UNIVERSITY OF LIVERPOOL

The Role of HPV16 E1 in Cervical Carcinogenesis

Thesis submitted in accordance with the requirements of the

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Doctor in Philosophy by

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I declare that this thesis entitled:

“The Role of HPV16 E1 in Cervical Carcinogenesis”

is entirely my own work

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The Role of HPV16 E1 in Cervical Carcinogenesis

Miss Fern Baedyananda

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biomedical Sciences and Biotechnology
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บทบาทของโปรตีน E1 ของไวรัสแปปิโลมาทัยป์ 16 ในการก่อมะเร็งปากมดลูก

นางสาวเฟิร์น แพทยานนท์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญา
วิทยาศาสตรดุษฎีบัณฑิต
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เฟิร์น แพทยานนท์ : บทบาทของโปรตีน E1 ของไวรัสแปปิโลมาทัยป์ 16 ในการก่อมะเร็งปากมดลูก (The Role of HPV16 E1 in Cervical Carcinogenesis) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.ภาวพันธ์ ภัทรโกศล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร.อาคม ไชยวงศ์คต, ดร.แชงคาร์ วาราดาราจัน, หน้า.

วัตถุประสงค์ การศึกษานี้มีเป้าหมายในการหารูปแบบการแสดงออกของยีน E1 ของไวรัสแปปิโลมาไทป์ 16 ในตัวอย่างเซลล์ปากมดลูก และบทบาทของโปรตีน E1 ในการก่อมะเร็งปากมดลูก นอกจากนี้งานนี้ยังมีเป้าหมายในการตรวจหาการซ้ำของ ยีน E1 ของไวรัสแปปิโลมาไทป์ 16 ขนาด 63 คู่เบสในประชากรไทยด้วย

วิธีทำ การศึกษานี้ใช้ตัวอย่างเซลล์ปากมดลูกที่มีไวรัสแปปิโลมาไทป์ 16 แบ่งเป็นเซลล์ปกติ เซลล์ผิดปกติระยะก่อนมะเร็ง (CIN) 1, 2/3 และ ระยะมะเร็ง (SCC) รวม 124 ตัวอย่าง วัดปริมาณการแสดงออกของ E1mRNA ด้วยวิธี ddPCR ศึกษาภาวะเมธิลเลชั่น ของโปรโมเตอร์ p97 และ p 670 ด้วยวิธี pyrosequencing ศึกษาลักษณะรูปแบบของสารพันธุกรรมและความเปลี่ยนแปลงของยีน E1 ด้วยวิธีปฏิกิริยาลูกโซ่โพลีเมอเรส ศึกษาคุณสมบัติของโปรตีน E1 ด้วยการนำพลาสมิดที่มียีน E1 (pEGFP-C1-HPV16 E1) เข้าในเซลล์ HEK293T หลังจากนั้นทำการศึกษาการเจริญเพิ่มจำนวนของเซลล์ โดยนับปริมาณเซลล์ที่มีชีวิตและตรวจการทำงานของเซลล์ ศึกษาการตายของเซลล์แบบ apoptosis และ necrosis ด้วยการย้อมด้วย Annexin V และ propidium iodine ในภาวะที่มีและไม่มีสารยับยั้ง caspase (QVD-OPH) และนับปริมาณเซลล์ด้วยการใช้เครื่อง flow cytometry ศึกษาการแสดงออกของ RNA ภายในเซลล์ที่มีการแสดงออกของโปรตีน E1 ด้วยวิธี microarray และยืนยันการแสดงออกของ RNA ด้วยวีธี Real-time reverse transcription (RT) PCR ใช้พลาสมิด pEGFP-C1 เป็นตัวควบคุมและเปรียบเทียบตลอดการศึกษานี้

ผลการทดลอง มีการเพิ่มขึ้นของปริมาณ E1mRNA สัมพันธ์เป็นเส้นตรงกับการพัฒนาของโรค (เซลล์ปกติ 0.18, CIN 1 0.41, CIN 2/3 0.65 และ SCC 0.79) อย่างมีนัยสำคัญ (r = 0.661, p =0.019) ไม่พบว่ามีความเกี่ยวข้องระหว่างลักษณะรูปแบบของสารพันธุกรรมกับการแสดงออกของ E1mRNA พบภาวะเมธิลเลชั่นของโปรโมเตอร์ p97 และ p 670 เพิ่มขึ้นอย่างมีนัยสำคัญในตัวอย่างเซลล์มะเร็งเมื่อเปรียบเทียบกับตัวอย่างเซลล์ปกติ และพบว่ามีความเปลี่ยนแปลงของยีน E1 ที่มีการซ้ำของ 63 คู่เบสเพียงร้อยละ 4.2 จากการนับจำนวนเซลล์ที่มีชีวิตและติดตามการเจริญเติบโตของเซลล์ HEK293T ที่มีการแสดงออกของโปรตีน E1 พบว่าเซลล์มีจำนวนลดลงอย่างมีนัยสำคัญที่ 24 ชั่วโมงหลังการนำเข้าพลาสมิด เมื่อเปรียบเทียบกับเซลล์ที่มีพลาสมิดตัวควบคุม (p < 0.0001) และยังพบว่าโปรตีน E1 เหนี่ยวนำให้เซลล์ตายด้วยกระบวนการของ apoptosis และ necrosis ที่ 48 ชั่วโมงภายหลังการนำเข้าพลาสมิด เมื่อใส่สาร QVD-OPH พบว่าเซลล์ตายลดลงแต่ไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติ เมื่อศึกษาการแสดงออกของ RNA ภายในเซลล์ที่มีการแสดงออกของโปรตีน E1 ด้วยวิธี microarray และยืนยันการแสดงออกของ RNA ด้วยวีธี Real-time reverse transcription (RT) PCR พบว่าโปรตีน E1 มีผลเปลี่ยนแปลงการแสดงออกของยีนหลายชนิดที่เกี่ยวข้องกับวิถีการสร้างโปรตีน (RPL36A) กลไกเมตะบอลิซึม (ALDOC) การเพิ่มจำนวนของเซลล์ (CREB5, HIF 1A, JMJD1C, FOXO3, NFKB1, PIK3CA, TSC22D3) การแตกหักของดีเอ็นเอ (ATR, BRCA1 และ CHEK1), และการตอบสนองทางภูมิคุ้มกัน (ISG20) อัตราการแสดงออกของยีนส่วนใหญ่ในเซลล์โฮสท์ที่มีการแสดงออกของโปรตีน E1 จะลดลงที่ 48 ชั่วโมงภายหลังการนำเข้าพลาสมิด

สรุป การตรวจหา E1mRNA และภาวะเมธิลเลชั่นของโปรโมเตอร์ p97 มีประโยชน์เป็นตัวบ่งชี้เพื่อพยากรณ์การเกิดมะเร็ง การมียีน E1 ซ้ำของ 63 คู่เบสในประชากรไทยพบสัมพันธ์กับรอยโรคระยะก่อนมะเร็ง โปรตีน E1 ของไวรัสแปปิโลมาไทป์ 16 สามารถเปลี่ยนแปลงวิถีสัญญาณภายในเซลล์หลายวิถีและขึ้นกับระยะเวลาที่แสดงออกของโปรตีน E1 ยีนที่ได้รับผลกระทบนี้เกี่ยวข้องกับการเพิ่มจำนวนของเซลล์และการตอบสนองต่อการแตกหักดีเอ็นเอของเซลล์โฮสท์ การศึกษานี้เสนอว่าโปรตีน E1 น่าจะมีบทบาทในการพัฒนาเซลล์กลายเป็นมะเร็ง อย่างไรก็ตาม ผลกระทบของโปรตีน E1 ภายในเซลล์ ในการศึกษานี้ไม่สามารถระบุวิถีที่จำเพาะเกี่ยวกับกลไกการเกิดมะเร็งได้ ซึ่งต้องมีการศึกษาต่อไป

สาขาวิชา ชีวเวชศาสตร์และชีวเทคโนโลยี ลายมือชื่อนิสิต .....

ปีการศึกษา 2560 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5674851930 : MAJOR BIOMEDICAL SCIENCES AND BIOTECHNOLOGY
KEYWORDS: HPV / E1 PROTEIN / CERVICAL CANCER / CARCINOGENESIS

FERN BAEDYANANDA: The Role of HPV16 E1 in Cervical Carcinogenesis. ADVISOR: ASSOC. PROF. PARVAPAN BHATTARAKOSOL, Ph.D., CO-ADVISOR: ARKOM CHAIWONGKOT, Ph.D., SHANKAR VARADARAJAN, Ph.D., pp.

Objectives: This study aimed to determine the expression profile of HPV-16 E1 in cervical samples and the role of E1 in cervical carcinogenesis. In addition, this work also aimed to determine whether the HPV-16 E1 63bp duplication is present in the Thai population.

Methods: One-hundred and twenty-four HPV16 positive cervical samples ranging from normal, CIN1, CIN2/3, and SCC lesions were studied. E1 mRNA expression was determined by ddPCR. Methylation of promoters p97 and p670 was quantified by pyrosequencing, while PCR and sequencing were used to determine the physical state and variations of HPV16 E1 genome. HEK 293T cells were transfected with pEGFP-C1 containing HPV16 E1. Cell proliferation of transfected cells was measured using cell viability count and cell metabolism assay. Apoptosis and necrosis was determined in transfected cells treated with or without QVD-OPH (pan-caspase inhibitor) by Annexin V and propidium iodide staining and quantitated using flow cytometry. RNA expression Microarray analysis was performed on FACS sorted HPV16 transfected cells. RNA expression was confirmed by Real-time reverse transcription (RT) PCR. Plasmid containing pEGFP-C1 was used as a vector control throughout the experiments.

Results: Increased E1mRNA expression related to disease progression (normal 0.18, CIN 1 0.41, CIN 2/3 0.65 and SCC 0.79) was demonstrated with significant positive correlation ($r = 0.661$, $p = 0.019$). No association between physical state and E1 expression was found. Methylation of p97and p670 promoters showed significant elevation in SCC compared to normal samples. Only 4.2% showed genomic variations of HPV16 E1 63 bp duplication. HPV16 E1 transfected HEK293T cells showed a significant decrease in number of viable cells and cell proliferation 24 h post-transfection ($p < 0.0001$). HPV16 E1 significantly induced both apoptotic and necrotic cell death 48 h post-transfection. Treatment of HPV16 E1 transfected cells with QVD-OPH showed a decreasing trend without statistical significance. Microarray and real-time RT PCR results revealed that E1 dysregulated genes involved in protein synthesis (RPL36A), metabolism (ALDOC), cell proliferation (CREB5, HIF 1A, JMJD1C, FOXO3, NFKB1, PIK3CA, TSC22D3), DNA damage (ATR, BRCA1 and CHEK1), and immune response (ISG20) pathways. Kinetic host gene expression in HPV16 E1 transfected cells indicated that most genes were downregulated after 48 h post-transfection.

Conclusion: Detection of E1 mRNA and p97 methylation were beneficial as cancer prognostic markers. The presence of 63 bp duplication in HPV16 E1 was observed in the Thai population and related to low grade lesions. The presence of HPV16 E1 protein alone was able to dysregulate many cellular pathways in a time dependent manner. Notable genes disrupted by HPV16 E1 included genes involved in cell proliferation and host DNA damage response. This study suggests that E1 may play a role in cancer development however HPV16 E1 transfection experiments did not provide a definite carcinogenic pathway which warrants further studies.

Field of Study: Biomedical Sciences and Biotechnology
Academic Year: 2017

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....
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AIF	Apoptosis inducible factor
Akt	Protein kinase b
APCs	Antigen presenting cells
Apf-1	Apoptotic protease activating factor 1
ASC-US	Atypical squamous cells of undetermined significance
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and rad3 related
BCL-2	B cell lymphoma 2
BH	Bcl-2 homology
bp	Base pairs
CDC25	Cell division cycle 25
CDKs	Cyclin dependent kinases
CHK1	Checkpoint kinases 1
CHK2	Checkpoint kinases 2
CIN	Cervical intraepithelial neoplasia
CO_2	Carbon dioxide
COX	Cyclooxygenase
CREB	Cyclic-AMP responsive element binding
CTLs	Cytotoxic Tlymphocytes
Cyt c	Cytochrome c
DBD	DNA binding domain
ddPCR	Droplet digital PCR
DIABLO	Direct inhibitors of apoptosis proteins binding protein with low pI
DISC	Death inducing signalling complex
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
DTT	Dithiothreitol
E6AP	E6 associated proteins
EGFR	Epidermal growth factor
ER	Endoplasmic reticulum
FADD	Fas associated protein via death domain
FBS	Fetal bovine serum
GILZ	Glucocorticoid-induced leucine zipper
h	Hour
HD	Helicase domain
HDAC	Histone deacetylase
HEK	Human embryonic kidney

HIF1α	Hypoxia inducible factor-1α
HPV	Human papillomavirus
HSIL	High-grade squamous intraepithelial lesion
IAPs	Inhibitors of apoptosis proteins
IFNs	Interferons
iPLA2	Calcium-independent phospholipase a2
L	Liter
LSIL	Low-grade intraepithelial lesion
M	Molar
mg	Milligram
$MgCL_2$	Magnesium chloride
min	Minute
ml	Milliliter
mM	Millimolar
MOMP	Mitochondria outer membrane potential
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NaCl	Sodium chloride
ND	N-terminal domain
NF-κB	Nuclear factor-κb
NLRs	Nod-like receptors
OD	Oligomerization domain
ORF	Open reading frame
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCD	Programmed cell death
PCR	Polymerase chain reaction
PDK	3-phosphoinositide-dependent protein kinase
PGs	Prostaglandins
PI3K	Phosphatidylinositol 3-kinases
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
pRB	Retinoblastoma protein
PRRs	Pattern recognition receptors
PS	Phosphatidylserine
RLRs	Rig-like receptors
RNA	Ribonucleic acid
RT	Reverse transcription
RTKs	Receptor tyrosine kinases
s	Second
SCC	Squamous cell carcinoma
SMAC	Second mitochondria-derived activator of caspase
SNORD	Small nucleolar RNA C/D box
SSIV	Superscript iv

TLRs	Toll-like receptors
TNF	Tumour necrosis factor
URR	Upstream regulatory region
VEGF	Vascular endothelial growth factor

CHAPTER I
GENERAL INTRODUCTION

Cervical cancer is the fourth most common cancer in women worldwide, and is the second most common cancer in women who reside in developing countries (Ferlay et al., 2012). More than 90% of cervical cancer cases are caused by the human papillomavirus (HPV), which is transmitted through intimate skin contact or sexual intercourse. HPV is currently the most common sexually transmitted infection. Although vaccines have been developed in an attempt to guard against the virus, the vaccines only protect against a few types of virus and do not protect people who have already been infected (Harper et al., 2004).

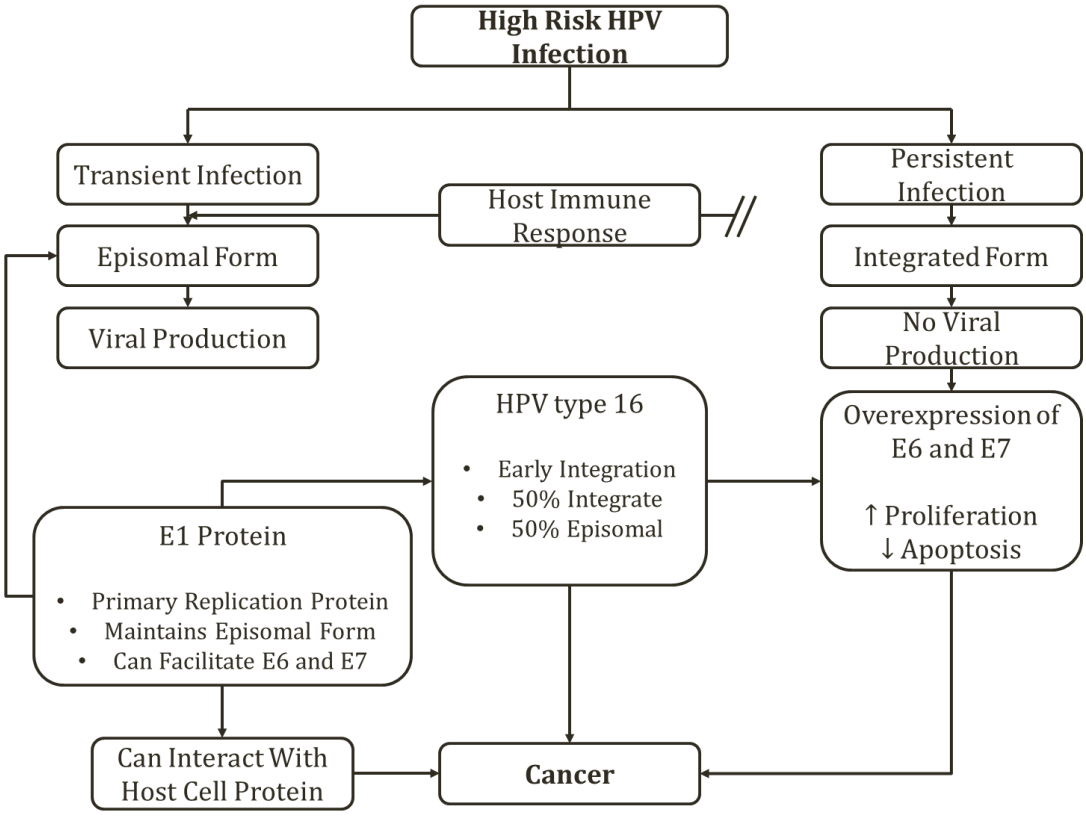
The human papillomavirus is a small DNA virus that infects the basal layer of the stratified epithelium of the skin and mucosa through wounds or small breaks in the skin and replicates as the cells differentiate. To this date more than 150 types of HPV have been discovered. The mucosal types of HPV can be classified into two major groups, low-risk (types 6, 11, 40, 42, 43, 44, 53, 54, 61, 72, 73 and 81) and high-risk (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68), based on their association with cancer. Of the high-risk types that are known to cause cancer, HPV type 16 (HPV-16) is the most common, being found in over 50% of all cancer cases. It is well established that the HPV types in the high-risk group are the cause of cervical cancer as well as other cancers including, anal, vulvar, and cancers of the oropharynx (Parkin & Bray, 2006). There are two main types of infection, transient and persistent infection. Transient infection is when HPV infects a host and goes through the normal mechanisms of producing new virions but after an amount of time the human immune system is able to clear the virus from the body. However in some cases persistent infection occurs due to the failure of the immune system to clear the virus and leads to the development of cervical cancer.

Once a persistent infection has been established HPV genome integration can occur therefore, the HPV genome copies can exist as episomal, integrated, or mixed forms within the cell. It was once generally believed that HPV integration was the

main mechanism leading to cervical carcinogenesis. Thus, HPV integration should be rarely found in precancerous lesions. This conclusion was demonstrated in cervical cancer with HPV-18 infection. Almost all of HPV-18 infections show complete integration by the time the cells have progressed to invasive cancer (Cullen et al., 1991). In contrast, HPV-16 which is responsible for over 50% of cervical cancer cases exhibits an integration pattern different from that of HPV-18. HPV-16 DNA integration occurs early after infection and only 50% has been shown to be integrated even at the carcinoma stage (Marongiu et al., 2014). HPV integration is followed by the overexpression of E6 and E7 due to a disruption in the E2 region during integration resulting in the loss of E2 function. E2 normally functions to regulate E6/E7 oncoprotein transcripts in conjunction with the E1 protein by binding to the E1 protein and regulating the replication and expression of the oncoproteins (Chen & Stenlund, 2002).

Replication of the HPV genome depends on the primary replication protein E1. E1 is an ATP-dependent helicase protein and is the only enzyme encoded by HPV. E1 is essential for the amplification of the viral episome in infected cells (Terenzi et al., 2008). Previous studies have shown that E1 does not only act as a helicase protein but is also involved in recruiting and interacting with other host proteins. E1 has also been deemed to drive host cell proliferation. However, the mechanism by which the E1 protein interacts with host cell proteins has not been described. In this study we aim to determine a possible mechanism in which E1 drives cell proliferation and possibly oncogenesis.

Conceptual Framework



Infection by high risk HPV types can lead to carcinogenesis. Transient infections where the virus replicates and produces virions are mostly cleared by the host. However, if a persistent infection occurs, the viral genome can become integrated into the host genome causing an overexpression of the integrated viral genes. HPV16 is the most common type of HPV associated with cervical cancer. However, detection of the HPV16 genome revealed that a significant percentage of cervical cancer cells contained the episomal form of the HPV16 genome. The HPV E1 protein is the main replication protein responsible for maintaining the episomal form of the viral genome. In addition, it is also often integrated into the host genome along with the known HPV oncoproteins E6 and E7. The E1 protein has also been shown to interact with host cell proteins and cause DNA damage. This study aims to determine if the HPV16 E1 protein plays a role in cervical carcinogenesis.

CHAPTER II
OBJECTIVES

Objectives

1. To determine the expression profile of HPV16 E1 in cervical samples
2. To determine if the Thai population contains the E1 63bp duplication
3. To determine the role of E1 in cervical carcinogenesis

Hypothesis

1. HPV16 E1 expression profile is different between the precancerous and cancerous stages.
2. The HPV16 E1 63bp duplication is present in the Thai population.
3. The HPV16 E1 protein is able to induce cell proliferation or inhibit apoptosis leading to cell immortalization.

CHAPTER III
LITERATURE REVIEW

Cervical cancer

Cervical cancer is the fourth most common cancer in women worldwide (Ferlay et al., 2013a). In 2012, there were more than 500,000 incidences of cervical cancer which caused over 200,000 deaths worldwide. Due to limited access to healthcare and lower rates of cervical cancer and HPV screening, cervical cancer prevalence in developing countries is much higher than developed countries (Arbyn et al., 2011; Ferlay et al., 2013b). Cervical cancer was ranked as one of the most common cancers, in developing countries such as Thailand (2nd) Myanmar (2nd), and Vietnam (4th). Although most of the global burden falls on developing countries, cervical cancer is still common in developed countries (Figure 1). Out of the most common cancers in women of developed countries such as the United Kingdom and United States of America, Japan, and Singapore, cervical cancer ranked 8th, 11th, 5th, and 6th respectively.

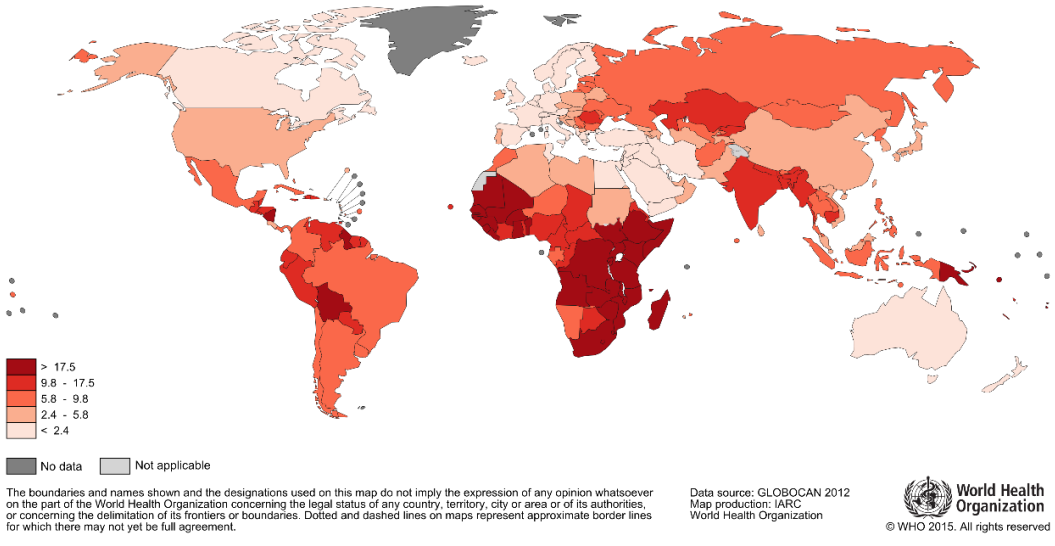


Figure 1. Graphical representation of cervical cancer mortality worldwide
Data represented as estimated age-standardised rates per 100,000 (Ferlay et al., 2013a).

Diagnosis

The American Cancer Society recommends routine screening for cervical cancer starting at 21 years of age (American Cancer Society, 2016). The main form of cervical cancer screening is the Papanicolaou test (Pap test, Pap smear). Pap smears are conducted by exfoliating cervical cells and placing them in a liquid preservative. The cells from the preservative are transferred to a microscopic slide and observed for abnormal cells by qualified medical personnel. Cytology results are reported as normal, uncertain (ASC-US: Atypical squamous cells of undetermined significance), and abnormal, i.e., low-grade intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL) and squamous cell carcinoma (SCC). The confirmatory diagnostic tests are colposcopy with biopsy, endocervical scraping, and cone biopsies. These methods are based on histological examination. The changes in a biopsy are called cervical intraepithelial neoplasia (CIN) or dysplasia (Figure 2). CIN can be graded into 3 stages, i.e., CIN1 (mild dysplasia), CIN2 (moderate dysplasia) and CIN3 (severe dysplasia) includes carcinoma *in situ*. If the cells are cancerous, they will be further identified as either squamous cell carcinoma (SCC), adenocarcinoma, or other types. Over 90% of all cervical cancer cases are caused by Human papillomavirus (HPV) (Walboomers et al., 1999). HPV testing is recommended following abnormal Pap smears, or if the patient is over 30 years of age (American Cancer Society, 2016).

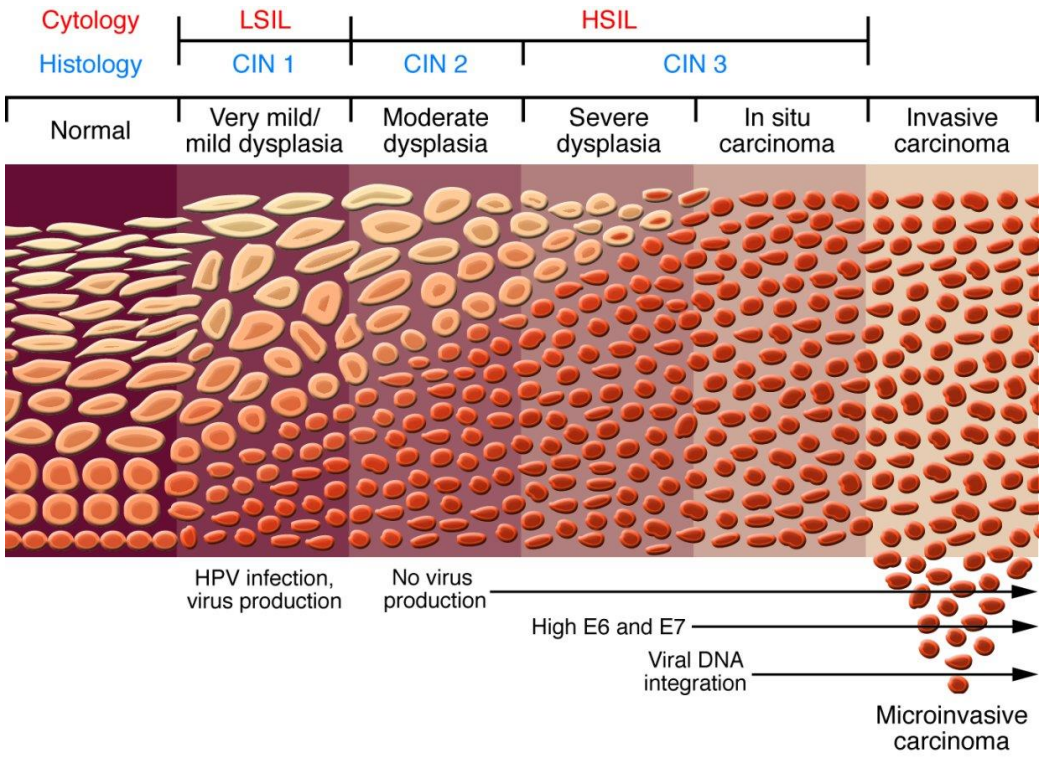


Figure 2. Schematic of HPV infection

Persistent HPV infection results in neoplasia and cancer. Cytologically detected lesions are classified as normal, low-grade intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL) and cancer. Histologically detected lesions are classified as normal, cervical intraepithelial neoplasia (CIN) 1-3 and cancer depending on severity and number of abnormal cells. LSIL corresponds to CIN 1, while HSIL corresponds to CIN 2 and 3 (Lowy & Schiller, 2006).

Human papilloma viruses

There are over 150 types of HPV, using genome similarity of the L1 gene HPV are classified into 5 genera: alpha, beta, gamma, mu, and nu (Figure 3). HPV in different genera share less than 60% similarity. Genomic similarity further separates HPV into species (60-70%), types (70-90%) and subtypes (>90%) (Burk et al., 2013; de Villiers et al., 2004). Depending on tissue tropism, HPV types in the alpha genus are classified into mucosal and cutaneous types. The mucosal types are separated further by each type's ability to cause cancer being termed low-risk and high-risk types (Doorbar et al., 2015). Low-risk mucosal types are typically associated with genital warts (HPV 6, 11, 40, 42, 43, 44, 53, 54, 61, 72, 73 and 81) whereas high-risk types are associated with cervical carcinoma (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68). Of the high-risk types, HPV16 and 18 are the most prevalent and HPV16 alone causes more than 50% of all cervical cancer cases.

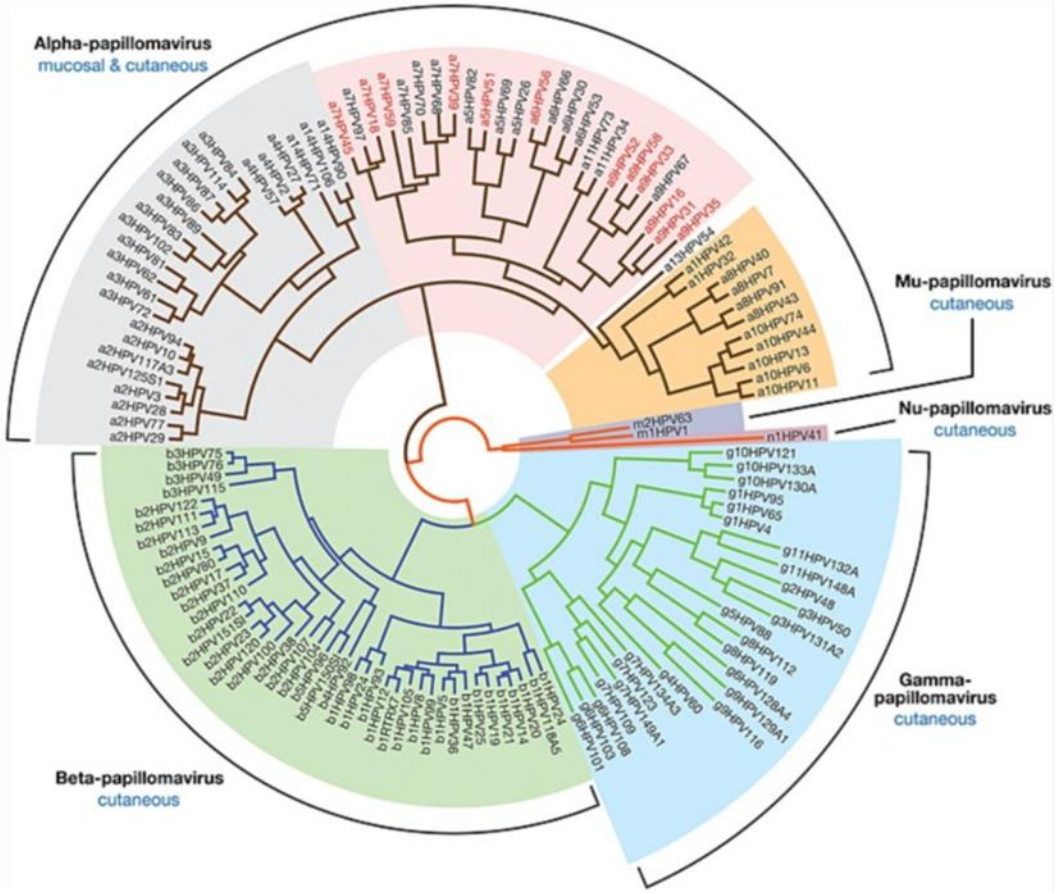


Figure 3. Phylogenetic tree of human papillomavirus types.

The five genera of human papillomaviruses are classified further into species, types and subtypes. Human papillomavirus types from the Alpha genus are often classified as low-risk cutaneous (grey), low-risk mucosal (orange), or high-risk mucosal (pink) (Doorbar et al., 2015).

HPV is a non-enveloped double-stranded DNA virus belonging to the family *Papillomaviridae* (Figure 4). HPV virions are approximately 55 nm in diameter. The HPV genome consists of circular double-stranded DNA approximately 8kb in size enclosed in an icosahedral capsid composed of 72 capsomeres (Veressimo Fernandes & Fernandes, 2012). The viral genome consists of the functional early (E) genes E1, E2, E4, E5, E6 and E7, and the structural late (L) genes, L1, and L2 (Figure 5). The early gene E1, encodes the primary protein responsible for viral replication, E2 is involved in transcriptional regulation, E4 is involved in virion release, E5 is responsible for the immune evasion of HPV, E6 binds and degrades the tumour suppressor protein p53 and E7 binds the retinoblastoma protein involved in regulating cell proliferation. The late proteins L1 and L2 are the major (80%) and minor (20%) capsid proteins respectively.

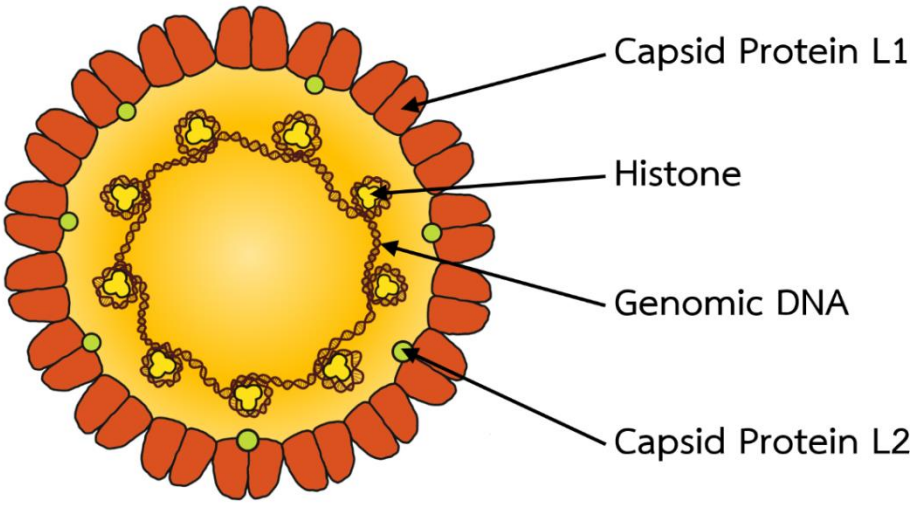


Figure 4. Structure of HPV.
Redrawn from (Swiss Institute of Bioinformatics, 2010).

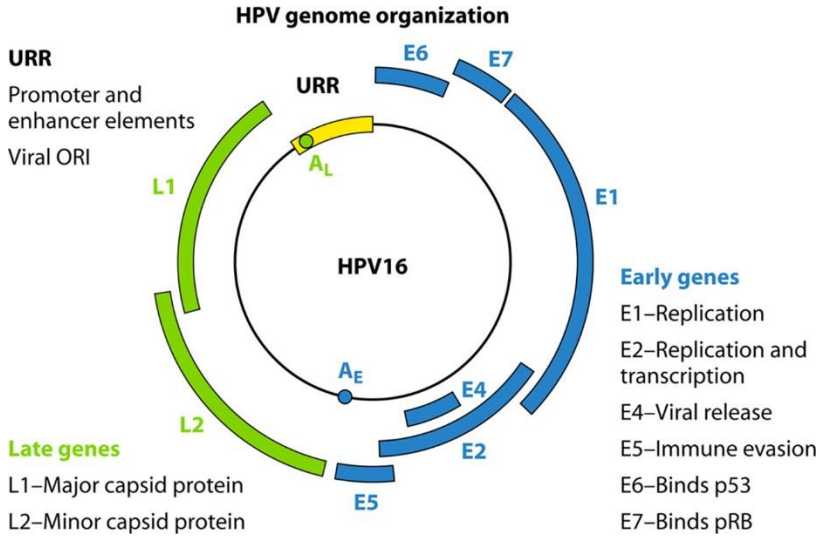


Figure 5. Diagram of HPV genome structure.

The HPV genome consists of early (E) and late (L) regions, which relate to their time of expression during the viral life cycle. The early region carries a number of genes which function at the level of viral replication and transcription. The late region encodes viral structural proteins and the upstream regulatory region (URR), contains promoters and the viral origin of replication (Stanley, 2012).

E1 protein

The E1 protein is the primary replication protein of HPV and is an ATP-dependent helicase protein that functions to bind to the viral origin of replication and as a helicase, unwinds the viral DNA and initiates replication (Hughes & Romanos, 1993). The E1 protein is the most conserved protein encoded by all papillomaviruses because the E1 helicase function is crucial for the viral episome replication. It is thought that the E1 protein is required in all phases of the viral replication cycle, including the establishment, maintenance and amplification phases. The E1 protein works in tandem with E2 in order to initiate replication (Berg & Stenlund, 1997). The E2 protein recruits E1 to the origin of replication by binding to the E2 binding sites near the origin of replication and binding to E1 (Frattini & Laimins, 1994). This interaction with the E2 protein increases the affinity and specificity of E1 binding to HPV DNA (Berg & Stenlund, 1997). However, it has been shown that E1 can also initiate viral replication independent of E2, although with lower efficiency (Bonne-Andrea et al., 1997).

The E1 protein can be divided into three main domains, each with a distinct and important function: The N-terminal regulatory domain, the DNA binding domain, and the helicase domain (Figure 6) (Bergvall et al., 2013). The N-terminal of the protein mainly contains the nuclear localization signal and the nuclear export signal, which allows for the shuttling of E1 in and out of the nucleus. The DNA binding domain is the domain that recognizes specific sequences near the origin of replication of the virus. There are a six E1 binding sites each with different affinities towards the E1 protein (Figure 7). The helicase domain of the E1 protein functions to bind ATP and forms a doughnut-shape around the viral DNA template (Enemark & Joshua-Tor, 2006).

Although the protein credited as being the major HPV transcriptional regulator protein is the E2 protein, E1 has been shown to also play a role in the transcriptional activation of HPV by forming a complex with E2 (King et al., 2011; Nishimura et al., 2000). This E1-E2 complex form extends E2 half-life by preventing ubiquitination (King et al., 2011). In addition to stabilizing E2, E1 itself is also able to act as a transcriptional activator. This additional function of E1 facilitates the expression of viral proteins by recruitment of host proteins during HPV integration where E2

protein is lost. As mentioned above the human papillomaviruses do not encode many proteins and rely heavily on host proteins in order to facilitate replication, transcription and translation. Therefore, another major role of the E1 protein is to interact with host cell proteins both in the episomal and integrated forms (Demeret et al., 1998; King et al., 2011). Several studies demonstrate that E1 can interact with proteins involved in cellular DNA replication machinery such as DNA polymerase alpha primase, replication protein A, and topoisomerase I, suggesting varied roles of E1 in viral replication (Loo & Melendy, 2004).

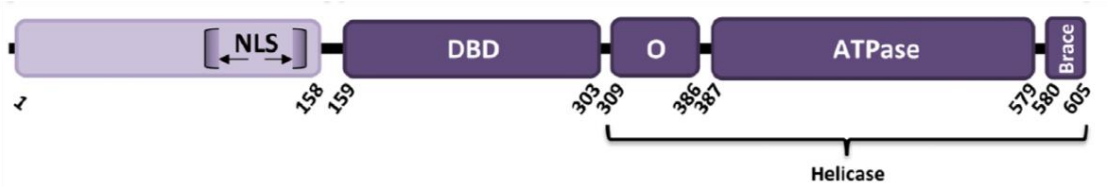


Figure 6. Diagram of E1 protein domains.

Diagram of the different functional domains of E1 including the bipartite nuclear localization signal (NLS), DNA binding domain (DBD), and the domains which construct the helicase domain: minimal oligomerization domain (O) ATPase domain and the C-terminal brace.

(Bergvall et al., 2013)

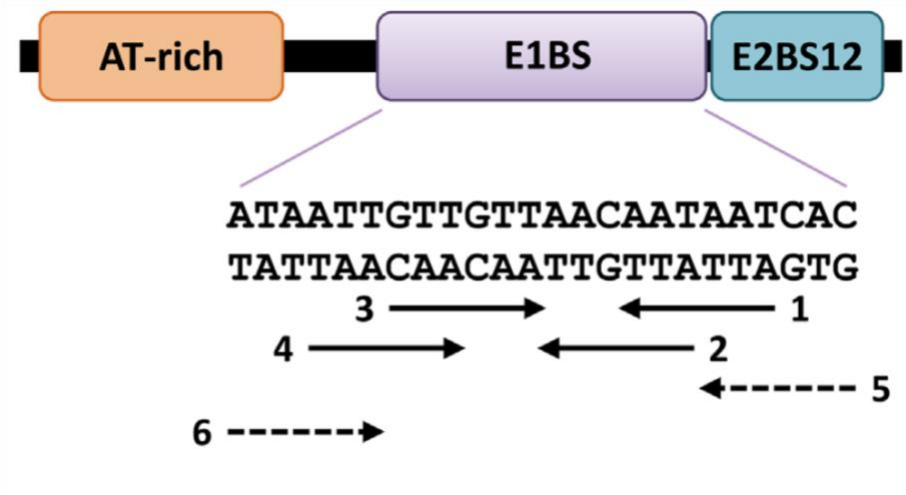


Figure 7. Diagram of the origin of replication for papillomavirus.

This diagram shows the 6 E1 binding sites (E1BS) and the E2 binding sites (E2BS).

(Bergvall et al., 2013)

HPV replication cycle

Papillomaviruses can infect a wide range of animal species, however each type of papillomavirus is highly host- and tissue-specific. HPV infects the basal layer of the epithelium through wounds or breaks in the epithelium and is maintained as an extrachromosomal element, or episome, in the nucleus of infected cells. In the lower strata of the epithelium only the early genes are expressed from the "early" promoter (Graham, 2017). As the infected cell proliferates, the HPV genome replicates and increases the episomal copy numbers in the cell. The viral genome is replicated along with host cell DNA replication, and after cell division occurs, the viral genome is also transferred to the daughter cells. During this phase, no new virion progeny are produced. When the infected cell proliferates and differentiates, HPV DNA replication increases resulting in a high episomal copy number (Burd, 2003). E1 is responsible for driving viral genome replication and acts as a helicase to unwind DNA (Hughes & Romanos, 1993). E2 helps recruit E1 to the viral origin of replication and is also the viral transcription regulator (Frattini & Laimins, 1994). E2 binds to specific E2 binding sites (E2BS) and activate the early promoter, however once high viral protein expression occurs, E2 binds to E2BS other to repress expression (Tan et al., 1994). The E6 and E7 proteins act to maintain the cell in the active state so that viral genome replication can continue to occur in contrast to the situation in uninfected cells where cells would no longer be in the active state. The E6 and E7 proteins therefore play an important role in maintaining the active state of the cell and preventing apoptosis by binding the p53 and retinoblastoma (pRB) proteins. Finally, in the upper strata of the epithelium, the "late" promoter is activated and the late proteins responsible for capsid formation, L1 and L2 are expressed and new virions are assembled and released in the upper strata of the epithelium (Longworth & Laimins, 2004b) (Figure 8). Most HPV infections are transient and are cleared within approximately 2 years, however if the host immune system is unable to clear the infection, a persistent infection occurs (Plummer et al., 2007).

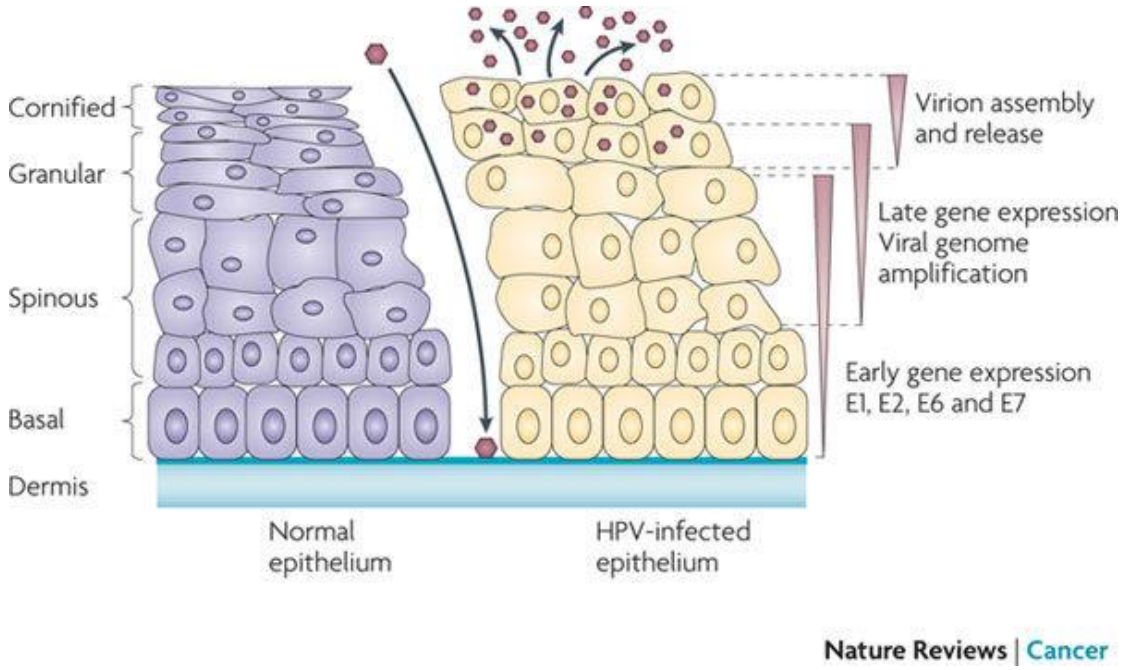


Figure 8. HPV replication cycle.

This figure shows uninfected epithelium to the left and HPV infected epithelium to the right. Upon infection HPV establishes in the nucleus as low-copy episomes and expresses the early HPV genes E1, E2, E6, and E7. The viral genomes are replicated along with host DNA. Once cell division occurs, one daughter cell containing the replicated HPV genome moves away from the basal layer and differentiates. The process of differentiation induces the productive phase of HPV where the viral genome is amplified and triggers the expression of the late genes, L1 and L2. In the upper layers of the epithelium the new virions are assembled and shed (Moody & Laimins, 2010).

HPV integration

Persistent infection of high-risk HPV can result in integration of the viral genome into the host genome. Integration can promote oncogenesis through the dysregulation of viral genes integrated into host DNA which function to increase proliferation and inhibit cell cycle checkpoints and apoptosis (Moody & Laimins, 2010). Integration patterns of HPV depend on virus type. For example, more than 90% of HPV18 associated cancers exhibit fully integrated HPV18 genomes. However, cancers caused by HPV 16 are much more varied in their integration pattern. HPV16 exhibits early integration but also keeps the episomal form of the genome as the cells have progressed to invasive cancer (Cullen et al., 1991).

HPV oncogenicity

HPV integration typically results in the disruption of the E2 ORF, therefore preventing the expression of the E2 protein. This results in the dysregulation of other integrated HPV genes (Jeon et al., 1995) especially E5, E6 and E7 expression. The E5 protein primarily localises in the endoplasmic reticulum, golgi apparatus and nuclear membrane. E5 has weak transformation capabilities when expressed alone. However, E5 has been reported to enhance the oncogenic properties of E6 and E7 by activating the epidermal growth factor (EGFR). E5 also plays a role in HPV immune evasion by decreasing the surface levels of MHC class I proteins, which inhibits clearance of infected cells by the immune response system (Figure 9). Overexpression of E6 causes a significant amount of the tumour suppressor protein p53 to be degraded by recruiting the cellular E3 ubiquitin ligase E6 associated proteins (E6AP) which triggers the degradation of p53. The protein p53 is known to play a crucial role in preventing genome mutation and maintaining genome stability, while degrading p53 causes the cell to proliferate unchecked (Figure 10) (Polager & Ginsberg, 2009). HPV does not cause a mutation in the p53 gene but E6 protein simply attacks and degrades it (Howie et al., 2009). Over expression of E7 causes cell immortalization because E7 binds the tumour suppressor protein, pRb, which normally binds and inactivates the transcription factor E2F. Because the oncoprotein E7 competes to bind with pRb, and thereby releasing E2F, the infected cells are able to increase proliferation. E7 also disrupts other cell cycle regulators such as histone deacetylase (HDAC) and facilitates

its removal to maintain the cell in an S phase environment required for viral genome amplification (Figure 11) (Longworth & Laimins, 2004a).

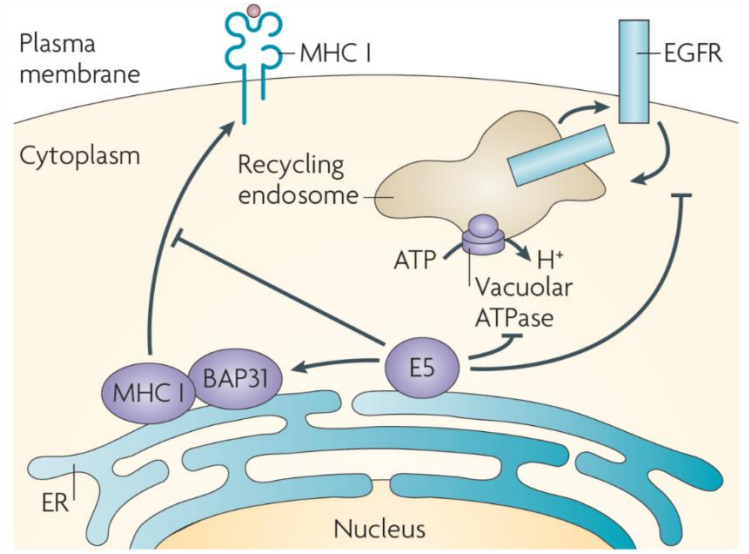


Figure 9. HPV E5 interactions with cellular pathways and factors.

E5 contributes to the actions of E6 and E7 by modulating the transit of signalling proteins through the endoplasmic reticulum (ER) and aiding in immune evasion. (Moody & Laimins, 2010)

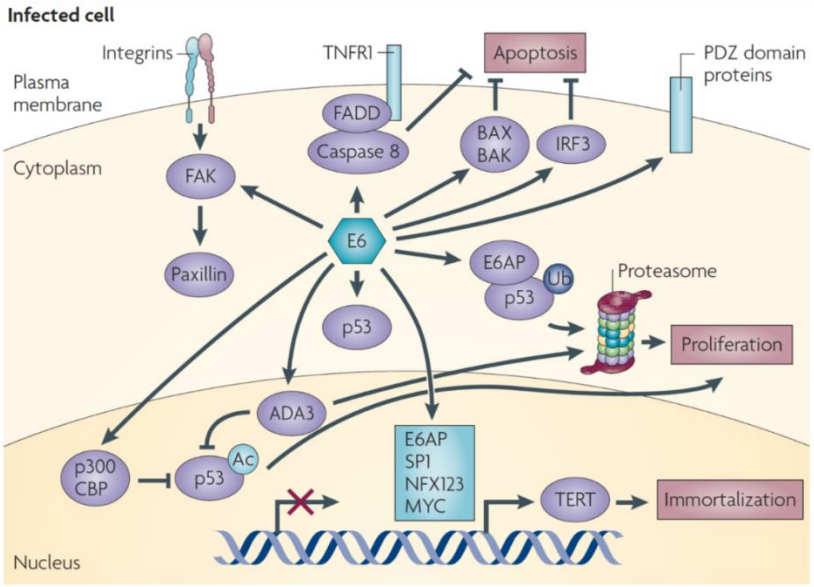


Figure 10. HPV E6 affects many cellular proteins and signalling pathways.
High-risk E6 proteins inhibit p53-dependent growth arrest and apoptosis in response to aberrant proliferation through several mechanisms, resulting in the induction of genomic instability and the accumulation of cellular mutations (Moody & Laimins, 2010).

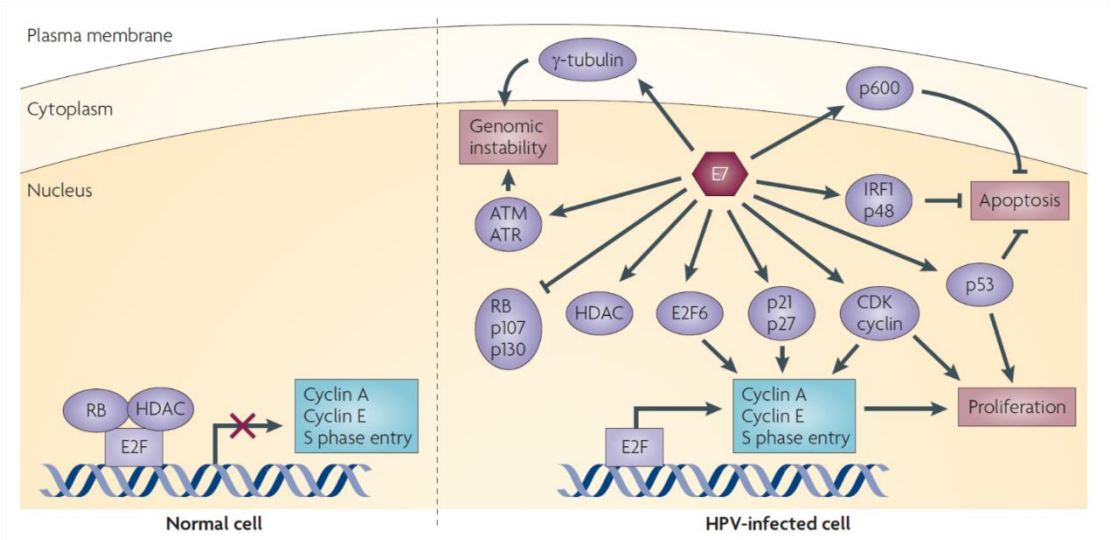


Figure 11. HPV E7 affects numerous cellular processes through interactions with multiple host cell proteins.
(Moody & Laimins, 2010)

DNA Helicases and possible roles in cancer

Genome instability is a characteristic of many cancers and has emerged to be a hallmark of cancer (Negrini et al., 2010). Many factors can cause genome instability including DNA damage, from external sources such as exposure to chemicals, and radiation (Langie et al., 2015) and other factors which include mutations in genes involved in DNA damage repair and tumour suppressor molecules (Negrini et al., 2010). DNA helicases are enzymes that function to bind and unwind complementary strands of DNA. This process is energy dependent and relies on nucleoside 5′-triphosphate hydrolysis (Figure 12). The helicases are found in prokaryotes, eukaryotes as well as viruses. They are involved in numerous processes such as DNA replication, repair, recombination, and transcription (Hall & Matson, 1999). Mutations and dysregulation of helicase expression has been associated with cell transformation and cancer development. Mutations in helicase encoding genes have detrimental effects, such as Werner syndrome and Bloom's syndrome. Werner syndrome is characterized by the appearance of premature aging features and early onset of age related diseases such as cardiovascular diseases, diabetes mellitus, and carcinoma (Martin, 1985). Patients with Bloom's syndrome are also predisposed to carcinogenesis (German, 1997). In addition, cells transformed by the Epstein-Barr virus and simian virus 40 have exhibited upregulated helicases expression. Genome instability leads to carcinogenesis (Kawabe et al., 2000).

It is known that HPV induces DNA damage and requires molecules involved in the DNA damage/DNA repair pathways in order to successfully replicate (Moody & Laimins, 2009). In addition, it has been shown that E1-E2 mediated replication of the HPV genome is not inhibited by the host DNA damage response pathway (King et al., 2010). The HPV E1 protein is a helicase that facilitates viral genome replication and also interacts with host cell proteins (Figure 13). DNA damage caused by HPV can be induced by the E1 protein alone. For example, the E1 protein of HPV 31 induces DNA damage (Fradet-Turcotte et al., 2011) and DNA damage at viral replication foci were observed in the presence of E1 and E2 (Sakakibara et al., 2011).

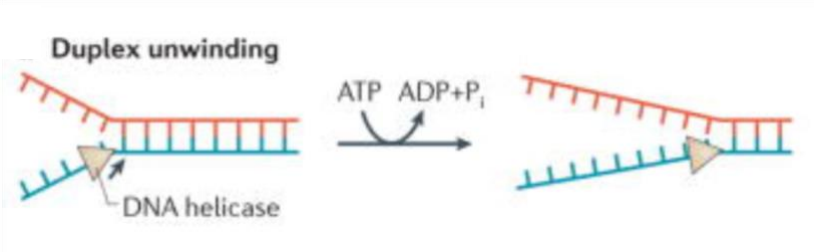


Figure 12 DNA helicases catalytically disrupt base pairs between complementary strands in an ATP-dependent manner.
(Brosh, 2013)

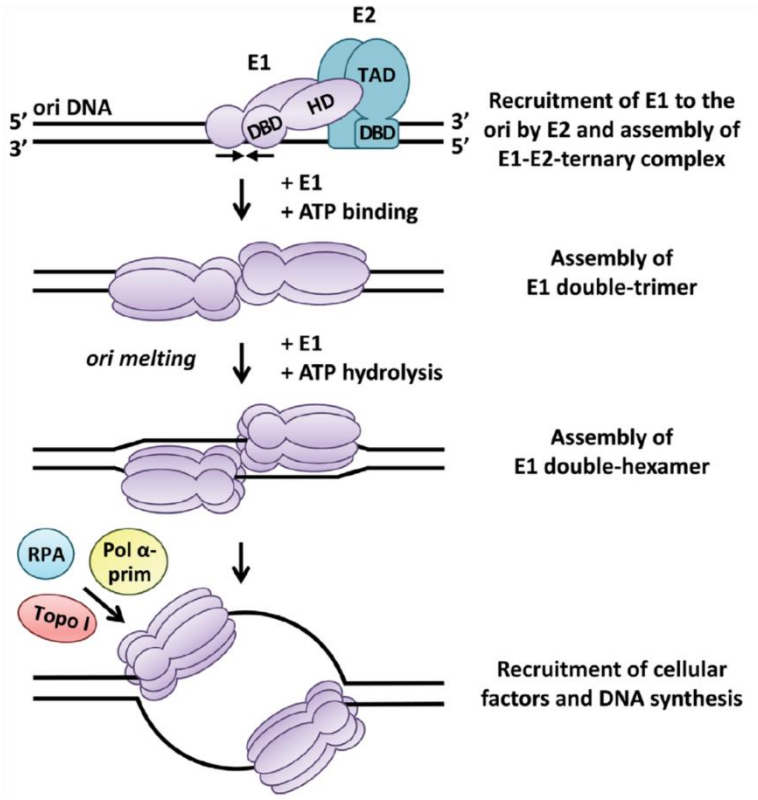


Figure 13. Schematic representation of the initiation of DNA replication the E1 protein.
(Bergvall et al., 2013)

Host immune response to HPV

To defend against foreign pathogens such as viruses and other microbes, the host utilizes a wide array of immune defence mechanisms. The first line of defence against HPV is the epithelial barrier. HPV infection occurs when there is a small wound in the epithelial barrier which allows the virus to attach and infect cells. Once infection occurs the next line of defence is the innate immune system which detects foreign pathogen-associated molecular patterns (PAMPs) rapidly and begins a signalling cascade for other immune response mechanisms (Turvey & Broide, 2010). The innate immune system consists of antigen presenting cells (APCs), phagocytes, natural killer (NK) cells and cytokines. Interferons (IFNs) are part of the innate immune response and are particularly important in defending against HPV infections. Interferon production can be stimulated through toll-like receptors (TLRs) that are located inside and outside of the cell (Nasu & Narahara, 2010). The most important TLRs in detecting viral PAMPs are intracellular TLR 3, 7, 8, and 9. These receptors sense foreign double stranded RNA, single stranded RNA and foreign DNA and signal the production of type I IFNs. Dendritic cells also sense pathogens using pattern recognition receptors (PRRs) and produce type I IFN (Turvey & Broide, 2010). Type I IFNs are a family of cytokines particularly important for viral infections, because they protect cells against viral infection by a signalling cascade that puts the cell in an antiviral state by inducing expression of hundreds of genes. In addition to TLRs, the innate immune system can also detect infection and cell damage in the cytosol through major classes of receptors called NOD-like receptors (NLRs), RIG-like receptors (RLRs) and cytosolic DNA sensors and the STING pathway which also promote inflammation and type I IFN production (Barber, 2015). In HPV, infection is strictly intraepithelial, and HPV does not spread through blood nor does it cause cytolysis or cell death. Therefore, the natural life cycle of HPV is optimal for immune evasion. Moreover, viral proteins such as HPV E6 and E7 aid in evasion of innate immune responses (Amador-Molina et al., 2013). Adaptive immunity is induced after the innate immunity but is much more specific than the innate immune response and can also memorize foreign pathogens that have previously been encountered (Abbas et al., 2014). The adaptive immune system consists of lymphocytes separated into 2 broad categories that facilitate humoral antibody (B

cells) and cell-mediated immune (T cell) responses. B cells are activated by foreign pathogens to secrete antibodies, which are proteins called immunoglobulins. Antibodies circulate the bloodstream and body fluids, and specifically bind to foreign antigens. When antibodies bind to viruses they block the pathogen's ability to bind to host cells and also serve as a homing device for the pathogens to be eliminated (Abbas et al., 2014). Cell mediated immune response is another integral part of the host immune system which consists of 2 main types of T cells: helper T cells and cytotoxic T lymphocytes (CTLs). CTLs recognize HPV specific peptides that are presented by MHC class I molecules on the surface of infected cells. CTLs kill infected cells but also activate nucleases which degrade viral genomes and secrete IFNγ which activate phagocytes. HPV has developed several adaptive immune response evasion mechanisms such as repression of MHC class I expression, absence of cell lysis and systemic viremia leading to low antigen levels available for presentation (Frazer, 2009). Despite the immune evasion mechanisms of HPV, most infections are cleared within 2 years (Plummer et al., 2007).

HPV diseases

HPV is a known cause of cervical cancers, with more than 90% of all cervical cancer cases being attributed to HPV. However other cancers have been linked to HPV as well such as vulvar, vaginal, penile, oropharyngeal, anal, and rectal cancers. In the United States, The Center for Disease Control estimated 13.5/100,000 persons of cancers in women and 9.7 of cancer in men were associated with HPV infection (Viens et al., 2016). In addition to cancer, HPV also causes benign cutaneous and mucosal lesions (Table 1). Several detection methods can diagnose HPV infection (Table 1). However, viral isolation of HPV is not possible because the virus cannot be propagated using normal tissue culture methods. Diagnosis of HPV can be done by observing the morphology of cells such as Pap smears and tissue samples. Cells infected with HPV exhibit a shrunken nucleus in large cytoplasmic vacuoles termed, koilocytes (Burd, 2003). The bulk of HPV diagnosis is done through molecular laboratory techniques. HPV DNA detection is done by two methods, the first is a polymerase chain reaction (PCR) using generic primers that can detect many HPV types (Husman et al., 1995). The second method used to detect HPV DNA is the

hybrid capture 2 method, which is based on hybridization of synthetic RNA probes, complementary to the DNA sequence of 13 high-risk (16, 18, 31, 33, 35, 39, 45, 51,52, 56, 58, 59, and 68) and five low-risk (6, 11, 42, 43,44) HPV types (Lorincz, 1996). HPV infection can also be tested by detecting HPV RNA. Testing for viral transcripts allows for the quantification of genome expression. The most common method to test for HPV RNA is reverse transcription quantitative real time PCR (Lamarcq et al., 2002). HPV infection can also be diagnosed using methods targeting viral proteins or HPV specific antibodies (Dias et al., 2005).

Table 1. HPV type and disease association
(Burd, 2003)

Disease	HPV type
Plantar warts	1,2,4,63
Common warts	2,1,7,4,26,27,29,41,57,65,77,1,3,4,10,28
Flat warts	3, 10, 26, 27, 28, 38, 41, 49, 75, 76
Other cutaneous lesions	6, 11, 16, 30, 33, 36, 37, 38, 41, 48, 60, 72, 73
(e.g., epidermoid cysts, laryngeal carcinoma)	
Epidermodysplasia verruciformis	2,3,10,5,8,9,12,14,15,17,19,20,21,22,23,24,25,36,37,
Recurrent respiratory papillomatosis	38,47,50
Focal epithelial hyperplasia of head & neck	6, 11
Conjunctival papillomas /carcinomas)	13, 32
Condyloma acuminata (genital warts)	6, 11, 16
Cervical intraepithelial neoplasia (CIN)	6, 11, 30, 42, 43, 45, 51, 54, 55, 70
Unspecified	
Low risk types	30, 34, 39, 40, 53, 57, 59, 61, 62, 64, 66, 67, 68, 69
High risk types	6, 11, 16, 18, 31, 33, 35, 42, 43, 44, 45, 51, 52, 74
	16, 18, 6, 11, 31, 34, 33, 35, 39, 42, 44, 45, 51, 52, 56, 58, 66
Cervical carcinoma	16, 18, 31, 45, 33, 35, 39, 51, 52, 56, 58, 66, 68, 70

HPV and methylation

Over the past few years, because HPV is an important cause of cervical cancer, co-testing of HPV DNA along with Pap smears has increased in healthcare practices. Infection plays an important role in cervical cancer diagnosis, prognosis, and treatment. HPV viral methylation has emerged in recent years as a new way to differentiate between women who have benign HPV infections and transformative infections (Chaiwongkot et al., 2013). DNA methylation is a normal process that occurs in humans and is a process that is used to regulate gene expression. Disruption of normal methylation sites has been linked to cancer (Stein, 2011). Regarding HPV, it is possible that the host defence mechanisms switch on and cause methylation of the HPV genome; it is also possible that methylation is an important process in which HPV regulates itself, by rendering some binding sites inaccessible, or signalling the virus to switch from a productive infection to a transformative infection (Vinokurova & von Knebel Doeberitz, 2011). For example, methylation of the HPV16 genome is high in CaSki cells with approximately 600 copies of integrated HPV16. In contrast, methylation of HPV16 in SiHa cells which have only 1-2 copies of integrated HPV16 is low (Chaiwongkot et al., 2013). With novel more sensitive and higher throughput technology emerging at a more cost-effective price point, the search for non-invasive reliable biomarkers for cancer development is growing. For example, methylation patterns in the L1 region of HPV-16 have been shown to be associated with increasing disease severity (Lorincz et al., 2013). Although methylation patterns have been suggested, a specific method that can be used to triage patients is still not defined.

Hallmarks of cancer

Cancer cells are different from normal cells because they have the ability to divide uncontrollably; this ability is accomplished through deregulating many cellular pathways involved in cell death, cell proliferation and invasion. Cancer cells therefore exhibit “hallmarks of cancer” that include: sustaining proliferative signalling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, and resisting cell death (Hanahan & Weinberg, 2011) (Figure 14). The most notable hallmark of cancer is the ability to proliferate

indefinitely. In normal cells, proliferation is controlled by many cellular signals that activate cell growth and signals that are involved in negative feedback loops. Cancer cells deregulate these signals, either by activating the cell proliferation signals or disrupting negative feedback signals and evade growth suppressor signals. Normal cells and cancer cells both require nutrients. Cancer cells activate angiogenesis to increase the amount of nutrients to help sustain expanding growth. In order to have a high replicative potential cancer cells obtain a high level of telomerase, which is a specialized DNA polymerase that adds telomere repeat segments to the ends of DNA. In normal cells, telomerase is present in low amounts in human somatic cells, which caps the ability of normal cells to proliferate. Another important ability of cancer cells is the ability to resist cell death. Normal cells are typically regulated by programmed cell death. Cancer cells resist programmed cell death by increasing expression of antiapoptotic regulators such as Bcl-2 or down-regulating proapoptotic factors such as Bax. Activation of invasion and metastasis occurs in cancer cells by disrupting expression of cell adhesion molecules and up-regulating molecules involved in migration of cells. Other important properties of cancer cells are genome instability, and inflammation. Recently studies have described emerging hallmarks which include reprogramming of energy metabolism and evading immune destruction.

These hallmarks of cancer occur due to transformation of normal cells into cancer cells by three major factors: spontaneous DNA mutations, environmental causes such as exposure to carcinogens, and microbial infections.

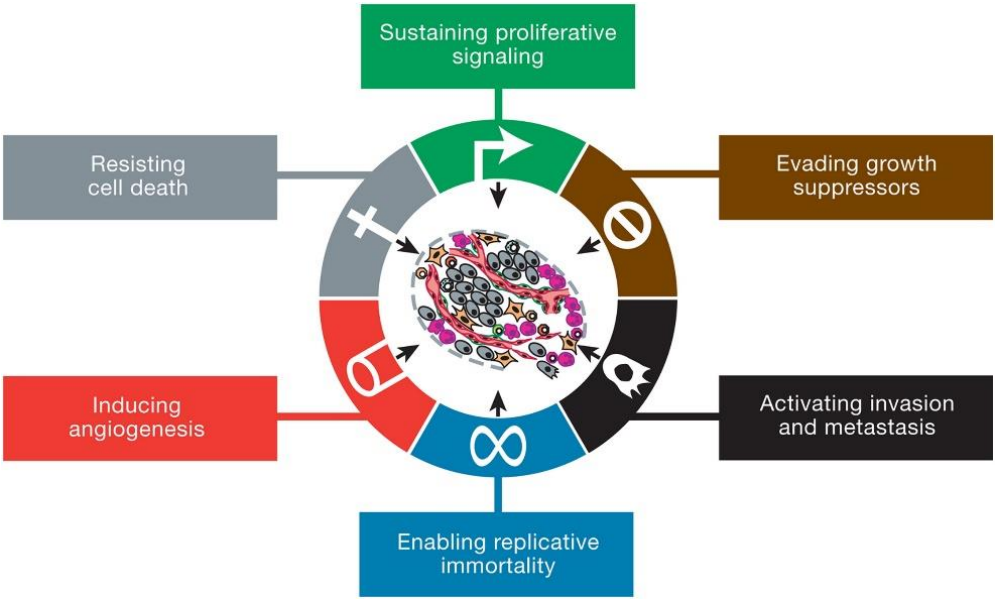


Figure 14. Hallmarks of cancer.

The hallmarks of cancer comprise six major biological capabilities. The six hallmarks of tumour cells are the ability to: sustain proliferative signalling, evade growth suppressors, resist cell death, enable replicative immortality, induce angiogenesis, and activate invasion and metastasis. (Hanahan & Weinberg, 2011)

Apoptosis

In order to maintain homeostasis, cells undergo programmed cell death (PCD) to maintain cell population in tissues, combat infections, and clear abnormal cells. There are two main pathways that initiate apoptosis: the extrinsic or death receptor mediated pathway, and the intrinsic or mitochondrial pathway (Figure 15). In both pathways initiator caspases are activated which in turn activate executioner caspases.

Initiation of apoptosis by extrinsic signalling pathways involves ligation and activation of transmembrane death receptors belonging to the tumour necrosis factor (TNF) receptor superfamily (Figure 16). Members of the TNF receptor family share similar cysteine-rich extracellular domains and have a cytoplasmic domain called the death domain that helps to initiate cell death by signalling death signals from the cell surface to intracellular molecules. Several receptors on the cell surface are known as decoy receptors which act to bind death signalling ligands but do not initiate cell death because they lack a functional death domain (Ashkenazi & Dixit, 1998). Death inducing ligands bind to their specific receptors and provided that a functional death domain is present, the receptors recruit death substrate proteins such as Fas associated protein via death domain (FADD) (Wajant, 2002). Proteins such as FADD induce apoptosis by activating the extrinsic initiator caspase 8 and forming the death inducing signalling complex (DISC). Death receptor-mediated apoptosis can be inhibited by proteins such as c-FLIP which binds to DISC and inactivates them (Figure 17) (Krueger et al., 2001).

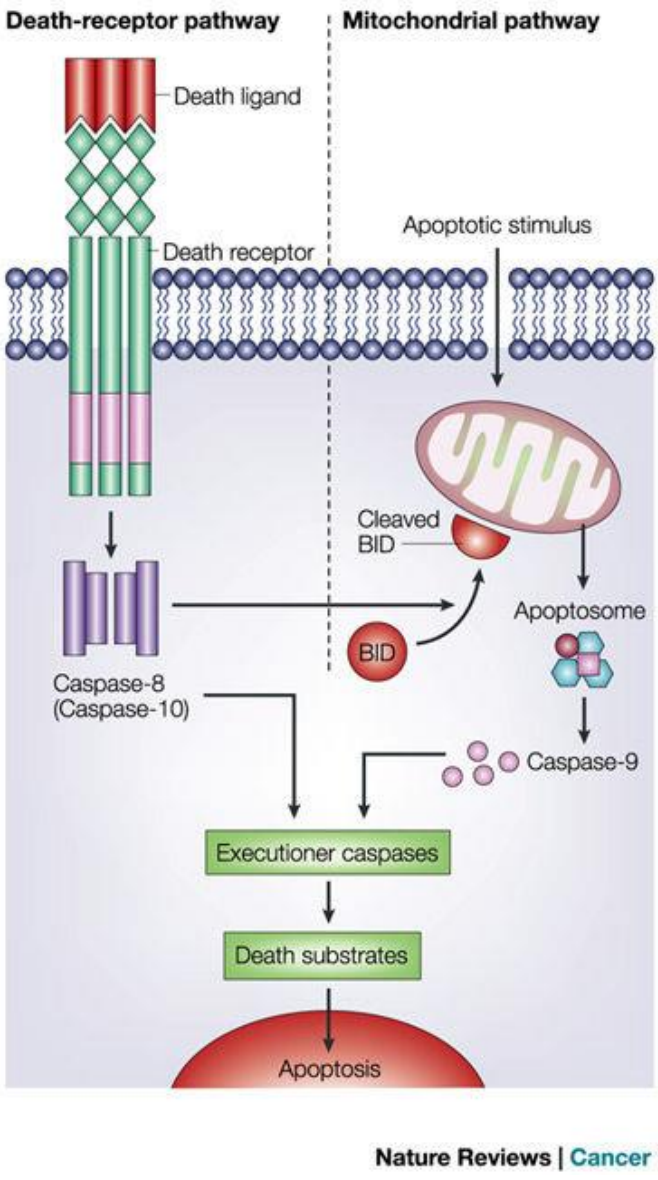


Figure 15. Death receptor and mitochondrial pathways of apoptosis.

Apoptosis can be initiated by two pathways: death receptors on the cell surface (extrinsic pathway) or mitochondria (intrinsic pathway). In both pathways, induction of apoptosis leads to activation of an initiator caspase which activate executioner caspases. Active executioner caspases cleave the death substrates resulting in apoptosis (Igney & Krammer, 2002).

Initiation of apoptosis by extrinsic signalling pathways involves ligation and activation of transmembrane death receptors belonging to the tumour necrosis factor (TNF) receptor superfamily (Figure 16). Members of the TNF receptor family share similar cysteine-rich extracellular domains and have a cytoplasmic domain called the death domain that helps to initiate cell death by signalling death signals from the cell surface to intracellular molecules. Several receptors on the cell surface are known as decoy receptors which act to bind death signalling ligands but do not initiate cell death because they lack a functional death domain (Ashkenazi & Dixit, 1998). Death inducing ligands bind to their specific receptors and provided that a functional death domain is present, the receptors recruit death substrate proteins such as Fas associated protein via death domain (FADD) (Wajant, 2002). Proteins such as FADD induce apoptosis by activating the extrinsic initiator caspase 8 and forming the death inducing signalling complex (DISC). Death receptor-mediated apoptosis can be inhibited by proteins such as c-FLIP which binds to DISC and inactivates them (Figure 17) (Krueger et al., 2001).

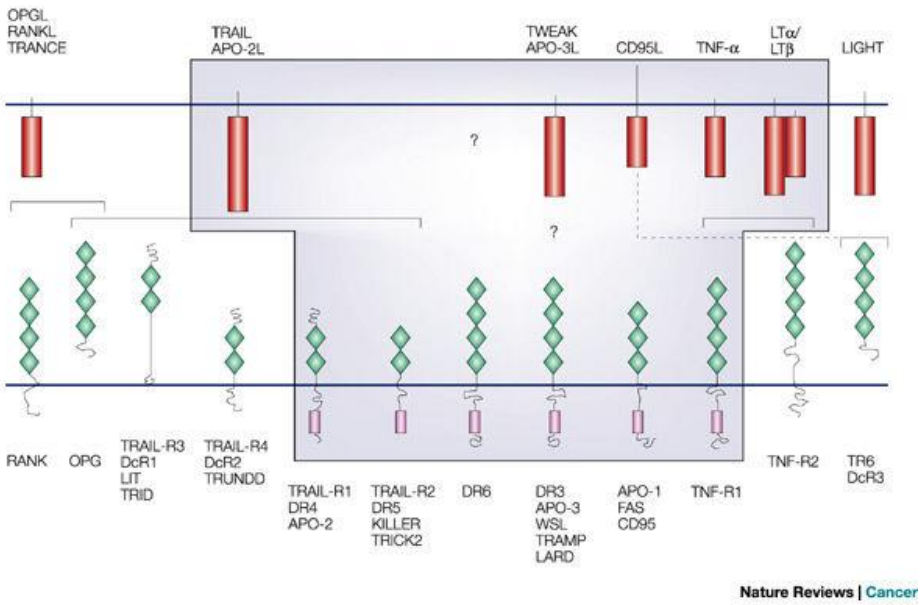


Figure 16. Death receptors and their respective death ligands.
(Igney & Krammer, 2002)

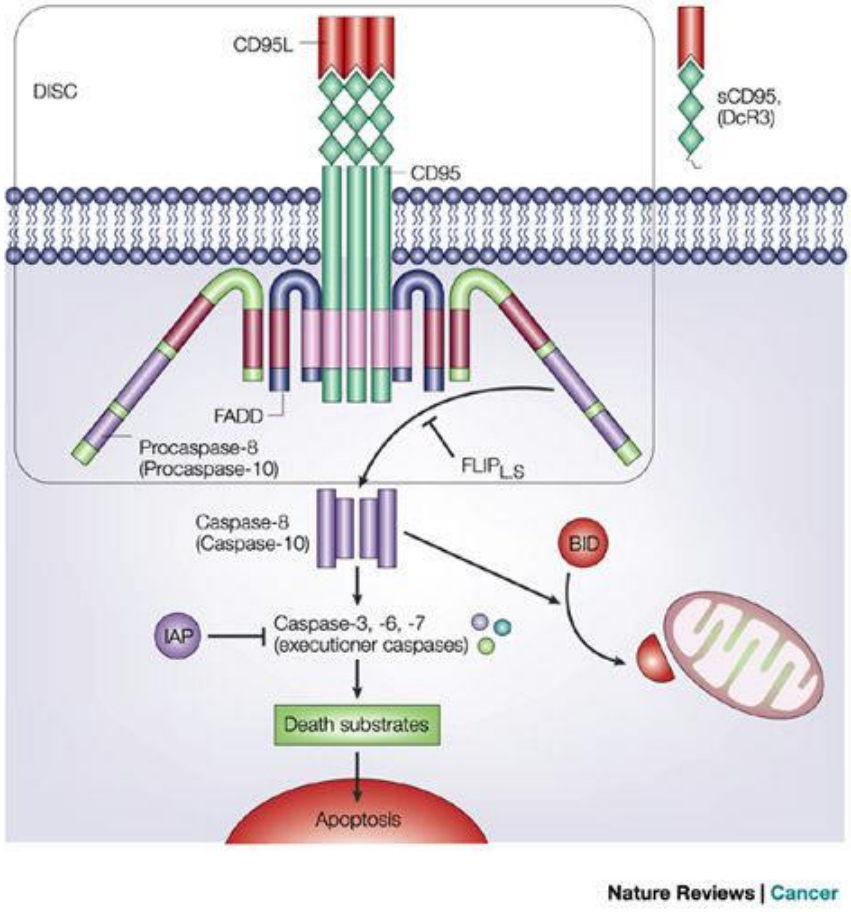


Figure 17. Initiation of death receptor mediated apoptosis.

An example of a death ligand (CD95L) binding to its receptor, recruiting Fas associated death domain protein and initiator caspase 8. This forms the death inducing signalling complex. Death-receptor-mediated apoptosis can be mediated by the competitive binding of Decoy receptor 3 or through inhibition by FLICE-inhibitory proteins (Igney & Krammer, 2002).

The intrinsic signalling pathways initiate apoptosis through the mitochondria (Figure 18). Intracellular signals can be positive or negative stimuli. Negative signals include the absence or down regulation of survival factors, growth factors, hormones, or molecules that would normally lead to suppression of apoptosis. Thus, the absence of these signals lead to initiation of apoptosis. Positive stimuli include cell damaging agents such as toxins, radiation, hypoxia, free radicals and viral infections (Igney & Krammer, 2002). Together these signals cause a change in mitochondria outer membrane potential (MOMP). Change in MOMP releases pro-apoptotic proteins normally kept in the intermembrane space of the mitochondria into the cytosol such as cytochrome c (Cyt c), second mitochondria-derived activator of caspase (SMAC) and direct inhibitors of apoptosis proteins binding protein with low pI (DIABLO). SMAC and DIABLO function to bind inhibitors of apoptosis proteins (IAPs) and drive apoptosis (Du et al., 2000; Verhagen et al., 2000). When cytochrome c is released it binds apoptotic protease activating factor 1 (Apaf-1) and together with ATP, recruits and activates intrinsic initiator caspase 9 forming the apoptosome. MOMP is mainly regulated by a family of pro and anti-apoptotic proteins belonging to the B cell lymphoma 2 (BCL-2) family (Green & Kroemer, 2004). The BCL-2 family of proteins can be subgrouped by function and structure according to their function and number of BCL-2 homology (BH) domains. The anti-apoptotic proteins with multiple homology domain proteins include BCL-2, BCL-XL, BCL-W, A1 and MCL-1. Pro-apoptotic multiple domain proteins consist of BAK, BAX, and BOK. Lastly, there are pro-apoptotic BCL-2 proteins that contain only BH3 homology, these proteins include BID, BIM, BIK, BMF, HRK, NOXA, PUMA and bNIP3 (Chipuk & Green, 2008).

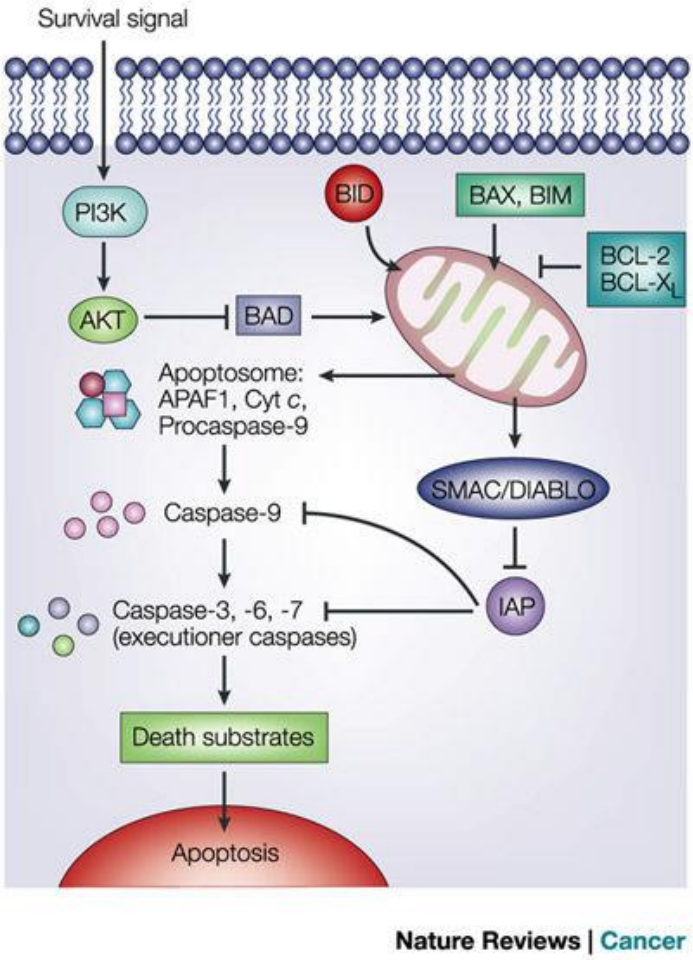


Figure 18. Initiation of mitochondrial apoptotic pathway.

Various stimuli signal pro-apoptotic BCL-2 proteins such as BAX, BID, BAD and BIM to activate mitochondrial release of cytochrome c (Cyt c) into the cytosol, where it binds apoptotic protease activating factor 1 (APAF1) and recruits and activates pro-caspase 9, forming the apoptosome. Apoptosis through the intrinsic pathway can be inhibited by survival signals, anti-apoptotic proteins, such as anti-apoptotic BCL2 family members, BCL-2 and BCL-XL and inhibitors of apoptosis proteins (IAPs), which are regulated by the proteins Second Mitochondria-derived Activator of Caspase (SMAC) and direct IAP binding protein with low pI (DIABLO) (Igney & Krammer, 2002).

Apoptosis can also be induced by extensive damage to cellular DNA (Figure 19). DNA damage has been shown to induce both extrinsic and intrinsic apoptosis (Roos & Kaina, 2013). When DNA damage occurs, the DNA repair pathway is normally initiated. The most prominent proteins involved in DNA damage detection and repair are: ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR). These proteins signal to checkpoint kinases 1 and 2 (CHK1, CHK2) and p53 proteins. Once these proteins are activated they phosphorylate and activate other proteins such as cell division cycle 25 (CDC25) which is a kinase involved in cell cycle regulation and p53 which induces expression of another cell cycle regulating protein p21 (Roos & Kaina, 2006). CDC25 and p21 are both S-phase checkpoint proteins and can induce cell cycle arrest where cellular resources can be directed towards repair of the damaged DNA. However, if the damaged DNA cannot be repaired, p53 can activate pro-apoptotic factors and induce apoptosis (Awasthi et al., 2015).

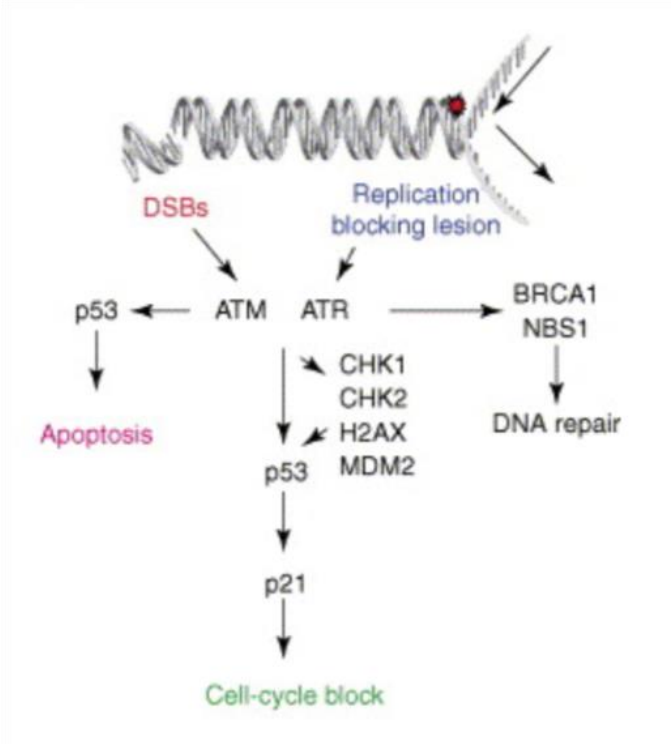


Figure 19. Activation of ATM or ATR in response to DNA damage.

ATM and ATR are proteins with DNA damage-binding and signalling activity. Depending on the downstream signalling targets of ATM and ATR, cells undergo apoptosis, DNA repair or cell-cycle arrest (Roos & Kaina, 2006).

Cell proliferation and the cell cycle

In order to replace damaged and dying cells, healthy cells need to grow and proliferate. The cell needs to replicate its DNA and divides into two daughter cells through a process called the cell cycle. The cell cycle consists of two main stages: 1) mitosis (M) separated into prophase, metaphase, anaphase, and telophase and 2) interphase separated into G0, G1, S, and G2. At the G1 phase cells decide whether or not they are going to divide. If conditions are suboptimal for cell growth cells withdraw from the cell cycle into the G0 phase until activated. However, if environmental conditions favour cell growth, then the cell progresses through all stages of the cell cycle and divides (Pardee et al., 1978). The cell cycle is tightly regulated and depends on many checkpoints and molecules to signal transition from each phase. E2F transcription factors are involved in controlling G1 to S phase progression. E2F transcription factors activate expression of a multitude of genes involved in DNA replication (Leone et al., 1998). The retinoblastoma protein family (pRB) is a negative regulator of E2F transcription factors and under suboptimal cell growth conditions, is able to inhibit cell cycle progression by binding to E2F. However, under optimal cell growth conditions pRB is inactivated by phosphorylation. This occurs through the activity of cyclin dependent kinases (CDKs). Activation of CDKs requires binding of regulatory subunits known as cyclins and also requires phosphorylation (Morgan, 1995). Cyclins are synthesized and destroyed at specific times during the cell cycle, therefore regulating CDK activity. Only a few CDKs are directly involved in driving the cell cycle. These include interphase CDKs: CDK2, CDK4 and CDK6, mitotic CDK1. CDKs form complexes with cyclins to drive cell proliferation. There are four different classes of cyclins: A, B, D, E that bind to cell cycle CDKs. The G1 – S phase transition involves CDK2, 4, and 6. Early in the G1 phase, CDK4 and 6 form complexes with Cyclin D and phosphorylate pRB and by doing so, free E2F transcription factors. CDK2 complexes with cyclin E and also phosphorylates pRB but also functions to recruit DNA helicases and polymerases for DNA replication (Massague, 2004). CDK activity is regulated by two families of inhibitors: INK4 proteins, including INK4A (p16), INK4B (p15), INK4C (p18) and INK4D (p19), and the Cip and Kip family, consisting of Cip1 (p21), Kip1 (p27) and Kip2 (p57). These proteins regulate cell cycle

progression by binding and inactivating cyclin and CDK proteins (Sherr & Roberts, 1999).

Cyclooxygenases

Cyclooxygenases (COX) comprise a family of enzymes which catalyse the formation of prostaglandins (PGs) from arachidonic acid. PGs are involved in cellular adhesion, growth ability and differentiation. There are two COX isoforms: COX-1 and COX-2. COX-1 is a constitutively-expressed enzyme and is responsible for prostaglandin production in most tissues. In contrast, COX-2 is an inducible enzyme, mostly expressed at sites of inflammation (Kirkby et al., 2012). Overexpression of COX-2 has been detected in a variety of tumours (Ristimaki et al., 2002; Secchiero et al., 2005). COX-2 has been shown to increase cell proliferation. Prostaglandin E2 is a major product of COX-2 which has been shown to increase cell proliferation and mediate apoptosis (Sheng et al., 1998). Prostaglandin E2 acts through different cell surface membrane receptors called EP receptors (EP1, EP2, EP3, and EP4) (Breyer et al., 2001). Prostaglandin E2 binding to EP2 and EP4 receptors are coupled with G proteins induce an increase of intracellular cAMP; cAMP is then able to activate cellular kinases such as protein kinase A (PKA) and phosphatidylinositol 3-kinases (PI3K); this is one pathway in which COX-2/ PGE2 increases cell proliferation (Choi et al., 2005).

PI3K/AKT

The phosphatidylinositol 3-kinases (PI3K)/AKT pathway regulates wide range of cellular processes involved in cell proliferation and growth (Figure 20). PI3K are members of a unique and conserved family of intracellular lipid kinases. These proteins bind and activate many intracellular signalling proteins. PI3K catalyses the formation of Phosphatidylinositol-3,4,5-trisphosphate (PIP3) in order to recruit a major downstream target, AKT, also known as protein kinase B (Stokoe et al., 1997). AKT is a serine/threonine kinase. There are three members of the AKT family: AKT1, AKT2 and AKT3, which are broadly expressed. AKT activation requires translocation to the plasma membrane and phosphorylation at Thr308 and Ser473.

Recruitment AKT to the plasma membrane is carried out by PIP3 while the necessary phosphorylation of AKT is carried out by 3-phosphoinositide-dependent protein kinase-1 and 2, (PDK1 and PDK2) (Stokoe et al., 1997). The PI3K/AKT pathway has been shown to increase cell proliferation by interacting with many proliferation and apoptotic proteins. For example, AKT targets mammalian target of rapamycin (mTOR) a protein known to drive cell proliferation (Nave et al., 1999). PI3K signalling also regulates angiogenesis and proliferation, by mechanisms including vascular endothelial growth factor (VEGF) transcriptional activation and hypoxia inducible factor-1α (HIF1α) (Skinner et al., 2004). AKT phosphorylates CDK inhibitors p21 and p27 resulting in their exclusion from the nucleus and subsequent degradation. With decreased levels of CDK inhibitors, then tightly controlled CDK-cyclin complexes are free to drive the cell cycle and proliferation.

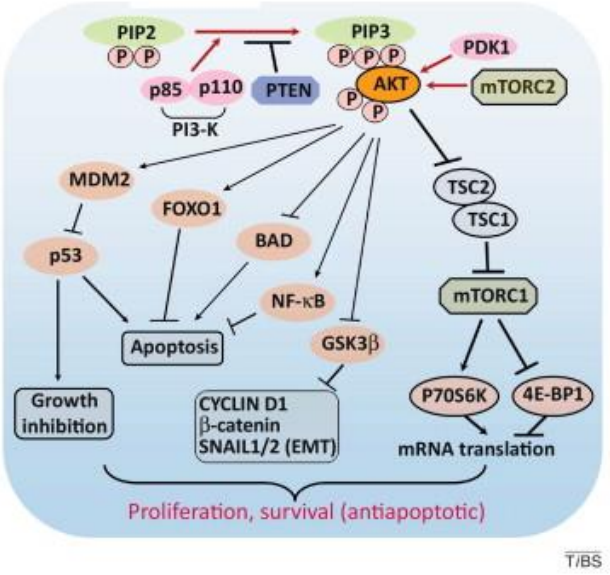


Figure 20. The phosphatidylinositol 3–kinase (PI3K) signalling pathway.
Activated receptor tyrosine kinases (RTKs) recruit and activate PI3K, leading to increased PIP3levels. PIP3 recruits many proteins to the membrane including AKT and activates AKT which phosphorylates a range of substrates. (Zhang, L. et al., 2013)

Summary and aims of this thesis

The majority of cervical cancer cases and a significant proportion of other cancers are attributed to HPV with HPV16 the most common type of HPV associated with cervical cancer (Li et al., 2011). The replicative helicase protein, E1 and the DNA binding protein E2, are required for viral genome replication, which is facilitated by the E1 protein following recruitment to the viral origin of replication by E2 (Frattini & Laimins, 1994)In addition, E2 acts as a transcriptional regulator for viral gene expression (Thierry, 2009). A hallmark of cervical cancer is viral genome integration into host cells which often results in the disruption of the HPV E2 ORF. Following integration, there is an increase in expression of the integrated viral genes which are no longer regulated by E2 (Moody & Laimins, 2010). The early viral genes often integrated into the host genome include E6, E7 and E1 (Woodman et al., 2007). The E6 protein of high risk HPV binds and degrades the tumour suppressor protein p53 which is a crucial genome maintenance protein and degradation of p53 causes cells to proliferate unchecked apoptosis is decreased (Howie et al., 2009). E7 binds to the retinoblastoma protein which is another tumour suppressor protein which acts to bind the transcription factor E2F causes E2F to be released and drive cell proliferation (Longworth & Laimins, 2004b). In addition to expression of E6 and E7 which decrease apoptosis and increase cell cycle activity, the E1 helicase may also promote DNA damage. Carcinogenesis is caused through the aberration of many pathways which includes inhibition of cell death, increase cell proliferation, and also DNA damage (Hanahan & Weinberg, 2011). Recently, studies have pointed that the E1 protein, which is responsible for the replication of HPV, could play a role in carcinogenesis (Castillo et al., 2014). HPV E1 has been shown to interact with various host proteins to facilitate viral replication and interference of cell proliferation pathways (Castillo et al., 2014) and DNA damage (Fradet-Turcotte et al., 2011). However, whether E1 plays a role in cancer development is still unknown. This study aims to determine whether HPV16 E1 is associated with cervical cancer progression and the functional roles in host cell pathways involved in carcinogenesis. In particular, it will:

- Quantitate E1 mRNA expression in a cohort of 124 HPV16 positive cervical samples.
- Determine the physical state of the HPV16 genome (i.e. episomal, intergrated or mixed) cervical samples.
- Establish if the HPV16 E1 virus in this cohort of Thai patients contains a previously reported 63bp duplication.
- Determine if expression of HPV16 E1 protein can induce proliferation or inhibit apoptosis of transfected cells.
- Determine if expression of HPV16 E1 affects host cell gene expression.

CHAPTER IV
MATERIAL AND METHODS

Part I. HPV16 E1 DNA and mRNA detection in clinical specimens

1. Clinical specimens and HPV DNA detection

Cervical samples in SurePath™ Liquid-based cytology system (BD, United States) were obtained from routine Pap smears at the Outpatient Department of Gynaecology, King Chulalongkorn Memorial Hospital, Bangkok, Thailand. The samples were from 2012 – 2014 and stored at -80°C. One mL of patient sample was used for HPV DNA detection at the Virology Unit, Department of Microbiology, King Chulalongkorn Memorial Hospital, Bangkok, Thailand by the Cobas 4800 high-risk HPV test (Roche, Switzerland). The use of left over specimens was approved by Institutional Review Board, Faculty of Medicine, Chulalongkorn University, a WHO certified ethics committee (COA No. 482/2015, IRB No. 298/58).

A total of 124 HPV16 positive samples were selected for this study. Pap smear results, i.e. normal, low/high-grade squamous intraepithelial lesion (LSIL/HSIL), and squamous cell carcinoma (SCC) and histological results from colposcopy (cervical intraepithelial neoplasia (CIN) 1, CIN 2/3 and SCC) were also obtained.

2. Cell Lines

The CaSki and SiHa cell lines were used in this study as HPV16 positive controls. CaSki cell contains approximately 600 copies of the HPV16 genome, whereas SiHa cell contains 1-2 copies of HPV16. The CaSki and SiHa cell lines were grown in Dulbecco's Modified Eagle's medium (DMEM) (GE Healthcare Life Sciences, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) and 100 units/ml penicillin and 100 µg/ml streptomycin (Gibco, USA) and incubated at 37 °C, 5% CO_2. The cells were passaged when 80-90% confluency was reached. In order to passage cells, the medium was removed, and the cells were washed twice with phosphate buffered saline (PBS) and trypsin-EDTA was added. The cells were then incubated at 37°C for 2-3 min. Growth medium was then added to the cells and the

cell suspension was mixed and subcultured at a ratio of 1:3 or 1:4 of the original cultures by adding an appropriate volume of culture medium to the flask.

3. Detection of HPV16 E1 DNA in Patient Samples

The presence of HPV16 E1 DNA in patient cervical samples was determined by PCR amplification of HPV16 E1 using the primer pair 1258f and 1404r (Integrated DNA Technologies, Singapore). The sequences of the primers were 5'-GCG GGT ATG GCA ATA CTG AA -3' and 5'- TAA CAC CCT CTC CCC CAC TT -3', respectively (Bogovac et al., 2011). Leftover nucleic acid extract from cervical samples was used as template DNA. Briefly, 10-50 ng of the template DNA, was mixed with the PCR master mix containing, 1X PCR buffer, 2.5 mM $MgCl_2$, 250 µM dNTP, 400 nM forward and reverse primers and 1.25 U high-fidelity grade DNA polymerase (Thermo Fisher Scientific, USA) using the following PCR conditions: initial denaturation at 95 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. PCR products were detected by 2% agarose gel electrophoresis stained with SYBR® Safe (Invitrogen, USA). The PCR products on the agarose gel were visualized by a UV transilluminator and photographed. The expected product size was 146 base pairs (bp).

4. 63 bp Duplication in HPV16 E1 genome

Because the primer pair used to detect the presence of HPV16 E1 also amplifies a region of interest in which a 63 bp duplication may occur, the presence of the duplication was simultaneously determined with the presence of the HPV16 E1 DNA. Wild-type samples yield a 146 bp product while samples containing the 63 bp duplication yield 209 bp. The PCR products were excised from the agarose gel and purified using a NucleoSpin PCR Cleanup and Gel Extraction kit (Macherey-Nagel, Germany). The amount and purity of purified DNA was measured using a biophotometer (Eppendorf, Germany). Samples exhibiting the 63 bp duplication phenotype were then sent for verification by Sanger sequencing (AIT Biotech, Singapore).

5. HPV16 physical state detection

PCR was used to determine the physical state of the HPV16 genome (episomal, integrated or mixed) by detecting a region of E2, which is deleted upon integration into the host genome. This was achieved using primers (Integrated DNA Technologies, Singapore) and PCR conditions as previously reported (Peitsaro et al., 2002). Briefly, 10-50 ng of the template DNA was mixed with the PCR master mix containing, 1X PCR buffer, 2.5 mM $MgCl_2$, 250 µM dNTP, 400 nM forward and reverse primers and 1.25 U high-fidelity grade DNA polymerase (Thermo Fisher Scientific, USA). Samples were amplified using the following PCR conditions: initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 55 °C for 1 min, 72 °C for 45 s and a final extension at 72 °C for 10 min. The amplified products of 82 bp (pure episomal/mixed form) or absence of a detectable PCR product (integrated form) were determined by 2% agarose gel electrophoresis.

In order to differentiate the physical state of the HPV16 genome into pure episomal, mixed and integrated form, quantitative real-time PCR (qPCR) detecting both E2 and E6 genes was performed using StepOnePlus Real-Time PCR System (Applied Biosystems, USA). External standard curves were determined for both E6 and E2 genes using full-length HPV16 plasmid, p1203 PML2d HPV16, a gift from Peter Howley (Addgene plasmid # 10869). Serial 10-fold dilutions containing 10^7 – 10^3 copies were amplified using the same primers as in the conventional PCR assay. In order to mimic mixed forms of the HPV16 genome, full-length HPV16 plasmid was mixed with SiHa DNA to create mixed forms with 20% - 80% integration (Nagao et al., 2002). The PCR reactions of E6 and E2 were run simultaneously in separate tubes under the same conditions as follows: 1X SsoAdvanced™ Universal SYBR® Green Supermix1X, 400 nM forward and reverse primers, and 10-50 ng of template DNA. PCR conditions were initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 sec, and 60 °C for 1 min. The ratio of E2/E6 copies was determined in each sample.

6. Quantification of HPV16 E1 mRNA expression in patient samples

6.1. RNA extraction:

Approximately 500 µL of clinical sample was centrifuged at 500g for 5 min. The cell pellet was washed once with PBS and suspended in PBS to a final volume of 200 µL. RNA extraction was performed using RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. The final elution volume was 40 µL. The amount and purity of the extracted RNA were determined using a biophotometer (Eppendorf, Germany).

6.2. Reverse transcription

Reverse transcription was performed according to manufacturer's protocol using Super Script IV (SSIV) (Invitrogen, USA). In order to synthesize cDNA, 1 µg of total RNA or a maximum of 11 µL from each patient sample was added to 2.5 µM Oligo d(T)20, and 0.5mM dNTP, and incubated at 65 °C for 5 min. The mixture was centrifuged to mix, and a final concentration of 200 U of reverse transcriptase, 40 U of RNaseOUT RNase Inhibitor, 100mM Dithiothreitol (DTT) and 1X of SSIV Buffer in a final volume of 20 µL was added and incubated at 50°C for 10 min. After that, the reaction was inactivated at 80°C for 10 min.

6.3. Droplet digital PCR (ddPCR)

In order to quantify HPV16 E1 mRNA expression in patient samples, cDNA from each patient was amplified using primers 1258f and 1404r (Integrated DNA Technologies, Singapore), as previously described (Bogovac et al., 2011), and quantitated by using a FAM-labelled TaqMan MGB probe 5' CCA TGT AGT CAG TAT AGT GG-3' FAM (Thermo Fisher Scientific, USA). β-actin mRNA levels were also determined using a primer assay set (catalogue no. Hs99999903_m1, Thermo Fisher Scientific, USA). The ddPCR reactions were performed according to the manufacturer's instructions. Briefly, 3 µL of sample cDNA was mixed with 900 nM (final concentration) of primers 1258f and 1404r, and 250nM of the E1 probe along with an 1x of the β-actin primer probe assay and

1x of ddPCR™ Supermix for Probes (Bio-Rad, USA) at a final volume of 20 µL. Samples were then emulsified with the Bio-Rad droplet generator oil using the QX200 droplet generator (Bio-Rad, USA). Droplets were then transferred to a 96-well PCR plate (Eppendorf, USA) and heat-sealed at 185 °C for 3 s. Amplification was carried out using a Bio-Rad C1000 Touch thermal cycler with the following parameters: initial denaturation at 95 °C for 10 min, followed by 40 cycles at 94 °C for 30 s, and 60 °C for 1 min, with a final step for enzyme inactivation at 98 °C for 10 min. The results were analysed by QuantaSoft software (Bio-Rad, USA). Only droplets above the minimum amplitude threshold level determined by a negative control were considered positive. The amount of HPV16 E1 mRNA was normalized to β-actin mRNA levels for each individual sample.

7. HPV16 methylation of early (p97) and late (p670) promoters by pyrosequencing

7.1. Preparation of bisulfite modified DNA

Leftover DNA extract from patient samples (1 µg) was bisulfite-treated following instructions from the EZ DNA-Gold Bisulfite Conversion Kit (Zymo Research, USA). Briefly, 130 µL of conversion reagent was added to 1 µg of DNA to a final volume of 150 µL. The mixture was then placed in a thermal cycler under the following conditions: 98 °C for 10 min, 64 °C for 2.5 h and 4 °C for 10 min. 600 µL M-Binding Buffer was then added to each column placed in collection tubes. Samples were then added to the column and mixed with the binding buffer before centrifugation at 10,000 g for 30 s. The flow-through was discarded and 100 µL of M-Wash Buffer was added to the column and centrifuged at 10,000g for s. Flow-through was again discarded and 200 µl of M-Desulphonation Buffer mixture was added to each column and incubated for 15 min. The columns were then centrifuged at 10,000 g for 30 s and washed again with 200 µl of M-Wash Buffer. Samples were then eluted with 20 µl of M-Elution Buffer and stored at – 20 °C. The bisulfite modified DNA was then used

to determine the methylation status of both the early (p97) and the late (p670) promoters.

7.2. PCR amplification

Bisulfite-modified HPV-16 DNA (4 µL) was amplified by PCR as follows: 1X PCR Buffer, 2.5 mM $MgCl_2$, 250 µM dNTP, 400 nM forward and reverse primers and 1.25 U high-fidelity grade DNA polymerase (Thermo Fisher Scientific, USA). The primers used were p97 Forward: 5'-TTG TAA AAT TGT ATA TGG GTG TG-3', Reverse: Biotin-5´-AAA TCC TAA AAC ATT ACA ATT CTC-3' (Rajeevan et al., 2006) (Integrated DNA Technologies, Singapore) and p670 Forward: 5'-TGG AAT AAT ATT AGA ATA GTA ATA TAA TAA A-3', Reverse: Biotin 5'-TTA TCC AAC TAA ACC ATC TAT TTC ATC C-3' (Integrated DNA Technologies, Singapore). PCR conditions were initial denaturation at 95 °C for 10 min, followed by 50 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min and a final extension at 72 °C for 10 min. PCR products were detected by 1.5% agarose and purified using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel).

7.3. Pyrosequencing

The purified products were denatured and mixed with 400 nM of sequencing primer and loaded into the PyroMark™ Q96 machine (Qiagen, Germany). Sequencing primers were: p97 5'-AAT TTA TGT ATA AAA TTA AGG G-3' (Rajeevan et al., 2006) and p670 5'-GTA AAG ATT TTA TAA TAT AAG GGG-3'(Integrated DNA Technologies, Singapore).

Part II. HPV16 E1 transfection in HEK 293T cells

1. Cell culture

Human embryonic kidney (HEK) 293T cell line was used in this study to carry out HPV16 E1 transfections. HEK 293T cells were grown in Dulbecco's Modified Eagle's Medium (DMEM), (GE Healthcare Life Sciences, USA) supplemented with 10% heat inactivated FBS (Gibco, USA), 100 unit/ml penicillin and 100 µg/ml streptomycin (Gibco, USA) and 1mM of sodium pyruvate (Gibco, USA) and incubated at 37°C, 5% CO_2. The cells were passaged when 80-90% confluency was reached. To passage cells, medium was removed, and the cells were washed twice with PBS and digested by trypsin-EDTA. The cells were then incubated at 37°C for 1-3 min. Growth medium was then added. Cells were resuspended and split at a ratio of 1:3 – 1:5, by addition of appropriate volumes of culture medium.

2. Plasmid construction

Plasmids containing codon optimized HPV16 E1 and its truncated forms were kindly provided by Dr. Seiichiro Mori (NIID, Japan). Truncated forms of E1 were generated by the addition of stop codons into the HPV16 E1 sequence. A total of 3 mutants were used in this study pEGFP-E1-184, pEGFP-E1-359 and pEGFP-E1-439, expressing amino acids 1 to 184, 1 to 359 and 1 to 439 of HPV16 E1, respectively. The primers used to amplify E1 were: forward using forward primer 5'- CTC CTC CTC GAG CTG CCG ACC CCG CTG GGA CG-3' and reverse 5'-GGT GGT GGT ACC CTA CAG TGT GTT GGT ATT TTG ACC-3'. The pEGFP-C1 vector (Figure 22) (Clontech, USA) was provided by Dr. Shankar Varadarajan (University of Liverpool, UK). The HPV16 E1 sequence as shown in Figure 21 was cloned into the pEGFP vector (Figure 23).

A) Amino acid sequence of HPV16E1

MADPAGTNGEEGTGCNGWFYVEAVVEKKTGDAISDDENENDSDTGEDLVDFIVNDNDYLT
QAETETAHALFTAQEAKQHRDAVQVLKRKYLGSPLSDISGCVDNNISPRLKAICIEKQSR
AAKRRLFESEDSGYGNTEVETQQMLQVEGRHETETPCSQYSGGSGGGCSQYSSGSGGEGV
SERHTICQTPLTNILNVLKTSNAKAAMLAKFKELYGVSFSELVRPFKSNKSTCCDWCIAA
FGLTPSIADSIKTLLQQYCLYLHIQSLACSWGMVVLLLVRYKCGKNRETIEKLLSKLLCV
SPMCMMIEPPKLRSTAAALYWYKTGISNISEVYGDTPEWIQRQTVLQHSFNDCTFELSQM
VQWAYDNDIVDDSEIAYKYAQLADTNSNASAFLKSNSQAKIVKDCATMCRHYKRAEKKQM
SMSQWIKYRCDRVDDGGDWKQIVMFLRYQGVEFMSFLTALKRFLQGIPKKNCILLYGAAN
TGKSLFGMSLMKFLQGSVICFVNSKSHFWLQPLADAKIGMLDDATVPCWNYIDDNLRNAL
DGNLVSMDVKHRPLVQLKCPPLLITSNINAGTDSRWPYLHNRLVVFTFPNEFPFDENGNP
VYELNDKNWKSFFSRTWSRLSLHEDEDKENDGDSLPTFKCVSGQNTNTL

B) Nucleotide sequence of HPV16E1

ATGGCCGACCCCGCTGGGACGAATGGGGAAGAAGGGACGGGGTGCAATGGCTGGTTC
TACGTCGAGGCGGTTGTGGAAAAAAAAACCGGCGATGCAATATCCGACGACGAAAAC
GAGAATGATTCAGACACAGGCGAAGACCTCGTCGATTTTATTGTTAACGATAATGAT
TATCTGACCCAAGCAGAAACAGAGACAGCCCACGCGCTTTTTACGGCCCAAGAGGCC
AAACAGCACAGAGATGCCGTGCAAGTGCTTAAGCGCAAATATCTCGGGTCACCATTG
TCCGATATATCAGGCTGTGTAGACAATAACATAAGCCCTAGACTTAAGGCAATCTGC
ATAGAAAAGCAAAGTCGCGCCGCCAAACGCAGGCTGTTCGAAAGCGAGGACTCCGGC
TACGGGAACACCGAGGTCGAGACCCAGCAGATGCTGCAGGTTGAAGGGCGCCATGAG
ACTGAGACTCCATGTAGTCAATATAGCGGAGGCTCAGGAGGCGGTTGCTCTCAGTAC
TCCTCAGGTAGCGGAGGGGAGGGAGTGTCAGAGCGGCACACCATCTGTCAGACTCCC
CTGACCAATATTCTTAACGTGCTTAAAACCTCTAACGCGAAGGCAGCCATGCTTGCT
AAATTCAAGGAGCTGTATGGAGTTAGCTTCTCAGAACTGGTTAGACCATTTAAAAGT
AACAAATCCACGTGCTGCGATTGGTGTATAGCAGCCTTCGGCCTCACTCCTTCAATC
GCCGATAGCATTAAAACACTCCTGCAACAGTATTGCCTCTATCTCCACATCCAGAGC
CTTGCTTGCAGTTGGGGGATGGTCGTGTTGCTTCTTGTGCGCTATAAGTGCGGCAAG
AACCGGGAGACGATTGAAAAACTTCTGAGCAAGCTTCTTTGCGTATCCCCAATGTGC
ATGATGATCGAGCCACCCAAGCTCCGGTCCACTGCTGCAGCGCTGTACTGGTATAAA
ACAGGTATAAGTAACATCTCCGAGGTTTACGGCGACACTCCCGAGTGGATCCAAAGA
CAGACTGTTCTTCAGCATTCCTTTAACGATTGCACGTTTGAACTGAGCCAGATGGTG
CAGTGGGCTTATGACAACGACATCGTTGATGACAGCGAAATTGCCTACAAGTACGCA
CAGTTGGCCGATACAAATAGCAATGCCAGTGCATTTCTTAAGAGTAATTCACAGGCG
AAAATTGTCAAGGACTGCGCTACAATGTGCCGACATTATAAGCGCGCAGAGAAGAAG
CAGATGAGCATGTCTCAGTGGATCAAGTATAGGTGTGACAGAGTTGATGATGGAGGA
GATTGGAAGCAGATTGTTATGTTCCTGCGATACCAGGGCGTGGAATTTATGTCTTTT
TTGACTGCATTGAAACGCTTTCTGCAGGGAATTCCCAAAAAAAATTGTATCCTCCTG
TACGGGGCGGCTAACACTGGCAAGTCCCTCTTTGGCATGTCACTGATGAAGTTCCTG
CAAGGAAGCGTCATCTGCTTCGTCAATTCAAAAAGTCACTTTTGGTTGCAGCCTCTC
GCAGATGCAAAAATCGGAATGCTCGACGATGCCACAGTGCCATGTTGGAATTATATC
GATGATAATCTGCGGAACGCTCTGGATGGTAATCTCGTCTCCATGGATGTGAAGCAT
CGCCCGCTGGTACAGCTCAAATGCCCTCCCTTGCTCATAACTTCCAACATCAATGCC
GGCACCGACTCCCGCTGGCCGTATTTGCATAACAGGCTTGTGGTCTTTACCTTCCCC
AATGAGTTTCCATTCGACGAGAATGGGAATCCCGTGTACGAACTGAACGACAAGAAT
TGGAAGTCTTTCTTCAGCAGGACTTGGTCACGGCTGTCTCTGCACGAGGACGAGGAC
AAAGAGAACGACGGTGATTCCCTCCCTACCTTCAAATGCGTATCTGGTCAAAATACC
AACACACTGTAG

Figure 21. HPV16 E1 sequence used to construct pEGFP-E1.

A) amino acid sequence B) nucleotide sequence.

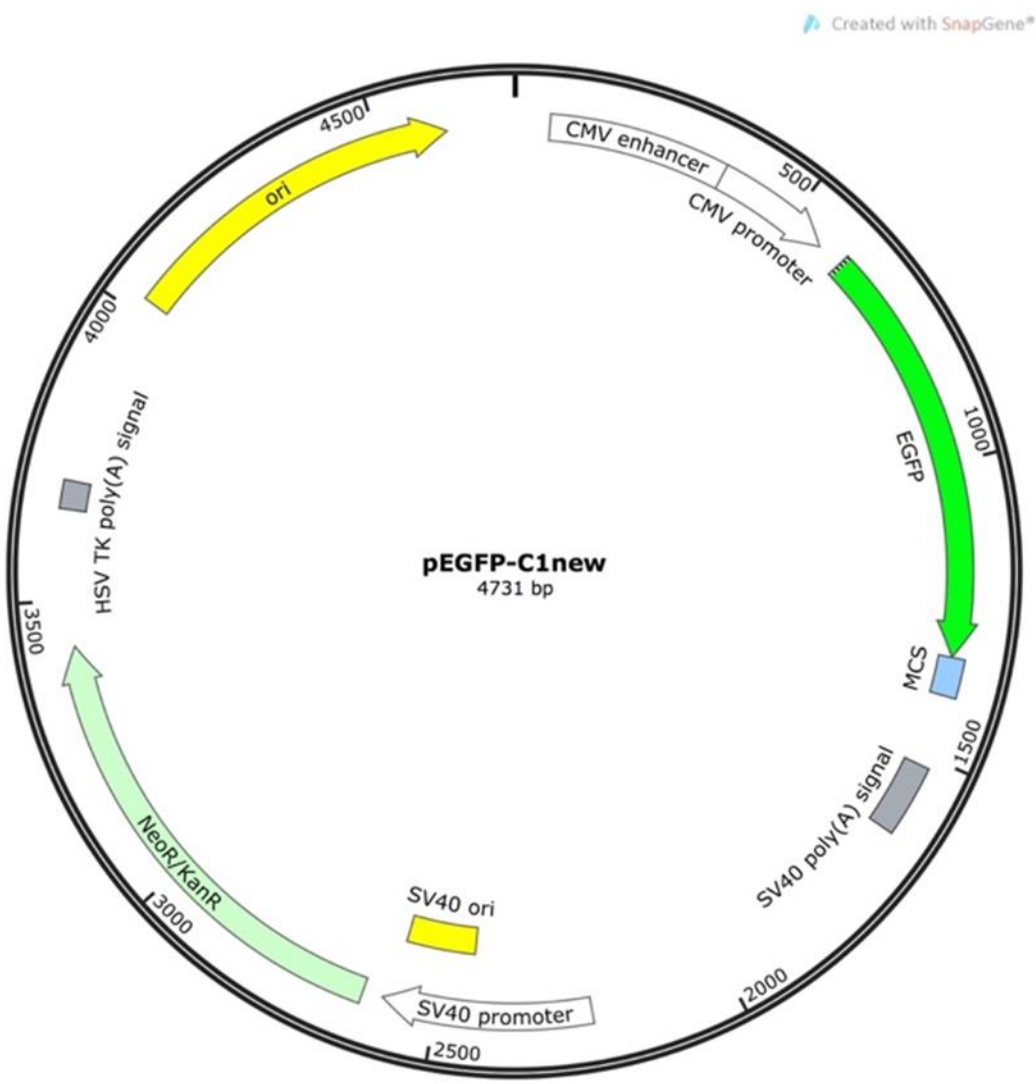


Figure 22. Schematic of pEGFP-C1 vector control plasmid.

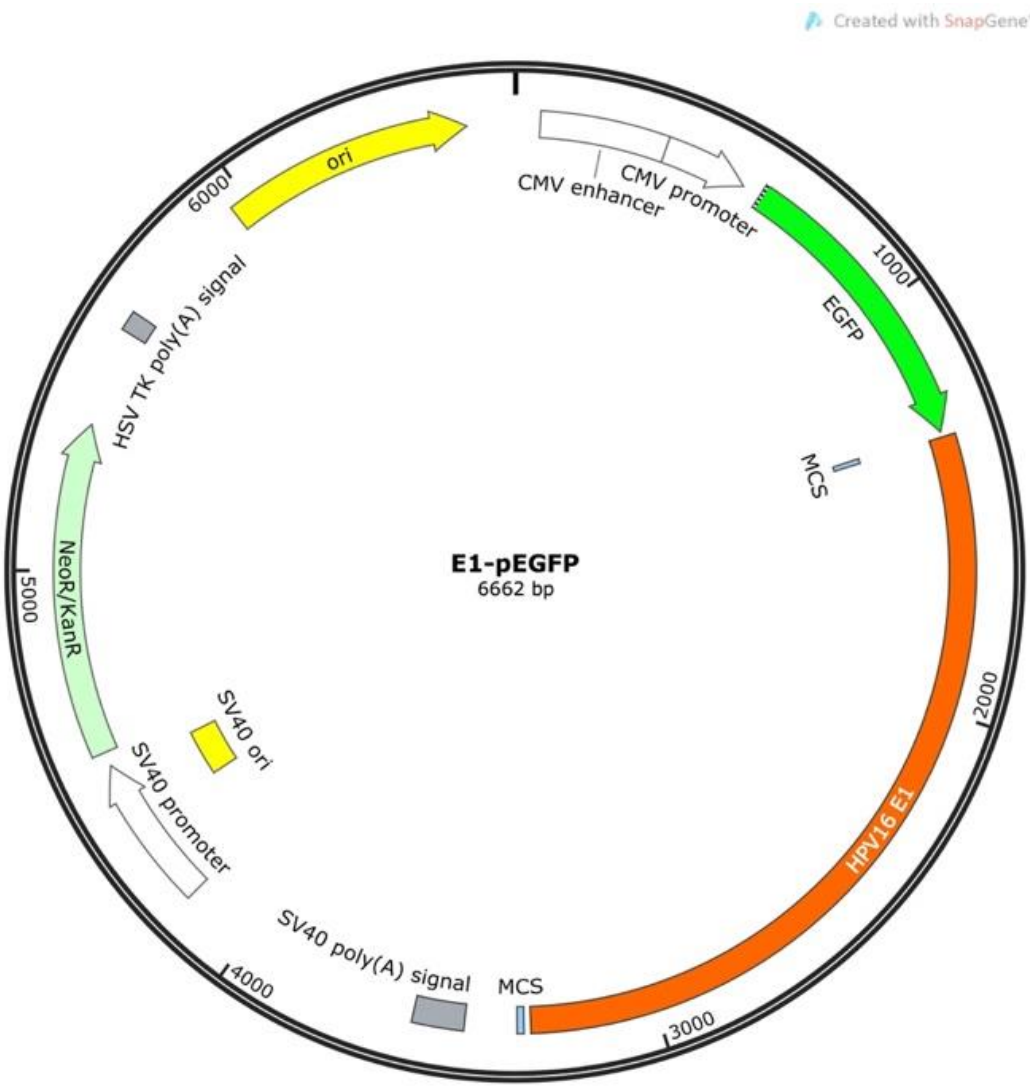


Figure 23. Schematic of inserted HPV16 E1 sequence in pEGFP-C1 plasmid.

3. Plasmid extraction

After the colonies were grown to the desired OD 600 (0.6-0.8), plasmid extraction was performed according to manufacturer's protocol using either NucleoSpin® Plasmid miniprep (Machery-Nagel, Germany) or NucleoBond® Xtra midiprep (Machery-Nagel, Germany) depending on the volume of bacterial culture. In brief, bacteria containing the desired plasmid were pelleted at 5000 g for 15 min. Resuspension buffer was added to the pellet and vortexed to ensure thorough mixing. Lysis buffer was added and mixed gently to ensure complete lysis while protecting the DNA from being damaged. Neutralization buffer was added and mixed gently. To clear the lysate, the solution was centrifuged (11,000 g) for 5 minutes. The supernatant was collected and applied to a NucleoSpin® Plasmid column, binding the DNA to the column. The column was then either centrifuged or allowed to elute by gravity flow. The flow through was discarded and the column was washed once with wash buffer. The plasmid DNA was then eluted and precipitated with isopropanol and centrifugation (15,000 g for 15 min). The precipitate was then washed with 70% ethanol and centrifuged again (15,000 g for 15 min). The pellet was resuspended in 10 mMTris-HCl, pH 8.5 for downstream use.

4. HPV16 E1 transfection and detection

HEK 293T cells were transfected with either pEGFP vector control plasmid or pEGFP-E1 plasmid using X-tremeGENE HP DNA Transfection reagent (Roche, USA). Transfection was performed according to manufacturer's protocol. Complexes were prepared in Opti-MEM™ I Reduced Serum Media (Gibco, USA). A 1:3 ratio of plasmid to transfection reagent was used for all experiments at a concentration of 1 µg plasmid/100 µL Opti-MEM™. GFP positive cells were detected using fluorescence microscopy and flow cytometry.

5. Cell proliferation assay by viable cell count

HEK 293T cells were seeded at a density of $5x10^4$ cells/mL of growth medium into each well of 24-well plate. After 24 h, cells were transfected with 0.25 µg of either pEGFP or pEGFP-E1. At 12, 24, 36, and 48 h post-transfection, cells were

harvested and stained with 0.4% trypan blue (Sigma-Aldrich, USA). Viable and non-viable cells were counted using a hemocytometer counting chamber. Two independent experiments with triplicate wells were performed.

6. Cell proliferation assay using CountBright™ Absolute Counting Beads

CountBright™ Absolute Counting Beads (Invitrogen, USA) are a calibrated suspension of microspheres approximately 7 µm in diameter that fluoresce brightly and have a wide range of excitation and emission wavelengths (UV to 635 nm excitation and 385-800 nm emission). Using flow cytometry, the microspheres can be separated from cells by either scatter or fluorescence threshold ratio of microspheres to cells in a known volume, which can then be used to calculate the absolute number of cells in a sample. HEK 293T cells were seeded at a density of $5x10^4$ cells/mL of growth medium into each well of 24-well plate. After 24 h, the cells were transfected with either pEGFP or pEGFP-E1. At 12, 24, 36, and 48 h post-transfection, the cells were harvested and 10µL of CountBright™ Absolute Counting Beads were added to each sample. Final cell count was determined by AxC/B where A = number of cell events, B = number of bead events and C = assigned bead count of the lot (beads/10µL).

7. Cell proliferation assay using TetraZ™ Cell Proliferation Kit

In order to quantify cellular proliferation, TetraZ™ Cell Counting Kit (BioLegend, USA) was used. The TetraZ™ Cell Counting solution is based on the dehydrogenase activity of viable cells. Upon addition of the TetraZ™ Cell Counting solution, viable cells produce a water soluble dye. Because the amount of dye produced is proportional to the amount of viable cells, cell proliferation can be determined by measuring absorbance. The transfection of either control plasmid or HPV16 E1 plasmid into the HEK 293T cells was performed as previously described. Twelve hours post-transfection, the culture medium was removed and replenished with new medium. At 12, 24, 36, and 48 h post-transfection, 10µL of TetraZ™ solution was added to each well. The cells were then placed in a 37°C, 5% CO_2

incubator for 2 h. Absorbance was measured at 450nm using a microplate reader (Perkin Elmer).

8. Cell viability assay using Zombie Yellow™ Fixable Viability Kit

To determine the cytotoxicity of E1 transfected cells, Zombie Yellow™ Fixable Viability Kit (BioLegend, USA) was used, which is an amine-reactive fluorescent dye that is permanent to cells with compromised membranes but non-permanent to live cells. The transfection of either control plasmid or HPV16 E1 plasmid into the HEK 293T cells was performed as previously described. The Zombie Yellow™ dye was diluted at a ratio of 1:100 in PBS. At 24, 48, and 72 h post-transfection, the cells were harvested, washed in PBS and resuspended with 100 µL of diluted Zombie Yellow™ solution. Cells were incubated at room temperature, in the dark, for 15 min. After incubation, the cells were washed and resuspended in PBS containing 2% FBS and then analysed by flow cytometry (BD FACSAria™ II). Two independent experiments with triplicate wells were performed.

9. Apoptosis

Apoptosis occurs normally in cells as a mechanism of programmed cell death. However, apoptosis can also occur as a defence mechanism when cells are under stress. A hallmark characteristic of apoptosis is the translocation of the phospholipid phosphatidylserine (PS) which is normally located in the inner plasma membrane leaflet of healthy cells to the outer membrane leaflet (Jacobs et al., 2012). The anticoagulant, annexin V is able to preferentially bind phosphatidylserine (Koopman et al., 1994). Therefore, APC-conjugated annexin V was used to quantitate apoptotic cells in this study. Propidium iodide is a nucleic acid-binding dye that is excluded from live cells but is permeable to late apoptotic and necrotic cells. In order to determine the extent of apoptosis in cells transfected with either pEGFP or pEGFP-E1, the transfected HEK 293T cells were harvested at 24, 48, and 72 h post-transfection and washed with PBS. Cells were resuspended in 100 µL of binding buffer (140 mM NaCl, 4 mM KCl, 0.75 mM $MgCl_2$, 10 mM HEPES and 2.5 mM CaCl2, pH 7.4) and stained with 5 µL of APC-conjugated annexin V (Biolegend,

USA) in the dark at room temperature for 15 min. Propidium iodide (final concentration 2.5 µg/mL) (Biolegend, USA) was added to the cells and incubated for 5 min at room temperature in the dark. Apoptosis and cell death were analysed by flow cytometry (BD FACSAria™ II). For confocal imaging of apoptosis, cells transfected with pEGFP were incubated with 5 µL of APC-conjugated annexin V for 15 min and imaged (Olympus FV 3000).

In order to confirm E1 induced apoptosis, QVD-OPH, a broad spectrum caspase inhibitor was used to block apoptosis (Caserta et al., 2003). HEK 293T cells in 24-well plate were treated with 2.5µM Q-VD-OPH (Sigma Aldrich, USA). After 24 h treatment, the cells were transfected with either pEGFP or pEGFP-E1. At 48 h post-transfection, cells were harvested and stained with Annexin V and PI, using the same protocol as previously described above. Apoptosis and cell death were analysed by flow cytometry (BD FACSAria™ II).

10. Microarray

HEK 293T cells were seeded ($2.0x10^6$ cells in 10 mL growth medium) into T75 flasks, and after 24 h, cells were transfected with either pEGFP or pEGFP-E1. Forty-eight hours after transfection, the cells were trypsinized. The GFP-positive cells were sorted by flow cytometry (BD FACSAria™ II). DNA, RNA, and protein were extracted from the sorted cells using NucleoSpin® TriPrep (Macherey-Nagel, Germany). Extracted RNA was then precipitated using 0.3M sodium acetate and 1 volume of isopropanol. The solution was chilled at -20°C for at least 1 h. The pellet was centrifuged for 15 min (15,000 g, 4 °C), washed with 70% ethanol and centrifuged again for 15 min (15,000 g, 4 °C). Precipitated RNA was then sent for microarray analysis (Illumina Human HT-12 platform) (Macrogen, Republic of Korea).

Bioinformatics analysis was performed by Macrogen. Significant difference in gene expression between pEGFP and pEGFP-E1 transfected cells was set at a difference of 2-fold.

11. Prime PCR

RNA was extracted from GFP-positive cells using NucleoSpin® RNA Plus (Macherey-Nagel, Germany). Extracted RNA (3 µg) was reverse transcribed using Super Script IV (Invitrogen, USA) according to manufacturer's protocol. The cDNA from pEGFP or pEGFP-E1 transfected cells was amplified by real-time PCR using PrimePCR™ 96 well as follows: 10µL of 2x SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, USA), 1µL of cDNA template, and 9µL of Nuclease-free H_2O for a final volume of 20µL. The PCR reaction was as follows: initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 sec. GAPDH was used as a reference gene. Gene expression analysis was calculated using the $2^{-\Delta\Delta CT}$ method.

CHAPTER V
RESULTS

Part I HPV16 E1 DNA and mRNA detection in clinical specimens

1. Patient demographic data

One-hundred and twenty-four HPV16 positive samples were used in this study. The samples were categorized using cytology (Pap smear) and histology (colposcopy) results. Samples positive for HPV16 with no abnormal cytological features were recruited as the "Normal" group. Colposcopy was performed in all patients with abnormal Pap smear results. Pap smear results perfectly correlated with colposcopy results, i.e., low grade intraepithelial lesion (LSIL) = CIN 1, high grade intraepithelial lesion (HSIL) = CIN 2/3, and SCC. From this information, this study presents categorized 124 samples as: Normal (n=22), CIN 1 (n=38), CIN 2/3 (n=27), and SCC (n=37). Mean age (years)±SD (range) for each group were: Normal; 44.35±8.03 (29-55), CIN I; 44.35±12.50 (16-56), CIN 2/3; 39.37±10.43 (21-57), and SCC; 51.45±14.89 (28-78), respectively.

2. HPV16 physical state

The ability of the HPV to cause cancer has been previously correlated with the physical state of the viral genome, categorized into: pure episomal, integrated, and mixed forms (Pirami et al., 1997; Williams et al., 2011). Viral genome integration often results in the disruption of the E2 gene, which leads to overexpression of the viral oncogenes E6 and E7 (Jeon et al., 1995), significantly increasing the pathogenicity of HPV due to the function of these known oncoproteins (Burke et al., 2012; Burke et al., 2014; Munger et al., 1992). In this study, the region of E2 that is deleted upon viral integration into the host genome was determined by PCR, and 111 out of 124 samples (89.5%) from different clinical stages were successfully amplified. Of these, integration of the viral genome was observed in 31 samples (28%), i.e.,

Normal 7/19 (37%), CIN 1 10/34 (29%), CIN2/3 6/24 (25%) and SCC 8/34 (24%) whereas the rest (72%) exhibited a mixed or pure episomal form.

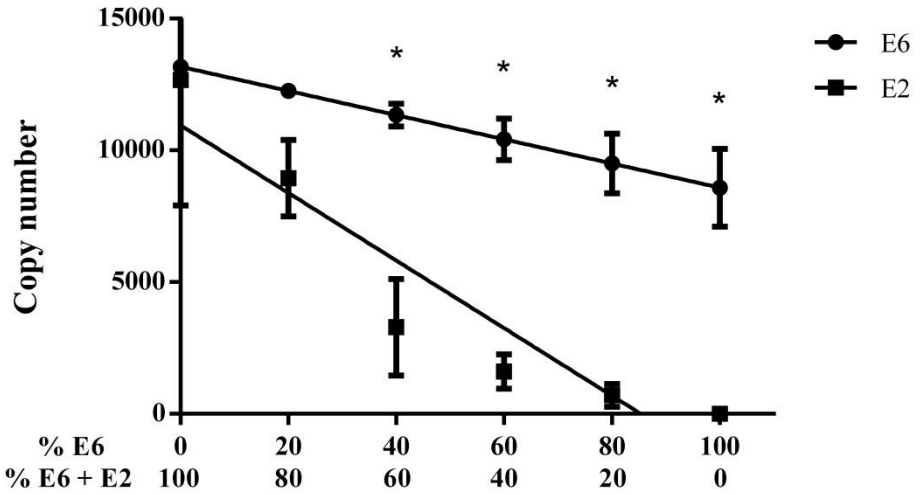


Figure 24. Establishment of a cut-off value for HPV16 physical state of episomal and mixed forms using HPV16 E2 and E6 genes.

Full-length HPV16 plasmid DNA was mixed with SiHa DNA to produce mixed forms of HPV16 episomal and integrated DNA. These forms represented episomal, integrated and mixed forms ranging from 20 to 80% integration. Differences in E2 and E6 copy number was determined by unpaired t-test. Bars indicate standard error and * indicates p-values < 0.05.

To distinguish the episomal from mixed form, we performed quantitative real-time PCR (qPCR) in 39 samples. To set a cut-off point between mixed and episomal forms, full-length plasmid DNA was mixed with 20-80% of SiHa DNA (absence of E2 gene) (Figure 24). Of the 39 samples, integrated, mixed and episomal forms were observed in 18%, 28%, and 54%, respectively (Table 2). From the qPCR results, the episomal form was observed in 33% of Normal, 88% of CIN 1, 83% of CIN 2/3 and 37% of SCC patients. Three discrepancies (integrated to mixed form) were found.

Table 2. Physical state determined by PCR and quantitative real-time PCR (qPCR).

Method	Physical State	NORMAL	CIN 1	CIN 2/3	SCC	Total
PCR	MIXED	3 (50%)	7 (88%)	6 (100%)	13 (68%)	74%
	INTEGRATED	3 (50%)	1 (12%)	0	6 (32%)	26%
qPCR	EPISOMAL	2 (33%)	7 (88%)	5 (83%)	7 (37%)	54%
	MIXED	2 (33%)	1 (12%)	1 (17%)	7 (37%)	28%
	INTEGRATED	2 (33%)	0	0	5 (26%)	18%
	DISCORDANCE (N)	1	1	0	1	

3. HPV16 E1 induces apoptosis and necrosis

It has been established that E1 is essential to the initiation of viral replication and could potentially instigate carcinogenesis (Castillo et al., 2014). Consequently, the correlation between E1 expression and carcinogenesis was first investigated. E1 mRNA expression was quantitatively determined and normalized to β-actin for each sample by Droplet Digital PCR (ddPCR) (Figure 25, Figure 26, and Figure 27). To establish the cut-off for E1 positive droplets, cDNA from the CaSki cell line was used (Figure 25). After E1 amplification, positive droplets were calculated for both E1 and β-actin.

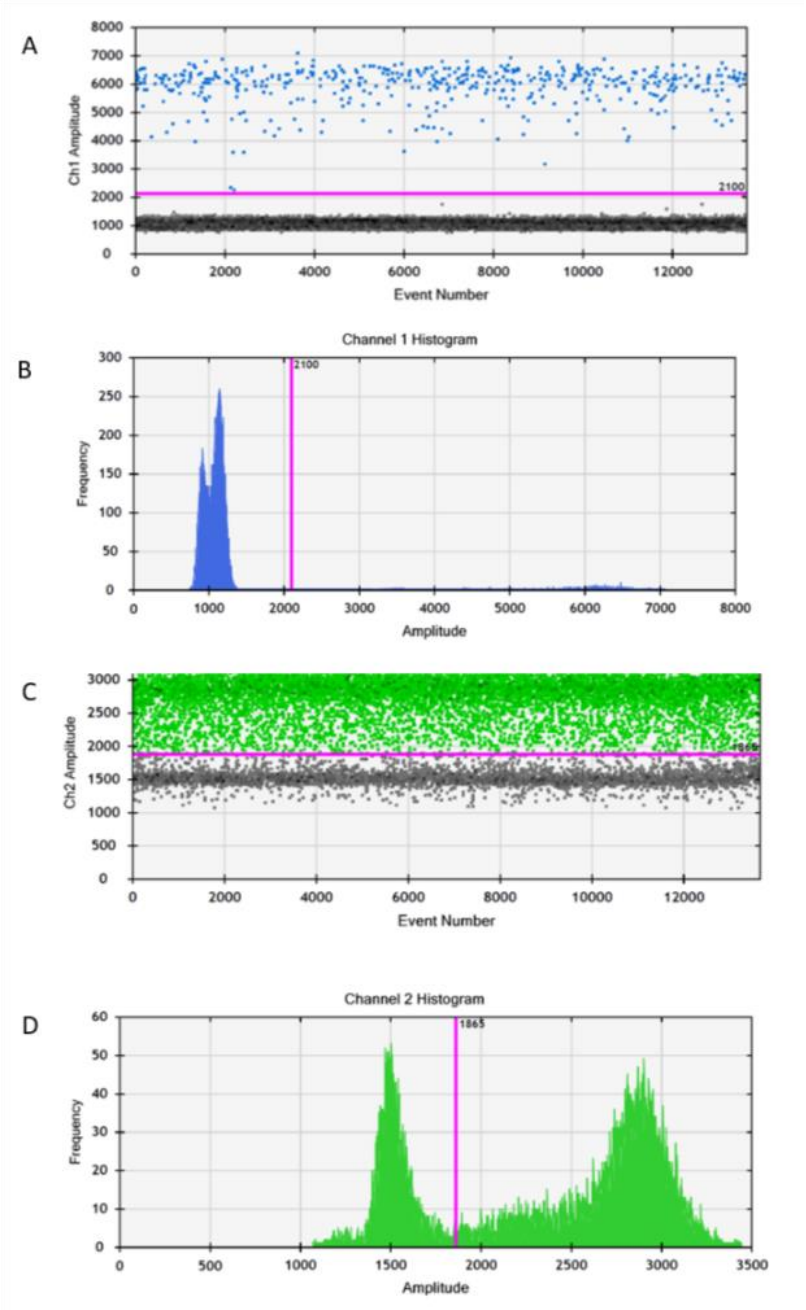


Figure 25. Graphical representation of amplified SiHa cDNA used for quantification of E1 and β-actin mRNAs by ddPCR.

A) Individual droplet amplitude of E1 mRNA B) Histogram of E1 mRNA C) Individual droplet amplitude of β-actin and D) Histogram of β-actin.

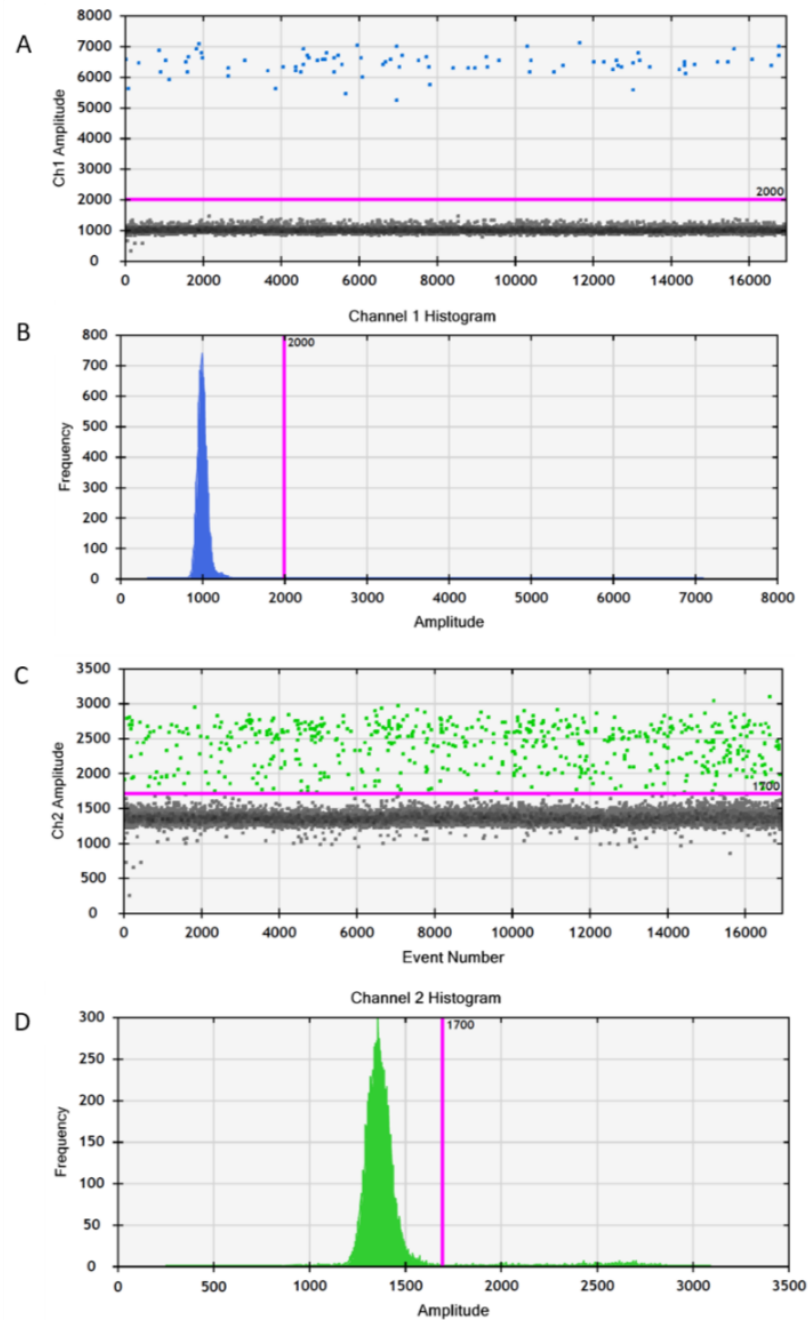


Figure 26. Graphical representation of amplified CaSki cDNA used for quantification of E1 and β-actin mRNAs by ddPCR.

A) Individual droplet amplitude of E1 mRNA B) Histogram of E1 mRNA C) Individual droplet amplitude of β-actin and D) Histogram of β-actin

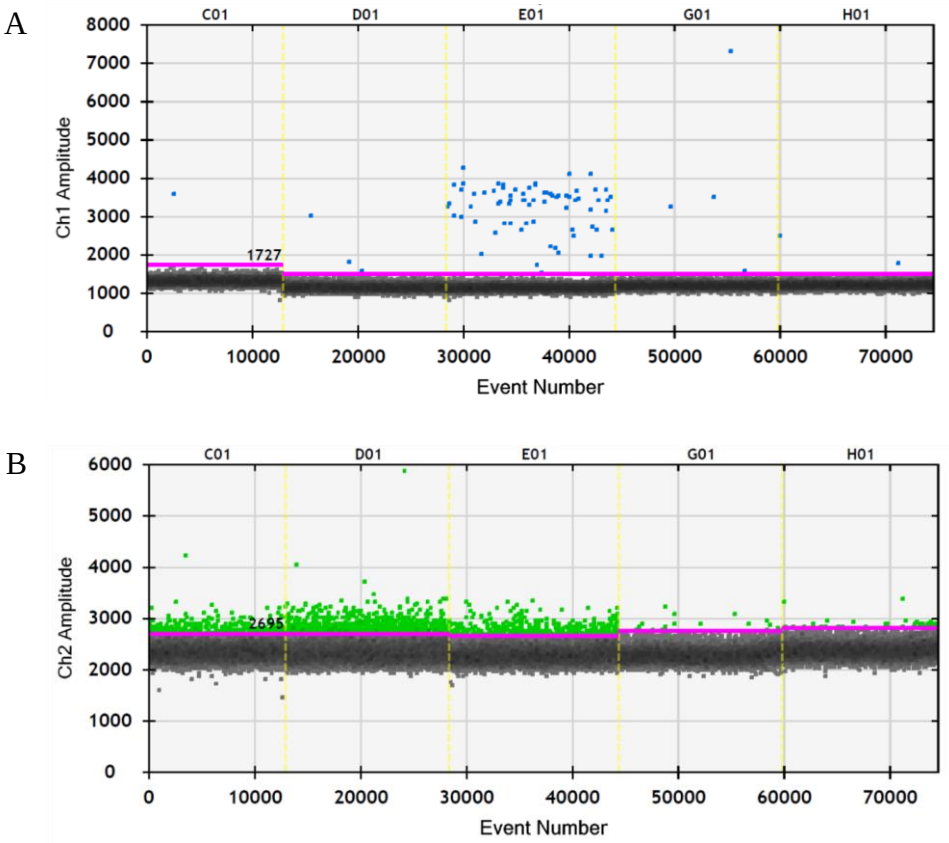


Figure 27. Graphical representation of amplified patient sample cDNA used for quantification of E1 and β-actin mRNAs by ddPCR.

A) Individual droplet amplitude of E1 mRNA B) Individual droplet amplitude of β-actin.

Of the 39 samples analysed, the mean relative E1 mRNA expression levels ±SE (range) were: Normal (n=5), 0.18±0.06 (0.01-0.33); CIN 1 (n=9), 0.42±0.07 (0.11-0.70); CIN 2/3 (n=7), 0.65±0.10 (0.29-1.10); and SCC (n=18), 0.79±0.12 (0.04-1.60), respectively (Figure 28). Relative E1 mRNA expression levels in CaSki and SiHa cervical cancer cell lines were 0.13 and 0.06, respectively. To determine if E1 expression was constant or changed throughout all clinical stages, the mean E1 mRNA level of all samples (0.60) was set as a cut-off point. The results revealed that the percentage of samples with E1 mRNA expression above the cut-off point were 0.0% (Normal), 25.0% (CIN 1), 71.4% (CIN 2/3), and 58.8% (SCC) indicating that E1 mRNA expression increased in relation to disease stage. A significant increase in E1 expression was found between SCC samples and normal samples (p-value = 0.014) and CIN 2/3 and normal samples (p-value = 0.003) (Figure 28). A significant positive correlation between E1 expression and clinical stage was therefore demonstrated (r = 0.661, p-value = 0.019).

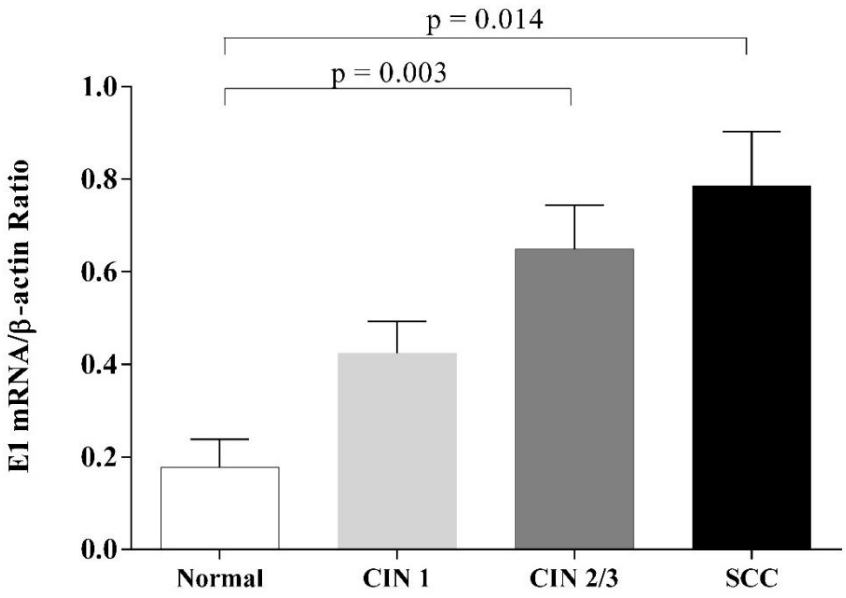


Figure 28. E1 mRNA expression increases with progression of cervical carcinoma.

Total cellular RNA was extracted from cervical samples, reverse transcribed and analysed by ddPCR. E1 expression was determined relative to clinical stage of each patient. E1 expression increases significantly with disease progression.

4. E1 expression is not related to physical state of the virus

In order to determine if E1 expression was related to the physical state of the virus, E1 mRNA expression was compared to HPV16 physical state determined by both PCR and qPCR. For PCR, no significant difference was found for E1 expression: 0.73±0.20 (mean ± SE) for integrated HPV and 0.65±0.08 for mixed/pure episomal form (p-value = 0.76). Similarly, qPCR showed no difference (p-value = 0.487) among episomal (0.97± 0.25), mixed (0.30±0.15) and integrated (0.74±0.19) (Figure 29)These results indicate that physical state may not be a crucial factor in regulating E1 expression or there were other underlying factors that correlated to the dynamic integration pattern of HPV16.

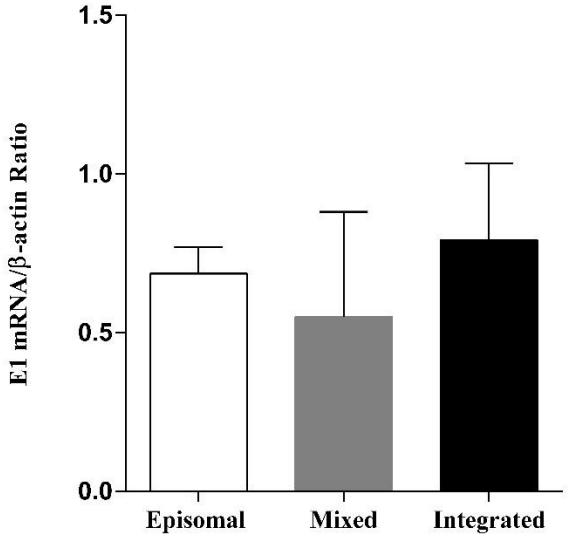


Figure 29. Physical state of the viral genome (episomal, mixed or integrated) was determined by qPCR.

E1 expression was compared to viral genome physical state. Data is presented as the ratio between E1 mRNA and β-actin. Bars represent the standard error.

5. Methylation of E2BS is associated with SCC

Gene expression is regulated by many different mechanisms, one of which is DNA methylation. Since E1 is controlled by both the early and late promoters, methylation in both promoter regions was explored. E2 binding sites (E2BS) responsible for transcriptional repression (nucleotide positions 37, 43, 52 and 58), in the early promoter region (p97) where the E2 protein binds resulting in negative regulation of viral gene expression, were examined by pyrosequencing.

In order to optimize the pyrosequencing conditions, SiHa and CaSki cell lines were used. It has been previously reported that SiHa cells have only 1 – 2 copies of HPV16 per cell, and that the HPV16 genome in SiHa cells lacks methylation while CaSki cells contain approximately 600 copies of HPV16 per cell and are extensively methylated (Badal et al., 2003).

Sixty-six samples (20 Normal, 13 CIN I, 14 CIN 2/3, and 19 SCC) were successfully amplified, % methylation of each CpG is shown in Table 3. Interestingly, mean methylation levels of all four CpG positions in the E2BS were higher in CIN 2/3 (12.60%) and SCC (12.96%) patients, than in Normal (1.29%) and CIN 1 (1.37%) patients (Table 3). The methylation status in SCC samples was significantly higher compared to Normal samples, for positions 37 (p-value =0.022), 43 (p-value =0.006), and 58 (p-value <0.001) (Figure 30 a, b, d). In addition, SCC samples could also be significantly differentiated from CIN 1 patients at positions 43 (p-value =0.011) and 58 (p-value <0.001) (Figure 30 b, d). E2BS methylation in SCC and CIN 2/3 samples was significantly higher compared with Normal (p-value < 0.001 and 0.002, respectively) and CIN 1 (p-value <0.001 and < 0.001, respectively) (Figure 31).

Methylation levels of the differentiation induced late promoter region (p670) at nucleotide positions 497, 504, 507, and 539 were also determined. Thirty samples (12 Normal, 9 CIN 1, 5 CIN 2/3 and 4 SCC) were investigated. Methylation levels of p670 were higher than those of E2BS in all stages (Figure 31). Methylation at each position in the p670 region (497, 504, 507 and 539), was not significantly different. However, statistically significant methylation of p670 in SCC was determined compared to Normal (p-value = 0.007) and CIN 2/3 (p-value = 0.001), but not CIN 1 (p-value = 0.2) (Figure 31). Interestingly, CaSki and SiHa cells, showed different

patterns of methylation. CaSki cells exhibited hypermethylation at all early and late promoter positions with a range of 56% - 82%, whereas SiHa cells exhibited hypomethylation with a range of 0% - 2%. Nevertheless, E1 expression from both cell lines was comparable (0.13 and 0.06, respectively).

Table 3. Early and late promoter methylation in patient cervical samples.

Group		Normal	CIN 1	CIN 2/3	SCC
Early promoter (p97)					
CpG 37	Mean	1.300	1.375	10.08	13.43
	SE	0.21	0.26	6.088	5.14
	Range	1-3	1-3	1-60	1-55
CpG 42	Mean	1.11	1.13	19.00	13.07
	SE	0.11	0.13	11.65	5.16
	Range	1-2	1-2	1-68	1-55
CpG 52	Mean	4.00	4.00	24.17	17.45
	SE	2.00	2.00	12.96	6.10
	Range	2-8	2-8	2-66	2-55
CpG 58	Mean	1.00	1.00	12.36	12.29
	SE	0.00	0.00	7.42	4.54
	Range	1-1	1-1	1-66	1-53
Total	Mean	1.29	1.37	12.60	12.96
Late Promoter (p670)					
CpG 497	Mean	21.00	29.00	21.20	45.50
	SE	2.52	4.16	2.34	7.50
	Range	16-24	20-39	14-28	38-53
CpG 504	Mean	46.67	61.80	46.75	62.50
	SE	9.97	8.89	4.70	1.5
	Range	31-57	42-85	35-58	61-64
CpG 507	Mean	25.00	34.80	19.80	44.00
	SE	5.51	5.03	2.56	5.00
	Range	14-31	22-47	13-28	39-49
CpG 539	Mean	38.50	37.20	36.00	46.00
	SE	1.50	5.57	6.43	2.00
	Range	37-40	23-51	26-48	44-48
Total	Mean	32.27	40.70	29.41	49.50

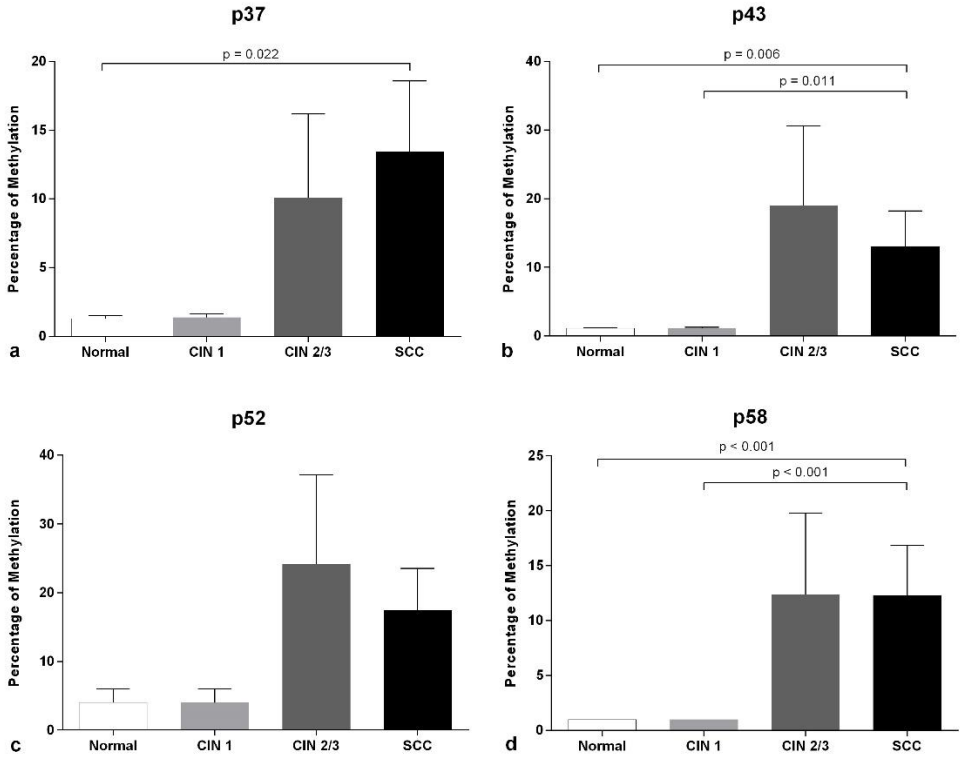


Figure 30. The percentage of methylation at different CpG positions of the E2BS. (a) p37 (b) p43 (c) p52 and (d) p58. Significant differences of methylation between cancer and normal patients were observed at 3 positions in the E2BS: p37, p43, p58. Data are represented as the mean of percentage methylation.

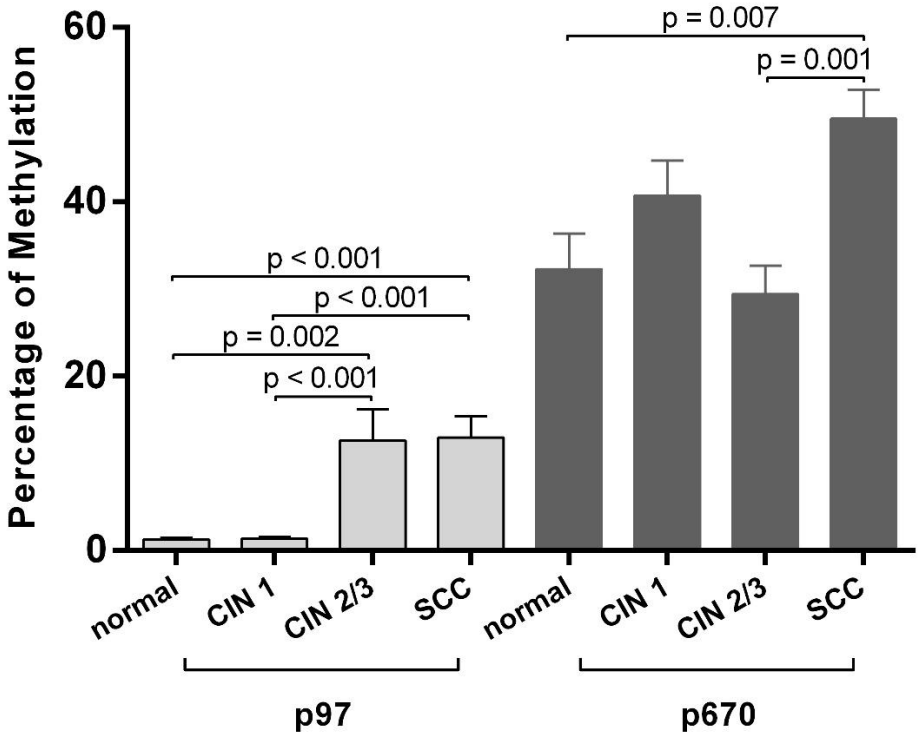


Figure 31. Methylation of all positions of E2BS showed a significant difference between SCC/CIN 2/3 patients and Normal/CIN 1 patients.

Late promoter methylation (p670) was consistently higher than E2BS methylation for all clinical stages. In the p670 region SCC patients exhibited significantly higher methylation than in normal patients.

6. Additional genetic events that may be involved in E1-associated carcinogenesis

In addition to measurements of E1 expression levels, genetic variation in the E1 gene was explored to determine if there was a possible correlation between genetic variance and disease progression. It has been previously reported that a 63 bp duplication in the E1 gene of HPV16 is associated with lower disease progression (Sabol et al., 2008). Therefore, the prevalence of the 63 bp duplication genomic variant of HPV16 E1 in HPV16 positive samples was determined by PCR (Figure 32). Ninety-five out of 124 samples (15 Normal, 22 CIN 1, 32 CIN 2/3 and 26 SCC) were successfully analysed. The 63 bp duplication was exhibited in 4 samples (4.2%), all of which were categorized as CIN. Nucleotide and amino acid sequences of those 4 samples were analysed using reference HPV16 sequences K02718 and NP_041327.2 (Figure 33).

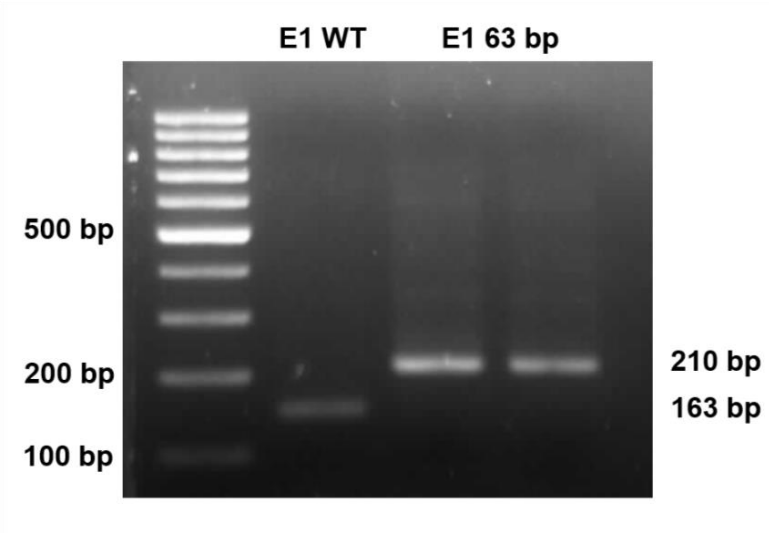


Figure 32. Gel electrophoresis of wild-type HPV16 E1 (WT) and HPV16 E1 containing the 63 bp duplication.

DNA from patient samples were amplified using primers specific to the region of interest in HPV16 E1. The amplified wild-type sequence was at 163 bp while the amplified fragments containing the duplication was at 210 bp.

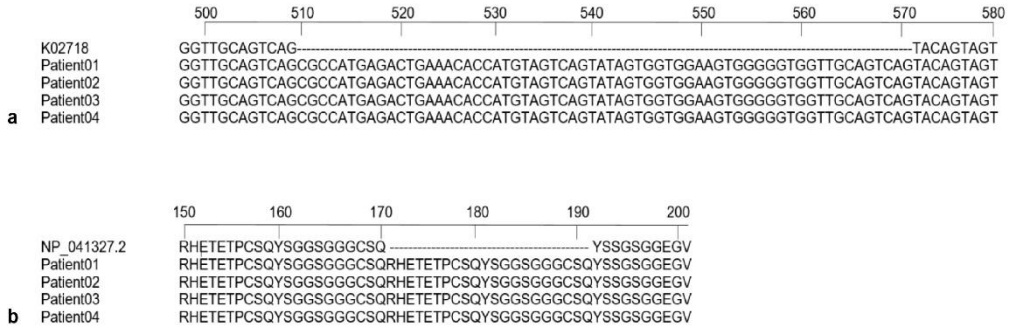


Figure 33. A 63 bp duplication was found in the E1 region in 4 clinical samples (patients 01-04).

Samples containing the 63 bp duplication in the E1 region are shown. (a) the nucleotide sequence of patients compared to the reference sequence K02718 and (b) the predicted amino acid sequence compared to that of the reference sequence NP_041327.2

Part II Functional role of HPV16 E1 transfection in HEK 293T cells

While a correlation between HPV16 E1 and carcinogenesis was observed in patient samples, the specific role of HPV16 E1 in carcinogenesis is still unknown. Experiments have determined E1 in various types of HPV to be critical in the maintenance of viral genome replication, and also been linked to DNA damage and pathways involved in carcinogenesis. The goal of the following experiments was to identify the pathways in which HPV16 E1 induces carcinogenesis.

1. Detection of HPV16 E1 transfection in HEK 293T cells

In order to determine the role of HPV16 E1, HEK 293T cells were transfected with either pEGFP (vector control) or pEGFP-E1 (eGFP tagged HPV16 E1). Detection of HPV16 E1 transfected cells was observed under fluorescence microscopy (Figure 34) and flow cytometry. The green fluorescence in E1 transfected HEK 293T cells was found mainly localized in the nucleus whereas the green fluorescence (of GFP alone) was observed both in the cytoplasm and nucleus of GFP transfected HEK 293T cells.

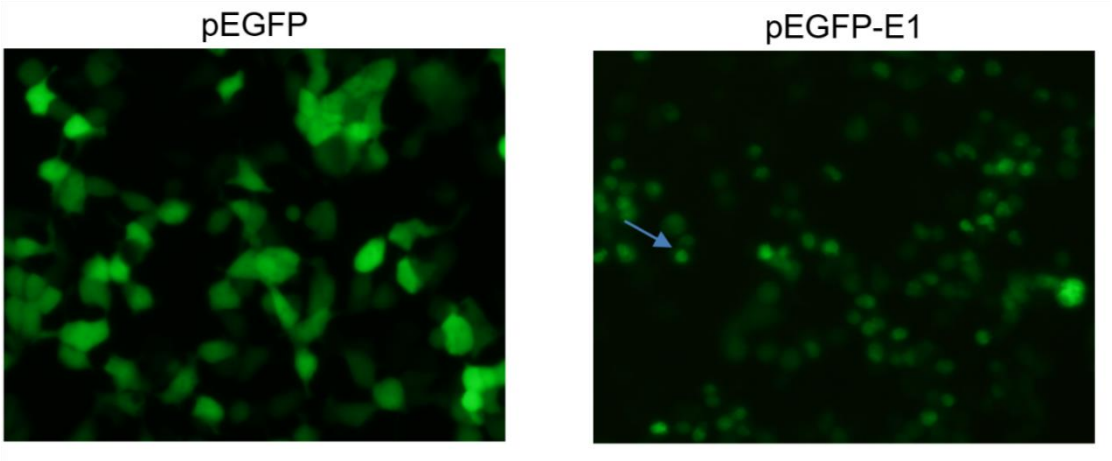


Figure 34. pEGFP and pEGFP-E1 transfect HEK 293T cells.

Green fluorescence was observed in HEK 293T cells transfected with pEGFP alone and with pEGFP-E1 transfected cells under fluorescence microscopy. Arrow denotes nuclear localization.

2. Full-length HPV16 E1 decreases cell proliferation

In order to determine if HPV16 E1 influenced cell growth, HEK 293T cells were transfected with either pEGFP or pEGFP-E1 and incubated at 37°C. Cell viability was quantitated using trypan blue staining technique. The results showed that the percentage of cell growth reduction in E1 transfected cells compared to GFP transfected cells was 12%, 39%, 38%, and 28% at 12, 24, 36, and 48 hours post-transfection, respectively. There was a significant difference in cell growth between GFP and E1 at 24 (p-value < 0.0001), 36 (p-value = 0.0001), and 48 h (p-value = 0.0004) (Table 4, Figure 35).

Table 4. Number of viable cells of GFP and HPV16 E1 transfected HEK 293T cells.

	Hours post-transfection			
	12	24	36	48
GFP	62400	124800	105600	112000
	67200	102400	124800	129600
	48000	97600	83200	105600
	68800	105600	108800	115200
	78400	100800	115200	124800
	70400	115200	123200	128000
Mean±SE	65867±4160	107733±4206	110133±6216	119200±3957
E1	46400	68800	75200	97600
	49600	59200	72000	96000
	67200	64000	73600	64000
	59200	72000	70400	88000
	57600	64000	60800	76800
	68800	65600	57600	89600
Mean±SE	58133±3687	65600±1801	68267±2969	85333±5220

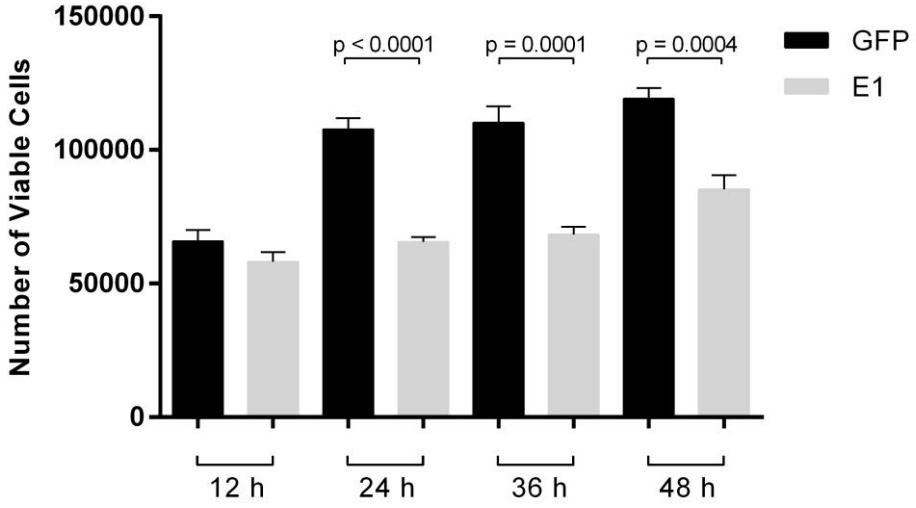


Figure 35. HPV16 E1 decreases cell growth.

Cell viability was quantitated using trypan blue. Statistically significant cell growth reduction was observed in E1 transfected cells at 24, 36, and 48 hours post transfection.

HPV16 E1 is comprised of 4 main domains: the N-terminal domain (ND) which contains the nuclear localization and export signals; the DNA binding domain (DBD) which binds to the viral replication origin; the oligomerization domain (OD) which is responsible for oligomerization and the ATPase helicase domain (HD). Plasmids containing the various domains of E1 were constructed i.e., pEGFP-E1-184 (ND) pEGFP-E1-359 (ND+DBD) pEGFP-E1-439 (ND+DBD+OD) (Figure 36) to explore which domain is important in cell growth reduction. HEK 293T cells were transfected with these 3 truncated forms of E1 along with full-length E1 and GFP vector control. Cell growth was measured using CountBright™ absolute counting beads and flow cytometry. Statistically significant cell growth reduction was observed in pEGFP-E1 (49%, p-value < 0.0001), pEGFP-E1-184 (11%, p-value < 0.01) and pEGFP-E1-359 (21%, p-value < 0.003) compared to pEGFP vector control (Figure 37 and Table 6).

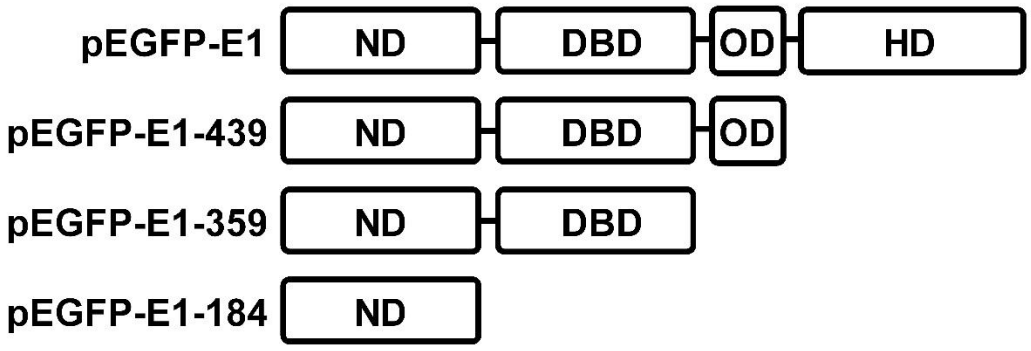


Figure 36. Truncated pEGFP-E1 plasmids and corresponding domains.

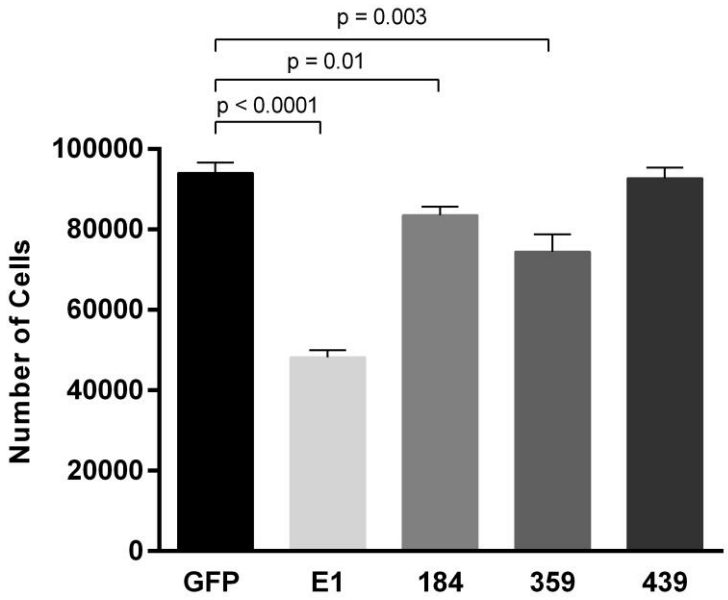


Figure 37. Full-length and partially truncated forms of E1inhibit cell proliferation.
HEK 293T cells were transfected with either the vector control (GFP), full-length HPV16 E1(E1) or one of the truncated forms (184, 359 and 439). Cell count was analysed using CountBright™ beads and flow cytometry

Table 5. Absolute cell count using CountBright™ beads for HEK 293T cells transfected with plasmids containing either the vector control, full-length E1 or truncated forms of E1.
* indicates p-value < 0.05

	GFP	E1	184	359	439
Absolute Cell Count	85221	45597	78494	63527	89922
	91307	43184	77710	63584	89346
	90674	45041	80928	83572	84399
	102763	53416	90091	75679	101537
	98297	50344	86288	71184	99177
	96115	51744	87634	89317	91828
Mean	94063	48221*	83524*	74477*	92701
SE	2550	1696	2109	4296	2637

To confirm the growth inhibitory effect of full-length and truncated forms of E1, cell proliferation was quantitated by the TetraZ™ Cell Counting Kit which is a colorimetric cell counting kit based on the ability of viable cells to convert tetrazolium salts to formazan, which can then be quantitated.

The results show that there was an increase in cell proliferation with incubation time for all truncated forms of HPV16 E1 and pEGFP. In contrast, full-length HPV16 E1 showed a significant reduction in cell proliferation at 24 ($p = 0.04$) and 36 hours ($p = 0.008$) post-transfection compared to the pEGFP vector control. The mean OD_{450} at 24 and 36 hours cells post-transfection was 1.83 and 1.98 while the mean OD_{450} for E1 transfected cells was 0.98 and 0.72. In conclusion, full-length E1 strongly inhibited cell proliferation at a statistically significant level in all experiments. It was also noted that only cells transfected with full-length HPV16 E1 exhibited decreased cell viability.

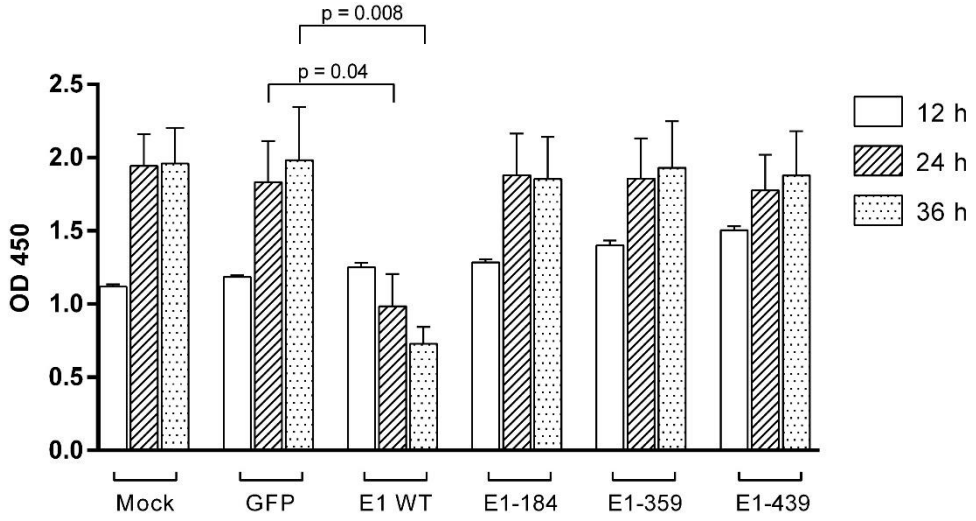


Figure 38. Cell proliferation of GFP vector control, full-length HPV16 E1 and truncated forms of HPV16 E1.

Cell proliferation was significantly decreased in full-length HPV16 E1 transfected cells.

Table 6. Full-length E1 decreases cellular proliferation at 24 and 36 hours post-transfection.

* indicated p-value < 0.05.

Hours PT		MOCK	GFP	E1 WT	184	359	439
12	Mean	1.12	1.18	1.25	1.29	1.40	1.50
	SE	0.04	0.03	0.07	0.05	0.08	0.06
	Min	1.06	1.14	1.146	1.21	1.29	1.41
	Max	1.17	1.22	1.35	1.35	1.51	1.59
24	Mean	1.94	1.83	0.98 *	1.88	1.86	1.78
	SE	0.22	0.28	0.22	0.28	0.27	0.24
	Min	1.42	1.18	0.47	1.22	1.19	1.21
	Max	2.57	2.57	1.56	2.61	2.63	2.40
36	Mean	1.96	1.98	0.73 *	1.85	1.93	1.88
	SE	0.24	0.36	0.12	0.29	0.32	0.30
	Min.	1.38	1.14	0.45	1.19	1.16	1.18
	Max	2.62	2.83	1.02	2.55	2.67	2.60

3. HPV16 E1 induces apoptosis and necrosis

Previous experiments indicated that transfection with full-length HPV16 E1 caused decreased proliferation in cells, when compared to transfection with the vector control. Decreases in cell proliferation can be induced by cell cycle arrest, but also cell death. It was noted from the previous experiments that HPV16 E1 may increase cell death. Subsequently the following experiments aimed to confirm if transfection with HPV16 E1 induced cell death in addition to decreasing cell proliferation.

HEK 293 T cells were transfected with either the GFP vector control or HPV16 E1, and cell death at 24, 48 and 72 hours after transfection was measured by staining with the viability dye, Zombie Yellow™, and analysed by flow cytometry. E1 induced significant cell death at 24 and 72 hours post-transfection (Figure 39). Death in vector control cells was less than 10% for the duration of the experiment. In contrast, the percentage of cell death increased over time in cells transfected with E1, i.e. 3.68% ± 0.75% at 24 hours, 13.13% ± 1.44% at 48 hours, and 34.63% ± 6.59% at 72 hours post-transfection.

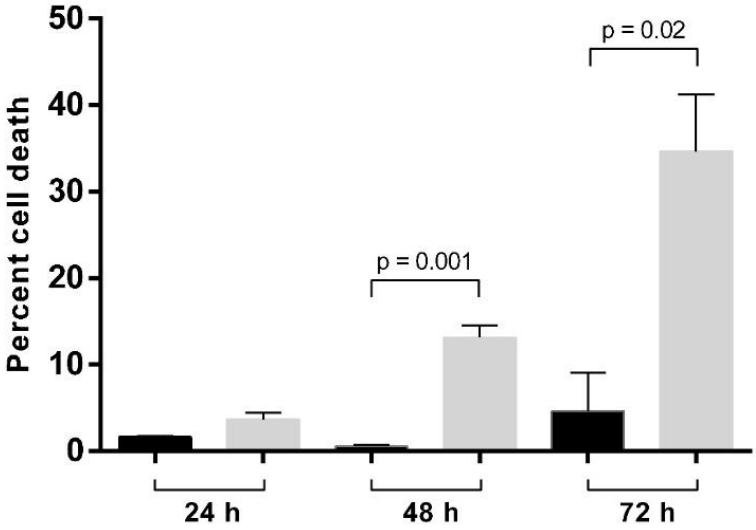


Figure 39. E1 induces cell death.

HEK 293 T cells were transfected with either pEGFP or pEGFP-E1. Cells were stained with Zombie Yellow and percent cell death was analysed by flow cytometry at 24, 48, and 72 hours post-transfection. Black bars indicate HEK 293T cells transfected with pEGFP vector control while grey bars indicate cells transfected with pEGFP-E1.

Since E1 also induced cell death, further exploration of the mechanism by which E1 induces death was undertaken. Cell death can occur naturally or can be induced by external factors, and it is also initiated through many pathways, the two most common being apoptosis and necrosis.

In order to determine whether HPV16 E1 induced cell death through apoptosis or necrosis, HEK 293T cells were transfected with either pEGFP or pEGFP-E1. Apoptosis and cell death were measured by annexin V and propidium iodide staining at 24, 48, and 72 hours post-transfection using flow cytometry (Figure 40). The results revealed that HPV16 E1 significantly induced apoptosis starting at 24 h (p-value < 0.001), 48 h (p-value < 0.001), and 72 h (p-value < 0.001) post-transfection. The difference in mean percentages of apoptotic cells between E1 transfected cells and the vector control were 10.97%, 26.44 % and 28.3 % at 24, 48, and 72 hours, respectively. A significant increase in mean percentage of necrotic cells was also observed in cells transfected with E1, i.e., 6.33 % (p-value = 0.007), 15.35% (p-value = 0.022), 26.20% (p-value < 0.001) at 24, 48, and 72 hours, respectively (Figure 40, Table 7). This experiment demonstrated that, in addition to decrease cell proliferation, E1 also causes cell death through both the apoptosis and necrosis pathways.

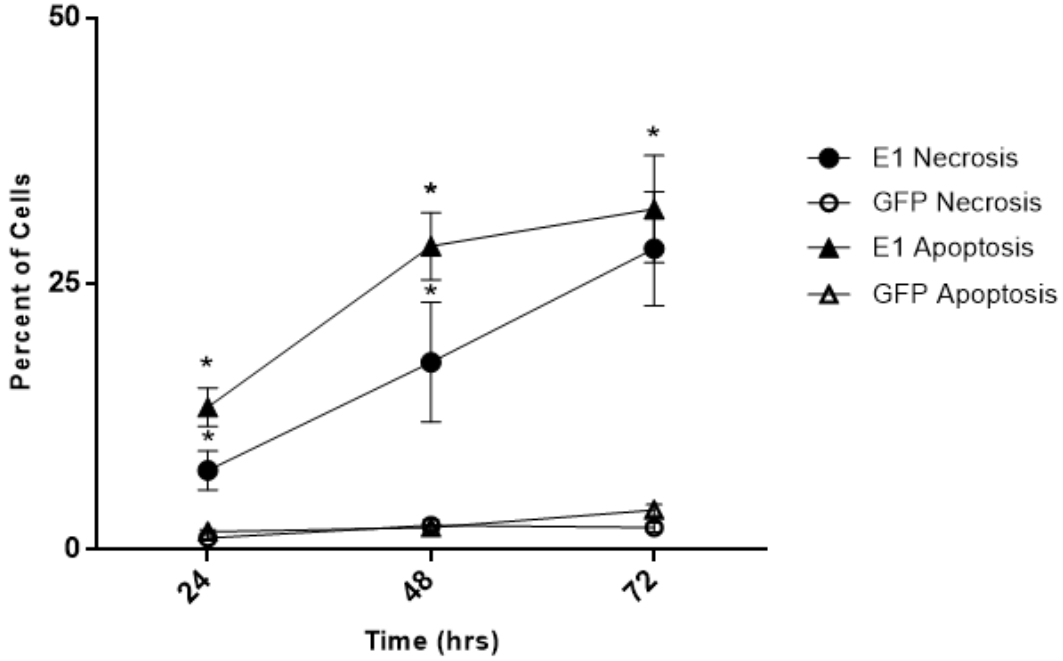


Figure 40. E1 induces both apoptosis and necrosis.

HEK 293 T cells were transfected with either pEGFP or pEGFP-E1. Apoptosis was measured using annexin V, necrosis was measured using propidium iodide. Results were analysed by flow cytometry. Significant difference between pEGFP and pEGFP-E1 transfected cells are denoted by *.

Table 7. Percentage of apoptotic cells at 24, 48 and 72 hours post-transfection.

Data presented as Mean ± SE.

	24 h	**48 h**	**72 h**
GFP			
Early Apoptosis	1.39 ± 0.22	1.64 ± 0.23	2.08 ± 0.22
Apoptosis	1.70 ± 0.21	2.10 ± 0.28	3.74 ± 0.53
Necrosis	1.12 ± 0.29	2.30 ± 0.49	2.10 ± 0.41
Dead	2.82 ± 0.23	4.39 ± 0.37	5.84 ± 0.43
E1			
Early Apoptosis	2.66 ± 0.68	4.46 ± 0.94	1.37 ± 0.063
Apoptosis	13.42 ± 1.80	28.54 ± 3.16	32.04 ± 5.04
Necrosis	7.45 ± 1.83	17.64 ± 5.64	28.30 ± 5.35
Dead	20.87 ± 2.80	46.19 ± 4.634	60.34 ± 3.68

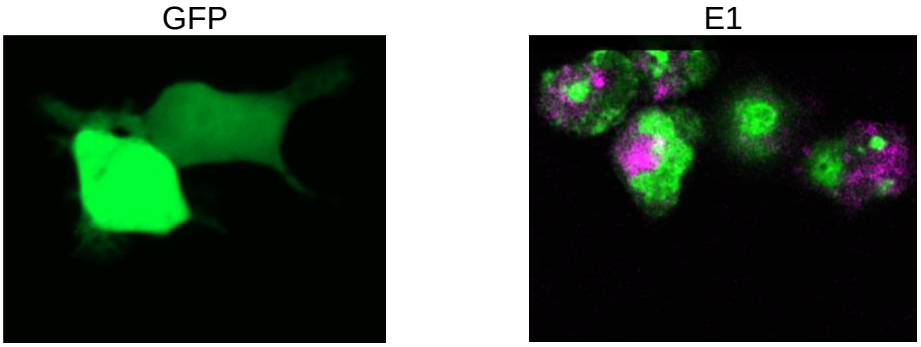


Figure 41. APC conjugated Annexin V staining in pEGFP or pEGFP E1transfected HEK 293T cells 48 hours post-transfection.

Subsequently, to confirm whether cell death caused by HPV16 E1 was due to apoptosis, the pan-caspase inhibitor, QVD-OPH was used to treat cells 24 hours prior to transfection. In order to determine the cytotoxicity of QVD-OPH on HEK 293T cells, the cells were treated with QVD-OPH for 72 hours. Cell death was then analysed using propidium iodide and flow cytometry. It was found that at concentrations higher than 2.5 µM QVD-OPH induced a significant amount of cell death (Figure 42). Mean cell death for cells treated with 5 µM and 10 µM of QVD-OPH was 15.4% (p-value = 0.04) and 15.7% (p-value = 0.001) respectively.

Additionally, cell viability was confirmed using TetraZ™ Cell Counting Kit. Average % viability was, 100% (CT), 97.7% (1.25 µM), 86% (2.5 µM), and 67% (5 µM), 34% (10 µM) (Figure 43). Because 5 µM and 10 µM concentrations negatively influenced cell viability in both experiments, 2.5 µM of QVD-OPH was used to treat cells. HEK 293T cells were treated with QVD-OPH, a pan-caspase inhibitor continuously, starting from 24 hours prior to transfection with either pEGFP or pEGFP-E1. Apoptosis and cell death were measured by annexin V and propidium iodide staining at 48 hours post-transfection by flow cytometry. There was a decreasing trend of apoptosis in cells treated with QVD-OPH, however the results were not statistically significant (p-value = 0.20) as shown in Figure 44.

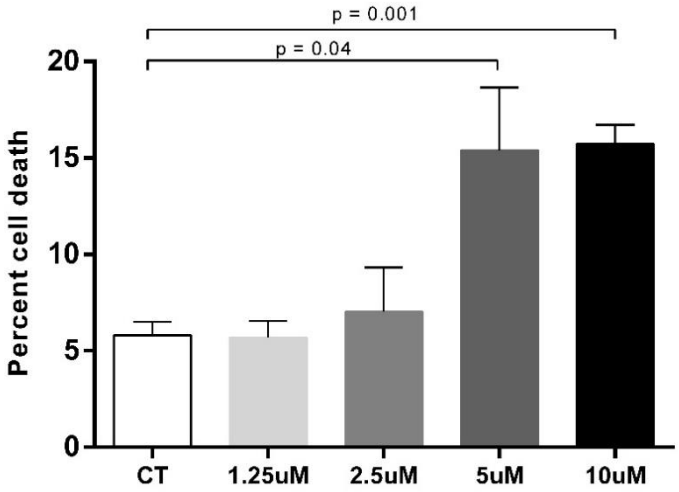


Figure 42. Percentage of cell death in cells treated with QVD-OPH.

HEK 293T cells were treated with QVD-OPH at the indicated concentrations for 72 hours. Cell death was determined by propidium iodide staining and analysed by flow cytometry.

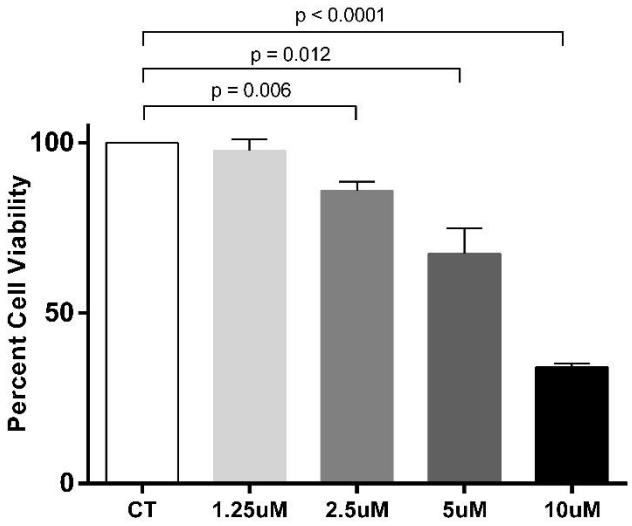


Figure 43. Percentage of viable cells treated with QVD-OPH.

Cells were treated with QVD-OPH for 72 hours. Cell viability was determined using TetraZ™ Cell Counting Kit and quantitated at 450 nm using a microplate reader.

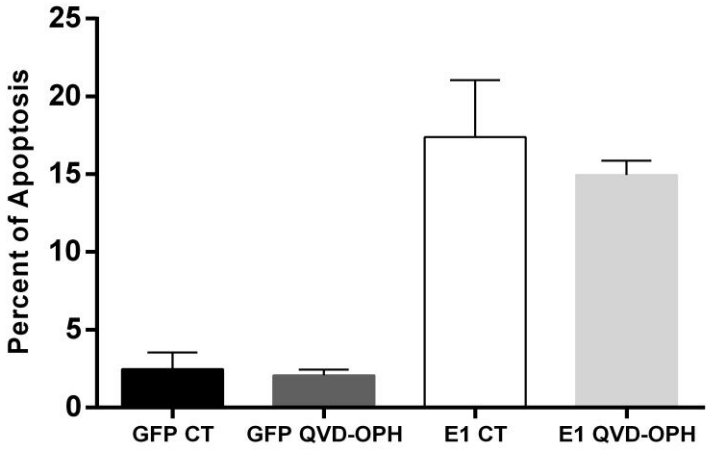


Figure 44. Treatment of E1 transfected cells with QVD-OPH showed a decreasing trend in apoptosis.

HEK 293 T cells were treated with QVD-OPH 24 hours prior to transfection with either pEGFP or pEGFP-E1. Apoptosis was determined using annexin V and quantitated by flow cytometry 48 hours after transfection.

4. HPV16 E1 affects many host cellular pathways

The previous experiments suggested that HPV16 E1 induced apoptosis but also caused cell death through other pathways. In order to further explore the role of HPV16 E1, HEK 293T cells were transfected with either pEGFP or pEGFP-E1. Microarray analysis was performed on RNA collected 48 hours post-transfection. Gene expression profiles revealed that E1 impacted gene expression in various pathways. In total, 416 genes were differentially expressed in HPV16 E1 transfected cells of these 238 were upregulated ($\geq$ 2-fold change), and 177 were downregulated ($\leq$ 2-fold change). Gene expression pattern was significantly different in non-transfected HEK 293T cells, pEGFP transfected cells and pEGFP E1 transfected cells as shown in the heatmap (Figure 45). The genes with the highest differential expression in E1 transfected cells were small nucleolar RNA C/D box A and C, SNORD3A (16.59-fold change) and SNORD3C (16.02-fold change); and SNORD84 (7.31-fold change). The next overexpressed gene was interferon stimulated gene 20, ISG20 (8.94-fold change) which is a gene known to exhibit antiviral properties. The fifth upregulated gene was variable charged X-link, VCX (6.21-fold change). In contrast, E1 suppressed the expression of many genes including inhibin beta E subunit, INHBE (-5.70-fold change). INHBE encodes for an inhibin beta subunit which is a member of transforming growth factor-beta superfamily. In addition, DNA Damage Inducible Transcript 4, DDIT4 (-4.43-fold change), normally upregulated during DNA damage (Ellisen et al., 2002), TSC22 domain family member 3, TSC22D3 (-3.90-fold change), encodes a glucocorticoid induced leucine zipper protein and interacts with FoxO3 were also suppressed. The most influenced pathways induced by E1 included ribosome, metabolism, transcriptional misregulation, cell proliferation and cell death (Table 8). Many genes involved in cell proliferation and death were significantly different between the vector control and E1 transfected cells. A total of 117 genes differentially expressed genes involved in cell growth/death were identified, 59 were upregulated while 58 were downregulated (Table 9).

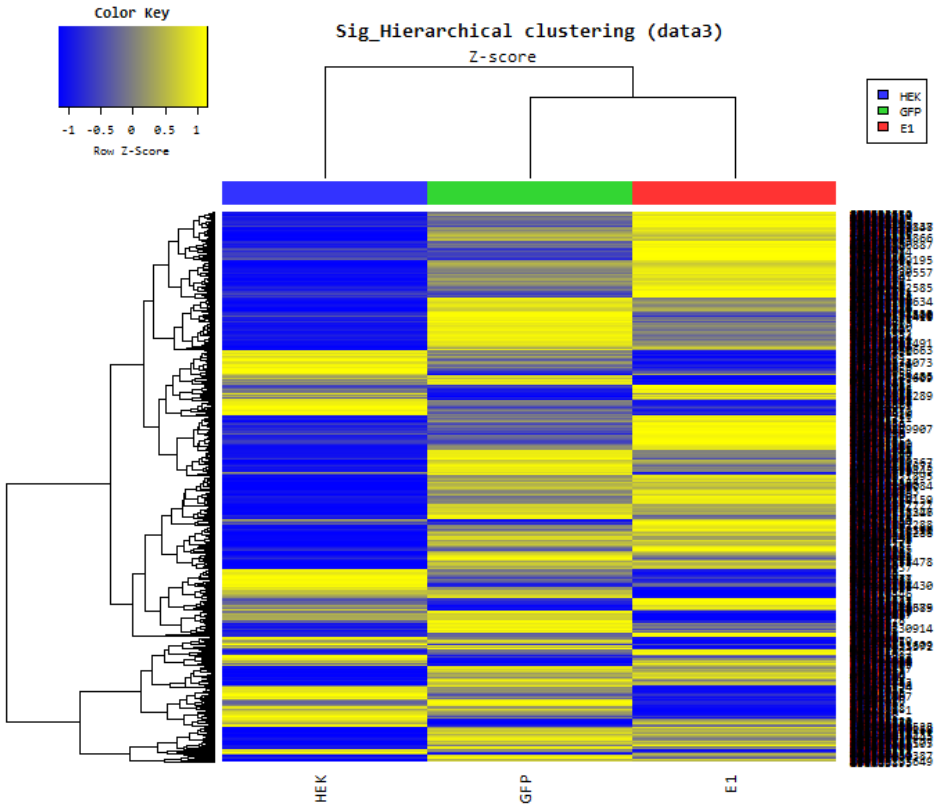


Figure 45.f Heat map showing gene expression patterns in non-transfected HEK 293T cells, pEGFP transfected cells and pEGFP-E1 transfected cells.

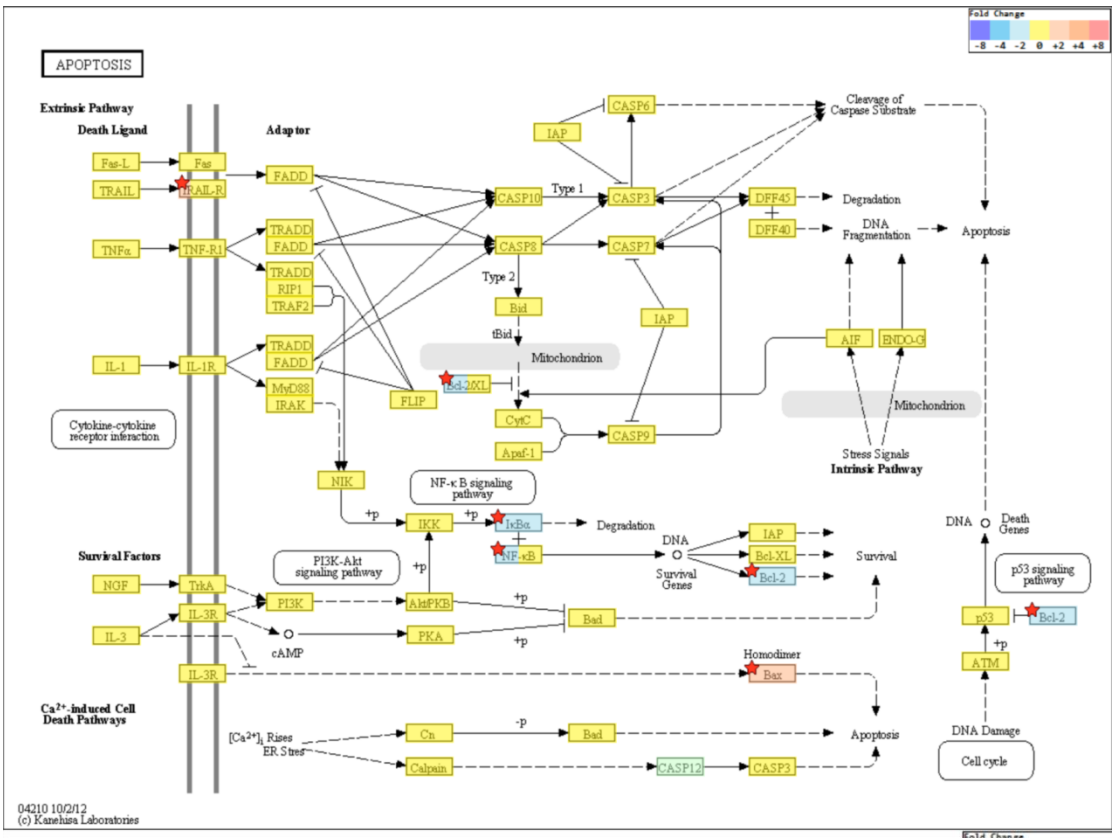
Table 8. Pathways significantly influenced by E1 from gene expression analysis.

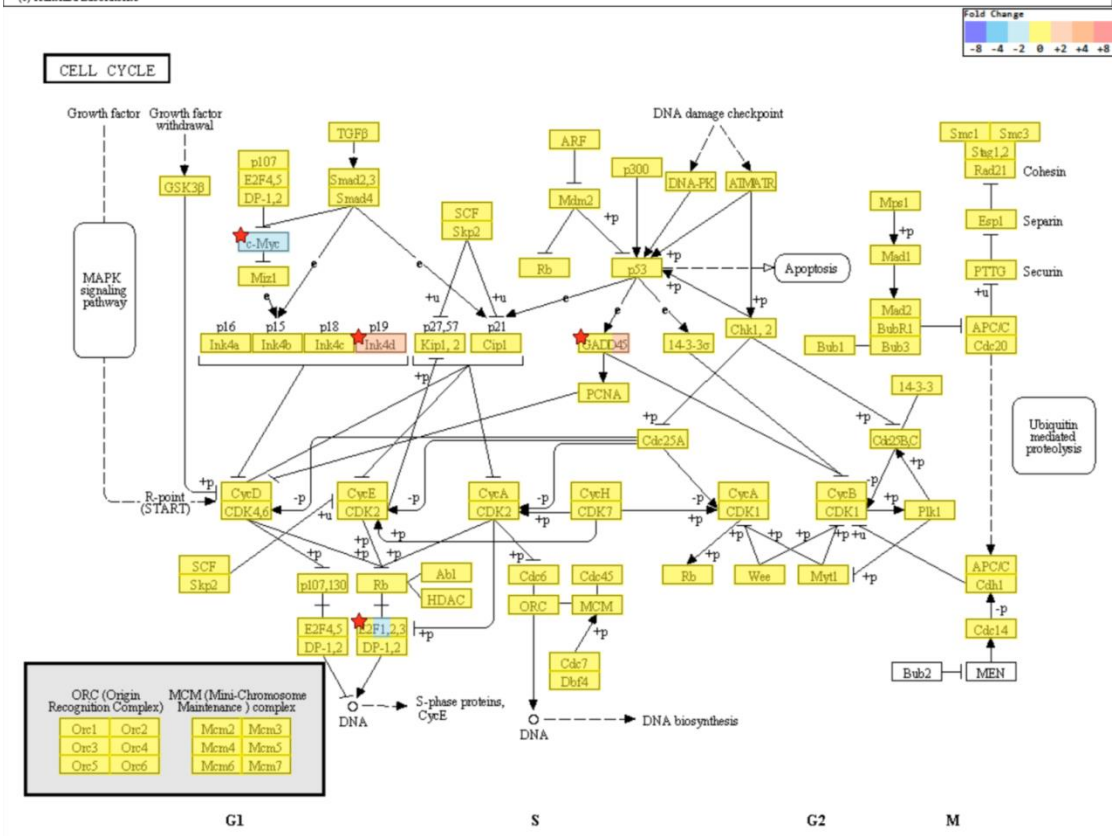
Pathway	Sig. Genes	p value	Pathway	Sig. Genes	p value
Ribosome	14	9.33E-15	Epithelial cell signaling in Helicobacter pylori infection	4	0.00208
FoxO signaling pathway	9	4.51E-08	Prolactin signaling pathway	4	0.00243
Hepatitis B	9	8.46E-08	Phagosome	5	0.00252
Neurotrophin signaling pathway	8	3.74E-07	Pertussis	4	0.00271
Transcriptional misregulation in cancer	9	3.75E-07	RNA transport	5	0.00362
MAPK signaling pathway	10	4.97E-07	Estrogen signaling pathway	4	0.00592
PI3K-Akt signaling pathway	11	6.39E-07	HIF-1 signaling pathway	4	0.00641
Small cell lung cancer	7	9.77E-07	Chagas disease (American trypanosomiasis)	4	0.00657
Pathways in cancer	11	2.12E-06	Toll-like receptor signaling pathway	4	0.00692
Legionellosis	6	2.41E-06	Alanine, aspartate and glutamate metabolism	3	0.00763
Protein processing in endoplasmic reticulum	8	3.33E-06	Bladder cancer	3	0.00887
Metabolic pathways	17	8.45E-06	Cell cycle	4	0.01049
Measles	7	0.00001	Osteoclast differentiation	4	0.01212
Non-alcoholic fatty liver disease (NAFLD)	7	0.00002	Amino sugar and nucleotide sugar metabolism	3	0.01362
Prostate cancer	6	0.00002	Malaria	3	0.01414
NF-kappa B signaling pathway	6	0.00002	Endocytosis	5	0.01427
Cocaine addiction	5	0.00004	Wnt signaling pathway	4	0.01442
HTLV-I infection	8	0.00005	Insulin signaling pathway	4	0.01442
Huntington's disease	7	0.00008	Signaling pathways regulating pluripotency of stem cells	4	0.01496
Toxoplasmosis	6	0.00009	Glutathione metabolism	3	0.01521
Colorectal cancer	5	0.00009	Pathogenic Escherichia coli infection	3	0.01746
Epstein-Barr virus infection	7	0.00010	Hippo signaling pathway	4	0.01845
Viral carcinogenesis	7	0.00011	NOD-like receptor signaling pathway	3	0.01863
Glycolysis / Gluconeogenesis	5	0.00012	Acute myeloid leukemia	3	0.01863
Biosynthesis of antibiotics	7	0.00014	cGMP-PKG signaling pathway	4	0.02270
Adipocytokine signaling pathway	5	0.00014	Pancreatic cancer	3	0.02430
Regulation of actin cytoskeleton	7	0.00014	Central carbon metabolism in cancer	3	0.02497
Ubiquitin mediated proteolysis	6	0.00016	Amphetamine addiction	3	0.02564
Chronic myeloid leukemia	5	0.00017	RIG-I-like receptor signaling pathway	3	0.02702
Apoptosis	5	0.00030	Thyroid hormone synthesis	3	0.02842
Alzheimer's disease	6	0.00039	Herpes simplex infection	4	0.02978
Influenza A	6	0.00049	Leishmaniasis	3	0.02985
Tuberculosis	6	0.00052	Biosynthesis of amino acids	3	0.02985
Alcoholism	6	0.00053	Chemokine signaling pathway	4	0.03100
TNF signaling pathway	5	0.00074	Cardiac muscle contraction	3	0.03280
MicroRNAs in cancer	7	0.00076	TGF-beta signaling pathway	3	0.03431
cAMP signaling pathway	6	0.00084	Salmonella infection	3	0.03901
Amyotrophic lateral sclerosis (ALS)	4	0.00094	ErbB signaling pathway	3	0.03982
Sphingolipid signaling pathway	5	0.00101	Rap1 signaling pathway	4	0.04068
AMPK signaling pathway	5	0.00114	Gap junction	3	0.04145
Non-small cell lung cancer	4	0.00122	Ras signaling pathway	4	0.04908
Shigellosis	4	0.00183			

Table 9. Genes differentially expressed in HPV16 E1 transfected cells involved in cell proliferation and cell death.

Gene	FC	Gene	FC	Gene	FC	Gene	FC
ISG20	8.94	GSTM3	2.26	BCL2	-2.05	ASNS	-2.35
SERTAD1	4.76	CX3CR1	2.23	MSH5	-2.07	SLC11A2	-2.35
SGK1	4.67	HSPA2	2.23	E2F2	-2.07	FOXO3	-2.36
RGS2	3.70	TNFRSF10D	2.22	MECOM	-2.07	H1F0	-2.40
KRTDAP	3.49	PSMD10	2.22	NFKB1	-2.09	JARID2	-2.45
CD14	3.32	SIRT4	2.21	SPEN	-2.10	TENM3	-2.45
GDF15	3.25	NPTX1	2.21	PPARGC1A	-2.10	NCOA1	-2.47
BIRC5	3.08	EOMES	2.20	SATB2	-2.10	KLF9	-2.48
HBEGF	3.06	KIF20A	2.19	SASS6	-2.10	TCF12	-2.50
ALDOC	2.97	TP53INP2	2.18	ASAP1	-2.10	FHIT	-2.50
IFI27	2.90	KRT17	2.17	NSMCE2	-2.11	BARD1	-2.50
HOXD1	2.84	KIAA1324	2.17	MYC	-2.16	KDM6A	-2.51
APOBEC3H	2.78	EGR1	2.16	HEY1	-2.16	PSD3	-2.53
TEX19	2.66	NMB	2.13	WDR7	-2.19	NGFRAP1	-2.54
FGFR3	2.63	THBS4	2.12	MKL1	-2.19	NFIB	-2.55
BAX	2.63	SLC9A3R1	2.12	ARID4B	-2.21	MEIS2	-2.56
PLK5	2.62	ZNF268	2.12	CTH	-2.22	FANCG	-2.60
LGALS1	2.62	LTA	2.11	FBXW7	-2.23	KLHL9	-2.63
ITGB2	2.53	FSCN1	2.10	FAF1	-2.25	RARB	-2.65
GADD45B	2.49	RPS15	2.09	FAM188A	-2.25	MAP2K5	-2.67
GAL	2.47	PGK1	2.09	HILPDA	-2.25	PBX3	-2.73
PLK2	2.45	SOX8	2.09	NFKBIA	-2.25	ARID5B	-2.83
CDK5R1	2.43	TAF7L	2.08	BCL11A	-2.28	RAPGEF6	-2.93
SLC9A1	2.42	PKNOX1	2.08	ATG7	-2.29	TSC22D3	-3.25
SOD2	2.41	HES5	2.07	RAD51D	-2.31	EXT1	-3.33
EVA1A	2.41	METRNL	2.03	PBX1	-2.31	DDIT4	-4.43
TCP1	2.30	CDKN2D	2.02	BTRC	-2.33	INHBE	-5.70
GABPA	2.29	BCL7C	2.01	NUPR1	-2.34		
CACYBP	2.29	PTH1R	2.01	SSH2	-2.35		

Because E1 influences many pathways involved in cell death and proliferation, the panel of genes comprised of hallmark genes in the cell proliferation, apoptosis, and DNA damage pathways, along with genes that were highly up/downregulated from these pathways were selected for validation by real-time RT PCR. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of apoptotic pathway and pathways involved in cell cycle and proliferation were analysed, genes with a differential expression of 2-fold were considered significant (Figure 46). A total of 38 genes were included in the panel (Table 10). In total, the real-time RT PCR identified 24 differentially expressed genes (3 upregulated and 21 downregulated) after 48 hours of transfection. The genes most affected by E1 transfection were genes involved in cell proliferation and DNA damage. Only 2 out of 8 genes, BAK1 (0.30) and CASP3 (0.23), in the apoptosis pathway showed any differential expression, whereas 16 out of 22 in the cell proliferation pathway and 5 out of 6 genes in the DNA damage pathways were found. Once it was confirmed that E1 had an effect on gene expression at 48 post-transfection the next experiment sought to determine if the effect on gene expression was time-dependent. Therefore, real-time RT PCR was performed on RNA collected from cells transfected with either vector control or HPV16 E1 at 12 and 24 hours post transfection. The results revealed that changes in expression occurred in a time-dependent manner. At 12 hours post-transfection only 5 genes out of the 38 in the panel were differentially expressed, while 12 genes were up/downregulated after 24 hours of transfection. The results from the real-time RT PCR concluded that E1 indeed has an effect on gene expression in a time-dependent manner; however it was noted that most genes were down regulated (Table 10).


APOPTOSIS
Extrinsic Pathway
Death Ligand
Adaptor
Fas-L
Fas
TRAIL
TRAIL-R
TNFα
TNF-R1
IL-1
IL-1R
FADD
TRADD
RIP1
TRAF2
MyD88
IRAK
FLIP
IAP
CASP6
CASP10
CASP8
CASP3
CASP7
Type 1
Type 2
Bid
tBid
Mitochondrion
Bcl-2/XL
CytC
Apaf-1
CASP9
DFF45
DFF40
Degradation
DNA Fragmentation
Apoptosis
Cleavage of Caspase Substrate
AIF
ENDO-G
Stress Signals
Intrinsic Pathway
Cytokine-cytokine receptor interaction
NIK
NF-κB signaling pathway
IKK
IκBα
NF-κB
Degradation
DNA
Survival Genes
IAP
Bcl-XL
Bcl-2
Survival
Survival Factors
NGF
TrkA
IL-3R
IL-3
PI3K-Akt signaling pathway
PI3K
Akt/PKB
PKA
Bad
cAMP
Homodimer
Bax
Ca2+-induced Cell Death Pathways
[Ca2+]i Rises ER Stres
Cn
Calpain
CASP12
CASP3
Apoptosis
DNA
Death Genes
p53 signaling pathway
p53
Bcl-2
ATM
DNA Damage
Cell cycle
04210 10/2/12
(c) Kanehisa Laboratories


CELL CYCLE
Growth factor
Growth factor withdrawal
MAPK signaling pathway
GSK3β
TGFβ
Smad2,3
Smad4
p107
E2F4,5
DP-1,2
c-Myc
Mirz1
SCF
Skp2
ARF
Mdm2
p300
DNA-PK
ATM/ATR
DNA damage checkpoint
Rb
p53
Apoptosis
p16
Ink4a
p15
Ink4b
p18
Ink4c
p19
Ink4d
p27,57
Kip1, 2
p21
Cip1
GADD45
14-3-3σ
Chk1, 2
Mps1
Mad1
Mad2
BubR1
Bub3
Bub1
Smc1
Smc3
Stag1,2
Rad21
Cohesin
Esp1
Separin
PTTG
Securin
APC/C
Cdc20
PCNA
14-3-3
Cdc25A
Cdc25B,C
Ubiquitin mediated proteolysis
R-point (START)
CycD
CDK4,6
CycE
CDK2
CycA
CDK2
CycH
CDK7
CycA
CDK1
CycB
CDK1
Plk1
Abl
HDAC
Cdc6
Cdc45
ORC
MCM
Rb
Wee
Myt1
APC/C
Cdh1
Cdc14
Bub2
MEN
Cdc7
Dbf4
E2F4,5
DP-1,2
E2F1,2,3
DP-1,2
DNA
S-phase proteins, CycE
DNA
DNA biosynthesis
ORC (Origin Recognition Complex)
Orc1
Orc2
Orc3
Orc4
Orc5
Orc6
MCM (Mini-Chromosome Maintenance) complex
Mcm2
Mcm3
Mcm4
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Mcm7
G1
S
G2
M

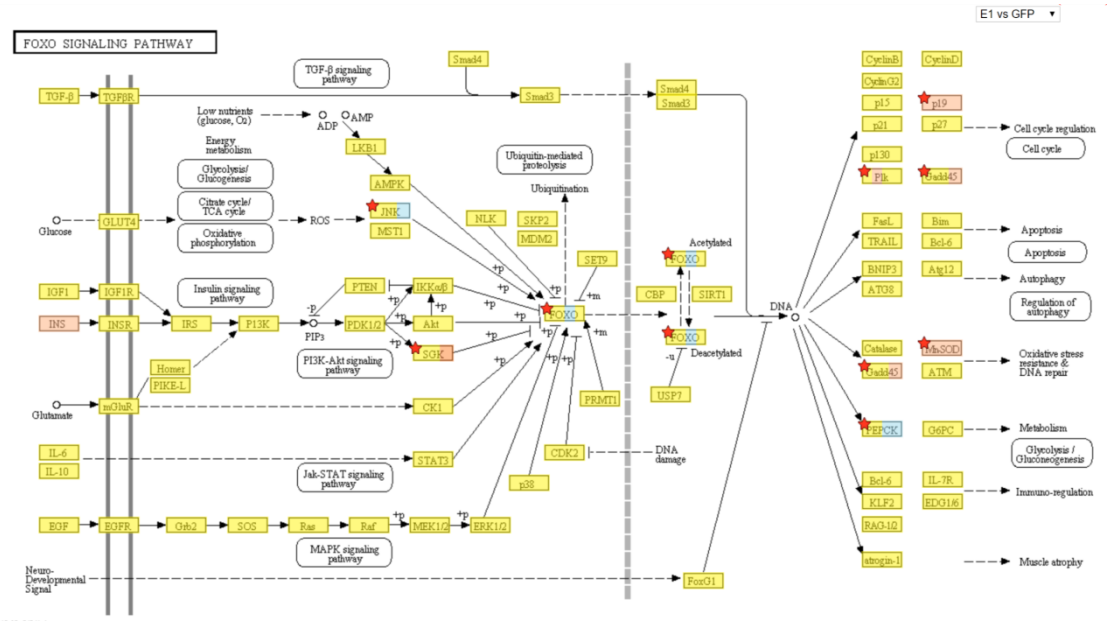

Figure 46. Example of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of apoptosis, cell cycle, and FOXO pathways.

Blue indicates decreased expression greater than 2-fold. Red indicates increased expression greater than 2-fold.

Table 10. Results obtained from Real-time RT-PCR at 12, 24 and 48 hours post-infection compared to the microarray results at 48 hours.

	Gene	12 (h)		24 (h)		48 (h)		Microarray
		Mean	SE	Mean	SE	Mean	SE	
Apoptosis	BAK1	0.94	0.02	0.76	0.03	0.30	0.11	0.93
	BAX	1.87	0.12	1.70	0.11	0.72	0.20	2.63
	BCL2	1.03	0.05	1.50	0.03	1.62	0.75	0.49
	CASP3	1.00	0.04	0.63	0.02	0.23	0.08	0.68
	CASP8	1.02	0.02	0.75	0.02	0.87	0.55	0.83
	CASP9	1.08	0.05	0.76	0.10	0.72	0.32	0.93
	TNFRSF10D*	2.17	0.08	3.70	0.17	0.96	0.30	2.22
	TP53	1.36	0.07	1.50	0.04	0.58	0.18	1.27
Cell Proliferation	AKT1	1.22	0.14	1.10	0.01	0.42	0.12	0.94
	BIRC5*	1.36	0.07	1.20	0.05	0.50	0.12	3.08
	CDC25C	1.17	0.09	0.53	0.00	0.23	0.08	0.81
	CDK1	1.44	0.06	0.99	0.01	0.39	0.13	no data
	CDKN2D	0.81	0.04	2.00	0.06	1.28	0.36	2.02
	CREB5*	0.48	0.01	0.23	0.00	0.13	0.06	0.33
	DDIT4*	1.17	0.21	1.40	0.21	0.34	0.11	0.23
	FOXO3*	0.66	0.00	0.45	0.01	0.23	0.09	0.42
	H1F0	0.77	0.03	0.59	0.03	0.14	0.04	0.42
	HIF1A	0.79	0.06	0.39	0.02	0.19	0.07	0.76
	INHBE*	1.97	0.13	1.30	0.03	0.16	0.04	0.18
	JMJD1C*	0.54	0.01	0.20	0.00	0.07	0.03	0.29
	MAP2K5	0.66	0.01	0.53	0.02	0.75	0.51	0.37
	MYC	0.64	0.03	0.64	0.01	0.27	0.11	0.46
	NFKB1	0.77	0.03	0.86	0.03	0.57	0.33	0.48
	PGK1	0.99	0.01	0.95	0.01	0.61	0.21	2.09
	PIK3CA	0.76	0.04	0.64	0.07	0.18	0.06	0.80
	PTGS2	0.82	0.01	1.50	0.04	1.97	0.98	1.13
	RASD2*	2.28	0.10	7.40	0.29	2.45	0.59	4.90
	SBSN*	3.90	0.11	3.80	0.00	2.18	0.97	4.81
	STAT3	1.14	0.08	0.72	0.01	0.31	0.12	0.86
	TSC22D3*	0.64	0.03	0.35	0.00	0.14	0.09	0.26
DNA Damage	ATM	1.15	0.05	0.88	0.01	0.70	0.30	0.83
	ATR	0.76	0.02	0.48	0.00	0.19	0.07	0.97
	BRCA1	0.80	0.02	0.65	0.02	0.38	0.17	0.63
	CHEK1	0.89	0.03	1.00	0.04	0.33	0.11	0.78
	CHEK2	1.10	0.02	0.90	0.02	0.38	0.13	1.07
	FANCG*	1.09	0.03	0.72	0.01	0.19	0.06	0.38
Immune Response	ISG20*	5.15	0.37	28.00	0.45	6.02	2.80	8.94
Metabolism	ALDOC*	1.07	0.02	2.30	0.01	1.57	0.61	2.97

	increase > 2-fold		decrease > 2-fold
	increase > 5-fold		decrease > 5-fold

CHAPTER VI
DISCUSSION

The major cause of cervical cancer is persistent HPV infection, although a minor population (<1%) of HPV-negative cancer has been reported (Taghizadeh et al., 2017; Walboomers et al., 1999). HPV16 causes the majority of all cervical cancer cases (zur Hausen, 2002) and the most extensively studied mechanism of HPV carcinogenesis involves the overexpression of the known oncoproteins, E6 and E7. These oncoproteins have been demonstrated to contribute towards malignant transformation by mainly targeting p53 and pRb tumour suppressor proteins (Mantovani & Banks, 2001; Munger et al., 1992). Recent studies have indicated that other proteins of HPV such as E5 and E1, may also work in conjunction with E6 and E7 to drive the transformation of host cells (Maufort et al., 2010).

E1 is a helicase and the only enzyme encoded by HPV (D'Abramo & Archambault, 2011). E1 can interact with the host DNA replication machinery (Clower et al., 2006; Liu et al., 1998; Loo & Melendy, 2004), induce DNA damage, and compromise host defence (Castillo et al., 2014; Fradet-Turcotte et al., 2011). Interestingly, functions ranging from cell cycle arrest and proliferation inhibition, to induction of DNA damage have been attributed to E1, independent of the E6 and E7 proteins (Fradet-Turcotte et al., 2011; Sakakibara et al., 2011). It is likely that E1 has a role in regulating the host cell cycle. In this thesis, to better understand the role of E1 in cervical carcinogenesis, the expression pattern of E1 was analysed by first determining the expression levels of E1 in HPV16 positive samples. HPV16 E1 mRNA was used to determine expression instead of E1 protein detection due to the absence of a good commercial E1 antibody. The results revealed that E1mRNA expression significantly increased with disease severity (Figure 28). These findings are similar to a previous study by Schmitt et al who demonstrated the levels of E1 mRNA in LSIL patients were lower than those in HSIL and cancer patients (Schmitt et al., 2011). Expression of mRNA might be expected to correlate with the copy number of HPV16 genome in the cells. However, CaSki cells (600 copies/cell) and SiHa cells (1-2 copies/cell) had almost the same levels of E1mRNA expression,

measured as 0.13 and 0.06 (E1mRNA/ β-actin ratio), respectively. Thus, there is no correlation between E1mRNA expression and number of copies of HPV16 genome. A similar observation by Wang-Johanning et al (Wang-Johanning et al., 2002) also demonstrated that the expression of E6 and E7 mRNA in CaSki and SiHa cells are nearly identical, indicating that mRNA expression is governed by factors other than viral copy numbers. Therefore, potential additional mechanisms that could account for E1 transcriptional activity were explored.

The physical state of the HPV genome has always been believed to be a key phenomenon in disease progression. This holds true for HPV18 in which 98-100% of all cancer cases show complete integration of the viral genome in carcinoma; however, it has been shown in numerous studies that HPV16 shows a different integration pattern in conjunction with disease. For example, Zhang, et al found a 27.7% integration rate of HPV16 in precancerous lesions (Zhang, R. et al., 2014), and also detected a high number of samples with fully integrated HPV16 in normal samples (2/6, 33%), similar to a finding reported by Dutta et al, (27% integration in normal samples) (Dutta et al., 2015). However, full integration was not found in precancerous stages (CIN1-3) and only at 26.32% in SCC (Table 2). Although early integration was present, the majority of our samples contained the HPV16 genome in the episomal form (32/39, 82.05%), which was present in 73.68% of SCC cases, suggesting that the episomal form is important for HPV16 cervical cancer progression. Although integration-derived transcripts are more stable, episome-derived oncogene transcripts that express E6 and E7 oncoproteins are sufficient to induce centrosome abnormalities and genomic instability in raft cultures (Duensing et al., 2001; Jeon & Lambert, 1995). Using qPCR to differentiate between episomal and mixed forms, a total of 3 discrepancies (integrated form to mixed form) were found when compared to PCR. This is as expected because qPCR has been shown to be more sensitive than conventional PCR, being able to detect low levels of E2 genes in the samples (Mackay et al., 2002). The physical state of the viral genome was compared to the expression levels of E1, and no significant correlation was found (Figure 29). Thus, the levels of E1 expressed from either the episomal or integrated form are enough to help maintain the viral episome and carcinogenic phenotype in cells that have already been transformed.

In response to this finding, other regulative factors that could influence E1 expression were further investigated. The methylation of the E2BS positions was of interest, because under normal conditions, E2 is able to bind to different binding sites and activate or repress the expression of viral genes, including E1. In this study the CpG positions near the repressor E2BS were explored. Methylation of these E2BS positions inhibits E2 protein binding and reduces the ability of E2 to regulate the transcription of genes (Leung et al., 2015). The results indicate that methylation of the E2BS, does in fact increase significantly with disease progression (Figure 30). Oncoprotein overexpression is one of the main causes of cervical carcinogenesis, and it is possible that the hypermethylation of the repressor E2BS is a factor that supports oncoprotein overexpression in cancer patients. As E1 is required to maintain the viral episomal form, it may indicate that the presence of the episomal form is important in HPV16 related cervical carcinogenesis. The data showed that the episomal form of HPV16 was present in all normal, CIN 1, CIN 2/3 and SCC stages (Table 2). It is possible that the presence of the episomal viral DNA in CIN 2/3 and SCC stages was able to induce hypermethylation of the viral genome compared to normal and CIN 1 samples, but these samples still contained unmethylated (i.e. active) copies of viral genes, which can be expressed. In addition to the physical state of the virus, viral copy number may also influence methylation status and gene expression. The CaSki cell line which has approximately 600 copies of integrated HPV16, had a mean E2BS methylation of 65.8% (data from 4 positions showed 56%, 70%, 62% and 74%), while the SiHa cell line, which contained only 1-2 integrated copies, had a mean E2BS methylation of 0.5% (data from 4 positions showed 1%, 0%, 1% and 0%). These results, suggest that the viral copy number affects methylation but not E1 expression, similar to the E6/E7 expression phenomenon in which the expression level did not depend on viral copy number (Wang-Johanning et al., 2002). It has been previously noted that SCC samples, which have multiple copies of HPV, are also highly- methylated (Chaiwongkot et al., 2013). It is possible that our highly-methylated samples also have multiple copies of the viral genome. Unfortunately, the viral copy number for each sample was not determined due to limited sample amounts. Overexpression of oncoproteins is the primary cause of HPV related cancers, and this study shows that the methylation in SCC samples for the repressor

E2BS increased by more than 3-fold compared to normal samples. The significant increase in methylation levels of the repressor E2BS, at either each position or combined positions, supports the expression of oncogenic transcripts in transformed epithelium (Figure 30 and Figure 31). In contrast to the early promoter, no significant difference in the late promoter (p670) methylation at each position was detected, whereas methylation levels of combined positions were related to disease progression (Figure 31).

In addition to the expression and regulation of E1, this study also explored additional genetic events which could possibly be associated with E1 carcinogenesis. A previous study in Europe determined a novel variant form of E1 which contained a 63 bp duplication within the E1 ORF. This variant form has been further investigated and found to be associated with lower disease progression (Sabol et al., 2008). The results presented in this thesis revealed that although the 63 bp genomic variant of HPV16 was observed in the Thai population, it is rare (4.2%). However, all 63 bp positive samples were categorized as CIN 1 supporting the theory that this variant form of E1 is less virulent.

Although elevated E1 expression and E2BS methylation were observed with disease progression, a direct correlation still remains to be established and studies measuring expression of full length E1 mRNA expression are warranted. A recent study discovered a novel promoter for HPV16 E1, p14, and it would be interesting to study the E1 expression pattern of this novel promoter, in normal and CIN 1 samples, in addition to the CIN2+ samples, as previously described (Fedorova et al., 2016). In addition, the lack of a reliable E1 antibody was a limiting factor in investigating E1 protein expression in different clinical stages. Despite these limiting factors, the results suggest that E1 expression is highly likely to play some role in carcinogenesis and therefore, E1 functional assays *in vitro* are needed.

Initial *in vitro* experiments were performed to determine the effect of HPV16 E1 on cell proliferation and viability using HEK 293T cells transfected with either pEGFP or pEGFP-E1. After transfection, cells containing HPV16 E1 showed a significant decrease in cell proliferation by approximately 2-fold within 24 h post-transfection (Figure 35, Table 4). A similar effect was demonstrated in a previous study, which revealed that HPV31 E1was able to decrease cell proliferation in assays

measured after 3 weeks of transfection (Fradet-Turcotte et al., 2010). E1 consists of 4 different functional domains: the N-terminal domain (ND) which contains the nuclear localization and export signals; the DNA binding domain (DBD) which binds to the viral replication origin; the oligomerization domain (OD) which is responsible for oligomerization and finally the ATPase helicase domain (HD). The role of each of these domains on cell proliferation was explored by mutagenesis. Transfections using various E1-truncated-plasmid constructs including the complete E1 plasmid, indicated that only full-length HPV16 E1 significantly decreased cell proliferation (Figure 38, Table 6). The requirement of full-length HPV16 E1 for decreased cell proliferation has been confirmed in another study by Fradet-Turcotte et al. (Fradet-Turcotte et al., 2011). Therefore, mechanisms in which HPV16 E1 causes this phenomenon in HEK 293 T cells were further explored.

Decreases in cell proliferation or cell growth can be induced by cell cycle arrest and/or by cell death. In this thesis, death of E1-expressing cells increased in a time-dependent manner post-transfection, but only about 30% of cells were dead at 72 h post-transfection (Figure 39). Cell death can occur through two major pathways of apoptosis (programmed cell death) and necrosis. The results obtained in this thesis indicated that E1 expressing cells induced both apoptotic and necrotic cell death mechanisms, although apoptotic cell death was slightly higher (Figure 40, Table 7). Unfortunately, while decreased apoptosis after treatment with a pan-caspase inhibitor (QVD-OPH) was observed, this decrease did not reach statistical significance (Figure 44). One proposed explanation concerning E1 function was that accumulation of HPV31 E1 in the nucleus, via mutation of its nuclear export signal, was deleterious to cell growth (Fradet-Turcotte et al., 2011). Another study demonstrated that HPV18 E1 induced double stranded DNA breaks compared to mock transfected cells (Reinson et al., 2013). Studies have shown that HPV recruits proteins involved in the ATM DNA damage pathway to nuclear foci and that activation of ATM is required for viral genome amplification (Gillespie et al., 2012; Moody & Laimins, 2009). In addition to causing DNA damage, another example of the negative effect of E1 on cell growth is that HPV31 E1 overexpression has been shown to cause S phase cell cycle arrest, leading to decreased cell growth (Fradet-Turcotte et al., 2011). In combination, these studies illustrate that HPV E1 proteins induce DNA damage, restrict cell growth and

recruit DNA repair protein to facilitate viral replication. Several studies have revealed that E1 is able to interact with various host cell proteins and so microarray analysis was performed on E1-transfected and vector-transfected cells in order to establish the role of E1 in regulation of gene expression in infected host cells (Castillo et al., 2014). Microarray results revealed that E1 significantly altered expression of many genes that are involved in cellular pathways such as ribosome biogenesis, MAPK, PI3K-Akt, FoxO, NF-kappa B, and apoptosis signalling pathways. The expression of a total of 14 genes was increased in the ribosome pathway (Table 8) in E1 over-expressed cells, which indicates an increase in protein synthesis. In addition to their role in protein synthesis, numerous ribosomal proteins also have extra-ribosomal functions (Warner, 2009). For example, RPL36A, which was overexpressed in the microarray analysis, is upregulated in hepatocellular carcinoma and has been shown to be involved in increased cell proliferation (Kim et al., 2004). In addition to the upregulation of ribosomal proteins, a family of noncoding RNAs involved in ribosome biogenesis, termed snoRNAs, were highly upregulated following expression of E1 (Table 8). Pathways involved in cell proliferation and carcinogenesis were also regulated by E1 over-expression. For example, many genes in the MAPK, PI3K-Akt, FoxO, NF-kappa B, and apoptosis signalling pathways were dysregulated (Table 8). Consequently, RT real-time PCR was conducted to confirm microarray results. Since E1 is involved in cell proliferation, apoptosis and DNA damage, gene expression of "hallmark" genes in those pathways were selected and quantitated in HEK 293 T transfected with E1 at 12, 24, and 48 hours post-transfection (Table 10). Interestingly, E1 changed host gene expression in a time-dependent manner but genes in which expression was increased by E1, peaked at 24 hours post-transfection, such as ISG20, TNFRSF 10D, and RASD2 (Table 10).

Interferons (IFNs) are produced in response to viral infections and increased production of interferons, stimulate the expression of genes that combat infection. ISG20 belongs to a group of interferon-stimulated genes and was the most intensively upregulated gene by E1 (28-fold at 24 hours and 6-fold at 48 hours). E1 also upregulated interferon alpha inducible protein 27 (IFI27) by 2.9-fold (Table 9). This

is as expected and confirms the ability of the viral protein in inducing the transfected cells to elicit an innate immune response.

TNFRSF10D, also known as Decoy Receptor 2 (DcR2), is a member of the tumour necrosis factor receptor superfamily. This protein functions as a TRAIL receptor but cannot induce apoptosis because it lacks the death domain (Degli-Esposti, Mariapia A. et al., 1997). Consequently, DcR2 is considered an anti-apoptotic protein because it competes with pro-apoptotic TRAIL receptors. DcR2 is also known to induce NF-κB activation and may promote cell growth (Degli-Esposti, M. A. et al., 1997). A recent study of cervical cancer cells treated with TRAIL and cisplatin showed that cancer cells with decreased decoy receptor expression showed higher activation of apoptosis than cells with normal levels of decoy receptor protein (Narayan et al., 2016). The data from both the RT real-time PCR and microarray analysis indicates that E1 elevates the expression TNFRSF10D mRNA from 12-48 h post transfection. These findings suggest that HPV16 E1 induces the expression of TNFRSF10D perhaps to promote cell survival. Another interesting gene upregulated by E1 is SBSN which codes for a novel oncoprotein, Suprabasin, which has been implicated as an oncoprotein in the highly-invasive glioblastoma and esophageal cancer. An *in vivo* study found that mice inoculated with cells over-expressing suprabasin had tumours that grew more rapidly and were heavier than those that developed in mice inoculated with vector-only expressing cells. Mice inoculated with suprabasin-silenced cells showed stunted tumour growth compared to the vector control (Zhu et al., 2016).

Another gene significantly upregulated by E1 is RASD2, which codes for the Rhes protein, that is preferentially expressed in the striatum. Rhes is involved in activating Akt by binding to PI3K and facilitating the translocation of Akt to the plasma membrane, where it is subsequently phosphorylated and activated (Bang et al., 2012). Upregulated RASD2 gene might facilitate several cellular functions by phosphorylation through Akt. These findings, together with those described above, suggest that E1 upregulated gene expression supports cell survival and growth between 12-24 h post-transfection. As it is well known that E1 is expressed very early after HPV infection, E1-induced cellular gene expression may be essential to support the viral infected cells to survive. Interestingly, activated expression of all

up-regulated genes declined after 48 h post-transfection (Table 10) suggesting that the cells were beginning to undergo cell death possibly due to high E1 overexpression in the cells.

E1 also suppressed several cellular genes over the time period from 12 h to 72 h post-transfection. These genes included CREB5, HIF 1A, JMJD1C, FOXO3, NFKB1, PIK3CA, TSC22D3, ATR, BRCA1 and CHEK1. Three of these genes, CREB5, HIF 1A, NFKB1 are transcriptional factors. Cyclic-AMP Responsive Element Binding Protein 5 (CREB5) belongs to a family of transcription factors and inhibition of CREB5 expression by miR-449a was shown to cause cell cycle arrest and inhibition of cell cycle transition and proliferation. (Zhang, X. et al., 2016). From the results in this thesis, low expression of CREB5 might be related to decreased cell proliferation due to cell cycle arrest, which leads to cell death via apoptosis. Hypoxia-inducible factor 1 alpha (HIF1A)) is unstable under normoxic conditions but is expressed at higher levels and is stable under hypoxic conditions (Semenza, 2004). Expression of HIF1A is also promoted by the PI3K and Akt signalling pathways. A common characteristic of solid tumours is hypoxia, and recent investigations into cancer biology have identified HIF1A as a tumour survival gene (Masoud, Georgina N. & Wei Li, 2015). HIF1A is able to aid tumorigenesis by inducing many downstream genes involved in metabolism and also pro-angiogenic genes such as vascular endothelial growth factor (VEGF) which stimulates blood vessel growth, bringing more oxygen to tumour cells leading to tumour growth (Masoud, G. N. & W. Li, 2015). Nuclear factor-κB Subunit 1 (NFKB1) encodes for the DNA binding subunit of Nuclear factor-κB (NF-κB) protein complex which is a transcription factor with a wide range of downstream target genes. The NF-κB protein is involved in cell proliferation and induction of immune response mechanisms and has also been shown to regulate tumour growth and angiogenesis. Although NF-κB protein is mostly regarded as a proliferation stimulating protein, it is also anti-tumorigenic in certain tumours such as hepatocellular carcinoma (Xia et al., 2014). Decreased expression/activity of transcription factors, especially those involved in cell growth by HPV16 E1, would be beneficial to viral growth.

Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), a subunit of Phosphatidylinositol 3-kinase which regulates many cellular

processes including cell growth, adhesion, apoptosis, and survival, is also involved in phosphorylating Akt. Expression of PIK3CA has been studied in many cancers, and a study of PI3KCA in cervical cancer indicated a high expression of PIK3CA in cervical tumour samples (Ma et al., 2000b). However, another study in lung cancer indicated that high expression of PIK3CA was correlated to increased patient survival after treatment with the tyrosine kinase inhibitor, Gefitinib (Cantley, 2002; Endoh et al., 2006; Ma et al., 2000a).

Another group of genes that were regulated by E1 expression are involved in the DNA damage pathway. Jumonji Domain Containing 1C (JMJD1C) is a novel histone demethylase which has been shown to be decreased or absent in breast cancer cells (Watanabe et al., 2013). The protein is involved in regulating the BRCA1 branch of the DNA damage response. BRCA1 is a type of tumour suppressor protein which is required for DNA repair and plays a role in ensuring the stability of each cell's genetic material(Wu et al., 2010)). ATM and ATR proteins as well as their downstream proteins are mainly involved in DNA damage response (Awasthi et al., 2015). Recent studies have shown that E1 induces DNA damage which increases expression of DNA damage response proteins, such as ATM, ATR, CHEK1 and CHEK2: however, the opposite was observed in this study. ATM, ATR, CHEK1 and CHEK2 expression remained relatively unchanged at 12 and 24 h post transfection but decreased significantly 48 h post-transfection. Glucocorticoid-induced leucine zipper (GILZ) protein encoded by the TSC22D3 gene is involved in the Akt pathway and expression of this gene was suppressed by E1 by more than 7-fold. GILZ was able to disrupt the mTOR2C/AKT pathway in drug-resistant chronic myeloid leukemia cells leading to increased susceptibility to treatment (Joha et al., 2012a). GILZ also activated FoxO3a transcription of the pro-apoptotic protein, Bim (Joha et al., 2012b). E1 also decreased mRNA expression of ataxia telangiectasia and Rad3-related (ATR) protein which is involved in DNA damage, but it did not have an effect on ataxia-telangiectasia-mutated protein (ATM) expression (Table 10).

E1 also decreased the expression of genes coding for tumour suppressor proteins. For example, FOXO3 belongs to the FOXO subclass of the Forkhead family of transcriptional regulators and active FOXO proteins inhibit cell proliferation. Therefore, inactivation of FOXO proteins for example, by Akt phosphorylation, leads

to cell survival and growth (Zhang, X. B. et al., 2011). Numerous studies have been conducted on the relationship of FOXO proteins and tumorigenesis (Yadav et al., 2018). For example, one study of colorectal cancer patients and found that FOXO3 expression was decreased in tumour tissue samples. Low FOXO3 expression was also correlated to decreased patient survival time (Bullock et al., 2013).

Moreover, some genes were suppressed after 24 h post-transfection such as CDC25C and DDIT4. Cell division cycle 25C (CDC25C) belongs to the CDC25 group of phosphatases that regulate cell cycle by activating cyclin dependent kinase 1 (CDK1) (Boutros et al., 2007). During DNA damage, CDC25C is inhibited by the DNA damage response proteins CHK1 and CHK2, in order to promote cell cycle arrest and inhibition of CDKs. Because CDC25 proteins are tightly regulated during the DNA damage response, misregulation of these proteins may lead to genome instability (Boutros et al., 2007). E1 decreased the expression of DDIT4 by approximately 3-fold. DDIT4 gene encodes for a protein called Regulated in Development and DNA damage response 1 (Redd1), which induces dephosphorylation of Akt (Dennis et al., 2014) and could lead to decreased cell growth and DNA repair, or even cell death. The most down-regulated gene by E1 was INHBE which codes for a preprotein which is processed to form the beta E subunit of either inhibin or activin. Both proteins are known to regulate a wide range of cellular processes including apoptosis, growth, and immune response (Mayo, 1994). Because it is not possible to determine the final product of INHBE (i.e. either inhibin or activin which has opposing actions) by measuring mRNA, the effect of the decrease in INHBE expression on the cell cannot be determined. Interestingly, early E1 transfection increased INHBE compared to vector control cells, and then expression markedly decreased at 48 h post-transfection. It is possible that the helicase function of E1 cause excessive damage to the host genome, inducing cell death. When DNA damage occurs the apoptosis and other response pathways are activated. For example, p53, a protein involved in DNA damage response and also a major tumour suppressor protein is activated following DNA damage. However, p53 is also targeted and degraded by the oncoprotein HPV E6. Therefore the combined effects of decreased apoptosis caused by E6 and DNA damage by E1 may possibly lead to genome instability and transformation of the infected cells. Collectively, gene expression

analysis following E1 transfection signified its importance in host gene expression and pathway regulation.

It is generally believed that carcinogenesis occurs as a result of inhibition of apoptosis (Hanahan & Weinberg, 2011). The E6 and E7 proteins of HPV have been termed oncoproteins because they function to block apoptosis and support cell growth (Moody & Laimins, 2010). Interestingly, a recent study suggested that apoptosis drives carcinogenesis by sending survival signals to surrounding cells (Ichim & Tait, 2016). For example, breast-, colorectal-, non-small cell lung-cancer patients with high expression of the anti-apoptotic protein BCL-2 are associated with favourable prognoses (Meterissian et al., 2001; Neri et al., 2006). In mice, breast cancer xenograph models showed that apoptotic tumour cells stimulate tumour regrowth and proliferation in a caspase-dependent manner. During apoptosis, caspases activate calcium-independent phospholipase A2 (iPLA2) which increases production of arachidonic acid, which is converted by cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2) to prostaglandin E2 (PGE2) (Greenhough et al., 2009). Moreover, cervical cancer (CaSki) mice xenograph models have also shown significantly increased COX-2, EGFR, and p-Akt (Yoysungnoen et al., 2016). Results in this thesis also found an increased expression of the COX-2 gene by E1 (Table 10) which increases proliferation through the MAPK, and PI3K-Akt pathway. E1 not only increased the expression of genes involved in cell survival, but it also induced cell death and apoptosis. This suggests that HPV16 E1 may also induce carcinogenesis in a similar manner, since E1 is expressed at higher levels in cervical cancer patients.

Although this study was able to determine that HPV16 E1 affects many host cell pathways there were limitations in this study:

1. The cell line used in the transfection system is not a cervical cancer cell line and therefore may have different properties and respond differently compared to cells originating from the cervix. Thus in this study HEK 293T cells may not truly represent cervical cells in terms of their mechanisms resist/promote apoptosis and/or DNA repair.
2. The changes in host gene expression were not confirmed at the protein level.

3. The expression level of E1 in transfected cells could not be controlled at levels that are found in cancer patients. To illustrate this, in CaSki cells which have 600 copies of HPV16 integrated into its genome, RNA seq found approximately 12,000 RNA transcripts of all early genes combined (Chaiwongkot, personal communication). However, for HEK 293T cells transfected with pEGFP-E1, RNA seq quantitated more than 3,000,000 transcripts of HPV16 E1 alone. Therefore, the precise function of E1 during viral infection and cancer could not be concluded.
4. It is unclear whether E1 would cause extensive cell death under normal carcinogenic conditions because this study did not co-transfect E1 with other proteins involved in HPV carcinogenesis. For example, HPV E6 acts to reduce apoptosis by binding and degrading p53 while HPV E7 promotes cell cycle progression. It is possible that under optimal conditions and the presence of other HPV proteins, E1 may act synergistically to the other oncoproteins in order to drive carcinogenesis.

However, a major advantage of this study is that gene expression experiments were carried out by sorting on only E1 positive cells without the need for chemical selection. In addition, kinetic expression of E1 was performed which revealed the effect of E1 distinguished host gene expression relative to time. In conclusion, the dysregulation of gene expression can be attributed to the role of E1 alone.

Conclusion

In conclusion, HPV16 E1 was demonstrated to have significant positive correlation with cancer progression by using the novel ddPCR method to detect and quantify the amount of HPV16 E1 mRNA in all stages of cervical specimens, i.e., normal, precancerous, and cancerous stages. The detection of HPV16 E1 mRNA was shown to be a promising biomarker for monitoring cervical cancer. The level of methylation at the E2BS (early promoter p97 of position (p) 37, 43 and 58) were also demonstrated to be beneficial as a prognostic marker. This study detected a high number of samples with fully integrated HPV16 in Normal samples (2/6, 33%), but no full integration was found in precancerous stages (CIN1-3) and only 26.32% in SCC. Although early integration was present, the majority of the samples contained the HPV16 genome in the episomal form (32/39, 82.05%). The episomal form was present in 73.68% of SCC cases, suggesting the episomal form is important for HPV16 cervical cancer progression. It was noted that no correlation between physical state of HPV genome and HPV16 E1 mRNA was found. The 63 bp duplication in HPV16 E1 was presented in the Thai population and related to low grade lesions. Role of HPV16 E1 protein was explored using in vitro transfection system. This study illustrated for the first time that the presence of HPV16 E1 protein alone was able to dysregulate many cellular signalling pathways early after protein expression (within 12 hours post-transfection). HPV16 E1 was able to upregulate genes involved in innate immune response, cell proliferation and survival, and downregulated genes involved in cell cycle control. However, overexpression of HPV16 E1 for long durations within the cell (after 24 hours post transfection) was detrimental to the host cell leading to cell death by either apoptosis or necrosis or both. Collectively, data from this study suggests that E1 may play a role in cancer development probably by deregulating cellular gene expression. In addition, HPV16 E1 itself may also interact with some cellular proteins to support the oncoprotein HPV16 E6 and E7 functions. However, HPV16 E1 transfection experiments did not provide a definitive carcinogenic pathway, which warrants further studies.

Further studies

In order to determine a clinically relevant cut-off point in using E1 as a biomarker for cervical cancer progression a larger cohort must be recruited.

To better understand the role of E1 in cervical carcinogenesis, HPV negative cervical cancer cells that do not have deficiencies in proteins involved in DNA damage response and apoptotic proteins should be used. In addition, the expression E1 should be controlled at a level similar to E1 protein expression in CIN/cancer cells. Expression control can be achieved through the use of a weaker promoter or translation control using a tunable synthetic translation inhibition (Stapleton et al., 2012). For example, a tunable synthetic translation inhibition system can be achieved by using a RNA/protein interaction translation switch (Figure 47). Once the expression level of E1 is controlled, the effect of E1 on host cell processes can be more accurately evaluated.

In addition, the presence of other viral proteins may also play an important role in carcinogenesis. Co-transfection experiments with E1 in the presence of either E6 or E7 or both E6 and E7 may give better insight into the synergistic effects of these proteins.

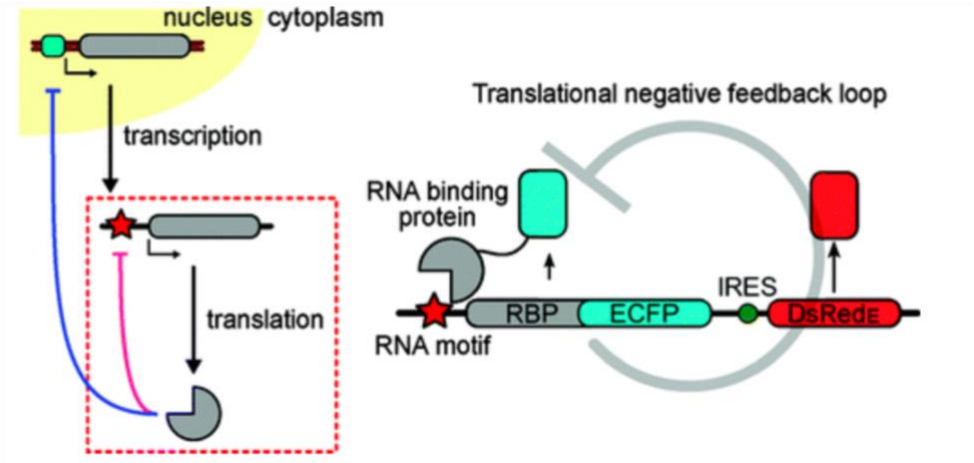

Figure 47. Schematic diagram of the feedback repression construct.
The protein of interest is linked to the protein L7Ae, which regulates the translation of its own mRNA which in turn regulates the fused protein of interest. (Stapleton et al., 2012).

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APPENDIX A
REAGENTS, MATERIALS AND INSTRUMENTS

Reagents

Absolute ethanol	(Merck, Germany)
Agarose	(Research organics, USA)
APC-conjugated annexin V	(Biolegend, USA)
Cell counting kit	(Biolegend, USA)
Dimethyl sulfoxide (DMSO)	(Sigma-Aldrich, Germany)
DMEM medium	(GE Healthcare Life Sciences, USA)
DNA Ladder	(Apsalagen, Germany)
DNase I	(Sigma-Aldrich, Germany)
DNase/RNase-free water	(Apsalagen, Thailand)
dNTP	(Fermentas, Canada)
Droplet digital PCR master mix	(Bio-rad, USA)
Ethylenediaminetetraacetic acid (EDTA)	(Sigma-Aldrich, Germany)
Fetal bovine serum	(Gibco, USA)
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	(Sigma-Aldrich, Germany)
Isopropanol	(Merck, Germany)
LB broth powder	(OXOID, UK)
LB agar powder	(OXOID, UK)
NaHCO3	(Bio Basic, USA)
Opti-MEM™ I Reduced Serum Media	(Gibco, USA)
Penicillin/Streptomycin	(Gibco, USA)
Phosphate buffered saline (PBS)	(Apsalagen, Germany)
Plasmid extraction	(Machery-Nagel, Germany)
Primers	(Integrated DNA Technologies, Singapore)
Reverse transcription kit	(Invitrogen, USA)

Sodium pyruvate	(Gibco, USA)
SYBR® Green gel stain	(Invitrogen, USA)
Transfection reagent	(Roche, USA)
Tris-boric acid EDTA buffer	(Research organics, USA)
Tris-HCL	(Machery-Nagel, Germany)
Trypan blue	(Sigma-Aldrich, Germany)
Trypsin	(Bio Basic, U.S.A.)
Trypsin-EDTA	(Gibco, USA)

Materials

Barrier tips	(Sorenson, USA)
Cryotubes (2 mL)	(Sarstedt, Germany)
DNase/RNase free clear tubes (1.5 mL)	(Axygen, USA)
Filters (0.22 µM and 0.45 µM)	(Merck Millipore, USA)
Pipette tips	(Sorenson, USA)
Round-bottom polystyrene tubes (5 mL)	(BD Falcon™, USA)
Sterile serological pipettes	(Eppendorf, Germany)
Sterile tubes (15 mL and 50 mL)	(Becton Dickinson, USA)
Tissue culture flasks	(Nunc™, Thermo Fisher Scientific, USA)
Tissue culture plates	(Nunc™, Thermo Fisher Scientific, USA)

Instruments

Autopipettes	(Thermo Fisher Scientific, USA)
BD FACSAria™ II flow cytometer	(BD Biosciences, USA)
Biosafety cabinet	(Labconco, USA)
Centrifuge	(Eppendorf, Germany)
Confocal microscope	(Olympus, Japan)
Droplet generator	(Bio-rad, USA)
Droplet reader	(Bio-rad, USA)
Electrophoresis chamber	(MiniRun GE100, China)
FlowJo® version 10	(FlowJo, LLC, USA)

Freezer (-80°C)	(Thermo Fisher Scientific, USA)
Incubator	(Thermo Fisher Scientific, USA)
Inverted fluorescence microscope (IX81)	(Olympus, Japan)
Inverted light microscope	(Nikon, Japan)
Liquid nitrogen tank	(Chart/MVE, USA)
Nalgene® Mr. Frosty cryopreserve box	(Sigma-Aldrich, Germany)
NanoDrop™ Spectrophotometer	(Eppendorf, USA)
Quantitative real time PCR	(Applied Bioscience, USA)
Refrigerated centrifuge (Allegra X-15R)	(Beckman Coulter, USA)
Thermal cycler	(Bio-rad, USA)
Vortex mixer	(Brand, Germany)
Water bath incubator	(Grant, UK)

APPENDIX B
PREPARATION OF REAGENTS

Cell culture

1. Complete culture medium for SiHa and CaSki cells

DMEM	180 mL
Heat inactivated fetal bovine serum	20 mL

2. Complete culture medium for HEK 293T cells

DMEM	180 mL
Heat inactivated fetal bovine serum	20 mL
100 mM sodium pyruvate	200 µL

3. 1M HEPES

HEPES	23.83 g
Deionized distilled water	100 ml

Sterilized by autoclaving and stored at 4 °C

4. 10% $NaHCO_3$

NaHCO3	10 g
Deionized distilled water	100 ml

Sterilized by autoclaving and stored at 4 °C

5. 1X PBS

10X PBS	100 ml
Deionized distilled water	900 ml

Sterilized by autoclaving and stored at room temperature

Plasmid amplification

1. LB broth

LB broth powder	30 g
Distilled water	1 L

Sterilized by autoclaving 1. LB broth

2. LB agar

LB agar powder	40 g
Distilled water	1 L

Sterilized by autoclaving