THE ROLE OF HUMAN PAPILLOMAVIRUS (HPV) E6 PROTEINS AS A RISK FACTOR FOR OESOPHAGEAL CANCER

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DEDICATION

To my fantastic husband

For his support, love, endless faith in me and for keeping me smiling.

Thank you

DECLARATION

I, Caryn Sarah Ross-Innes, hereby declare that the work on which this dissertation is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Caryn Sarah Ross-Innes
15 August 2007

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ABBREVIATIONS

11E6	Human papillomavirus 11 E6 protein
18E6	Human papillomavirus 18 E6 protein
BLASTn	Nucleotide-nucleotide basic local alignment search too
BPE	Bovine pituitary extract
bp	Base pairs
BCA	Bicinchoninic acid
BL	Burkitt's lymphoma
BSA	Bovine serum albumin
^{o}C	Degrees celsius
cDNA	Complementary deoxyribonucleic acid
CYP3A5	Cytochrome P450 3A5
DAPI	4',6-Diamidino-2-phenylindole
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E6AP	E6-associated protein
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetra acetic acid
EGF	Epidermal growth factor
EPC2	EPC2-hTERT
FBS	Foetal bovine serum
FISH	Filter *in situ* hybridisation
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HPV	Human papillomavirus
HPV11	Human papillomavirus type 11
HPV18	Human papillomavirus type 18
ISH	*In situ* hybridisation
IST	Instituto Nazional per la Ricerca Sul Cancro
kDa	Kilodaltons

Kb	Kilobase
KSFM	Keratinocyte serum-free media
LB	Luria broth
LCR	Long control region
M	Molar
mRNA	Messenger RNA
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCBI	National Center for Biotechnology Information
NISH	Non-isotopic *in situ* hybridisation
OD	Optical density
OSCC	Oesophageal squamous cell carcinoma
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pcDNA3-11E6	pcDNA3 vector with 11E6 gene insert
pcDNA3-18E6	pcDNA3 vector with 18E6 gene insert
PFA	Paraformaldehyde
PNA	Peptide nucleic acid
PNA_{18}	HPV18 E6 anti-gene peptide nucleic acid
PNA_{mut}	Mutant peptide nucleic acid
PNAEμ	PNA targeting enhancer Eμ intronic sequence
pRb	Retinoblastoma protein
qRT-PCR	Quantitative reverse transcriptase-polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase-polymerase chain reaction
SBH	South blot hybridisation
SCC	Squamous cell carcinoma
SDS	Sodium dodecyl sulphate
Seq	DNA sequencing
Taq	*Thermus aquaticus* polymerase

TBE	Tris borate EDTA
TBS-T	Tris-buffered saline containing 0.05 % Tween-20
TE	Tris-EDTA
U	Units
V	Volts

ABSTRACT

Oesophageal squamous cell carcinoma (OSCC) is a major cancer in South Africa, affecting mainly black males. Several risk factors for OSCC have been reported but this study focuses on the role of human papillomavirus (HPV) in the development of OSCC. HPV is a well-known risk factor for cervical cancer resulting in its classification into low- and high-risk HPV types. The role of the different HPV types in OSCC development is not known, but in cervical cancer the critical HPV transforming gene has been shown to be E6. In this project, the effects of HPV11 E6, a low-risk type, and HPV18 E6, a high-risk type, were investigated by transfecting HPV-negative cell lines (EPC2-hTERT, MCF12A and Rat1) with HPV11 and HPV18 E6. It was observed that the HPV18 E6 protein caused a decrease in p53 protein levels but the HPV11 E6 protein did not. The HPV11 E6 gene, when transcribed at higher levels, caused a decrease in cell growth, an increase in the proportion of cells in G2 and a 4-fold increase in the percentage of cells with DNA content greater than G2. HPV18 E6 did not appear to affect the growth rate or the cell cycle profile of the cells. In addition, an HPV-positive cell line (HeLa) was used to determine the effects of down regulating HPV E6 using peptide nucleic acids (PNAs). PNAs targeting the HPV18 E6 gene (PNA_{18}) were used to determine the effects of down regulating HPV18 E6 expression. Treatment with PNA_{18} caused a 15-35% decrease in HPV18 E6 mRNA levels as well as a decrease in the growth rate of the HeLa cells. However, this may have been caused by non-specific toxicity since treatment with a mutant PNA had similar effects. In conclusion, it appears that HPV11 or HPV18 E6 has different biological effects and that down regulating HPV18 E6 expression may negatively impact on the cells by decreasing the growth rate.

CHAPTER ONE

Introduction

1.1 Oesophageal cancer

Oesophageal cancer is the eighth most common cancer (Parkin *et al.*, 2005) and the sixth leading cause of cancer death worldwide (Tew *et al.*, 2005; Parkin *et al.*, 2005). In South Africa, it is the third leading cancer in males with a lifetime risk of developing the disease being 1 in 73 (Mqoqi *et al.*, 2004). There are two common types of oesophageal cancer, namely adenocarcinoma and squamous cell carcinoma. Adenocarcinoma originates in glandular tissue that is not usually present in the lining of the oesophagus and can only develop once glandular cells have replaced a section of squamous epithelial cells. This condition is called Barrett's oesophagus and develops when there is chronic acid reflux from the stomach. This type of oesophageal cancer is more commonly found in developed countries and is rare in developing countries such as South Africa (Kwong, 2005).

Squamous cell carcinoma of the oesophagus originates in the squamous epithelium that lines the oesophagus. Figure 1.1 shows that the OSCC protrudes from the submucosal layer causing obstruction of the oesophageal lumen. This type of oesophageal cancer is more prevalent in developing countries such as South Africa and other countries such as China, Japan, Iran, India and Brazil (Kwong, 2005; Parkin *et al.*, 2001).

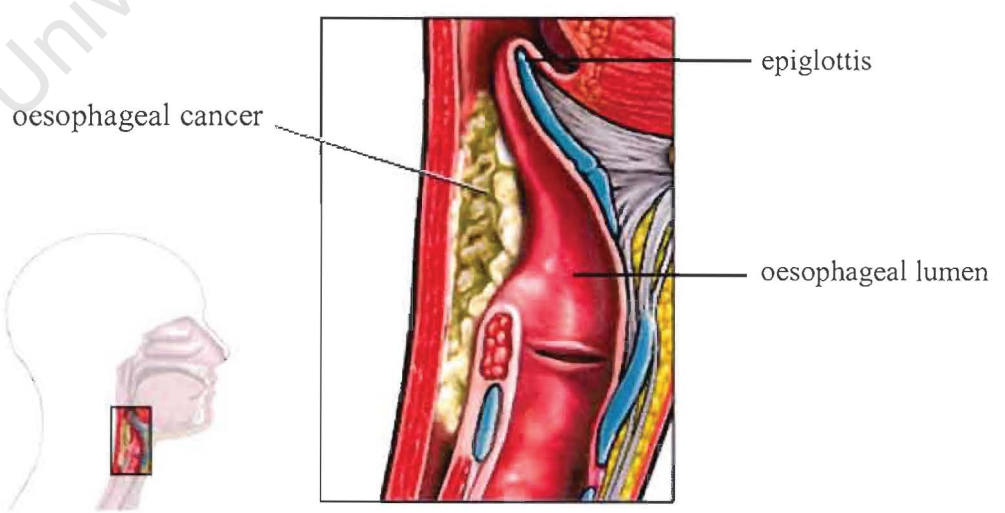

Figure 1.1: Diagram showing squamous cell carcinoma of the oesophagus. This diagram shows a longitudinal section of an OSCC in the upper third of the oesophagus. The tumour protrudes from the submucosal layer causing obstruction of the oesophageal lumen (adapted from Carson-DeWitt, 2005).

Oesophageal squamous cell carcinoma shows great variability in its distribution worldwide and is generally a disease of developing or underdeveloped countries and this is illustrated in Figure 1.2. Areas that have a high risk for OSCC are clustered along the eastern coasts of both Africa and South America and in most of Asia and Eastern Europe. In South Africa, OSCC is highest in the Transkei region in the Eastern Cape Province. A study published by Somdyala *et al.* (2003) showed that the mean annual age-standardised incidence rate of OSCC in Transkei was 76.6/100 000 for males and 36.5/100 000 for females, compared to a global age standardised rate of 11.5/100 000 for males and 4.7/100 000 for females (Parkin *et al.*, 2005).

The incidence of OSCC shows a gender difference in its distribution but is generally more common in males than females (Parkin *et al.,* 2005; Somdyala *et al.*, 2003). It is recorded to be about 7-fold more common in males in Eastern Europe (Parkin *et al.*, 2005). In Africa, the high incidence OSCC belt stretches along the east coast from South Africa, through Mozambique and Tanzania and up to Kenya and incidentally these regions are predominantly maize-consuming areas (Sammon, 1992; Wakhisi *et al.*, 2005).

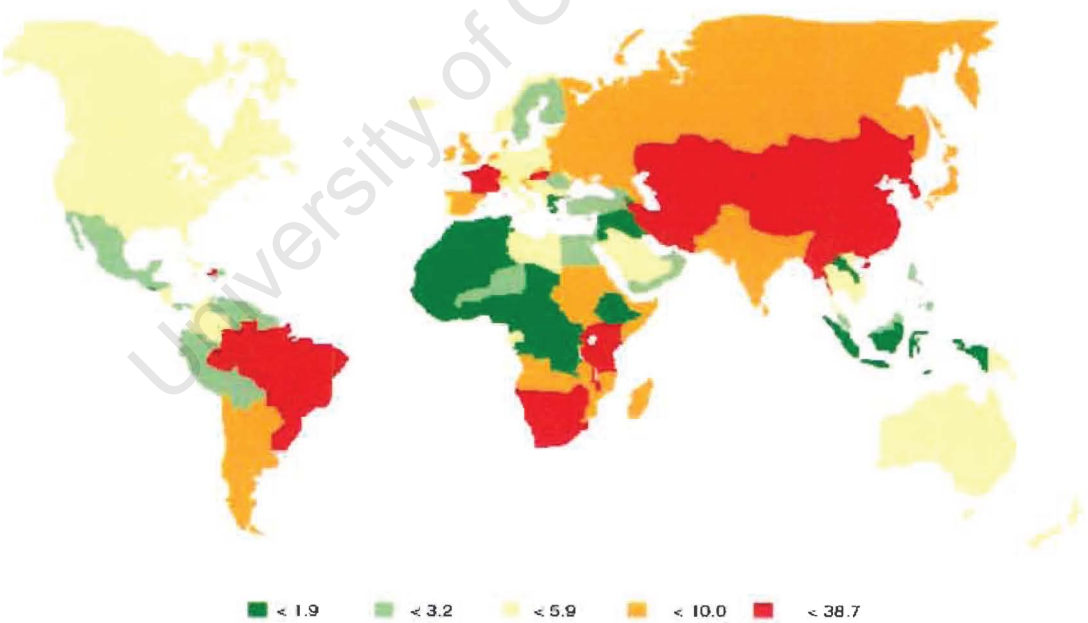

Figure 1.2: The global incidence of oesophageal cancer in males. The different colours show the age-standardised rate of OSCC in males (Parkin *et al.*, 2001).

The mortality rate for OSCC is very high with a 5-year overall global survival rate estimated at around 14% (Tew *et al.*, 2005). There are many reasons for the low survival rate and poor prognosis for the disease, the most critical being late detection of the cancer.

This is due to lack of early symptoms and no screening programmes. It is estimated that over 50% of OSCC patients present with unresectable disease and distant metastases (Tew *et al.*, 2005). In most cases the patients become aware of the tumour when it starts to obstruct passage of food and also when they experience difficulties or pain in swallowing. At this late stage the tumour would have grown very large and often would have spread to other organs. In order to improve early detection of OSCC and treatment, it is important to understand the factors that are associated with this disease.

1.2 Risk factors associated with oesophageal squamous cell carcinoma

The development of OSCC is a complex, multistep process with a multifactorial aetiology. The disease is associated with several environmental, dietary and genetic risk factors. Excessive tobacco smoking and alcohol consumption have been associated with OSCC in many different populations (Wu *et al.*, 2006; Pacella-Norman *et al.*, 2002; Syrjanen, 2002; Pinto *et al.*, 2003; Dandara *et al.*, 2005, Dandara *et al.*, 2006). It has been observed that with respect to tobacco smoking, the critical factors are duration of smoking and amount of tobacco smoked. Individuals who started smoking at an earlier age, smoked for a long duration, or smoked large amounts of cigarettes per day, had a significantly increased risk of developing OSCC (Wu *et al.*, 2006; Dandara *et al.*, 2006). Similarly, individuals that start consuming alcohol at a young age and consume it for a long duration have an increased risk of developing OSCC (Wu *et al.*, 2006).

The association of high incidence OSCC areas with maize consumption is one factor that has received considerable attention. It is thought that contaminants in the maize used for making home-brewed beer could be the source as the maize is sometimes contaminated with fungal toxins produced by *Fusarium* species. From this observation it has been postulated that fungal toxins such as aflatoxins and fumonisins could be risk factors for developing OSCC (Mqoqi *et al.*, 2004). In some areas consumption of very hot beverages or food have been associated with increased risk as this is thought to irritate and cause inflammation in the oesophagus (Castellsague *et al.*, 2000; Phukan *et al.*, 2001, Hung *et al.*, 2004).

Nutritional deficiencies have also been associated with increased risk of developing several cancers including OSCC, but nutrition is difficult to quantitate (Sur and Cooper, 1998).

Generally it has been reported that individuals regularly eating fresh fruit and vegetables have some protection from developing OSCC. A study by De Stefani *et al.* (2005), conducted in Uruguay, showed that consumption of total fruits were more protective than total vegetables, and furthermore, citrus fruits compared to other fruits had an associated reduced risk for OSCC. Conversely, in Dafeng, China, high consumption of pickled and dried fish and seafood products and eating fast was associated with significantly elevated risk of developing OSCC (Wu *et al.,* 2006).

Commenting on the distribution of OSCC in the world, there is a clear picture that the risk of OSCC is inversely associated with socioeconomic status (Wu *et al.,* 2006). High incidence areas are mostly inhabited by the poorest communities in the world (Figure 1.2). These areas are occupied by people in low socio-economic groups, characterised by low levels of education. There are also other lifestyle factors, such as the use of wood and charcoal for cooking or keeping warm, which have been shown to be associated with increased risk of OSCC (Dandara *et al.*, 2006). Families in lower socio-economic groups regularly burn wood and charcoal inside their homes, frequently exposing themselves to many different compounds released during combustion of the wood. There is a need to identify the causative agent(s) that may be released during the burning of the wood and may affect the oesophageal epithelium, ultimately leading to the development of squamous cell carcinoma.

Surprisingly, in areas where people are exposed to certain types of carcinogens, not all individuals exhibit the same risk profiles, some tend to be more susceptible to developing cancer. Most environmental carcinogens need prior activation in order to exert their effects. The system of enzymes responsible for this activation have been shown to exhibit genetic polymorphisms resulting in certain individuals carrying certain genetic variants that make them more susceptible to OSCC. Different variants have been shown to be associated with OSCC risk in different populations (Pinto *et al.,* 2003; Lin *et al.*, 1998; Dandara *et al.*, 2005; Dandara *et al.*, 2006).

For example, cytochrome P450 enzymes oxidise several tobacco-smoke and alcohol-related carcinogens making them more reactive and able to bind to cellular macromolecules, a process that can lead to cancer formation. However, the body expresses other enzymes such as glutatione S-transferases that are able to remove the activated

intermediates from the body. Thus, individuals carrying variants of cytochrome P450 that increase formation of activated intermediates and variants of glutatione S-transferases that decrease conjugation are likely to be at an increased risk of OSCC (Taningher *et al.*, 1999).

A study by Dandara *et al.* (2005) showed that in some genetic backgrounds or environmental conditions, potentially high cytochrome P450 3A5 (CYP3A5) activity may increase OSCC risk, possibly due to the formation of reactive intermediates. The study further reported that the normal CYP3A5 genotype was significantly associated with increased risk for OSCC among cigarette smokers and alcohol consumers, while no difference was observed among non-smokers and non-alcohol consumers. This observation supports the possible involvement of CYP3A5 in the activation of carcinogenic constituents of tobacco smoke and alcohol.

In different areas there are several cultural practices that have been associated with increased risk of OSCC and these include areca-chewing among Taiwanese (Wu *et al.*, 2004) and the habit of self-induced vomiting as a cleansing ritual among Black South Africans (Sammon, 1992). Infectious agents have also been associated with OSCC in many different geographical regions, particularly infection with the human papillomavirus (HPV) (Matsha *et al.*, 2002; Cooper *et al.*, 1995, Williamson *et al.*, 1991; Chang *et al.*, 1990; 1992; 2000; Farhadi *et al.*, 2005; Katiyar *et al.*, 2005; Furihata *et al.*, 1993). HPV infection and its role in OSCC susceptibility is a focus of this project.

1.3 Human papillomavirus and oesophageal squamous cell carcinoma

Human papillomavirus is a well-known aetiological agent for cervical cancer. There are more than 100 types of HPVs, with some of them being classified as low- and high-risk types depending on their association with causing cervical cancer (Motoyama *et al.*, 2004). Epidemiological studies are starting to show that there could also be a role for HPV in OSCC. The first association between HPV infection and OSCC was suggested in 1982 by Syrjanen *et al.* (1982). Since then, many studies have been published with conflicting results showing that the percentage of OSCC with HPV DNA present in the tumour cells varies from 5.6 to 71%. Table 1.1 shows some of the results and the methods used for HPV detection.

gene as PCR is more sensitive than *in situ* hybridisation and the HPV E6 gene is always conserved after viral integration (Sur and Cooper, 1998).

A study by Williamson *et al.* (1991) using nested PCR to detect HPV L1 DNA showed that 71% (10/14) of the OSCC biopsies harboured HPV DNA compared to only 15% (6/41) of biopsies from patients without oesophageal malignancy. The sample size for the study was very small (only 14 OSCC biopsies were tested) therefore the results may not be a true reflection on the population studied. However, they further showed that 43% (6/14) of the OSCC biopsies contained HPV and 66% (6/9) of the adjacent tissue were HPV positive. They noted that no tumours were HPV positive without the adjacent tissue being positive. Moreover, in some cases the tumour cells were negative for HPV DNA but the adjacent tissue was positive. This could indicate that the viral DNA in the tumour cells had integrated in the host genome, via disruption of the HPV L1 gene, therefore giving a false negative result. They hypothesised that the viral DNA in the normal adjacent tissue is still episomal with the HPV L1 gene intact.

The association between HPV infection and OSCC is high in areas with a high incidence of OSCC such as South Africa, China, and Iran and low in low incidence areas such as USA (Turner *et al.*, 1997), UK (Morgan *et al.*, 1997), Hong Kong (Loke *et al.*, 1990) and the Netherlands (Kok *et al.*, 1997). The variation in the association between HPV and OSCC may be due to a combination of environmental factors, geographic location or genetic susceptibility to oesophageal HPV infection in different populations (Sur and Cooper, 1998).

1.4 Human papillomavirus

Human papillomaviruses are small, double-stranded DNA viruses that infect mucosal and cutaneous epithelial tissue. They are non-enveloped viruses with icosohedral capsids (Longworth and Laimins, 2004). The HPV genome is approximately 8 kb in size encoding 6 early proteins, E1, E2, E4, E5, E6 and E7, and two late proteins, L1 and L2 (Figure 1.3). The proteins are expressed from polycistronic mRNAs which are transcribed from a single DNA strand (Jo and Kim, 2005).

The HPV E1 and E2 genes are the first to be expressed and encode proteins that are vital for extrachromosomal DNA replication and the completion of the viral life cycle (Motoyama *et al.*, 2004). HPV E1 and E2 form a complex and bind to sequences at the viral origin of replication. This binding acts to recruit cellular polymerases and accessory proteins that are necessary to mediate replication of the viral DNA. In addition, HPV E1 exhibits helicase activity and functions to separate viral DNA strands ahead of the replication complex (Longworth and Laimins, 2004). HPV E2 also plays an important role in regulation of viral transcription from the early promoter. At low concentrations, HPV E2 binds adjacent to the early promoter and activates transcription, while at high levels, it represses transcription of the early genes, including HPV E6 and E7, by blocking the binding of cellular transcription factors. This allows HPV E2 to control the viral copy number in undifferentiated cells as both HPV E1 and E2 are expressed from the early promoter (Longworth and Laimins, 2004).

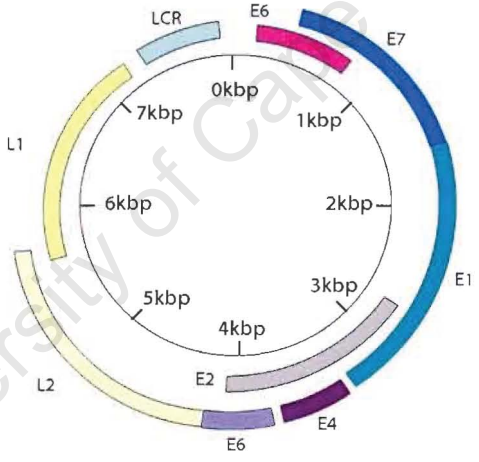

Figure 1.3: Organisation of the human papillomavirus genome. The HPV genome is 8 kb in size and is divided into three regions, the non-coding long control region (LCR), and the protein-coding early (E) and late (L) gene regions. The virus encodes six early and two late proteins. Most HPV mRNAs are polycistronic, and many carry three or more open reading frames (Jo and Kim, 2005).

The functions of the HPV E4 and E5 proteins are not fully understood, however they have both been implicated in regulation of late viral function (Longworth and Laimins, 2004). HPV E4 is only expressed in the later stages of infection when complete virions are being assembled (Jo and Kim, 2005) and it is thought to play a role in maturation and replication of the virus. HPV E5 is often deleted in cervical carcinoma cells, indicating that its

function may not be important in maintaining the malignant transformation of the host cell (Motoyama *et al.*, 2004).

The HPV E6 and E7 genes encode oncoproteins that are involved in the transformation of host cells (will be discussed later). In addition, HPV E6 and E7 proteins have been shown to be necessary for maintaining the HPV genome extrachromosomally in undifferentiated basal cells (Longworth and Laimins, 2004). The two late genes, HPV L1 and L2, encode for the viral structural proteins that form the icosahedral viral capsid consisting of 72 capsomers (Jo and Kim, 2005). The virions are packaged in the cell and then released from the uppermost layers of the epithelium. The non-coding, long control region (LCR) of 400 to 1 000 bp has many overlapping binding sites for activators and repressors of transcription and this region is thought to play a role in determining the host range for specific HPV types (Motoyama *et al.,* 2004). The LCR regulates transcription of the early and late regions, therefore controlling viral protein and virion production (Jo and Kim, 2005).

1.5 The role of human papillomavirus in cancer

Human papillomavirus is associated with several cancers, the most common and best studied being cervical cancer. It is now accepted that HPV is the causative factor for cervical cancer as at least 99% of the cervical cancer cases have an HPV aetiology (Longworth and Laimins, 2004). Reports have been published supporting an etiologic role for HPV in other cancers including breast cancer (de Villiers *et al.*, 2005), squamous cell carcinoma of the head and neck (Ferris *et al.*, 2005) and anogenital cancer (Finzer *et al.*, 2002).

Approximately 118 different types of HPV have been identified, with limited DNA homology (Jo and Kim, 2005). The different HPV types are divided into two categories, the high- and low-risk types, depending on their association with causing cervical cancer (Longworth and Laimins, 2004). The high-risk types include types 16, 18, 31, 33, 35, 39, 45, 50, 51, 53, 55, 56, 58, 59, 64 and 68 and these types are commonly detected in carcinomas and dysplasias (Motoyama *et al.*, 2004). The low-risk types include types 1, 6 and 11 and these are usually associated with causing benign warts and lesions which rarely progress to cancer (Li *et al.*, 2005; Sur and Cooper, 1998).

However, the so-called "low-risk" types, specifically types 6 and 11, have also been identified in penile cancer (Dianzani *et al.*, 1998), laryngeal and bronchogenic carcinomas (Reidy *et al.*, 2004) as well as OSCC (Cooper *et al.*, 1995; Matsha *et al.*, 2002; Chen *et al.*, 1994). Reidy *et al.* (2004) detected HPV11 in 100% (9/9) of patients with laryngeal or bronchogenic carcinomas that had developed due to recurrent respiratory papillomatosis. They concluded that HPV11 is an "aggressive virus in laryngeal cancer".

A study by Chen *et al.* (1994) using specimens from Fuzhou, a high-risk area in Southern China, found that 24/40 (60%) of the oesophageal biopsies tested were HPV positive and out of the HPV-positive tumours, 50% contained HPV 6 DNA. HPV16 was detected in 8% (2/24) of the HPV-positive biopsies and dual infections with HPV types 6 and 16 were detected in 17% (4/24) of the HPV-positive biopsies. The HPV type was unknown in 25% (6/24) of the samples. Furthermore, a study performed by Matsha *et al.* (2002) showed that HPV11 was the dominant type accounting for 48% (11/23) of the HPV-positive OSCC patients in Transkei, South Africa. The other HPV types identified in that study were HPV39 (30%), HPV16 (9%), HPV52 (4%) and unknown (9%). It is therefore necessary to determine whether the categorised "low-risk" HPV types play a role in the development of tumours or whether they are merely bystanders in the tumorigenic process.

1.6 The human papillomavirus lifecycle

Human papillomaviruses that infect the genital region are mostly sexually transmitted, but infection is not limited to the genital tract as approximately 20% of cancers of the oropharynx contain high-risk HPV DNA. It is estimated that two-thirds of individuals who have sexual intercourse with an HPV-infected partner will contract the virus, however most people clear the infection within a year (Longworth and Laimins, 2004). The receptor for entry of the virus into cells is currently unknown, however heparin sulphate (Longworth and Laimins, 2004) and stabilising proteoglycans (Jo and Kim, 2005) have been suggested to mediate the initial attachment of virions to epithelial cells.

If the virus initially infects differentiated, more superficial cells, the infection will usually be transient, since the viral DNA will be lost as the infected cells are shed during terminal differentiation. However, if the virus infects proliferating basal cells of the squamous epithelium, the infection is usually persistent (Snijders *et al.*, 2006). It is thought that HPV

accesses and infects the basal layer cells through microwounds of the epithelium that expose the basal cells (Longworth and Laimins, 2004). In oesophageal epithelium, microwounds could be due to consumption of very hot drinks, the use of emetics and exposure to tobacco smoke which may constantly irritate and damage the oesophageal cells allowing HPV entry into the basal epithelial cells.

The viral lifecycle of HPV is linked to the differentiation programme of the infected host cell, the keratinocyte, and can be separated into two stages, the non-productive and productive stage. In persistent infections, the infected basal cells usually have monomeric episomes that co-replicate with the genome of the host cells (the non-productive stage). There are usually between 20 and 100 extrachromosomal copies of viral DNA per cell (Longworth and Laimins, 2004). When the infected cell undergoes differentiation, the productive stage of the life cycle starts (Figure 1.4). Viral transcription, particularly of the HPV E6 and E7 oncogenes, is markedly increased and vegetative DNA replication and assembly of new virions occur.

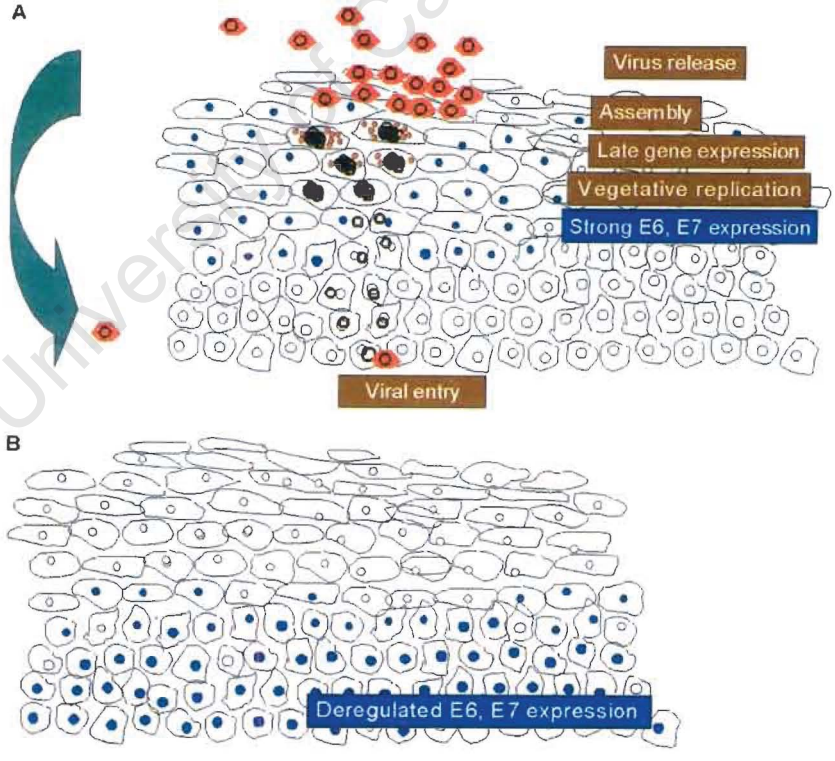

Figure 1.4: Schematic representation of the HPV lifecycle. A) Productive HPV lifecycle. Nuclei expressing HPV E6 and E7 are indicated in blue. Viral episomes are indicated as black circles and viral capsid proteins are indicated in red. Virions are depicted as black circles in red, icosahedral structures. **B)** Deregulated HPV E6 and E7 expression in proliferating basal cells associated with cell transformation and failure of the viral life cycle (Picture taken from Snijders *et al.*, 2006).

Once the terminally differentiated cells are shed, the newly packaged viruses are simultaneously released (Figure 1.4A). If the HPV E6 and E7 expression remains differentiation-dependent there is a very low-risk of the cells becoming transformed as the oncogenes will only be expressed in cells that are terminally differentiated. However transformation of cells may occur when there is an increase in HPV E6 and E7 expression in proliferating cells (Figure 1.4B). The mechanisms behind this deregulated expression are poorly understood (Snijders *et al.*, 2006).

Deregulation of HPV E6 and E7 expression in the basal cells is associated with cell transformation and failure of the viral life cycle. The viral genome may integrate into the host genome usually disrupting the HPV E1 and E2 open reading frames. This leads to an increase in HPV E6 and E7 levels as E2 can no longer repress transcription of the oncogenes (Finzer *et al.*, 2002). HPV integration terminates the viral lifecycle because large portions of the viral genome are disrupted and are therefore functionally inactive (Jo and Kim, 2005). The majority of HPV-positive cervical cancer tumours have integrated HPV DNA and once the viral DNA has integrated into the host genome it is more oncogenic due to HPV E6 and E7 overexpression (Motoyama *et al.*, 2004). HPV integration sites appear to be randomly distributed over the whole genome. There is currently no evidence supporting targeted disruption of critical cellular genes by the integrated viral genomes (Jo and Kim, 2005).

A study in South Africa using non-isotopic *in situ* hybridisation showed that all HPV DNA-positive oesophageal cancer biopsies harboured the virus in an integrated state (Cooper *et al.*, 1995). This indicates that the presence of HPV in the oesophageal cells may play a role in the development of a tumour as the viral DNA has integrated and is therefore more oncogenic as the E6 and E7 oncoproteins will be overexpressed. The deregulation of HPV E6 and E7 expression in proliferating cells can cause many changes in the cells and ultimately lead to the development of tumours (Snijders *et al.*, 2006). Since it has been shown that HPV E6 expression alone can lead to transformation of the host cell (Gao *et al.*, 1997; Sedman *et al.*, 1992; Kiyono *et al.*, 2001), this project focussed solely on the affects of HPV E6 from either the high-risk HPV18 or the low-risk HPV11 to determine whether there are any differences in their affects on cellular transformation.

1.7 The effects of high- and low-risk HPV oncoproteins on cellular transformation

The two oncoproteins encoded by HPV, namely E6 and E7, are involved in transformation of host cells. The HPV E6 and E7 proteins are about 160 and 100 amino acids in size, respectively (Munger and Howley, 2002). The HPV E7 oncoprotein has been shown to bind to the retinoblastoma protein, pRb and inactivate it by preventing it from binding the transcription factor E2F (Dyson *et al.*, 1989). pRb in its active form is hypophosphorylated and inhibits S phase entry by binding the transcription factor, E2F. Figure 1.5 shows that during normal G1 progression, pRb is sequentially phosphorylated by cyclin D1/cdk4 and 6 and cyclin E/cdk2 complexes causing pRb to dissociate from E2F. E2F is therefore free to promote expression of its target genes involved in S-phase progression and the cell enters S phase (Munger and Howley, 2002; Jo and Kim, 2005).

HPV E7 inactivates pRb function by binding to hypophosphorylated pRb, therefore preventing it from binding E2F, resulting in S phase entry (Figure 1.5). HPV-E7-expressing cells therefore continually traverse the cell cycle, leading to an increase in cellular proliferation (Jo and Kim, 2005). HPV E7 also induces degradation of pRb via the ubiquitin-proteasome pathway indicating a second mechanism of how the viral HPV E7 protein inactivates pRb function resulting in S phase entry (Boyer *et al.*, 1996).

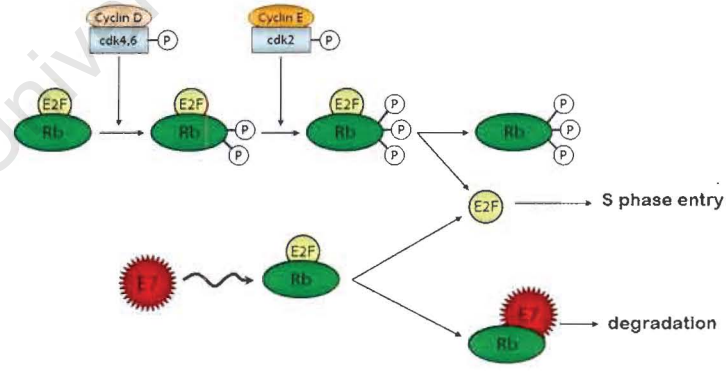

Figure 1.5: HPV E7 inactivates the retinoblastoma protein (pRb). Sequential phosphorylation of pRb by cyclin/cdk complexes prevents pRb binding the transcription factor, E2F. This results in the dissociation of the pRb/E2F complex resulting in S phase entry. HPV E7 binds hypophosphorylated pRb and similarly disrupts the complex between pRb and E2F resulting in the liberation of E2F. This allows the cell to enter S phase of the cell cycle (Picture from Jo and Kim, 2005).

Malanchi *et al.* (2004) recently showed that HPV E6 is also capable of inactivating pRb function through a different method to that of HPV E7. They showed that both the low-risk (HPV1) and high-risk (HPV16) E6 proteins promote phosphorylation of the pRb protein, therefore preventing it from binding E2F and allowing E2F to activate its target genes. Thus both the low and high risk E6 proteins had the ability to functionally inactivate the pRb pathway in HPV E6-expressing primary human cells and therefore alter regulation of the cell cycle.

The high-risk HPV E6 oncoproteins have also been shown to form a trimeric complex with the cellular ubiquitin ligase, E6-associated protein (E6AP) (Zanier *et al.*, 2005), and the tumour suppressor protein, p53 (Werness *et al.*, 1990) (Figure 1.6). The formation of this trimeric complex causes the E6AP to polyubiquitinate the p53 protein therefore increasing the rate of degradation of p53 via proteasomal degradation by the 26S proteasome (Werness *et al.*, 1990). This is detrimental to the cell as the role of p53 is to safeguard the integrity of the genome by inducing G1 cell cycle arrest or apoptosis in the presence of DNA damage or cell stress. These stresses will therefore result in increased p53 levels in normal cells (Kumar and Klark, 2002). As p53 levels cannot be increased in HPV-infected cells, the cells continue to proliferate regardless of the integrity of their DNA, allowing accumulation of DNA mutations (Havre, 1995; Kessis *et al.*, 1993) and chromosomal instability (Schaeffer *et al.*, 2004; Thomas and Laimins, 1998) which is thought to be an essential part of malignant transformation.

The low-risk HPV E6 oncoproteins have been shown to bind with very low affinity to p53, if at all, but not increase its rate of degradation (Slebos *et al.*, 1995). In HPV11-E6-expressing cells, accumulation of p53 after DNA damage is still intact and the cells are able to undergo G_1 cell cycle arrest in response to DNA damage (Slebos et al., 1995). Futhermore, Zanier *et al.* (2005), showed that low-risk HPV E6 proteins do not bind E6AP and form the trimeric E6/E6AP/p53 complex like the high-risk HPV E6 proteins.

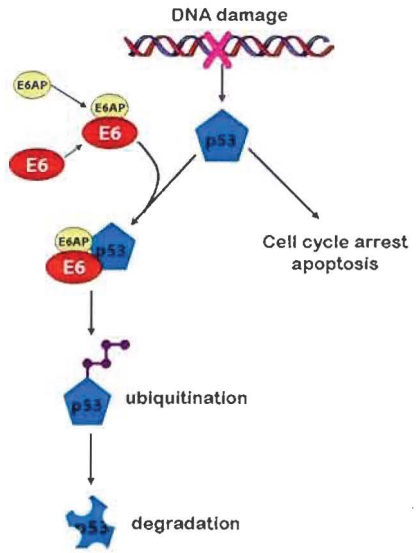

Figure 1.6: HPV E6 causes increased degradation of p53. DNA damage induces p53 activation, leading to cell cycle arrest or apoptosis. The high-risk HPV E6 binds E6AP and p53, forming a trimeric complex. The complex formation results in E6AP-mediated ubiquitination and rapid proteasomal degradation of p53, therefore preventing growth arrest or apoptosis in response to DNA mutations (Picture from Jo and Kim, 2005).

The high-risk HPV E6 protein, HPV16 E6, also prevents apoptosis in a p53-independent mechanism. This is achieved by the HPV16 E6 protein decreasing Bax mRNA levels, shortening the half-life of the Bax protein and stimulating the degradation of the Bax protein in human keratinocytes (Magal *et al.*, 2005). Since Bax is a proapoptotic protein that enhances apoptosis (Kumar and Clark, 2002), the HPV E6 protein binds and inhibits Bax-induced apoptosis thus preventing programmed cell death and resulting in accumulation of DNA mutations (Magal *et al.*, 2005)

The high-risk HPV E6 proteins also target several other cellular proteins for degradation in a manner similar to that of p53 degradation. The high-risk E6 proteins contain a PDZ domain protein binding motive and can bind a number of cellular PDZ-domain-containing proteins such as hDlg (the human homologue of the *Drosophilia melangaser* tumor suppressor protein discs large) (Kiyono *et al.*, 1997), MUPP1 (a multi-PDZ domain protein) (Lee *et al.*, 2000) and hScrib (the human homologue of the *Drosophilia melangaser* tumor suppressor scribble) and target them for proteolysis via E6AP-mediated ubiquitination. The low-risk HPV E6 proteins lack the PDZ binding site and therefore cannot target PDZ-domain-containing proteins for degradation (for review see Munger and Howley, 2002).

High risk HPV E6 mRNA transcripts are alternatively spliced leading to the production of a smaller HPV E6 protein, named E6* (Ingaki *et al*, 1988; Vaeteewoottachern *et al.,* 2005; Stacey *et al.,* 1995; Sedman *et al.,* 1991; Schneider-Gadicke *et al.*, 1988). This phenomenon has been observed in the high risk HPV types, but no study has shown alternatively spliced HPV E6 mRNA in the low-risk HPV types, including HPV11 E6 (Stacey *et al.,* 1995; Sedman *et al.,* 1991; Schneider-Gadicke *et al.*, 1988). Comparison of the nucleotide sequence necessary for splicing for the HPV E6 mRNA by Schneider-Gadicke *et al.* (1988) showed that the low risk HPV types, i.e. HPV1, 6 and 11, are unlikely to be spliced as they lack the essential dinucleotides (GT) of splice consensus sequences that are present in the high risk types, HPV16, 18, 31 and 33.

The HPV18 E6* protein is only 6.5 kDa in size compared to the full-length E6 proteins which are roughly 18.9 kDa (Schneider-Gadicke *et al.,* 1988). The alternatively spliced HPV E6 mRNA has been reported to be in abundance, with the full length HPV E6 transcripts present at very low levels (Zheng and Baker, 2006; Smotkin and Wettstein, 1986). The E6* proteins negatively regulate the expression of the active, full-length HPV E6 proteins therefore contributing to low HPV E6 protein levels (Sedman *et al.,* 1991).

Since only the high-risk HPV E6 mRNA is spliced, it was previously thought that perhaps the HPV E6* may be associated with transforming activity. However, several studies have shown the opposite (Pim *et al.*, 1997; Sedman *et al.,* 1991). Pim *et al.* (1997) showed that the HPV E6* protein inactivated the function of the full length HPV18 E6 protein. They showed that the HPV18 E6* protein can bind to both the full-length HPV E6 protein and the E6AP, *in vitro*. This binding results in the E6* protein inactivating the function of the full-length HPV18 E6 protein by preventing it from binding p53, *in vitro* and *in vivo*. The trimeric complex consisting of HPV18 E6, E6AP and p53 is therefore unable to form and hence p53 will not be polyubiquitinated and degraded.

Pim *et al.* (1997) also showed that increased expression of HPV E6* in HPV-positive transformed cells lead to an increase in transcriptional activity of p53 resulting in a decrease in transformed cell growth. Sedman *et al.* (1991) reported that the HPV16 E6* protein had no transforming activity and functioned to facilitate HPV E7 translation from the bicistronic mRNA and decrease HPV E6 translation, rather than generate biologically

active E6* proteins. However, Stacey *et al.* (1995) showed conflicting results and reported that the alternatively spliced HPV E6 mRNA did not increase HPV E7 translation.

The high-risk HPV E6 oncoproteins are capable of immortalising the host cell. Normal human keratinocytes have a finite proliferative lifespan and enter senescence within 50-100 cell divisions due to telomere shortening (Snijders *et al.,* 2006). HPV E6 prevents telomere shortening and hence senescence by activating hTERT, the catalytic subunit of telomerase, the enzyme which is responsible for maintaining telomere length at the 3' end of the chromosome. HPV E6 achieves this by forming a complex with the E6AP and binding directly to the hTERT promoter thus activating transcription of the gene (Seo *et al.*, 2004; Gewin *et al.,* 2004). Furthermore, it has recently been proposed that the E6/E6AP complex targets NFX1-91, a newly identified repressor of telomerase, for ubiquitination and degradation and in so doing activates telomerase activity (Gewin *et al.,* 2004). The low-risk HPV11 genome is unable to immortalise cells, however it has been shown to be capable of extending the lifespan of the cells, grown as monolayers, to over three months without undergoing senescence (Thomas *et al.*, 2001).

Thomas *et al.* (2001) also showed that HPV11 altered the patterns of differentiation, similar to high-risk HPVs. In normal keratinocytes, nuclei are lost when cells differentiate, while in cells expressing high-risk HPVs, nuclei are maintained throughout the suprabasal layers. Thomas *et al.* (2001) reported that cells containing the low-risk HPV11 DNA similarly demonstrated an altered differentiation pattern compared to cells lacking the virus. However, the changes appeared less severe than those seen in cells infected with the high-risk HPV31. In the HPV11-expressing cells, nuclei were maintained throughout all differentiated layers, however the degree to which nuclei were retained depended on the HPV11 copy number within the cells. In addition, the cells with a higher HPV11 copy number were more likely to amplify HPV11 DNA, as was observed in cells containing high-risk HPV DNA, than cells with a lower copy number.

The same HPV11-expressing cells were used for microarray analysis to identify genes that were differentially expressed in cells containing low- (HPV11) or high-risk (HPV31) HPV types. Five genes that were repressed and 76 genes that were activated (by 2-fold or more) by HPV11 were identified. Only a few sets of genes were similarly activated by both the high- and low-risk genomes during their productive lifecycles indicating that the low- and

high-risk HPV types target distinct sets of cellular genes and pathways. Some of the genes that were highly activated by HPV11 were members of the interferon-inducible family. Other genes found to be markedly increased in HPV11-containing cells included transforming growth factor β-induced 86 kDa protein, defensin, desmoglein, Stat-1 and COX2 (Thomas *et al.*, 2001). This study only looked at the gene expression levels in proliferating monolayer cultures and not differentiating cells, so it is possible that additional changes in cellular gene expression occur during differentiation.

Research by Shen *et al.* (2000; 2001; 2003; 2004) has demonstrated that the high-risk HPV18 E6 and E7 proteins together are able to immortalise and malignantly transform human embryonic oesophageal epithelial cells. Their results showed that the HPV18 E6 and E7 oncoproteins are sufficient to cause telomerase activation, changes in cell morphology, anchorage-independent growth, increased cell proliferation, chromosomal instability and malignant transformation of oesophageal epithelial cells. This leads one to believe that HPV may play a causative role in the development of OSCC. A review by Sur and Cooper (1998) suggested that there may be a synergistic action between HPV and other carcinogenic agents and that the exposure to carcinogens in the diet or environment may augment the transformation process initiated by the viral HPV E6 and E7 proteins. It is known that exposure to cigarette smoke, alcohol, nitrosamines and mycotoxins can result in inactivation of the p53 and pRB genes through different mechanisms such as gene rearrangements, allelic losses or missense mutation and this may work together with the virus to ultimately form a tumour (Sur and Cooper, 1998).

Besides studies investigating the roles of different HPV proteins in cancer development, there is a large area of research concentrating on investigating the effects of knocking out HPV genes in HPV-positive cells. Several methods are being used and these include short interfering RNA and the use of peptide nucleic acids (PNAs).

1.8 Peptide nucleic acids as a potential antiviral agent

The treatment for OSCC is usually individualised with respect to the patient's tumour stage, site of the tumour, general health of the patient and the patient's desire for treatment (Mackay *et al.*, 2006). Cisplatin-based chemoradiation or surgery are frequently administered to patients with resectable, localised tumours (stage I-III) that have not

metastasised (Tew *et al.*, 2005). However, it has recently been shown that preoperative chemoradiation provides a good treatment strategy for patients with a good performance status (i.e. those who can handle aggressive treatment) (Schneider and Urba, 2006). Chemotherapy is used to shrink large, inoperable tumours, and to treat micrometastases early in the course of the disease and thus minimise the risk of distant relapse (Mackay *et al.*, 2006).

Patients with metastatic or unresectable oesophageal cancer (stage IV) constitute more than 50% of cases (Tew *et al.*, 2005), and chemotherapy or radiation is administered only to shrink the tumour and improve the quality of life (Mackay *et al.*, 2006). Another intervention used for late-stage patients is the use of stents which help keep the oesophagus distended to allow passage of food (Boyce, 1993). Many new therapies for OSCC are currently in various phase I/II clinical trials and these include monoclonal antibodies targeting either the Her2/Neu receptors, the epidermal growth factor receptor or the vascular endothelial growth factor as well as signal transduction/tyrosine kinase inhibitors targeting epidermal growth factor receptor (Tew *et al.*, 2005).

Peptide nucleic acids (PNAs) designed to target different genes upregulated in cancers are also promising with regards to targeted cancer therapies. PNAs were first described by Nielsen *et al.* (1991) and are synthetic structural homologues of nucleic acids with the deoxyribose phosphate backbone replaced by repeating *N*-(2-aminoethyl)glycine units and the nucleobases attached through methylenecarbonyl linkers (Hyrup and Nielsen, 1996) (Figure 1.7a). The PNA molecules are more closely related to peptides and proteins than to oligonucleotides (Nielsen, 2001) but can be used to target and down regulate transcription or translation of a chosen target gene.

Peptide nucleic acids were first used as antisense drugs, targeting RNA and preventing translation of a specific protein. More recently, anti-gene PNA are being used to prevent transcription of a chosen gene (Cutrona *et al.*, 2000; Cutrona *et al.*, 2003, Boffa *et al.*, 2007; Braun *et al.*, 2004). PNAs have an uncharged polyamide backbone instead of a negatively charged phosphate-sugar backbone and this is thought to increase the thermal stability of PNA-DNA duplexes relative to the corresponding DNA-DNA duplexes as there is no electrostatic repulsion between the two strands (Hyrup and Nielsen, 1996; Pooga *et al.*, 2000).

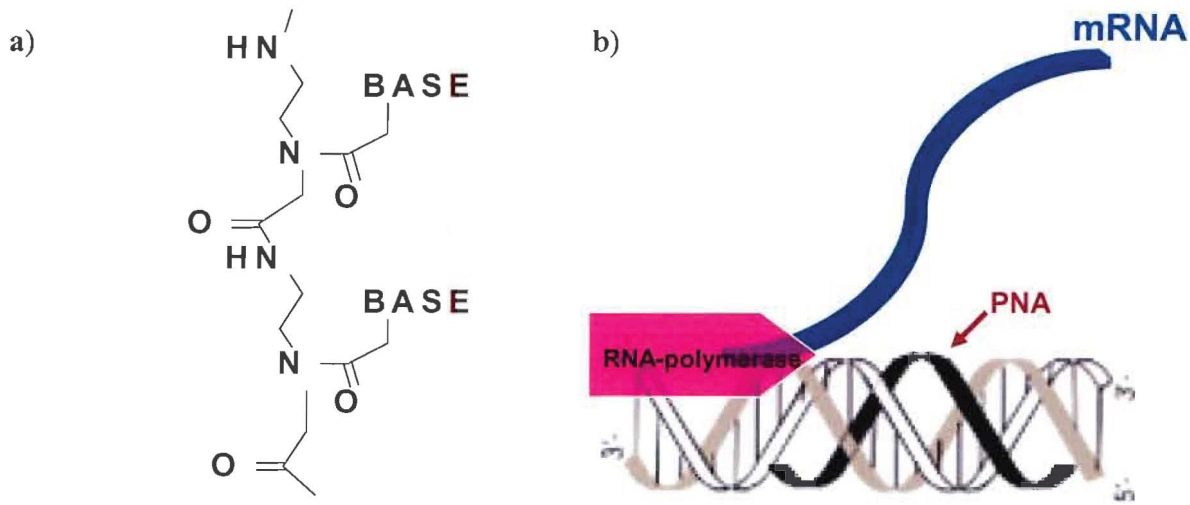

Figure 1.7: Peptide nucleic acids. a) Schematic of PNA oligomers. PNAs are synthetic structural homologues of nucleic acids with the deoxyribose phosphate backbone replaced by repeating *N*-(2-aminoethyl)glycine units and the nucleobases attached through methylenecarbonyl linkers. **b) Representation of PNA binding to complementary sequence in double-stranded DNA.** PNA binds to its target gene forming a strong triple-stranded helix that DNA helicase is unable to unwind. RNA polymerase therefore cannot transcribe the gene leading to a decrease in protein production.

Peptide nucleic acids recognise and bind to their complementary target in double-stranded DNA thus preventing transcription of the targeted gene (Nielsen, 2001) (Figure 1.7b). This binding of PNA to DNA may be mediated either through triplex formation or strand displacement. It is thought that PNA-mediated arrest of transcription is due to the inability of the DNA helicase to unwind the strong heteroduplexes (Pooga *et al.*, 2000) hence preventing transcription and ultimately protein production. PNAs are most effective when designed to bind specifically to the target gene as close to the 5' end of the gene as possible and to be between 16 and 18 bases in length. The PNAs must also not form any secondary structures (Personal communication, Prof. Lidia Boffa, IST, Genoa, Italy).

As PNAs are synthetic pseudopeptides, they are not recognised by either nucleases or proteases (Hyrup and Nielsen, 1996; Pooga *et al.*, 2000) and are stable in serum and cell extracts (Dean, 2000). This is favourable for their use as potential therapeutic agents. Braun *et al.* (2004) tested four different PNAs, three designed to target the HPV18 E6 gene, and one to target the HPV18 E7 gene, on HeLa-S suspension cells, which are known to contain HPV18 DNA (Boshart *et al.*, 1984). Their results showed that PNA targeting only HPV E7 did not decrease the proliferation rate of HeLa-S cells. Furthermore, PNAs targeting only HPV18 E6 were found to decrease the growth rate of the cells but did not

induce apoptosis. The most effective PNA treatment was using a mixture of three PNAs, two that target HPV18 E6 and one that targets HPV18 E7. Treatment with the mixture of PNAs resulted in a decrease in cell proliferation and an increase in apoptosis. An increase in pRb and p53 protein levels was also observed indicating that knocking out HPV E6 and E7 is able to restore p53 and pRb function. The cells treated with the mixture of PNAs had an altered morphology and lost the ability to grow non-adherently (Braun *et al.*, 2004). All the observed changes indicated that the PNA-treated cells were reverting back to a "normal", untransformed phenotype.

A group in China (Wang *et al.*, 2004), tested PNAs designed to target human telomerase mRNA. Most tumour cells activate telomerase expression to immortalise the cells and prevent senescence, however telomerase is rarely activated in normal cells making it a good marker for many malignant tumours. The PNA designed to bind telomerase RNA was tested on a gastric cancer cell lines, SGC7901, and was found to successfully decrease telomerase protein levels. This decrease lead to a change in morphology of the PNA-treated cells as well as a decrease in cell proliferation and colony formation.

Other studies have looked at the use of PNA in treating Burkitt's Lymphoma (BL). In BL cells, it has been observed that the c-*myc* oncogene is often translocated close to the Eμ enhancer of the Ig gene locus. This translocation causes hyperexpression of c-*myc* resulting in increased cell proliferation (Kuppers and Dalla-Favera, 2001). Cutrona *et al.* (2003) designed PNAs complementary to the enhancer Eμ intronic sequence (PNAEμ), that would selectively block expression of the c-*myc* oncogene under Eμ control but not of other c-*myc* alleles. The PNAEμ had an added nuclear localisation signal to aid uptake into the nucleus.

The PNAEμ was tested in SCID mice where a human tumour was established by inoculation of cells from a BL cell line. Treatment with PNAEμ was able to block tumour growth in SCID mice inoculated with the human BL cell lines (Boffa *et al.*, 2007). The tumours in the mice treated with PNAEμ contained areas of cell necrosis particularly around the blood vessels. The inhibition of tumour growth was specific as it was not observed in mice treated with PNAEμmut (carrying sequence mutations) or in BL cell lines when c-*myc* was not translocated and therefore not under control of the Eμ enhancer (Boffa *et al.*, 2007). The PNAEμ was found to have a relatively long life *in vivo* in tissues,

particularly in the BL tumour mass, which is favourable for potential therapeutic drugs (Boffa *et al.*, 2005).

The above examples show some advances in PNA studies and suggest that PNAs show great promise as potential therapeutic agents. In this project, PNAs targeting the HPV18 E6 gene were used to investigate the effect on cell proliferation.

1.9 Project objectives and aims

The main objective of this project was to investigate the role of the low risk HPV11 E6 and compare it to the high-risk HPV18 E6 in susceptibility to OSCC. This objective was achieved by introducing either the HPV11 or HPV 18 E6 genes into HPV-negative cell lines, determining whether the gene is transcribed and expressed in the selected stable transfectants, and investigating the consequent effects on the cells. HPV18 E6 expression was knocked down in the HPV-positive cell line, HeLa, using PNA to determine if this has any effect on the transformed phenotype.

CHAPTER TWO

Materials and Methods

2.1 Materials

2.1.1 Expression vectors and peptide nucleic acids

Human papillomavirus 11 and 18 E6 genes cloned into pcDNA3.1(+) expression vector (Invitrogen, Paisley, Scotland) (Appendix A.3) were provided by Dr Lawrence Banks at The International Centre for Genetic Experimentation and Biotechnology, Triest, Italy. The peptide nucleic acids were designed and synthesised by Prof Lidia Boffa at the Istituto Nazionale per la Ricerca Sul Cancro (IST), Genoa, Italy. Appendix A contains information regarding the DNA and protein molecular weight markers used in this study. Appendix B contains the details of all solutions used in the following methods.

2.1.2 Cell lines and media

Four different cell lines, namely EPC2-hTERT (EPC2), a telomerase-immortalised oesophageal epithelial cell line (Harada *et al.*, 2003), MCF-12A, a non-tumorigenic breast epithelial cell line established from tissue taken at reduction mammoplasty from a mulliparous patient with fibrocystic breast disease (Paine *et al.*, 1992), Rat1 fibroblasts (ATCC CRL-2210) and HeLa, a known HPV18-positive cervical cancer cell line (Boshart *et al.*, 1984.), were selected to determine the effects of HPV11 and HPV18 E6. EPC2 cells were grown in Keratinocyte Serum-Free Media (KSFM) (Invitrogen, Paisley, UK) supplemented with 50 µg/ml Bovine Pituitary Extract (BPE) (Invitrogen, Paisley, UK), 1 ng/ml epidermal growth factor (EGF) (Invitrogen, Paisley, UK), 100 U/ml penicillin and 100 µg/ml streptomycin.

MCF12A cells were grown in 50% Hams F12 medium, 50% Dulbecco's Modified Eagle Medium (DMEM), 5% heat-inactivated foetal bovine serum (FBS) (Invitrogen, Paisley, UK), 50 U/ml penicillin, 50 µg/ml streptomycin, 100 ng/ml cholera toxin (Sigma, Ayrshire, UK), 20 ng/ml mouse EGF (Sigma, Steinheim, Germany), 10 µg/ml bovine insulin (Sigma, Steinheim, Germany) and 500 ng/ml hydrocortisone (Sigma, Missouri, USA). Rat1 fibroblasts and HeLa cells were grown in DMEM containing 10%

heat-inactivated FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. The oesophageal cancer cell lines, WHCO1, WHCO3, WHCO5, WHCO6, KYSE30, KYSE70, KYSE180 and KYSE520 (Veale *et al.*, 1989; Shimata *et al.*, 1992) as well as the known HPV16-positive cervical cancer cell lines, CaSki (Baker *et al.*, 1987) were grown in DMEM containing 10% heat-inactivated FBS, 100 U/ml penicillin and 100 µg/ml streptomycin.

2.2 Methods

2.2.1 Cell culture

Generally, cells were cultured to between 80 and 100% confluence after which they were trypsinised using 0.05% trypsin-EDTA. After the cells had detached, the trypsin was neutralised with either DMEM containing 10% FBS for all cells except for EPC2 cells for which 250 µg/ml of trypsin inhibitor Type 1-S from soybean (Sigma, Steinheim, Germany) diluted in sterile phosphate-buffered saline (PBS) was used. Cells were collected by centrifugation for 5 minutes at 1000 g (Beckman Model TJ-6 Centrifuge, California, USA), resuspended in the corresponding growth medium and replated at a dilution of between 1:4 and 1:10.

Preparation of frozen stocks involved steps described above, however, instead of replating the cells, the cells were counted using a haemocytometer (Marienfeld, Mannheim, Germany), centrifuged and resuspended in cell freeze medium at a concentration of $1x10^6$ cells/ml. The cell freeze medium contained 10% dimethyl sulphoxide (DMSO), 20% FBS and 70% DMEM except for the EPC2 cells which were frozen in medium containing 10% DMSO and 90% FBS. One millilitre samples were aliquoted into ampules and slow freezed to -80°C before transferring to liquid nitrogen for long term storage. When required, frozen stocks were thawed in a 37°C water bath followed by centrifugation for 5 minutes at 1000 g and the pellet resuspended in 2 ml medium for plating in either 60 mm (EPC2) or 100 mm dishes (all other cell lines).

2.2.2 Preparation and quantification of genomic DNA

Genomic DNA was prepared from the cells lines, using the method described by Strauss (1998), and the presence of HPV DNA was detected using the polymerase chain reaction (PCR). The cells were plated in 100 mm dishes and incubated at 37°C in an incubator containing 5% CO_2. Once the cells were 80-100% confluent, they were harvested by trypsinisation, neutralised and then centrifuged for 5 minutes at 1000 g (Beckman Model TJ-6 Centrifuge, California, USA) to pellet the cells. The cells were then washed twice by resuspension in 10 ml ice-cold PBS and resuspended in 600 µl digestion buffer. The samples were then incubated overnight in a shaking water bath at 50°C.

An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added and mixed by vortexing. Samples were centrifuged for 10 minutes at 1 700 g and the resulting aqueous layer was transferred to a new tube. The phenol:chloroform:isoamyl alcohol extraction was repeated if a large, white interphase was observed. A half volume of 7.5 M ammonium acetate and 2 volumes of 100% ethanol were added after which the DNA was recovered by centrifugation at 1 700 g for 2 minutes. The pellet was rinsed in 70% ethanol and air dried. DNA was resuspended in Tris-EDTA (TE) buffer, and DNase-free Ribonuclease A (Fermentas, Maryland, USA) was added to a final concentration of 1 µg/ml and incubated at 37°C for 1 hour to degrade contaminating RNA.

The quality and quantity of DNA was determined spectrophotometrically by measuring absorbance (OD) at 260 and 280 nm. The formula: DNA concentration (µg/ml) = OD_{260} x 50 µg/ml x dilution factor, was used to calculate the DNA yield. A known amount of DNA was electrophoresed on 1% agarose containing 0.5 µg/µl ethidium bromide to further confirm the quality. DNA molecular weight markers were routinely included in all agarose gels to determine the approximate sizes of the DNA bands. Appendix A.1 shows the different DNA molecular weight markers used and the corresponding sizes of the bands.

2.2.3 PCR detection of HPV DNA sequences

The integrity of the extracted DNA was validated by the amplification of either human or rat serum albumin. For the human serum albumin gene, primers 5′-GCC CTC TGC TAA CAA GTC CTA-3′ and 5′-GCC CTA AAA AGA AAA TCC CCA ATC-3′ amplified a

93 bp fragment and for the rat serum albumin gene, the primers 5′-TGC CTT TTC TAG GGG TGT GT-3′ and 5′-CCT GGC TTC CGT TAC TAC GA-3′ amplified a 340 bp fragment. Generally the reaction mixture contained 20 pmol of each primer, GoTaq® Flexi Buffer, 150 ng DNA, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1.25 U GoTaq® DNA Polymerase and H_2O to a final volume of 25 µl.

The PCR conditions for amplifying rat and human serum albumin consisted of an initial denaturation at 95°C for 1 minute followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C (55°C for rat serum albumin) for 30 seconds and extension at 72°C for 30 seconds, followed by a final elongation step of 7 minutes at 72°C (GeneAmp® PCR system 2700, Applied Biosystems, California, USA). The PCR products were resolved on 2% agarose containing ethidium bromide.

For the detection of HPV, either the HPV L1 or E6 gene was amplified. Amplification of the HPV L1 gene involved using the consensus, degenerate primers MY09 (5′-CGT CCM ARR GGA WAC TGA TC-3') and MY11 (5′-GCM CAG GGW CAT AAY AAT GG-3′) [M=A+C, R=A+G, W=A+T, Y=C+T, S=G+C] to amplify at 450 bp fragment for a wide range of HPV types followed by nested PCR with a second set of primers (Manos *et al.*, 1989). The PCR mixture for the first amplification reaction consisted of GoTaq® Flexi Buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 20 pmol of each of the MY09 and MY11 primers, 1.25 U GoTaq® DNA Polymerase, 400 ng genomic DNA and H_2O to a final volume of 50 µl. A hot-start PCR was performed by withholding the inclusion of the MY11 primer until the PCR mixture reached 94°C for 1 minute. The samples were then denatured for a further 9 minutes at 94°C, followed by 40 cycles of 1 minute at 94°C, 1 minute at 55°C and 1.5 minutes at 72°C, followed by a final elongation step of 5 minutes at 72°C.

The nested PCR reaction was done in the presence of the non-degenerate $GP5^+$ and $GP6^+$ primer set to further improve detection. The no template control from the first amplification reaction was reamplified using the nested primers to check for any cross contamination. The PCR mixture consisted of GoTaq® Flexi Buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 50 pmol of each of the primers ($GP5^+$: 5′–TTT GTT ACT GTG GTA GAT ACT AC-3′, $GP6^+$: 5′–CTT ATA CTA AAT GTC AAA TAA AAA G-3′), 1.25 U GoTaq® DNA Polymerase, 1 µl of PCR product from the MY09/MY11

amplification reaction and H_2O to a final volume of 50 µl. The DNA was first denatured for 5 minutes at 94°C, followed by 30 cycles of 1 minute at 94°C, 2 minutes at 44°C and 1.5 minutes at 72°C, followed by a final elongation step of 5 minutes at 72°C. The products from the nested PCR were resolved on 2% agarose gels containing 0.5 µg/µl ethidium bromide to detect the 150 bp band.

For detection of the HPV E6 gene, PCR using the WD set of primers, which consists of three forward and two reverse primers, was used. The sequences of the primers are as follows: WD72: 5′-CGG TCG GGA CCG AAA ACG G-3′, WD76: 5′–CGG TTS AAC CGA AAM CGG- 3′, WD154: 5′–TCC GTG TGG TGT GTC GTC C-3′, WD67: 5′-WGC AWA TGG AWW GCY GTC TC-3′, WD66: 5′–AGC ATG CGG TAT ACT GTC TC-3′. [M= A+C, R=A+G, W-A+T, Y=C+T, S=G+C]. The PCR consisted of GoTaq® Flexi Buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 10 pmol of WD72, 10 pmol of WD66, 10 pmol of WD154, 40 pmol of WD76, 40 pmol WD67, 2.5 U GoTaq® DNA Polymerase, 200 ng of genomic DNA and H_2O to a final volume of 50 µl. The DNA was first denatured for 5 minutes at 94°C, followed by 40 cycles of 30 seconds at 94°C, 30 seconds at 60°C and 30 seconds at 72°C, followed by a final elongation step of 5 minutes at 72°C. A positive result was indicated by the amplification of a 240 bp product.

Polymerase chain reaction was used to determine whether the transfected cells contained the HPV11 or HPV18 E6 gene. Primers specifically amplifying a section of either the HPV11 or HPV18 E6 genes were designed using Primer3 (Rozen and Skaletsky, 2000). The PCR mix used to amplify the HPV18 E6 gene consisted of GoTaq® Flexi Buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 20 pmol of each of the primers (forward 5'-GCG ACC CTA CAA GCT ACC TG-3' and reverse 5'-GAG TCG TTC CTG TCG TGC TC-3'), 1 U GoTaq® DNA Polymerase and 150 ng of genomic DNA in a final volume of 25 µl. The PCR conditions involved denaturation for 5 minutes at 95°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 66°C and 45 seconds at 72°C, followed by a final elongation step of 7 minutes at 72°C. The PCR products were analysed on a 1.5% agarose gel containing 0.5 µg/µl ethidium bromide at 3.9 V/cm to determine the presence of a 423 bp band.

The PCR mixture used to amplify the HPV11 E6 gene consisted of GoTaq® Flexi Buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 20 pmol of each of the primers (forward 5'-CCT

CCA CGT CTG CAA CAT C-3' and reverse 5'-ATC TCT GCG GTG GTC AGT G-3'), 1 U GoTaq® DNA Polymerase and 150 ng of genomic DNA in a final volume of 25 µl. The DNA was first denatured for 5 minutes at 95°C, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 60°C and 30 seconds at 72°C, followed by a final elongation step of 7 minutes at 72°C. The PCR products were analysed on 2% agarose containing 0.5 µg/µl ethidium bromide at 3.9 V/cm to determine the presence of a 112 bp band.

2.2.4 Propagation of HPV11 and HPV18 E6 clones in *E. coli* DH5α

The pcDNA3 vector containing either the HPV11 or HPV18 E6 genes were received from Dr Lawrence Banks at The International Centre for Genetic Experimentation and Biotechnology, Triest, Italy, however we needed to confirm the presence of the correct insert. The confirmation was done by transforming *E. coli* DH5α bacteria with the vectors, extracting plasmid DNA from the transformed bacteria and confirming the insert by restriction enzyme analysis and DNA sequencing. The pcDNA3 vector constructs were transformed into DH5α bacteria to produce sufficient amounts of the plasmids for further experiments. Competent bacteria were produced according to the protocol by Maniatis *et al.* (1982) and stocks of the competent cells were made by adding glycerol to a final concentration of 10% in Luria broth (LB) and stored at -80°C.

Competent bacteria (~200 µl) were transformed by adding 50 ng of pcDNA3 plasmid DNA (carrying either the HPV11 or HPV18 E6 gene or no insert), mixing gently and keeping them on ice for 30 minutes. The cells were then heat shocked for 50 seconds in a 42°C water bath and then placed on ice for 2 minutes. One millilitre of LB was added to the cells, which were incubated at 37°C for a minimum of 1 hour. Thereafter, 50–150 µl of the transformed bacteria was plated on Luria agar plates containing 100 µg/ml of ampicillin (Sigma, Steinheim, Germany). Colonies containing the transformed plasmid were picked after incubation of the plates for 12-15 hours at 37°C. Negative control plates contained no plasmid DNA.

2.2.5 Small-scale preparation of plasmid DNA

Bacterial colonies were picked and grown overnight in 5 ml of LB containing 100 µg/ml ampicillin. The three pcDNA3 vector constructs, pcDNA3, pcDNA3-11E6 or pcDNA3-18E6, were harvested using the QIAGEN® Plasmid Mini Kit (QIAGEN, Hilden, Germany). The protocol was followed as described by the manufacturer. After the extraction, 20 µg/ml DNase-free Ribonuclease A was added to the plasmid DNA to digest any remaining RNA. The plasmid DNA was then quantified as described in section 2.2.2 and 500 ng was electrophoresed on a 1% agarose gel containing 0.5 µg/µl ethidium bromide at 3.9 V/cm to determine the quality of the extracted plasmid DNA.

2.2.6 Restriction enzyme mapping

The plasmid DNA was analysed by restriction enzyme digestion to confirm the presence of the correct insert in the pcDNA3 vectors. DNAman was used to select a restriction enzyme that would differentiate between the HPV11 and HPV18 E6 inserts as well as the vector lacking an insert. The restriction enzyme *Aha*III (Amersham Biosciences, Buckinghamshire, UK) was chosen as it cuts the HPV11 and HPV18 E6 genes at different places producing different sized fragments that can be used to identify the genes. Digestions were set up containing 2 µg plasmid DNA, 4 U *Aha*III, Buffer M (Amersham Biosciences, Buckinghamshire, UK) and H_2O to a final volume of 30 µl. The samples were digested at 37°C for 2 hours and then resolved on a 1.4% agarose gel containing 0.5 µg/µl ethidium bromide at 3.9 V/cm to determine the size of the resulting products.

2.2.7 Sequencing of the pcDNA3 inserts

DNA sequencing was performed using the BigDye Terminator v3.1 kit (Applied Biosystems, California, USA). The reaction mixture for the sequencing contained Ready Reaction Premix, BigDye Sequencing Buffer, 3.2 pmol T_7 primer, 500 ng of template plasmid DNA and H_2O to a final volume of 20 µl. The PCR consisted of 25 cycles of 30 seconds at 96°C, 15 seconds at 50°C and 4 minutes at 60°C. The PCR products were analysed by capillary electrophoresis on an ABI 3100 Genetic Analyser. The identification of the sequence of the insert was determined using the Nucleotide-Nucleotide Basic Local

Transfectin™ Lipid Reagent was used for transfecting both MCF12A and Rat1 cells. For the MCF12A transfection, 120 000 cells were set up in triplicate in 6-well plates, and incubated overnight at 37°C in an incubator containing 5% CO_2. One microlitre of Transfectin™ Lipid Reagent was added to 99 µl of DMEM, lacking any supplements. After incubating the mixture for 5 minutes at room temperature, it was added to eppendorf tubes containing 500 ng of either the pcDNA3, pcDNA3-11E6 or pcDNA3-18E6 constructs diluted in 100 µl DMEM. After mixing gently and incubating at room temperature for 20 minutes, the mixture was added drop-wise to the cells and left for 5 hours at 37°C in an incubator with 5% CO_2. The medium was changed after 48 hours and G418 was added to the cells at a final concentration of 350 µg/ml to select for cells containing the pcDNA3 vector constructs.

For Rat1 fibroblasts, cells were plated in triplicate in 100 mm dishes and transfected when 50 to 60% confluent. For each 100 mm dish, 6 µl Transfectin™ Lipid Reagent, 3 µg of either pcDNA3, pcDNA3-11E6 or pcDNA3-18E6 were added to 50 µl serum-free DMEM. After mixing gently and incubating at room temperature for 20 minutes, the mixture was added drop-wise to the cells and processed as described for the MCF12A cells, with the exception that G418 was added at a final concentration of 400 µg/ml.

2.2.9 Selection for transfected cells

Transfected cells were selected for G418 resistance. To determine the concentration of G418 necessary for selection of the various cell lines, 50 000 cells were plated per well in a 24-well plate and allowed to settle overnight at 37°C in an incubator containing 5% CO_2. Different concentrations of G418, ranging from 100 to 1000 µg/ml for MCF12A and Rat1 cells and 10 to 200 µg/ml G418 for EPC2 cells, were used to determine the best dose. The concentration of G418 that caused substantial cell death within the first 4-5 days and killed all the untransfected cells within 2 weeks was used to select for transfected cells.

For each transfection, G418 was added to a plate of untransfected cells to determine whether the drug killed all the cells lacking the pcDNA3 vector. Once all the untransfected cells were dead, the concentration of G418 added to the medium was decreased to a maintenance dose. The cells were maintained continuously in medium containing the maintenance concentration of G418 and trypsinised every 3 to 4 days, when confluent.

Once sufficient cells were obtained, freezes of the cells were made as described in section 2.2.1. Cells were plated for selection of stable clones by seeding 1 cell per well in 96-well plates.

2.2.10 RNA extraction

Total RNA was extracted from the transfected EPC2 and Rat1 pools 8 to 10 passages after transfection to determine the relative HPV11 and HPV18 E6 mRNA levels. HeLa cells, known to contain HPV18 DNA, were included as a positive control and cells containing only the pcDNA3 vector were included as a negative control. Briefly, 500 000 cells were plated in triplicate in 60 mm dishes and incubated until 80-90% confluent. The cells were washed once with PBS to remove residual medium, trypsinised and neutralised with DMEM containing 10% FBS for Rat1 cells and soybean trypsin inhibitor for EPC2 cells. The cells were centrifuged at 1000 g for 5 minutes and resuspended in 2 ml PBS. The cells were then counted using a haemocytometer and 1.2×10^6 cells were aliquotted into clean microcentrifuge tubes, centrifuged and resuspended in 200 µl PBS. Total RNA was then harvested using the High Pure RNA Isolation Kit (Roche, Mannheim, Germany) according to the manufacturer's protocol.

The extracted RNA was quantified by measuring the absorbance at 260 nm and 280 nm using a Beckmann Du650 spectrophotometer (California, USA). The following formula was used to calculate the RNA concentration: RNA concentration (µg/ml) = OD_{260} x 40 µg/ml x dilution factor. One microgram of total RNA was diluted in RNA loading dye and electrophoresed on a formaldehyde-containing 1.5% agarose gel dissolved in MOPS buffer containing 0.5 µg/µl ethidium bromide. The gel was electrophoresed at a voltage of 3.6 V/cm for 30 minutes.

2.2.11 cDNA synthesis

The RNA/primer mix was prepared by diluting 1 µg of total RNA to a final volume of 7 µl in diethylpyrocarbonate (DEPC)-treated water and adding 500 ng oligo $dT_{(15)}$ (Promega, Wisconsin, USA). The mixture was incubated at 70°C for 10 minutes, centrifuged briefly and placed immediately on ice to cool. To the RNA/primer mix was added first strand buffer (Promega, Wisconsin, USA), 10 mM 1.4-dithiothreitol (DTT), 1 U/µl RNAsin®

flame-sterilised coverslips (Deckglaser, Germany) and incubated in normal growth medium at 37°C in an incubator containing 5% CO_2 until 70-80% confluent. Medium was then removed and the cells were fixed using 4% paraformaldehyde (PFA) in PBS for 20 minutes at room temperature. Thereafter, the cells were washed three times in PBS (each wash lasting 5 minutes). The cells were permeabilised in 0.5% Triton X-100 in PBS for 5 minutes and then washed three times, as before. The free aldehyde groups were subsequently quenched by the addition of 50 mM NH_4Cl in PBS for 5 minutes and the cells then blocked in 0.2% gelatin (from porcupine skin) in PBS, for 30 minutes.

The coverslips, with the cell-side facing down, were lowered onto a 1:50 dilution of HPV16/18 E6 (C1P5) sc-480 mouse monoclonal IgG antibody (SantaCruz Biotechnology, California, USA) in blocking solution (0.2% gelatine in PBS) and incubated in a humidified chamber for 45 minutes at room temperature. The cells were then washed three times and a 1:250 dilution of the Cy3-labelled affinity purified antibody to mouse IgG (H + L) (Xtra Serum Adsorbed, KPL, Maryland, USA) in blocking solution was added to the Rat1 cells and a 1:400 dilution of the Alexa 488-labelled goat anti-mouse IgG antibody (Invitrogen, Ontario, Canada) was added to the EPC2 cells. The cells were incubated as for the primary antibody and kept in the dark for further steps.

The cells were washed once in PBS for 5 minutes and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma, Mannheim, Germany) was then added at a final concentration of 100 ng/ml in PBS and incubated for 5 minutes. The cells were washed, dipped in distilled water and mounted on slides using Mowiol® 4-88 Reagent (Calbiochem, California, USA). The slides were viewed using the Zeis Immunofluorescence microscope. HeLa cells were routinely included as a positive control and cells containing the pcDNA3 vector only were included as negative controls. Cells exposed only to the secondary antibody were included to check for any non-specific binding of the secondary antibody.

2.2.14 Detection of p53 by Western blot analysis

p53 protein levels could not be determined in the transfected Rat1 pools as no rat p53 antibody was available. Protein was extracted from the transfected EPC2 pools, 8 to 10 passages after transfection. The transfected cells were grown in 100 mm dishes until

80-90% confluent and then placed on ice and washed three times using PBS. RIPA buffer containing Complete protease inhibitor cocktail (Roche, Mannheim, Germany) was added to the cells and scraped using a cell scraper. The protein lysates were sonicated for 10 seconds and the samples were then centrifuged at 10 000 rpm for 10 minutes at 4°C. The supernatants were transferred to clean eppendorf tubes for quantification.

2.2.14.1 Protein quantification

The BCA™ (Bicinchoninic Acid) Protein Assay Kit (Pierce, Illinois, USA) was used to quantify the protein lysates extracted from the transfected EPC2 pools. The protocol was followed as described by the manufacturer. Briefly, a standard curve was constructed using concentrations between 0 and 2000 µg/ml of bovine serum albumin (BSA) diluted in RIPA buffer. The protein lysates were diluted in RIPA buffer to a 1:10 dilution and 25 µl of test sample and the standard were pipetted into a Sero-Wel 96-well plate. RIPA buffer was used as the blank.

A 200 µl volume of BCA working solution (50 parts A: 1 part B) was added to each well and incubated at 37°C for 30 minutes. Reagent A (sodium carbonate, sodium bicarbonate, BCA detection reagent, sodium tartrate in 0.1 M NaOH) and Reagent B (4% $CuSO_4.5H_2O$) were supplied. After cooling to room temperature, the absorbance at 595 nm was recorded for each sample using a 96-well plate reader (EL800, BioTek Instruments, Winooski, USA). The concentration of the protein samples was extrapolated from the standard curve.

2.2.14.2 Denaturing gel electrophoresis

Protein lysates from cells were electrophoresed on 10% SDS polyacrylamide gels. The protein lysates (30 µg) were added to Laemmli buffer, boiled for 10 minutes and loaded on a SDS-polyacrylamide gel. A protein molecular weight marker (PRN800 molecular weight marker, Amersham Biosciences, Buckinghamshire, UK) (Appendix A.2) was included for the determination of protein sizes. The polyacrylamide gel was prepared using 4% stacking gel on top of a 10% resolving gel. The gel tank apparatus (Bio-Rad Mini-Protean 3 Cell Assembly) was filled with running buffer and a voltage of 200 V was applied to the gel for 45 minutes.

2.2.14.3 Electroblotting and immunodetection

The resolved proteins were transferred onto a Hybond-ECL nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK) for 1 hour at 100 V in Transfer Buffer. After transfer, the membrane was stained with Ponceau S for 5 minutes, destained in water for a few minutes until red protein bands could be visualised and a photocopied image was taken of the membrane as an indication of the loading. The membrane was further destained using water and then finally rinsed with Tris-buffered saline containing 0.05% Tween-20 (TBS-T). After transfer, the gel was treated with fixing solution for 15 minutes, with shaking. Thereafter, the gel was incubated in Rapid Coomassie Blue for 15 minutes, with shaking, and then destained in 10% acetic acid, with shaking, until the protein bands were clearly visible. The gel was then dried on a Drygel Sr Slab Gel Dryer SE 1160, (Hoefer Scientific Instruments, San Francisco, USA) to check for loading.

The nitrocellulose membrane containing the bound proteins was blocked in 5% non-fat milk powder for 1 hour, with shaking, to prevent non-specific binding of the antibodies to the membrane. Thereafter, a 1:500 dilution of the monoclonal mouse anti-human p53 protein clone DO-7 (DakoCytomation, Glostrup, Denmark) in TBS-T was added to the membrane and incubated overnight at 4°C with shaking. The membrane was washed 3 times for 10 minutes each in TBS-T. A 1:1 000 dilution in TBS-T of the stabilised goat anti-mouse HRP-conjugated antibody (Pierce, Illinois, USA) was added and incubated at room temperature for 1 hour, with shaking. The membrane was subjected to three 10 minute washes in TBS-T, to remove non-specific antibody binding. LumiGLO Reserve™ Chemiluminescent substrate kit (KPL, Maryland, USA) was used to visualise the protein bands as described by the manufacturer. The relative protein levels were analysed by densitometric scanning.

2.2.15 Cell morphology analysis

EPC2 and Rat1 cells transfected with pcDNA3, pcDNA3-11E6 or pcDNA3-18E6 were plated on 22 mm diameter, sterile coverslips and incubated in normal growth medium at 37°C in an incubator containing 5% CO_2 until 70-80% confluent. The medium was removed from the cells and the cells were fixed using 4% PFA in PBS for 20 minutes at room temperature. Thereafter the cells were washed three times in PBS for 5 minutes.

The coverslips were dipped in water and mounted on slides using Mowiol® 4-88 Reagent. The slides were then viewed using the Zeis microscope at 100X or 200X magnification.

2.2.16 Cell proliferation analysis

The MTT Cell Proliferation Kit (Roche, Mannheim, Germany) was used to determine cell growth rate. The assay involved plating 1 500 and 2 000 Rat1 and EPC2 cells, respectively. Cells were plated in quadruplicate for every time point (i.e. 0, 1, 2, 3, 4 and 5 days) in 96-well plates and medium was changed on day 3. After 3 hours at 37°C, 10 µl of MTT reagent was added to each well. After at least 4 hours of incubation with MTT at 37°C, 100 µl of solubilisation buffer was added to each well and incubated overnight at 37°C in an incubator containing 5% CO_2. The following day, absorbance readings at 595 nm were obtained to measure cell viability using a 96-well plate reader. This process was repeated for each time point. Changes observed in cell proliferation using the MTT assay were confirmed by counting the number of cells using a coulter counter.

2.2.17 Cell cycle analysis

Half a million transfected EPC2 or Rat1 cells were plated in triplicate in 60 mm dishes and harvested when 80% confluent. To harvest the floating cells, the medium was removed and kept in a 12-ml tube. The adherent cells were harvested by rinsing twice with PBS (which was added to the medium in the 12-ml tube) and trypsinised using 1 ml of trypsin-EDTA. The trypsin was neutralised using 1 ml DMEM containing FBS for the Rat1 cells and soybean trypsin inhibitor for the EPC2 cells and the cells were added to the 12-ml tube. The harvested cells were pelleted by centrifugation at 1000 g for 5 minutes and resuspended in 2 ml medium, and counted using a haemocytometer. Cells were fixed by adding ice-cold ethanol (95%) to a final volume of 10 ml and left at -20°C for up to 2 weeks.

After fixation, the cells were centrifuged at 1000 g for 5 minutes, the supernatant removed and discarded. The pellet was rinsed twice using 1 ml PBS and on the second rinse, 0.5-1.0 x 10^6 cells were aliquotted into a clean eppendorf. The cells were pelleted and 200 µl of 50 µg/ml RNase A (Fermentas, Maryland, USA) diluted in PBS was added to the cells which were then incubated for 30 minutes at room temperature. Thereafter, 1 ml per

1 x 10^6 cells of propidium iodide stain solution was added to the cells 20 minutes before analysis on a Beckman Coulter FACS-calibur flow cytometer (Buckinghamshire, UK). Ten thousand events were acquired on the flow cytometer after excluding doublets and debris. Subsequent data analysis was done using Flow Jo software.

2.2.18 Peptide nucleic acid (PNA) studies

The PNAs were designed and synthesised by Prof Lidia Boffa (IST, Genoa, Italy). The sequence of the HPV18 E6 anti-gene peptide nucleic acid (PNA_{18}) was VKRKKKP-aataaagatacagaac-NH_2. A mutant PNA (PNA_{mut}), VKRKKKP-tgaaaagtgaagata-NH2, was included in all the experiments to control for non-specific or cytotoxic effects. The PNAs were dissolved in sterile H_2O and the pH was corrected to 7.0 using NaOH. HeLa was chosen as a model system as it is a known HPV18-positive cervical cancer cell line (Boshart *et al.*, 1984). For all PNA experiments, the PNA_{18} or PNA_{mut} were added at a final concentration of 10 µM

HeLa cells (500 000) were plated, in triplicate, in 60 mm dishes and allowed to settle overnight at 37°C in an incubator containing 5% CO_2. The next day, PNA_{18} or the PNA_{mut} was added to the HeLa cells in triplicate. Untreated HeLa cells were also included as a control. Total RNA was extracted using QUIzol (QIAGEN, Hilden, Germany) 24 and 48 hours after the addition of the PNAs. The cells were washed three times with PBS on ice and 1 ml of QUIzol was added per 60 mm dish. The cells were scraped using a cell scraper and stored at -80°C until further processing.

For the RNA extraction, 200 µl of chloroform was added and shaken vigorously by hand for 15 seconds. The samples were then incubated on ice for 10 minutes and centrifuged at 8 000 rpm for 15 minutes at 4°C. The aqueous phase was added to a sterile tube and 500 µl of isopropanol was added to the aqueous phase. This was incubated at -20°C overnight to precipitate the RNA. The samples were centrifuged at 8 000 rpm for 30 minutes at 4°C and the supernatant removed. One millitre of 75% ethanol was added the RNA pellet and gently resuspended. After centrifugation at 8 000 rpm for 30 minutes at 4°C the RNA pellet was air dried for about 10 minutes. The pellet was then dissolved in 20-30 µl DEPC-treated water and quantified as described in section 2.2.10. Two micrograms of total RNA was resolved on a formaldehyde-containing 1.5% agarose gel containing 0.5 µg/µl

CHAPTER 3

Results

The role of human papillomavirus (HPV) in the development of OSCC is poorly understood. It is therefore necessary to determine whether HPV E6 is a risk factor for the development of OSCC. This study looked at the effects of two HPV E6 proteins on cellular transformation, namely HPV11 E6 and HPV18 E6. HPV11 E6, a low-risk HPV type, was chosen as it was found by Matsha *et al.* (2002) to be the most common HPV type in the South African OSCC patients. HPV18 E6 was chosen as an example of a high-risk HPV type that is commonly found in cervical cancer tumours (Snijders *et al.*, 2006). The effects of the HPV E6 proteins were determined by, 1) introducing them into HPV-negative cells and investigating the effects on cellular transformation, 2) knocking out HPV E6 in an HPV-positive cell line and determining the resultant effects.

3.1 Human papillomavirus status of cultured cell lines

Since the work to be done in this study depends on knowing the HPV status of the cells that are to be used, several cell lines were screened for the presence of HPV DNA. Genomic DNA was extracted from the cell lines and the serum albumin gene was amplified in order to determine the quality of the DNA. Figure 3.1 shows a representative picture of the amplification of human serum albumin.

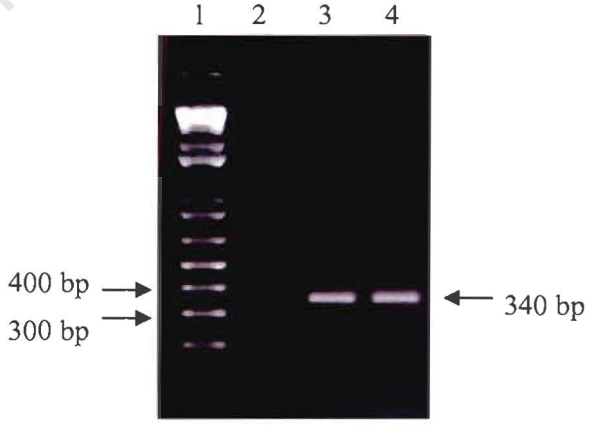

Figure 3.1: PCR amplification of the human serum albumin gene. The DNA extracted from the cell lines was validated by the amplification of the human serum albumin gene as described in section 2.2.3 and resolved on a 1.5% agarose gel containing ethidium bromide. Lane 1: 1 Kb Plus Marker, lane 2: no template control, lane 3: EPC2 DNA, and lane 4: MCF12A DNA.

After confirmation of the quality of the DNA for each of the cell lines, PCR was performed to amplify either the HPV L1 or E6 gene. It was necessary to check for both the HPV L1 and E6 genes as the L1 gene may be disrupted when the viral DNA integrates into the host genome. Amplification of the HPV L1 gene involved nested PCR using the degenerate consensus primers, MY09 and MY11, and the non-degenerate $GP5^+$ and $GP6^+$ primers (section 2.2.3). None of the cell lines tested positive for HPV L1 except the positive controls, HeLa and CaSki. Amplification of the HPV E6 gene also revealed that none of the cell lines were positive for HPV E6 except HeLa and CaSki (Table 3.1).

Table 3.1: HPV status of selected cell lines. DNA was extracted from the cell lines and PCR was performed to amplify either the HPV L1 or E6 gene.

Cell line	Origin of cell line	HPV L1	HPV E6	Reference
EPC2	Normal oesophageal epithelium	-	-	Harada *et al.*, 2003
MCF12A	Normal breast epithelium	-	-	Paine et al., 1992
Rat1	Rat fibroblast	-	-	ATCC CRL-2210
WHCO1	OSCC	-	-	Veal *et al.*, 1989
WHCO3	OSCC	-	-	Veal *et al.*, 1989
WHCO5	OSCC	-	-	Veal *et al.*, 1989
WHCO6	OSCC	-	-	Veal *et al.*, 1989
KYSE30	OSCC	-	-	Shimada *et al.*, 1992
KYSE70	OSCC	-	-	Shimada *et al.*, 1992
KYSE180	OSCC	-	-	Shimada *et al.*, 1992
KYSE520	OSCC	-	-	Shimada *et al.*, 1992
HeLa	Cervical SCC	+	+	Boshart *et al.*, 1984
CaSki	Cervical SCC	+	+	Baker *et al.*, 1987

After the characterisation of the different cell lines with respect to their HPV status, three were chosen for transfection studies, i.e. MCF12A, EPC2 and Rat1.

3.2 Transfection of cell lines with HPV E6

The HPV11 and HPV18 E6 genes were transfected into the EPC2, Rat1 and MCF12A cell lines to determine whether the low- and high-risk HPV E6 proteins have different effects on cellular transformation. It was first necessary to determine whether the pcDNA3 constructs used for the transfection studies contained the correct HPV E6 gene.

3.2.1 Confirmation of the HPV E6 insert in the pcDNA3 constructs

The pcDNA3 vector containing the appropriate HPV E6 insert was subjected to restriction enzyme analysis to confirm the presence of the insert. Digestion with the enzyme, *Aha*III, resulted in the appropriate digestion products that allowed distinction between the HPV11 and HPV18 E6 genes (Figure 3.2). The pcDNA3 vector generated fragments of 3365, 1250, 692, 102 and 19 bp (of which only the three larger bands can be see on this gel), while the HPV11 E6 clone generated fragments of 3525, 1543, 692 and 19 bp and the HPV18 E6 clone generated fragments of 3695, 1397, 692 and 19 bp.

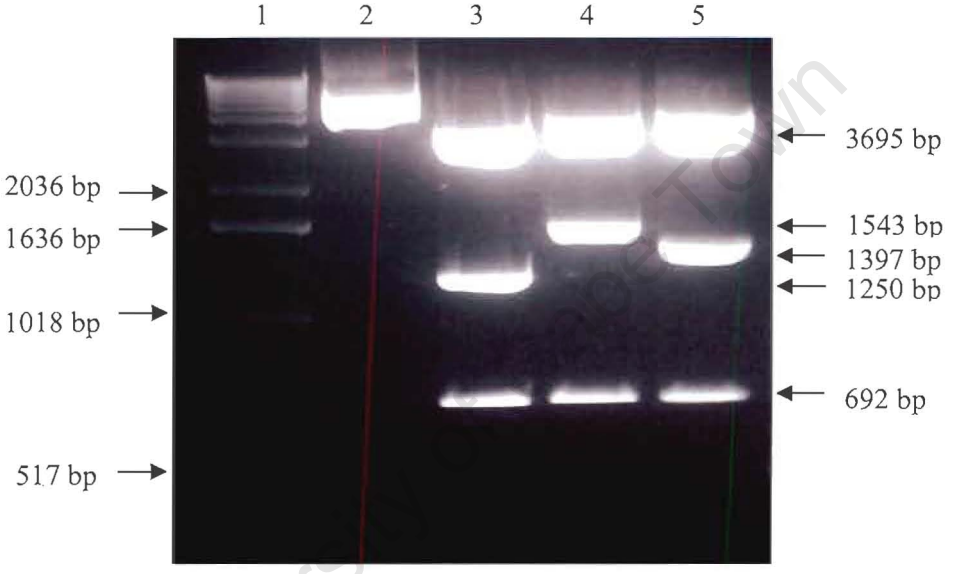

Figure 3.2: Restriction enzyme analysis of the pcDNA3-HPV-E6 constructs. DNA was digested with *Aha*III and resolved on a 1.4% agarose gel containing ethidium bromide as described in section 2.2.6. Lane 1: Marker X, lane 2: undigested pcDNA3 vector, lane 3: pcDNA3 digested with *Aha*III, lane 4: pcDNA3-11E6 digested with *Aha*III, and lane 5: pcDNA3-18E6 digested with *Aha*III. The 102 and 19 bp fragments could not be visualised as they were too small to be retained on the gel.

After restriction digestion the inserts in the pcDNA3-11E6 and pcDNA3-18E6 constructs were sequenced and the Nucleotide-Nucleotide Basic Local Alignment Search Tool (BLASTn) used to search the National Center for Biotechnology Information (NCBI) database to determine the identities of the gene. The sequencing alignments obtained confirmed the two HPV E6 genes present in the pcDNA3 vectors (Figure 3.3). After this confirmation, MCF12A, EPC2 and Rat1 cells were transfected with either the empty vector (pcDNA3 only), pcDNA3-11E6 or pcDNA3-18E6.

a)

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Insert:GGTTATATATAAACCAGCCCAAAAAATTAGCAGACGAGGCATTATGGAAAGTAAAGATGC   60
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
11E6:  GGTTATATATAAACCAGCCCAAAAAATTAGCAGACGAGGCATTATGGAAAGTAAAGATGC  118

       CTCCACGTCTGCAACATCTATAGACCAGTTGTGCAAGACGTTTAATCTTTCTTTGCACAC  120
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
       CTCCACGTCTGCAACATCTATAGACCAGTTGTGCAAGACGTTTAATCTTTCTTTGCACAC  178

       TCTGCAAATTCAGTGCGTGTTTTGCAGGAATGCACTGACCACCGCAGAGATATATGCATA  180
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
       TCTGCAAATTCAGTGCGTGTTTTGCAGGAATGCACTGACCACCGCAGAGATATATGCATA  238

       TGCCTATAAGAACCTAAAGGTTGTGTGGCGAGACAACTTTCCCTTTGCAGCGTGTGCCTG  240
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
       TGCCTATAAGAACCTAAAGGTTGTGTGGCGAGACAACTTTCCCTTTGCAGCGTGTGCCTG  298

       TTGCTTAGAACTGCAAGGGAAAATTAACCAATATAGACACTTTAATTATGCTGCATATGC  300
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
       TTGCTTAGAACTGCAAGGGAAAATTAACCAATATAGACACTTTAATTATGCTGCATATGC  358

       ACCTACAGTAGAAGAAGAAACCAATGAAGATATTTTAAAAGTGTTAATTCGTTGTTACCT  360
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
       ACCTACAGTAGAAGAAGAAACCAATGAAGATATTTTAAAAGTGTTAATTCGTTGTTACCT  418

       GTGTCACAAGCCGTTGTGTGAAATAGAAAAACTAAAGCACATATTGGGAAAGGCACGCTT  420
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
       GTGTCACAAGCCGTTGTGTGAAATAGAAAAACTAAAGCACATATTGGGAAAGGCACGCTT  478

       CATAAAACTAAATAACCAGTGGAAGGGTCGTTGCTTACACTGCTGGACAACATGCATGGA  480
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
       CATAAAACTAAATAACCAGTGGAAGGGTCGTTGCTTACACTGCTGGACAACATGCATGGA  538
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b)

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Insert:AAGATGTGAGAAACACACCACAATACTATGGCGCGCTTTGAGGATCCAACACGGCGACCC   60
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
18E6:  AAGATGTGAGAAACACACCACAATACTATGGCGCGCTTTGAGGATCCAACACGGCGACCC  342

       TACAAGCTACCTGATCTGTGCACGGAACTGAACACTTCACTGCAAGACATAGAAATAACC  120
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
       TACAAGCTACCTGATCTGTGCACGGAACTGAACACTTCACTGCAAGACATAGAAATAACC  402

       TGTGTATATTGCAAGACAGTATTGGAACTTACAGAGGTATTTGAATTTGCATTTAAAGAT  180
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
       TGTGTATATTGCAAGACAGTATTGGAACTTACAGAGGTATTTGAATTTGCATTTAAAGAT  462

       TTATTTGTGGTGTATAGAGACAGTATACCGCATGCTGCATGCCATAAATGTATAGATTTT  240
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
       TTATTTGTGGTGTATAGAGACAGTATACCGCATGCTGCATGCCATAAATGTATAGATTTT  522

       TATTCTAGAATTAGAGAATTAAGACATTATTCAGACTCTGTGTATGGAGACACATTGGAA  300
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
       TATTCTAGAATTAGAGAATTAAGACATTATTCAGACTCTGTGTATGGAGACACATTGGAA  582

       AAACTAACTAACACTGGGTTATACAATTTATTAATAAGGTGCCTGCGGTGCCAGAAACCG  360
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
       AAACTAACTAACACTGGGTTATACAATTTATTAATAAGGTGCCTGCGGTGCCAGAAACCG  642

       TTGAATCCAGCAGAAAAACTTAGACACCTTAATGAAAAACGACGATTTCACAACATAGCT  420
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
       TTGAATCCAGCAGAAAAACTTAGACACCTTAATGAAAAACGACGATTTCACAACATAGCT  702

       GGGCACTATAGAGGCCAGTGCCATTCGTGCTGCAACCGAGCACGACAGGAACGACTCCAA  480
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
       GGGCACTATAGAGGCCAGTGCCATTCGTGCTGCAACCGAGCACGACAGGAACGACTCCAA  762

       CGACGCAGAGAAACACAAGTATAATATTAAGTATGCATGGACCTAAGGCAACATTGCAAG  540
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
       CGACGCAGAGAAACACAAGTATAATATTAAGTATGCATGGACCTAAGGCAACATTGCAAG  822
```


Figure 3.3: Sequence alignment of the pcDNA3 HPV E6 inserts. The pcDNA3 inserts were sequenced as described in section 2.2.7 to confirm the presence of the correct HPV E6 gene. **a) Sequence alignment of the HPV11 E6 insert**. The sequenced insert showed 100% (514/514) homology with the HPV11 E6 gene (gi|333026|gb|M14119.1|PPH11). **b) Sequence alignment of the HPV18 E6 insert.** The sequenced insert showed 100% (540/540) homology with the HPV18 E6 gene (emb|X04354.1|PARHPVE6).

3.2.2 Transfection of the EPC2, Rat1 and MCF12A cell lines

Generally very low transfection efficiencies were observed for the EPC2, MCF12A and Rat1 cell lines. Transfectin™ Lipid Reagent gave better transfection efficiencies (compared to FuGENE®6 Transfection Reagent) for the MCF12A and Rat1 cells. However, despite the better efficiency associated with it, Transfectin™ Lipid Reagent could not be used on the EPC2 cells as it was too toxic and killed all cells within 5 hours. Instead, FuGENE®6 Transfection Reagent was used to transfect the EPC2 cells.

After transfection of the cells using the conditions outlined in Table 3.2, G418 was used to select for cells containing the pcDNA3 constructs. The concentration of G418 that caused substantial cell death within the first 4-5 days and killed all untransfected cells within 2 weeks was chosen to select for successfully transfected cells. The G418 selection concentration for the EPC2, MCF12A and Rat1 cells was 30, 350 and 400 μg/ml, respectively. Once the drug had killed all untransfected cells, the concentration of G418 was reduced to a maintenance dose. The G418 maintenance dose for the EPC2, MCF12A and Rat1 cells was 30, 150 and 200 μg/ml, respectively.

Table 3.2: Transfection of EPC2, MCF12A and Rat1 cells. Different transfection reagents and conditions were tested on the cell lines to optimise for transfection efficiency. This table shows the conditions used.

Cell line	Reagent used	Dish size (mm)	Volume of reagent added (μl)	Amount of plasmid added (μg)	Incubation time before adding G418 (hours)
EPC2	FuGENE®6	60	14	2.21	96
MCF12A	Transfectin™	35	1	0.5	48
Rat1	Transfectin™	100	6	3	48

After G418 selection, pools of transfected cells were used for further analysis since isolation of individual cells proved difficult for the epithelial cells. Three pools of cells containing the pcDNA3 vector, 7 pools of cells containing pcDNA3-11E6, and 8 pools of cells containing pcDNA3-18E6 were used for subsequent analysis.

3.2.3 Confirmation of the presence of HPV E6 genes in selected pools

Prior to performing the subsequent experiments on the transfected pools, it was important to confirm the presence of the HPV E6 genes in each of the pools. DNA was extracted and PCR amplification of the HPV E6 genes confirmed their presence. In all cases, the quality of the DNA was checked by amplifying a small fragment of either the human or rat serum albumin gene. Figure 3.4 shows that the DNA extracted from the pools of each cell line was of good quality.

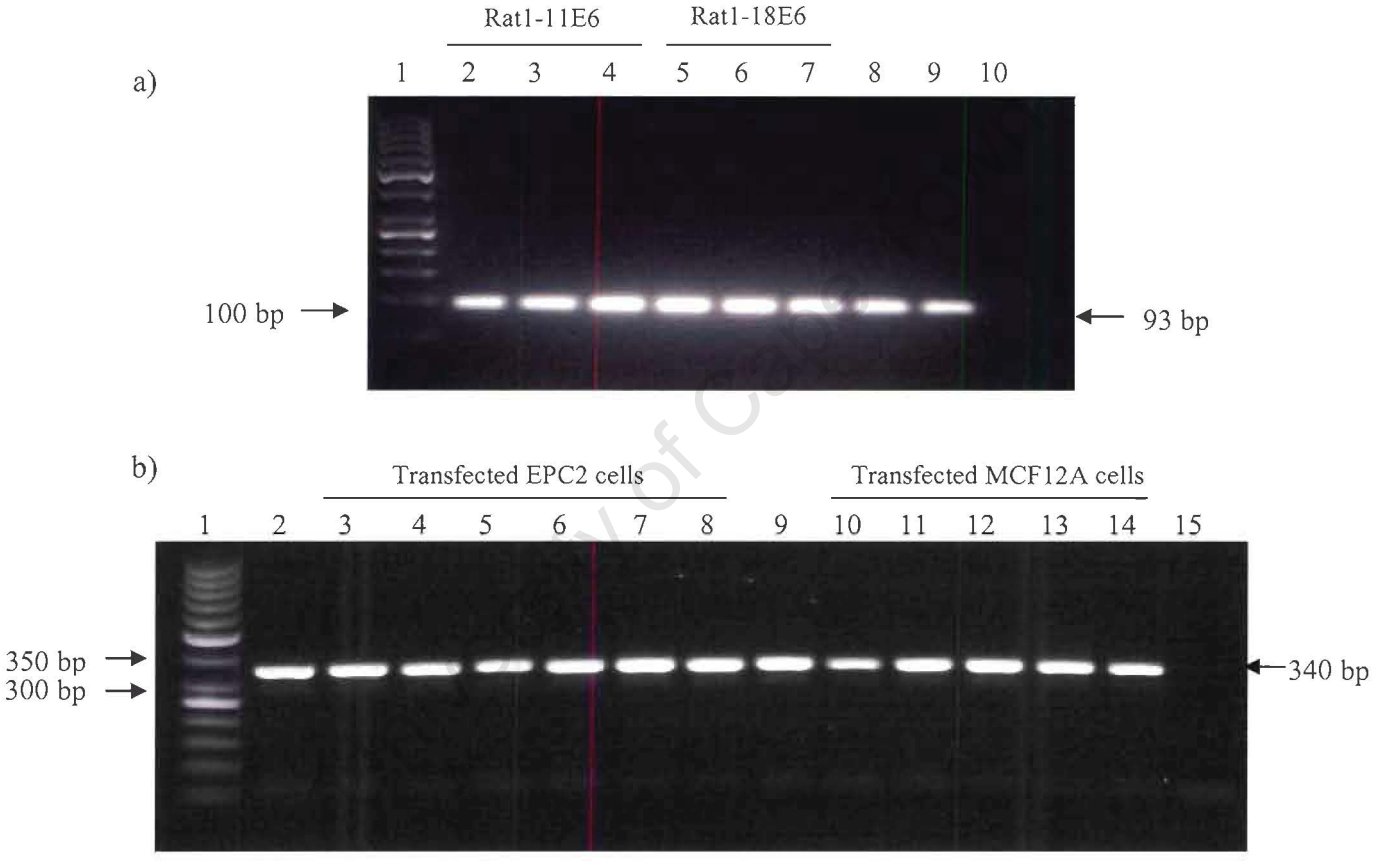


Figure 3.4: Integrity of extracted DNA. Genomic DNA extracted from the transfected cells was validated by the amplification of the serum albumin gene as described in section 2.2.3 and the products were separated on 2% agarose gel and visualised by staining with ethidium bromide. **a) PCR amplification of the rat serum albumin gene from extracted DNA.** Lane 1: 50 bp DNA ladder, lane 2: Rat1-11E6 pool 1, lane 3: Rat1-11E6 pool 2, lane 4: Rat1-11E6 pool 3, lane 5: Rat1-18E6 pool 1, lane 6: Rat1-18E6 pool 2, lane 7: Rat1-18E6 pool 3, lane 8: Rat1-pcDNA3, lane 9: Rat1, and lane 10: no template control. b) **PCR amplification of the human serum albumin gene from extracted DNA**. Lane 1: 50 bp DNA ladder, lane 2: EPC2, lane 3: EPC2-pcDNA3, lane 4: EPC2-11E6 pool 1, lane 5: EPC2-11E6 pool 2, lane 6: EPC2-18E6 pool 1, lane 7: EPC2-18E6 pool 2, lane 8: EPC-18E6 pool 3, lane 9: MCF12A, lane 10: MCF12A-pcDNA3, lane 11: MCF12-11E6 pool 1, lane 12: MCF12A-11E6 pool 2, lane 13: MCF12A-18E6 pool 1, lane 14: MCF12A-18E6 pool 2,and lane 15: no template control.

The DNA from each pool was used to amplify HPV11 or HPV18 E6 in all the transfected cells (Figure 3.5 and 3.6). For Rat1, pools 1, 2 and 3 transfected with the pcDNA3-11E6 construct were all positive for the HPV11 E6 gene (Figure 3.5a). Similarly, pools 1, 2 and 3 transfected with the pcDNA3-18E6 construct were all positive for the HPV18 E6 gene (Figure 3.5b) with no cross amplification between HPV11 and 18 E6 (compare lanes 2-4 with 5-7 in Figure 3.5 a and b). EPC2-11E6 pool 1 was positive for HPV11 E6 but pool 2 was negative (Figure 3.6a). Neither of the MCF12A-11E6 pools contained the HPV11 E6 gene. All three of the EPC2-18E6 pools were positive for the HPV18 E6 gene, however MCF12A-18E6 pool 1 was positive for the HPV18E6 gene but pool 2 was not (Figure 3.6b). The MCF12A transfected cells were discontinued from further analysis as all the transfected cells, including the cells containing pcDNA3, became spindly in shape and stopped growing. The EPC2-11E6 pool 2 was also discontinued from the study as it did not contain the HPV11 E6 gene.

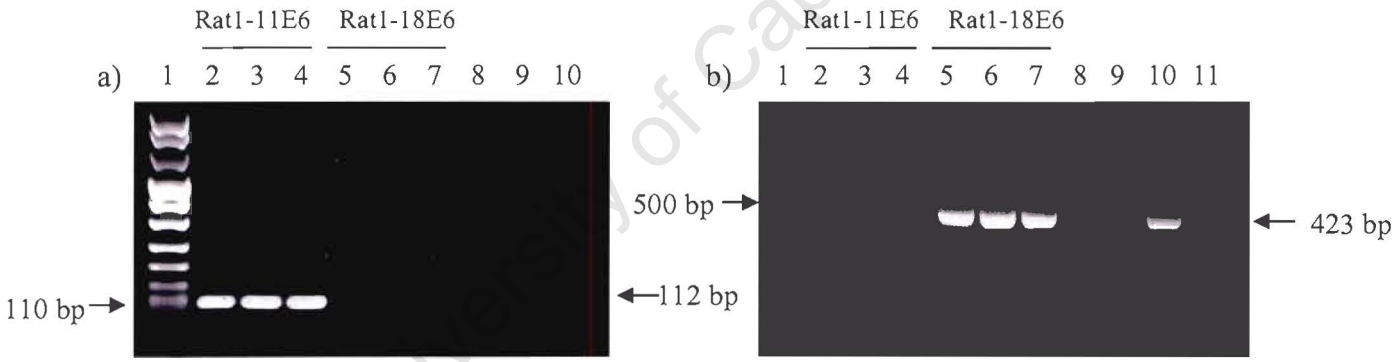


Figure 3.5: Amplification of HPV E6 genes in Rat1 transfected cells. The presence of the HPV11 and 18 E6 genes was validated by the amplification of a 112 bp region of the HPV11 E6 gene and a 423 bp region of the HPV18 E6 gene from DNA extracted from the pools as described in section 2.2.3. The PCR products were resolved on 2% (HPV11 E6) and 1.5% (HPV18 E6) agarose gel, and visualised by staining with ethidium bromide. **a) HPV11 E6 PCR.** Lane 1: Marker VIII, lane 2: Rat1-11E6 pool 1, lane3: Rat1-11E6 pool 2, lane 4: Rat1-11E6 pool 3, lane 5: Rat1-18E6 pool 1, lane 6: Rat1-18E6 pool 2, lane 7: Rat1-18E6 pool 3, lane 8: Rat1-pcDNA3, lane 9: Rat1, and lane 10: no template control. **b) HPV18 E6 PCR**. Lane 1: 50 bp DNA ladder, lane 2: Rat1-11E6 pool 1, lane3: Rat1-11E6 pool 2, lane 4: Rat1-11E6 pool 3, lane 5: Rat1-18E6 pool 1, lane 6: Rat1-18E6 pool 2, lane 7: Rat1-18E6 pool 3, lane 8: Rat1-pcDNA3, lane 9: Rat1, lane 10: HeLa as a positive control, and lane 11: no template control.

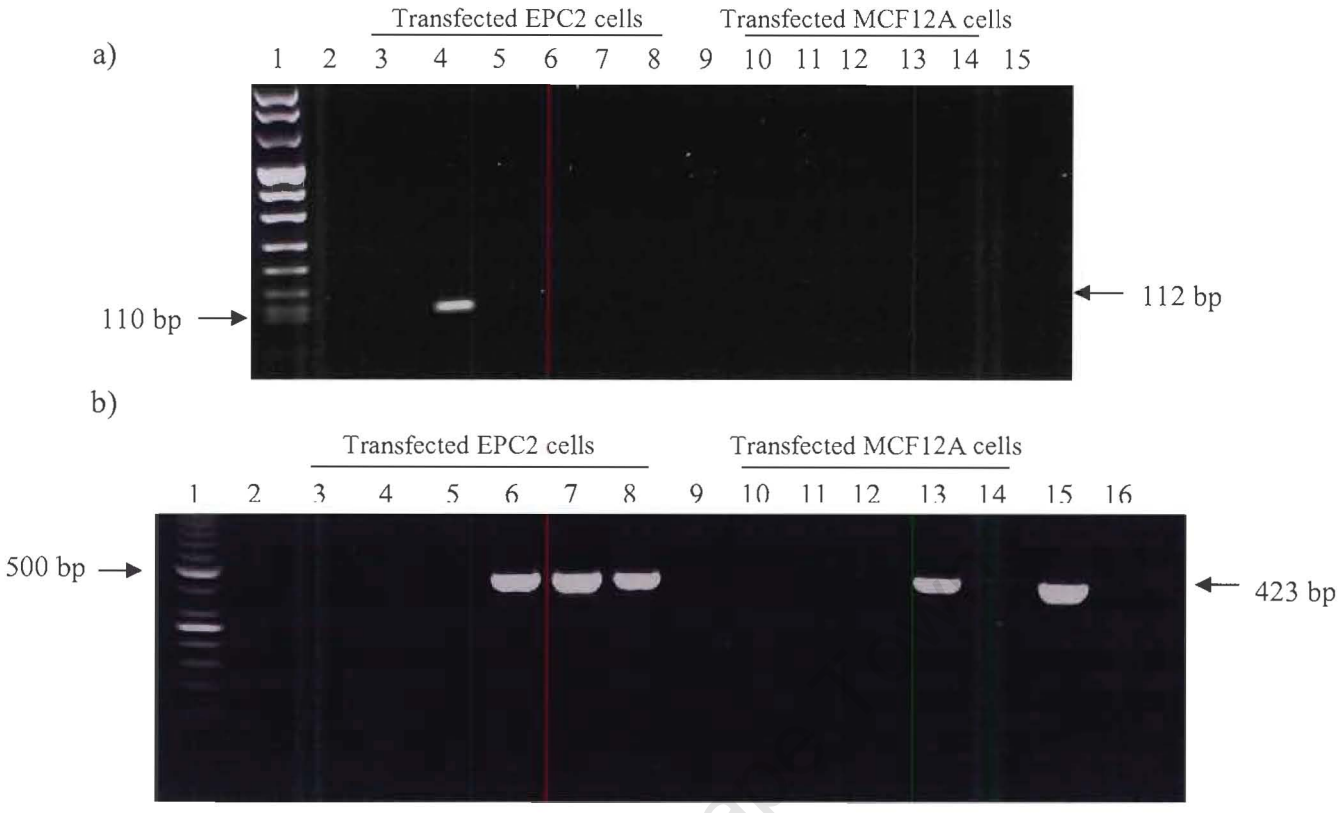


Figure 3.6: Amplification of HPV E6 genes in transfected EPC2 and MCF12A cells. The presence of the HPV11 and 18 E6 genes was validated by the amplification of a 112 bp region of the HPV11 E6 gene and a 423 bp region of the HPV18 E6 gene from DNA extracted from the pools as described in section 2.2.3. The PCR products were resolved on 2% (HPV11 E6) and 1.5% (HPV18 E6) agarose gel and visualised by staining with ethidium bromide. **a) HPV11 E6 PCR.** Lane 1: Marker VIII, lane 2: EPC2, lane 3: EPC2-pcDNA3, lane 4: EPC2-11E6 pool 1, lane 5: EPC2-11E6 pool 2, lane 6: EPC2-18E6 pool 1, lane 7: EPC2-18E6 pool 2, lane 8: EPC-18E6 pool 3, lane 9: MCF12A DNA, lane 10: MCF12A-pcDNA3, lane 11: MCF12-11E6 pool 1, lane 12: MCF12A-11E6 pool 2, lane 13: MCF12A-18E6 pool 1, lane 14: MCF12A-18E6 pool 2, and lane 15: no template control. **b) HPV18 E6 PCR.** Lane 2: EPC2, lane 3: EPC2-pcDNA3, lane 4: EPC2-11E6 pool, lane 5: EPC2-11E6 pool 2, lane 6: EPC2-18E6 pool 1, lane 7: EPC2-18E6 pool 2, lane 8: EPC-18E6 pool 3, lane 9: MCF12A, lane 10: MCF12A-pcDNA3, lane 11: MCF12-11E6 pool 1, lane 12: MCF12A-11E6 pool 2, lane 13: MCF12A-18E6 pool 1, lane 14: MCF12A-18E6 pool 2, lane 15: HeLa, and lane 16: no template control.

3.2.4 Transcription of the HPV E6 genes in the transfected pools

After confirming the presence of the HPV E6 genes in the transfected pools, it was necessary to investigate whether the genes were transcribed and translated before investigating any effects on the cell. Figure 3.7 shows an example of the integrity of the extracted RNA where the 28S and 18S ribosomal RNA bands were clearly seen in all samples

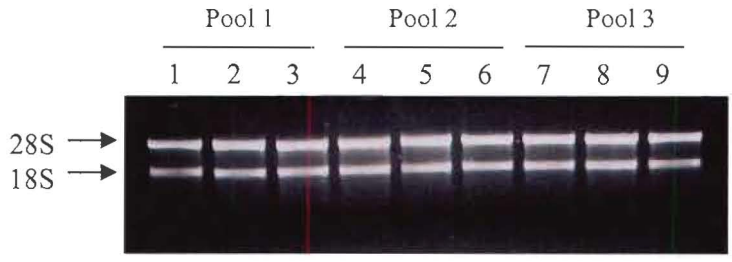


Figure 3.7: Integrity of extracted total RNA. Total RNA was extracted from all the transfected pools and 1 μg of RNA from each pool was electrophoresed on a formaldehyde-containing agarose gel to determine whether the RNA was intact as described in section 2.2.10. Lanes 1-3: RNA from Rat1-11E6 pool 1, lanes 4-6: RNA from Rat1-11E6 pool 2 and lanes 7-9: RNA extracted from Rat1-11E6 pool 3.

After confirming the integrity of the extracted RNA, cDNA was synthesised for quantitative RT-PCR analysis. Relative standard curves were constructed to determine the efficiencies of the different amplification reactions using either human GAPDH, rat GAPDH, HPV11E6 or HPV18 E6 specific primers. Different efficiencies were obtained for the reactions. The amplification reaction using the HPV18 E6 primers had the highest efficiency (1.96), followed by the reaction containing the HPV11 E6 primers (1.85). The efficiencies of the reactions containing the rat and human GAPDH primers were lower at 1.82 and 1.78, respectively. It was necessary to calculate the efficiencies in order to use the normalisation with calibrator method (section 2.2.12) for analysing the relative mRNA levels in the transfected cells.

The relative HPV11 and HPV18 E6 mRNA levels were calculated for the different transfected pools using either rat GAPDH (for the Rat1 pools) or human GAPDH (for the EPC2 pools) as the normaliser. The three Rat1-11E6 pools showed different HPV11 E6 mRNA levels with pool 1 containing the lowest compared to pools 2 and 3 (Figure 3.8a). Pool 3 had about 6-fold higher HPV11 E6 mRNA compared to pool 1. A smaller difference in the HPV E6 mRNA levels was observed in the three Rat1-18E6 pools with pool 3 having slightly lower HPV18 E6 mRNA levels than pools 1 and 2 (Figure 3.8b).

For the EPC2 pools, there was only one pool obtained containing the HPV11 E6 gene and therefore RT-PCR without quantification was performed on that pool. The HPV11 E6 gene was transcribed within the pool (Figure 3.9a). In the EPC2-18E6 pools, pool 3 had about 2-fold higher HPV18 E6 mRNA levels than pool 1 (Figure 3.9b). It was not possible to determine the HPV 18E6 mRNA level in pool 2 as the cells died and could not be grown

past passage 7. This pool was therefore discontinued from further analysis. On the whole, the HPV11 and HPV18 E6 mRNA levels in the EPC2 and Rat1 transfected cells were comparable. However, it should be noted that HPV11 E6 was transcribed at substantially lower levels than HPV18 E6.

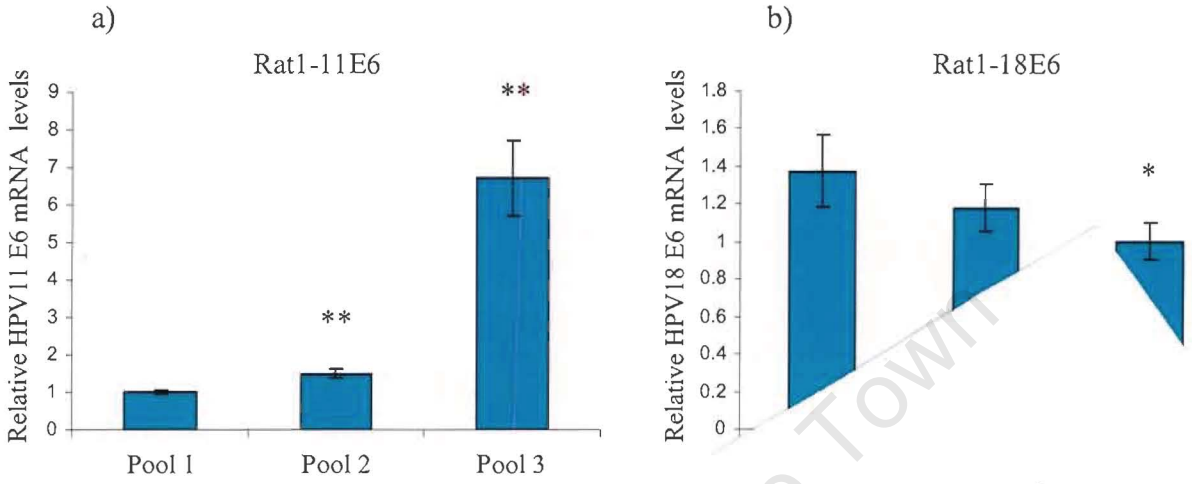


Figure 3.8: HPV E6 mRNA levels in the the Rat1 pools and quantitative RT-PC mRNA levels were determined **a) HPV11 E6 mRNA levels in mRNA levels in Rat1-18E6 poo** triplicate samples. Error bars repr lowest expressing pool. No HPV E6

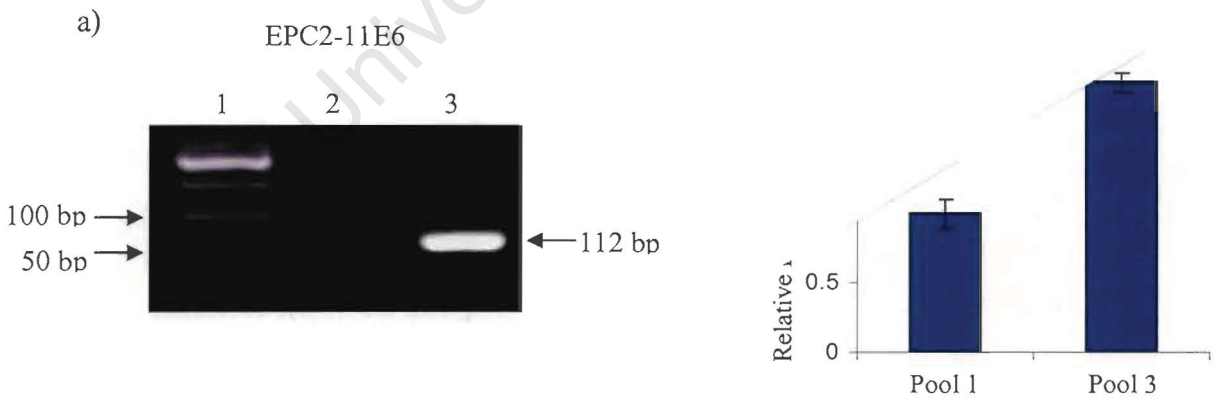


Figure 3.9: HPV E6 mRNA levels in EPC2 transfected cells. Total RNA was extracted from the EPC2 pools and RT-PCR or quantitative RT-PCR was performed as described in section 2.2.12. Relative mRNA levels were determined and the lowest expressing pool was assigned a value of 1. **a) RT-PCR on EPC2-11E6 pool 1**. Lane 1: 50 bp DNA ladder, lane 2: no-reverse transcriptase control to check for DNA contamination, and lane 3: amplification of cDNA from EPC2-11E6 pool 1. **b) Relative HPV18 E6 mRNA levels in two EPC2-18E6 pools**. Each bar represents the mean of triplicate samples. Error bars represent standard deviation. ** $p < 0.01$ compared to lowest expressing pool. No HPV E6 mRNA was detected in EPC2-pcDNA3 cells.

It is known that a proportion of the HPV18 E6 mRNA transcripts in HeLa cells are spliced giving rise to a smaller E6 protein (Ingaki *et al.,* 1988). This was similarly found to be the case in the HPV18-E6-transfected EPC2 and Rat1 cells (Figure 3.10). The spliced HPV18 E6 mRNA seemed to be more abundant than the unspliced mRNA.

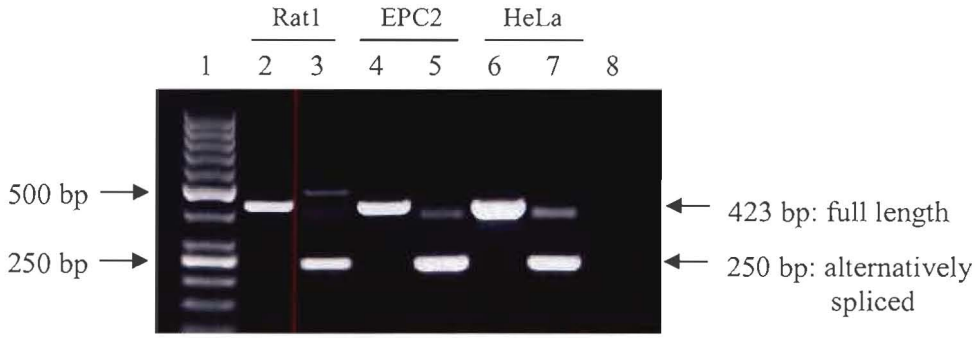


Figure 3.10: Splicing of HPV18 E6 mRNA. RNA was extracted from the transfected cells and PCR, using primers that amplify both full length (resulting in a 423 bp product) and alternatively spliced (resulting in a 250 bp product) HPV18 E6 mRNA, was performed as described in section 2.2.12. Lane 1: 50 bp DNA ladder. Lanes 2, 4 and 6: Amplification of DNA from Rat1, EPC2 and HeLa cells, respectively. Lanes 3, 5 and 7: Amplification of cDNA from Rat1, EPC2 and HeLa cells, respectively. Lane 8: no template control.

3.2.5 Detection of HPV E6 protein in the transfected cells

After confirming that the HPV E6 genes were transcribed, it was necessary to confirm that the mRNA was translated into protein in the Rat1 and EPC2 pools. This was done using immunofluorescence. There is no commercially available antibody to detect HPV11 E6 expression therefore the assays were performed for HPV18 E6 only. Figure 3.11 shows the images that were captured under the fluorescent microscope confirming the presence of the HPV18 E6 protein in the Rat1-18E6 and EPC2-18E6 pools.

All cells in the Rat1-18E6 and EPC-18E6 pools expressed HPV18 E6. In some cells, HPV18 E6 was observed only in the cytoplasm and in others it was observed in both cytoplasm and to a lesser extent in the nucleus (Figure 3.11a). The Rat1-pcDNA3 pool gave no signal in the Cy3 channel indicating that the labelling is specific for HPV18 E6. Similarly, the EPC2-pcDNA3 pool gave no signal indicating that the green signal is specific for HPV18 E6 expression (Figure 3.11b). For the EPC2-18E6 pools, staining was seen in both nucleus and cytoplasm.

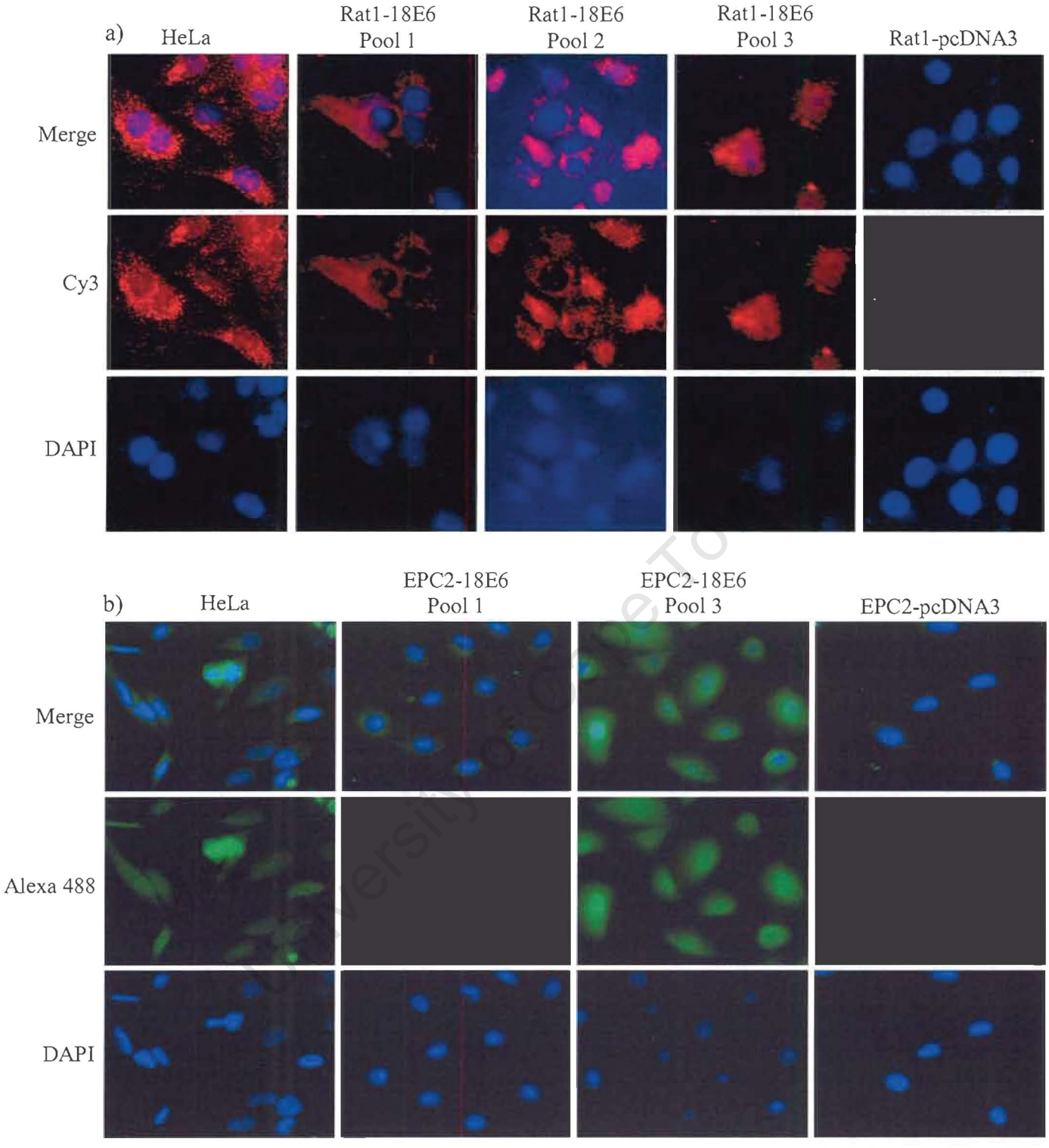


Figure 3.11: Localisation of the HPV18 E6 protein in the transfected pools. Cells were cultured on sterile coverslips, fixed and probed with an antibody to detect for HPV18 E6 protein as described in section 2.2.13. DAPI was used for nuclear staining and images were viewed at a magnification of 200 times. **a) Transfected Rat1 cells.** The Cy3 channel was used to detect for HPV18 E6 expression. **b) Transfected EPC2 cells.** The Alexa 488 channel was used to detect for HPV18 E6 expression.

3.3 The effects of HPV E6 on the transfected cells

After confirming expression of the HPV E6 gene, the next step was to investigate the effect of these proteins on the transfected cells. HPV E6 is known to interact with the tumour suppressor, p53, thus it was necessary to check the effects of HPV E6 on p53 protein levels.

3.3.1 Effect of HPV E6 on p53 levels

Western blot analysis was performed on whole cell lysates prepared from the transfected EPC2 cells. This could not be performed on the transfected Rat1 cells as the human p53 antibody does not cross-react with rat p53. Figure 3.12 shows that p53 levels were significantly decreased in the cells producing HPV18 E6 protein compared to the levels in the cells containing the pcDNA3 vector only (Figure 3.12a). The HPV18 E6 proteins resulted in a 45-65% reduction in the p53 levels in the EPC2 cells (Figure 3.12b). However, HPV11 E6 did not affect the p53 levels as there were no differences between cells transfected with HPV11 E6 and those with the vector only (Figure 3.12c).

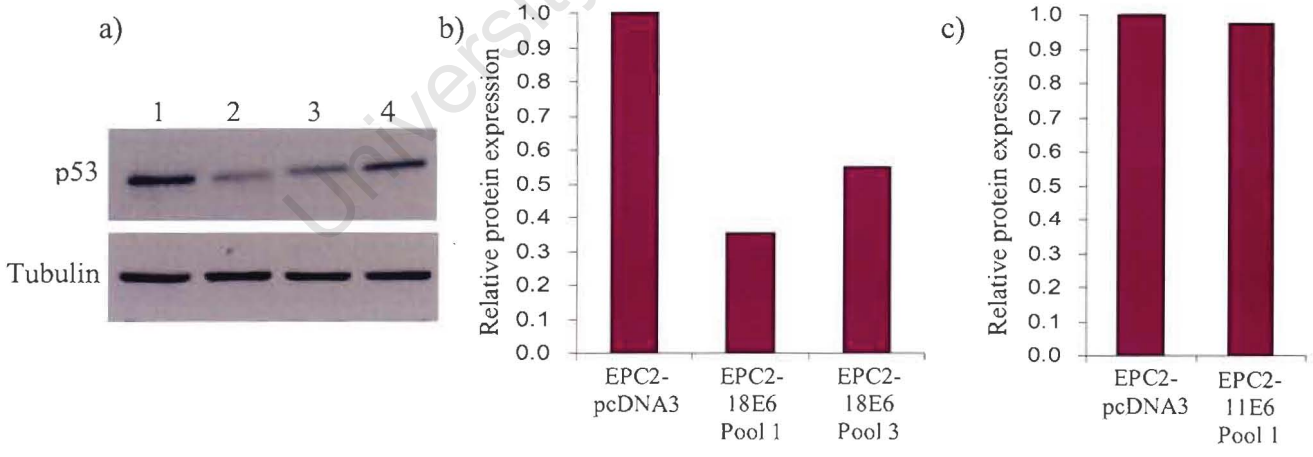


Figure 3.12: p53 protein levels in transfected EPC2 cells. p53 protein levels were determined using western blot analysis as described in section 2.2.14. Tubulin was included to correct for loading. **a) Autoradiograph showing the p53 and tubulin protein levels** in the different samples. Lane 1: EPC2-pcDNA3, lane 2: EPC2-18E6 pool 1, lane 3: EPC2-18E6 pool 3, and lane 4: EPC2-11E6 pool 1. **b) and c); the relative p53 protein levels**, analysed using densitometry, in the EPC2 cells containing HPV18 E6 or 11 E6, respectively. p53 protein level in EPC2-pcDNA3 cells was assigned a value of 1. This result is representative of two independent experiments.

3.3.2 Effect of HPV E6 on cell morphology

The transfected EPC2 and Rat1 cells were monitored for any morphological changes that might occur in response to expression of the HPV11 or HPV18 E6 genes. No changes in morphology were observed in the transfected Rat1 or EPC2 cells. Figure 3.13 shows a representative picture of the morphologies of the different pools.

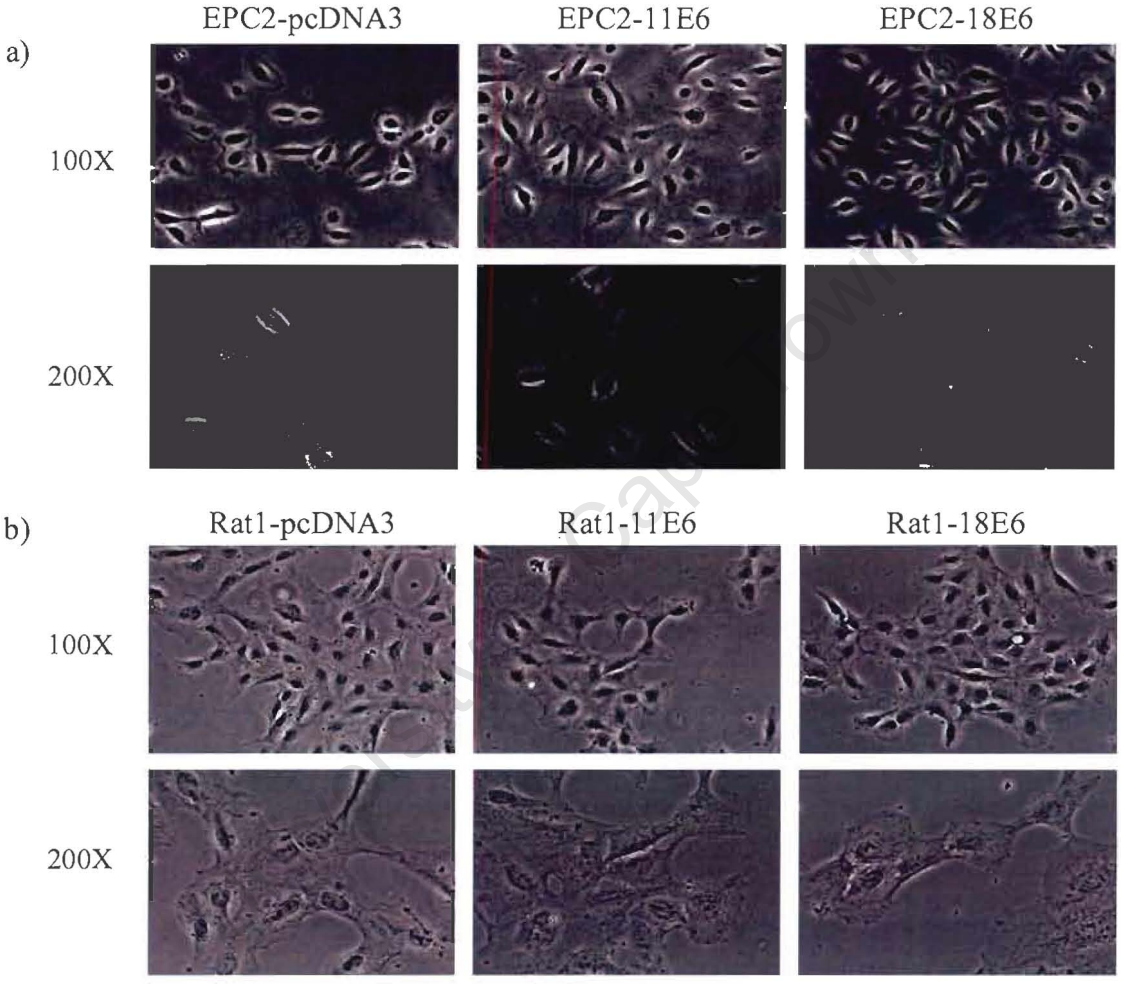


Figure 3.13: Morphology of transfected cells. Cells were plated on sterile coverslips, fixed and viewed under a microscope to investigate any morphological changes as described in section 2.2.15. **a) Morphology of the EPC2 cells** transfected with pcDNA3, pcDNA3-11E6 or pcDNA3-18E6. **b) Morphology of the Rat1 cells** transfected with pcDNA3, pcDNA3-11E6 or pcDNA3-18E6.

3.3.3 Effect of HPV E6 on cell growth rate

The growth rate of the transfected EPC2 and Rat1 cells was investigated. Figure 3.14 a and b show the growth rates of the transfected Rat1 cells. All the Rat1-11E6 and Rat1-18E6 pools had similar growth rates as Rat1-pcDNA3 cells, except for Rat1-11E6

pool 3 which showed a significantly slower growth rate of almost 70% after 4 days. This result was confirmed by counting the number of cells over a period of 5 days using a coulter counter and a 50% decrease in the growth rate of the Rat1-11E6 pool 3 was seen (Figure 3.15). Rat1-11E6 pool 3 also had the highest HPV11 E6 mRNA levels (Figure 3.8a). The growth rate of the EPC2 cells remained unaltered in the presence of either the HPV11 or HPV18 E6 protein (Figure 3.14 c and d).

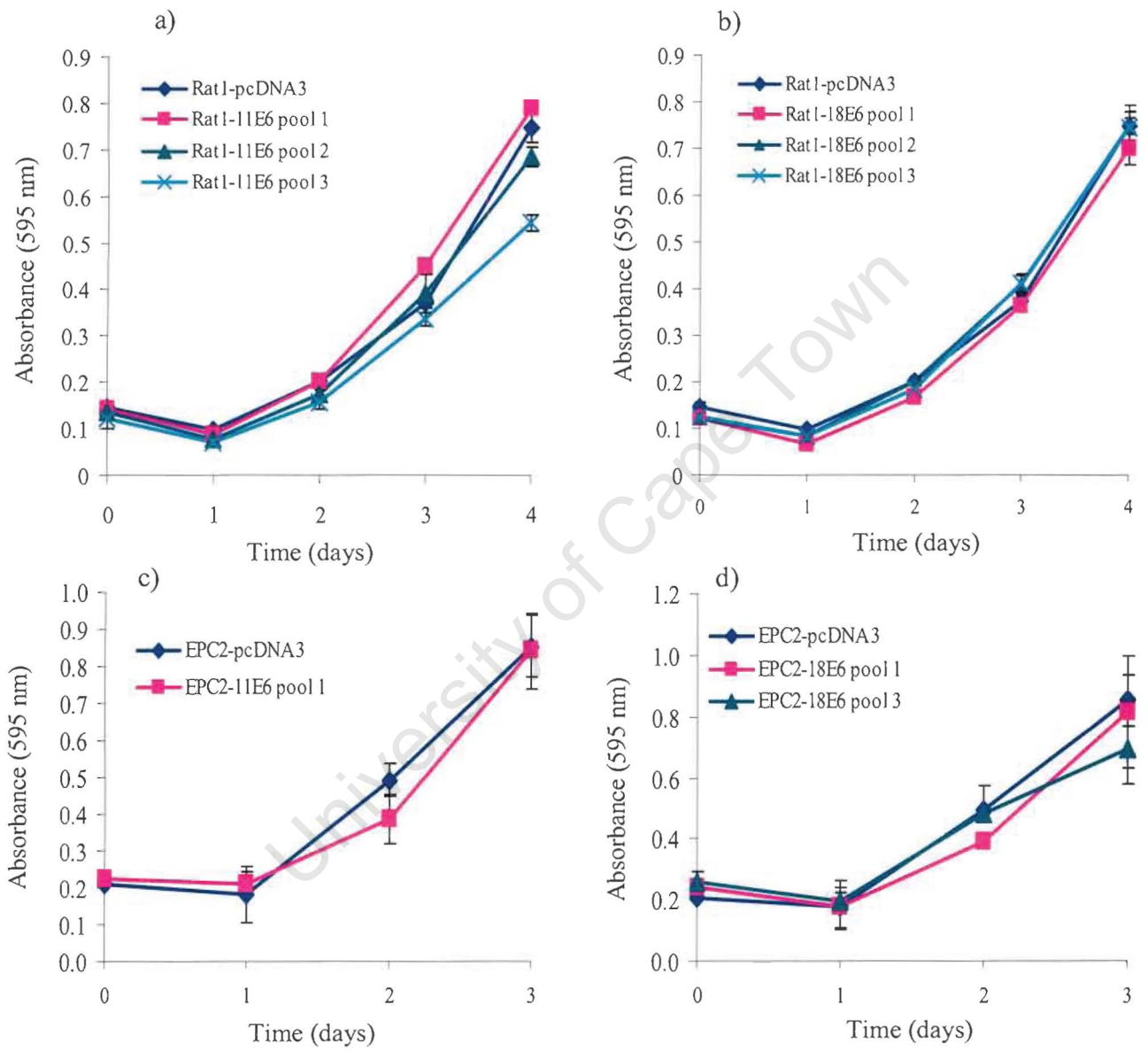


Figure 3.14: Growth rates of HPV E6 transfected cells. The growth rate of the transfected cells was determined using the MTT Cell Proliferation assay as described in section 2.2.16. **a) Growth rate of the three Rat1-11E6 pools** compared to the growth rate of the Rat1-pcDNA3 cells. **b) Growth rate of the three Rat1-18E6 pools** compared to the growth rate of the Rat1-pcDNA3 cells**. c) Growth rate of the EPC2-11E6** pool 1 compared to the growth rate of EPC2-pcDNA3 cells. **d) Growth rate of the two EPC2-18E6 pools** compared to the growth rate of EPC2-pcDNA3 cells. Each point represents the mean of four wells, with each experiment done at least twice. The error bars indicate standard deviations.

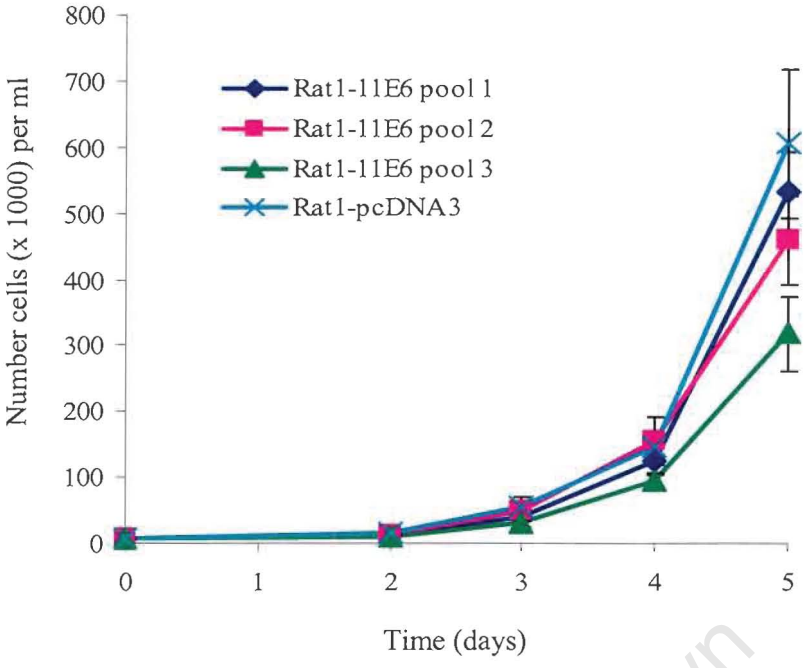
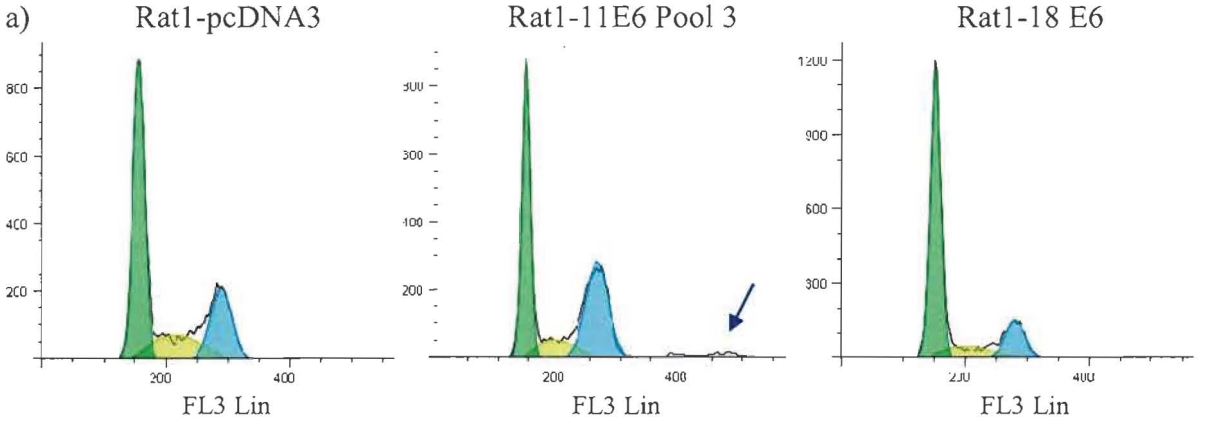


Figure 3.15: Confirmation of the growth rate of the Rat1-11E6 pools. The growth rate of the three Rat1-11E6 pools compared to the growth rate of the Rat1-pcDNA3 cells was investigated using a coulter counter to count the number of cells over time. Each point represents the mean of three wells. The error bars indicate standard deviations.

3.3.4 Effect of HPV E6 on cell cycle progression

To further investigate the effects of HPV E6, cell cycle analysis was performed. Figure 3.16 a-c shows the cell cycle distribution profiles of Rat1 transfected cells. Rat1-11E6 pool 3, the slower growing pool with the highest HPV11 E6 mRNA levels, had a substantially altered cell cycle profile compared to the Rat1-pcDNA3 cells. An increase in the percentage of cells in G2 and a 4-fold increase in the percentage of cells with DNA content greater than 4N was observed (i.e. there was an emergence of a small aneuploid population of cells indicated by the arrow in Figure 3.16a). The cell cycle distribution profiles of the Rat1-11E6 pool 1 and 2 were the same as those of the Rat1-pcDNA3 cells.



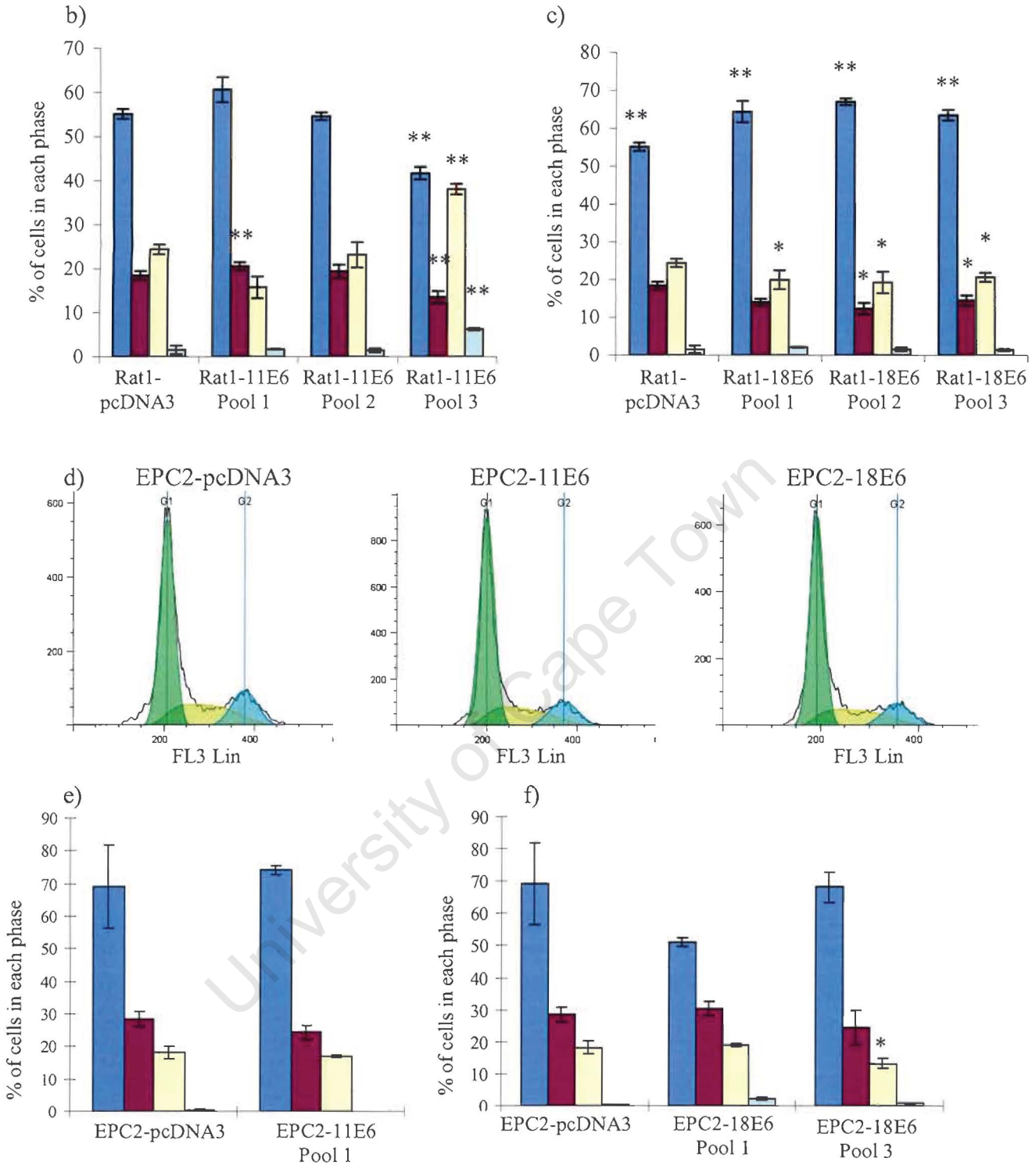


Figure 3.16: Cell cycle analysis of HPV E6 transfected cells. Cells were harvested, permeabilised and the DNA stained with propidium iodide to determine the percentage of cells in each phase of the cell cycle (section 2.2.17). a) Cell cycle distribution profiles for Rat1-pcDNA3, Rat1-11E6 pool 3 and a representative profile for the Rat1-18E6 transfectants. b) and c) show graphical representations of the distributions of the cell cycle in the Rat1-11E6 and 18E6 pools, respectively. d) Cell cycle distribution profiles obtained for EPC2-pcDNA3, EPC2-11E6 and EPC2-18E6. e) and f) show graphical representations of the distributions of the cell cycle in the EPC2-11E6 and 18E6 pools, respectively. Each bar represents the mean of triplicate samples, with each experiment done at least twice. The error bars indicate standard deviation. * $p < 0.05$, ** $p < 0.01$ compared to cells transfected with pcDNA3. %G1 %S %G2 %>G2

There was no difference in the cell cycle distribution profiles of the three Rat1-18E6 pools (Figure 3.16c). A significant increase in the percentage of cells in G1 and a slight decrease in the percentage of cells in G2, compared to Rat1-pcDNA3 cells, was observed for all three pools. There was no increase in the percentage of cells with DNA content greater than G2 compared to what was observed in the Rat1-pcDNA3 cells. For the transfected EPC2 cells (Figure 3.16 d-f), there was no change in the cell cycle profiles for the pools containing HPV11 or HPV18 E6, compared to the EPC2-pcDNA3 cells.

3.4 HPV E6 knockout using peptide nucleic acids

The aim of this study was to investigate whether down regulating HPV18 E6 expression in cells already expressing this gene had any effect on the transformed phenotype. All the oesophageal cancer cell lines tested in our laboratory were negative for HPV DNA (Table 3.1), thus HeLa (a known HPV18-positive cell line) was used as a model system. The experiments involved the use of peptide nucleic acids (PNAs) that were designed to target and down regulate expression of the HPV18 E6 gene.

3.4.1 HPV18 E6 mRNA levels in PNA-treated cells

Quantitative RT-PCR was used to determine the effects of the HPV18 E6 anti-gene PNA (PNA_{18}) on HPV18 mRNA levels in HeLa cells. A non-specific, mutant PNA (PNA_{mut}) designed not to bind to HPV18 E6 was included to control for non-specific or cytotoxic effects. Total RNA was extracted from PNA_{18}-treated, PNA_{mut}-treated and untreated cells. The quality and integrity of extracted RNA was tested as described earlier and Figure 3.17 shows an agarose gel of total RNA.

RNA was isolated 24 and 48 hours after PNA treatment and cDNA synthesis and qRT-PCR were performed to determine relative HPV18 E6 mRNA levels. PNA_{18} was effective in decreasing the relative HPV18 E6 mRNA levels in HeLa cells by approximately 20%, compared to untreated cells, 24 hours after treatment (Figure 3.18a). A larger decrease (35%) was detected 48 hours after treatment (Figure 3.18b). However, the mutant also affected the HPV18 mRNA levels and caused a larger decrease than the PNA_{18} at both time points. This was unexpected and will be alluded to in the discussion.

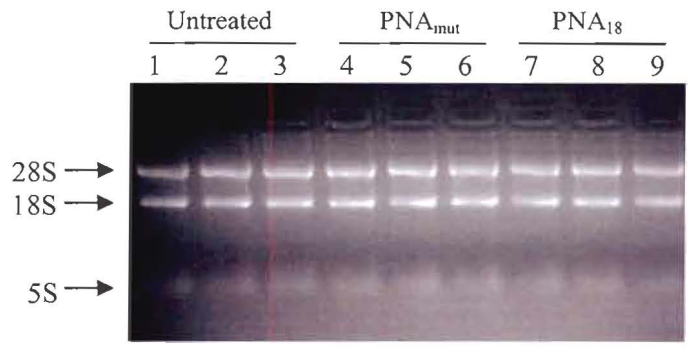


Figure 3.17: Integrity of extracted RNA. Total RNA was extracted from PNA-treated cells and 2 μg of RNA from each sample was electrophoresed on a formaldehyde-containing agarose gel to determine whether the RNA was intact as described in section 2.2.10. Lanes 1-3: RNA from untreated HeLa cells, lanes 4-6: RNA from HeLa cells treated with PNA$_{mut}$ for 24 hours, and lanes 7-9: RNA extracted from HeLa cells treated with PNA$_{18}$ for 24 hours.

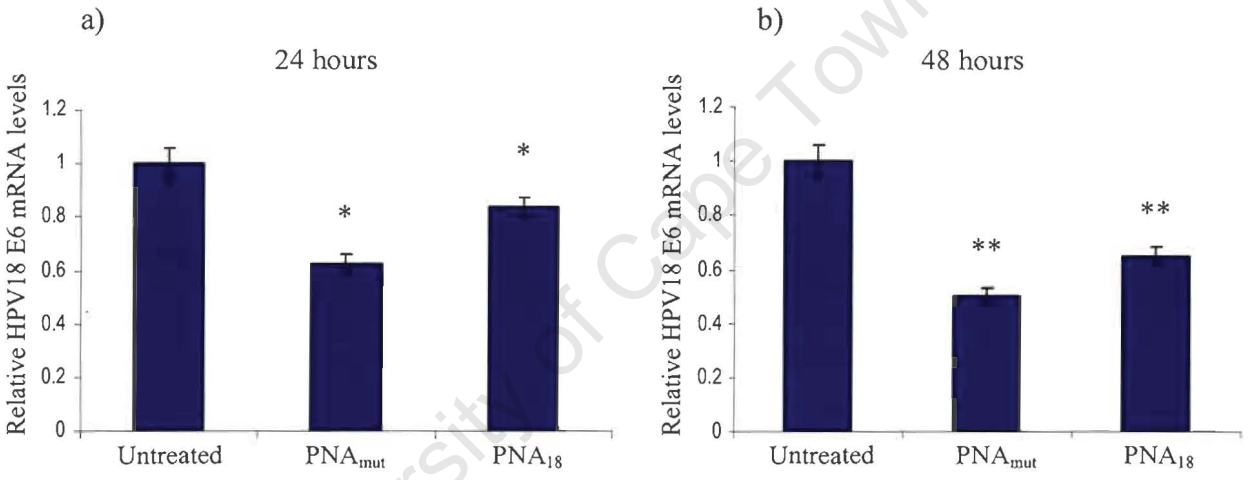


Figure 3.18: Relative HPV18 mRNA levels in PNA-treated HeLa cells. Cells were treated with PNA$_{18}$, PNA$_{mut}$ or left untreated and qRT-PCR was performed as described in section 2.2.12. The HPV18 E6 mRNA levels were determined and plotted relative to untreated HeLa cells that were assigned a value of 1. **a) Relative HPV18 mRNA levels 24 hours after treatment, b) relative HPV18 E6 mRNA levels 48 hours after treatment.** Each point represents the mean of triplicate samples, showing standard deviation. This result is representative of four independent experiments. * $p<0.05$, ** $p<0.01$ compared to untreated.

3.4.2 Cell proliferation in PNA-treated cells

Cell proliferation was investigated to determine whether the decrease in HPV18 E6 mRNA levels observed in the presence of PNA resulted in decreased cell growth. Figure 3.19 shows the growth of the untreated and PNA-treated HeLa cells with a decrease in cell growth observed in the presence of PNA$_{18}$. Unfortunately, PNA$_{mut}$ also appeared to cause a decrease in cell growth. A 40-45% decrease in the growth rate of the cells was observed in both PNA$_{18}$- and PNA$_{mut}$-treated samples.

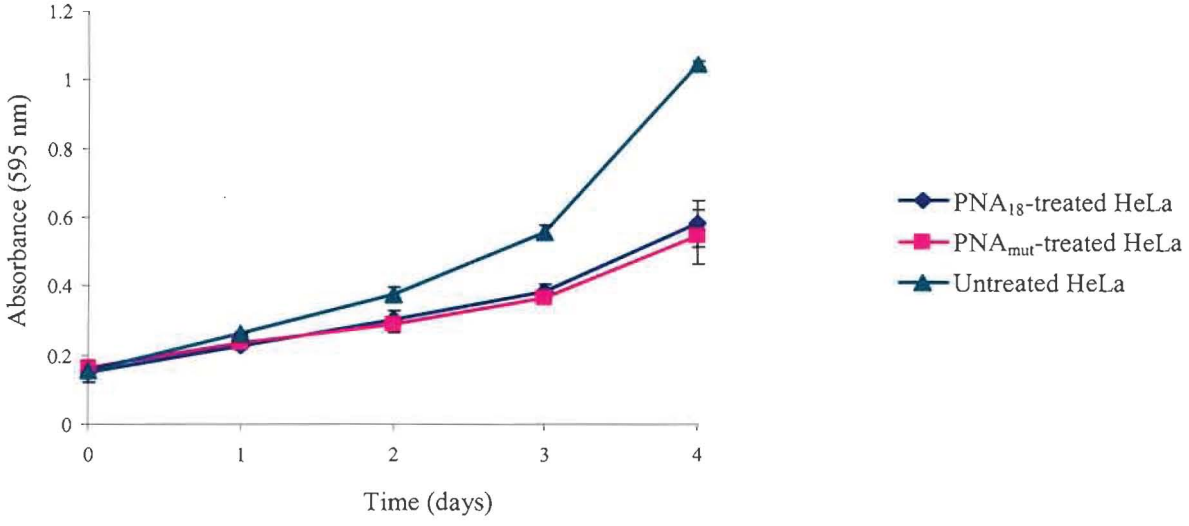


Figure 3.19: Growth rate of HeLa cells after PNA treatment. PNA_{18} and PNA_{mut} were added to HeLa cells and a MTT cell proliferation assay was performed as described in section 2.2.18. Each point represents the mean of 4 wells showing the standard deviation. This is representative of three independent experiments.

The observed decreased cell growth rates in both PNA_{18}- and PNA_{mut}-treated cells pointed to the possibility of general toxicity to the cells. In order to confirm this, the PNA experiment was repeated in an HPV-negative cell line, WHCO1. Figure 3.20 shows that both the PNA_{18} and PNA_{mut} decreased the growth rate of the WHCO1 cells. This confirms that the PNAs are generally cytotoxic when added at a final concentration of 10 μM. The decrease in cell growth seen in Figure 3.19 could therefore not be attributed to a decrease in HPV18 E6 expression.

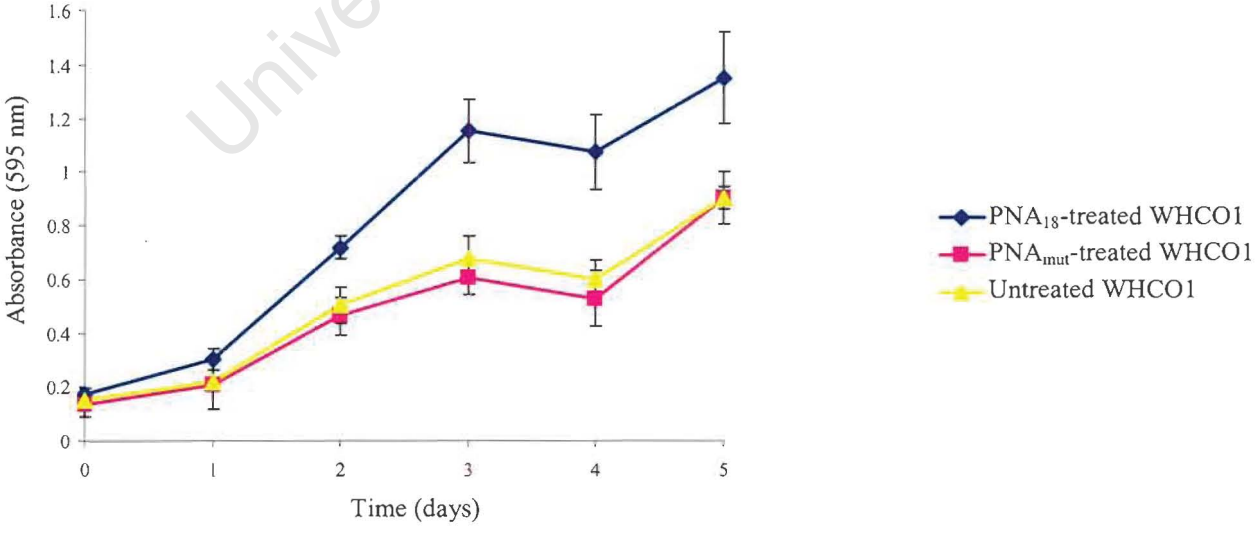


Figure 3.20 Growth rate of WHCO1 cells after treatment with PNA. PNA_{18} and PNA_{mut} were added to WHCO1 cells (known to be HPV negative) and the MTT cell proliferation assay was performed as described in section 2.2.18. Each point represents the mean of 4 wells showing the standard deviation.

CHAPTER 4

Discussion and Conclusions

4.1 Discussion

Oesophageal squamous cell carcinoma is a concern worldwide and particularly in South Africa. The cause of OSCC is not known, but different risk factors are associated with OSCC, such as excessive alcohol consumption and tobacco smoking (Wu *et al.*, 2006; Pacella-Norman *et al.*, 2002; Syrjanen, 2002; Pinto *et al.*, 2003), nutritional deficiencies (Sur and Cooper, 1998), genetic polymorphisms (Pinto *et al.*, 2003; Lin *et al.*, 1998; Dandara *et al.*, 2005; 2006) and infection with the human papillomavirus (Matsha *et al.*, 2002; Farhadi *et al.*, 2005; Katiyar *et al.*, 2005; Furihata *et al.*, 1993, Chang *et al.*, 1990; 1992; 2000). The association between HPV and OSCC is poorly understood and has been the focus of this study.

Human papillomaviruses are categorised into high- and low-risk types depending on their association with causing cervical cancer (Motoyama *et al.*, 2004). The so called "low-risk" HPV11 was found to be the most common type in South African OSCC patients (Matsha *et al.*, 2002). It is therefore necessary to determine whether HPV11 is a risk factor for the development of OSCC or merely a bystander. HPV18 was not detected in the cohort of OSCC patients in the study by Masha *et al.* (2002), but this type has been identified in oesophageal tumour cells in other studies (Cooper *et al.*, 1995; Lavergne and de Villiers, 1999). In this project, the role of HPV E6 proteins in OSCC was investigated by evaluating the effects of either introducing HPV E6 genes into HPV-negative cell lines or by knocking out the expression of E6 genes in an HPV-positive cell line.

4.1.1 Introducing HPV E6 genes into HPV-negative cell lines

Two different HPV E6 genes, HPV11 and HPV18 E6, were transfected into cells to determine whether these proteins play a role in the development of OSCC or are merely spectators. HPV11 E6, a low-risk HPV type, was chosen as this was the most common type found in South African OSCC patients and HPV18 E6 was chosen as a reference to compare with a high-risk type. EPC2 and MCF12A cells were selected as examples of "normal" oesophageal and breast epithelial cell lines, respectively. Rat1 cells were also

included as they are routinely used in transfection studies and also because several studies have looked at the effects of HPV proteins in fibroblasts (Malanchi *et al.,* 2004; Bedell *et al.,* 1987). A comparative analysis between different cell types may therefore be useful.

In the transfection studies, three of the MCF12A pools and one of the EPC2 pools unfortunately did not contain the HPV E6 gene. Lack of the HPV E6 gene in the EPC2 pool could have been due to the very low G418 concentration used to select for transfected EPC2 cells (30 μg/ml). This might have allowed for the emergence of spontaneously resistant cells. Use of higher G418 concentrations to select for successfully transfected EPC2 cells were attempted (between 50 to 100 μg/ml) but always resulted in complete cell death of the transfected cells. The G418 concentration used to select for transfected MCF12A cells was much higher (450 μg/ml). However, we speculated that the transfected MCF12A cells lacking the HPV E6 gene may have come about due to excision of the E6 gene, but maintaining the neomycin resistance gene. Alternatively, in both the transfected EPC2 and MCF12A cells lacking the HPV E6 genes, the gene may have integrated in a transcriptionally inactive portion of the genome. All transfected pools lacking the HPV E6 gene were excluded from the study.

In the pools that contained the HPV11 or HPV18 E6 genes, transcription of the genes occurred at varying levels. This may have been due to the copy number of pcDNA3 constructs present in each of the pools or the sites of integration of the HPV E6 genes within the genome. It was not possible to detect HPV E6 protein expression by western blot analysis in this study. There were no commercially available antibodies to detect HPV11 E6 and therefore it was not possible to confirm that the HPV11 E6 protein was produced in the EPC2-11E6 and Rat1-11E6 pools. Attempts to produce polyclonal antibodies against HPV11 E6 and HPV18 E6 proteins fused to glutathione-S-transferase tags in our laboratory were unsuccessful.

It has been observed that HPV E6 proteins are expressed at very low levels making them difficult to detect. In fact, in many studies that look at HPV E6 proteins, they only show E6 mRNA using RT-PCR or Nothern blot analysis (Horner *et al.,* 2004; Slebos *et al.,* 1995) because of the known difficulties in detecting HPV E6 proteins. Other studies add a green fluorescent tag or a HA (hemagglutinin) or Flag tag onto the HPV E6 proteins to aid detection (Yamato *et al.,* 2006; Vaeteewoottachern *et al.,* 2005). In retrospect, a tag could

have been added to the HPV E6 proteins in this study. This would also have allowed the detection of HPV11 E6 expression without the use of specific HPV11 E6 antibodies.

The monoclonal antibody to detect HPV18 E6 expression within the HPV18-E6-positive cells did not work well for western blot analysis, but gave good results when used in immunofluorescence. Immunofluorescence confirmed that all cells in the Rat1-18E6 and EPC2-18E6 pools expressed the HPV18 E6 protein, and that it was localised to the cytoplasm of some cells and the cytoplasm and nucleus (to a lesser extent) of other cells. This observation is in agreement with what is reported in the literature (Vaeteewoottachern *et al.*, 2005; Li *et al.*, 2005).

We observed alternatively spliced HPV18 E6 mRNA in cells transfected with HPV18 E6. The alternatively spliced HPV18 E6 mRNA was observed in the three Rat1-18E6 and three EPC2-18E6 pools, indicating that the splicing event was not cell specific. Our observation is in agreement with that of Ingaki *et al.* (1988) who showed that HPV18 E6 transcripts present in HeLa cells were alternatively spliced, leading to the production of a smaller E6 protein, named E6*. The HPV18 E6* protein is 6.5 kDa in size instead of 18.9 kDa because the splicing causes a change in the reading frame leading to the introduction of a premature stop codon (Schneider-Gadicke *et al.,* 1988).

Our results appear to show that the alternatively spliced HPV18 E6 mRNA was in abundance (compared to unspliced HPV18 E6 mRNA), confirming the findings in other studies that the full length HPV E6 transcripts are in extremely low abundance (Zheng and Baker, 2006; Smotkin and Wettstein, 1986). This could help to explain why the HPV18 E6 protein was difficult to detect in this study. It has been shown that the E6* proteins negatively regulate the expression of the active, full-length E6 proteins therefore contributing to low HPV E6 protein levels (Sedman *et al.,* 1991). Splicing of HPV E6 mRNA has also been observed for HPV16 E6, another high-risk HPV type, but no study has shown this phenomenon in the low-risk HPV types, including HPV11 E6 (Stacey *et al.,* 1995; Sedman *et al.,* 1991; Schneider-Gadicke *et al.*, 1988).

The observation in our study that HPV18 E6 resulted in decreased p53 protein levels, while HPV11 E6 did not, is in line with what is reported in the literature. The high-risk

HPV E6 proteins (e.g. HPV18 E6) form a trimeric complex by binding p53 and the E6-associated protein (E6AP) (Zanier *et al.,* 2005; Werness *et al.,* 1990). This complex formation increases the rate of degradation of p53, which is associated with decreased p53 protein levels in HPV-infected cells. In contrast, the low-risk HPV E6 proteins (e.g. HPV11 E6) are unable to bind E6AP and p53 and therefore do not result in decreased p53 levels (Zanier *et al.,* 2005; Malanchi *et al.*, 2004; Slebos *et al.*, 1995). The decreased p53 levels in transfected EPC2 cells indicated that the HPV18 E6 protein was functionally active. The difference in the effects of HPV11 and HPV18 E6 proteins show that the low- and high-risk HPV E6 proteins possibly act via different pathways. It would be interesting for future work to identify the different pathways altered by the low- and high-risk HPV E6 proteins using microarray analysis.

We also investigated the effects of HPV11 and HPV18 E6 proteins on cell growth. Previous studies have shown that both the high- and low-risk HPV E6 proteins are associated with increased growth rates of cells (Malachi *et al.*, 2004; You *et al.*, 2002). In contrast, most of the transfected Rat1 and EPC2 pools did not show any change in overall growth rate, except for one of the Rat1-11E6 pools. Rat1-11E6 pool 3, the pool with highest HPV11 E6 mRNA levels, grew significantly slower than the cells containing the pcDNA3 vector only. This pool also had a markedly different cell cycle distribution profile than the cells containing pcDNA3. There was an emergence of a small aneuploid population and an increase in the proportion of cells in G2. Some cells in this slower-growing pool may be cycling between 4N and 8N. Some of the cells were probably undergoing crisis and therefore growing slower. More detailed analysis needs to be performed to confirm the mechanisms that result in an increase in the DNA content in the cells.

An increase in the percentage of cells with DNA content greater than G2 was not observed in the EPC2-11E6 pool. This may be because the expression levels of HPV11 E6 were lower than that of the slower-growing Rat1-11E6 pool. There was only one pool of EPC2 cells containing the HPV 11E6 gene so this result would need to be confirmed by generating several pools of transfectants. There was no increase in the percentage of cells with DNA content greater than G2 observed in the HPV18 E6-transfected cells, as was observed in HPV11 E6 transfected Rat1 cells. In the literature, there are contradictory findings as to whether HPV E6 alone can cause aneuploidy or whether simultaneous HPV

E7 expression is required. The difference seems to depend on the different growth conditions used (Patel *et al.*, 2004; Schaeffer *et al.*, 2004). It is possible that no change in the cell cycle and growth rate of the cells was observed in this study due of the lack of HPV E7 expression.

Some studies have reported that HPV E6 proteins affect cell morphology (You *et al.*, 2002; Malanchi *et al.*, 2004), however no change in the morphology of the transfected EPC2 or Rat1 cells was observed. A study by Bedell *et al.* (1987) showed distinct morphological changes in Rat1 cells expressing both HPV18 E6 and E7. This indicates that expression of both the HPV oncoproteins is required to alter the morphology of Rat1 cells. Future work should include introducing both HPV E6 and HPV E7 into an HPV-negative cell line to determine whether the expression of both oncoproteins are required to alter the cell morphology, growth rate and cell cycle profile of a previously HPV-negative cell.

The discrepancy between our results and that in literature is unlikely to be due to passage number. The assays were carried out on cells 8 to 10 passages after transfection, which is similar to the study by Kinjo *et al.* (2003), who reported effects 10 passages after transfection. The best method for investigating the effects at a higher passage number requires the use of single clones. The increase in the proportion of cells with DNA content greater than G2, seen 10 passages after transfection, in the slower-growing Rat1-11E6 pool, was not observed 20 passages after transfection (data not shown), implying the possibility that the faster growing cells in the pool out competed the slower growing cells (cells with a DNA content greater than 4N).

4.1.2 Knocking out HPV E6 using peptide nucleic acids

A second approach to investigating the role of HPV E6 genes was to knockout HPV E6 expression in cells expressing the gene. There are several ways of knocking out genes such as the use of short interfering RNA or PNA. Peptide nucleic acids designed to target the HPV18 E6 gene (PNA_{18}) were used in this study. In addition, a mutant PNA (PNA_{mut}) was also designed to control for non-specific effects and toxicity to cells. Previous work by Braun *et al.* (2004) demonstrated that treating HeLa-S suspension cells with 3 PNAs, two that target HPV18 E6 and one that targets HPV18 E7, resulted in a decrease in cell

proliferation and an increase in apoptosis. The PNA-treated HeLa-S cells also had altered cell morphology and lost the ability to grow non-adherently. This current study sought to design and test a single PNA that targeted the HPV18 E6 gene to determine whether a single PNA could be effective in decreasing HPV18 E6 expression. Previous studies (Cutrona *et al.*, 2000; 2003; Boffa *et al.,* 2007; Wang *et al.*, 2004) have shown that the addition of a single PNA can be effective in decreasing the expression of its target gene.

Since none of the eight OSCC cell lines that are routinely used in our laboratory tested positive for HPV L1 or E6 DNA, HeLa cells were chosen as the model system for the PNA studies as they are known to contain HPV18 DNA and to produce the HPV E6 protein.

The PNA_{18} used in this study decreased HPV18 E6 mRNA levels, indicating that the PNA interacted with the HPV18 E6 gene and decreased transcription of the gene. The observed decrease was small (i.e. 15% and 35% after 24 and 48 hours of treatment, respectively) and this may be due to many different factors, including the design of the PNA and the poor uptake of the PNA into the nucleus of the cells. The design of the PNA is critical for their effectiveness. It has been observed that the closer the PNAs bind to the 5′ end of the coding region of the gene, the more effective they are (Personal communication, Prof Lidia Boffa, IST, Genoa, Italy). PNA_{18} was designed to bind at the 75^{th} base in the HPV18 E6 exon, which is a distance from the start of the exon.

Cutrona *et al.* (2000) showed that the addition of the nuclear localisation signal (NLS) (VKRKKKP) to the N terminus of the PNA was effective for uptake into the nucleus of Burkitt's lymphoma cells. However, a study (Bendifallah *et al.*, 2006) using HeLa cells, tested the addition of the same NLS to PNA and showed that there was almost no activity of the PNA at a concentration of 10 μM (same as the concentration used in this study and by Cutrona *et al.*, 2000). Therefore, perhaps the NLS is effective for uptake in some cell lines, but not in others such as HeLa, but this needs to be confirmed in different cell lines.

A study performed in HeLa cells (Wolf *et al.*, 2006), showed that the best cell-penetrating peptide (CPP) to add to PNA to aid uptake was the KLA fragment (KLAL KLAL KAL KAAL KLA – NH2) added onto the N terminus of the PNA. They observed that a positive charge, helicity and amphipathicity were required to aid uptake. In contrast, in this project the NLS is positively charged but unlikely to form a helix (analysed using

http://www.embl-heidelberg.de/services/serrano/agadir) and is not amphipathic. Many other CPP have been tested, for example Transportan and oligo-arginine (R_{7-9}), to determine which is most effective (Morris *et al.,* 2007; Bendifallah *et al.*, 2006; Wolf *et al.*, 2006).

In this study the PNA_{mut} caused a larger decrease in HPV18 E6 mRNA levels than PNA_{18}. This was an unexpected result as the PNA_{mut} should not bind to the HPV18 E6 gene. However, after using the BLASTn programme to search in the NCBI database, it revealed that both the PNA_{mut} and the PNA_{18} were homologous to many human genes, some with 100% homology. Furthermore the PNA_{mut} also caused a decrease in the growth rate of HeLa cells. To determine whether the decrease in growth rate was HPV18 E6 specific, the HPV-negative cell line, WHCO1, was treated with the PNA_{18} and PNA_{mut} and a cell proliferation assay was performed. Treatment with either PNA_{18} or PNA_{mut} resulted in a decrease in the growth rate of WHCO1 cells indicating that the observed decrease is due to cytotoxic effects of the PNAs. Furthermore, dead cells were observed 48 hours after treatment with either of the PNAs.

All PNA experiments were performed using a final concentration of 10 μM which was the concentration used by Cutrona *et al.* (2000). Some studies have shown that PNA are cytotoxic at higher concentrations, such as 10 μM. For example, the study by Wolf *et al.* (2006) showed that treatment with PNA at a final concentration of 4 μM caused 80% cell death (assessed by MTT assay). Future experiments using PNA should optimise the cell-specific concentration used to obtain a specific decrease in growth rate and decrease cytotoxic effects.

To improve the effectiveness of the PNAs, the following recommendations are made;

1) addition of a different cell penetrating peptide onto the PNA to increase cell uptake,
2) designing of the PNA to bind closer to the 5' end of the gene,
3) ensuring that the PNA_{mut} and PNA_{18} do not bind to any other human genes.

4.2 Conclusion

Oesophageal cancer is a multifactorial disease with variability in its geographical and gender distribution. While this study concentrated on HPV, the role of other risk factors such as genetic polymorphisms in xenobiotic metabolising enzymes, alcohol consumption and tobacco smoking is also important. From our observations, it appears that HPV11 and HPV18 E6 affect cells differently, however more work is required to determine whether HPV11 E6 plays a role in the development of OSCC. Studies introducing the HPV11 E6 gene into embryonic oesophageal cell lines, similar to the studies by Shen *et al.* (2000; 2001; 2003; 2004), and looking at the resultant effects over time may be able to answer some of the questions better than the systems used in this study.

It is also necessary to look at the effects of HPV E7 on the oesophageal cells as perhaps both HPV E6 and E7 expression are required to transform the cells. In addition, the correlation between HPV E6 and exposure to carcinogens in the diet and the environment may be required in conjunction with HPV E6 expression to transform the cells. Suitable PNAs targeting HPV18 E6 may be a possible treatment option for HPV-positive cancers. It has been shown that continuous HPV E6 expression is required in cervical cancer cells to maintain the transformed phenotype (Horner *et al.,* 2004) therefore, in decreasing HPV18 E6 protein levels, it may be possible to revert tumour cells back to their untransformed phenotype.

APPENDIX A

Additional Figures

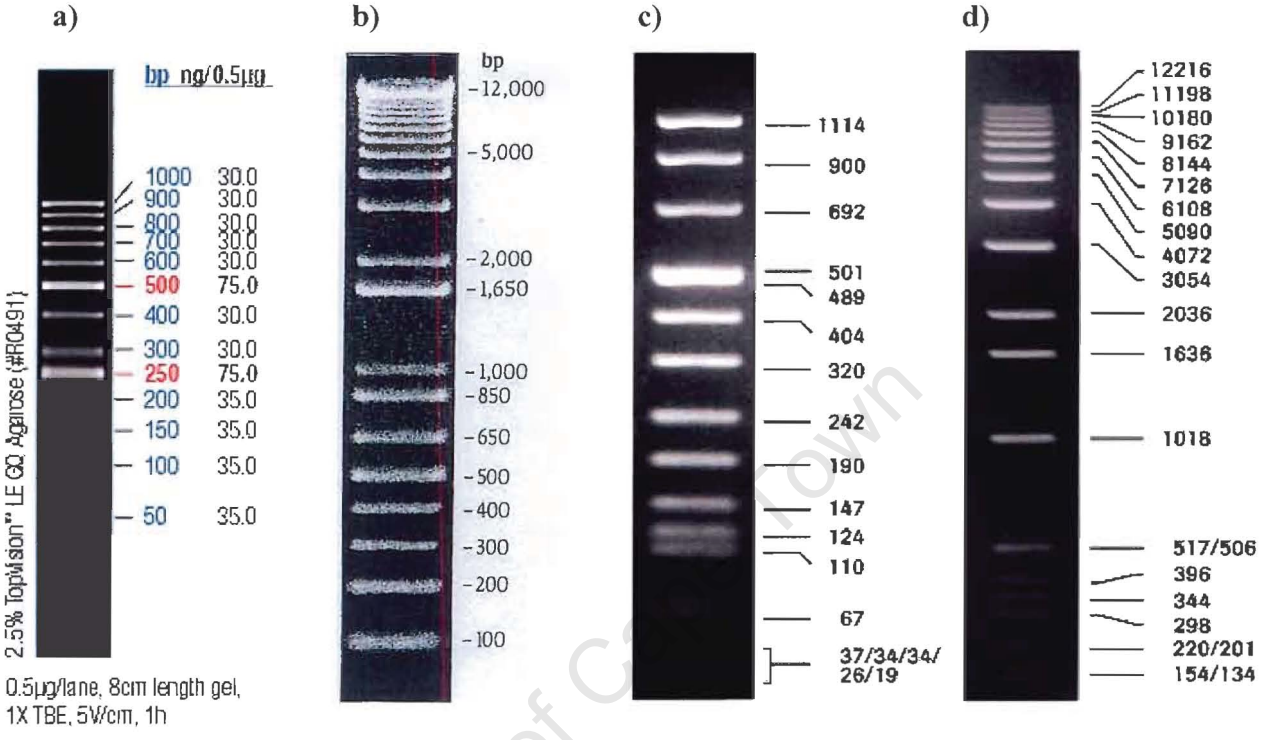


Figure A.1: DNA Molecular Weight Markers. a) GeneRuler 50 bp DNA Ladder (Fermentas, Maryland, USA) **b) 1 Kb Plus Marker** (Invitrogen, Paisley, UK). **c) Marker VIII** (Roche, Mannheim, Germany), mixture of pUCBM21 DNA cleaved with *Hpa*11, and pUCBM21 DNA cleaved with *Dra*1 and *Hind*III. **d) Marker X** (Roche, Mannheim, Germany), mixture containing a 1018 bp fragment derived from the 2 µ plasmid of the yeast *Saccharomyces cerevisiae*, multimers of this fragment, and cleavage products from pBR322 DNA. These markers were used to estimate the size of DNA fragments electrophoresed on agarose gels. The sizes of the bands (number of base pairs) are indicated.

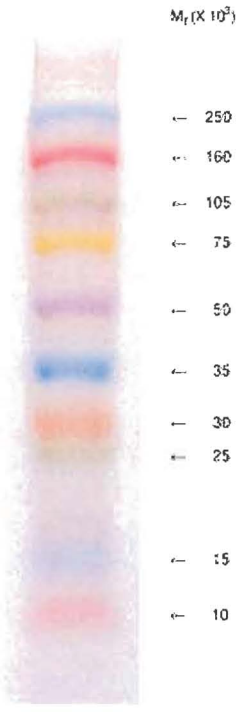


Figure A.2: PRN800 Molecular Weight Marker (Amersham Biosciences, Buckinghamshire, England). This protein molecular weight marker was used to estimate the size of proteins electrophoresed on denaturing polyacrylamide gels.

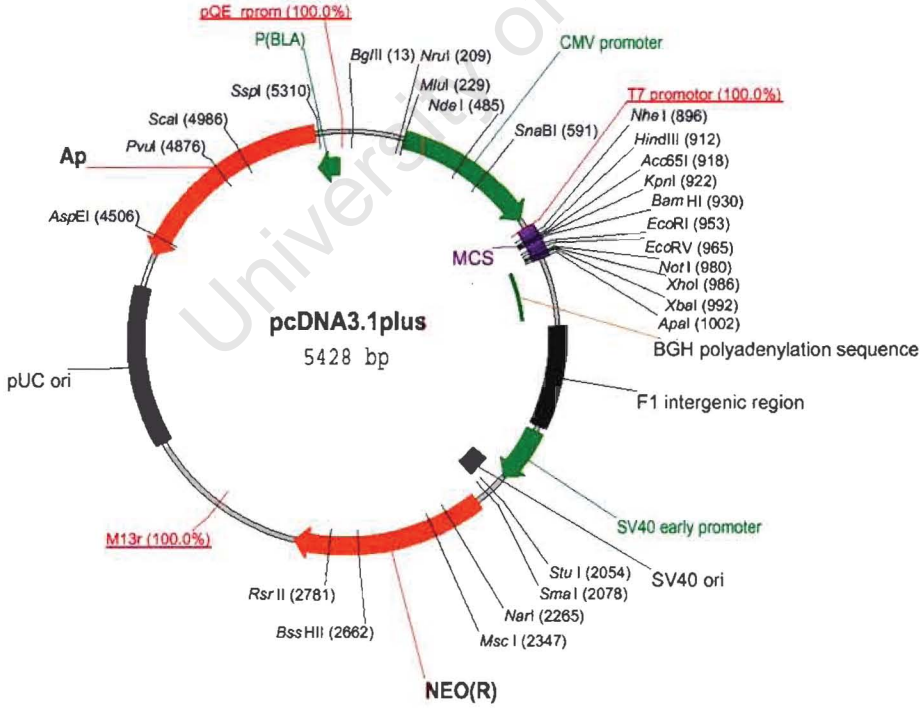


Figure A.3: pcDNA3.1 plus expression vector (Invitrogen, Paisley, UK). The HPV11 E6 and HPV18 E6 genes were cloned into the pcDNA3 vector to allow protein expression in a mammalian system.

APPENDIX B

Solutions

Digestion buffer
100 mM NaCl
10 mM Tris-Cl, pH 8.0
25 mM EDTA, pH 8.0
0.5% SDS
0.1 mg/ml proteinase K

Fixing solution
25% (v/v) isopropanol
10% (v/v) acetic acid

Formaldehyde-containing agarose gel
1.5% agarose in MOPS buffer
0.5 µg/µl ethidium bromide
2% formaldehyde

Laemmli buffer
62.5 mM Tris, pH 6.8
1.5% SDS
0.00125% bromophenol blue
10% glycerol
2.5% β-mercaptoethanol

Luria broth (LB)
10g/*l* tryptone powder
5g/*l* yeast extract powder
85.5 mM NaCl

Luria Agar
LB containing 14 g/*l* agar

MOPS Buffer
0.02 mM MOPS, pH 5.5-7.0
5 mM Sodium acetate
1 mM EDTA

Mowiol mounting solution
2.4 g Mowiol® 4-88 Reagent
6ml glycerol
Leave at room temperature for at least 4 hours
Add 12 ml 0.2 M Tris (pH 8.5)
Incubate at 50°C for 1 hour with occasional stirring
Store at –20°C

4% Paraformaldehyde (PFA) in PBS
2.5 ml 16% PFA dissolved in water
1 ml 10X PBS
6.5 ml distilled water
Store at –20°C

25:24:1 (v/v/v) Phenol:Chloroform:Isoamyl alcohol
50 ml phenol
48ml chloroform
2 ml isoamyl alcohol
Store at 4°C

Phosphate-buffered saline (PBS)
137 mM NaCl
2.7 mM KCl
4.3 mM Na_2HPO_4
1.4 mM KH_2PO_4
pH to 7.5

Ponceau S stain
1 g/*l* Ponceau S
10% glacial acetic acid

Propidium iodide stain solution
0.1% Triton X-100
2 mM $MgCl_2$
100 mM NaCl
10 mM PIPES, pH 6.8
10 µg/ml propidium iodide

Rapid Coommasie blue
10% acetic acid
0.006% (w/v) Coomassie brilliant blue

RNA loading buffer
8% formamide
2.8% formaldehyde
0.83% glycerol
0.04% bromophenol blue in MOPS

RIPA buffer
150 mM NaCl
10 mM Tris, pH 7.2
0.1% SDS
1% Triton X-100
1% NaDeoxycholate

Running buffer
53.3 mM glycine
52.2 mM Tris
1% SDS

Transfer buffer
2.5 mM Tris
1.92 mM glycine
20% methanol

Tris-buffered saline containing 0.05% Tween-20 (TBS-T)
50 mM Tris, pH 7.5
150 mM NaCl
0.05% Tween-20

Tris-EDTA (TE) buffer
10 mM Tris-Cl, pH 8.0
1 mM EDTA pH 8.0

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