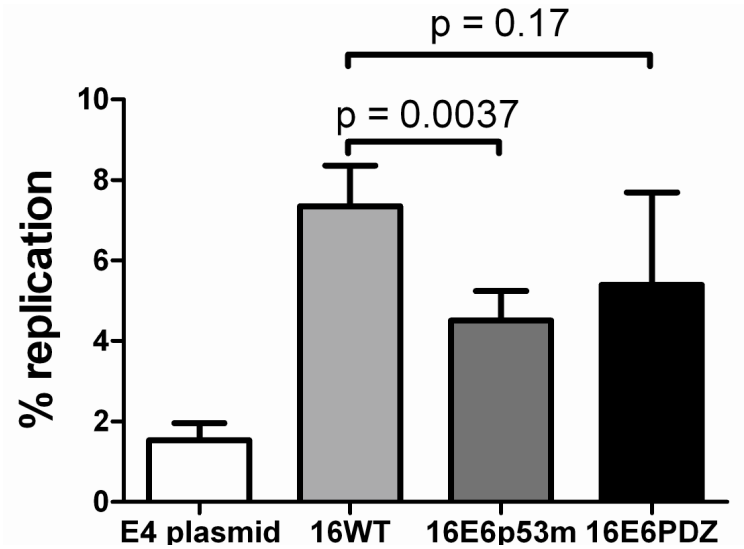


Figure 4.3: Transient replication of wild-type and mutant HPV16 genomes
NIKS cells were transfected with the 16WT, 16E6p53m or 16E6PDZ genomes, or the E4 plasmid. Episomal DNA was extracted at four days post-transfection and the number of E4 copies was measured by qPCR in undigested and digested samples. The bar chart shows the mean percentage replication and the standard deviation of four replicates.

4.2.2. HPV DNA cannot persist in E6-expressing cells

Having ascertained that the 16E6p53m and 16E6PDZ mutant genomes are able to replicate in NIKS cells and yet are unable to persist in them, I wondered whether this was due to a *cis*- or a *trans*-acting effect of these mutations. In the case of the 16E6p53m genome for example, which was shown above to replicate less efficiently than the 16WT genome, a *cis*-acting effect could be one where the mutation within the E6 ORF reduces the expression of E1 or E2 genes. A *trans*-acting effect would be one where the mutant E6 protein is not able to carry-out its normal activities in the cell to facilitate persistence.

To investigate this, I wanted to test whether ectopic expression of wild-type E6 protein in NIKS cells would restore the persistence capabilities of the mutant genomes. This could be done by carrying out the persistence assays in NIKS cells that constitutively express E6WT (LXSN-E6WT cells, described in Chapter 3). If the mutant genomes could persist in these cells at levels similar to the 16WT genomes, that would imply that the E6 mutations had a *trans* effect on persistence.

Before carrying out these persistence assays using the mutant genomes, it was necessary to ensure that the 16WT genomes could persist episomally in the LXSN-E6WT cells. To do this, I chose two LXSN-E6WT clonal lines that express different levels of E6 (Fig. 4.4A), and transfected them with the 16WT genomes, and the pcDNA6 plasmid. In accordance to the method described in Chapter 3, following antibiotic selection (4 days), the cells were cultured as a population for several passages and DNA was collected at each passage. The DNA was then analysed by qPCR to determine the number of HPV copies per cell. Importantly, HPV-negative LXSN cells were also transfected in parallel to act as a positive control. Figure 4.4B shows the number of HPV copies per cell measured at each passage. The graph also includes the copy number at “passage zero” (p0) which represents the cells that survived antibiotic selection, prior to being passed onto a new plate. The HPV copies detected in the different transfectants at p0 are at comparable numbers and they all quickly drop to much lower levels within one passage. As expected, within an additional four passages, the HPV16 DNA in LXSN cells started increasing in numbers and continued to increase, with some stabilisation seen at later passages.

Unexpectedly however, the copy numbers of HPV16 DNA in both of the E6-expressing cell lines never recovered and remained very low, through all the passages.

To test whether the difference between the persistence of HPV16 DNA in LXSN cells and lack of persistence in E6-expressing cells could be due to differences in the transfection efficiencies between cells, I repeated the transfection but replaced the pcDNA6 plasmid with the pMV10 plasmid that expresses β-galactosidase (β-gal). The levels of β-gal in each cell type are shown in Figure 4.5. The two LXSN-E6WT clonal lines were found to have lower transfection efficiencies compared to the LXSN cells. Although this could potentially affect the results of the persistent assay, as mentioned above, in the experiment described in Figure 4.4B I observed that the copy numbers at p0 and p1 were similar between the different transfectants. This suggests that the observations regarding the persistence of 16WT genomes in these cells are unlikely to be due to differences in transfection efficiencies. This will be addressed further below.

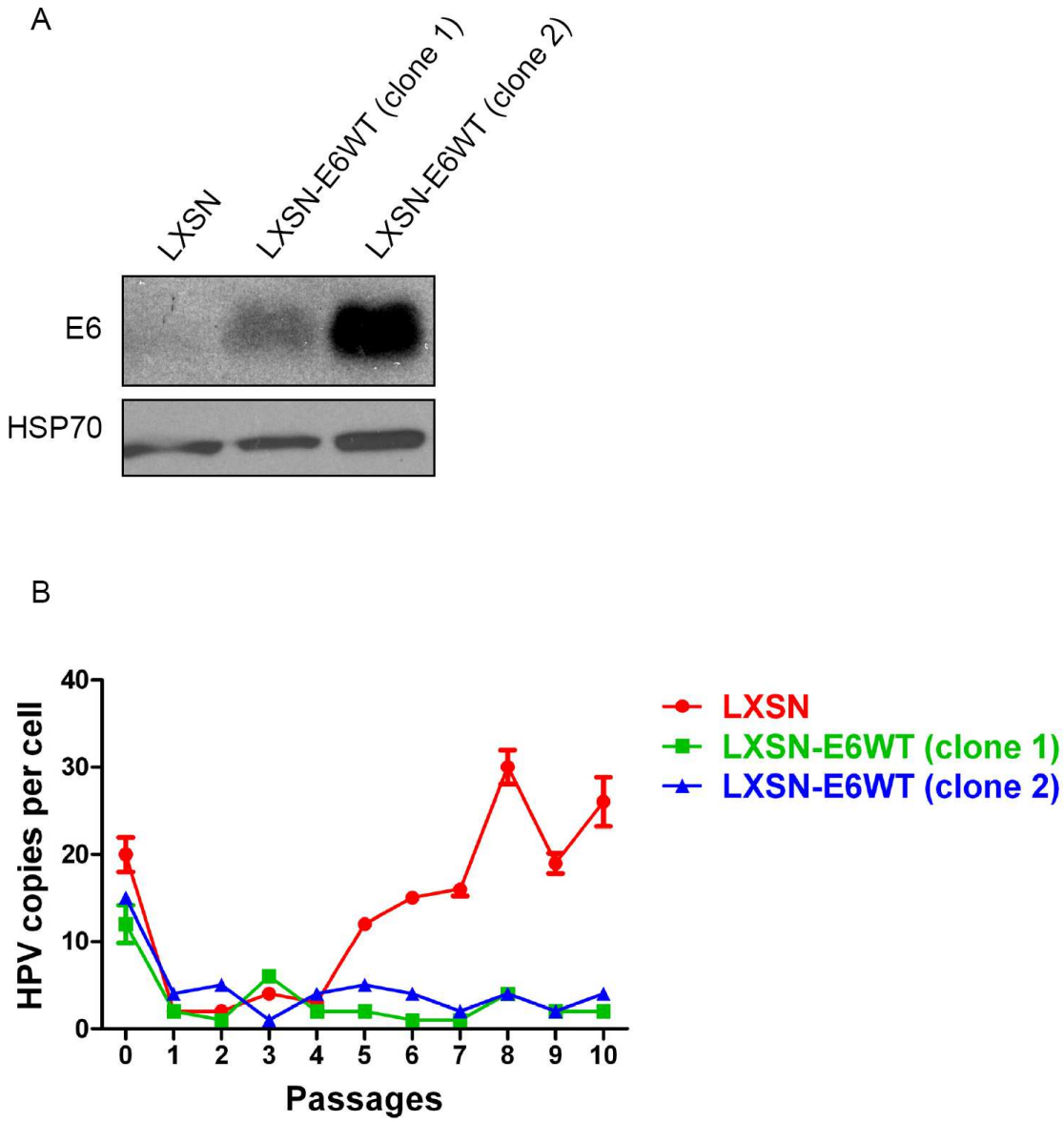


Figure 4.4: 16WT genomes cannot persist in LXSN-E6WT clonal lines

A) NIKS cells were infected with retroviruses bearing the wild-type E6 gene. The cells were selected with antibiotics and cloned. Whole-cell extracts were prepared and the levels of E6 in two individual clones were determined by western blotting. HSP70 was used as a loading control. B) LXSN cells and two LXSN-E6WT clones were co-transfected with 16WT genomes, and the pcDNA6 plasmid. Following antibiotic selection, the cells were grown as populations and DNA was extracted at each passage. The DNA was analysed by qPCR to determine the number of HPV copies per cell. The error bars represent +/- the standard deviation of the qPCR triplicates.

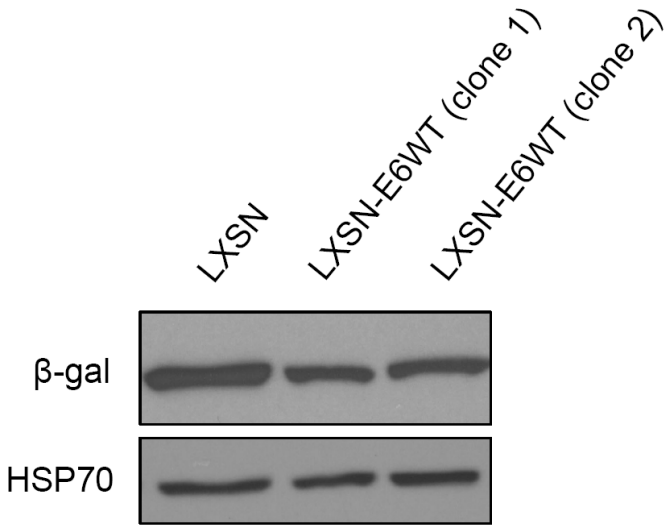


Figure 4.5: Transfection efficiencies of LXSN and LXSN-E6WT clonal lines
LXSN cells and LXSN-E6WT clones were co-transfected with 16WT genomes and the pMV10 plasmid and whole-cell extracts were prepared at 48 h post-transfection. The levels of β-gal were determined by western blotting. HSP70 was used as a loading control.

The experiment described above yielded some unexpected but interesting results as it suggested that viral genomes (even wild-type ones) cannot persist in cells that already express the E6 protein. This could have important implications when considering what factors contribute to making a permissible cellular environment for HPV infections, as well as when looking at the possibility of re-infection of HPV-positive cells. Furthermore, it was intriguing to find that a protein that is necessary for episomal persistence is in fact also inhibitory to it, if expressed prior to the entry of the viral DNA into the cell.

However, there were some caveats in the previous experiment that needed to be addressed prior to making any firm conclusions. The first was that the E6-expressing cell lines used in the experiment were clones, whereas the LXSN cells were not. It was possible that the cloning process may have inadvertently selected for some characteristics which were not desirable in this experiment. Furthermore, as seen in Figure 4.5, both of these cell lines had somewhat lower transfection efficiency than the LXSN cells, and this may have affected the ability of the viral genomes to persist in these cells.

To address both of these issues, I made new LXSN and LXSN-E6WT cell populations (omitting the cloning step), and used early passages of these cells for the persistence assay. I repeated the persistence assay in duplicate for each cell population (labelled LXSN (1) and (2) or LXSN-E6WT pop. (1) and (2)), and also repeated it in one of the LXSN-E6WT clones (clone 2) used in the previous experiment, as an additional control. The levels of E6 are shown in Figure 4.6A. The levels of p53 were also determined and, as expected, were much lower in the E6-expressing cell population and cell clone (Fig. 4.6A) compared to the LXSN cells. The transfection efficiencies of the cells were measured as described above (Fig. 4.6B) and the transfection efficiency of the LXSN-E6WT clone was again found to be lower than that of the LXSN cells. However, the transfection efficiency of the LXSN-E6WT cell population was found to be comparable to that of the LXSN cells.

As in the previous experiment, the 16WT genomes persisted in the LXSN cells but not in the LXSN-E6WT clone (Fig. 4.7A and B). Interestingly, the 16WT

genomes were also unable to persist in the LXSN-E6WT cell population (Fig. 4.7A and B). I was unfortunately unable to follow the LXSN-E6WT pop. (2) transfection further than p6. However, up to that point it closely resembled the phenotype observed with the LXSN-E6WT pop. (1), that is, it did not appear to support the persistence of 16WT genomes.

The HPV copy numbers for this experiment were also determined at p0. This is the time-point when antibiotic selection had stopped (cells were treated with antibiotics for six days), but prior to passing the cells onto new plates. At this time-point the copy numbers were found to be much higher, and therefore could not be presented meaningfully on the same graph as the copy number values of the subsequent passages. The data from this time-point alone are presented in Figure 4.7C. This shows that high levels of HPV genomes were detected in all transfected cell types at this time-point, indicating that the transfections were successful. Although there was some variation in the copy numbers in the different transfectants, there was no consistency between copy number at p0 and ability to sustain HPV persistence at later passages. This is indicated by the fact that the lowest and one of the highest copy number values measured were both found in the two LXSN cell populations which were the only cells that supported persistence of the viral DNA.

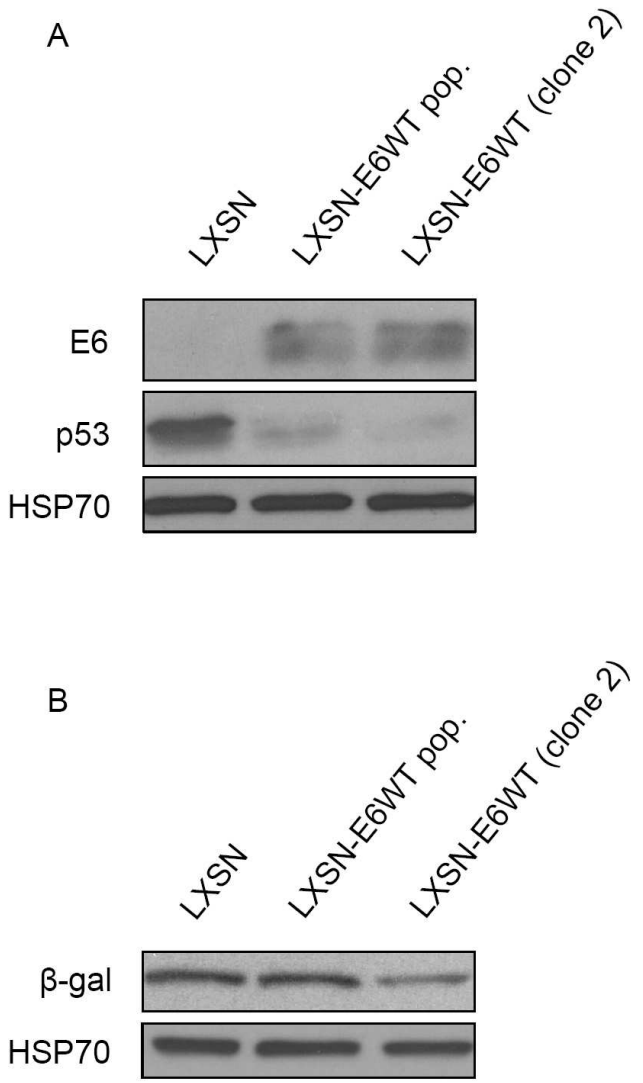
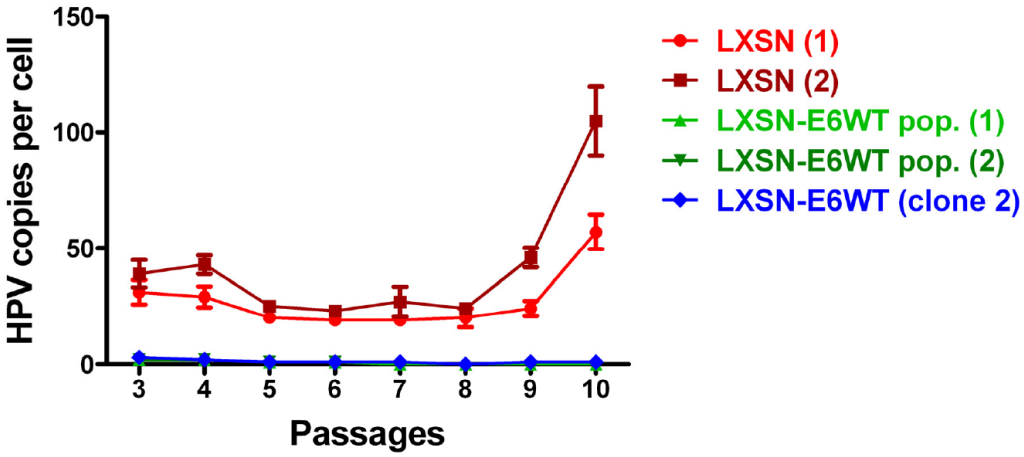


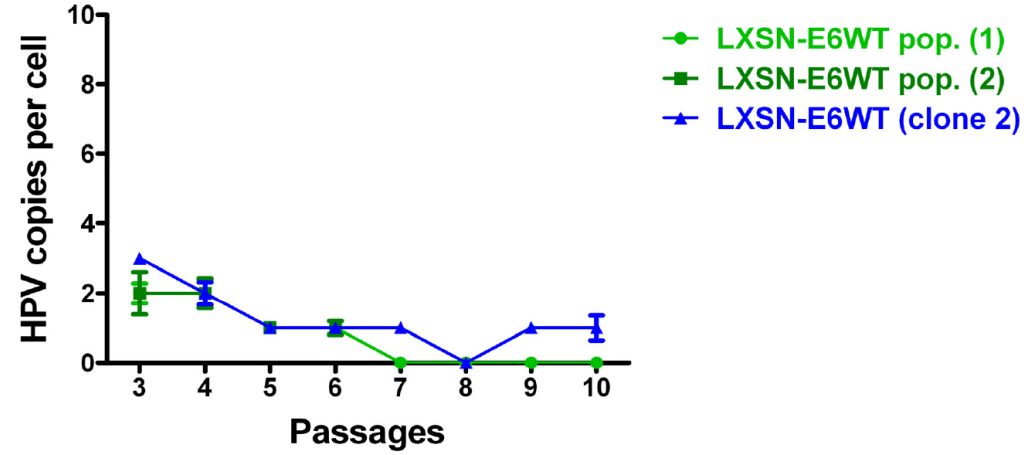
Figure 4.6: Expression of E6 in an LXSN-E6WT cell population and LXSN-E6WT clone and transfection efficiencies of LXSN and LXSN-E6WT cells

A) NIKS cells were infected with retroviruses bearing the wild-type E6 gene and the cells were grown as a population (LXSN-E6WT pop.). Whole-cell extracts were prepared from this cell population as well as from an LXSN-E6WT clone and the levels of E6 and p53 were determined by western blotting. HSP70 was used as a loading control. B) LXSN and LXSN-E6WT cell populations and an LXSN-E6WT clone were co-transfected with 16WT genomes and the pMV10 plasmid and whole-cell extracts were prepared 48 h post-transfection. The levels of β-gal were determined by western blotting. HSP70 was used as a loading control.

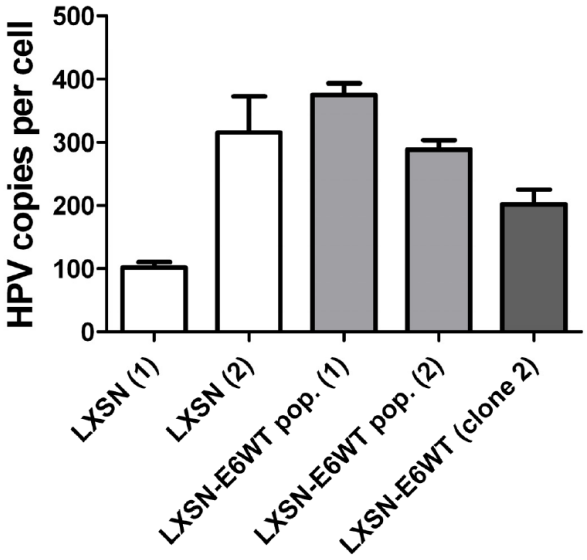
A



B



C



(legend on page 156)

Figure 4.7: 16WT genomes cannot persist in LXSN-E6WT cell populations

LXSN and LXSN-E6WT cell populations and an LXSN-E6WT clone were co-transfected with 16WT genomes and the pcDNA6 plasmid. Following antibiotic selection, the cells were grown as populations and DNA was extracted at each passage. The DNA was analysed by qPCR to determine the number of HPV copies per cell. The transfection of the LXSN and the LXSN-E6WT cell populations was repeated in duplicate. A) The mean number of copies per cell between p3 and p10 for all five transfections. B) The mean number of copies per cell between p3 and p10 for the transfection of the LXSN-E6WT cell populations and LXSN-E6WT clone. C) The mean number of copies per cell measured at p0 for all five transfections. The error bars represent the standard deviation of the qPCR triplicates.

4.2.3. HPV DNA cannot persist in cells that have low levels of p53

The unexpected inability of even wild-type HPV16 DNA to persist in E6-expressing cells precludes the use of these cells to test whether supplementation of E6 in *trans* will restore the ability of 16E6p53m and 16E6PDZ genomes to persist in cells. I would therefore need to adopt an alternative approach to answer this question.

As mentioned in Chapter 3, the observation that the 16E6p53m genomes do not persist in NIKS cells does not conclusively prove that p53-degradation is necessary for persistence, as it is conceivable that this mutant may be unable to perform some other activity of E6 which has not yet been attributed to this region of the E6 protein. To test this, I wanted to compensate for the loss of this activity in 16E6p53m genomes, by constitutively knocking down the levels of p53 in NIKS cells. If the 16E6p53m genomes were able to persist in these cells, it would prove that p53-degradation is necessary for persistence. Moreover, it would prove that the mutation exerted a *trans*-acting effect instead of an unexpected *cis* effect on the viral genome.

To knock down the levels of p53 in NIKS cells, I used the retroviral expression system to stably express an shRNA construct against p53 (NIKS-shp53 cells). To obtain homogeneous NIKS-shp53 cell lines, I cloned the retrovirus-infected cells. The reduced levels of p53 in three such clones are shown in Figure 4.8A. Clone 3 exhibited the highest efficiency in reducing levels of endogenous p53 protein.

Before using the cells in the intended experiment with 16E6p53m genomes, I wanted to ascertain that these cells were competent in supporting wild-type HPV16 DNA replication and persistence in the first place. To do this, I co-transfected NIKS-shp53 (3) cells with the 16WT genomes and the pcDNA6 plasmid. Following antibiotic selection (4 days), the cells were cultured as a population for a few passages and DNA was collected at each passage. The DNA was then analysed by qPCR to determine the number of HPV copies per cell (Fig. 4.8B). Surprisingly, 16WT genomes failed to persist in NIKS-shp53 cells.

This was truly an unexpected outcome as the current understanding of the effect of p53 on HPV replication would not have predicted a detrimental effect of reduced levels of p53 on HPV DNA persistence. After all, HPV16 E6 naturally reduces the levels of endogenous p53 in the cell. This curious observation mirrors that described above regarding the inability of wild-type HPV16 DNA to persist when introduced into cells that already express the E6 protein constitutively. In combination, it would appear that the inability of the E6-expressing cells to sustain the persistence of ectopically-introduced HPV DNA may be due at least in part to the low levels of p53 in these cells (seen in Fig 4.6A) at the time of HPV DNA entry. This suggestion is intriguing as it implies a positive role for p53 in the early stages of HPV16 persistence. Further implications of this will be discussed later, but in regards to my experiments, it is clear that I cannot use these cells to address the role of p53-degradation in HPV16 DNA persistence in NIKS cells.

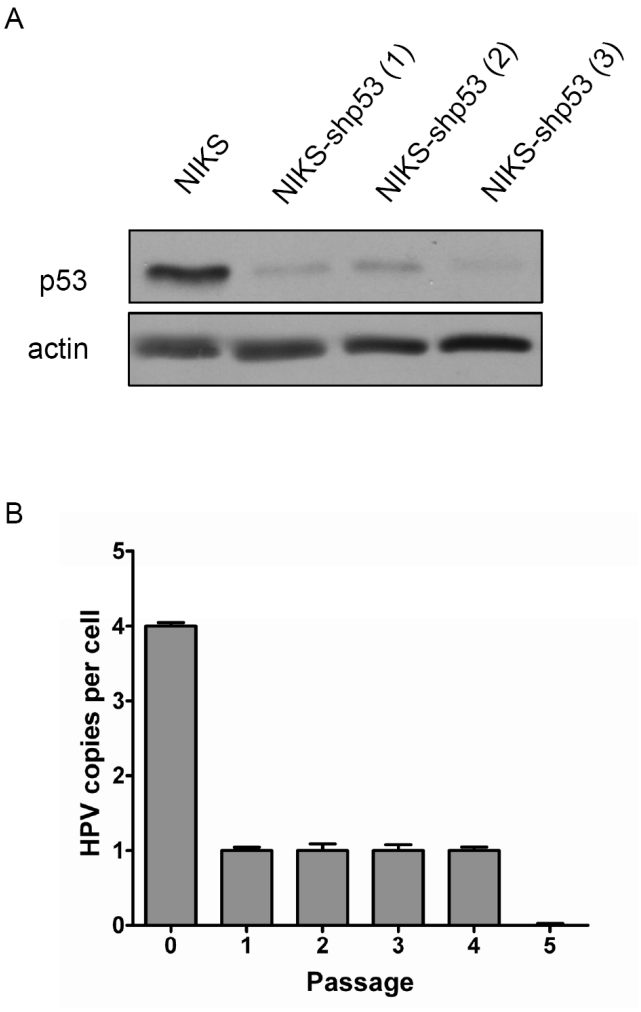


Figure 4.8: 16WT genomes cannot persist in NIKS-shp53 cells

A) NIKS cells were infected with retroviruses bearing an shRNA construct against p53. The cells were selected with antibiotics and cloned. Cell lysates were collected and the levels of p53 in three clones were determined by western blotting. Actin was used as a loading control. B) The NIKS-shp53 (3) cell line was co-transfected with 16WT genomes and the pcDNA6 plasmid. Following antibiotic selection, the cells were grown as populations and DNA was extracted at each passage. DNA was analysed by qPCR to determine the number of HPV copies per cell. The error bars represent the standard deviation of the qPCR triplicates.

4.3. Discussion

In this chapter, I aimed to address different factors that could potentially contribute to the inability of the 16E6p53m and 16E6PDZ mutant genomes to persist in NIKS cells. Previous studies have highlighted the inhibitory effect of p53 on the replication of different viruses, including PVs (Brown *et al.*, 2008; Ilves *et al.*, 2003; Lepik *et al.*, 1998), suggesting that E6-mediated degradation of p53 may be necessary to allow efficient replication of the HPV genomes. This effect has been suggested to come about via the interaction of p53 with the viral replication protein E2 (Brown *et al.*, 2008). I therefore wanted to determine whether the mutant HPV16 genomes were able to replicate in NIKS cells, and if so, whether they could replicate with similar efficiency as the wild-type genomes.

Data from the qPCR-based transient replication assay is consistent with this view as they show that the 16E6p53m genome, which is unable to degrade p53, is compromised in replication. Importantly, in my study I investigated replication in the context of the entire HPV16 genome, instead of the reporter plasmids used in the studies mentioned above, which generally include only the PV origin of replication in the presence of ectopically-expressed E1 and E2 proteins. This is an important consideration as other viral proteins may have an effect on replication by regulating the levels of viral and cellular proteins.

It is important to note however, that in both my study and the ones mentioned above, the HPV genomes or reporter plasmids were introduced into the cells by transfection and not by infection. Infection with HPV is thought to only introduce a small number of genomes into each cell, which are then amplified to 10 - 200 copies per cell (Doorbar, 2005). When transfection is used to introduce viral genomes into cells, a much larger number of genomes is expected to enter each cell. It is therefore not known if amplificational/establishment replication follows the same mechanism in these transfected cells as it would in cells that have been naturally infected. It is worth mentioning however that viral DNA has previously been shown to replicate faster than cellular DNA following transfection, thus suggesting that amplificational/establishment replication must also take place in transfected cells (Lusky & Botchan, 1986). A more detailed

analysis would be required to study this in the transfected NIKS cells and to determine the timing of the switch between amplificational replication and maintenance replication. At this stage it is not clear whether the replication levels measured by the qPCR assay solely reflect amplificational replication, or if they partly reflect maintenance replication as well.

In any case, my data do show that the 16E6p53m genomes are replication-competent with reduced efficiency compared to the 16WT genomes. Although this difference was found to be statistically significant, this assay cannot tell me whether it is also biologically significant and if it is the sole reason for the inability of the 16E6p53m genomes to persist in NIKS cells (as observed in Chapter 3). The 16E6PDZ mutant genomes are also replication-competent and their replication efficiency is not statistically different to that of the 16WT genomes.

My transient replication data with regards to the p53-degradation mutant genome is consistent with previous studies that also looked at viral genome persistence. In the Thomas *et al*. study, the authors used HPV31 p53-degradation mutant genomes (with a different mutation than the 16E6p53m genomes used in this study) and noted that these do not replicate as efficiently as the HPV31 wild-type genomes (Thomas *et al.*, 1999). Moreover, in the Park and Androphy study, HPV31/16 E6-mutant hybrid genomes were used that carried the same mutation as the 16E6p53m genomes used in this study (Park & Androphy, 2002). Like me, these authors concluded that the mutant genomes were replication-competent. Although the authors did not comment on this, their Southern blot data also suggest that the replication efficiency of the mutant genomes was impaired compared to that of the wild-type genomes. This observation is again consistent with my results. On the other hand, the data available on the transient replication of PDZ-binding mutant genomes are different to mine, as reduced replication was observed with HPV31 mutant genomes that lack the PDZ-binding motif (Lee & Laimins, 2004). Variations may be due to the fact that the abovementioned studies used a different HPV type than I did. Moreover, in the earlier studies the replication of the genomes was assessed by Southern blotting, which is less sensitive and quantitative than

qPCR. A further advantage of my study is that the transient replication assays were carried out in the same cell line as the persistence assays, in NIKS cells.

I also wanted to carry out some experiments to determine if the mutations I have been working with inhibit persistence in *cis* or in *trans*. These yielded some very interesting results in that I found that wild-type HPV16 DNA was unable to persist in cells that already expressed the E6 protein. Studies in the 1980s had looked at the ability of BPV1 genomes to persist in cells that already harboured viral genomes (Berg *et al.*, 1986a; Berg *et al.*, 1986b). The results were fascinating in that wild-type BPV1 genomes failed to replicate when introduced into cells that already harboured mutant BPV1 genomes (with mutations in the E6 or E7 ORFs). As a result, the newly-introduced wild-type genomes persisted at a very low copy number in these cells. However, when the two genomes (wild-type and mutant) were introduced into the cells at the same time, they were both able to persist at high copy numbers (Berg *et al.*, 1986a; Berg *et al.*, 1986b). This suggests that the persistence of viral genomes is subjected to a very ordered and well timed expression of viral proteins in cells. Similarly, my data show that the expression of E6 prior to HPV DNA entry into cells is detrimental to persistence. In my experiments I did not use a cell line that harboured mutant viral genomes. Rather, I used a cell line that expressed one viral protein alone (E6), which represents aberrant expression of the protein.

The notion that pre-existing expression of certain viral proteins prevents re-infection with new virus is very important, as it implies that an already infected cell may be refractive to being successfully infected by other HPVs. In support of this is a study from the 1990s, that showed that wild-type and mutant BPV1 genomes were unable to co-exist in the same cells, even though both were able to persist independently (Stewart *et al.*, 1994).

What is interesting about my results is that E6, which is necessary for the persistence of HPV16, is also detrimental to it when expressed at the wrong time (prior to infection) or at the wrong amount. In line with the older studies mentioned above, this suggests that the regulation and timing of viral protein expression is very important and complex in the viral life-cycle.

We do not yet know which activity of E6 causes the inhibition of persistence in the LXSN-E6WT cells. However my experiments suggest a role for p53-degradation. This is consistent with a hypothesis proposed by Lepik *et al*. that perhaps p53-mediated inhibition of replication may be necessary at the early stages of infection in order to ensure that viral replication is kept under control and that the genomes do not replicate uncontrolled and cause harm to the cell (Lepik *et al.*, 1998). An alternative explanation might be that cells that express E6, or have low-levels of p53 (due to the expression of shRNA against p53) may grow too quickly for the viral DNA to establish itself. Although little is know about the requirements for establishment, it is perceivable that too rapid cell proliferation may not allow time for the build up of viral proteins to the required level that is crucial for viral DNA replication and segregation. It has to be noted however that the persistence assay in the NIKS-shp53 cell line is lacking the proper control, which in this case would be the transfection of 16WT genomes in cells that express an unrelated shRNA construct such as shGFP. The ability of 16WT to persist in these cells would prove that the lack of persistence in the NIKS-shp53 cells was due to the low levels of p53, and not an effect of the shRNA expression, such as induction of an interferon response (Bridge *et al.*, 2003). Such a response could inhibit the persistence of HPV DNA, for example by inducing the expression of p56, which in turn down-regulates the replication of HPV DNA (Terenzi *et al.*, 2008).

In conclusion, the results presented in this chapter show that the 16E6p53m genome is compromised in its ability to replicate in NIKS. This suggests a mechanism by which p53-degradation may be important for viral genome persistence. As no significant impairment of viral DNA replication was observed for the 16E6PDZ mutant genome, I hypothesise that the reason why this genome cannot persist is due to a different mechanism.

Chapter 5: Stabilisation of E6 by PDZ proteins

5.1. Introduction

In Chapter 4 I showed that the 16E6p53m mutant genomes do not replicate as efficiently as the 16WT genomes, and I suggested that this could be the reason why the 16E6p53m genomes do not persist in NIKS cells. The same was not true for the 16E6PDZ mutant genomes, as these were shown to replicate with a similar efficiency as the 16WT genomes. Therefore, I concluded that the role of the PDZ-binding motif of E6 in persistence is not related to HPV DNA replication, but to another process or processes.

As described in Chapter 1, the PDZ-binding motif is found on most high-risk HPV types, and is absent from the low-risk types (Kiyono *et al.*, 1997; Lee *et al.*, 1997; Nakagawa & Huibregtse, 2000). Previous studies have shown that this motif mediates an interaction between the scaffolding PDZ proteins and high-risk E6 proteins, and E6 has been shown to induce the proteasomal degradation of some of these PDZ proteins (Gardiol *et al.*, 1999; Glaunsinger *et al.*, 2000; Nakagawa & Huibregtse, 2000; Thomas *et al.*, 2002). Interestingly, HPV16 and HPV18 E6 proteins have been shown to target PDZ proteins with different affinities, with hScrib being a preferential target of HPV16 E6 and hDlg being a preferential target of HPV18 E6 (Thomas *et al.*, 2005). The absence of a PDZ-binding motif from low-risk HPV types suggests a potential role for this motif in the transformation of cells and the oncogenicity of the high-risk E6 proteins (Howley & Lowy, 2007). However, the role for this interaction in the productive life-cycle of HPVs is not well understood. From the work presented in Chapter 3 and from the work of Lee and Laimins on HPV31 (Lee & Laimins, 2004), it is now clear that the PDZ-binding motif of E6 is necessary for viral episomal persistence.

The PDZ proteins are members of multi-protein structures in the cell and many of their interactions occur via their multiple PDZ domains. Cellular PDZ-binding proteins include the tumour suppressor adenomatous polyposis coli (APC),

which is an integral member of the Wnt signalling pathway as part of the complex that controls the degradation of β-catenin (Logan & Nusse, 2004). This protein interacts with both hScrib and hDlg (Matsumine *et al.*, 1996; Takizawa *et al.*, 2006).

Another cellular tumour suppressor protein, PTEN, interacts with the PDZ proteins MAGI-1, -2 and -3 (Kotelevets *et al.*, 2005; Wu *et al.*, 2000b; Wu *et al.*, 2000c). Interaction with MAGI-2 has been shown to stabilise PTEN (Valiente *et al.*, 2005; Wu *et al.*, 2000b), which provides evidence that binding to PDZ proteins can have an effect on the steady-state levels of the PDZ-binding protein. In support of this are also data from a study of the *Drosophila* PDZ protein InaD, which has been shown to stabilise other proteins by way of its PDZ domains (Tsunoda *et al.*, 1997).

Interestingly, a recent study identified a novel and important feature of E6's interaction with the cellular ubiquitin ligase E6AP; it demonstrated that by associating with E6AP, the E6 protein is stabilised (Tomaic *et al.*, 2009b). Until this report, the interaction between E6 and E6AP had been studied primarily within the context of p53 protein degradation (Scheffner *et al.*, 1993). This new finding suggests that the outcomes of E6-E6AP interaction are not confined to the use of E6AP's ubiquitin ligase activity, but also extend to the stabilisation of the E6 protein itself.

These studies prompted me to question whether the interactions of E6 with other cellular proteins may also have an impact on E6 stability. Recent developments in E6 detection by western blotting in the laboratory allowed me to address this question and specifically test whether the E6-PDZ protein interactions have any effect on the E6 protein.

5.2. Results

5.2.1. Detection of E6 protein after transient transfection of NIKS cells

As stated in the introduction, the aim of this part of the study was to investigate the relationship of E6 with its binding partners, the PDZ proteins, in light of a recent report that suggested that E6 can be stabilised by its binding partners (Tomaic *et al.*, 2009b). Previous studies have only investigated this relationship from the perspective of the degradation of PDZ proteins by E6. I have also done this in Chapter 3, where I characterised the wild-type and mutant E6 proteins in terms of their ability to degrade hScrib. To do this I co-transfected NIKS cells with plasmids that express E6WT, E6p53m or E6PDZ, and a plasmid that expresses HA-tagged hScrib, and analysed the levels of HA-hScrib by western blotting. One such experiment was presented in Figures 3.4B and C and the results were consistent with the published data (Nakagawa & Huibregtse, 2000) in that E6WT and E6p53m proteins were able to degrade hScrib, whereas the E6PDZ protein, which lacks the PDZ-binding motif, was not.

Whilst carrying out the abovementioned experiments, I came across some unexpected and very interesting results with regards to the levels of E6 in the transfected cells. The E6PDZ mutant protein was consistently found to be present at significantly lower levels than the E6WT and the E6p53m proteins. One such example is shown in Figure 5.1. These observations with regards to the levels of E6 protein were novel. Previous studies that focused on the levels of PDZ proteins in the presence of wild-type and mutant E6 proteins, did not determine the levels of E6 in their experiments. This may be partly due to the difficulty in detecting the E6 protein by western blotting.

The difference in the levels of the E6WT and E6PDZ proteins was very intriguing as it could imply a role for the PDZ-binding motif of E6 in the stability of the protein and consequently in the protein's role in the viral life-cycle. I was thus interested to investigate this further.

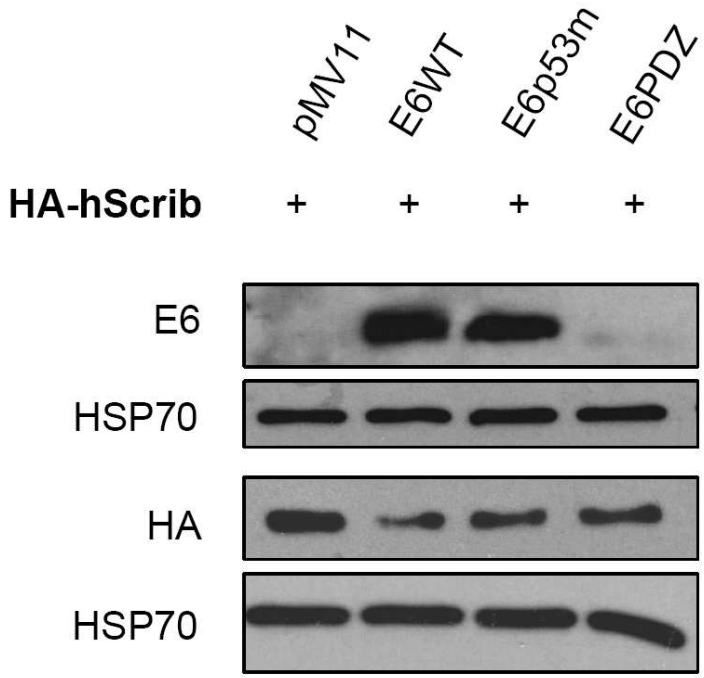


Figure 5.1: Expression of wild-type and mutant E6 proteins in NIKS cells
NIKS cells were transiently co-transfected with pMV11-E6WT, pMV11-E6p53m, pMV11-E6PDZ or control pMV11 plasmid, and the pcDNA-HA-hScrib plasmid and extracts were prepared 48 h post-transfection. Western blots show the levels of the wild-type or mutant E6 proteins as well as the levels of the HA-hScrib protein. HSP70 was used as a loading control.

5.2.2. The difference in the levels of E6WT and E6PDZ proteins is not due to variations in their solubilities or in the transfection efficiencies of the plasmids

We first considered the possibility that the difference in the levels of E6WT and E6PDZ proteins may be caused by variations in the solubility of the E6 proteins. The lysates analysed in the western blot presented in Figure 5.1 were prepared in a RIPA buffer that contained a relatively low concentration of anionic detergent. Therefore, these lysates were not whole-cell extracts but were RIPA-soluble extracts. As mentioned in Chapter 1, E6 has been shown to have cytoplasmic binding partners, such as E6AP (Huibregtse *et al.*, 1991), as well as nuclear ones, such p53 (Werness *et al.*, 1990). As the solubility of a protein can change depending on its localisation in the cell, I hypothesised that there may be pools of E6 that are not RIPA-soluble, and may have been excluded from the analysis.

To address any potential differences in the solubility of E6WT and E6PDZ, I measured the levels of E6 protein in whole-cell extracts (prepared in a RIPA buffer containing 6% of SDS). These extracts were of NIKS cells that were co-transfected with vectors expressing E6 proteins (wild-type or mutant) and HA-hScrib. Once again the levels of E6WT were found to be significantly higher than those of E6PDZ (Fig. 5.2A). The intensities of the three E6WT and three E6PDZ bands were measured using ImageJ software and normalised to the loading controls (HSP70). An unpaired t-test showed that the levels of the E6WT protein were statistically higher than those of the E6PDZ protein ($p=0.0034$), while no significant difference was observed between the levels of E6WT and E6p53m proteins (Fig. 5.2B).

To test whether the difference between the levels of E6WT and E6PDZ proteins may be caused by variations in the transfection efficiencies of their respective plasmids, I repeated the transfections as before but with the inclusion of pMV10, a plasmid that expresses β-galactosidase (β-gal) (Forrester *et al.*, 1992), in the transfection mix. The levels of β-gal protein were analysed by western blotting and were found to be similar between the two sets of transfections (Fig. 5.2C) indicating that the difference in the levels of the two E6 proteins was not due to differences in the transfection efficiencies of the

plasmids. Furthermore, as in Figure 3.4C, the levels of HA-hScrib were lower in the presence of E6WT than E6PDZ, which is consistent with what I would expect based on the literature (Nakagawa & Huibregtse, 2000).

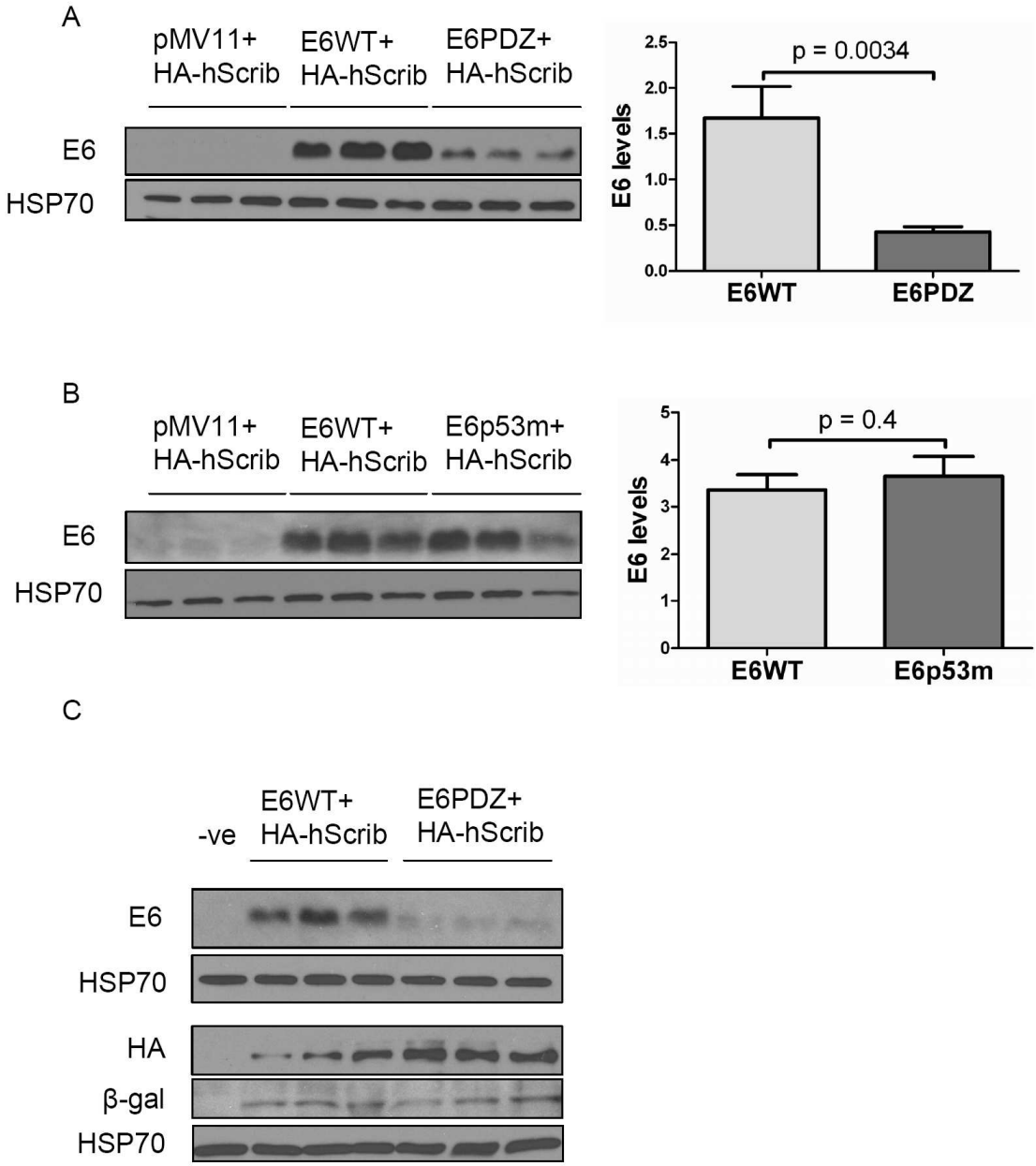


Figure 5.2: Levels of E6PDZ protein but not E6p53m protein are lower than levels of E6WT protein

NIKS cells were transiently co-transfected with pMV11-E6WT, pMV11-E6p53m, pMV11-E6PDZ, or control pMV11 plasmid and pcDNA-HA-hScrib plasmid and whole-cell extracts were prepared 48 h post-transfection. Each transfection was carried out in triplicate. A) Levels of E6WT and E6PDZ. B) Levels of E6WT and E6p53m. The levels of E6 were measured using ImageJ software and normalised to the loading controls. The bar charts show the mean levels of E6 protein (in arbitrary units) and the standard deviation. Unpaired t-tests were used to compare the levels of E6 and p values are shown above the graphs. C) Levels of E6WT, E6PDZ, HA and β –gal. HSP70 was used as a loading control.

5.2.3. The lower levels of the E6PDZ protein are not cell line dependent or antibody-dependent

To ascertain whether my observation with regards to the lower levels of E6PDZ mutant protein was specific to NIKS cells, or if it is an intrinsic characteristic of the protein, I repeated the transfections in two different cell lines. The cell lines used were HT1080 cells, a human fibrosarcoma cell line, and 293T cells, a human embryonic kidney cell line, which expresses the SV40 T-antigen. The cells were co-transfected with pMV11-E6WT or pMV11-E6PDZ plasmids, and pcDNA-HA-hScrib plasmid and the levels of E6 were assessed (Fig. 5.3A). Once again, the levels of E6PDZ protein were much lower than those of E6WT protein in both HT1080 and 293T cells, in line with the observations in NIKS cells. These results confirm that my previous observations were not specific to NIKS cells but were likely to be characteristic of a wide variety of cell types, possibly including the cells that HPV normally resides in.

To exclude the possibility that the observations made so far were caused by the specific antibody I have been using, which recognises an epitope in the C-terminal half of E6, I repeated the western blot, using a different antibody (Fig. 5.3B). The 1E-6F4 antibody clone, also from Euromedex, was used. This recognises an epitope in the N-terminus of E6. The levels of E6PDZ protein were again lower than the levels of E6WT protein. This confirms that my previous results were not due to differences in the detection of the two proteins by the antibody I have been using.

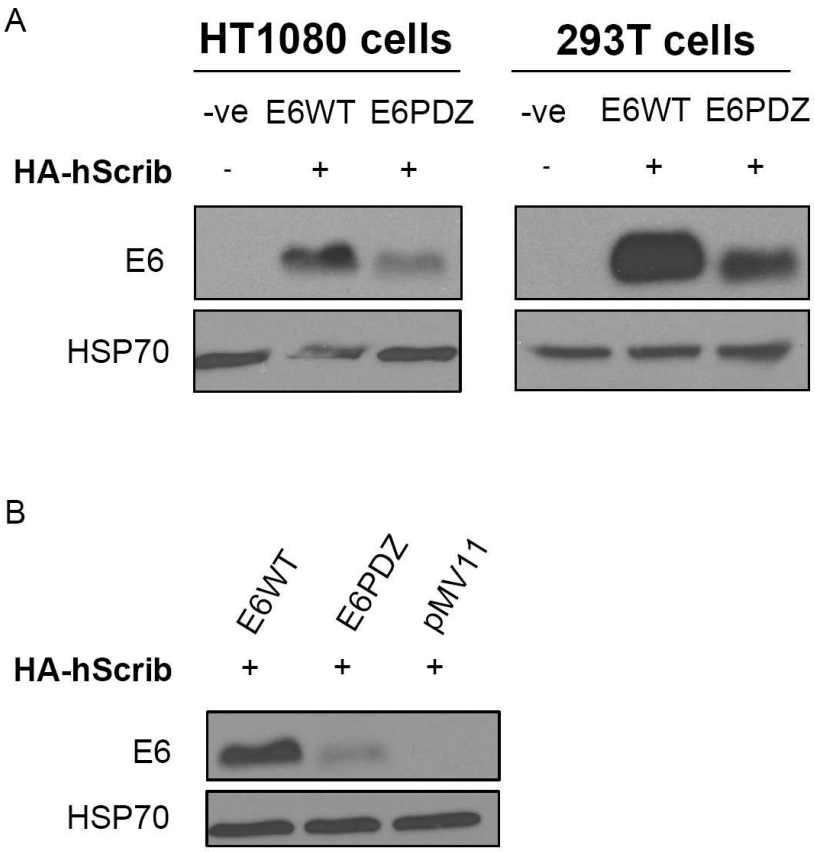


Figure 5.3: The difference in the levels of E6WT and E6PDZ is not cell line or antibody dependent

A) HT1080 and 293T cells or B) NIKS cells were transiently co-transfected with pMV11-E6WT, pMV11-E6PDZ or control pMV11 plasmid, and the pcDNA-HA-hScrib plasmid, and whole-cell extracts were prepared 48 h post-transfection. The western blots show the levels of E6WT, E6PDZ and HA-hScrib detected with an antibody that recognises an epitope in the A) C-terminus or B) N-terminus of E6. HSP70 was used as a loading control.

5.2.4. The difference in the levels of E6WT and E6PDZ proteins is not due to differences in their transcription levels

One process that can affect the turn-over rate of a protein (and thus its steady-state levels) is the rate at which it is produced. Therefore, the difference in the levels of the wild-type and mutant E6 proteins may be a reflection of a difference in their respective mRNA levels.

To address this, NIKS cells were again co-transfected in triplicate with pMV11-E6WT or pMV11-E6PDZ plasmid, and pcDNA-HA-hScrib plasmid and RNA was extracted from the cells 48 hours post-transfection. cDNA was made by reverse transcription and used as a template for qPCR. Primers against the E6 ORF were used to detect full-length E6 transcripts and primers against β-actin were used as a control. The levels of E6 transcripts were normalised to those of β-actin transcripts for each sample (Fig. 5.4). An unpaired t-test showed that the difference in the levels of E6WT and E6PDZ transcripts was not statistically significant ($p = 0.14$) and if anything, the E6PDZ transcript levels appear higher.

I thus conclude that the difference in the levels of E6WT and E6PDZ proteins cannot be explained by differences in their respective trascription levels.

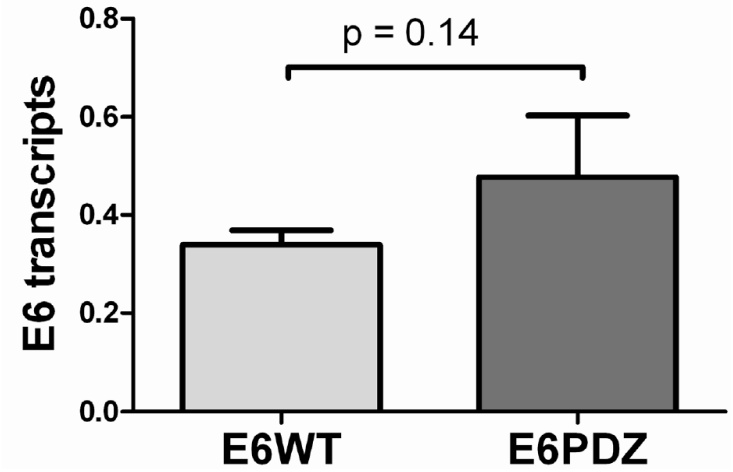


Figure 5.4: E6WT and E6PDZ have similar transcription levels

NIKS cells were transiently co-transfected with pMV11-E6WT or pMV11-E6PDZ plasmid, and pcDNA-HA-hScrib plasmid. RNA was extracted 48 h post-transfection and analysed by RT-qPCR. The transfections were carried out in triplicate and the bar chart shows the mean levels of full-length E6 transcripts (normalised to β-actin transcripts) and the standard deviation. An unpaired t-test was used to compare the levels of E6 transcripts and the p value is shown above the graph.

5.2.5. The difference in the levels of E6WT and E6PDZ is due to protein stability

Having established that the difference in the levels of E6WT and E6PDZ protein is not due to differences in the transcription levels of the two plasmids, I next wanted to assess the relative stability of these two proteins. To investigate this, NIKS cells were co-transfected with pMV11-E6WT or pMV11-E6PDZ plasmids, and the pcDNA-HA-hScrib plasmid. At 46 hours post-transfection the cells were treated with 50 µg/ml of cycloheximide, an inhibitor of protein biosynthesis, for 60 or 120 minutes, and then harvested for protein analysis. The levels of E6 were assessed by western blotting, and presented relative to the levels at the "0 minutes" time-point (Fig. 5.5).The rate of loss of the E6PDZ protein is greater than that of the E6WT protein, which demonstrates that the wild-type E6 protein is more stable than the mutant one. An unpaired t-test analysis showed that the levels of the two proteins were significantly different at time-point 120' ($p=0.032$). Moreover, from these data, the half-lives of the two proteins were calculated to be 113 minutes for E6WT and 47 minutes for E6PDZ.

As previous studies have shown that E6 is degraded by the proteasome (Kehmeier *et al.*, 2002; Stewart *et al.*, 2004), I hypothesised that the PDZ-binding motif of E6WT may protect the protein from proteasomal degradation. To investigate this, NIKS cells were co-transfected with pMV11-E6WT or pMV11-E6PDZ plasmids, and the pcDNA-HA-hScrib plasmid and at 46 hours post-transfection were treated with the proteasome inhibitor MG-132 (final concentration 40 µM) or with an equivalent volume of DMSO as a control, for two hours. Whole-cell extracts were prepared and the levels of E6 protein were analysed by western blotting (Fig. 5.6). A paired t-test showed that the levels of E6WT protein did not vary significantly in MG-132- or DMSO-treated cells ($p=0.62$). On the contrary, the levels of E6PDZ protein were significantly increased in the MG-132-treated cells, compared to the untreated control cells ($p=0.0046$). The accumulation of E6PDZ protein in the 2-hour treatment with the inhibitor indicates that this protein is more susceptible to proteasomal degradation than the E6WT protein.

Furthermore, the data presented in Figure 5.6 also appear to show a higher degree of degradation of HA-hScrib in the presence of E6WT than in the

presence of E6PDZ, consistent with the current understanding of degradation of the hScrib protein by E6 (Nakagawa & Huibregtse, 2000).

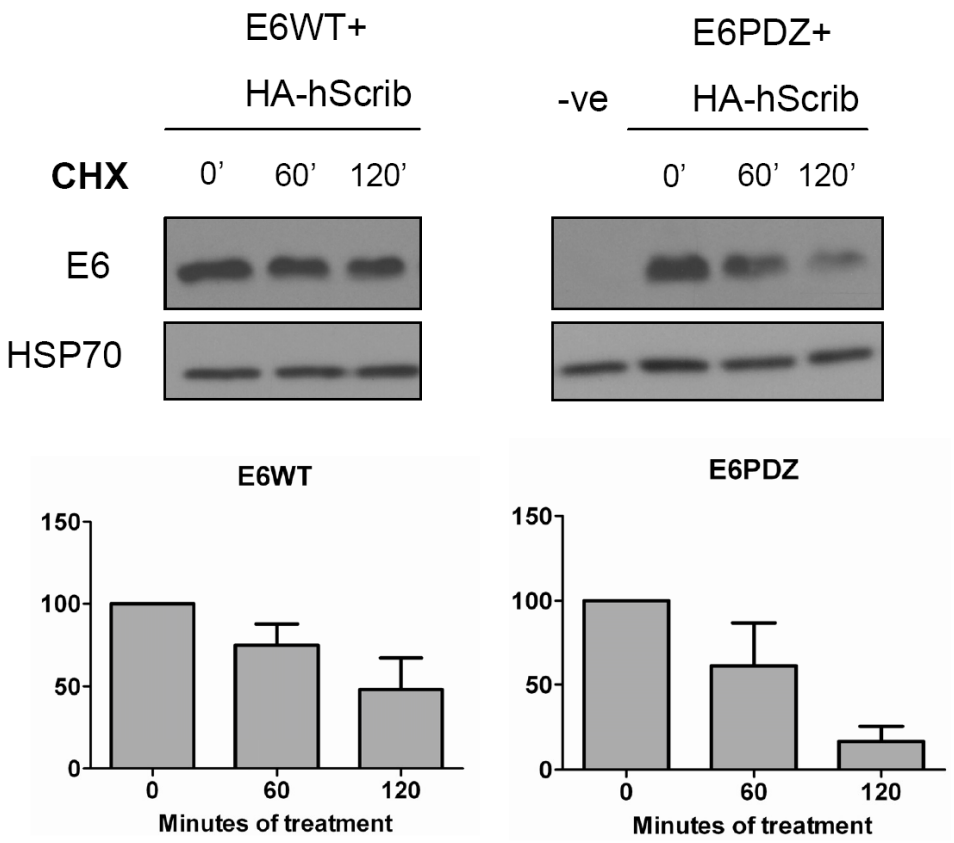


Figure 5.5: E6WT protein is more stable than E6PDZ protein

NKS cells were transiently co-transfected with pMV11-E6WT or pMV11-E6PDZ plasmid, and pcDNA-HA-hScrib plasmid. At 46 h post-transfection, cells were treated with 50 µg/ml of cycloheximide for 60 or 120 minutes and whole-cell extracts were prepared for each time-point. Extracts were also prepared from untreated cells to be used as the "0 minutes" time-point. The levels of E6 were analysed by western blotting and measured using ImageJ software. The transfections were carried out in duplicate and the bar charts show the mean levels of E6, relative to the levels at the "0 minutes" time-point, and the standard deviation. HSP70 was used as a loading control.

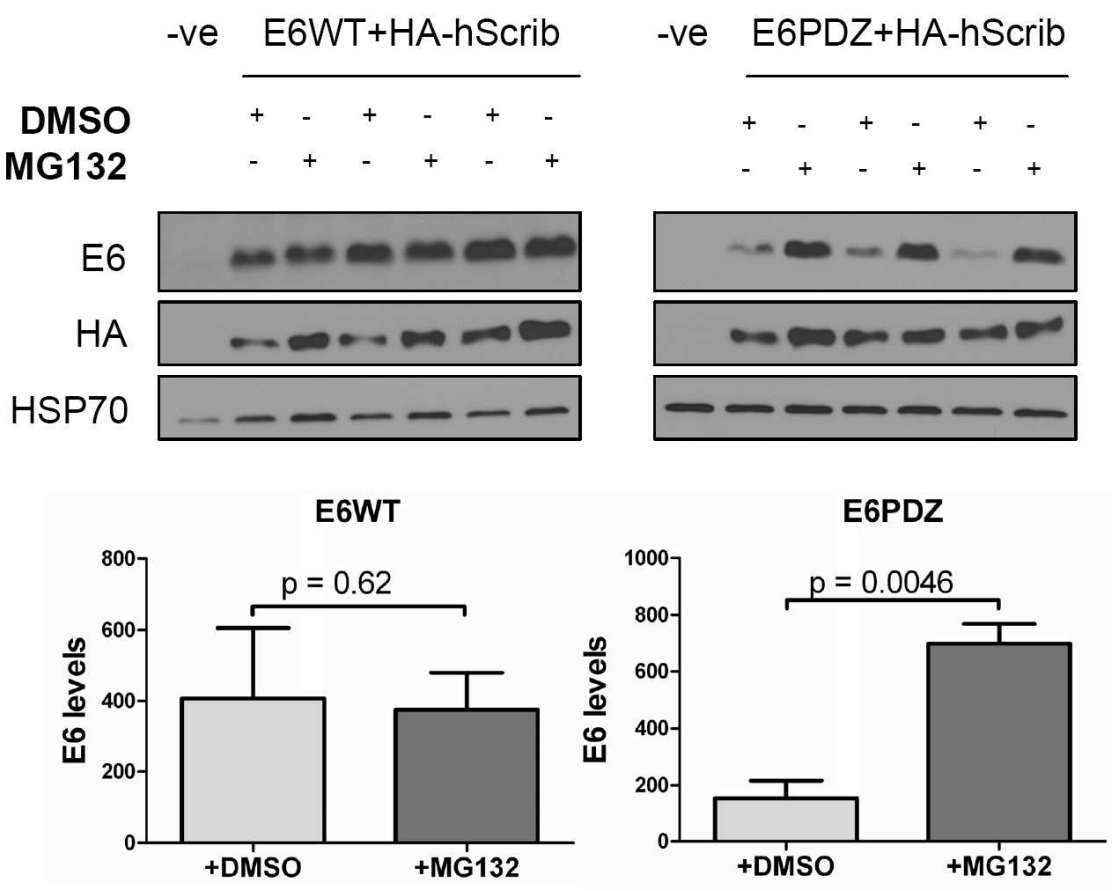


Figure 5.6: E6PDZ is more susceptible to proteasomal degradation than E6WT in the presence of HA-hScrib

NIKS cells were transiently co-transfected with pMV11-E6WT or pMV11-E6PDZ plasmid, and pcDNA-HA-hScrib plasmid. At 46 h post-transfection, cells were treated with 40 μM of MG-132 or with DMSO as a control, for two hours, and whole-cell extracts were prepared. Each transfection was carried out in triplicate. The levels of E6 were measured using ImageJ software and the bar charts show the mean levels of E6 protein (in arbitrary units) and the standard deviation. A paired t-test was used to compare the levels of E6 with and without MG-132, and the p values are shown above the graphs. HSP70 was used as a loading control.

5.2.6. Levels of HPV16 E6WT, but not HPV16 E6PDZ or HPV11 E6WT proteins, are higher in the presence of exogenous hScrib.

The data so far showed that the E6PDZ protein is more unstable than the E6WT protein. The only difference in primary structure between these two proteins is the absence of the PDZ-binding motif on the C-terminus of E6PDZ mutant protein. As described above, this is the motif that interacts with the PDZ domains of hScrib (Nakagawa & Huibregtse, 2000). As such, it has been presumed that the difference in the stability of these two proteins is owed to their ability (or not) to interact with PDZ proteins. Hence, all the experiments presented so far had been carried out in the presence of exogenously-expressed hScrib. To test whether this presumption is erroneous or correct, the following experiments were carried out.

NIKS cells were transiently co-transfected in triplicate with pMV11-E6WT (Fig. 5.7) or pMV11-E6PDZ plasmid (Fig. 5.8), and pcDNA-HA-hScrib or control pcDNA plasmid. The levels of E6 proteins were measured and normalised to those of the loading control (HSP70). The results were very informative from two perspectives; firstly, I observed that the levels of E6WT were very much higher in the presence of exogenous hScrib compared to the control cells, in which the empty pcDNA vector was co-transfected with the E6-expression vector ($p=0.0013$) (Fig. 5.7). This validated the notion that the stability of the E6 protein is affected by the presence of exogenous hScrib. Secondly, this hScrib-dependent augmentation of the E6 protein level did not apply to the E6PDZ protein, as the levels of this mutant protein remained unaffected by the presence of exogenous hScrib ($p=0.79$) (Fig. 5.8).

From this section I can conclude that the difference in the stabilities of E6WT and E6PDZ proteins is due to stabilisation of E6WT by hScrib. Thus, the PDZ-binding motif of E6 plays a role in stabilising the E6 protein. If this is correct, it would stand to reason that hScrib would have no effect on the levels of the low-risk HPV11 E6 protein, which lacks a PDZ-binding motif (Kiyono *et al.*, 1997; Lee *et al.*, 1997; Nakagawa & Huibregtse, 2000). To test this, NIKS cells were co-transfected with pGWI-HA-11E6 (a plasmid that expresses HA-tagged HPV11 E6), kindly provided by Dr. Lawrence Banks (Glaunsinger *et al.*, 2000), or control pMV11 plasmid, and pcDNA-HA-hScrib or control pcDNA plasmid.

The transfections were carried out in triplicate and whole-cell extracts were prepared. The protein levels of E6 were measured by western blotting and normalised to the HSP70 loading controls (Fig 5.9). As expected, the levels of low-risk E6 protein were not altered in the presence of HA-hScrib. This confirms my prediction that the absence of a PDZ-binding motif in HPV11 E6 prevents hScrib-mediated stabilisation of the E6 protein.

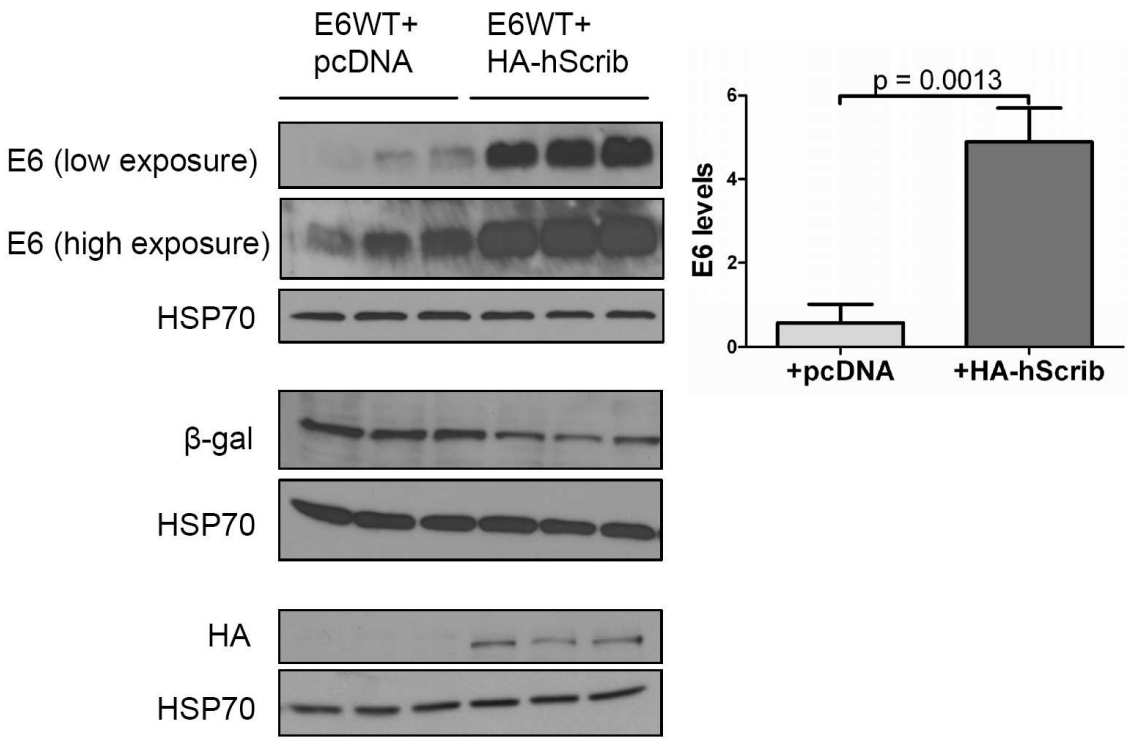


Figure 5.7: Levels of E6WT protein are higher in the presence of HA-hScrib

NIKS cells were transiently co-transfected with pcDNA-HA-hScrib or control pcDNA plasmid, and pMV11-E6WT plasmid and whole-cell extracts were prepared 48 h post-transfection. Each transfection was carried out in triplicate. The levels of E6 were measured using ImageJ software and normalised to the loading control. The bar chart shows the mean levels of E6 protein (in arbitrary units) and the standard deviation. An unpaired t-test was used to compare the levels of E6 and the p value is shown above the graph. β-gal expression was used as a transfection control and HSP70 was used as a loading control.

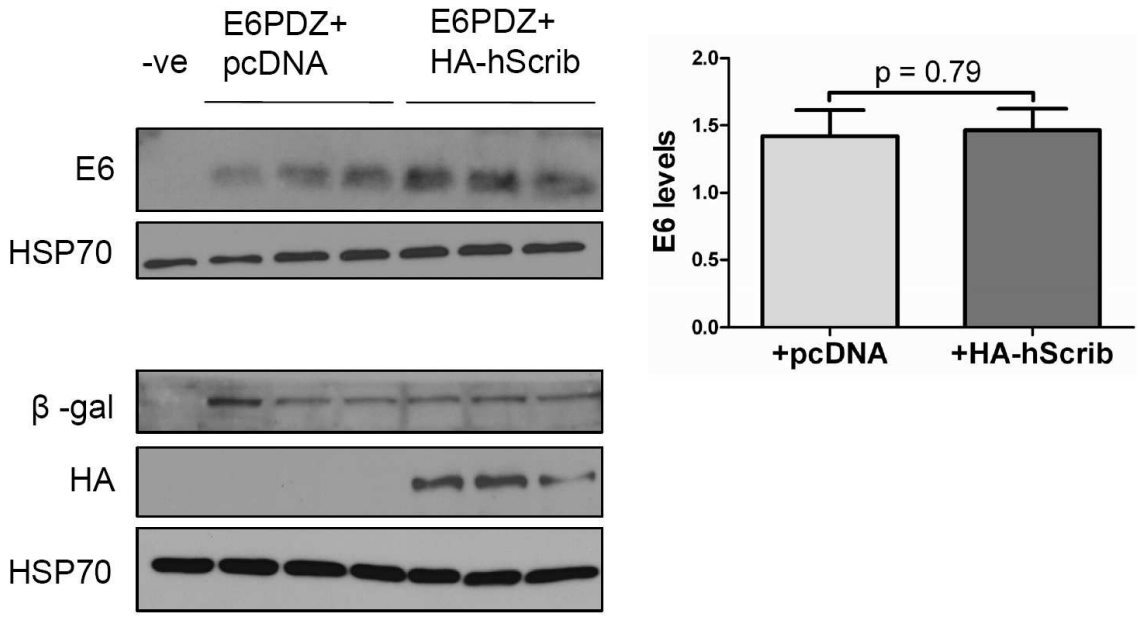


Figure 5.8: Levels of E6PDZ protein are unaffected by the presence of HA-hScrib

NIKS cells were transiently co-transfected with pcDNA-HA-hScrib or control pcDNA plasmid, and pMV11-E6PDZ plasmid and whole-cell extracts were prepared 48 h post-transfection. Each transfection was carried out in triplicate. The levels of E6 were measured using ImageJ software and normalised to the loading controls. The bar chart shows the mean levels of E6 protein (in arbitrary units) and the standard deviation. An unpaired t-test was used to compare the levels of E6 and the p value is shown above the graph. β-gal expression was used as a transfection control and HSP70 was used as a loading control.

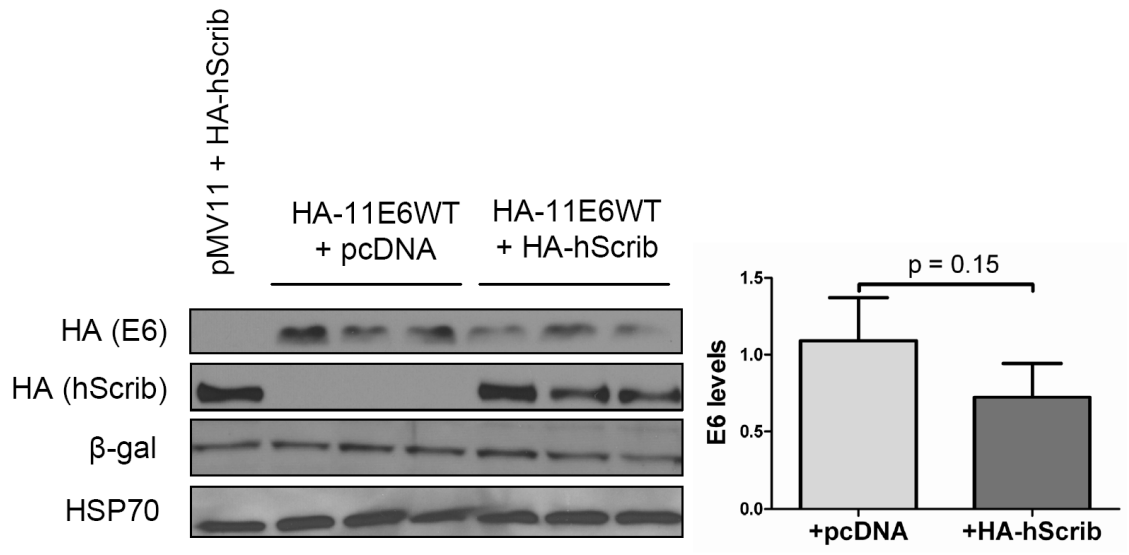


Figure 5.9: Levels of low-risk E6 protein are unaffected by the presence of HA-hScrib

NIKS cells were transiently co-transfected with pGW1-HA-11E6 or control pMV11 plasmid, and pcDNA-HA-hScrib or control pcDNA plasmid and whole-cell extracts were prepared 48 h post-transfection. Each transfection was carried out in triplicate. The levels of E6 were measured using ImageJ software and normalised to the loading controls. The bar chart shows the mean levels of E6 protein (in arbitrary units) and the standard deviation. An unpaired t-test was used to compare the levels of E6 and the p value is shown above the graph. β-gal expression was used as a transfection control and HSP70 was used as a loading control.

5.2.7. The PDZ domains of hScrib are involved in the stabilisation of wild-type E6 protein

As E6 has been shown to bind to hScrib via the PDZ domains of the latter (Nakagawa & Huibregtse, 2000), I wanted to investigate whether the PDZ domains on the hScrib protein are necessary for the stabilisation of E6. To test this, I made use of a plasmid that expresses a mutant hScrib protein, pcDNA-HA-hScribΔPDZ (Thomas *et al.*, 2005), which was kindly provided by Dr. Lawrence Banks. This plasmid encodes an HA-tagged hScrib protein that lacks the whole of the PDZ domain region (deleted between amino acids 724 and 1224) and was shown to not be degraded by HPV16 E6 (Thomas *et al.*, 2005). Its localisation was also investigated using a GFP-tagged protein, and the mutant protein was found to resemble the wild-type in localising to adherens junctions at the basolateral membrane (Thomas *et al.*, 2005).

For my experiments, NIKS cells were co-transfected in triplicate with pcDNA-HA-hScribΔPDZ or control pcDNA plasmid, and pMV11-E6WT plasmid. The levels of E6WT protein were analysed by western blotting (Fig. 5.10) and were found not to increase in the presence of mutant hScrib protein ($p=0.28$), confirming that the PDZ domains of hScrib are necessary for the stabilisation of E6WT protein.

5.2.8. Endogenous hScrib cannot stabilise exogenously expressed E6 as efficiently

Having observed that exogenously-expressed hScrib increases the levels of wild-type E6 protein in a manner that is dependent on the PDZ-binding motif of E6 and the PDZ domains of hScrib, I went on to test whether endogenous hScrib protein can do the same to E6. NIKS cells were transiently co-transfected with pMV11-E6WT or pMV11-E6PDZ plasmid, and control pcDNA plasmid. The transfections were carried out in triplicate and whole-cell extracts were prepared. The levels of E6 were measured by western blotting and normalised to the protein levels of HSP70 in the respective lanes (Fig. 5.11).

Although the levels of E6WT protein appeared to be higher than those of E6PDZ protein, the difference was much smaller than that observed in the

presence of exogenous HA-hScrib. This result may suggest that the effect of the endogenous hScrib protein on the levels of E6WT protein is less significant than the effect of the exogenous hScrib protein (compare Figure 5.11 to 5.2C). However, it is important to note that in the previous experiments, both hScrib and E6 were expressed from CMV promoters. In this experiment however, hScrib was expressed solely from its own promoter, and the total levels of hScrib protein in transfected cells would therefore be lower. The effect of hScrib on E6 may thus be masked by the sheer amount of E6 protein present.

To get around this problem, I decided to knock-down the hScrib protein in HPV16-positive episomal cell lines, using a retroviral expression system, and to observe whether this would have any effect on the levels of the E6 protein. I firstly tested two different shRNA constructs against hScrib (Dow *et al.*, 2007) and one shRNA construct with a scrambled sequence (Dow *et al.*, 2008) (kindly provided by Dr. Patrick Humbert) in NIKS cells. I found that shScrib 1 (shScrib 6 in Dow *et al.*, 2007) did not knock down hScrib protein levels in these cells (compared to the levels of hScrib observed with shScramble). On the other hand, shScrib 2 (shScrib 7 in Dow *et al.*, 2007) did so very efficiently (Fig. 5.12A). Retroviruses expressing the shScrib 2 and shScramble constructs were then used to infect an HPV-positive NIKS cell line (in duplicate). However, the shRNA did not work as efficiently in these cells as it did in the NIKS cells (Fig. 5.12B). This was repeated with similar results.

The reason for the above results is not clear. However, at the same time as this work was being carried out, our collaborators in Dr. Lawrence Banks's laboratory (ICGEB, Trieste) successfully knocked down hScrib in HeLa cells using siRNA, and observed a reduction in the levels of E6 (Nicolaides *et al.*, 2011). This demonstrates that the E6 protein in HeLa cells is stabilised by endogenous hScrib protein. Furthermore, as HeLa cells contain integrated copies of HPV18, these results also suggest that the stability of E6 protein by hScrib holds true for other HPV types as well.

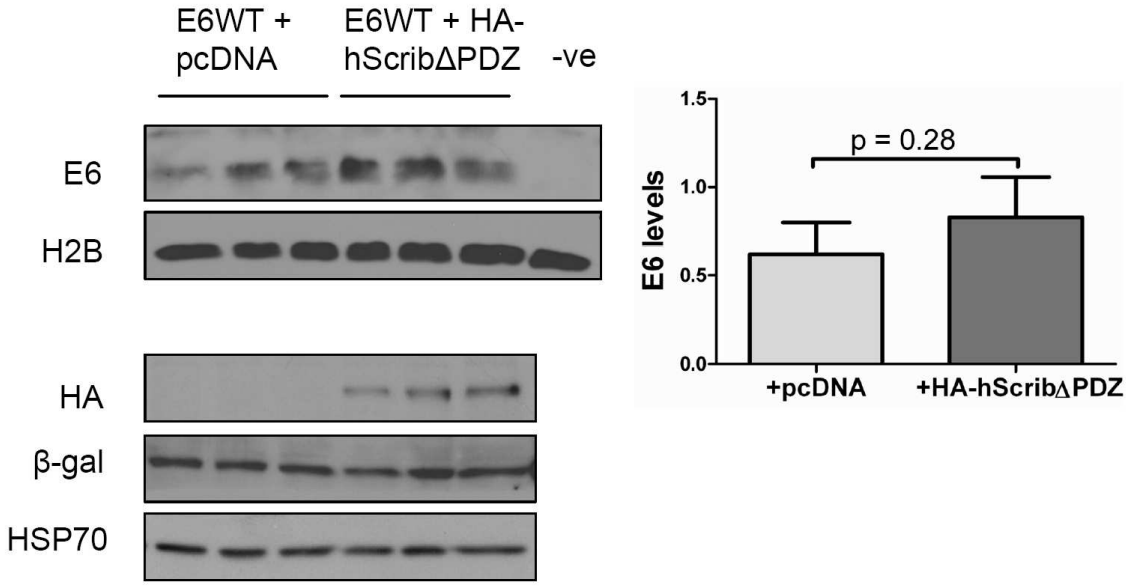


Figure 5.10: Levels of E6WT protein are unaffected by the presence of mutant HA-hScrib

NIKS cells were transiently co-transfected with pcDNA-HA-hScribΔPDZ or control pcDNA plasmid, and pMV11-E6WT plasmid and whole-cell extracts were prepared 48 h post-transfection. Each transfection was carried out in triplicate. The levels of E6 were measured using ImageJ software and normalised to the loading control. The bar chart shows the mean levels of E6 protein (in arbitrary units) and the standard deviation. An unpaired t-test was used to compare the levels of E6 and the p value is shown above the graph. β-gal expression was used as a transfection control and Histone H2B (H2B) and HSP70 were used as loading controls.

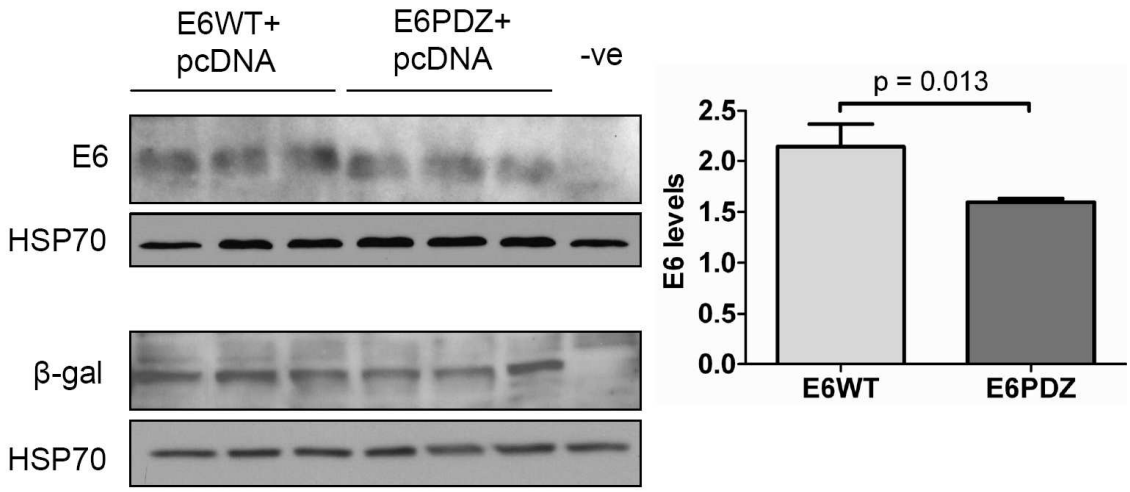


Figure 5.11: The difference between the levels of E6WT and E6PDZ proteins is marginal in the absence of exogenous hScrib

NIKS cells were transiently co-transfected with pMV11-E6WT or pMV11-E6PDZ plasmid, and pcDNA plasmid and whole-cell extracts were prepared 48 h post-transfection. Each transfection was carried out in triplicate. The levels of E6 were measured using ImageJ software and normalised to the loading control. The bar chart shows the mean levels of E6 protein (in arbitrary units) and the standard deviation. An unpaired t-test was used to compare the levels of E6 and the p value is shown above the graph. β-gal expression was used as a transfection control and HSP70 was used as a loading control.

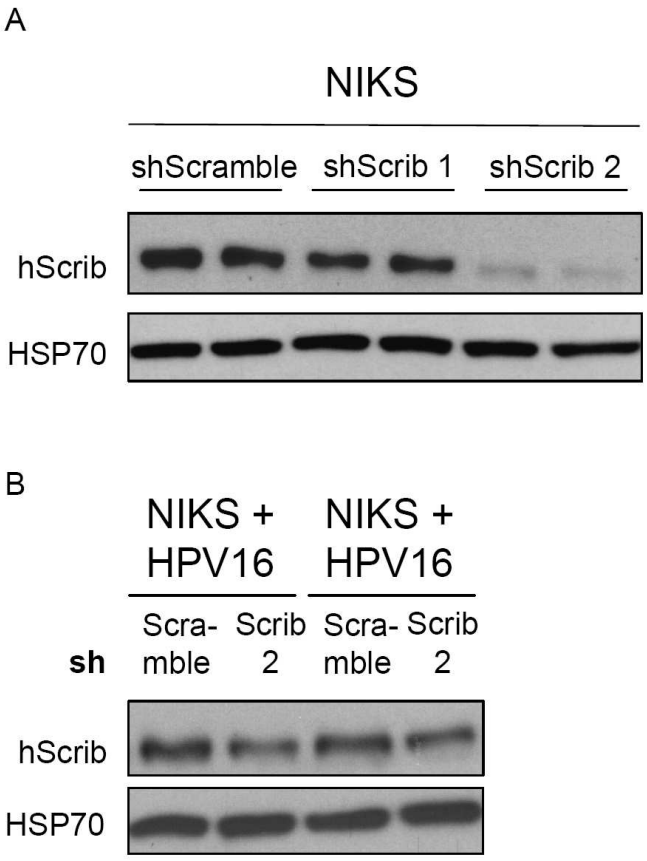


Figure 5.12: shRNA against hScrib

A) NIKS cells or B) a NIKS + HPV16 clonal cell line were infected with retroviruses expressing shRNA against hScrib or a scrambled sequence. Two different shScrib constructs were used to knock down hScrib in NIKS cells (A), whilst only the most efficient one (shScrib 2) was used in the HPV-positive NIKS cell line (B). In both A) and B) the infections were carried out in duplicate. HSP70 was used as a loading control.

5.2.9. E6WT protein is also stabilised by other PDZ proteins

We were also interested to determine whether other PDZ proteins were able to stabilise E6 in the way that hScrib has been shown to do. In addition to hScrib, targets of E6 include other MAGUK proteins such as hDlg (Gardiol *et al.*, 1999; Kiyono *et al.*, 1997; Lee *et al.*, 1997) and MAGI-1, -2 and -3, (Glaunsinger *et al.*, 2000; Thomas *et al.*, 2002) as well as the non-MAGUK protein MUPP-1 (Lee *et al.*, 2000).

Most of the abovementioned targets of E6 were found to be more efficiently targeted by HPV18 than HPV16 E6 (Gardiol *et al.*, 1999; Pim *et al.*, 2000; Thomas *et al.*, 2001; Thomas *et al.*, 2002). The reason why I initially chose to study the relationship between E6 and hScrib in this study, instead of any of the other PDZ proteins, was because hScrib was found to be more efficiently targeted for degradation by HPV16 rather than HPV18 E6 (Thomas *et al.*, 2005). This may indirectly suggest that HPV16 E6 binds more efficiently to hScrib than any of the other PDZ proteins.

We decided to look at whether hDlg and MAGI-1, which have also been shown in the literature to bind to HPV16 E6 (Glaunsinger *et al.*, 2000; Kiyono *et al.*, 1997), have the same effect on the steady-state levels of E6 as hScrib does. NIKS cells were co-transfected in triplicate with pMV11-E6WT or pMV11-E6PDZ plasmid, and pGW1-HA-Dlg (Gardiol *et al.*, 1999) or pcDNA-FLAG-MAGI-1c (Glaunsinger *et al.*, 2000) plasmid, both of which were kindly provided by Dr. Lawrence Banks. The levels of E6 protein were assessed by western blotting (Fig. 5.13A). The presence of exogenous MAGI-1 had a similar effect as hScrib on the levels of E6; that is, the levels of E6WT protein were much higher than those of E6PDZ protein. This is not surprising as MAGI-1, like hScrib, has been shown to bind strongly to HPV16 E6 (Glaunsinger *et al.*, 2000) and is also efficiently targeted for degradation in HPV16-positive cancer cell lines (Kranjec & Banks, 2010). In the presence of exogenous HA-Dlg, E6WT protein levels were also higher than E6PDZ protein levels; however the difference was not as striking as the one observed in the presence of hScrib or MAGI-1.

To verify that these plasmids do indeed express the respective proteins, I transfected them into NIKS cells and analysed the lysates by western blotting. I

was not able to detect either of these proteins on the blots. A repeat of the transfection in 293T cells however demonstrated that these plasmids can indeed express the respective PDZ proteins (Figure 5.13B). This apparent anomaly is almost certainly due to the poor transfection efficiency of the NIKS cells compared to the 293T cells.

These data suggest that the stabilisation of E6 may be a common characteristic of several PDZ proteins and not just of hScrib. However, different PDZ proteins appear to stabilise E6 to different degrees. This may be simply due to a difference in the expression levels of these PDZ proteins from their respective plasmids. However, I consider it more likely that this is reflective of the variation in the binding affinities of the E6 protein to different PDZ proteins. Whether other PDZ proteins have a similar effect on E6 protein levels remains to be established.

From the experiments described so far, I can conclude that E6WT protein is stabilised by its cellular binding partners, the PDZ proteins. This stabilisation is dependent on the PDZ-binding motif of E6 as well as the PDZ domains of the PDZ proteins and comes about by the protection of the E6WT protein from proteasomal degradation.

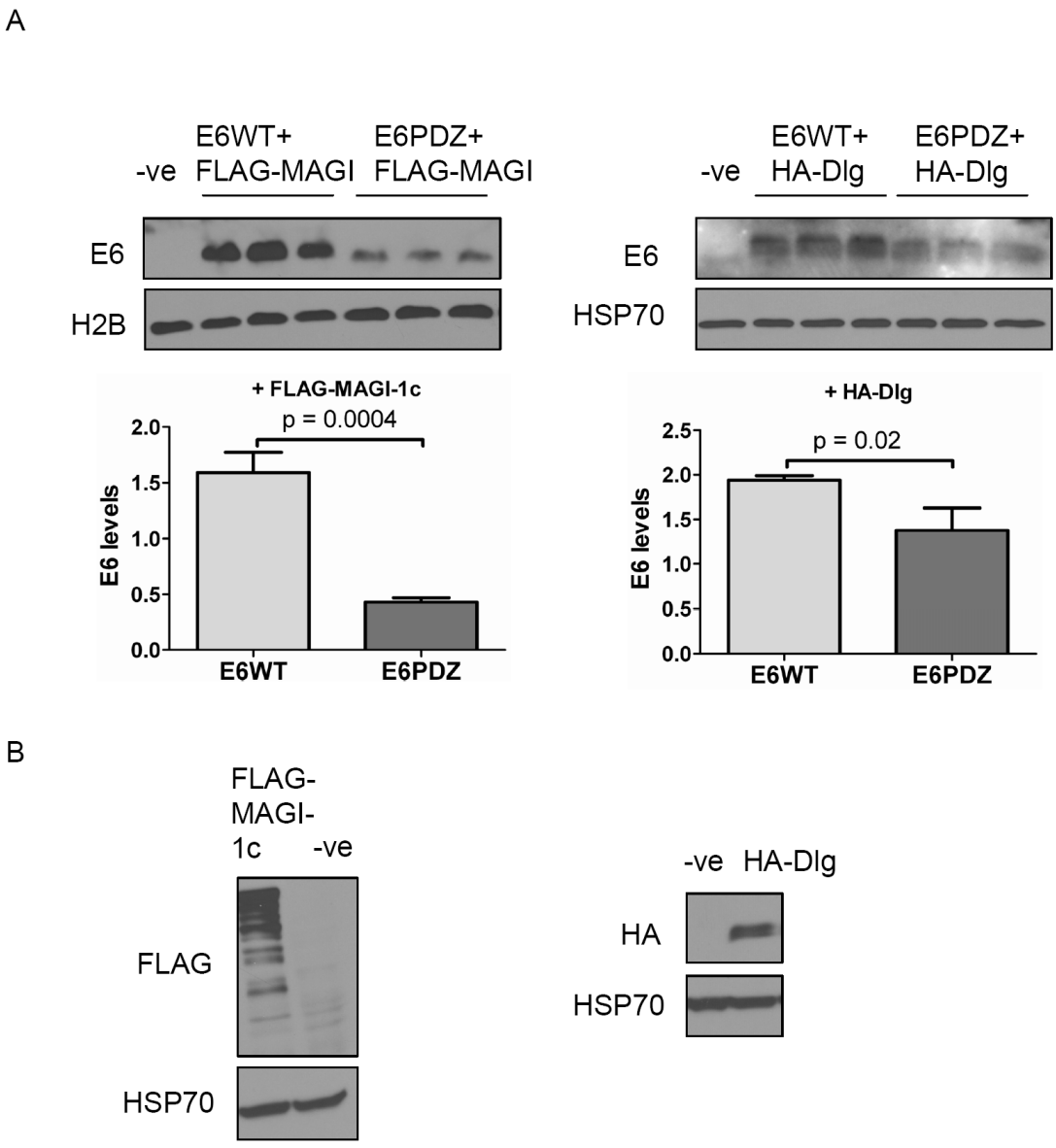


Figure 5.13: Multiple PDZ proteins can stabilise E6WT

A) NIKS cells were transiently co-transfected with pMV11-E6WT or pMV11-E6PDZ plasmid, and pcDNA-FLAG-MAGI-1c or pGW1-HA-Dlg plasmid and whole-cell extracts were prepared. Each transfection was carried out in triplicate. The levels of E6 were measured using ImageJ software and normalised to the loading control. The bar charts show the mean levels of E6 protein (in arbitrary units) and the standard deviation. Unpaired t-tests were used to compare the levels of E6 and the p values are shown above the graphs. Histone H2B (H2B) and HSP70 were used as loading controls. B) 293T cells were transfected with FLAG-MAGI-1c or pGW1-HA-Dlg plasmid. HSP70 was used as a loading control.

All of the experiments carried out so far have looked at the levels of E6 protein when expressed from the CMV promoter of the pMV11 plasmid. E6 expressed from a CMV promoter is expected to be at much higher levels than E6 expressed from the HPV genomes. Therefore, the levels of E6 in these experiments do not accurately reflect the levels present in the cells following an infection with the virus. That being said, the use of the over-expression system was deemed necessary as the levels of E6 protein expressed from the HPV16 genomes when transiently transfected into NIKS cells, have proven very difficult to detect by western blotting. This difficulty was compounded by the relatively low transfection efficiency of NIKS, as well as the fact that it was imperative to assess the levels of E6 shortly after transfection, since I have previously shown that the 16E6PDZ genomes are unable to persist in NIKS cells. This highly-restricted time-frame meant that I could not utilise any antibiotic selection method to enrich for cells that contain the HPV genomes. Nevertheless, it was important to assess whether the stabilisation of E6 by PDZ proteins holds true in the endogenous system as well.

5.2.10. The levels of E6 transcripts are significantly lower when expressed from the HPV16 genomes than the pMV11 plasmid

Unsurprisingly, the initial attempts, using western blots, to detect the E6 protein expressed from the HPV genomes in a population of transfected NIKS cells, were unsuccessful. This was attempted using varying amounts of protein on the western blots, but when high amounts were loaded on the gel, the blot had significant background that made it difficult to detect individual bands. Different anti-E6 antibodies were used with similar results (see Chapter 2). I also co-transfected the pcDNA-HA-hScrib plasmid with the HPV16 genomes in an attempt to augment the levels of E6WT in transfected cells, but to no avail as the detection of E6 was still unsuccessful (Fig. 5.14).

I then wanted to ascertain the difference in the levels of E6 transcripts that were produced from HPV16 genomes and those produced from pMV11-E6WT plasmids. NIKS cells were transfected with wild-type HPV16 genomes (16WT) or pMV11-E6WT plasmids. RNA was extracted 48 hours post-transfection and cDNA was made and used as a template for qPCR in order to detect the levels

of full-length E6 transcripts (Fig. 5.15). For the synthesis of cDNA, I chose to use oligo-dT as this primer will only initiate reverse-transcription of mature mRNAs and will thus give a more accurate indication of the levels of mRNA that are “ready” for translation. β-actin transcript levels were used as a control. As expected, the levels of E6 transcripts were much lower when expressed from the 16WT genomes, compared to the pMV11-E6WT plasmids. Therefore, in order to be able to detect the E6 protein when expressed from the HPV genomes, I would need to use very sensitive western blotting detection methods or to enrich the population of transfected cells for E6-expressing cells. It should be noted however that the above results were obtained by measuring the levels of E6 transcripts and may not necessarily accurately reflect the difference in the protein levels.

I also wanted to determine the time-point post-transfection at which the levels of E6 transcripts were highest, as this could in turn indicate the best time-point for E6 protein analysis. NIKS cells were transfected with 16WT genomes and RNA was extracted every 24 hours for 4 days post-transfection. cDNA was prepared (using oligo-dT) and the levels of full-length E6 transcripts were measured and normalised to the levels of β-actin transcripts (Fig. 5.16). The highest levels of E6 transcripts were detected at 24 and 48 hours post-transfection. This could be because after this time-point, the majority of the HPV genomes may be lost from the cells, before their number is stabilised. I therefore decided that 48 hours post-transfection would be the appropriate time-point to assess the levels of E6 protein (as I have been doing thus far), as I considered that the 24-hour time-point might be too early for the detection of E6 protein.

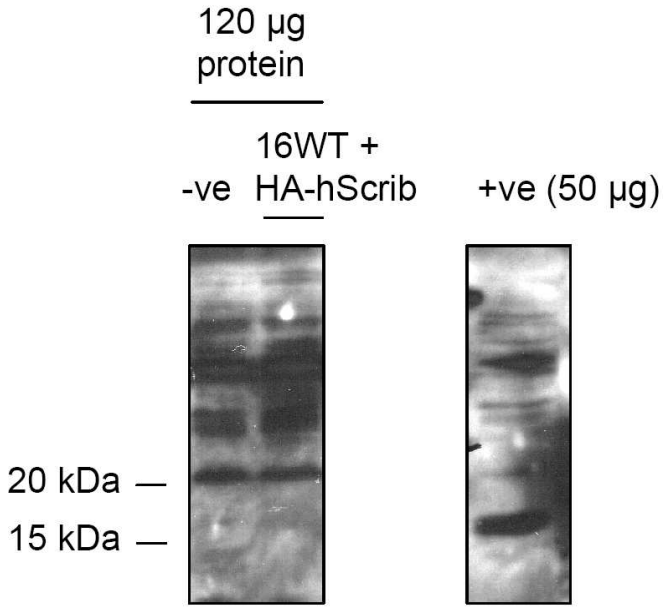


Figure 5.14: Attempts to detect E6 expressed from HPV16 genomes

NIKS cells were co-transfected with 16WT genomes and pcDNA-HA-hScrib plasmid and 120 µg of protein were loaded on a 15% gel. The blot was probed with anti-E6 antibody. The positive control is cell extract from cells transfected with pMV11-E6PDZ.

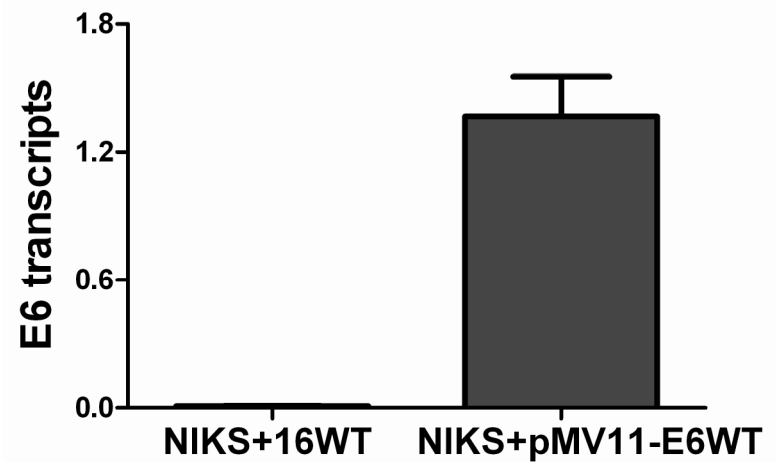


Figure 5.15: qPCR analysis of E6 transcripts from HPV16 genomes or pMV11 plasmids

NIKS cells were transiently transfected with 16WT genomes or pMV11-E6WT plasmids. RNA was extracted 48 h post-transfection and analysed by RT-qPCR. The transfections were carried out in duplicate and the bar chart shows the mean levels of full-length E6 transcripts (normalised to β-actin transcripts) and the standard deviation. The transfection was not carried out in an equimolar ratio. The values from the qPCR were adjusted so that the graph represents the values expected from the equimolar ratio.

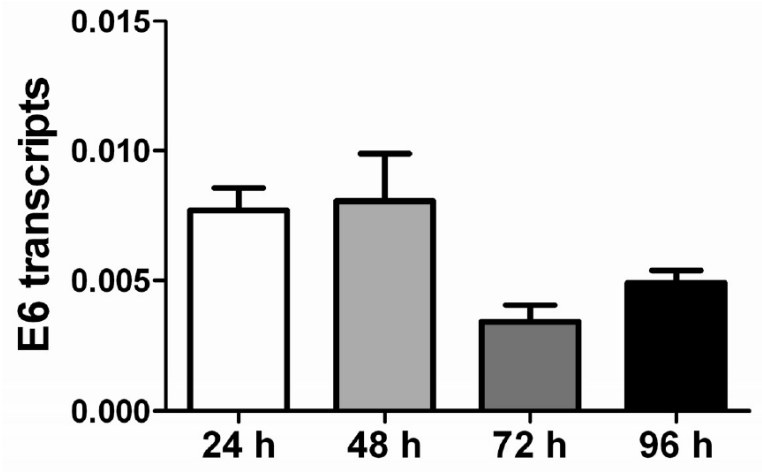


Figure 5.16: qPCR analysis of E6 transcripts from HPV16 genomes at 24-hour intervals post-transfection

NIKS cells were transfected with 16WT genomes and RNA was extracted at 24-hour intervals for 96 hours and analysed by RT-qPCR. The transfections were carried out in duplicate and the bar chart shows the mean levels of full-length E6 transcripts (normalised to β-actin transcripts) and the standard deviation.

5.2.11. Optimising the anti-E6 western blot

From the experiments described in the previous section, it became clear that in order to be able to detect E6 expressed from the HPV16 genomes, I would need to optimise the western blot to make it more sensitive in detecting much lower levels of E6 protein than had been necessary thus far. Loading higher levels of protein and using more sensitive ECL reagents resulted in blots with very high background and with multiple unspecific bands, including some in the 15-20 kDa range, which is where the E6 protein runs. Several things were tested in order to increase the sensitivity of the western blots whilst decreasing the background of the blots. These are outlined in Table 5.1.

Table 5.1: Outline of attempts to detect E6 expressed from HPV16 genomes by western blotting

Reduce background on the western blots	
More stringent washing of western blots; added 0.1% SDS and 0.25 M NaCl to washing buffer	No signal detected on the blot
Lower anti-E6 antibody concentration; tried dilutions of 1:2000 to 1:8000	Decreased overall background but still had unspecific bands in the 15-20 kDa region with high sensitivity ECL
Try to eliminate or shift the location of the unspecific bands	
Used different protein extraction buffers; used a urea buffer	Similar background as with RIPA 6% SDS buffer
Pre-adsorb the antibody with protein lysate from NIKS cells; pre-incubated the antibody solution with a blot that only had NIKS cell lysate on it, or added crude NIKS cell lysate together with the blot	Unspecific bands were still visible
Used gels with different compositions; used the 16% Novex® Tricine gels from Invitrogen, commonly used to separate low-molecular weight proteins	Unspecific bands were still visible

I also considered the possibility that the secondary antibody may be contributing to the unspecific bands. Incubating a membrane containing NIKS lysate with the GE Heatlhcare anti-mouse secondary antibody alone, I observed that even in the absence of primary antibody there is significant background on the blot, including in the 15-20 kDa region. To try to eliminate the background contributed from the secondary antibody, I repeated the experiment using a secondary anti-mouse antibody from Pierce instead. The background was similar as with the former secondary antibody.

5.2.12. Enriching the transfected cell population for E6-expressing cells

From the results presented above, it became clear that in order to be able to detect E6 protein that is expressed from the viral genomes after transient transfection of NIKS cells, I would need to enrich from the population of cells, those that were successfully transfected. To do this, I decided to co-transfect the HPV16 genomes with a GFP-expressing plasmid (pCI-EGFP) (Tuting *et al.*, 1999) and use Fluorescent Activated Cell Sorting (FACS) to sort cells on the basis of their GFP-expression. As co-transfected plasmids are thought to enter cells together, I reasoned that the GFP-positive cells would be cells that were also transfected with the HPV16 genomes. Therefore the sorted population of cells would be enriched not only for GFP-positive cells but also for E6-expresssing cells.

To test this, and to determine if I could indeed detect E6 in these cells by western blotting, I first carried out an experiment in which only the expression of E6 from the 16WT genomes was assessed. Cells were co-transfected with 16WT genomes and the pCI-EGFP plasmid. Alternatively, cells were transfected with the pMV10 plasmid to be used for setting up the GFP-negative gate for the sorting. A subset of the 16WT+pCI-EGFP-transfected cells was used to set the GFP-positive gate (Fig. 5.17A). The cells were sorted 48 hours post-transfection and the sorting was carried out by Graham Preece at the Flow Cytometry Facility at the NIMR. Both GFP-negative and GFP-positive cells were collected, pelleted, washed once with PBS, pelleted again and the pellets frozen at -80^{o}C.

For the detection of E6 potein, whole-cell extracts were prepared, quantified and used for western blotting (Fig. 5.17B). Un-transfected cells were used as a negative control for E6 and cells transfected with pMV11-E6WT and pCI-EGFP plasmids were used as a positive control. As can be seen, the GFP-positive sorted population had very high levels of GFP whereas the GFP-negative sorted population had undetectable levels of GFP, thus confirming that the sorting enriched the population for GFP-positive cells. More importantly, the E6 band was detectable in the GFP-positive cells and no bands were detected in the un-transfected cells in the 15-20 kDa region of the gel, thereby suggesting that the enrichment for GFP-positive cells worked in enriching for E6-expressing cells as well.

This result suggests that this type of experiment could be used to compare the levels of E6WT and E6PDZ proteins expressed from the HPV16 genomes. In order to improve the quality of the blot, in the following experiment the important samples to be analysed were loaded with a separating lane in between, to avoid any interference between signals from different samples. In addition, the fact that the E6 band in the GFP-positive sample was very faint suggested that more sample may need to be loaded on the gel. Hence in the following experiment I transfected a larger number of cells in order to obtain a higher concentration of enriched protein lysate, and consequently load more protein on the gel.

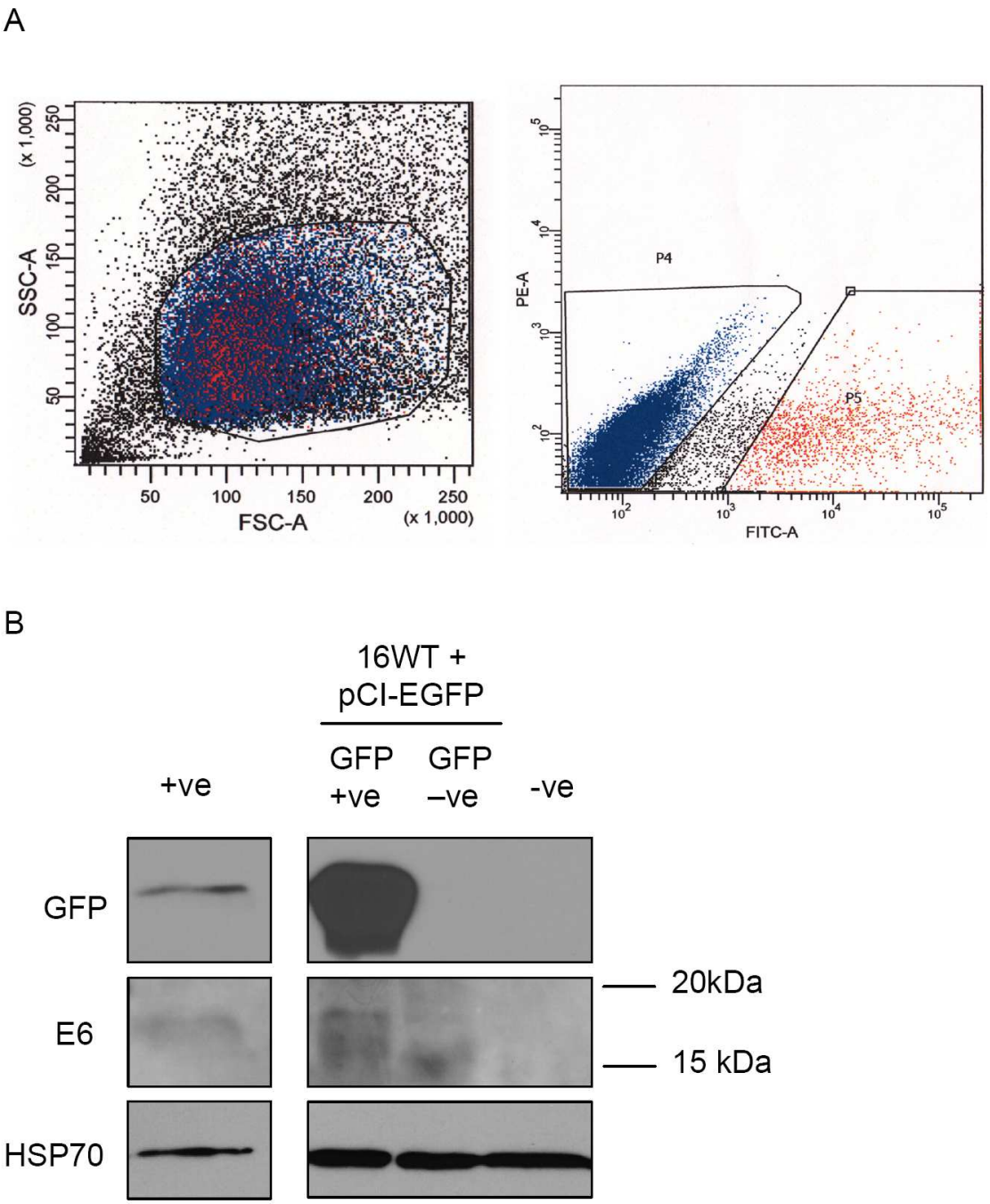


Figure 5.17: Sorted cells are enriched for E6-expressing cells

NIKS cells were transiently co-transfected with 16WT genomes and pCI-EGFP plasmid and were subjected to FACS 48 h post-transfection, to sort on the basis of GFP-expression. A) The left panel shows the Forward and Side Scatter (FSC/SSC) set-up. The right panel shows the FITC set-up. P5 was the GFP-positive gate and P4 was the GFP-negative gate. B) Whole-cell extracts from FACS-sorted GFP-positive and GFP-negative cells were analysed by western blot for GFP and E6 expression. HSP70 was used as a loading control.

5.2.13. E6WT protein is more stable than E6PDZ protein when these are expressed from HPV16 genomes

Having confirmed that the sorting method was able to enrich the population of transfected cells for E6-expressing cells, I wanted to use this method to investigate the relative stability of the wild-type and mutant E6 proteins when these are expressed from their respective HPV16 genomes.

To do this I co-transfected NIKS cells with re-circularised 16WT or 16E6PDZ genomes, and pCI-EGFP plasmid. Thirty-five wells of a 6-well plate were transfected with each of the genomes and pCI-EGFP. The sorting procedure was carried out as described above. Whole-cell extracts were obtained, quantified and used for western blotting (Fig. 5.18A). The sorting procedure was again able to enrich for E6-expressing cells, as both E6WT and E6PDZ proteins were detectable on this gel, with no interfering background bands in the negative controls. Most importantly however, the levels of E6WT protein were much higher than those of E6PDZ protein. To ensure that the difference in the protein levels was not due to a difference in the expression of E6 transcripts, these were assessed by RT-qPCR (Fig. 5.18B) and were found not to be significantly different ($p=0.33$).

As no exogenous PDZ proteins were expressed in this experiment, the data suggest that the higher levels of E6WT compared to E6PDZ protein are due to the stabilisation of the wild-type protein by endogenous PDZ proteins. This is of particular importance as it confirms that the results I have presented so far, using an over-expression system, also hold true for the endogenous system, in which PDZ proteins and E6 are expressed at more physiological levels.

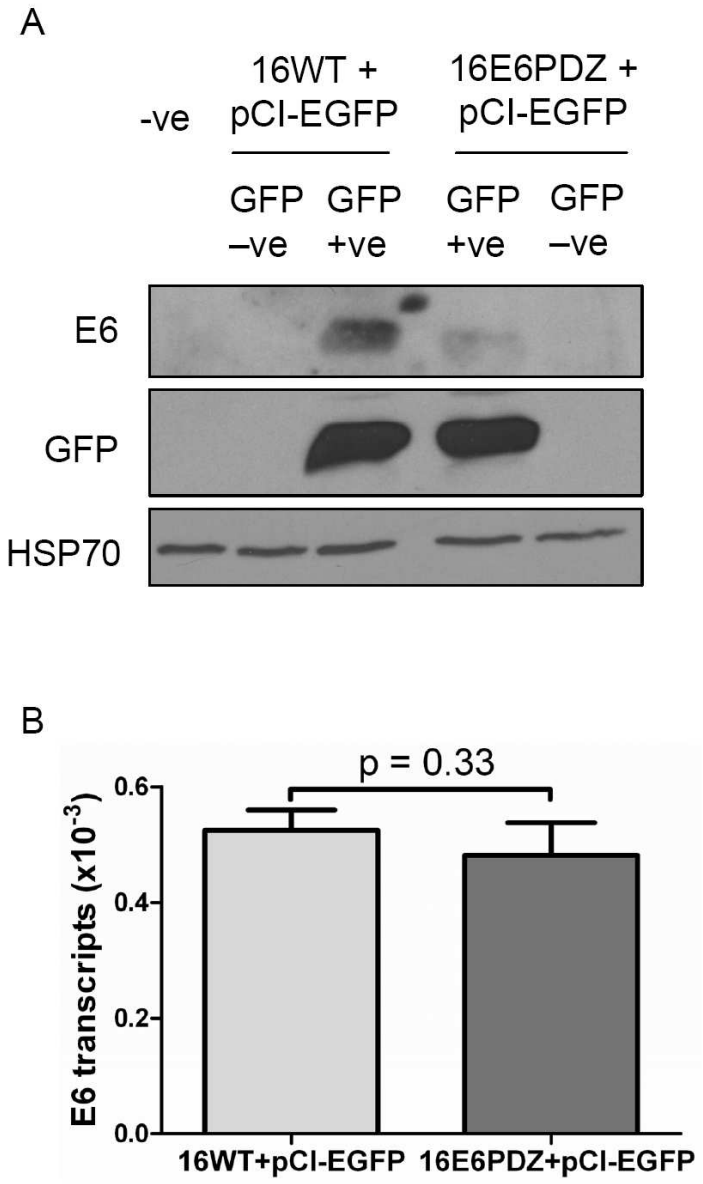


Figure 5.18: The levels of E6WT protein are higher than those of E6PDZ protein when expressed from the HPV16 genomes

NIKS cells were transiently co-transfected with 16WT or 16E6PDZ genomes and pCI-EGFP plasmid. A) At 48 h post-transfection the cells were subjected to FACS to sort on the basis of GFP-expression. Whole-cell extracts from the sorted GFP-positive and GFP-negative cells were analysed by western blotting for GFP and E6 expression. HSP70 was used as a loading control. B) RNA was extracted from the total cell population at 48 h post-transfection and analysed by RT-qPCR. The transfection was carried out in triplicate and the bar chart shows the mean levels of full-length E6 transcripts (normalised to β-actin transcripts) and the standard deviation. An unpaired t-test was used to compare the levels of E6 transcripts and the p value is shown above the graph.

5.2.14. Levels of p53 are similarly diminished in NIKS cells transfected with 16WT or 16E6PDZ genomes

I have shown so far that the E6PDZ protein is unstable compared to the E6WT protein when these are expressed from heterologous promoters or from the HPV promoter. This is important when considered in the context of my earlier findings, as it would suggest that the reason why the 16E6PDZ genomes were unable to persist (as presented in Chapter 3) may be because the levels of E6 protein in these cells were insufficient. In Chapter 3 I also presented data that suggested that E6-mediated p53-degradation is necessary for viral genome persistence. Therefore, I hypothesised that the reason why the 16E6PDZ genomes were unable to persist may be because the E6PDZ protein was unable to efficiently degrade p53.

I was thus interested to determine whether the levels of p53 were higher in cells transfected with 16E6PDZ genomes compared to cells transfected with 16E6WT genomes. To do this, I transfected cells with 16WT or 16E6PDZ genomes or pMV10 plasmid, and the pCI-EGFP plasmid, and FACS-sorted them as described above. Whole-cell extracts from GFP-positive cells were analysed for the levels of p53 protein (Fig. 5.19A). Cells transfected with 16WT genomes had lower levels of p53 protein than cells transfected with pMV10 plasmid. Interestingly, the levels of p53 protein in cells transfected with 16E6PDZ genomes were similar to those in cells transfected with 16WT genomes. The bar graph in Figure 5.19A shows the intensities of the p53 bands (normalised to the loading control). p53 levels were also measured in the samples presented in section 5.2.13. The average of the two experiments is presented in Figure 5.19B.

I also wanted to determine whether E6 had any effect on endogenous hScrib at this early time-point post-transfection. I therefore analysed the levels of hScrib in the transfected and FACS-sorted cells (Fig. 5.19C). The levels of hScrib were found not to be different between the cells that had been transfected with pMV10 plasmid or either of the HPV16 genomes. This suggests that the E6 protein, even the wild-type, had no effect on the levels of endogenous hScrib protein at this early time-point.

In conclusion, despite the difference in their stabilities, E6WT and E6PDZ proteins are able to degrade p53 protein with similar efficiencies. Neither however seems to have had an effect on endogenous hScrib protein in the time-frame in which the testing was carried out.

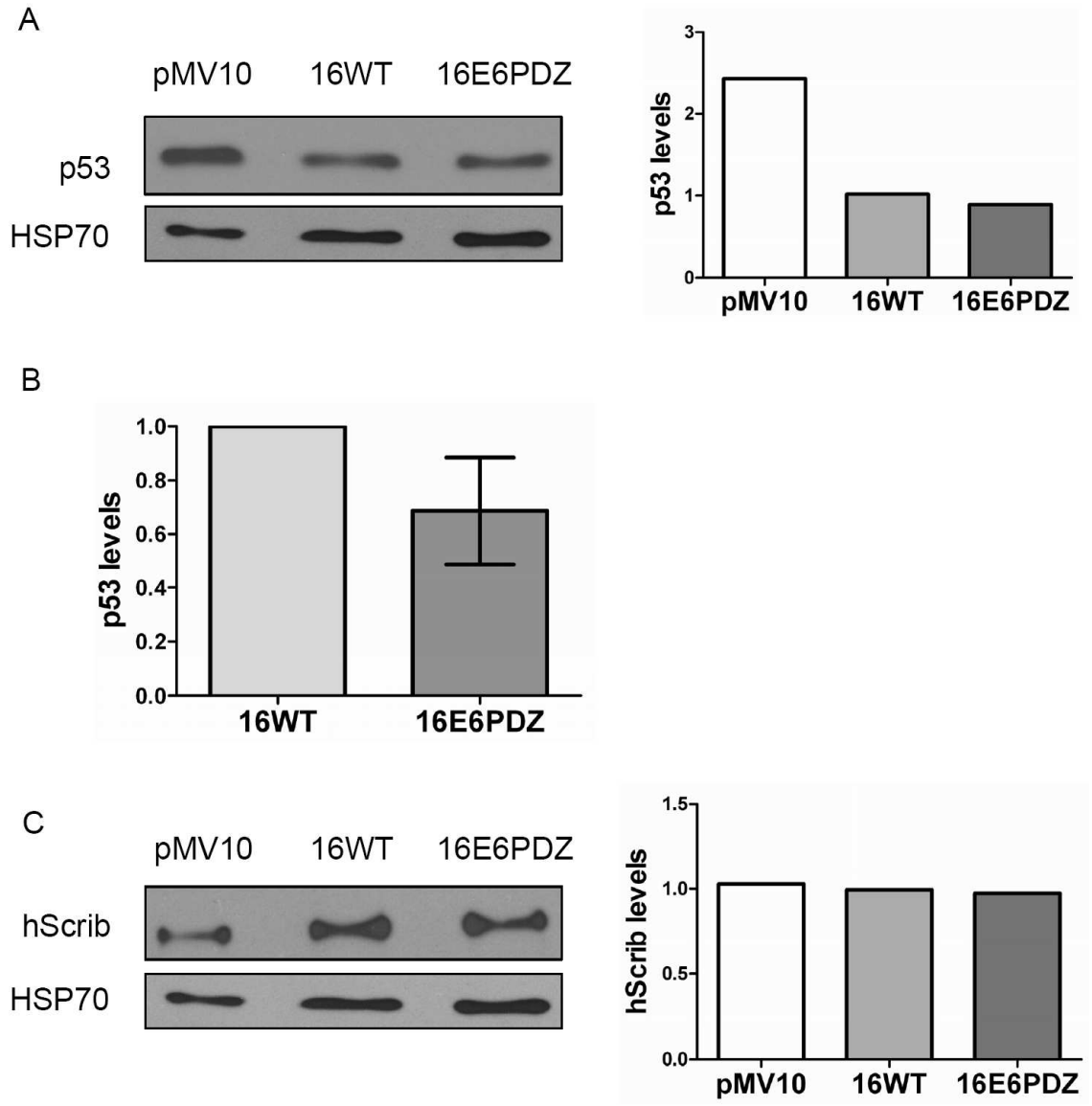


Figure 5.19: p53 and hScrib levels in transfected and FACS-sorted cells

NIKS cells were transiently co-transfected with 16WT or 16E6PDZ genomes or pMV10 plasmid, and pCI-EGFP plasmid, and subjected to FACS 48 h post-transfection, to sort on the basis of GFP-expression. Whole-cell extracts from the sorted GFP-positive cells were analysed by western blotting for A) p53 and C) hScrib. The levels of p53 and hScrib were measured using ImageJ software and normalised to the loading controls and are shown in the bar charts (in arbitrary units). HSP70 was used as a loading control. B) The levels of p53 in cells transfected with 16WT or 16E6PDZ genomes were measured in two experiments. The bar chart shows the mean levels of p53 in cells transfected with 16E6PDZ genomes (in arbitrary units), normalised to the levels of p53 in cells transfected with16WT genomes, and the range.

5.3. Discussion

The experiments presented in this chapter have conclusively shown that the HPV16 E6 protein is stabilised by way of its interaction with the PDZ protein hScrib. This stabilisation was found to be dependent on the PDZ-binding motif of the E6 protein as well as the PDZ domains on the hScrib protein. The resulting stabilisation of the E6 protein is brought about by the protection of the E6 protein from proteasomal degradation. My data also show that this stabilisation of the E6 protein can be afforded not only by hScrib but also by other PDZ proteins, namely hDlg and MAGI. Only hDlg and MAGI were investigated in this study and it would be interesting to determine if other PDZ proteins have a similar effect, as well as whether this effect is true for E6 proteins from other high-risk types. Different levels of E6 protein stabilisation were observed with the different PDZ proteins. This supports previous studies that showed that E6 proteins from different HPV types target PDZ proteins with varying efficiencies (Thomas *et al.*, 2005).

Importantly, in this study I was able to demonstrate that the stabilisation of E6, observed when E6 and PDZ proteins are over-expressed from heterologous promoters, also holds true when E6 is expressed from the HPV16 promoter and the PDZ proteins from their endogenous promoters. This is significant as it shows that E6 is stabilised by PDZ proteins under physiological conditions. The difficulty in carrying out these experiments has been highlighted in this chapter and is also reflected by the fact that previous studies have used tags and over-expression systems in order to study the E6 protein. To the best of my knowledge, this is the first time that the levels of un-tagged E6 protein have been analysed by western blotting following the transient transfection of keratinocytes and prior to any antibiotic selection processes or prolonged cell growth. This is important, as I have already shown in Chapter 3 that prolonged growth of cells transfected with mutant HPV genomes may result in loss of the genomes, which would inevitably influence the results. Furthermore, prolonged cell growth may inadvertently select for cells that express higher levels of E6 protein, as these are expected to have a growth advantage over other cells in the population. This selection may mask the differences in the levels of wild-type and mutant E6 protein.

The finding that PDZ proteins stabilise wild-type E6 suggests a way in which the PDZ-binding motif of E6 may be involved in viral episomal persistence. Its role may be to stabilise enough E6 protein, to facilitate the E6 activities that are necessary for persistence. One obvious activity is p53-degradation, the need for which in persistence was shown in Chapter 3. The lack of a PDZ-binding motif may mean that insufficient E6 protein is present to efficiently degrade p53. If this were the case, the levels of p53 would be expected to be higher in E6PDZ-expressing cells than in E6WT-expressing cells. However, my experiments suggest that this is not the case, as the levels of p53 were found to be similarly reduced in cells transfected with 16WT or 16E6PDZ genomes. This had also been suggested in a previous study, in which an E6 mutant that is deficient for PDZ-binding (6 amino acid deletion) was able to induce efficient degradation of p53 (Foster *et al.*, 1994). A more recent study also showed that a similar E6 mutant affected the cellular response to DNA damage in a comparable way as the wild-type protein, thus again implying similar reduction in p53 levels in cells expressing wild-type or mutant E6 (Nguyen *et al.*, 2003). However, in both of these studies the E6 protein was over-expressed in cells in the absence of exogenous expression of hScrib (or other PDZ proteins) and the levels of the E6 protein were not assessed. Therefore the levels of wild-type and mutant E6 proteins in these studies may not actually have been different. My results on the other hand show that the efficiency of p53-degradation is similar when E6 is expressed from the wild-type or mutant HPV16 genomes, even when the steady-state levels of E6WT protein are clearly higher than those of E6PDZ protein.

As insufficient p53-degradation does not appear to be the reason why the 16E6PDZ genomes do not persist, this suggests that other activities of E6, apart from p53-degradation must also be necessary. These activities may be compromised by the instability of the E6PDZ protein, consequently resulting in the lack of viral episomal persistence seen with the 16E6PDZ mutant genomes. One such activity which has recently been shown to be necessary for persistence is the ability of E6 to bind to and degrade E6TP1 (Lee *et al.*, 2007). It is therefore possible that the E6PDZ mutant protein is unable to degrade E6TP1 efficiently, due to its lower levels. Moreover, the *Lee et al*. study suggested that the localisation of E6 to the nucleus may also be important for

viral episomal persistence (Lee *et al.*, 2007). An earlier study identified three nuclear localisation signals on the HPV16 E6 protein, none of which overlapped with the PDZ-binding motif (Tao *et al.*, 2003). However, it is possible that deletion of the PDZ-binding motif disrupts an as yet unidentified nuclear localisation signal of E6 or induces a conformational change in E6 that prevents its localisation to the nucleus, thereby inhibiting episomal persistence. More ways in which the levels of E6 protein may impact persistence will be discussed later. It is important to note however, that my results on the instability of the E6PDZ protein do not preclude the possibility that the degradation of PDZ proteins is also necessary for viral episomal persistence, in addition to the stability of E6.

It is also of interest that hScrib, which has previously been shown to be degraded by E6 (Nakagawa & Huibregtse, 2000), is also able to stabilise it. This apparent paradox will be further discussed later. As mentioned earlier, this is not a novel concept as previous studies have also shown a similar relationship between E6 and E6AP. The E6AP protein, which is targeted by E6 for degradation (Kao *et al.*, 2000) also stabilises the E6 protein (Tomaic *et al.*, 2009b). These observations raise interesting questions as to whether E6AP is directly necessary for certain activities of E6 (such as for p53-degradation) or whether it is needed primarily to stabilise sufficient amounts of E6. Similarly, my data indicate that care must be taken when working with E6 mutants that lack the PDZ-binding motif, as results could potentially be affected by the lower levels of E6 protein instead of by changes in the levels of PDZ proteins. This could also be true when looking retrospectively at studies in which such E6 mutants were used. For example, the binding of E6 to PDZ proteins has been attributed roles in cell growth as well as in tissue hyperproliferation (Lee & Laimins, 2004). It is possible however that these effects were not due to the impact of E6 on PDZ proteins but rather due to the impact of the PDZ proteins on the stability of E6. Similarly, the reduced hyperplasia observed in mice that express PDZ-binding mutant E6 protein compared to ones that express wild-type E6 protein, might also be explained by the instability of the mutant E6 protein (Nguyen *et al.*, 2003). Future studies using such mutants would benefit from a thorough investigation of the levels of E6 prior to attributing any effects to the degradation of PDZ proteins.

Chapter 6: Final Discussion

6.1. Two regions of the E6 ORF are necessary for episomal persistence of HPVs

Previous studies have suggested a role for E6 in the persistence of HPV episomes (Thomas *et al.*, 1999; and Ken Raj, unpublished data). In this study my aim was to build on these observations and investigate what the role of HPV16 E6 is in this function. My results show that two regions of the E6 protein are necessary for the episomal persistence of HPV16 DNA. The first region has been associated with the degradation of p53 (Kiyono *et al.*, 1998; Klingelhutz *et al.*, 1996), whereas the second region is the PDZ-binding motif of the E6 protein, which is found at the C-terminus of the protein (Kiyono *et al.*, 1997; Lee *et al.*, 1997; Nakagawa & Huibregtse, 2000). I show that HPV16 genomes that harbour mutations in these regions are unable to persist in NIKS cells.

Previous studies have also investigated the ability of mutant genomes (with mutations in the E6 ORF) to persist in cells (Lee & Laimins, 2004; Park & Androphy, 2002; Thomas *et al.*, 1999). These had various unresolved issues, which I took care to address in my study. Firstly, whereas the previous studies only used Southern blotting to assess persistence, I also assessed it by qPCR, by measuring the number of HPV copies per cell in successive passages. I believe that the qPCR analysis provides higher resolution, as it allows me to determine smaller and quantitative differences between samples, and to analyse earlier time-points post-transfection as it requires significantly lower amounts of sample than Southern blotting.

Secondly, I took care to prevent other factors from interfering with the results. One such factor is the potential growth competition that may exist in a population of cells that was transfected with mutant HPV16 genomes. As discussed in Chapter 3, I considered the possibility that cells harbouring the 16E6p53m or 16E6PDZ mutant genomes may have a growth disadvantage compared to cells that do not contain any viral genomes, and may be outgrown. This would result in an apparent loss of mutant viral genomes from cells.The

experimental approach was designed in a way that would minimise this possibility, in that I isolated individual cells soon after transfection (thereby removing them from the growth competition), and analysed the resulting clones for the presence of HPV DNA. Moreover, I directly assessed potential growth differences between cells that express mutant E6 protein (together with E7 protein) compared to control cells. All of the above led me to conclude that the reason why the mutant genomes are unable to persist in NIKS cells is not due to a growth disadvantage of the cells that harbour them.

Thirdly, unlike the previous studies, I carried out the persistence assays in an immortalised cell line (NIKS) (Allen-Hoffmann *et al.*, 2000), which supports the HPV life-cycle (Flores *et al.*, 1999). The decision to use immortalised cells instead of primary cells was made because some mutant genomes may be unable to immortalise keratinocytes upon transfection (as discussed in Thomas et al., 1999). The limited life-span of primary cells would thus prevent long-term persistence of the viral DNA and may even interfere with short-term persistence. By using the NIKS cells, I hoped to study factors that solely affect episomal persistence, without interference from other factors that may be required for cellular immortalisation. A further advantage of NIKS cells over primary cells is that they provide an isogenic background for all the experiments.

In conclusion, I have shown that two regions of the E6 protein are necessary for the episomal persistence of HPV16 DNA. As discussed in Chapter 3 however, I have not conclusively shown that the activities of E6 that have been associated with these regions of the protein (i.e. p53-degradation and binding/degradation of PDZ proteins) are necessary for persistence. An experiment aimed at determining whether p53-degradation was indeed necessary for viral DNA persistence was presented in Chapter 4 (using shRNA against p53). While this experiment yielded unexpected and interesting results, it did not prove to be suitable for answering the original question. More experiments to this end will be discussed in section 6.7.1.

6.2. 16E6p53m and 16E6PDZ genomes: two different persistence-defective phenotypes

The aims of this study were not only to investigate which activities of E6 are necessary for viral persistence, but also to determine why they are so. To this end, I set out to investigate what persistence mechanisms may be affected by the mutations I introduced into the HPV16 genome.

My experiments show that the replication efficiency of the 16E6p53m genomes is significantly lower than that of the 16WT genomes. Although this result does not conclusively show that inefficient replication is the reason why the 16E6p53m genomes are unable to persist, it suggests that this may be a contributing factor. This was not found to be the case for the 16E6PDZ genomes, which replicate at a similar efficiency to the 16WT genomes.

My results also show that the E6PDZ mutant protein is unstable, being more susceptible to proteasomal degradation than the E6WT protein. Consequently its steady-state levels are significantly lower than those of the E6WT protein. This is not the case for the E6p53m protein. This suggests that the mechanism by which the PDZ-binding motif of E6 is involved in persistence may be through stabilisation of the E6 protein, rather than through regulation of the levels of the PDZ proteins.

In observing the lower levels of the E6PDZ protein, compared to the E6WT protein, my initial hypothesis was that degradation of p53 by the E6PDZ protein may be inefficient. This could explain the lack of persistence of the 16E6PDZ mutant genomes. However, my results show that, in fact, p53 is degraded at similar efficiencies in cells expressing E6WT and E6PDZ proteins, despite the much reduced levels of the E6PDZ protein, suggesting that this is not the reason why the 16E6PDZ genomes cannot persist. This was in support of the observation that the two genomes behave differently in the transient replication assay.

Moreover, close analysis of the results from Chapter 3 hint at the existence of two different persistence-defective phenotypes. Cells containing 16E6p53m genomes exhibit gradual loss of the viral genomes. Cells containing 16E6PDZ

genomes exhibit either complete loss of the viral DNA, or loss of the viral episomes and integration of the viral DNA into the cellular DNA at high copies. All of the above suggest that the E6 protein is involved in at least two processes that function in permitting the extrachromosomal persistence of the viral DNA during successive cell divisions.

6.3. E6PDZ protein is less stable than E6WT protein in the endogenous system

A significant aspect of this study was that I was able to demonstrate the instability of the E6PDZ protein compared to the E6WT protein, both in an over-expression system, but more importantly in the endogenous system, when these proteins are expressed from the HPV genome. These results suggest that endogenous PDZ proteins can stabilise the E6WT protein during a productive infection and emphasise the fact that future studies should take care to assess the levels of E6 in their experiments, when using mutants of the E6 protein. For example, phenotypes that have so far been attributed to the binding/degradation of PDZ proteins by E6 may in fact be due to the instability of the E6PDZ protein. The importance of these results is also highlighted by the difficulty in obtaining them, as described in Chapter 5.

In addition to the importance of studying the stability of E6 in the endogenous system, an interesting observation was also made with regards to the detection of different E6 species. Although not discussed in the results chapters, several of the E6 western blots shown in this study suggest the existence of different E6 species, as determined by the detection of more than one specific band by the anti-E6 antibody. The antibody used in this study is unable to detect the known E6* splice variants, as it recognises an epitope in the C-terminus of E6. We thus speculate that the alternative E6 species detected in these blots could either be as-yet-unidentified splice variants, or full-length E6 protein that has undergone post-translational modification (for example, phosphorylation). Post-translational modifications of E6 have not been well studied. It would thus be interesting to address the presence of these E6 variants in future studies and attempt to characterise them and to determine whether they are differentially stabilised by interaction with PDZ proteins.

6.4. What other activities of E6 may be necessary for persistence?

The lower steady-state levels of the E6PDZ protein may have implications on the ability of this mutant protein to carry out its activities. In Chapter 5 I discussed an activity of E6, that of binding to and degrading E6TP1 (Gao *et al.*, 1999), that has already been linked to viral DNA persistence (Lee *et al.*, 2007). This activity may be compromised by the lower levels of E6PDZ protein. However, there may be other activities of E6 that are also important but have not yet been identified as being necessary for persistence. These may also be compromised by the low levels of E6PDZ protein. Some of these are highlighted below.

6.4.1. Interaction of E6 with E2

A recent study has shown interplay between the E6 protein and the viral replication and transcription protein E2 (Grm *et al.*, 2005). The two proteins were shown to affect each other's cellular distribution and activities. For example E6 augments E2-mediated viral gene transcription and inhibits E2-mediated viral DNA replication (Grm *et al.*, 2005). Lower levels of the E6PDZ protein may interfere with the regulation of these pathways, potentially leading to lower transcription of other viral proteins, and/or uncontrolled replication which may consequently result in loss of episomes or integration.

E2 has also been shown to interact with topoisomerase II-binding protein 1 (TopBP1) (Boner *et al.*, 2002). Topoisomerase II (Topo II) is involved in decatenation of DNA following replication, including viral DNA (Snapka *et al.*, 1988), and it is possible that Topo II is also required for the decatenation of HPV episomes. This may be mediated by an interaction between Topo II, TopBP1 and E2, and E6 may also play a role by way of its interaction with E2. If the above is true, then lower levels of E6PDZ protein may fail to elicit the decatenation of HPV episomes. This could affect the equal segregation of the genomes into the two daughter cells or result in the integration of multiple copies of HPV DNA (as concatamers) in the cellular chromosomes.

Moreover, E2 is the only HPV protein that has so far been attributed a role in episomal segregation (Abbate *et al.*, 2006; Bastien & McBride, 2000; Ilves *et al.*,

1999; Lehman & Botchan, 1998; Oliveira *et al.*, 2006; Skiadopoulos & McBride, 1998; Van Tine *et al.*, 2004). It is conceivable that the interaction between E6 and E2 may facilitate this process, suggesting that lower levels of E6PDZ may fail to elicit proper segregation of viral episomes, thus preventing their persistence. Moreover, several cellular proteins have been implicated in episomal segregation, such as Brd4 (Abbate *et al.*, 2006; Baxter *et al.*, 2005; You *et al.*, 2004; You *et al.*, 2005), Chlr1 (Parish *et al.*, 2006a) and TopBP1 (Donaldson *et al.*, 2007). It is plausible that E6 may affect the expression of these proteins (as it does with many other cellular proteins). Alternatively, E6 may mediate their interaction with the cellular chromosomes and with the episome-bound E2. For example, E6 may be a co-factor of segregation by facilitating the already demonstrated interaction between E2 and TopBP1 (Boner *et al.*, 2002). It is worth noting however, that most work on episomal segregation has been carried out using BPV1 and studies suggest that variations exist between the different PVs (McPhillips *et al.*, 2006; Oliveira *et al.*, 2006). The mechanism employed by HPV16 has not yet been elucidated.

6.4.2. E6 and replication

In the transient replication assay the 16E6PDZ mutant genomes were not found to replicate significantly less efficiently than the 16WT genomes. However, the possibility still exists that even minor or statistically insignificant differences in replication efficiency may have a biological effect on the ability of the mutant genomes to persist. Moreover, the transient replication assay was a short-term assay, carried out over the course of 4 days. It is therefore possible that subtle differences in the replication efficiencies of the 16WT and 16E6PDZ genomes do exist but were not identified by this assay. Hence, possible ways in which the levels of E6 may impact viral DNA replication, and consequently persistence, should be considered.

One cellular pathway that has been shown to inhibit viral DNA replication is the IFN- signalling pathway, which inhibits the replication of both HBV (Hayashi & Koike, 1989) and HPV (Terenzi *et al.*, 2008). HPVs down-regulate the expression of IFN-inducible genes, such as Stat-1 (Chang & Laimins, 2000), and this is at least in part due to the activities of E6 (Li *et al.*, 1999; Nees *et al.*,

2001). It is therefore plausible that E6 functions to reduce the IFN-mediated inhibition of viral DNA replication. If the levels of E6 protein are lower, as is the case with the E6PDZ mutant protein, the levels of IFN-inducible proteins may increase, thereby impeding viral DNA replication, and resulting in the loss of viral episomes, as seen with the transfection of 16E6PDZ mutant genomes. Interestingly, several studies have looked at the effect of IFN-treatment on BPV- and HPV-positive cells (Chang *et al.*, 2002; Herdman *et al.*, 2006; Turek *et al.*, 1982). In all studies, treatment with IFN resulted in loss of viral episomes. Importantly, Herdman *et al*. also reported an increase in HPV integrants following IFN-treatment of HPV16-positive cells (Herdman *et al.*, 2006).

Another replication-related protein that is regulated by E6 is minichromosome maintenance 7 (MCM7), which has a role in ensuring that DNA only replicates once per cell-cycle (reviewed in Chong *et al.*, 1996). It has been suggested that E6 degrades MCM7 by interaction with E6AP (Kuhne & Banks, 1998). HPVs rely on cellular replication machinery to replicate their DNA and it is likely that the activities of MCM7 will have an effect on HPV DNA replication as well. It is therefore reasonable to consider that E6-mediated degradation of MCM7 may be necessary to allow amplificational/establishment replication to take place. The lower levels of E6PDZ protein may impinge on this.

6.4.3. E6 and DNA damage

A recent report identified an interaction between the tight junction protein Par3 (partitioning-defective 3) and two of the subunits of the DNA-PK complex (involved in double-strand break repair), Ku70 and Ku80, and demonstrated a role for Par3 in this pathway (Fang *et al.*, 2007). Par3 is a PDZ protein and has been shown to interact with the HPV18 E6 protein, as well as the Rhesus papillomavirus type 1 E7 protein, which also has a PDZ-binding motif (Tomaic *et al.*, 2009a). Interestingly, Ku80 has been suggested to play a role in the persistence of extrachromosomal DNA in cells, as transfected plasmids are rapidly lost in cells that lack the Ku80 protein (Liang & Jasin, 1996). Moreover, depletion of Ku70 in episomal HPV16-positive cells was shown to promote episome loss and generation of integrants (Winder *et al.*, 2007). The interaction of E6 with Par3, and Par3's interaction with Ku70 and Ku80 may therefore play

a role in Ku70/80-mediated stabilisation of extrachromosomal DNA, thereby also supporting the persistence of viral episomal DNA.

6.4.4. E6 and the immune response

In addition to down-regulating IFN-inducible genes (see section 6.4.2.), E6 also interferes with the immune system by down-regulating the expression of E-cadherin (Matthews *et al.*, 2003). Potential consequences of this would be reduced recognition of HPV-positive cells by the immune system, lack of clearance, and consequent persistence of HPV DNA in cells. Although this would be relevant in the persistence of HPV DNA in infected tissues, where an immune system is present, it cannot explain the loss of episomes in cultured cells in the absence of an immune system, as have been used in this study.

6.4.5. E6* splice variants

As mentioned earlier, high-risk E6 ORFs contain an intron, the excision of which has been shown to generate several alternatively spliced E6 transcripts (Zheng & Baker, 2006) collectively referred to as E6*. In addition to the alternatively spliced transcripts, an E6* protein product has also been identified, the function of which remains unclear. In the study presented here, the role of individual E6 protein species (full-length or spliced) in persistence was not assessed. It would thus be important to determine whether the full-length and splice variants of the E6 protein have different roles in persistence, as this could potentially identify a role for E6* in the viral life-cycle. Such studies would however involve the mutation of the splice donor and acceptor sites within the E6 ORF, which could interfere with the splicing of other transcripts and therefore the expression of other viral proteins. In fact, one study did try to investigate the role of the E6 intron in the persistence of HPV31 genomes (Thomas *et al.*, 1999). The authors reported that HPV31 splice-donor or splice-acceptor mutant genomes were replication-defective, and demonstrated that this was due to disruption of the expression of E1 and E2 replication proteins.

6.5. E6 cannot be expressed prior to entry of HPV DNA into cells

One of the more unexpected results obtained from this study is that the E6 protein, although necessary for persistence, is detrimental to it if expressed prior to the entry of HPV DNA into cells, or at abnormally high levels. The experiments using cell lines that constitutively express E6 (LXSN-E6WT cells) were carried out in order to establish if a *cis*- or *trans*-acting factor may contribute to the inability of the 16E6p53m and 16E6PDZ mutant genomes to persist. However, the results from the experiments show that not even the wild-type genomes can persist in LXSN-E6WT cells, and suggest an inhibitory role for E6 in persistence when it is expressed at this early stage.

The reason for this inhibitory effect is unknown. p53 has been shown to inhibit amplificational/establishment replication (Ilves *et al.*, 2003; Lepik *et al.*, 1998). We could thus speculate that the levels of p53 need to be kept above a certain threshold, so as to prevent uncontrolled replication. This control on replication would be beneficial to the virus, as it would ensure that the virus does not harm the cell, causing cell death and ending the viral life-cycle. The involvement of p53 in the inhibition of viral DNA persistence is supported by the observation that 16WT genomes cannot persist in NIKS-shp53 cells either. Importantly, these results could have significant implications when considering whether HPV virions can infect cells that already harbour HPV DNA (“super-infection”).

Moreover, if very low levels of p53 are detrimental to episomal persistence, this may also be true in high-grade lesions. As the lesions become more severe, the levels of p53 decrease (Kurvinen *et al.*, 1996). This could lead to an environment that is not conducive to episomal persistence, and could promote integration of the viral DNA into cellular chromosomes, as is often seen in cancers.

6.6. E6 and PDZ proteins: Degradation or stabilisation?

An interesting question that arises from my results is how PDZ proteins, which are degraded by wild-type E6 protein (Gardiol *et al.*, 1999; Glaunsinger *et al.*, 2000; Nakagawa & Huibregtse, 2000; Thomas *et al.*, 2002), are also able to stabilise it. As discussed in Chapter 5, this apparent paradox is not a novel

concept, as a similar relationship has already been shown for E6 and E6AP (Kao *et al.*, 2000; Tomaic *et al.*, 2009b). Although, the mechanism of how this degradation and stabilisation is regulated remains unknown, the results imply that the relationship of E6 and its binding partners is complex.

One aspect of this question that should be addressed is how does the PDZ-binding motif of E6 confer stability on the E6 protein? As mentioned in Chapter 5, the stabilisation of PDZ-binding proteins via interaction with PDZ proteins has been shown before, with MAGI-2 stabilising PTEN (Valiente *et al.*, 2005; Wu *et al.*, 2000b) and the *Drosophila* PDZ protein InaD stabilising some of its binding partners (Tsunoda *et al.*, 1997). This suggests that a common mechanism may exist.

As the mechanism is not known we are free to speculate as to how this is brought about. PDZ proteins are generally large proteins (hScrib is >200 kDa) that are often found as part of even larger, multi-protein complexes (Thomas *et al.*, 2008). The E6 protein on the other hand is small (~15 kDa). It is therefore possible that by binding to PDZ proteins, E6 finds itself stabilised as part of a large structure. Moreover, the complexes that the PDZ proteins are often found in form the adherens or tight junctions (Thomas *et al.*, 2008) at the cell membrane. Re-localisation of E6 to these sites, upon binding to PDZ proteins, may make it less accessible to the proteasome. Binding to PDZ proteins could also change the structure of the E6 protein, making it more stable. In addition to interacting with cellular proteins, E6 has been shown to form stable oligomers (Garcia-Alai *et al.*, 2007). It may be that oligomerisation is somehow mediated by binding to PDZ proteins.

In its discussion, the Tomaic *et al*. report suggested that the binding of E6AP to E6 may conceal the ubiquitination sites of E6 (Tomaic *et al.*, 2009b), thereby making E6 refractive to proteasomal degradation. This could also be true for the interaction of PDZ proteins with E6. Other possibilities include mediating the binding of E6 to the deubiquitylating enzyme USP15, which was recently shown to augment the levels of E6 protein (Vos *et al.*, 2009), or preventing the binding of E6 to the as-yet-unknown ubiquitin ligase that regulates its levels.

What determines whether PDZ proteins are degraded by E6 or whether they stabilise E6 remains unclear. We hypothesise that many factors can affect the outcome of the interaction of E6 with PDZ proteins and these include the levels and localisation of the proteins in the cells. Interestingly, the degradation of PDZ proteins by E6 has so far only been shown in cancer cell lines (Kranjec & Banks, 2010; Massimi *et al.*, 2004) or using over-expression systems (Gardiol *et al.*, 1999; Nakagawa & Huibregtse, 2000 and Chapter 3). In these cases, the levels of E6 protein are higher than those found during productive infections. In fact, no difference was observed in the levels of PDZ proteins between cells harbouring HPV31 episomes and control cells (Lee & Laimins, 2004). Moreover, in my experiments I did not observe a difference in the levels of hScrib in cells that had been transfected with 16WT genomes or control plasmid (Chapter 5). It is therefore possible that during a productive infection, when the levels of E6 are not high, the interaction of E6 with PDZ proteins leads to stabilisation of the E6 protein. This dynamic may change during neoplasia, when the levels of E6 increase. It is important to note however, that stabilisation of E6 by hScrib was also observed in HeLa cells, (Nicolaides *et al.*, 2011), suggesting that the two processes may not be mutually exclusive.

Furthermore, it has been shown that PDZ proteins exist in different cellular pools (Garcia-Mata *et al.*, 2007; Massimi *et al.*, 2004; McLaughlin *et al.*, 2002) and the same appears to be true for E6, as the protein has been reported to be nuclear, cytoplasmic and membrane-associated (Grossman *et al.*, 1989; Tosi *et al.*, 1993). Interestingly, the cellular localisation of E6 has been shown to affect its stability (Grossman *et al.*, 1989). Moreover, the targeting of PDZ proteins by E6 is dependent on the cellular localisation of the PDZ proteins, as well as on their phosphorylation state (Massimi *et al.*, 2004; Massimi *et al.*, 2006; Narayan *et al.*, 2009a). Therefore, the outcome of the interaction between E6 and PDZ proteins may depend on the different cellular pools of the proteins that are interacting. This hypothesis is supported by the observations that the pool of E6 that is stabilised by PDZ proteins is not the same as the one involved in p53-degradation (Chapter 5). Whether the PDZ-bound E6 protein is still able to interact with its other binding partners, and perform its known activities, remains of interest.

6.7. Future experiments

6.7.1. To determine whether p53-degradation is indeed necessary for persistence

It remains important to conclusively determine whether p53-degradation is indeed necessary for persistence. My initial plan of using NIKS-shp53 cells to answer this question proved to be unsuitable. Therefore alternative means would need to be employed in order to address this. One option would be to use chemicals to inhibit p53 in NIKS cells (such as the p53-inhibitor Pifithrin-α (Komarov *et al.*, 1999)) and to establish whether 16E6p53m genomes are able to persist in treated cells. The inhibitor will need to be applied to the cells after transfection with HPV DNA, as my results so far suggest that low levels of p53 at the time of HPV entry into cells are detrimental for persistence. Before using this chemical for such an assay, preliminary experiments to determine whether the drug confers inhibitory effects on the persistence of the wild-type genomes will be required.

Alternatively we could co-transfect the 16E6p53m genomes with a plasmid that expresses a dominant negative form of p53. A similar experiment was discussed in a previous study (Thomas *et al.*, 1999). The authors mentioned that they co-transfected an HPV31 E6null genome with a plasmid that expresses a dominant negative form of p53, to determine whether degradation of p53 is sufficient for persistence (Thomas *et al.*, 1999). The E6null genomes were unable to persist in this experiment. It is now clear from the work presented here (Chapter 3) and that of others (Lee & Laimins, 2004; Lee *et al.*, 2007), that p53-degradation is not sufficient for persistence. Therefore, the experiment proposed here, using 16E6p53m mutant genomes instead of 16E6null genomes, would be a refined version of the experiment in the Thomas *et al*. study (Thomas *et al.*, 1999), in trying to conclusively determine whether p53-degradation is in fact necessary for persistence.

To further elucidate the requirement for E6 in persistence, we could co-transfect the two mutant genomes, 16E6p53m and 16E6PDZ, to determine whether they

can complement each other. This experiment would determine whether the two mutations disrupt a common function of E6 that is necessary for persistence.

6.7.2. What other activities of E6 are necessary for persistence?

The finding that the E6PDZ protein is less stable has opened doors for the exploration of other activities of E6 that may be necessary for persistence, and which may be compromised by the lower steady-state levels of the E6PDZ protein. Several possible activities of E6 were discussed in section 6.4. and experiments to address some of these are discussed here.

The ability of E6 to target E6TP1 for degradation has already been shown to be necessary for persistence (Lee *et al.*, 2007). It would thus be important to determine whether the levels of E6TP1 are different in cells that harbour 16WT or 16E6PDZ genomes (using FACS-sorted cells from experiments similar to the ones described in Chapter 5). Alternatively, we could assess whether the 16E6PDZ mutant genomes can persist in cells that have low levels of E6TP1 (generated using shRNA against E6TP1).

The localisation of E6 to the nucleus, has also been implicated in persistence (Lee *et al.*, 2007), and it would therefore be important to determine whether this localisation is compromised by the deletion of the PDZ-binding motif. This could be addressed by immunocytochemistry, or cell-fractionation followed by western blotting, in order to determine whether the localisation of E6PDZ protein is different to that of E6WT protein. Aberrant localisation of the E6PDZ protein would more likely be due to the deletion of the PDZ-binding motif, rather than to the lower steady-state levels of the protein itself. Therefore these experiments could be carried out in cells that stably express the wild-type and mutant E6 proteins (for example the LXSN-E6WT and LXSN-E6PDZ cells), rather than in FACS-sorted cells, as the former are easier to obtain. As both the degradation of E6TP1 and the nuclear localisation of E6 have been attributed roles in persistence (Lee *et al.*, 2007), disruption of either process due to the deletion of the PDZ-binding motif would suggest the pathway by which this mutation disrupts persistence.

The abovementioned activities or properties of E6 have already been linked to persistence (Lee *et al.*, 2007), and are therefore important to address. However, as discussed earlier, other activities may potentially have a role in persistence One such activity that may be significant is interference with the IFN-signalling pathway (Nees *et al.*, 2001), as IFN-signalling has been shown to down-regulate HPV replication (Terenzi *et al.*, 2008) Therefore, looking at the expression levels of different IFN-inducible genes, such as Stat-1 or p56, in the 16E6WT- and 16E6PDZ-positive FACS-sorted cells, could help us identify any potential differences that may suggest an interferon-mediated inhibition of viral DNA replication.

Moreover, assessing the levels of MCM7 in the FACS-sorted cells could also give an indication of whether the degradation of this protein is compromised by the lower levels of E6PDZ protein, and suggest a potential pathway for persistence.

I have also discussed a potential role for E6 in the segregation of viral episomes, although the segregation pathway of HPV16 episomes is not well understood. This could be addressed using fluorescence *in situ* hybridisation to look at the segregation of viral episomes in cells that have been transfected with 16WT or 16E6PDZ genomes. This would allow the detection of differences in the localisation of the viral DNA during cell division, and consequently potentially identify a role for E6 in viral genome segregation.

6.7.3. Can HPV infections be established in cells that already harbour HPV episomes?

My results have also suggested a role for E6 in inhibiting “super-infection” which could have potential implications when considering treatments of HPV-induced lesions. It would be interesting to investigate whether HPV-positive cells are indeed refractive to the establishment of a new infection by a different HPV type. To do this we could transfect episomal HPV-positive cell lines with genomes of different HPV types and assess the ability of the newly-introduced genomes to persist. These experiments would determine whether certain HPV types would not be able to persist together in the same cells.

6.7.4. Is degradation of PDZ proteins also necessary for episomal persistence?

Our results suggest that the reason why the 16E6PDZ mutant genomes cannot persist may be due to the lower stability of the E6PDZ protein. However, they do not preclude a role for the degradation of PDZ proteins in persistence. PDZ proteins are signalling proteins involved in the control of cell polarity and cell proliferation (Thomas *et al.*, 2008) and may have a role in establishing and maintaining a cellular environment that is conducive to the persistence of HPV episomes.

PDZ proteins are a large group of proteins and it is not currently know which of these may be involved in HPV persistence. To address this, we could systematically knock-down individual PDZ proteins, or combinations of proteins, that have been identified as targets of E6. We could assess the persistence of the 16E6PDZ genomes in stable NIKS cell lines in which these proteins have been knocked-down, to identify which, if any, are inhibitory to persistence.

Similarly, we could individually knock-down PDZ proteins in HPV-positive cells, to identify which ones are able to stabilise E6 (in addition to hScrib, hDlg and MAGI-1, which were identified in this study). In this way, we could also look for any variations in the stabilisation of E6 proteins from different HPV types, thereby contributing to the current understanding of the different affinities of E6 proteins for PDZ proteins.

6.7.5. Is there a balance between the stabilisation of E6 by PDZ proteins and the E6-mediated degradation of PDZ proteins

We hypothesised earlier that the levels and localisation of E6 and PDZ proteins in the cell may determine whether PDZ proteins stabilise E6 or are targeted by it for degradation. For example, at low levels of E6 (such as in productive infections), E6 may be stabilised by PDZ proteins, whereas as at high-levels (such as during neoplasia) PDZ proteins may be degraded by E6. Investigation of this is important, as it could contribute to the current understanding of the molecular events that promote the development of neoplasia.

In the laboratory we have NIKS-based cell lines that stably harbour HPV16 episomes. These could be used in combination with the control NIKS cells in order to study the effect of episomally-expressed E6 on PDZ proteins and vice versa. In addition, we have an integrated HPV16-positive cell line that was made by growing one of the episomal HPV16-positive cell lines without feeder cells for multiple passages (made by Dr. Deborah Jackson). This line could provide an important tool in this study, as it is isogenic to the NIKS cells and the episomal HPV16-positive cell line. As the levels of E6 in the integrated cell line are expected to be much higher than those in the episomal cell line, the two could be used to compare the effects of low- versus high-levels of E6 on PDZ proteins and vice-versa.

Furthermore, to determine whether only some cellular pools of E6 are stabilised by PDZ proteins, we could carry out cell fractionation experiments, and assess the levels of E6 in the different fractions. Alternatively, it would be interesting to use immunofluorescence to visualise the localisation of E6 and PDZ proteins, and to determine whether these change when the two proteins are expressed together or individually.

6.8. Final remarks

This study has shed light on the regulation of episomal persistence by the viral protein E6. Importantly, I have not only looked at regions of E6 that are necessary for the episomal persistence of HPV DNA, but also highlighted that regulation of timing and/or amount of E6 expression is critical for persistence.

Most of the current knowledge of the E6 protein has been derived from studies of cellular cancer lines, or other systems in which the viral life-cycle is not supported. Results from such studies are useful for identifying the oncogenic activities of E6. It is however important to recognise that the functions of the E6 protein are unlikely to have evolved to promote its oncogenicity, as this would imply that E6 is fundamentally detrimental to the virus. Rather, E6 has essential roles in the viral life-cycle, one of which being episomal persistence. The oncogenic effects of E6 are therefore likely to be unfortunate consequences of its essential life-cycle functions.

Interestingly, low-risk HPVs, whose E6 proteins cannot degrade p53 (Foster *et al.*, 1994; Scheffner *et al.*, 1990) and do not have a PDZ-binding motif (Kiyono *et al.*, 1997; Lee *et al.*, 1997; Nakagawa & Huibregtse, 2000), do not persist very well in cell culture (Oh *et al.*, 2004, and observations of others in the laboratory). This suggests that the activities of high-risk E6 proteins may have evolved to enable the persistence of viral genomes under certain conditions, such as in proliferating basal cells, often seen in high-risk HPV-induced lesions (Middleton *et al.*, 2003).

Insights gained from my study also have implications for future studies using mutants of the E6 ORF, as I have shown that mutant proteins may have lower stability than the wild-type protein. Therefore, interesting phenotypes which may be wrongly attributed to the mutated region of E6 may in fact be due to the lower steady-state levels of the protein itself. Detailed protein analysis of E6 has so far been hindered by the low-levels of the protein and the lack of sensitive detection methods. As shown in this study, current methods allow a more thorough analyses and this should be taken into consideration in future studies.

Lastly, it would be tempting to propose the inhibition of E6 activities as a therapeutic means to rid cells of their HPV episomes. However, care must be taken when such methods are put forward. As seen by my results, although inhibiting the activities of E6, or causing mutations in the E6 ORF, can indeed have the desired effect of eliminating viral episomes, in some cases this could result in the integration of viral genomes into the cellular chromosomes. Such an event can be even more detrimental to the cell and to the organism as a whole.

References

Abbate, E. A., Voitenleitner, C. & Botchan, M. R. (2006). Structure of the papillomavirus DNA-tethering complex E2:Brd4 and a peptide that ablates HPV chromosomal association. *Mol Cell* **24**, 877-889.

Abramson, A. L., Nouri, M., Mullooly, V., Fisch, G. & Steinberg, B. M. (2004). Latent Human Papillomavirus infection is comparable in the larynx and trachea. *J Med Virol* **72**, 473-477.

Allen-Hoffmann, B. L., Schlosser, S. J., Ivarie, C. A., Sattler, C. A., Meisner, L. F. & O'Connor, S. L. (2000). Normal growth and differentiation in a spontaneously immortalized near-diploid human keratinocyte cell line, NIKS. *J Invest Dermatol* **114**, 444-455.

Amtmann, E. & Sauer, G. (1982). Bovine papilloma virus transcription: polyadenylated RNA species and assessment of the direction of transcription. *J Virol* **43**, 59-66.

Androphy, E. J., Lowy, D. R. & Schiller, J. T. (1987). Bovine papillomavirus E2 trans-activating gene product binds to specific sites in papillomavirus DNA. *Nature* **325**, 70-73.

Angeletti, P. C., Kim, K., Fernandes, F. J. & Lambert, P. F. (2002). Stable replication of papillomavirus genomes in Saccharomyces cerevisiae. *J Virol* **76**, 3350-3358.

Antinore, M. J., Birrer, M. J., Patel, D., Nader, L. & McCance, D. J. (1996). The human papillomavirus type 16 E7 gene product interacts with and trans-activates the AP1 family of transcription factors. *EMBO J* **15**, 1950-1960.

Arroyo, M., Bagchi, S. & Raychaudhuri, P. (1993). Association of the human papillomavirus type 16 E7 protein with the S-phase-specific E2F-cyclin A complex. *Mol Cell Biol* **13**, 6537-6546.

Baeg, G. H., Matsumine, A., Kuroda, T., Bhattacharjee, R. N., Miyashiro, I., Toyoshima, K. & Akiyama, T. (1995). The tumour suppressor gene product APC blocks cell cycle progression from G0/G1 to S phase. *EMBO J* **14**, 5618-5625.

Band, V., De Caprio, J. A., Delmolino, L., Kulesa, V. & Sager, R. (1991). Loss of p53 protein in human papillomavirus type 16 E6-immortalized human mammary epithelial cells. *J Virol* **65**, 6671-6676.

Barbera, A. J., Chodaparambil, J. V., Kelley-Clarke, B., Joukov, V., Walter, J. C., Luger, K. & Kaye, K. M. (2006). The nucleosomal surface as a docking station for Kaposi's sarcoma herpesvirus LANA. *Science* **311**, 856-861.

Barnard, P. & McMillan, N. A. (1999). The human papillomavirus E7 oncoprotein abrogates signaling mediated by interferon-alpha. *Virology* **259**, 305-313.

Bastien, N. & McBride, A. A. (2000). Interaction of the papillomavirus E2 protein with mitotic chromosomes. *Virology* **270**, 124-134.

Baxter, M. K., McPhillips, M. G., Ozato, K. & McBride, A. A. (2005). The mitotic chromosome binding activity of the papillomavirus E2 protein correlates with interaction with the cellular chromosomal protein, Brd4. *J Virol* **79**, 4806-4818.

Bechtold, V., Beard, P. & Raj, K. (2003). Human papillomavirus type 16 E2 protein has no effect on transcription from episomal viral DNA. *J Virol* **77**, 2021-2028.

Berg, L., Lusky, M., Stenlund, A. & Botchan, M. R. (1986a). Repression of bovine papilloma virus replication is mediated by a virally encoded trans-acting factor. *Cell* **46**, 753-762.

Berg, L. J., Singh, K. & Botchan, M. (1986b). Complementation of a bovine papilloma virus low-copy-number mutant: evidence for a temporal requirement of the complementing gene. *Mol Cell Biol* **6**, 859-869.

Bernard, B. A., Bailly, C., Lenoir, M. C., Darmon, M., Thierry, F. & Yaniv, M. (1989). The human papillomavirus type 18 (HPV18) E2 gene product is a repressor of the HPV18 regulatory region in human keratinocytes. *J Virol* **63**, 4317-4324.

Bernard, H. U., Calleja-Macias, I. E. & Dunn, S. T. (2006). Genome variation of human papillomavirus types: phylogenetic and medical implications. *Int J Cancer* **118**, 1071-1076.

Bernard, H. U., Burk, R. D., Chen, Z., van Doorslaer, K., Hausen, H. & de Villiers, E. M. (2010). Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. *Virology* **401**, 70-79.

Bilder, D., Li, M. & Perrimon, N. (2000). Cooperative regulation of cell polarity and growth by Drosophila tumor suppressors. *Science* **289**, 113-116.

Bilder, D. & Perrimon, N. (2000). Localization of apical epithelial determinants by the basolateral PDZ protein Scribble. *Nature* **403**, 676-680.

Bilder, D. (2004). Epithelial polarity and proliferation control: links from the Drosophila neoplastic tumor suppressors. *Genes Dev* **18**, 1909-1925.

Boner, W., Taylor, E. R., Tsirimonaki, E., Yamane, K., Campo, M. S. & Morgan, I. M. (2002). A Functional interaction between the human papillomavirus 16 transcription/replication factor E2 and the DNA damage response protein TopBP1. *J Biol Chem* **277**, 22297-22303.

Bosch, F. X., Manos, M. M., Munoz, N. & other authors (1995). Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. International biological study on cervical cancer (IBSCC) Study Group. *J Natl Cancer Inst* **87**, 796-802.

Bousarghin, L., Touze, A., Sizaret, P. Y. & Coursaget, P. (2003). Human papillomavirus types 16, 31, and 58 use different endocytosis pathways to enter cells. *J Virol* **77**, 3846-3850.

Bouvard, V., Storey, A., Pim, D. & Banks, L. (1994). Characterization of the human papillomavirus E2 protein: evidence of trans-activation and trans-repression in cervical keratinocytes. *EMBO J* **13**, 5451-5459.

Boyer, S. N., Wazer, D. E. & Band, V. (1996). E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteasome pathway. *Cancer Res* **56**, 4620-4624.

Brehm, A., Nielsen, S. J., Miska, E. A., McCance, D. J., Reid, J. L., Bannister, A. J. & Kouzarides, T. (1999). The E7 oncoprotein associates with Mi2 and histone deacetylase activity to promote cell growth. *EMBO J* **18**, 2449-2458.

Bridge, A. J., Pebernard, S., Ducraux, A., Nicoulaz, A. L. & Iggo, R. (2003). Induction of an interferon response by RNAi vectors in mammalian cells. *Nat Genet* **34**, 263-264.

Brimer, N., Lyons, C. & Vande Pol, S. B. (2007). Association of E6AP (UBE3A) with human papillomavirus type 11 E6 protein. *Virology* **358**, 303-310.

Brown, C., Kowalczyk, A. M., Taylor, E. R., Morgan, I. M. & Gaston, K. (2008). P53 represses human papillomavirus type 16 DNA replication via the viral E2 protein. *Virol J* **5**, 5.

Brown, D. R., Shew, M. L., Qadadri, B., Neptune, N., Vargas, M., Tu, W., Juliar, B. E., Breen, T. E. & Fortenberry, J. D. (2005). A longitudinal study of genital human papillomavirus infection in a cohort of closely followed adolescent women. *J Infect Dis* **191**, 182-192.

Brumby, A. M. & Richardson, H. E. (2003). scribble mutants cooperate with oncogenic Ras or Notch to cause neoplastic overgrowth in Drosophila. *EMBO J* **22**, 5769-5779.

Brummelkamp, T. R., Bernards, R. & Agami, R. (2002). Stable suppression of tumorigenicity by virus-mediated RNA interference. *Cancer Cell* **2**, 243-247.

Bryan, J. T. & Brown, D. R. (2001). Transmission of human papillomavirus type 11 infection by desquamated cornified cells. *Virology* **281**, 35-42.

Buck, C. B., Pastrana, D. V., Lowy, D. R. & Schiller, J. T. (2005). Generation of HPV pseudovirions using transfection and their use in neutralization assays. *Methods Mol Med* **119**, 445-462.

Burghardt, E. & Ostor, A. G. (1983). Site and origin of squamous cervical cancer: a histomorphologic study. *Obstet Gynecol* **62**, 117-127.

Campo, M. S., Graham, S. V., Cortese, M. S., Ashrafi, G. H., Araibi, E. H., Dornan, E. S., Miners, K., Nunes, C. & Man, S. (2010). HPV-16 E5 down-regulates expression of surface HLA class I and reduces recognition by CD8 T cells. *Virology* **407**, 137-142.

Camus, S., Menendez, S., Cheok, C. F., Stevenson, L. F., Lain, S. & Lane, D. P. (2007). Ubiquitin-independent degradation of p53 mediated by high-risk human papillomavirus protein E6. *Oncogene* **26**, 4059-4070.

Chan, S. Y., Chew, S. H., Egawa, K., Grussendorf-Conen, E. I., Honda, Y., Rubben, A., Tan, K. C. & Bernard, H. U. (1997). Phylogenetic analysis of the human papillomavirus type 2 (HPV-2), HPV-27, and HPV-57 group, which is associated with common warts. *Virology* **239**, 296-302.

Chan, W. K., Chong, T., Bernard, H. U. & Klock, G. (1990). Transcription of the transforming genes of the oncogenic human papillomavirus-16 is stimulated by tumor promotors through AP1 binding sites. *Nucleic Acids Res* **18**, 763-769.

Chang, Y. E. & Laimins, L. A. (2000). Microarray analysis identifies interferon-inducible genes and Stat-1 as major transcriptional targets of human papillomavirus type 31. *J Virol* **74**, 4174-4182.

Chang, Y. E., Pena, L., Sen, G. C., Park, J. K. & Laimins, L. A. (2002). Long-term effect of interferon on keratinocytes that maintain human papillomavirus type 31. *J Virol* **76**, 8864-8874.

Chellappan, S. P., Hiebert, S., Mudryj, M., Horowitz, J. M. & Nevins, J. R. (1991). The E2F transcription factor is a cellular target for the RB protein. *Cell* **65**, 1053-1061.

Cheng, S., Schmidt-Grimminger, D. C., Murant, T., Broker, T. R. & Chow, L. T. (1995). Differentiation-dependent up-regulation of the human papillomavirus E7 gene reactivates cellular DNA replication in suprabasal differentiated keratinocytes. *Genes Dev* **9**, 2335-2349.

Chiang, C. M., Dong, G., Broker, T. R. & Chow, L. T. (1992a). Control of human papillomavirus type 11 origin of replication by the E2 family of transcription regulatory proteins. *J Virol* **66**, 5224-5231.

Chiang, C. M., Ustav, M., Stenlund, A., Ho, T. F., Broker, T. R. & Chow, L. T. (1992b). Viral E1 and E2 proteins support replication of homologous and heterologous papillomaviral origins. *Proc Natl Acad Sci U S A* **89**, 5799-5803.

Chien, W. M., Parker, J. N., Schmidt-Grimminger, D. C., Broker, T. R. & Chow, L. T. (2000). Casein kinase II phosphorylation of the human papillomavirus-18 E7 protein is critical for promoting S-phase entry. *Cell Growth Differ* **11**, 425-435.

Chong, J. P., Thommes, P. & Blow, J. J. (1996). The role of MCM/P1 proteins in the licensing of DNA replication. *Trends Biochem Sci* **21**, 102-106.

Clifford, G. M., Smith, J. S., Plummer, M., Munoz, N. & Franceschi, S. (2003). Human papillomavirus types in invasive cervical cancer worldwide: a meta-analysis. *Br J Cancer* **88**, 63-73.

Clower, R. V., Fisk, J. C. & Melendy, T. (2006). Papillomavirus E1 protein binds to and stimulates human topoisomerase I. *J Virol* **80**, 1584-1587.

Coleman, N., Birley, H. D., Renton, A. M., Hanna, N. F., Ryait, B. K., Byrne, M., Taylor-Robinson, D. & Stanley, M. A. (1994). Immunological events in regressing genital warts. *Am J Clin Pathol* **102**, 768-774.

Cotter, M. A., 2nd & Robertson, E. S. (1999). The latency-associated nuclear antigen tethers the Kaposi's sarcoma-associated herpesvirus genome to host chromosomes in body cavity-based lymphoma cells. *Virology* **264**, 254-264.

Crook, T., Wrede, D. & Vousden, K. H. (1991). p53 point mutation in HPV negative human cervical carcinoma cell lines. *Oncogene* **6**, 873-875.

Daniels, P. R., Sanders, C. M. & Maitland, N. J. (1998). Characterization of the interactions of human papillomavirus type 16 E6 with p53 and E6-associated protein in insect and human cells. *J Gen Virol* **79 (Pt 3)**, 489-499.

Danos, O., Engel, L. W., Chen, E. Y., Yaniv, M. & Howley, P. M. (1983). Comparative analysis of the human type 1a and bovine type 1 papillomavirus genomes. *J Virol* **46**, 557-566.

Davies, R., Hicks, R., Crook, T., Morris, J. & Vousden, K. (1993). Human papillomavirus type 16 E7 associates with a histone H1 kinase and with p107 through sequences necessary for transformation. *J Virol* **67**, 2521-2528.

Davy, C. & Doorbar, J. (2007). G2/M cell cycle arrest in the life cycle of viruses. *Virology* **368**, 219-226.

Davy, C. E., Jackson, D. J., Wang, Q. & other authors (2002). Identification of a G(2) arrest domain in the E1 wedge E4 protein of human papillomavirus type 16. *J Virol* **76**, 9806-9818.

Day, P. M., Roden, R. B., Lowy, D. R. & Schiller, J. T. (1998). The papillomavirus minor capsid protein, L2, induces localization of the major capsid protein, L1, and the viral transcription/replication protein, E2, to PML oncogenic domains. *J Virol* **72**, 142-150.

de Villiers, E. M., Fauquet, C., Broker, T. R., Bernard, H. U. & zur Hausen, H. (2004). Classification of papillomaviruses. *Virology* **324**, 17-27.

DeCaprio, J. A., Ludlow, J. W., Figge, J., Shew, J. Y., Huang, C. M., Lee, W. H., Marsilio, E., Paucha, E. & Livingston, D. M. (1988). SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. *Cell* **54**, 275-283.

Degenhardt, Y. Y. & Silverstein, S. (2001). Interaction of zyxin, a focal adhesion protein, with the e6 protein from human papillomavirus type 6 results in its nuclear translocation. *J Virol* **75**, 11791-11802.

Del Vecchio, A. M., Romanczuk, H., Howley, P. M. & Baker, C. C. (1992). Transient replication of human papillomavirus DNAs. *J Virol* **66**, 5949-5958.

Demers, G. W., Halbert, C. L. & Galloway, D. A. (1994). Elevated wild-type p53 protein levels in human epithelial cell lines immortalized by the human papillomavirus type 16 E7 gene. *Virology* **198**, 169-174.

Desaintes, C. & Demeret, C. (1996). Control of papillomavirus DNA replication and transcription. *Semin Cancer Biol* **7**, 339-347.

Dollard, S. C., Wilson, J. L., Demeter, L. M., Bonnez, W., Reichman, R. C., Broker, T. R. & Chow, L. T. (1992). Production of human papillomavirus and modulation of the infectious program in epithelial raft cultures. *Genes Dev* **6**, 1131-1142.

Donaldson, M. M., Boner, W. & Morgan, I. M. (2007). TopBP1 regulates human papillomavirus type 16 E2 interaction with chromatin. *J Virol* **81**, 4338-4342.

Donjerkovic, D. & Scott, D. W. (2000). Regulation of the G1 phase of the mammalian cell cycle. *Cell Res* **10**, 1-16.

Doorbar, J. & Gallimore, P. H. (1987). Identification of proteins encoded by the L1 and L2 open reading frames of human papillomavirus 1a. *J Virol* **61**, 2793-2799.

Doorbar, J., Parton, A., Hartley, K., Banks, L., Crook, T., Stanley, M. & Crawford, L. (1990). Detection of novel splicing patterns in a HPV16-containing keratinocyte cell line. *Virology* **178**, 254-262.

Doorbar, J., Ely, S., Sterling, J., McLean, C. & Crawford, L. (1991). Specific interaction between HPV-16 E1-E4 and cytokeratins results in collapse of the epithelial cell intermediate filament network. *Nature* **352**, 824-827.

Doorbar, J. (2005). The papillomavirus life cycle. *J Clin Virol* **32 Suppl 1**, S7-15.

Doorbar, J. (2006). Molecular biology of human papillomavirus infection and cervical cancer. *Clin Sci (Lond)* **110**, 525-541.

Dow, L. E., Brumby, A. M., Muratore, R., Coombe, M. L., Sedelies, K. A., Trapani, J. A., Russell, S. M., Richardson, H. E. & Humbert, P. O. (2003). hScrib is a functional homologue of the Drosophila tumour suppressor Scribble. *Oncogene* **22**, 9225-9230.

Dow, L. E., Kauffman, J. S., Caddy, J., Zarbalis, K., Peterson, A. S., Jane, S. M., Russell, S. M. & Humbert, P. O. (2007). The tumour-suppressor

Scribble dictates cell polarity during directed epithelial migration: regulation of Rho GTPase recruitment to the leading edge. *Oncogene* **26**, 2272-2282.

Dow, L. E., Elsum, I. A., King, C. L., Kinross, K. M., Richardson, H. E. & Humbert, P. O. (2008). Loss of human Scribble cooperates with H-Ras to promote cell invasion through deregulation of MAPK signalling. *Oncogene* **27**, 5988-6001.

DuBridge, R. B., Tang, P., Hsia, H. C., Leong, P. M., Miller, J. H. & Calos, M. P. (1987). Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. *Mol Cell Biol* **7**, 379-387.

Duensing, S., Lee, L. Y., Duensing, A., Basile, J., Piboonniyom, S., Gonzalez, S., Crum, C. P. & Munger, K. (2000). The human papillomavirus type 16 E6 and E7 oncoproteins cooperate to induce mitotic defects and genomic instability by uncoupling centrosome duplication from the cell division cycle. *Proc Natl Acad Sci U S A* **97**, 10002-10007.

Duensing, S., Duensing, A., Flores, E. R., Do, A., Lambert, P. F. & Munger, K. (2001). Centrosome abnormalities and genomic instability by episomal expression of human papillomavirus type 16 in raft cultures of human keratinocytes. *J Virol* **75**, 7712-7716.

Durst, M., Kleinheinz, A., Hotz, M. & Gissmann, L. (1985). The physical state of human papillomavirus type 16 DNA in benign and malignant genital tumours. *J Gen Virol* **66 (Pt 7)**, 1515-1522.

Dutta, A., Ruppert, J. M., Aster, J. C. & Winchester, E. (1993). Inhibition of DNA replication factor RPA by p53. *Nature* **365**, 79-82.

Dyson, N., Howley, P. M., Munger, K. & Harlow, E. (1989). The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* **243**, 934-937.

Dyson, N. (1998). The regulation of E2F by pRB-family proteins. *Genes Dev* **12**, 2245-2262.

Egawa, K., Inaba, Y., Yoshimura, K. & Ono, T. (1993). Varied clinical morphology of HPV-1-induced warts, depending on anatomical factors. *Br J Dermatol* **128**, 271-276.

Egawa, K. (2003). Do human papillomaviruses target epidermal stem cells? *Dermatology* **207**, 251-254.

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicol Pathol* **35**, 495-516.

Evander, M., Frazer, I. H., Payne, E., Qi, Y. M., Hengst, K. & McMillan, N. A. (1997). Identification of the alpha6 integrin as a candidate receptor for papillomaviruses. *J Virol* **71**, 2449-2456.

Fahraeus, R., Chen, W., Trivedi, P., Klein, G. & Obrink, B. (1992). Decreased expression of E-cadherin and increased invasive capacity in EBV-LMP-transfected human epithelial and murine adenocarcinoma cells. *Int J Cancer* **52**, 834-838.

Fang, L., Budgeon, L. R., Doorbar, J., Briggs, E. R. & Howett, M. K. (2006). The human papillomavirus type 11 E1/\E4 protein is not essential for viral genome amplification. *Virology* **351**, 271-279.

Fang, L., Wang, Y., Du, D., Yang, G., Tak Kwok, T., Kai Kong, S., Chen, B., Chen, D. J. & Chen, Z. (2007). Cell polarity protein Par3 complexes with DNA-PK via Ku70 and regulates DNA double-strand break repair. *Cell Res* **17**, 100-116.

Favre-Bonvin, A., Reynaud, C., Kretz-Remy, C. & Jalinot, P. (2005). Human papillomavirus type 18 E6 protein binds the cellular PDZ protein TIP-2/GIPC, which is involved in transforming growth factor beta signaling and triggers its degradation by the proteasome. *J Virol* **79**, 4229-4237.

Favre, M., Ramoz, N. & Orth, G. (1997). Human papillomaviruses: general features. *Clin Dermatol* **15**, 181-198.

Feeney, K. M. & Parish, J. L. (2009). Targeting mitotic chromosomes: a conserved mechanism to ensure viral genome persistence. *Proc Biol Sci* **276**, 1535-1544.

Fehrmann, F., Klumpp, D. J. & Laimins, L. A. (2003). Human papillomavirus type 31 E5 protein supports cell cycle progression and activates late viral functions upon epithelial differentiation. *J Virol* **77**, 2819-2831.

Filippova, M., Parkhurst, L. & Duerksen-Hughes, P. J. (2004). The human papillomavirus 16 E6 protein binds to Fas-associated death domain and protects cells from Fas-triggered apoptosis. *J Biol Chem* **279**, 25729-25744.

Firestein, B. L. & Rongo, C. (2001). DLG-1 is a MAGUK similar to SAP97 and is required for adherens junction formation. *Mol Biol Cell* **12**, 3465-3475.

Flores, E. R., Allen-Hoffmann, B. L., Lee, D., Sattler, C. A. & Lambert, P. F. (1999). Establishment of the human papillomavirus type 16 (HPV-16) life cycle in an immortalized human foreskin keratinocyte cell line. *Virology* **262**, 344-354.

Flores, E. R., Allen-Hoffmann, B. L., Lee, D. & Lambert, P. F. (2000). The human papillomavirus type 16 E7 oncogene is required for the productive stage of the viral life cycle. *J Virol* **74**, 6622-6631.

Florin, L., Sapp, C., Streeck, R. E. & Sapp, M. (2002). Assembly and translocation of papillomavirus capsid proteins. *J Virol* **76**, 10009-10014.

Forrester, A., Farrell, H., Wilkinson, G., Kaye, J., Davis-Poynter, N. & Minson, T. (1992). Construction and properties of a mutant of herpes simplex virus type 1 with glycoprotein H coding sequences deleted. *J Virol* **66**, 341-348.

Foster, S. A., Demers, G. W., Etscheid, B. G. & Galloway, D. A. (1994). The ability of human papillomavirus E6 proteins to target p53 for degradation in vivo correlates with their ability to abrogate actinomycin D-induced growth arrest. *J Virol* **68**, 5698-5705.

Fradet-Turcotte, A., Moody, C., Laimins, L. A. & Archambault, J. (2010). Nuclear export of human papillomavirus type 31 E1 is regulated by Cdk2 phosphorylation and required for viral genome maintenance. *J Virol* **84**, 11747-11760.

Frattini, M. G. & Laimins, L. A. (1994). The role of the E1 and E2 proteins in the replication of human papillomavirus type 31b. *Virology* **204**, 799-804.

Friedman, P. N., Kern, S. E., Vogelstein, B. & Prives, C. (1990). Wild-type, but not mutant, human p53 proteins inhibit the replication activities of simian virus 40 large tumor antigen. *Proc Natl Acad Sci U S A* **87**, 9275-9279.

Funk, J. O., Waga, S., Harry, J. B., Espling, E., Stillman, B. & Galloway, D. A. (1997). Inhibition of CDK activity and PCNA-dependent DNA replication by p21 is blocked by interaction with the HPV-16 E7 oncoprotein. *Genes Dev* **11**, 2090-2100.

Gage, J. R., Meyers, C. & Wettstein, F. O. (1990). The E7 proteins of the nononcogenic human papillomavirus type 6b (HPV-6b) and of the oncogenic HPV-16 differ in retinoblastoma protein binding and other properties. *J Virol* **64**, 723-730.

Gannon, J. V. & Lane, D. P. (1987). p53 and DNA polymerase alpha compete for binding to SV40 T antigen. *Nature* **329**, 456-458.

Gao, Q., Srinivasan, S., Boyer, S. N., Wazer, D. E. & Band, V. (1999). The E6 oncoproteins of high-risk papillomaviruses bind to a novel putative GAP protein, E6TP1, and target it for degradation. *Mol Cell Biol* **19**, 733-744.

Gao, Q., Kumar, A., Singh, L., Huibregtse, J. M., Beaudenon, S., Srinivasan, S., Wazer, D. E., Band, H. & Band, V. (2002). Human papillomavirus E6-induced degradation of E6TP1 is mediated by E6AP ubiquitin ligase. *Cancer Res* **62**, 3315-3321.

Garcia-Alai, M. M., Dantur, K. I., Smal, C., Pietrasanta, L. & de Prat-Gay, G. (2007). High-risk HPV E6 oncoproteins assemble into large oligomers that allow localization of endogenous species in prototypic HPV-transformed cell lines. *Biochemistry* **46**, 341-349.

Garcia-Mata, R., Dubash, A. D., Sharek, L., Carr, H. S., Frost, J. A. & Burridge, K. (2007). The nuclear RhoA exchange factor Net1 interacts with proteins of the Dlg family, affects their localization, and influences their tumor suppressor activity. *Mol Cell Biol* **27**, 8683-8697.

Gardiol, D., Kuhne, C., Glaunsinger, B., Lee, S. S., Javier, R. & Banks, L. (1999). Oncogenic human papillomavirus E6 proteins target the discs large

tumour suppressor for proteasome-mediated degradation. *Oncogene* **18**, 5487-5496.

Gardiol, D., Zacchi, A., Petrera, F., Stanta, G. & Banks, L. (2006). Human discs large and scrib are localized at the same regions in colon mucosa and changes in their expression patterns are correlated with loss of tissue architecture during malignant progression. *Int J Cancer* **119**, 1285-1290.

Garnett, T. O., Filippova, M. & Duerksen-Hughes, P. J. (2006). Accelerated degradation of FADD and procaspase 8 in cells expressing human papilloma virus 16 E6 impairs TRAIL-mediated apoptosis. *Cell Death Differ* **13**, 1915-1926.

Gewin, L. & Galloway, D. A. (2001). E box-dependent activation of telomerase by human papillomavirus type 16 E6 does not require induction of c-myc. *J Virol* **75**, 7198-7201.

Giannini, S. L., Hubert, P., Doyen, J., Boniver, J. & Delvenne, P. (2002). Influence of the mucosal epithelium microenvironment on Langerhans cells: implications for the development of squamous intraepithelial lesions of the cervix. *Int J Cancer* **97**, 654-659.

Gissmann, L., Diehl, V., Schultz-Coulon, H. J. & zur Hausen, H. (1982). Molecular cloning and characterization of human papilloma virus DNA derived from a laryngeal papilloma. *J Virol* **44**, 393-400.

Glaunsinger, B. A., Lee, S. S., Thomas, M., Banks, L. & Javier, R. (2000). Interactions of the PDZ-protein MAGI-1 with adenovirus E4-ORF1 and high-risk papillomavirus E6 oncoproteins. *Oncogene* **19**, 5270-5280.

Gloss, B. & Bernard, H. U. (1990). The E6/E7 promoter of human papillomavirus type 16 is activated in the absence of E2 proteins by a sequence-aberrant Sp1 distal element. *J Virol* **64**, 5577-5584.

Gottlieb, T. M. & Oren, M. (1996). p53 in growth control and neoplasia. *Biochim Biophys Acta* **1287**, 77-102.

Graham, F. L., Smiley, J., Russell, W. C. & Nairn, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol* **36**, 59-74.

Grassmann, K., Rapp, B., Maschek, H., Petry, K. U. & Iftner, T. (1996). Identification of a differentiation-inducible promoter in the E7 open reading frame of human papillomavirus type 16 (HPV-16) in raft cultures of a new cell line containing high copy numbers of episomal HPV-16 DNA. *J Virol* **70**, 2339-2349.

Greer, C. E., Wheeler, C. M., Ladner, M. B., Beutner, K., Coyne, M. Y., Liang, H., Langenberg, A., Yen, T. S. & Ralston, R. (1995). Human papillomavirus (HPV) type distribution and serological response to HPV type 6 virus-like particles in patients with genital warts. *J Clin Microbiol* **33**, 2058-2063.

Grm, H. S. & Banks, L. (2004). Degradation of hDlg and MAGIs by human papillomavirus E6 is E6-AP-independent. *J Gen Virol* **85**, 2815-2819.

Grm, H. S., Massimi, P., Gammoh, N. & Banks, L. (2005). Crosstalk between the human papillomavirus E2 transcriptional activator and the E6 oncoprotein. *Oncogene* **24**, 5149-5164.

Grossman, S. R., Mora, R. & Laimins, L. A. (1989). Intracellular localization and DNA-binding properties of human papillomavirus type 18 E6 protein expressed with a baculovirus vector. *J Virol* **63**, 366-374.

Halbert, C. L., Demers, G. W. & Galloway, D. A. (1991). The E7 gene of human papillomavirus type 16 is sufficient for immortalization of human epithelial cells. *J Virol* **65**, 473-478.

Hampson, L., Li, C., Oliver, A. W., Kitchener, H. C. & Hampson, I. N. (2004). The PDZ protein Tip-1 is a gain of function target of the HPV16 E6 oncoprotein. *Int J Oncol* **25**, 1249-1256.

Han, Y., Loo, Y. M., Militello, K. T. & Melendy, T. (1999). Interactions of the papovavirus DNA replication initiator proteins, bovine papillomavirus type 1 E1 and simian virus 40 large T antigen, with human replication protein A. *J Virol* **73**, 4899-4907.

Harwood, C. A. & Proby, C. M. (2002). Human papillomaviruses and non-melanoma skin cancer. *Curr Opin Infect Dis* **15**, 101-114.

Hasan, U. A., Bates, E., Takeshita, F. & other authors (2007). TLR9 expression and function is abolished by the cervical cancer-associated human papillomavirus type 16. *J Immunol* **178**, 3186-3197.

Haupt, Y., Maya, R., Kazaz, A. & Oren, M. (1997). Mdm2 promotes the rapid degradation of p53. *Nature* **387**, 296-299.

Hayashi, Y. & Koike, K. (1989). Interferon inhibits hepatitis B virus replication in a stable expression system of transfected viral DNA. *J Virol* **63**, 2936-2940.

He, W., Staples, D., Smith, C. & Fisher, C. (2003). Direct activation of cyclin-dependent kinase 2 by human papillomavirus E7. *J Virol* **77**, 10566-10574.

Heck, D. V., Yee, C. L., Howley, P. M. & Munger, K. (1992). Efficiency of binding the retinoblastoma protein correlates with the transforming capacity of the E7 oncoproteins of the human papillomaviruses. *Proc Natl Acad Sci U S A* **89**, 4442-4446.

Hennings, H., Michael, D., Cheng, C., Steinert, P., Holbrook, K. & Yuspa, S. H. (1980). Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell* **19**, 245-254.

Herber, R., Liem, A., Pitot, H. & Lambert, P. F. (1996). Squamous epithelial hyperplasia and carcinoma in mice transgenic for the human papillomavirus type 16 E7 oncogene. *J Virol* **70**, 1873-1881.

Herbst, L. H., Lenz, J., Van Doorslaer, K., Chen, Z., Stacy, B. A., Wellehan, J. F., Jr., Manire, C. A. & Burk, R. D. (2009). Genomic characterization of two novel reptilian papillomaviruses, Chelonia mydas papillomavirus 1 and Caretta caretta papillomavirus 1. *Virology* **383**, 131-135.

Herdman, M. T., Pett, M. R., Roberts, I., Alazawi, W. O., Teschendorff, A. E., Zhang, X. Y., Stanley, M. A. & Coleman, N. (2006). Interferon-beta treatment of cervical keratinocytes naturally infected with human papillomavirus 16 episomes promotes rapid reduction in episome numbers and emergence of latent integrants. *Carcinogenesis* **27**, 2341-2353.

Heselmeyer, K., Schrock, E., du Manoir, S., Blegen, H., Shah, K., Steinbeck, R., Auer, G. & Ried, T. (1996). Gain of chromosome 3q defines the transition from severe dysplasia to invasive carcinoma of the uterine cervix. *Proc Natl Acad Sci U S A* **93**, 479-484.

Hoffmann, R., Hirt, B., Bechtold, V., Beard, P. & Raj, K. (2006). Different modes of human papillomavirus DNA replication during maintenance. *J Virol* **80**, 4431-4439.

Honda, R., Tanaka, H. & Yasuda, H. (1997). Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS Lett* **420**, 25-27.

Howie, H. L., Katzenellenbogen, R. A. & Galloway, D. A. (2009). Papillomavirus E6 proteins. *Virology* **384**, 324-334.

Howley, P. M. & Lowy, D. R. (2007). Papillomaviruses. In *Fields Virology*, pp. 2299-2354. Edited by D. M. Knipe & P. M. Howley. Philadelphia, PA: Lippincott Williams and Wilkins.

Hubert, P., Caberg, J. H., Gilles, C., Bousarghin, L., Franzen-Detrooz, E., Boniver, J. & Delvenne, P. (2005). E-cadherin-dependent adhesion of dendritic and Langerhans cells to keratinocytes is defective in cervical human papillomavirus-associated (pre)neoplastic lesions. *J Pathol* **206**, 346-355.

Hughes, F. J. & Romanos, M. A. (1993). E1 protein of human papillomavirus is a DNA helicase/ATPase. *Nucleic Acids Res* **21**, 5817-5823.

Huibregtse, J. M., Scheffner, M. & Howley, P. M. (1991). A cellular protein mediates association of p53 with the E6 oncoprotein of human papillomavirus types 16 or 18. *EMBO J* **10**, 4129-4135.

Humbert, P., Russell, S. & Richardson, H. (2003). Dlg, Scribble and Lgl in cell polarity, cell proliferation and cancer. *Bioessays* **25**, 542-553.

Hummel, M., Hudson, J. B. & Laimins, L. A. (1992). Differentiation-induced and constitutive transcription of human papillomavirus type 31b in cell lines containing viral episomes. *J Virol* **66**, 6070-6080.

Ide, N., Hata, Y., Nishioka, H. & other authors (1999). Localization of membrane-associated guanylate kinase (MAGI)-1/BAI-associated protein (BAP) 1 at tight junctions of epithelial cells. *Oncogene* **18**, 7810-7815.

Iftner, T., Elbel, M., Schopp, B., Hiller, T., Loizou, J. I., Caldecott, K. W. & Stubenrauch, F. (2002). Interference of papillomavirus E6 protein with single-strand break repair by interaction with XRCC1. *EMBO J* **21**, 4741-4748.

Ilves, I., Kivi, S. & Ustav, M. (1999). Long-term episomal maintenance of bovine papillomavirus type 1 plasmids is determined by attachment to host chromosomes, which Is mediated by the viral E2 protein and its binding sites. *J Virol* **73**, 4404-4412.

Ilves, I., Kadaja, M. & Ustav, M. (2003). Two separate replication modes of the bovine papillomavirus BPV1 origin of replication that have different sensitivity to p53. *Virus Res* **96**, 75-84.

Ingle, A., Ghim, S., Joh, J., Chepkoech, I., Jenson, A. B. & Sundberg, J. P. (2010). Novel Laboratory Mouse Papillomavirus (MusPV) Infection. *Vet Pathol*.

Ishidate, T., Matsumine, A., Toyoshima, K. & Akiyama, T. (2000). The APC-hDLG complex negatively regulates cell cycle progression from the G0/G1 to S phase. *Oncogene* **19**, 365-372.

Iso, Y., Sawada, T., Okada, T. & Kubota, K. (2005). Loss of E-cadherin mRNA and gain of osteopontin mRNA are useful markers for detecting early recurrence of HCV-related hepatocellular carcinoma. *J Surg Oncol* **92**, 304-311.

Jackson, S., Harwood, C., Thomas, M., Banks, L. & Storey, A. (2000). Role of Bak in UV-induced apoptosis in skin cancer and abrogation by HPV E6 proteins. *Genes Dev* **14**, 3065-3073.

James, M. A., Lee, J. H. & Klingelhutz, A. J. (2006). HPV16-E6 associated hTERT promoter acetylation is E6AP dependent, increased in later passage cells and enhanced by loss of p300. *Int J Cancer* **119**, 1878-1885.

Javier, R. T. (2008). Cell polarity proteins: common targets for tumorigenic human viruses. *Oncogene* **27**, 7031-7046.

Jeong, K. W., Kim, H. Z., Kim, S., Kim, Y. S. & Choe, J. (2007). Human papillomavirus type 16 E6 protein interacts with cystic fibrosis transmembrane regulator-associated ligand and promotes E6-associated protein-mediated ubiquitination and proteasomal degradation. *Oncogene* **26**, 487-499.

Jha, S., Vande Pol, S., Banerjee, N. S., Dutta, A. B., Chow, L. T. & Dutta, A. (2010). Destabilization of TIP60 by human papillomavirus E6 results in attenuation of TIP60-dependent transcriptional regulation and apoptotic pathway. *Mol Cell* **38**, 700-711.

Jing, M., Bohl, J., Brimer, N., Kinter, M. & Vande Pol, S. B. (2007). Degradation of tyrosine phosphatase PTPN3 (PTPH1) by association with oncogenic human papillomavirus E6 proteins. *J Virol* **81**, 2231-2239.

Johnson, K. M., Kines, R. C., Roberts, J. N., Lowy, D. R., Schiller, J. T. & Day, P. M. (2009). Role of heparan sulfate in attachment to and infection of the murine female genital tract by human papillomavirus. *J Virol* **83**, 2067-2074.

Jones, D. L., Alani, R. M. & Munger, K. (1997). The human papillomavirus E7 oncoprotein can uncouple cellular differentiation and proliferation in human keratinocytes by abrogating p21Cip1-mediated inhibition of cdk2. *Genes Dev* **11**, 2101-2111.

Jones, D. L. & Munger, K. (1997). Analysis of the p53-mediated G1 growth arrest pathway in cells expressing the human papillomavirus type 16 E7 oncoprotein. *J Virol* **71**, 2905-2912.

Joyce, J. G., Tung, J. S., Przysiecki, C. T., Cook, J. C., Lehman, E. D., Sands, J. A., Jansen, K. U. & Keller, P. M. (1999). The L1 major capsid protein of human papillomavirus type 11 recombinant virus-like particles interacts with heparin and cell-surface glycosaminoglycans on human keratinocytes. *J Biol Chem* **274**, 5810-5822.

Kadaja, M., Silla, T., Ustav, E. & Ustav, M. (2009). Papillomavirus DNA replication - from initiation to genomic instability. *Virology* **384**, 360-368.

Kanda, T., Watanabe, S., Zanma, S., Sato, H., Furuno, A. & Yoshiike, K. (1991). Human papillomavirus type 16 E6 proteins with glycine substitution for cysteine in the metal-binding motif. *Virology* **185**, 536-543.

Kao, W. H., Beaudenon, S. L., Talis, A. L., Huibregtse, J. M. & Howley, P. M. (2000). Human papillomavirus type 16 E6 induces self-ubiquitination of the E6AP ubiquitin-protein ligase. *J Virol* **74**, 6408-6417.

Kehmeier, E., Ruhl, H., Voland, B., Stoppler, M. C., Androphy, E. & Stoppler, H. (2002). Cellular steady-state levels of "high risk" but not "low risk" human papillomavirus (HPV) E6 proteins are increased by inhibition of proteasome-dependent degradation independent of their p53- and E6AP-binding capabilities. *Virology* **299**, 72-87.

Kim, K. & Lambert, P. F. (2002). E1 protein of bovine papillomavirus 1 is not required for the maintenance of viral plasmid DNA replication. *Virology* **293**, 10-14.

Kiyono, T., Hiraiwa, A., Fujita, M., Hayashi, Y., Akiyama, T. & Ishibashi, M. (1997). Binding of high-risk human papillomavirus E6 oncoproteins to the human homologue of the Drosophila discs large tumor suppressor protein. *Proc Natl Acad Sci U S A* **94**, 11612-11616.

Kiyono, T., Foster, S. A., Koop, J. I., McDougall, J. K., Galloway, D. A. & Klingelhutz, A. J. (1998). Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. *Nature* **396**, 84-88.

Klingelhutz, A. J., Foster, S. A. & McDougall, J. K. (1996). Telomerase activation by the E6 gene product of human papillomavirus type 16. *Nature* **380**, 79-82.

Klumpp, D. J. & Laimins, L. A. (1999). Differentiation-induced changes in promoter usage for transcripts encoding the human papillomavirus type 31 replication protein E1. *Virology* **257**, 239-246.

Komarov, P. G., Komarova, E. A., Kondratov, R. V., Christov-Tselkov, K., Coon, J. S., Chernov, M. V. & Gudkov, A. V. (1999). A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. *Science* **285**, 1733-1737.

Kotelevets, L., van Hengel, J., Bruyneel, E., Mareel, M., van Roy, F. & Chastre, E. (2005). Implication of the MAGI-1b/PTEN signalosome in stabilization of adherens junctions and suppression of invasiveness. *Faseb J* **19**, 115-117.

Kranjec, C. & Banks, L. (2010). A systematic analysis of human papillomavirus (HPV) E6 PDZ substrates identifies MAGI-1 as a major target of HPV type 16 (HPV-16) and HPV-18 whose loss accompanies disruption of tight junctions. *J Virol* **85**, 1757-1764.

Kuhne, C. & Banks, L. (1998). E3-ubiquitin ligase/E6-AP links multicopy maintenance protein 7 to the ubiquitination pathway by a novel motif, the L2G box. *J Biol Chem* **273**, 34302-34309.

Kukimoto, I., Aihara, S., Yoshiike, K. & Kanda, T. (1998). Human papillomavirus oncoprotein E6 binds to the C-terminal region of human minichromosome maintenance 7 protein. *Biochem Biophys Res Commun* **249**, 258-262.

Kumar, A., Zhao, Y., Meng, G. & other authors (2002). Human papillomavirus oncoprotein E6 inactivates the transcriptional coactivator human ADA3. *Mol Cell Biol* **22**, 5801-5812.

Kurvinen, K., Syrjanen, K. & Syrjanen, S. (1996). p53 and bcl-2 proteins as prognostic markers in human papillomavirus-associated cervical lesions. *J Clin Oncol* **14**, 2120-2130.

Lace, M. J., Anson, J. R., Thomas, G. S., Turek, L. P. & Haugen, T. H. (2008). The E8--E2 gene product of human papillomavirus type 16 represses early transcription and replication but is dispensable for viral plasmid persistence in keratinocytes. *J Virol* **82**, 10841-10853.

Lace, M. J., Yamakawa, Y., Ushikai, M., Anson, J. R., Haugen, T. H. & Turek, L. P. (2009). Cellular factor YY1 downregulates the human papillomavirus 16 E6/E7 promoter, P97, in vivo and in vitro from a negative element overlapping the transcription-initiation site. *J Gen Virol* **90**, 2402-2412.

Laimins, L. A. (1993). The biology of human papillomaviruses: from warts to cancer. *Infect Agents Dis* **2**, 74-86.

Lambert, P. F., Ozbun, M. A., Collins, A., Holmgren, S., Lee, D. & Nakahara, T. (2005). Using an immortalized cell line to study the HPV life cycle in organotypic "raft" cultures. *Methods Mol Med* **119**, 141-155.

Lane, D. P. & Crawford, L. V. (1979). T antigen is bound to a host protein in SV40-transformed cells. *Nature* **278**, 261-263.

LaPorta, R. F. & Taichman, L. B. (1982). Human papilloma viral DNA replicates as a stable episome in cultured epidermal keratinocytes. *Proc Natl Acad Sci U S A* **79**, 3393-3397.

Laurson, J., Khan, S., Chung, R., Cross, K. & Raj, K. (2010). Epigenetic repression of E-cadherin by human papillomavirus 16 E7 protein. *Carcinogenesis* **31**, 918-926.

Lechner, M. S., Mack, D. H., Finicle, A. B., Crook, T., Vousden, K. H. & Laimins, L. A. (1992). Human papillomavirus E6 proteins bind p53 in vivo and abrogate p53-mediated repression of transcription. *EMBO J* **11**, 3045-3052.

Lee, C. & Laimins, L. A. (2004). Role of the PDZ domain-binding motif of the oncoprotein E6 in the pathogenesis of human papillomavirus type 31. *J Virol* **78**, 12366-12377.

Lee, C., Wooldridge, T. R. & Laimins, L. A. (2007). Analysis of the roles of E6 binding to E6TP1 and nuclear localization in the human papillomavirus type 31 life cycle. *Virology* **358**, 201-210.

Lee, S. S., Weiss, R. S. & Javier, R. T. (1997). Binding of human virus oncoproteins to hDlg/SAP97, a mammalian homolog of the Drosophila discs large tumor suppressor protein. *Proc Natl Acad Sci U S A* **94**, 6670-6675.

Lee, S. S., Glaunsinger, B., Mantovani, F., Banks, L. & Javier, R. T. (2000). Multi-PDZ domain protein MUPP1 is a cellular target for both adenovirus E4-ORF1 and high-risk papillomavirus type 18 E6 oncoproteins. *J Virol* **74**, 9680-9693.

Lehman, C. W. & Botchan, M. R. (1998). Segregation of viral plasmids depends on tethering to chromosomes and is regulated by phosphorylation. *Proc Natl Acad Sci U S A* **95**, 4338-4343.

Leone, G., DeGregori, J., Yan, Z., Jakoi, L., Ishida, S., Williams, R. S. & Nevins, J. R. (1998). E2F3 activity is regulated during the cell cycle and is required for the induction of S phase. *Genes Dev* **12**, 2120-2130.

Lepik, D., Ilves, I., Kristjuhan, A., Maimets, T. & Ustav, M. (1998). p53 protein is a suppressor of papillomavirus DNA amplificational replication. *J Virol* **72**, 6822-6831.

Li, S., Labrecque, S., Gauzzi, M. C., Cuddihy, A. R., Wong, A. H., Pellegrini, S., Matlashewski, G. J. & Koromilas, A. E. (1999). The human papilloma virus (HPV)-18 E6 oncoprotein physically associates with Tyk2 and impairs Jak-STAT activation by interferon-alpha. *Oncogene* **18**, 5727-5737.

Li, X. & Coffino, P. (1996). High-risk human papillomavirus E6 protein has two distinct binding sites within p53, of which only one determines degradation. *J Virol* **70**, 4509-4516.

Liang, F. & Jasin, M. (1996). Ku80-deficient cells exhibit excess degradation of extrachromosomal DNA. *J Biol Chem* **271**, 14405-14411.

Liang, X. H., Volkmann, M., Klein, R., Herman, B. & Lockett, S. J. (1993). Co-localization of the tumor-suppressor protein p53 and human papillomavirus E6 protein in human cervical carcinoma cell lines. *Oncogene* **8**, 2645-2652.

Lichtig, H., Gilboa, D. A., Jackman, A., Gonen, P., Levav-Cohen, Y., Haupt, Y. & Sherman, L. (2010). HPV16 E6 augments Wnt signaling in an E6AP-dependent manner. *Virology* **396**, 47-58.

Liu, J., Lian, Z., Han, S. & other authors (2006). Downregulation of E-cadherin by hepatitis B virus X antigen in hepatocellullar carcinoma. *Oncogene* **25**, 1008-1017.

Liu, X., Yuan, H., Fu, B. & other authors (2005). The E6AP ubiquitin ligase is required for transactivation of the hTERT promoter by the human papillomavirus E6 oncoprotein. *J Biol Chem* **280**, 10807-10816.

Liu, X., Dakic, A., Zhang, Y., Dai, Y., Chen, R. & Schlegel, R. (2009). HPV E6 protein interacts physically and functionally with the cellular telomerase complex. *Proc Natl Acad Sci U S A* **106**, 18780-18785.

Logan, C. Y. & Nusse, R. (2004). The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol* **20**, 781-810.

Longworth, M. S., Wilson, R. & Laimins, L. A. (2005). HPV31 E7 facilitates replication by activating E2F2 transcription through its interaction with HDACs. *EMBO J* **24**, 1821-1830.

Lue, R. A., Marfatia, S. M., Branton, D. & Chishti, A. H. (1994). Cloning and characterization of hdlg: the human homologue of the Drosophila discs large tumor suppressor binds to protein 4.1. *Proc Natl Acad Sci U S A* **91**, 9818-9822.

Lusky, M. & Botchan, M. R. (1986). Transient replication of bovine papilloma virus type 1 plasmids: cis and trans requirements. *Proc Natl Acad Sci U S A* **83**, 3609-3613.

Maglennon, G. A., McIntosh, P. & Doorbar, J. (2011). Persistence of viral DNA in the epithelial basal layer suggests a model for papillomavirus latency following immune regression. *Virology* **414**, 153-163.

Maldonado, E., Cabrejos, M. E., Banks, L. & Allende, J. E. (2002). Human papillomavirus-16 E7 protein inhibits the DNA interaction of the TATA binding transcription factor. *J Cell Biochem* **85**, 663-669.

Mantovani, F., Massimi, P. & Banks, L. (2001). Proteasome-mediated regulation of the hDlg tumour suppressor protein. *J Cell Sci* **114**, 4285-4292.

Marechal, V., Dehee, A., Chikhi-Brachet, R., Piolot, T., Coppey-Moisan, M. & Nicolas, J. C. (1999). Mapping EBNA-1 domains involved in binding to metaphase chromosomes. *J Virol* **73**, 4385-4392.

Marshall, T., Pater, A. & Pater, M. M. (1989). Trans-regulation and differential cell specificity of human papillomavirus types 16, 18, and 11 cis-acting elements. *J Med Virol* **29**, 115-126.

Marte, B. M. & Downward, J. (1997). PKB/Akt: connecting phosphoinositide 3-kinase to cell survival and beyond. *Trends Biochem Sci* **22**, 355-358.

Massimi, P., Pim, D., Storey, A. & Banks, L. (1996). HPV-16 E7 and adenovirus E1a complex formation with TATA box binding protein is enhanced by casein kinase II phosphorylation. *Oncogene* **12**, 2325-2330.

Massimi, P., Pim, D. & Banks, L. (1997). Human papillomavirus type 16 E7 binds to the conserved carboxy-terminal region of the TATA box binding protein and this contributes to E7 transforming activity. *J Gen Virol* **78 (Pt 10)**, 2607-2613.

Massimi, P., Pim, D., Bertoli, C., Bouvard, V. & Banks, L. (1999). Interaction between the HPV-16 E2 transcriptional activator and p53. *Oncogene* **18**, 7748-7754.

Massimi, P., Gammoh, N., Thomas, M. & Banks, L. (2004). HPV E6 specifically targets different cellular pools of its PDZ domain-containing tumour suppressor substrates for proteasome-mediated degradation. *Oncogene* **23**, 8033-8039.

Massimi, P., Narayan, N., Cuenda, A. & Banks, L. (2006). Phosphorylation of the discs large tumour suppressor protein controls its membrane localisation and enhances its susceptibility to HPV E6-induced degradation. *Oncogene* **25**, 4276-4285.

Massimi, P., Shai, A., Lambert, P. & Banks, L. (2008). HPV E6 degradation of p53 and PDZ containing substrates in an E6AP null background. *Oncogene* **27**, 1800-1804.

Matsumine, A., Ogai, A., Senda, T. & other authors (1996). Binding of APC to the human homolog of the Drosophila discs large tumor suppressor protein. *Science* **272**, 1020-1023.

Matter, K. & Balda, M. S. (2003). Signalling to and from tight junctions. *Nat Rev Mol Cell Biol* **4**, 225-236.

Matthews, K., Leong, C. M., Baxter, L., Inglis, E., Yun, K., Backstrom, B. T., Doorbar, J. & Hibma, M. (2003). Depletion of Langerhans cells in human papillomavirus type 16-infected skin is associated with E6-mediated down regulation of E-cadherin. *J Virol* **77**, 8378-8385.

McIntosh, P. B., Martin, S. R., Jackson, D. J. & other authors (2008). Structural analysis reveals an amyloid form of the human papillomavirus type 16 E1--E4 protein and provides a molecular basis for its accumulation. *J Virol* **82**, 8196-8203.

McLaughlin-Drubin, M. E., Christensen, N. D. & Meyers, C. (2004). Propagation, infection, and neutralization of authentic HPV16 virus. *Virology* **322**, 213-219.

McLaughlin-Drubin, M. E. & Munger, K. (2009). The human papillomavirus E7 oncoprotein. *Virology* **384**, 335-344.

McLaughlin, M., Hale, R., Ellston, D., Gaudet, S., Lue, R. A. & Viel, A. (2002). The distribution and function of alternatively spliced insertions in hDlg. *J Biol Chem* **277**, 6406-6412.

McPhillips, M. G., Veerapraditsin, T., Cumming, S. A., Karali, D., Milligan, S. G., Boner, W., Morgan, I. M. & Graham, S. V. (2004). SF2/ASF binds the human papillomavirus type 16 late RNA control element and is regulated during differentiation of virus-infected epithelial cells. *J Virol* **78**, 10598-10605.

McPhillips, M. G., Oliveira, J. G., Spindler, J. E., Mitra, R. & McBride, A. A. (2006). Brd4 is required for e2-mediated transcriptional activation but not genome partitioning of all papillomaviruses. *J Virol* **80**, 9530-9543.

Middleton, K., Peh, W., Southern, S. & other authors (2003). Organization of human papillomavirus productive cycle during neoplastic progression provides a basis for selection of diagnostic markers. *J Virol* **77**, 10186-10201.

Milligan, S. G., Veerapraditsin, T., Ahamet, B., Mole, S. & Graham, S. V. (2007). Analysis of novel human papillomavirus type 16 late mRNAs in differentiated W12 cervical epithelial cells. *Virology* **360**, 172-181.

Mole, S., McFarlane, M., Chuen-Im, T., Milligan, S. G., Millan, D. & Graham, S. V. (2009a). RNA splicing factors regulated by HPV16 during cervical tumour progression. *J Pathol* **219**, 383-391.

Mole, S., Milligan, S. G. & Graham, S. V. (2009b). Human papillomavirus type 16 E2 protein transcriptionally activates the promoter of a key cellular splicing factor, SF2/ASF. *J Virol* **83**, 357-367.

Moody, C. A. & Laimins, L. A. (2009). Human papillomaviruses activate the ATM DNA damage pathway for viral genome amplification upon differentiation. *PLoS pathogens* **5**, e1000605.

Morris, P. J., Dent, C. L., Ring, C. J. & Latchman, D. S. (1993). The octamer binding site in the HPV16 regulatory region produces opposite effects on gene expression in cervical and non-cervical cells. *Nucleic Acids Res* **21**, 1019-1023.

Mounts, P., Shah, K. V. & Kashima, H. (1982). Viral etiology of juvenile- and adult-onset squamous papilloma of the larynx. *Proc Natl Acad Sci U S A* **79**, 5425-5429.

Munger, K., Phelps, W. C., Bubb, V., Howley, P. M. & Schlegel, R. (1989a). The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. *J Virol* **63**, 4417-4421.

Munger, K., Werness, B. A., Dyson, N., Phelps, W. C., Harlow, E. & Howley, P. M. (1989b). Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. *EMBO Jl* **8**, 4099-4105.

Munoz, N., Bosch, F. X., de Sanjose, S., Herrero, R., Castellsague, X., Shah, K. V., Snijders, P. J. & Meijer, C. J. (2003). Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med* **348**, 518-527.

Nagasaka, K., Nakagawa, S., Yano, T. & other authors (2006). Human homolog of Drosophila tumor suppressor Scribble negatively regulates cell-cycle progression from G1 to S phase by localizing at the basolateral membrane in epithelial cells. *Cancer Sci* **97**, 1217-1225.

Nakagawa, S. & Huibregtse, J. M. (2000). Human scribble (Vartul) is targeted for ubiquitin-mediated degradation by the high-risk papillomavirus E6 proteins and the E6AP ubiquitin-protein ligase. *Mol Cell Biol* **20**, 8244-8253.

Nakahara, T., Peh, W. L., Doorbar, J., Lee, D. & Lambert, P. F. (2005). Human papillomavirus type 16 E1circumflexE4 contributes to multiple facets of the papillomavirus life cycle. *J Virol* **79**, 13150-13165.

Narayan, N., Massimi, P. & Banks, L. (2009a). CDK phosphorylation of the discs large tumour suppressor controls its localisation and stability. *J Cell Sci* **122**, 65-74.

Narayan, N., Subbaiah, V. K. & Banks, L. (2009b). The high-risk HPV E6 oncoprotein preferentially targets phosphorylated nuclear forms of hDlg. *Virology* **387**, 1-4.

Navarro, C., Nola, S., Audebert, S. & other authors (2005). Junctional recruitment of mammalian Scribble relies on E-cadherin engagement. *Oncogene* **24**, 4330-4339.

Nees, M., Geoghegan, J. M., Hyman, T., Frank, S., Miller, L. & Woodworth, C. D. (2001). Papillomavirus type 16 oncogenes downregulate expression of interferon-responsive genes and upregulate proliferation-associated and NF-kappaB-responsive genes in cervical keratinocytes. *J Virol* **75**, 4283-4296.

Nguyen, C. L. & Munger, K. (2008). Direct association of the HPV16 E7 oncoprotein with cyclin A/CDK2 and cyclin E/CDK2 complexes. *Virology* **380**, 21-25.

Nguyen, M. L., Nguyen, M. M., Lee, D., Griep, A. E. & Lambert, P. F. (2003). The PDZ ligand domain of the human papillomavirus type 16 E6 protein is required for E6's induction of epithelial hyperplasia in vivo. *J Virol* **77**, 6957-6964.

Nicolaides, L., Davy, C., Raj, K., Kranjec, C., Banks, L. & Doorbar, J. (2011). Stabilization of HPV16 E6 protein by PDZ proteins, and potential implications for genome maintenance. *Virology* **414**, 137-145.

Obenauer, J. C., Denson, J., Mehta, P. K. & other authors (2006). Large-scale sequence analysis of avian influenza isolates. *Science* **311**, 1576-1580.

Offord, E. A., Chappuis, P. O. & Beard, P. (1993). Different stability of AP1 proteins in human keratinocyte and fibroblast cells: possible role in the cell-type specific expression of human papillomavirus type 18 genes. *Carcinogenesis* **14**, 2447-2455.

Oh, S. T., Longworth, M. S. & Laimins, L. A. (2004). Roles of the E6 and E7 proteins in the life cycle of low-risk human papillomavirus type 11. *J Virol* **78**, 2620-2626.

Oliveira, J. G., Colf, L. A. & McBride, A. A. (2006). Variations in the association of papillomavirus E2 proteins with mitotic chromosomes. *Proc Natl Acad Sci U S A* **103**, 1047-1052.

Oren, M., Maltzman, W. & Levine, A. J. (1981). Post-translational regulation of the 54K cellular tumor antigen in normal and transformed cells. *Mol Cell Biol* **1**, 101-110.

Ozbun, M. A. & Meyers, C. (1997). Characterization of late gene transcripts expressed during vegetative replication of human papillomavirus type 31b. *J Virol* **71**, 5161-5172.

Ozbun, M. A. & Meyers, C. (1999). Two novel promoters in the upstream regulatory region of human papillomavirus type 31b are negatively regulated by epithelial differentiation. *J Virol* **73**, 3505-3510.

Palefsky, J. M., Gillison, M. L. & Strickler, H. D. (2006). Chapter 16: HPV vaccines in immunocompromised women and men. *Vaccine* **24 Suppl 3**, S3/140-146.

Parish, J. L., Bean, A. M., Park, R. B. & Androphy, E. J. (2006a). ChlR1 is required for loading papillomavirus E2 onto mitotic chromosomes and viral genome maintenance. *Mol Cell* **24**, 867-876.

Parish, J. L., Kowalczyk, A., Chen, H. T., Roeder, G. E., Sessions, R., Buckle, M. & Gaston, K. (2006b). E2 proteins from high- and low-risk human papillomavirus types differ in their ability to bind p53 and induce apoptotic cell death. *J Virol* **80**, 4580-4590.

Parish, J. L., Rosa, J., Wang, X., Lahti, J. M., Doxsey, S. J. & Androphy, E. J. (2006c). The DNA helicase ChlR1 is required for sister chromatid cohesion in mammalian cells. *J Cell Sci* **119**, 4857-4865.

Park, J. S., Kim, E. J., Kwon, H. J., Hwang, E. S., Namkoong, S. E. & Um, S. J. (2000). Inactivation of interferon regulatory factor-1 tumor suppressor protein by HPV E7 oncoprotein. Implication for the E7-mediated immune evasion mechanism in cervical carcinogenesis. *J Biol Chem* **275**, 6764-6769.

Park, P., Copeland, W., Yang, L., Wang, T., Botchan, M. R. & Mohr, I. J. (1994). The cellular DNA polymerase alpha-primase is required for

papillomavirus DNA replication and associates with the viral E1 helicase. *Proc Natl Acad Sci U S A* **91**, 8700-8704.

Park, R. B. & Androphy, E. J. (2002). Genetic analysis of high-risk e6 in episomal maintenance of human papillomavirus genomes in primary human keratinocytes. *J Virol* **76**, 11359-11364.

Patel, D., Huang, S. M., Baglia, L. A. & McCance, D. J. (1999). The E6 protein of human papillomavirus type 16 binds to and inhibits co-activation by CBP and p300. *EMBO J* **18**, 5061-5072.

Pecoraro, G., Morgan, D. & Defendi, V. (1989). Differential effects of human papillomavirus type 6, 16, and 18 DNAs on immortalization and transformation of human cervical epithelial cells. *Proc Natl Acad Sci U S A* **86**, 563-567.

Peden, K. W., Pipas, J. M., Pearson-White, S. & Nathans, D. (1980). Isolation of mutants of an animal virus in bacteria. *Science* **209**, 1392-1396.

Peh, W. L., Brandsma, J. L., Christensen, N. D., Cladel, N. M., Wu, X. & Doorbar, J. (2004). The viral E4 protein is required for the completion of the cottontail rabbit papillomavirus productive cycle in vivo. *J Virol* **78**, 2142-2151.

Peto, J., Gilham, C., Fletcher, O. & Matthews, F. E. (2004). The cervical cancer epidemic that screening has prevented in the UK. *Lancet* **364**, 249-256.

Petti, L., Sample, C. & Kieff, E. (1990). Subnuclear localization and phosphorylation of Epstein-Barr virus latent infection nuclear proteins. *Virology* **176**, 563-574.

Pfister, H. (2003). Chapter 8: Human papillomavirus and skin cancer. *J Natl Cancer Inst Monogr*, 52-56.

Phelps, W. C. & Howley, P. M. (1987). Transcriptional trans-activation by the human papillomavirus type 16 E2 gene product. *J Virol* **61**, 1630-1638.

Piirsoo, M., Ustav, E., Mandel, T., Stenlund, A. & Ustav, M. (1996). Cis and trans requirements for stable episomal maintenance of the BPV-1 replicator. *EMBO J* **15**, 1-11.

Pim, D., Massimi, P. & Banks, L. (1997). Alternatively spliced HPV-18 E6* protein inhibits E6 mediated degradation of p53 and suppresses transformed cell growth. *Oncogene* **15**, 257-264.

Pim, D., Thomas, M., Javier, R., Gardiol, D. & Banks, L. (2000). HPV E6 targeted degradation of the discs large protein: evidence for the involvement of a novel ubiquitin ligase. *Oncogene* **19**, 719-725.

Pim, D., Tomaic, V. & Banks, L. (2009). The human papillomavirus (HPV) E6* proteins from high-risk, mucosal HPVs can direct degradation of cellular proteins in the absence of full-length E6 protein. *J Virol* **83**, 9863-9874.

Pittayakhajonwut, D. & Angeletti, P. C. (2010). Viral trans-factor independent replication of human papillomavirus genomes. *Virol J* **7**, 123.

Potten, C. S. & Loeffler, M. (1990). Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. *Development* **110**, 1001-1020.

Praetorius, F. (1997). HPV-associated diseases of oral mucosa. *Clin Dermatol* **15**, 399-413.

Rasheed, S., Nelson-Rees, W. A., Toth, E. M., Arnstein, P. & Gardner, M. B. (1974). Characterization of a newly derived human sarcoma cell line (HT-1080). *Cancer* **33**, 1027-1033.

Remm, M., Brain, R. & Jenkins, J. R. (1992). The E2 binding sites determine the efficiency of replication for the origin of human papillomavirus type 18. *Nucleic Acids Res* **20**, 6015-6021.

Remmink, A. J., Walboomers, J. M., Helmerhorst, T. J., Voorhorst, F. J., Rozendaal, L., Risse, E. K., Meijer, C. J. & Kenemans, P. (1995). The presence of persistent high-risk HPV genotypes in dysplastic cervical lesions is associated with progressive disease: natural history up to 36 months. *Int J Cancer* **61**, 306-311.

Reuver, S. M. & Garner, C. C. (1998). E-cadherin mediated cell adhesion recruits SAP97 into the cortical cytoskeleton. *J Cell Sci* **111 (Pt 8)**, 1071-1080.

Richards, R. M., Lowy, D. R., Schiller, J. T. & Day, P. M. (2006). Cleavage of the papillomavirus minor capsid protein, L2, at a furin consensus site is necessary for infection. *Proc Natl Acad Sci U S A* **103**, 1522-1527.

Ristriani, T., Masson, M., Nomine, Y., Laurent, C., Lefevre, J. F., Weiss, E. & Trave, G. (2000). HPV oncoprotein E6 is a structure-dependent DNA-binding protein that recognizes four-way junctions. *J Mol Biol* **296**, 1189-1203.

Roberts, J. M. & Weintraub, H. (1988). Cis-acting negative control of DNA replication in eukaryotic cells. *Cell* **52**, 397-404.

Roberts, S., Calautti, E., Vanderweil, S., Nguyen, H. O., Foley, A., Baden, H. P. & Viel, A. (2007). Changes in localization of human discs large (hDlg) during keratinocyte differentiation are [corrected] associated with expression of alternatively spliced hDlg variants. *Exp Cell Res* **313**, 2521-2530.

Romanczuk, H., Thierry, F. & Howley, P. M. (1990). Mutational analysis of cis elements involved in E2 modulation of human papillomavirus type 16 P97 and type 18 P105 promoters. *J Virol* **64**, 2849-2859.

Ronco, L. V., Karpova, A. Y., Vidal, M. & Howley, P. M. (1998). Human papillomavirus 16 E6 oncoprotein binds to interferon regulatory factor-3 and inhibits its transcriptional activity. *Genes Dev* **12**, 2061-2072.

Rosenberger, S., De-Castro Arce, J., Langbein, L., Steenbergen, R. D. & Rosl, F. (2010). Alternative splicing of human papillomavirus type-16 E6/E6*

early mRNA is coupled to EGF signaling via Erk1/2 activation. *Proc Natl Acad Sci U S A* **107**, 7006-7011.

Rotenberg, M. O., Chiang, C. M., Ho, M. L., Broker, T. R. & Chow, L. T. (1989). Characterization of cDNAs of spliced HPV-11 E2 mRNA and other HPV mRNAs recovered via retrovirus-mediated gene transfer. *Virology* **172**, 468-477.

Ruesch, M. N., Stubenrauch, F. & Laimins, L. A. (1998). Activation of papillomavirus late gene transcription and genome amplification upon differentiation in semisolid medium is coincident with expression of involucrin and transglutaminase but not keratin-10. *J Virol* **72**, 5016-5024.

Sakurai, T., Suzuki, M., Sawazaki, T., Ishii, S. & Yoshida, S. (1994). Anti-oncogene product p53 binds DNA helicase. *Exp Cell Res* **215**, 57-62.

Sarnow, P., Ho, Y. S., Williams, J. & Levine, A. J. (1982). Adenovirus E1b-58kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54 kd cellular protein in transformed cells. *Cell* **28**, 387-394.

Scheffner, M., Werness, B. A., Huibregtse, J. M., Levine, A. J. & Howley, P. M. (1990). The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* **63**, 1129-1136.

Scheffner, M., Munger, K., Byrne, J. C. & Howley, P. M. (1991). The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. *Proc Natl Acad Sci U S A* **88**, 5523-5527.

Scheffner, M., Huibregtse, J. M., Vierstra, R. D. & Howley, P. M. (1993). The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. *Cell* **75**, 495-505.

Schiffman, M. & Kjaer, S. K. (2003). Chapter 2: Natural history of anogenital human papillomavirus infection and neoplasia. *J Natl Cancer Inst Monogr*, 14-19.

Schiller, J. T., Day, P. M. & Kines, R. C. (2010). Current understanding of the mechanism of HPV infection. *Gynecol Oncol* **118**, S12-17.

Schlegel, R., Phelps, W. C., Zhang, Y. L. & Barbosa, M. (1988). Quantitative keratinocyte assay detects two biological activities of human papillomavirus DNA and identifies viral types associated with cervical carcinoma. *EMBO J* **7**, 3181-3187.

Schmidt, M. T., Olejnik, A. K. & Gozdzicka-Jozefiak, A. (2005). The HPV16 E2 transcriptional regulator mode of action depends on the physical state of the viral genome. *Acta Biochim Pol* **52**, 823-832.

Schmitt, A., Rochat, A., Zeltner, R., Borenstein, L., Barrandon, Y., Wettstein, F. O. & Iftner, T. (1996). The primary target cells of the high-risk cottontail rabbit papillomavirus colocalize with hair follicle stem cells. *J Virol* **70**, 1912-1922.

Schneider-Gadicke, A. & Schwarz, E. (1986). Different human cervical carcinoma cell lines show similar transcription patterns of human papillomavirus type 18 early genes. *EMBO J* **5**, 2285-2292.

Schneider-Gadicke, A., Kaul, S., Schwarz, E., Gausepohl, H., Frank, R. & Bastert, G. (1988). Identification of the human papillomavirus type 18 E6 and E6 proteins in nuclear protein fractions from human cervical carcinoma cells grown in the nude mouse or in vitro. *Cancer Res* **48**, 2969-2974.

Sekaric, P., Cherry, J. J. & Androphy, E. J. (2008). Binding of human papillomavirus type 16 E6 to E6AP is not required for activation of hTERT. *J Virol* **82**, 71-76.

Sherman, L., Alloul, N., Golan, I., Durst, M. & Baram, A. (1992). Expression and splicing patterns of human papillomavirus type-16 mRNAs in pre-cancerous lesions and carcinomas of the cervix, in human keratinocytes immortalized by HPV 16, and in cell lines established from cervical cancers. *Int J Cancer* **50**, 356-364.

Sherman, L. & Schlegel, R. (1996). Serum- and calcium-induced differentiation of human keratinocytes is inhibited by the E6 oncoprotein of human papillomavirus type 16. *J Virol* **70**, 3269-3279.

Sherr, C. J. (1993). Mammalian G1 cyclins. *Cell* **73**, 1059-1065.

Shope, R. E. & Hurst, E. W. (1933). Infectious Papillomatosis of Rabbits : With a Note on the Histopathology. *J Exp Med* **58**, 607-624.

Skiadopoulos, M. H. & McBride, A. A. (1998). Bovine papillomavirus type 1 genomes and the E2 transactivator protein are closely associated with mitotic chromatin. *J Virol* **72**, 2079-2088.

Smotkin, D. & Wettstein, F. O. (1986). Transcription of human papillomavirus type 16 early genes in a cervical cancer and a cancer-derived cell line and identification of the E7 protein. *Proc Natl Acad Sci U S A* **83**, 4680-4684.

Smotkin, D., Prokoph, H. & Wettstein, F. O. (1989). Oncogenic and nononcogenic human genital papillomaviruses generate the E7 mRNA by different mechanisms. *J Virol* **63**, 1441-1447.

Snapka, R. M., Powelson, M. A. & Strayer, J. M. (1988). Swiveling and decatenation of replicating simian virus 40 genomes in vivo. *Mol Cell Biol* **8**, 515-521.

Song, S., Pitot, H. C. & Lambert, P. F. (1999). The human papillomavirus type 16 E6 gene alone is sufficient to induce carcinomas in transgenic animals. *J Virol* **73**, 5887-5893.

Spalholz, B. A., Yang, Y. C. & Howley, P. M. (1985). Transactivation of a bovine papilloma virus transcriptional regulatory element by the E2 gene product. *Cell* **42**, 183-191.

Spalholz, B. A., Lambert, P. F., Yee, C. L. & Howley, P. M. (1987). Bovine papillomavirus transcriptional regulation: localization of the E2-responsive elements of the long control region. *J Virol* **61**, 2128-2137.

Spanos, W. C., Hoover, A., Harris, G. F. & other authors (2008). The PDZ binding motif of human papillomavirus type 16 E6 induces PTPN13 loss, which allows anchorage-independent growth and synergizes with ras for invasive growth. *J Virol* **82**, 2493-2500.

Spink, K. M. & Laimins, L. A. (2005). Induction of the human papillomavirus type 31 late promoter requires differentiation but not DNA amplification. *J Virol* **79**, 4918-4926.

Srivenugopal, K. S. & Ali-Osman, F. (2002). The DNA repair protein, O(6)-methylguanine-DNA methyltransferase is a proteolytic target for the E6 human papillomavirus oncoprotein. *Oncogene* **21**, 5940-5945.

Stambolic, V., Suzuki, A., de la Pompa, J. L. & other authors (1998). Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* **95**, 29-39.

Stanley, M. A., Browne, H. M., Appleby, M. & Minson, A. C. (1989). Properties of a non-tumorigenic human cervical keratinocyte cell line. *Int J Cancer* **43**, 672-676.

Stanley, M. A. (2009). Immune responses to human papilloma viruses. *The Indian J Med Res* **130**, 266-276.

Steger, G. & Corbach, S. (1997). Dose-dependent regulation of the early promoter of human papillomavirus type 18 by the viral E2 protein. *J Virol* **71**, 50-58.

Steinberg, B. M., Topp, W. C., Schneider, P. S. & Abramson, A. L. (1983). Laryngeal papillomavirus infection during clinical remission. *N Engl J Med* **308**, 1261-1264.

Stewart, A. C., Jareborg, N., Simonsson, M., Alderborn, A. & Burnett, S. (1994). Segregation properties of bovine papillomaviral plasmid DNA. *J Mol Biol* **236**, 480-490.

Stewart, D., Kazemi, S., Li, S., Massimi, P., Banks, L., Koromilas, A. E. & Matlashewski, G. (2004). Ubiquitination and proteasome degradation of the E6 proteins of human papillomavirus types 11 and 18. *J Gen Virol* **85**, 1419-1426.

Stoler, M. H. & Broker, T. R. (1986). In situ hybridization detection of human papillomavirus DNAs and messenger RNAs in genital condylomas and a cervical carcinoma. *Hum Pathol* **17**, 1250-1258.

Stoppler, H., Stoppler, M. C., Johnson, E., Simbulan-Rosenthal, C. M., Smulson, M. E., Iyer, S., Rosenthal, D. S. & Schlegel, R. (1998). The E7 protein of human papillomavirus type 16 sensitizes primary human keratinocytes to apoptosis. *Oncogene* **17**, 1207-1214.

Storrs, C. H. & Silverstein, S. J. (2007). PATJ, a tight junction-associated PDZ protein, is a novel degradation target of high-risk human papillomavirus E6 and the alternatively spliced isoform 18 E6. *J Virol* **81**, 4080-4090.

Stubenrauch, F., Hummel, M., Iftner, T. & Laimins, L. A. (2000). The E8E2C protein, a negative regulator of viral transcription and replication, is required for extrachromosomal maintenance of human papillomavirus type 31 in keratinocytes. *J Virol* **74**, 1178-1186.

Sturzbecher, H. W., Brain, R., Maimets, T., Addison, C., Rudge, K. & Jenkins, J. R. (1988). Mouse p53 blocks SV40 DNA replication in vitro and downregulates T antigen DNA helicase activity. *Oncogene* **3**, 405-413.

Sun, L., Zhang, G., Lei, T., Huang, C., Song, T. & Si, L. (2008). Two different HPV-11E6 fusion proteins trap p53 in the cytoplasm and induce apoptosis. *Cancer Biol Ther* **7**, 1909-1915.

Sun, Q., Tsutsumi, K., Kelleher, M. B., Pater, A. & Pater, M. M. (1992). Squamous metaplasia of normal and carcinoma in situ of HPV 16-immortalized human endocervical cells. *Cancer Res* **52**, 4254-4260.

Sun, Y., Jiang, X., Chen, S., Fernandes, N. & Price, B. D. (2005). A role for the Tip60 histone acetyltransferase in the acetylation and activation of ATM. *Proc Natl Acad Sci U S A* **102**, 13182-13187.

Sverdrup, F. & Khan, S. A. (1994). Replication of human papillomavirus (HPV) DNAs supported by the HPV type 18 E1 and E2 proteins. *J Virol* **68**, 505-509.

Sykes, S. M., Mellert, H. S., Holbert, M. A., Li, K., Marmorstein, R., Lane, W. S. & McMahon, S. B. (2006). Acetylation of the p53 DNA-binding domain regulates apoptosis induction. *Mol Cell* **24**, 841-851.

Takizawa, S., Nagasaka, K., Nakagawa, S. & other authors (2006). Human scribble, a novel tumor suppressor identified as a target of high-risk HPV E6 for ubiquitin-mediated degradation, interacts with adenomatous polyposis coli. *Genes Cells* **11**, 453-464.

Tang, S., Tao, M., McCoy, J. P., Jr. & Zheng, Z. M. (2006a). The E7 oncoprotein is translated from spliced E6*I transcripts in high-risk human papillomavirus type 16- or type 18-positive cervical cancer cell lines via translation reinitiation. *J Virol* **80**, 4249-4263.

Tang, Y., Luo, J., Zhang, W. & Gu, W. (2006b). Tip60-dependent acetylation of p53 modulates the decision between cell-cycle arrest and apoptosis. *Mol Cell* **24**, 827-839.

Tao, M., Kruhlak, M., Xia, S., Androphy, E. & Zheng, Z. M. (2003). Signals that dictate nuclear localization of human papillomavirus type 16 oncoprotein E6 in living cells. *J Virol* **77**, 13232-13247.

Taylor, E. R. & Morgan, I. M. (2003). A novel technique with enhanced detection and quantitation of HPV-16 E1- and E2-mediated DNA replication. *Virology* **315**, 103-109.

Terenzi, F., Saikia, P. & Sen, G. C. (2008). Interferon-inducible protein, P56, inhibits HPV DNA replication by binding to the viral protein E1. *EMBO J* **27**, 3311-3321.

Thomas, J. T., Hubert, W. G., Ruesch, M. N. & Laimins, L. A. (1999). Human papillomavirus type 31 oncoproteins E6 and E7 are required for the maintenance of episomes during the viral life cycle in normal human keratinocytes. *Proc Natl Acad Sci U S A* **96**, 8449-8454.

Thomas, M. & Banks, L. (1998). Inhibition of Bak-induced apoptosis by HPV-18 E6. *Oncogene* **17**, 2943-2954.

Thomas, M. & Banks, L. (1999). Human papillomavirus (HPV) E6 interactions with Bak are conserved amongst E6 proteins from high and low risk HPV types. *J Gen Virol* **80 (Pt 6)**, 1513-1517.

Thomas, M., Glaunsinger, B., Pim, D., Javier, R. & Banks, L. (2001). HPV E6 and MAGUK protein interactions: determination of the molecular basis for specific protein recognition and degradation. *Oncogene* **20**, 5431-5439.

Thomas, M., Laura, R., Hepner, K., Guccione, E., Sawyers, C., Lasky, L. & Banks, L. (2002). Oncogenic human papillomavirus E6 proteins target the MAGI-2 and MAGI-3 proteins for degradation. *Oncogene* **21**, 5088-5096.

Thomas, M., Massimi, P., Navarro, C., Borg, J. P. & Banks, L. (2005). The hScrib/Dlg apico-basal control complex is differentially targeted by HPV-16 and HPV-18 E6 proteins. *Oncogene* **24**, 6222-6230.

Thomas, M., Narayan, N., Pim, D., Tomaic, V., Massimi, P., Nagasaka, K., Kranjec, C., Gammoh, N. & Banks, L. (2008). Human papillomaviruses, cervical cancer and cell polarity. *Oncogene* **27**, 7018-7030.

Todaro, G. J. & Green, H. (1963). Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *J Cell Biol* **17**, 299-313.

Tomaic, V., Gardiol, D., Massimi, P., Ozbun, M., Myers, M. & Banks, L. (2009a). Human and primate tumour viruses use PDZ binding as an evolutionarily conserved mechanism of targeting cell polarity regulators. *Oncogene* **28**, 1-8.

Tomaic, V., Pim, D. & Banks, L. (2009b). The stability of the human papillomavirus E6 oncoprotein is E6AP dependent. *Virology* **393**, 7-10.

Tomaic, V., Pim, D., Thomas, M., Massimi, P., Myers, M. P. & Banks, L. (2011). Regulation of the HPV-18 E6/E6AP Ubiquitin Ligase Complex by the HECT domain-containing protein EDD. *J Virol* **85**, 3120-3127.

Tommasino, M., Adamczewski, J. P., Carlotti, F., Barth, C. F., Manetti, R., Contorni, M., Cavalieri, F., Hunt, T. & Crawford, L. (1993). HPV16 E7 protein associates with the protein kinase p33CDK2 and cyclin A. *Oncogene* **8**, 195-202.

Tong, X. & Howley, P. M. (1997). The bovine papillomavirus E6 oncoprotein interacts with paxillin and disrupts the actin cytoskeleton. *Proc Natl Acad Sci U S A* **94**, 4412-4417.

Tosi, P., Pallini, V., Cintorino, M. & other authors (1993). Use of antibodies against a synthetic peptide of the E6 protein of human papillomavirus (HPV) type 16 for the diagnosis of genital HPV lesions. *Cytopathology* **4**, 3-15.

Tsunoda, S., Sierralta, J., Sun, Y., Bodner, R., Suzuki, E., Becker, A., Socolich, M. & Zuker, C. S. (1997). A multivalent PDZ-domain protein assembles signalling complexes in a G-protein-coupled cascade. *Nature* **388**, 243-249.

Turek, L. P., Byrne, J. C., Lowy, D. R., Dvoretzky, I., Friedman, R. M. & Howley, P. M. (1982). Interferon induces morphologic reversion with elimination of extrachromosomal viral genomes in bovine papillomavirus-transformed mouse cells. *Proc Natl Acad Sci U S A* **79**, 7914-7918.

Tuting, T., Gambotto, A., Robbins, P. D., Storkus, W. J. & DeLeo, A. B. (1999). Co-delivery of T helper 1-biasing cytokine genes enhances the efficacy of gene gun immunization of mice: studies with the model tumor antigen beta-galactosidase and the BALB/c Meth A p53 tumor-specific antigen. *Gene Ther* **6**, 629-636.

Valiente, M., Andres-Pons, A., Gomar, B., Torres, J., Gil, A., Tapparel, C., Antonarakis, S. E. & Pulido, R. (2005). Binding of PTEN to specific PDZ domains contributes to PTEN protein stability and phosphorylation by microtubule-associated serine/threonine kinases. *J Biol Chem* **280**, 28936-28943.

van den Heuvel, S. J., van Laar, T., The, I. & van der Eb, A. J. (1993). Large E1B proteins of adenovirus types 5 and 12 have different effects on p53 and distinct roles in cell transformation. *J Virol* **67**, 5226-5234.

Van Tine, B. A., Dao, L. D., Wu, S. Y., Sonbuchner, T. M., Lin, B. Y., Zou, N., Chiang, C. M., Broker, T. R. & Chow, L. T. (2004). Human papillomavirus (HPV) origin-binding protein associates with mitotic spindles to enable viral DNA partitioning. *Proc Natl Acad Sci U S A* **101**, 4030-4035.

Veldman, T., Horikawa, I., Barrett, J. C. & Schlegel, R. (2001). Transcriptional activation of the telomerase hTERT gene by human papillomavirus type 16 E6 oncoprotein. *J Virol* **75**, 4467-4472.

Vogelstein, B., Lane, D. & Levine, A. J. (2000). Surfing the p53 network. *Nature* **408**, 307-310.

Vos, R. M., Altreuter, J., White, E. A. & Howley, P. M. (2009). The ubiquitin-specific peptidase USP15 regulates human papillomavirus type 16 E6 protein stability. *J Virol* **83**, 8885-8892.

Wai, L. K. (2004). Telomeres, telomerase, and tumorigenesis--a review. *MedGenMed* **6**, 19.

Walboomers, J. M., Jacobs, M. V., Manos, M. M. & other authors (1999). Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J Pathol* **189**, 12-19.

Wang, E. H., Friedman, P. N. & Prives, C. (1989). The murine p53 protein blocks replication of SV40 DNA in vitro by inhibiting the initiation functions of SV40 large T antigen. *Cell* **57**, 379-392.

Wang, H. K., Duffy, A. A., Broker, T. R. & Chow, L. T. (2009). Robust production and passaging of infectious HPV in squamous epithelium of primary human keratinocytes. *Genes Dev* **23**, 181-194.

Wang, Q., Griffin, H., Southern, S. & other authors (2004). Functional analysis of the human papillomavirus type 16 E1=E4 protein provides a mechanism for in vivo and in vitro keratin filament reorganization. *J Virol* **78**, 821-833.

Watson, R. A., Rollason, T. P., Reynolds, G. M., Murray, P. G., Banks, L. & Roberts, S. (2002). Changes in expression of the human homologue of the Drosophila discs large tumour suppressor protein in high-grade premalignant cervical neoplasias. *Carcinogenesis* **23**, 1791-1796.

Welters, M. J., de Jong, A., van den Eeden, S. J. & other authors (2003). Frequent display of human papillomavirus type 16 E6-specific memory t-Helper cells in the healthy population as witness of previous viral encounter. *Cancer Res* **63**, 636-641.

Welters, M. J., van der Logt, P., van den Eeden, S. J. & other authors (2006). Detection of human papillomavirus type 18 E6 and E7-specific CD4+ T-helper 1 immunity in relation to health versus disease. *Int J Cancer* **118**, 950-956.

Werness, B. A., Levine, A. J. & Howley, P. M. (1990). Association of human papillomavirus types 16 and 18 E6 proteins with p53. *Science* **248**, 76-79.

Whyte, P., Buchkovich, K. J., Horowitz, J. M., Friend, S. H., Raybuck, M., Weinberg, R. A. & Harlow, E. (1988). Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. *Nature* **334**, 124-129.

Wilcock, D. & Lane, D. P. (1991). Localization of p53, retinoblastoma and host replication proteins at sites of viral replication in herpes-infected cells. *Nature* **349**, 429-431.

Wilson, R., Fehrmann, F. & Laimins, L. A. (2005). Role of the E1--E4 protein in the differentiation-dependent life cycle of human papillomavirus type 31. *J Virol* **79**, 6732-6740.

Wilson, R., Ryan, G. B., Knight, G. L., Laimins, L. A. & Roberts, S. (2007). The full-length E1E4 protein of human papillomavirus type 18 modulates differentiation-dependent viral DNA amplification and late gene expression. *Virology* **362**, 453-460.

Winder, D. M., Pett, M. R., Foster, N., Shivji, M. K., Herdman, M. T., Stanley, M. A., Venkitaraman, A. R. & Coleman, N. (2007). An increase in DNA double-strand breaks, induced by Ku70 depletion, is associated with human papillomavirus 16 episome loss and de novo viral integration events. *J Pathol* **213**, 27-34.

Woodman, C. B., Collins, S. I. & Young, L. S. (2007). The natural history of cervical HPV infection: unresolved issues. *Nat Rev Cancer* **7**, 11-22.

Woods, D. F. & Bryant, P. J. (1989). Molecular cloning of the lethal(1)discs large-1 oncogene of Drosophila. *Dev Biol* **134**, 222-235.

Woods, D. F., Hough, C., Peel, D., Callaini, G. & Bryant, P. J. (1996). Dlg protein is required for junction structure, cell polarity, and proliferation control in Drosophila epithelia. *J Cell Biol* **134**, 1469-1482.

Woodworth, C. D., Doniger, J. & DiPaolo, J. A. (1989). Immortalization of human foreskin keratinocytes by various human papillomavirus DNAs corresponds to their association with cervical carcinoma. *J Virol* **63**, 159-164.

Wu, H., Ceccarelli, D. F. & Frappier, L. (2000a). The DNA segregation mechanism of Epstein-Barr virus nuclear antigen 1. *EMBO Rep* **1**, 140-144.

Wu, X., Hepner, K., Castelino-Prabhu, S. & other authors (2000b). Evidence for regulation of the PTEN tumor suppressor by a membrane-localized multi-PDZ domain containing scaffold protein MAGI-2. *Proc Natl Acad Sci U S A* **97**, 4233-4238.

Wu, Y., Dowbenko, D., Spencer, S., Laura, R., Lee, J., Gu, Q. & Lasky, L. A. (2000c). Interaction of the tumor suppressor PTEN/MMAC with a PDZ domain of MAGI3, a novel membrane-associated guanylate kinase. *J Biol Chem* **275**, 21477-21485.

You, J., Croyle, J. L., Nishimura, A., Ozato, K. & Howley, P. M. (2004). Interaction of the bovine papillomavirus E2 protein with Brd4 tethers the viral DNA to host mitotic chromosomes. *Cell* **117**, 349-360.

You, J., Schweiger, M. R. & Howley, P. M. (2005). Inhibition of E2 binding to Brd4 enhances viral genome loss and phenotypic reversion of bovine papillomavirus-transformed cells. *J Virol* **79**, 14956-14961.

You, J., Srinivasan, V., Denis, G. V., Harrington, W. J., Jr., Ballestas, M. E., Kaye, K. M. & Howley, P. M. (2006). Kaposi's sarcoma-associated herpesvirus

latency-associated nuclear antigen interacts with bromodomain protein Brd4 on host mitotic chromosomes. *J Virol* **80**, 8909-8919.

Zanier, K., Ruhlmann, C., Melin, F. & other authors (2010). E6 proteins from diverse papillomaviruses self-associate both in vitro and in vivo. *J Mol Biol* **396**, 90-104.

Zerfass-Thome, K., Zwerschke, W., Mannhardt, B., Tindle, R., Botz, J. W. & Jansen-Durr, P. (1996). Inactivation of the cdk inhibitor p27KIP1 by the human papillomavirus type 16 E7 oncoprotein. *Oncogene* **13**, 2323-2330.

Zhan, L., Rosenberg, A., Bergami, K. C., Yu, M., Xuan, Z., Jaffe, A. B., Allred, C. & Muthuswamy, S. K. (2008). Deregulation of scribble promotes mammary tumorigenesis and reveals a role for cell polarity in carcinoma. *Cell* **135**, 865-878.

Zhang, B., Chen, W. & Roman, A. (2006). The E7 proteins of low- and high-risk human papillomaviruses share the ability to target the pRB family member p130 for degradation. *Proc Natl Acad Sci U S A* **103**, 437-442.

Zhao, K. N., Hengst, K., Liu, W. J., Liu, Y. H., Liu, X. S., McMillan, N. A. & Frazer, I. H. (2000). BPV1 E2 protein enhances packaging of full-length plasmid DNA in BPV1 pseudovirions. *Virology* **272**, 382-393.

Zheng, Z. M., Tao, M., Yamanegi, K., Bodaghi, S. & Xiao, W. (2004). Splicing of a cap-proximal human Papillomavirus 16 E6E7 intron promotes E7 expression, but can be restrained by distance of the intron from its RNA 5' cap. *J Mol Biol* **337**, 1091-1108.

Zheng, Z. M. & Baker, C. C. (2006). Papillomavirus genome structure, expression, and post-transcriptional regulation. *Front Biosci* **11**, 2286-2302.

zur Hausen, H. (2009). Papillomaviruses in the causation of human cancers - a brief historical account. *Virology* **384**, 260-265.

Appendix I