The carcinogenic properties of naturally occurring human papillomavirus 16 E6 oncogene variants

A thesis presented to

The Faculty of Graduate Studies

of

Lakehead University

by

SARAH NICCOLI

In partial fulfillment of requirements
for the degree of
Master of Science in Biology

July 19, 2012

ABSTRACT

Human papillomavirus is the causative agent of the 2nd highest occurring cancer in women, cervical cancer, which is the result of expression of the E6 and E7 oncogenes. Here we challenged the dogma that the oncogenic protein E6 requires co-expression of E7 for malignant transformation of human keratinocytes using common HPV16 E6 variants that are highly associated with cervical cancer: the Asian-American E6 variant (AAE6) and L83V variant of HPV16. The E6 variants containing the L83V amino acid mutation are more frequently detected in cervical cancer than the corresponding prototype HPV 16 as evidenced by independent epidemiological data. We set out to analyze in a cell culture study how these variants in the absence of E7 perform during vital steps of carcinogenesis and assessed their ability to immortalize and transform primary human foreskin keratinocytes (PHFKs). Their migration ability, a hallmark for invasiveness and/or metastasis, was also investigated. Immortalization capability was based on population doublings, number of passages, surpassing Mortality Stages 1 and 2, telomerase reverse transcriptase expression and the ability to overcome G1 arrest via p53 degradation. Transformation and migration efficiency were analyzed by a combination of functional cell-based assays. For the first time, we observed that all E6 proteins alone were sufficient to immortalize PHFKs, with the AAE6 variant being the most proficient. The AAE6 variant protein alone also pushed PHFKs through transformation and significantly increased their migration ability over that of the PHFKs expressing L83V and E6 prototype proteins. Our findings are in line with epidemiological data that the AAE6 variant confers an increased risk over the prototype for cervical cancer as evidenced by a superior immortalization, transformation and metastasis potential.

LAY SUMMARY

Faculty and students in the Department of Biology are bound together by a common interest in explaining the diversity of life, the fit between form and function, and the distribution and abundance of organisms. The purpose of the study undertaken here was to investigate the cancer-causing ability of different gene variants of human papillomavirus 16, a cause of many types of cancers today. The variations occur among the cancer-causing gene called E6 and the ones which were studied are the Asian-American variant, the L83V variant, and the European prototype. This is a field which contains a large knowledge gap regarding the function of these variants and the resulting diversity in cervical cancer in the human population. Experiments revealing the ability of these different viral genes to immortalize and transform cells into a cancerous phenotype were completed and showed that the Asian-American gene variant was more powerful at completing these steps. This correlates with epidemiological studies which found this viral variant over the others in more cervical cancers. These results are of academic interest in that they assist in clarifying the function of these viral genes in the process of cancerous development, which in turn can be built upon for translational research.

DEDICATION

To my family for their love and support.

ACKNOWLEDGMENTS

First, I would like to thank my supervisor, Dr. Ingeborg Zehbe for this opportunity. The past two years have enhanced my respect for and my interest in research and increased my enthusiasm for cancer research. It would not have been possible without your guidance, knowledge and support. Also thank you for hosting so many fun after-lab events! I think we are all in a little better shape because of the outdoor adventures you took us on!

I would also like to thank my committee members, Drs. Dave Law and Heidi Schraft, for their knowledge, encouraging words and their support during some stressful times. You guys were great!

To the TBRRI lab members, especially Rob, Melissa, Suraj and Dexter. You have all helped me countless ways in the past two years, from bouncing ideas for experiments off of each other to making me smile every day. Rob: your statistics-powered brain ensured I did my analyses properly! Melissa: thank you for lending an ear during those stressful days! Suraj: your suggestions for experiments were excellent and really rounded out my thesis. You were also extremely helpful when it came down to editing my thesis and preparing for my defense. Thank you! Dexter: You are just great. I don't need to say much more than that!

To the scientists of the TBRRI for making this a great learning environment with all of your combined interdisciplinary knowledge. It was a real pleasure working with everyone in different fields of study and getting a chance to participate in project collaborations!

Thanks to Dr. Dave Law and his lab members for allowing me to use their Experion for my experiments!

Also thanks to Dr. Greg Pyle for showing me the wonderful world of statistics! Because of Biostats, I will never think of experimental design the same way! Also thanks to Sir

Billiam of Canterbury (Bill Dew) for answering all of my ridiculously titled emails about stats. I could not have conquered R without you!

PERSONAL ACKNOWLEDGEMENTS

To my best friend Nat: I think we were twins in another life. I am so glad we became as close as we are now! Something good came out of all of our mutual friends leaving us for big cities! I think you are the only person I know who enjoys imagining your future home and decorating it in your mind as much as I do ("oh wow, imagine this on an end table…"). We are such Marthas! It is even more awesome that we both share an extreme (probably unhealthy) love of food and baking. I can't wait to expand our pseudo-cupcake business! Also, thank you for always being there for me, and going on Martha adventures with me. I hope we keep making lists together for years to come!

To Mike: thank you for always making me smile, and for your encouraging words. And thank you for reminding me there is life outside of the lab! Most of all, thanks for being there for me.

To Rob: oh lab husband, where do I start? So many hilarious quotes on the whiteboard ("McBraids in cryo", "Spin me, Jackson", "a sick obsession with low confluency" etc.). Thanks for rapping Kanye with me throughout the lab, and thanks for slurping your pop extremely loudly. You were a key part in my every day lab life and I couldn't have done it without you!

To the rest of my good friends: Thank you all so much simply for being your awesome selves! Specifically, thank you Heidi for being my Biostats crew partner in crime, especially when Mike and Rob were watching Archer while we worked our butts off! Thanks Esa for our many sushi-filled hang outs, they were delightful and delicious! And thank you Alicia and Tones! You made the lab so excellent and happy, and were always up for a good time. I miss you guys! Also thanks to Caroline, Kristin, Rim (DB for life),

Bromontana, Meghan, Tyne, Sam, Crystal and Duska for being a constant source of encouragement and hilariousness! So many good times, not enough room here to talk about them all!

Lastly, thank you to my family, the most important people in my life. I am so lucky to have such a great family who support me in everything I do. You mean the world to me. I love you and thank you! And thanks to my kittens, for being cuddly and cute.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AAE6	Asian American HPV16 E6 gene variant
AD	Actinomycin D
BSA	Bovine serum albumin
C33A	A cervical cancer cell line without HPV
CAIX	Carbonic anhydrase IX
cDNA	Complementary deoxyribonucleic acid
Ct	Cycle threshold
DDW	Double distilled water
DMEM	Dulbecco‟s minimum essential medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotides
DU145	A prostate cancer cell line
E. coli	*Escherichia coli*
E1-E7	Early genes of HPV
E6-AP	E6-Associated Protein
EMEM	Eagle‟s minimum essential medium
EMT	Epithelial-mesenchymal transition
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
G418	Gentamicin
GFP	Green fluorescent protein
HeLa	A cervical cancer cell line from Henrietta Lacks
HIF-1	Hypoxia inducible factor 1
HPRT1	Hypoxanthine phosphoribosyltransferase
HPV	Human papillomavirus
HRP	Horseradish peroxidase
hTERT	Telomerase reverse transcriptase
IDH	Isocitrate dehydrogenase

IFN	Interferon
KGM	Keratinocyte growth medium
L1/L2	Late genes of HPV
LCR	Long control region
M1	Mortality Stage 1
M2	Mortality Stage 2
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHFK	Primary human foreskin keratinocytes
PMSF	Phenylmethylsulfonyl fluoride
pRb	Retinoblastoma protein
PVDF	Polyvinylidene difluoride
qRT	Quantitative real time
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	Small interfering ribonucleic acid
TBST	Tris-Buffered Saline with Tween 20
TCA	Tri-carboxylic acid
TEMED	Tetramethylethylenediamine

1.0 INTRODUCTION

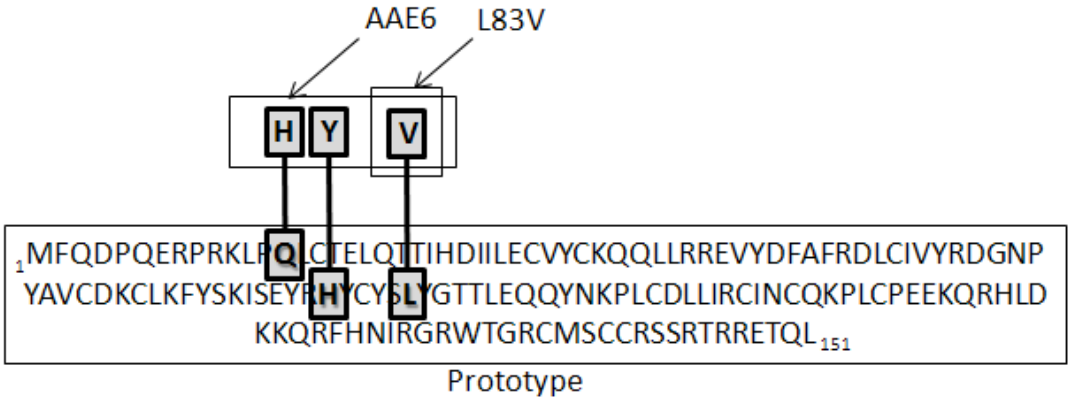
In 2008 alone there were over 529,000 new cases of cervical cancer diagnosed, with most occurring in developing nations. Cervical cancer leads to the third highest mortality rate among all cancers in women, after breast and lung cancer (58). Etiological studies have shown a strong association between human papillomavirus (HPV) infection and cervical cancer (97). Hence it is important to improve our understanding of HPV-induced carcinogenesis and appropriately elucidate the underlying molecular and cellular mechanisms involved. This will help develop improved screening procedures, better diagnostic and treatment approaches, as well as accelerate development of appropriate druggable targets.

1.1 Human Papillomavirus

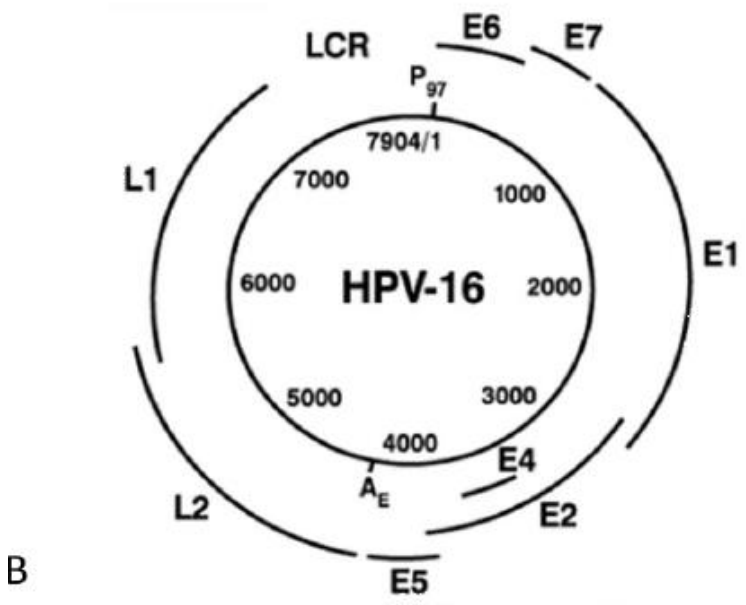
Human Papillomavirus (HPV) is a double-stranded DNA virus belonging to the family *Papillomaviridae*. Members of this family infect the squamous epithelial cells of the skin and mucous membranes in humans. HPV infection is implicated in virtually all cervical cancers worldwide (97), making it the most important focus of studies regarding cervical cancer.

There are over 100 types of HPV which can be divided into low- and high-risk categories based on their oncogenic potential. High-risk HPV types are prevalent in malignant tumours due to a better ability to immortalize and transform the host keratinocytes while low-risk HPV types are prevalent in benign lesions (68). HPV 16 is the most commonly known high-risk type (97), and within this type variation occurs in the genome sequence. The prevalence of these variants differs among populations (11, 93), and the variants are distributed between the continents: European (E), Asian-American (AA), African, and Asian (5) (Fig. 1 a). Epidemiological studies show differences in the ability of these variants to cause malignancies, and through these studies it has been suggested that the AA variant is most aggressive (2, 5, 12, 83, 91, 92, 95). The HPV genome consists of three regions: the long control region (LCR), the upper

regulatory region, and the early (E) and late (L) gene region (Fig. 1 b). The LCR controls the production of viral proteins by regulating transcription of the early and late regions. The late region encodes structural proteins; L1 and L2 proteins are the major and minor capsid proteins, respectively. Both are expressed later during the infection in the upper epithelial layers during differentiation and they encapsulate the virus (96). The early region contains six open reading frames and is expressed in the basal layer. E1 and E2 proteins are required for the initiation of viral genome replication. The E4 protein binds to proteins within the infected cells and contributes to the release of virions, and the E5 protein has been shown to prevent apoptosis of DNA damaged cells (96). E6 and E7 are the major proteins involved in the carcinogenesis of the HPV virus. E6 is an oncoprotein that is found in the nucleus and cytoplasm of HPV infected cells (69, 96) and it interacts with p53, a tumour suppressor gene that is activated by DNA damage. p53 activation leads to cell cycle arrest and apoptosis of the damaged cells which assists in lowering the levels of possible mutations and therefore also the spread of viral infections (96). E6 binds to p53 in a ternary complex with E6 Associated Protein (E6-AP) (78) and tags the p53 for degradation by ubiquitylation. This results in failure of p53‟s function which allows DNA replication to continue after damage has occurred (96). Because of this, E6 can overcome cell cycle arrest which contributes to its oncogenic potential. E7 is another oncoprotein of HPV which can cooperate with E6 but can also act independently. It binds to the retinoblastoma protein (pRb) which releases E2F transcription factors. This leads to constitutive activation of E2F proteins which activate genes required for DNA synthesis. Therefore, by binding to pRb and effectively blocking it, E7 overcomes cell cycle arrest (96). Overall, both E6 and E7 function in surpassing growth arrest induced by DNA damage, and create chromosomal instability. Both of these conditions create the basis for the formation of a tumour mass.



A



B

Figure 1. The HPV genome. (A) The amino acid sequence of the European prototype E6 oncoprotein with changes resulting in the Asian-American (AAE6), and L83V variants highlighted. The sequence has amino acids 1-151 and the AAE6 variant corresponds to the following changes: a glutamine (Q) to a histidine (H) change at the 14th amino acid, a histidine to a tyrosine (Y) change at the 78th amino acid, and a leucine (L) to a valine (V) change at the 83rd amino acid. Only the last change corresponds to the L83V variant. (B) This depicts the organization of the early and late genes of the HPV 16 genome. The early genes are E1, E2, E4, E5, E6 and E7. The late genes are L1 and L2. The Long Control Region (LCR) regulates the transcription of these genes. Taken from Beutner *et al.* (6).

About one-third of the known types of HPV infect the genital epithelia (26). The HPV viral genome begins its non-productive stage in the basal layer where it enters the cells. Here, it is established as a low copy number episome having its DNA replicated once per cell cycle. Afterward, it enters the productive stage in the suprabasal layer. Here, the DNA replication method switches from bidirectional theta replication to the rolling circle model which causes the DNA to reach a high copy number. The L1 and L2 capsid proteins are then synthesized and viral assembly occurs after which virions are released into the environment (Fig. 2) (22). Before HPV infection causes cervical cancer, HPV DNA must become integrated into the host genome. During this integration, the E2 gene expression is interrupted, and E6 and E7 expression is dysregulated (37). Cells containing integrated viral DNA proliferate more than cells containing episomal viral DNA (37), suggesting an increased potential for tumour formation, and therefore of cervical carcinoma. However, DNA integration only occurs after persistent infection from a single virus or multiple HPV types. Even after DNA integration neoplasias can be eliminated by the immune system so that only 0.8% of initial high-risk HPV infections develop into invasive cancer (96). One of the initial steps of the carcinogenesis process following infection with HPV 16 is cellular immortalization which was found to be mediated through the E6 and E7 oncogenes (32, 41, 56).

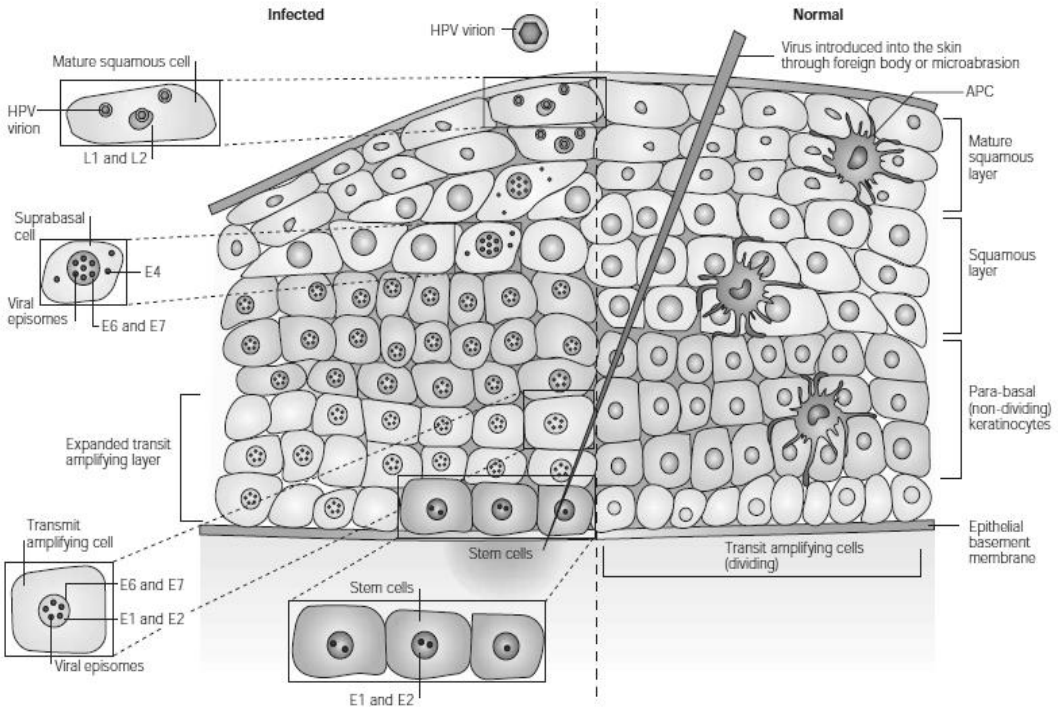


Figure 2. The expression of the various genes of HPV 16 during the infection process. In the basal layer, E1 and E2 initiate viral replication and the E6 and E7 oncogenes are expressed. Once infected cells reach the suprabasal layer, E4 is expressed and readies the cell for virion release. Once in the squamous cells, L1 and L2 are expressed and viral particles can be released to the environment. Taken from Frazer *et al* (27).

1.2 The Role of HPV16 E6 and E7 Oncogenes in Cellular Immortalization

In 1989, several researchers published reports originally showing that the E6 and E7 oncogenes were the cause of cellular immortalization in HPV 16 positive primary human keratinocytes (32, 41, 56). These articles showed that when keratinocytes were

transfected with E6 or E7 oncogenes or both, it was necessary that E6 and E7 oncogenes cooperated to accomplish immortalization.

Over the years, immortalization has been defined in numerous ways. During the time when Hawley-Nelson *et al* (32), Munger *et al* (56), and Kaur *et al* (41) published their papers, little was known about the causes of cell immortalization. As a result, these articles defined immortalization only as „extension of lifespan" and their experimental methods were suboptimal as gene technology at that time was rudimentary. In their studies, direct transfection was employed because amphotropic high titre virus technology was not yet developed for transducing primary human foreskin keratinocytes (PHFKs) (55) which would mimic conditions occurring *in vivo.* As more research was conducted through the years, the process of cell immortalization was partially resolved. In 1985, (29) Greider *et al.* discovered the enzyme telomerase, but it was not until 1992 when Counter *et al* showed the link between telomerase expression and immortalization (15). This seminal report provided the foundation to further expand cell immortalization research. It is now known that the point when cells become immortalized is actually defined by an immortalization crisis, referred to as mortality stage 2, "M2" which takes place after a period of replicative senescence, mortality stage 1, "M1" (9, 74, 75, 77, 87). During M1, cells can remain viable for months then escape from this stage, resulting in extended lifespan which can be confused for immortalization. This can occur when cells express certain viral particles as in the case of high risk HPV type 16 infections (9). M2 will occur once telomeres reach a critically short length and apoptosis is induced (9, 53, 66). The process of escaping from this stage has been associated with the stabilization of telomere length through reactivation of telomerase (9, 53, 66), which was observed to be promoted by the E6 oncoprotein of HPV 16 (48, 54, 63, 77, 82).

1.3 The Role of HPV16 E6 and E7 Oncogenes in Cellular Transformation

Cellular transformation is another important part of the HPV-induced carcinogenic process. During this time, PHFKs attain a malignant phenotype which allows for formation of colonies that are resistant to harsh conditions, therefore producing

a microenvironment conducive to tumour growth (56). This can be seen when transformed cells successfully avoid detachment-induced death, or „anoikis" (28). When normal keratinocytes lose their ability to adhere to a substratum and to each other, terminal differentiation is induced (1) and cells commit to anoikis (28). Overcoming this could be supported by HPV 16 infection, whereby the E6 protein may counteract the pro-apoptotic effects of p53 (71). Detachment conditions can be recreated *in vitro* through the use of semisolid medium (1). In addition, the ability of the cells to withstand serum starved conditions that can promote cell colony formation indicates the transformational ability of the cells (25).

1.3.1 Invasion and Migration

To complete the picture of carcinogenesis, invasion and migration ability must be assessed. Invasion of the tumour into the basement membrane is the first step in the metastasis cascade, the most deadly part of carcinogenesis. Following invasion, intravasation occurs when the primary tumour cells enter either lymphatic or blood microvessels. Here, they have access to nutrients and oxygen. If the cells enter blood microvessels, they may then be transported to distant sites throughout the body. However, this is a very hostile environment where cells may not survive. If the cells make it through the circulation, they can become trapped within vessels of other tissues and organs after which they must extravasate through interactions between vessel walls to form micrometastases. Eventually, certain micrometastases may acquire the ability to colonize the tissue/organ they have become trapped in, and a macrometastasis is formed. Once this occurs, the probability of developing more macrometastases from the first one is heightened since this secondary tumour has already acquired the ability to colonize tissues and organs (85). Before the metastasis cascade can proceed, the cells must undergo an epithelial-mesenchymal transition (EMT) which involves cells acquiring a mesenchymal phenotype in preparation for detachment from the epithelial sheets (85). One important gene that is down-regulated during this process is E-cadherin which mediates cell-cell attachment. It has been shown by Matthews *et al.* (51) that E6 gene expression down-regulates expression of E-cadherin as well, which potentially induces an EMT, suggesting this gene is an interesting subject for further study.

1.4 Mechanisms Causing Differences in Oncogenic Potential

As previously mentioned, certain E6 oncogene variants are found more often than others in different populations (2, 5, 12, 83, 91, 92, 95) and through these studies, it is suggested that the AAE6 variant is more aggressive than others. In an effort to reveal the mechanisms behind the differing prevalence and oncogenic potential of these E6 oncogene variants, metabolic pathways were studied. Carbonic anhydrase IX (CAIX) was chosen as an enzyme of interest since it is known to be regulated by hypoxia inducible factor 1 (HIF-1), a transcription factor affected by isocitrate dehydrogenase (IDH) (72). This was an enzyme found to be expressed differentially between the E6 gene variants in the presence of E7 (67). IDH is necessary for α-ketoglutarate production which is then used for hydroxylation of HIF-1α in normoxic conditions. Therefore, a decrease in IDH as was seen by Richard *et al.* (67) would result in an increase of HIF-1α and therefore HIF-1 (72). So, as a downstream gene in the HIF-1 pathway, CAIX is a viable target for observation as an indicator of enhanced metabolism and therefore oncogenic potential.

1.5 Current Understanding of HPV 16-Induced Carcinogenesis

Many researchers have followed the perception that E6 and E7 must work together to induce immortalization in human keratinocytes (18, 39, 43, 48, 49, 82), while Halbert *et al.* (31) observed that E7 alone was sufficient for immortalization. However, none of these articles were able to show a complete picture of immortalization as they relied only on one segment of the complex process as an indicator, such as extension of lifespan (31), inactivation of the retinoblastoma protein (39), or increased telomerase expression (18, 43, 48, 49, 82). Also, there has not been a study focused on the differences in immortalization and transformation ability between the different E6 oncogene variants alone.

Previously, the differences between AAE6 and prototype E6 gene variants along with E7 have been extensively studied through 2D-gel electrophoresis (67). The findings

have shown differences in expression of eight proteins involved in metabolic functions between the prototype E6 variant and variants containing the L83V mutation. These include enzymes of amino acid metabolism, the tri-carboxylic acid (TCA) cycle, and glycolytic pathways (67). These changes are some of what gives the AAE6 variant a clear advantage in *in vitro* immortalization and transformation over the prototype E6 because a higher metabolism indicates increased proliferative potential and survival (67). This opened the potential for further study into metabolic pathway changes between the different E6 oncogene variants.

The purpose of this study was to observe the ability of E6 oncogene expression alone to immortalize and transform keratinocytes, and thereby increase the ability of the primary keratinocytes to invade and migrate into surrounding tissue. Clarifying the ability of the E6 gene alone to perform the initial steps in cervical cancer progression will allow for more focused future work on HPV 16-induced carcinogenesis.

1.6 Hypotheses and Rationale

1) The E6 oncogene alone can immortalize PHFKs through activation of telomerase mRNA synthesis and deactivation of p53 protein, irrespective of the HPV 16 variant.

   Until now, no long-term study has been performed to challenge the concept that E6 and E7 are needed for immortalization, or to highlight the differences that may be seen between individual naturally occurring E6 variants without the help of E7. We propose a complete definition of the immortalization process using the keratinocyte model: PHFKs in culture surpassing M1 followed by a clear crisis (M2) and an increase in hTERT expression.

2) Expression of the AAE6 oncogene alone will cause the PHFKs to show the first signs of malignant transformation.

Previously, the E6 and E7 oncogenes have been shown to be necessary for PHFK transformation (56) but the ability of the different E6 oncogene variants alone to transform PHFKs has not been observed, and the model used in the current study, PHFKs transduced by a viral particle, is more conducive to what is occurring *in vivo* than the model used by Munger *et al.* (56), PHFKs transfected with a plasmid. Results previously seen by our group show that the AAE6 oncogene in the presence of E7 formed colonies in semisolid medium (67), suggesting they are transformed.

3) Expression of the AAE6 and L83V E6 oncogenes alone can increase PHFK‟s invasive and migratory ability compared to the E6 prototype-transduced PHFKs.

Because metastatic tumours are usually found in vital organs, more than 90% of deaths from cancer are caused by these metastases (85). It is therefore extremely important to characterize the E6 oncogene‟s ability to increase invasion and migration capabilities of PHFKs. It has been shown previously in epidemiological studies (2, 95) that the HPV16 E6 oncogenes containing the L83V mutation in the presence of E7 are found more frequently than the E6 prototype in invasive carcinomas. In addition the AA variant has been found 20 times more often in cervical cancer than the prototype in the Americas (5).

2.0 MATERIALS AND METHODS

2.1 Cell Culture and Propagation

2.1.1 Cell Lines

All cultures were incubated at 37^{o}C in a humidified atmosphere of 95% air and 5% CO_2. Cells were grown in flasks of sizes 12.5 cm^2, 25 cm^2, or 75 cm^2.

Primary Human Epidermal Keratinocytes (PHFKs, Cell Applications Inc, San Diego CA, cat. # 102-05n) were cultured in Keratinocyte Growth Medium, Serum-Free (Cell Applications Inc, San Diego, CA, cat. # 131-500). To passage these, the cells were incubated with trypsin which was neutralized with trypsin neutralizer (Cell Applications Inc, San Diego, CA, cat. # 080-100). To start the next 75 cm^2 flask, 300,000 cells were seeded.

Various other cancerous cell lines used as controls in a number of experiments were cultured as follows: A prostate cancer cell line DU145 (American Type Culture Collection (ATCC), Manassas VA, cat. # HTB-81) was cultured in Eagle"s Minimum Essential Medium with Earle"s Balanced Salt Solution (EMEM, Cedarlane, Burlington ON, cat. # 30-2003) and cervical cancer lines HeLa (HPV 18, ATCC, Manassas VA, cat. # CCL-2), and C33A (HPV negative, ATCC, Manassas VA, cat. # CRM-HTB-31) were cultured in Dulbecco"s Minimum Essential Medium (DMEM, Sigma, St. Louis MO, cat. # D5796) supplemented with 10% fetal bovine serum (FBS, Sigma, St. Louis MO, cat. # F6178) and 1% antibiotics/antimycotics (Invitrogen, Carlsbad CA, cat. # 15240-062). To passage these, the cells were incubated with trypsin which was neutralized with media. A dilution of 1:10 was performed for the next starting flask. Phoenix cells used for viral transductions (a gift from Dr. Garry P. Nolan) were cultured following the same procedure as HeLa and C33A.

2.1.2 Cryopreservation of Cells

Left over cells were resuspended in 1 mL of freezing solution (10% dimethyl sulphoxide (DMSO, Sigma, St. Louis MO, cat. # 472301), and 90% respective culture medium). Cells were put into cryotubes then into an isopropanol-containing freezing unit overnight in the -80^{o}C freezer. The cells were then transferred to liquid nitrogen storage.

2.1.3 Thawing Cells

The cells were thawed from liquid nitrogen storage in a 37^{o}C water bath. All contents of the cryotube were added to 9 mL respective culture medium and the solution was centrifuged for 5 minutes at 25 x g (Beckman GS-6KR Centrifuge, Mississauga,

ON). The supernatant was aspirated and the cell pellet was resuspended in the appropriate culture medium and transferred to a flask.

2.1.4 Protein Extraction for Western Blotting

After storage at -80^{o}C the cell pellet was washed with 1 X Dulbecco‟s Phosphate Buffered Saline (PBS, Invitrogen, Carlsbad CA, cat. # 14190) and resuspended in 110 µL of lysis buffer (1 mL nuclei buffer (0.25 M Sucrose, 0.2 M NaCl, 10 mM Tris-HCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 1% TritonX-100, and water to 1 L), 10 µL phenylmethylsulfonyl fluoride (PMSF, Sigma, St. Louis MO, cat. # P7626), and 1 µL protease inhibitor cocktail (Sigma, St. Louis MO, cat. # P8340)), and left on ice for 20 minutes. The solutions were centrifuged for 1 minute at 16,000 x g (Eppendorf Centrifuge 5415C, Mississauga, ON) to remove cell debris and the supernatant was obtained. A Bradford assay was done using Bio-Rad Protein Assay (Bio-Rad, Hercules CA, cat. # 500-0006) to determine the protein concentrations of the samples for further use in western blotting.

2.2 Transformation

2.2.1 Selective Agar Plates and LB Broth

LB agar tablets (Sigma, St. Louis MO, cat. # L7025) were mixed with double distilled water (DDW) for 2 minutes and autoclaved on liquid cycle (20 minutes at 121^{o}C). The agar was cooled to 54^{o}C using a water bath and ampicillin (Sigma, St. Louis MO, cat. # A9393) was added to a concentration of 100 µg/mL agar. Plates were poured in the tissue culture hood and allowed to dry before use in growing up bacteria for plasmid DNA amplification. Unused plates were stored at 4^{o}C.

LB broth tablets (Sigma, St. Louis MO, cat. # L7275) were mixed with DDW for 2 minutes and autoclaved on the liquid cycle (20 minutes at 121^{o}C). Extra broth was stored at room temperature.

2.2.2 Transformation of E. coli Cells

In order to generate E6 and E7 viral particles, 5-alpha competent *E. coli* cells (New England BioLabs, Ipswich, MA, USA cat. # C2992H) were transformed with plasmid DNA carrying either E6 or E7 gene inserts. Briefly, 1µL of plasmid DNA (E6 prototype, AAE6 variant, pLxSN (empty vector), L83V, or green fluorescent protein (GFP)) was added to each tube of *E. coli* cells and mixed. Each mixture was put on ice for 30 minutes then heat shocked at 42^{o}C in a water bath for 30 seconds to facilitate the entry of the plasmid into the bacteria. They were then placed on ice for another 5 minutes and 950µL room temperature SOC medium (New England BioLabs, Ipswich MA) was added to each. The mixtures were put in a shaker at 37^{o}C for 60 minutes at 250 rpm, and selection plates were warmed to 37^{o}C at this time. 75 µL of each mixture was spread onto one selection plate each and incubated overnight at 37^{o}C in the bacterial incubator. The bacteria that grew should contain the plasmid due to the selection characteristics of the plate (the plate contained ampicillin, the plasmid contained an ampicillin resistant gene). This was later confirmed by expression of the E6 and E7 oncogenes through RT-PCR.

The next day, 5mL LB broth was aliquoted into a 15 mL falcon tube for each construct and ampicillin was added to a concentration of 100 µg/mL. A single colony from each selection plate was selected and added to the broth. The cell suspension was put into the shaker at 37^{o}C and shaken at 300 rpm for 8 hours to allow the bacteria to grow, therefore allowing the plasmid to multiply.

After 8 hours, 500 µL from each cell suspension was added to 250 mL LB broth containing 100 µg/mL ampicillin in a 1L flask. The new cell suspensions were put into the shaker overnight at 37^{o}C and 300 rpm. The next day, the suspensions were divided among 50mL falcon tubes and centrifuged at 6000 x g (Beckman Coulter Allegra™ 25R Centrifuge) for 15 minutes. The supernatants were decanted and the pellets were stored at -20^{o}C.

2.3 Maxi Prep

A maxi prep was performed on the pellets of the overnight growth bacteria to remove the plasmids from the *E. coli* cells. This was done using the EndoFree® Plasmid Maxi Kit (Qiagen, Hilden Germany, cat. # 12362), and was completed according to the manufacturer‟s protocol without any changes. DNA concentrations were read using a Synergy 4 plate reader. The final product of each construct was stored at -20^{o}C.

2.4 Retroviral Infection

2.4.1 Calcium Phosphate Transduction Method

To obtain viral particles for infection of keratinocytes, a calcium phosphate transfection was performed on Phoenix cells in a 75 cm^2 flask using a kit from Clontech (Mountain View, CA). First, 5 mL fresh DMEM with serum and 5 µl chloroquin was added to each flask. Next, DNA mixtures were made up for each variant comprised of 10 µg DNA, water making the solution up to 438 µl, and 62 µl of calcium solution (0.248 M final concentration). The mixture for each variant was added drop-wise to 500 µl HEPES-buffered saline, mixed, and was then added to the cells drop-wise. The flasks were then incubated at 37^{o}C, 95% oxygen, 5% CO_2 for 10 hours, washed with 5 mL 1 x PBS, and 10mL fresh medium was added. After 24 hours, the cells were washed again with 1 x PBS and 5mL fresh media was added. Another 24 hours later, the media was drawn through a syringe with a 0.2 µm filter and kept at -80^{o}C.

2.4.2 Transfection Efficiency Check

The flask containing Phoenix cells transfected with GFP plasmid DNA was observed under a Zeiss fluorescent microscope. Pictures were taken at a 10X objective with an exposure time of 10 milliseconds for halogen light and 50 ms for fluorescence (longer exposure to correct for the difference between what is seen under the microscope and what is seen on the computer screen). The amount of cells seen with the halogen light was counted as well as the amount of cells seen containing fluorescence and a transfection efficiency percentage was obtained.

2.4.3 Transduction

In order to obtain a biological model of HPV 16 infected keratinocytes, PHFKs were grown to passage three in 12.5 cm^2 flasks. There were three flasks for each construct, one for a selection control, and one to continue the untransduced PHFKs. These were seeded at $0.75x10^5$ cells per flask, and the transduction was performed when the cells reached 60-70% confluency. The viral supernatants from the transfected Phoenix cells (55) were used for the following constructs: prototype E6, AAE6, L83V, E7, and pLxSN. E7 viral supernatant was obtained from Phoenix cells transfected with E7 DNA obtained from a personal contact of Dr. Zehbe. In each flask, the medium was aspirated and 2mL of the respective viral supernatant was added in the presence of polybrene (a cationic polymer used to increase the transduction efficiency, 1 µg/mL viral supernatant). A co-transduction was also performed using 1 mL of E7 supernatant and 1 mL of prototype E6 supernatant in three flasks to create E6E7. The flasks were incubated at 37°C and 5% CO_2 atmosphere for 3 hours, after which Keratinocyte Growth Medium was added 1:1. This was done to avoid supernatant serum-induced differentiation from the DMEM of the viral supernatant. The flasks were incubated for another 3 hours and the media mixture was aspirated. 5 mL of Keratinocyte Growth Medium was added back to the flasks and the cells were placed back into the incubator.

2.4.4 Selection

The day after transduction, a selection was performed to ensure all cells growing contained the viral constructs. The media was replaced in each flask with Keratinocyte Growth Medium containing 100 µg/mL gentamicin (G418, Roche Applied Science, Laval QC, cat. # 11059467001). The cells were incubated at 37°C and 5% CO_2 atmosphere for 48 hours, after which the media was replaced with more Keratinocyte Growth Medium containing 100 µg/mL G418. This was continued for 4 days, and then the medium was changed to medium containing 50 µg/mL G418 for a maintenance selection. This was continued for one week after which the medium was changed to regular medium without G418 to end the selection.

2.5 Analysis of Cellular Immortalization

2.5.1 DNA and RNA Extraction

For future PCR and quantitative real time (qRT-) PCR, DNA and RNA were extracted from the infected cells at passages 6, 16 and 30. DNA was extracted following the Animal Blood (Spin-Column) protocol in the DNeasy® Blood and Tissue Kit (Qiagen, Hilden Germany, cat. # 69504) and the concentration was measured using a Synergy 4 plate reader after which the DNA was stored at -20^{o}C. RNA was extracted using the Arcturus PicoPure RNA Isolation Kit (Applied Biosystems, Carlsbad CA, cat. # KIT0204). The quality and purity was assessed using the Bio-Rad Experion Automated Electrophoresis system with the StdSens Analysis Kit (Bio-Rad, Hercules CA, cat. # 700-7111) and the quantity was measured using a Synergy 4 plate reader after which the RNA was stored at -80^{o}C.

2.5.2 Reverse transcription

For future qRT-PCR, the RNA was reverse-transcribed using the High Capacity cDNA Archive kit (Applied Biosystems, Carlsbad CA). Samples were made up to 30 µl with nuclease-free water and 30 µl master mix (containing 10x RT buffer, 10x primers, 25x dNTPs, and multiscribe) was added. The parameters for the thermocycler were 25^{o}C for 10 minutes, 37^{o}C for two hours, and 85^{o}C for 5 minutes.

2.5.3 PCR and qRT-PCR

In order to ensure E6 and E7 oncogenes were being expressed in the infected PHFKs, PCR and qRT-PCR were performed. Briefly, for PCR, 100 ng DNA was used along with the master mix containing 1x PCR buffer, 1 mM $MgCl_2$, 200 µM dNTPs, 0.5µM of forward and reverse primer, and 1unit of Taq polymerase. The thermocycler parameters were 40 cycles of 94^{o}C for 1 minute, 55^{o}C for 1 minute, 72^{o}C for 2 minutes and a final extension at 72^{o}C for 7 minutes. The resulting amplified DNA was imaged on a 1.5% agarose gel. For qRT-PCR, 150 ng of cDNA was mixed with 45 µl TaqMan master mix, 4.5 µl of the gene assay (either hTERT, HPRT1, E6, or E7; Applied

Biosystems) and nuclease-free water to make it up to 90 µl. Triplicates of 25 µl each were pipetted into a 96-well plate and analyzed by a 7500 ABI cycler.

2.5.4 Cell Cycle Analysis

In order to determine if the infected cells could overcome induced cell cycle arrest, actinomycin D (AD, Sigma, St. Louis MO, cat. # A9415) was added to one of two dishes of cells at a concentration of 0.5 nM to induce p53 expression through DNA damage (24). To the other dish, 100% DMSO was added as a control. The cells were incubated for 24 hours then were harvested and washed once with 1xPBS and were resuspended in 200 µL of 1xPBS. While vortexing the cell suspension, 1.8mL of ice cold ethanol (70%) was added drop-wise to fix the cells. They were stored at 4^{o}C for at least 4 hours, or until flow cytometry could be done. This was completed at passage 6, 16 and 30 and analysis was performed using the CellQuest Pro program with the FACS flow cytometer. Briefly, the cells were spun down at 25 x g (Beckman GS-6KR Centrifuge) for 5 minutes, and the ethanol was decanted. The pellet was then washed in 1xPBS after which the supernatant was aspirated and each pellet was resuspended in 0.6mL of the following mixture: 100 µL of 10% Triton X-100, 100 µL of RNase A (Qiagen, Hilden Germany, cat. # 19101), 200 µL of 1 mg/mL propidium iodide, and 9.75 mL of 1xPBS. The standard flow cytometry protocol was followed from here on. To analyze, peaks representing the different cell cycle phases of G1, S, and G2/M were gated within the CellQuest Pro program for the DMSO-treated samples. For each corresponding AD-treated sample, the gates were not adjusted. The percentage of cells in each of the G1 and S phases in the AD-treated samples was standardized to the percentage of cells in each of those phases in the DMSO-treated cells (AD:DMSO). A G1:S ratio was then made based on AD:DMSO ratio for each phase.

2.5.5 Inducing Anoikis

To observe if the infected PHFKs were transformed, anoikis was induced using semisolid medium. To make semisolid medium, 3.37 g of methylcellulose (Sigma, St. Louis MO, cat. # M0512) was autoclaved for 20 minutes with a magnetic stir bar. 200 mL serum-free medium (Ham‟s F12 (Invitrogen, Carlsbad CA, cat. # 21700-075) and

DMEM at a ratio of 3:1 as well as calcium at a final concentration of 0.66 mM) was made up and half of it was heated to 60^{o}C. The heated medium was then added to the autoclaved methylcellulose and mixed for 20 minutes at room temperature. Next, the rest of the medium was added and stirred at 4^{o}C for 1 hour. The medium was then aliquoted into 50 mL falcon tubes and centrifuged for 90 minutes at 15,000 x g (Beckman Coulter Allegra™ 25R Centrifuge) to remove non-dissolved methylcellulose fibres. Once the cells reached passage 6 (subcultured six times after transduction), 200,000 cells were seeded into 15mL falcon tubes (in triplicates for each flask) and resuspended in semisolid medium at a density of 1x10^{6} cells/mL. 100,000 cells were also seeded into each well of a 6 well plate as a negative control (one for each flask). The tubes and plate were incubated at 37°C and 5% CO_2 atmosphere for 24 hours, after which the plate wells were overlaid with semisolid medium and incubated for another 24 hours. The tubes were spun down for 5 minutes at 25 x g (Beckman GS-6KR Centrifuge) and the medium aspirated. The pellets were washed three times with 1xPBS, once with regular Keratinocyte Growth Medium, and then twice more in 1xPBS. The pellets were stored at -20^{o}C until flow cytometry could be done. After the control cells had incubated for 24 hours, they were harvested and processed following the same method that was done for the tubes. This was repeated when the cells reached passages 16 and 30. To analyze the effect of the semisolid medium on the cells, flow cytometry was performed. Briefly, pellets were resuspended in 1x10^{6} cells/mL binding buffer and moved to 5 mL round bottom tubes. Each volume was split in two for technical replicates. A portion of each control was combined for an unstained sample to calibrate the cytometer. From an Annexin V-FITC kit (Sigma, St. Louis MO, cat. # APOAF-50TST), 2.5 µL of annexin V-FITC reagent and 5 µL of propidium iodide was added to each sample and they were incubated in the dark for 10 minutes at room temperature. The suspension was analyzed by flow cytometry using the CellQuest Pro program on a FACSCalibur flow cytometer (Becton Dickinson). To analyze, the percentage of cells in each quadrant is observed. The top right quadrant contains cells that are annexin V-FITC positive and propidium iodide positive, signifying that they are dead. The lower right quadrant contains cells that are annexin V-FITC positive and propidium iodide negative, which shows that they are in early apoptosis. The

lower left quadrant contains cells that are annexin V-FITC negative, and propidium iodide negative, meaning that they are alive.

2.6 Protein Expression Analysis

2.6.1 Gel Electrophoresis

SDS-PAGE gels were made for protein analysis. First, the separating gel was made at various concentrations depending on the protein of interest. This mixture contained 53.5% DDW, 0.375 M Tris - HCl (pH 8.8), 8% acrylamide (Bio-Rad, Hercules CA, cat. # 161-0146), 0.1% sodium dodecyl sulphate (SDS), 0.06% ammonium persulfate (APS, Sigma, St. Louis MO, cat. # A9164), and 0.1% TEMED (Sigma, St. Louis MO, cat. # T7024). This was allowed to polymerize for 15 minutes. The stacking gel was made which contained 63.6% DDW, 0.126 M Tris - HCl (pH 6.8), 4% acrylamide, 0.1% SDS, 0.08% APS, and TEMED. This was added and the gel combs were put in the gel to make the wells for the samples. The gel was allowed to polymerize for 30 minutes. Once the gel had polymerized, samples containing SDS were prepared. Each sample was boiled for 5 minutes and the standard was boiled for 2 minutes (Bio-Rad Prestained SDS-PAGE standards Low Range, or Precision Plus Streptactin –HRP conjugate, Hercules CA, cat. # 161-0305 or 161-0380). These were then spun down for 30 seconds at 16,000 x g (Eppendorf Centrifuge 5415C). A noted amount of protein was loaded into each well based on the known concentrations of each sample. The gel was run at 100V in 1X running buffer (144 g glycine, 30.3 g Tris Base, 50 mL 10% SDS into 1 L of DDW, then 100 mL of this into 900 mL of DDW) until the samples ran into the separating gel, then the voltage was turned up to 120V. Once the samples had run to the bottom of the gel, indicated once the dye front had disappeared, the electrophoresis was stopped.

2.6.2 Western Blotting

The gel was transferred to PVDF membrane in 1X transfer buffer. This was done for 1 hour at 100V. The membrane was cut to size and soaked for 10 minutes in 1X Tris-Buffered Saline with Tween 20 (TBST), and then blocked in an appropriate percentage of

milk for 1 hour at room temperature. The membrane was then put into primary antibody solution (Antibody diluted in milk. Used CAIX (1:5000 dilution, ab15086, Abcam) and E-cadherin (1:200 dilution, M3612, DAKO)) overnight with agitation at 4^{o}C. The next day, the membrane was washed 4 times for 10 minutes each in 1X TBST. It was then put into secondary antibody solution (made in milk, antibody specific to primary) for 1 hour at room temperature. Afterwards the membrane was washed again 4 times for 10 minutes each time in 1X TBST. Chemiluminescence was done with Western Lightning Plus-ECL (PerkinElmer, Inc.) solutions. One mL of each of the two solutions was mixed together and was put onto the membrane for 2 minutes. Pictures were then taken using the Biospectrum 410® Imaging System (UVP).

2.6.3 Normalization and Densitometry

The housekeeping gene actin was used as a loading control to normalize the results. Each western blot was stripped of its previous primary/secondary antibody complex by incubation with stripping solution (7.507 g glycine and 5.844 g NaCl, into 1 L of DDW (pH 2.2) and autoclaved for 20 minutes at 121^{o}C) for 1.5 hours. Each blot was then probed with actin primary antibody (actin goat polyclonal IgG. Santa Cruz Biotechnology, Inc.) at a concentration of 1:1000 in 5% milk, and a secondary antibody (Peroxidase-conjugated AffiniPure Donkey Anti-Goat IgG. Jackson ImmunoResearch Laboratories, Inc.) at a concentration of 1:2000 in 5% milk. The density of the actin and the protein bands were calculated for each sample using the UVP VisionWorks®LS Analysis Software. These densities were compared as ratios and these ratios were then compared between samples.

2.7 Transformation Study

2.7.1 Clonogenic Assays

High passage keratinocyte cultures (passage 65) containing the various E6 genes, as well as negative and positive control cells (parental PHFKs from passage 3 and HeLa cells, respectively), were assessed for their ability to form colonies in the presence of basement membrane proteins mimicking *in vivo* conditions through a modified

clonogenic assay described by (25). Briefly, 6-well plates were immobilized with and without 20 µg/mL Matrigel (BD Biosciences) and blocked with 1 % Bovine Serum Albumin (BSA) to prevent non-specific binding of cells to the substratum. Cells were seeded on the plates and grown for 11 days in serum-free DME/F12 media. The cells were replenished with fresh serum-free media every third day and on the eleventh day fixed with cold methanol and stained with 0.1 % Crystal violet Gram Stain (Sigma Aldrich) and imaged under low magnification. Colonies were observed and categorized into scattered, closed, or mixed types.

2.7.2 Adhesion and Viability Assays

To determine whether the basement membrane proteins promote cell adhesion capacity, an adhesion assay was performed. Briefly, 96-well plates were immobilized with different concentrations of matrigel (0 µg/mL, 5 µg/mL, 10 µg/mL, 20 µg/mL, and 40 µg/mL) in technical replicates of 4, and blocked with 1% BSA for at least 10 minutes. About 100,000 cells were seeded per well, after which plates were incubated at 37^{o}C for 40 minutes. The cells were washed with Serum free DME/F12 until cells in the BSA wells stopped detaching. The cells were fixed within the wells using ice cold methanol for 10 minutes at -20^{o}C. Methanol was aspirated and residual methanol was washed away with 1xPBS. The PBS was aspirated and cells were stained with 0.1% crystal violet Gram Stain. Filtered 2% SDS was added to all wells and the plates were incubated with agitation at room temperature for 30 minutes, after which absorbance readings at 550 nm were taken using the PowerWave XS plate reader (BioTek, Vermont, USA). In order to determine if the matrigel affects the cell proliferation a resazurin based fluorimetric assay (R & D Systems, Minnesota, USA) was performed. After the 96-well plates were immobilized with matrigel as described above, 4000 cells were seeded per well and allowed to grow in their normal growth medium until BSA wells reached a confluence of 80%. Resazurin solution was added to each well as described by the manufacturer‟s protocol and plates were incubated at 37^{o}C for 2 hours after which the plates were incubated with agitation in the dark at room temperature for 20 minutes. The fluorescence intensities were measured using the FLx800 plate reader at excitation of 540 nm and emission of 590 nm.

2.8 Migration Study

2.8.1 Invasion and Migration Assays

High passage (passage 70) keratinocyte cultures containing the various E6 genes, as well as negative and positive controls (PHFKs, HeLa and L83V/E7 and prototype E6/E7 and AAE6/E7, respectively) were assessed for their ability to migrate through a porous membrane. A CytoSelect™ 24-well Cell Haptotaxis Assay (Cell Biolabs) coated with collagen I was used for this study. Ten percent FBS in KGM was added to the bottom chamber of two out of three wells per variant. The third well was left without FBS to control for random migration. Each variant was seeded at 150 000 cells per insert and the plate was incubated at 37^{o}C for 16 hours, the time chosen by observing what other groups have used in similar experiments (3, 10, 33, 64, 81). Migratory cells were stained, and imaged using an inverted microscope at 200X magnification. Three fields per insert were imaged, the stain was extracted and the absorbance reading at 560nm was taken using the PowerWave XS plate reader (BioTek, Vermont, USA).

2.9 Statistics

All statistical analyses were performed using open-source statistical software called R (version 2.12.1, R Development Core Team. 2008. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org). All data were tested for normality and homogeneity of variances using a Shapiro-Wilks test and a Bartlett‟s test, respectively, before choosing a suitable parametric or non-parametric statistical test. A resulting p-value of less than 0.05 was considered to be significant. Parametric data sets were analysed using student‟s t tests and one and two-way ANOVAs followed by a post-hoc such as a Tukey HSD. Non-parametric data sets were analysed using two-way ANOVAs or one-way Kruskal Wallis ANOVA followed by a Nemenyi‟s post hoc, and Friedman‟s tests followed by a pairwise comparison using a Wilcoxon signed rank test with a

Bonferroni adjustment. Cell population doublings were calculated based on the method described by Willey *et al.* (86).

3.0 RESULTS

In the current study, three HPV16 E6 oncogene variants have been characterized with respect to their ability to immortalize, transform, and increase invasion/migration ability in PHFKs. A similar study was previously completed by our group (data not published, manuscript submitted) and the results from the current study were used to confirm the results seen before. The previous study had transduced PHFKs grown until passage 60 whereas the current study examined preliminary signs that PHFKs from a separate donor were exhibiting characteristics seen in the first donor through growth until passage 30. Transduced PHFKs grown until passage 60 were considered fully immortalized and were therefore used to test other parameters to assess the E6 oncogene‟s role in cellular transformation and induction of invasion and migration.

3.1 The E6 oncoprotein alone is sufficient for PHFK lifespan extension and escape from the immortalization crisis

3.1.1 Confirmation of E6 Oncogene Expression

Conventional and qRT-PCR both confirmed the presence and expression of the E6 genes in each variant at checkpoints of passages 6, 16 and 30 (Fig. 3). All E6 variants were devoid of any E7 expression, E7 was devoid of any E6 expression and pLXSN and parental PHFKs were clear of any E6 or E7 expression.

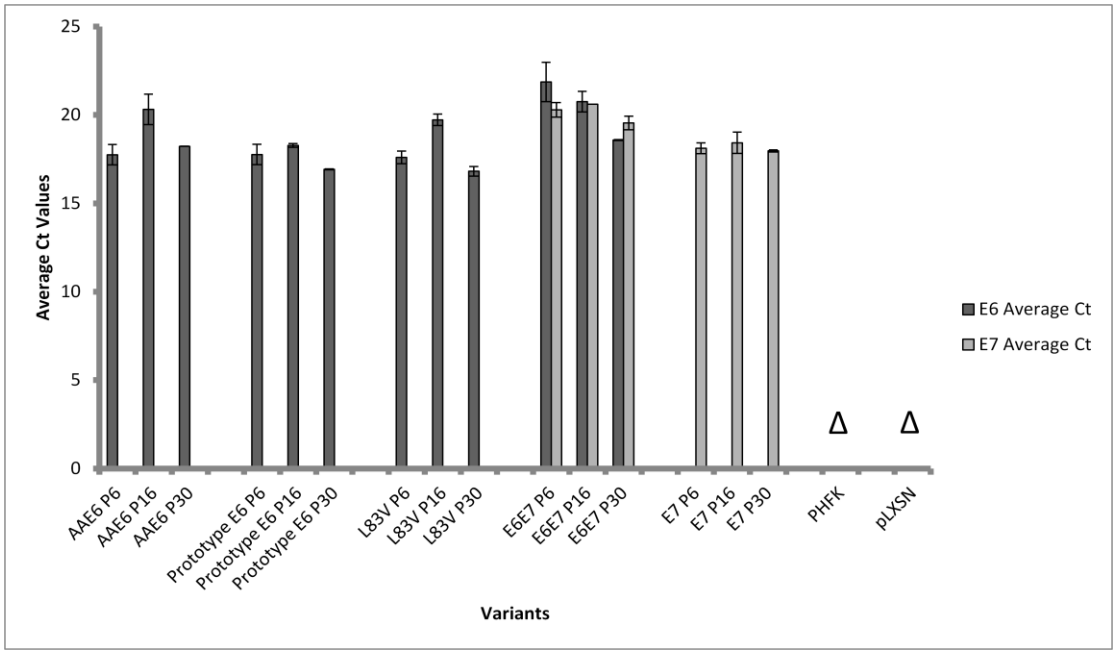


Figure 3. E6 and E7 oncogene expression in transduced primary human keratinocytes. In order to ensure E6 gene expression was present in the transduced PHFKs, a real-time PCR using TaqMan gene assays for HPV 16 E6 and E7 in the ABI-7500 system was performed. E6 gene expression was present in all variants at all passages and the expression was not significantly different between variants or passages. Cycle thresholds are shown rather than relative expression levels because the Ct values of E6 and E7 are so low compared to HPRT1 mRNA that a negative value would occur. PHFKs: Primary human foreskin keratinocytes, pLXSN: empty vector. The symbol Δ denotes Ct values above the threshold of 35 Ct.

3.1.2 Extension of Lifespan through E6 Oncogene Expression

In order to create a model to observe immortalization and confirm results seen in previous work by our group (data not published, manuscript submitted), PHFKs were transduced with the E6 gene variants or E7 as well as E6 with E7 (positive control) and were grown up to passage 30. Untransduced PHFKs survived until passage 12 while pLXSN-transduced PHFKs senesced at passage 7 (Fig. 4 a). The PHFKs completed 14

population doublings while by passage 30, the AAE6, prototype E6 and L83V variants completed 55, 50, and 55 doublings, respectively. The positive control E6E7 completed 71 doublings while E7 completed 65 (Fig. 4 b). The population doubling times were calculated based on a method described by Willey *et al.* (86): AAE6 reached passage 30 in 126 days, prototype E6 in 138 days and L83V in 155 days with a difference in population doubling time being 67.54 hours ± 24.67 (AAE6), 82.89 hours ± 34.02 (E6 prototype), and 80.73 hours ± 37.17 (L83V). The positive control of E6E7 took 130 days with a population doubling time of 51.42 hours ± 18.21 while E7 took 168 days with a population doubling time of 90.95 ± 90.34 (Table 1). The positive control E6E7 had significantly more population doublings than all E6 variants ($p<0.05$), and also had significantly shorter population doubling times ($p<0.05$) compared to prototype E6 and L83V. Therefore, the AAE6 population doubling time is comparable to the positive control. The differences seen here between the E6 oncogene variants could be attributed to variations in metabolic enzymes as suggested by Richard *et al.* (67). To test this notion, we performed western blotting on high passage transduced PHFKs to examine CAIX expression levels.

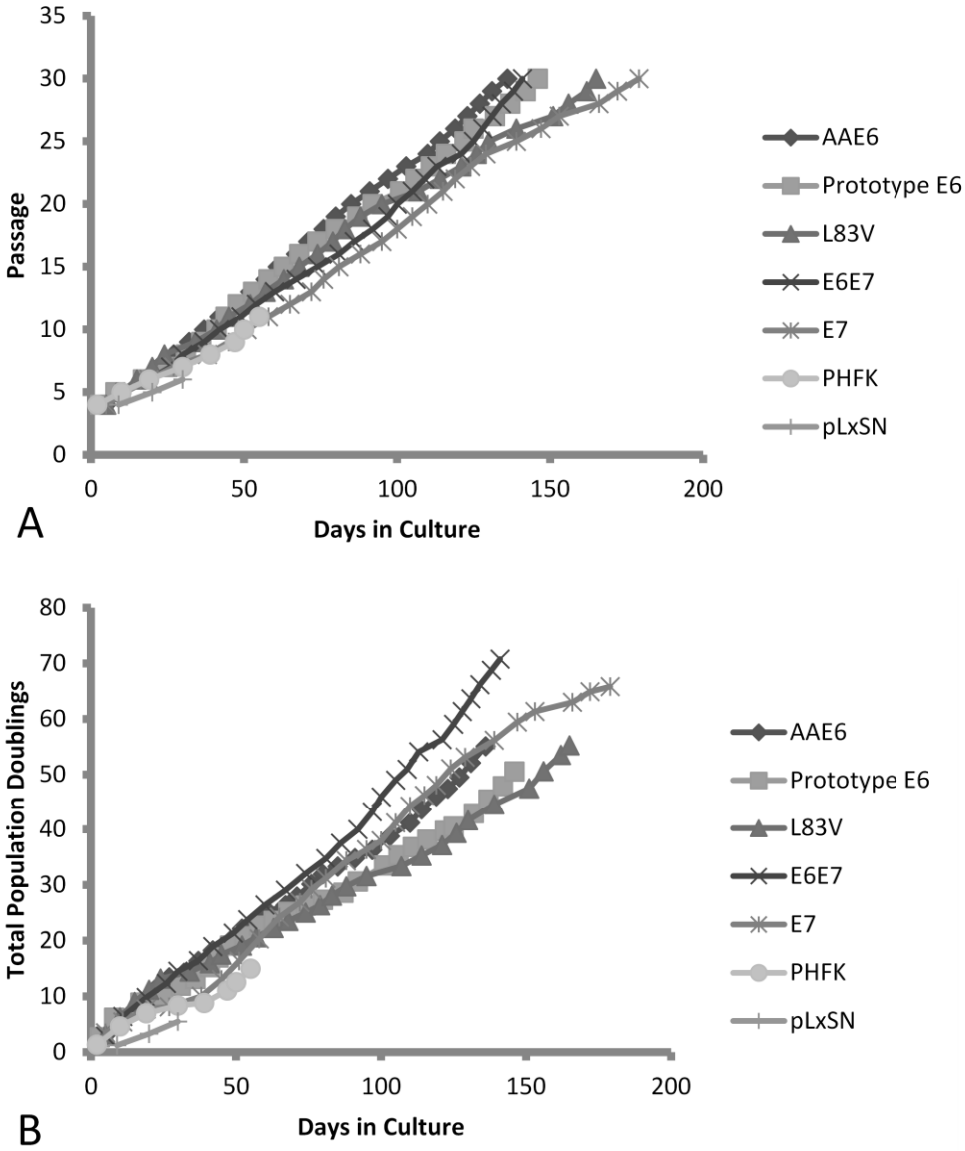


Figure 4. Immortalization of primary human keratinocytes (PHFKs). Retrovirally transduced keratinocytes were grown in serum-free KGM and passaged until passage 30. (A) Transduced PHFKs were subcultured and the number of passages vs. the days in culture was plotted on a graph. It was observed that there was no significance regarding how many days the variants took to reach passage 30 ($p > 0.05$, Friedman test followed by pair wise comparison using Wilcoxon signed rank test with Bonferroni adjustment, $n = 3$). (B) Total population doublings of transduced PHFKs were determined based on the method described Willey *et al.* (86). There was no significant difference regarding population doublings between the variants but E6E7 had significantly more population doublings than all variants ($p < 0.05$, one way ANOVA, $n = 27$).

	Passage 6-16		Passage 17-30		Passage 6-30	
Variant	**Doubling Time (h)**	**Passages (d)**	**Doubling Time (h)**	**Passages (d)**	**Doubling Time (h)**	**Passages (d)**
AAE6	73.63 ± 28.82	57	62.76 ± 20.71	69	67.54 ± 24.67	126
Prototype E6	83.26 ± 38.01	60	82.60 ± 32.01	78	82.89 ± 34.02	138
L83V	87.95 ± 41.91	64	75.06 ± 33.48	91	80.73 ± 37.17	155
E6E7	61.79 ± 15.64	70	43.27 ± 16.17	60	51.42 ± 18.21	130
E7	97.55 ± 117.18	77	85.77 ± 66.61	91	90.95 ± 90.34	168

Table 1. Population doubling time in hours for transduced primary human keratinocytes. PHFKs containing the E6 oncogene variants were passaged up to 30 times. The population doubling times in hours were calculated using a standard formula (3.32(log Nt - log No) = tf) where Nt is the number of cells counted and No is the number of cells plated. E6E7 has significantly lower population doubling time compared to prototype E6 and L83V ($p<0.05$, one-way ANOVA, n=27).

3.1.3 The E6 variants activate CAIX at different expression levels to enhance carcinogenesis

In order to determine a mechanism behind the differences seen in oncogenic potential in the different E6 oncogene variants, the protein expression level of CAIX in the E6 transduced PHFKs was investigated through western blotting. CAIX is a metalloenzyme which is known to function in pH regulation and cellular adhesion. It regulates pH by converting carbon dioxide to bicarbonate facilitating the transportation of the bicarbonate from extracellular to intracellular spaces. This is necessary for the cells to survive acidic pH conditions that are formed in a typical tumour microenvironment by neutralizing the intracellular space (60). The enzyme controls cell adhesion through its proteoglycan domain containing specific amino acid sequences that allow it to mediate cell attachment to non-adhesive solid support. It is known to be upregulated in many cancers and is indicative of a poor patient prognosis (60). CAIX expression was highest in AAE6 over the other variants and HeLa cells but this was not significant (Fig. 5 a). The CAIX expression differences were also studied in the AAE6 and prototype E6 variants in the presence of E7 since these variants were the focus of the study by Richard *et al.* (67). It was found that the AAE6/E7 had significantly increased expression of CAIX over the prototype E6/E7 variant ($p<0.001$) (Fig. 5 b), suggesting that E7 is necessary for stabilization of CAIX expression and also that CAIX expression is one means by which the oncogenic potential in the AAE6 variant is increased over the other variants.

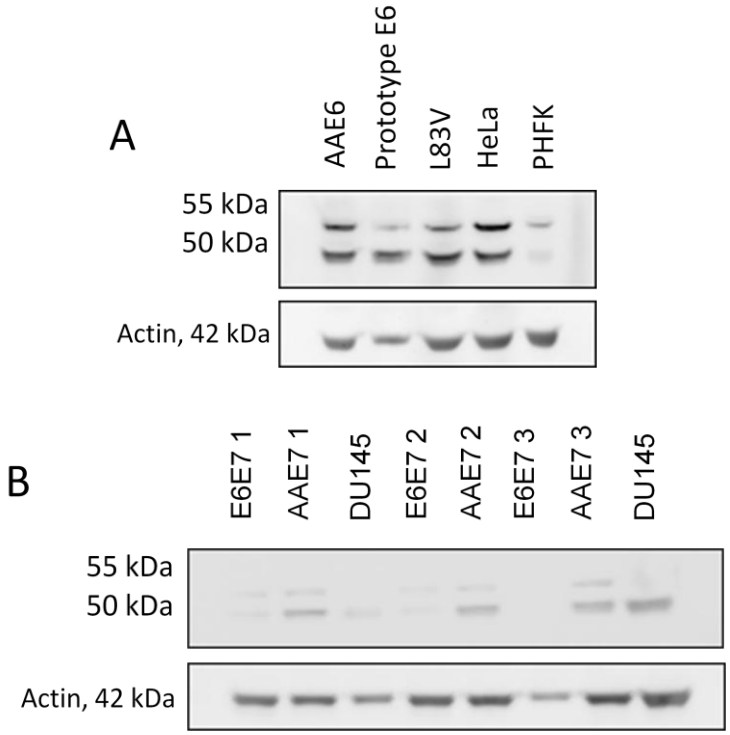


Figure 5. Carbonic anhydrase IX expression levels in the presence of different E6 oncogene variants. Western blot results on high passage transduced PHFKs, in triplicates. 55kDa and 50kDa signify the molecular weight of the protein heterodimers. (A) Carbonic anhydrase IX (CAIX) protein expression was found to be highest in AAE6 over prototype E6 and L83V however this was not significant ($p > 0.05$, one-way ANOVA n = 3). (B) Carbonic anhydrase IX protein expression in AAE6 and prototype E6 variants both in the presence of E7. AAE6/E7 had significantly higher CAIX expression compared to prototype E6/E7 ($p<0.001$, one-way ANOVA, n=6). DU145, a prostate cancer line, was used as a positive control. kDa: kilodaltons.

3.1.4 Overcoming Mortality Stage 1

The first stage that cells must overcome before immortalization can occur is the replicative senescence stage (M1). At this point, telomeres have reached a critically short length and chromosomal DNA begins to become damaged (14). Following DNA damage

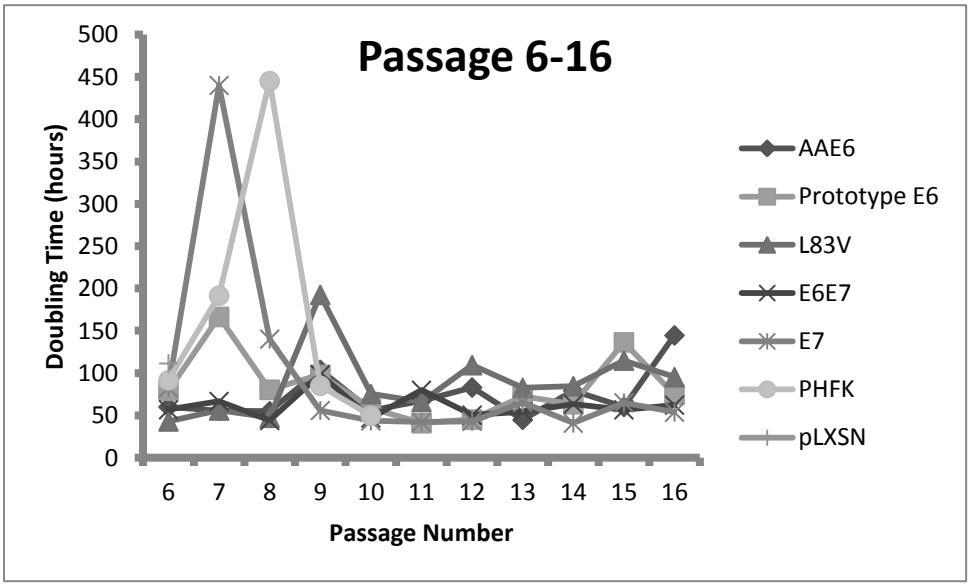
under normal cellular conditions, p53 is elevated which in turn activates p21. This leads to a downregulation of cyclin-dependent kinases that is essential for cell cycle progression resulting in G1 arrest (34). In the absence of p53, it has been shown that cells bypass M1 (88). Since E6 is known to abolish p53 activity, its presence in PHFKs should be enough to bypass cell cycle arrest and consequently, M1. To test this notion, actinomycin D was used to induce DNA damage through strand breaks by interfering with cellular topoisomerase activity (24). This results in an increased p53 expression, which will lead to G1 arrest (40). The PHFKs containing the E6 variants were expected to block this induced p53 activity, and this cell cycle stage was analyzed by flow cytometry. G1:S ratios were calculated by comparing the number of cells in G1 phase with the number of cells in S phase. The ratios approaching or below one were considered to reflect cells overcoming the cell cycle arrest. Already at passage 6, it was observed that expression of any of the E6 proteins significantly contributed to cells‟ ability to overcome G1 arrest when compared to PHFKs ($p<0.01$) (Table 2). E7-expressing PHFKs were only able to overcome arrest once in passage 30. This shows that the E6 oncoprotein was functional and the transduced PHFKs were overcoming M1.

	G1:S		
Variant	**Passage 6**	**Passage 16**	**Passage 30**
AAE6	0.88	0.80	0.85
Prototype E6	0.95	0.92	0.83
L83V	0.88	1.02	0.94
E6E7	1.05	1.01	0.99
E7	2.32	1.34	0.89
PHFK	2.60	N/A	N/A
pLXSN	2.60	N/A	N/A

Table 2. Cell cycle stage analysis of transduced primary human keratinocytes. Flow cytometry was performed to observe the effect of the E6 oncogene on induced cell cycle arrest. PHFKs containing the E6 oncogene variants were exposed to actinomycin D and a G1:S ratio was calculated. This experiment showed that the E6 oncogene expression resulted in overcoming G1 cell cycle arrest, leading to a significantly higher number of cells in S phase when compared to PHFKs ($p<0.01$, two-way ANOVA, n=3).

3.1.5 Overcoming Mortality Stage 2 Leading to Immortalization

Once keratinocytes bypass M1, they are characterized by an extension of lifespan after which an immortalization crisis, M2 ensues (73, 87). All E6 PHFK cultures exhibited some level of crisis during the culture period from which the immortalized subpopulation emerged. AAE6 had a shorter major crisis time (100 h) compared to the prototype E6 (175 h) and L83V (200 h) variants. E7-expressing PHFKs had two major crises, each being much longer than any E6 variant crises (450 h and 275 h, respectively), while the positive control E6E7 had one crisis of 100 h (Fig. 6). Overcoming this stage has been attributed to combating telomere shortening. In normal cells, telomeres are constantly shortened until they reach a critical length and in the process important chromosomal DNA is lost, leading to cell death. Immortalized cells have ways to overcome this condition through transient expression of telomerase which acts to restore telomere length (29). It is composed of a catalytic subunit termed telomerase reverse transcriptase (hTERT) and a template RNA. This enzyme is present in normal cells but is inactive. However, it is still detectable at very low levels (14, 30, 94). E6 has been shown to induce telomerase activity through an increase in hTERT transcription in keratinocytes, a hallmark for immortalization (44, 48, 54, 57, 63, 77, 82). To further assess immortalization status of the variants, qRT-PCR for hTERT was performed on transduced PHFKs as well as control PHFKs. The expression of the telomerase subunit hTERT was standardized to hypoxanthine phosphoribosyltransferase 1 (HPRT1) expression in the E6 PHFK cultures as previous results indicate HPRT1 expression is unaffected by HPV infection (16). As expected, it was observed that as the passage number increased from 16 to 30, hTERT expression steadily increased in a time-dependent manner (Fig. 7) showing that this mechanism for immortalization was consistently being up-regulated. Once at passage 30, hTERT expression was highest in all E6 variants as well as E6E7. E7 expression did not induce hTERT expression, leading to the conclusion that E7 oncogene expression cannot immortalize PHFKs.



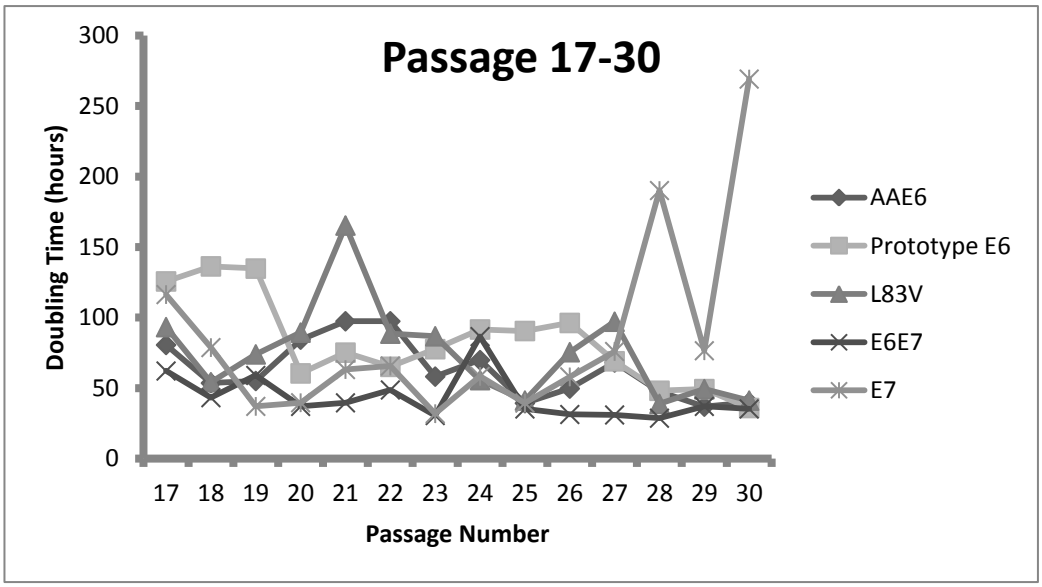


Figure 6. Average population doubling time in hours per passage in primary human keratinocytes transduced with E6 oncogene variants. To highlight the different points of immortalization crisis (M2) within each culture, doubling time in hours was plotted against passage number for each variant from each donor. An extreme increase in doubling time suggests a point of crisis in culture growth.

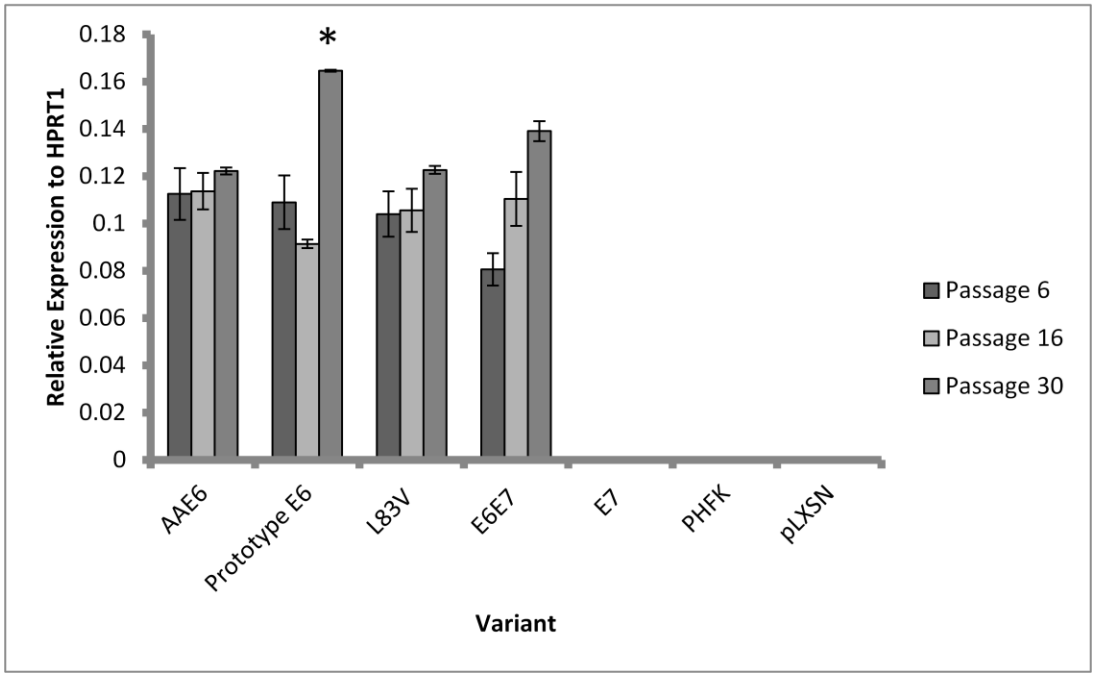


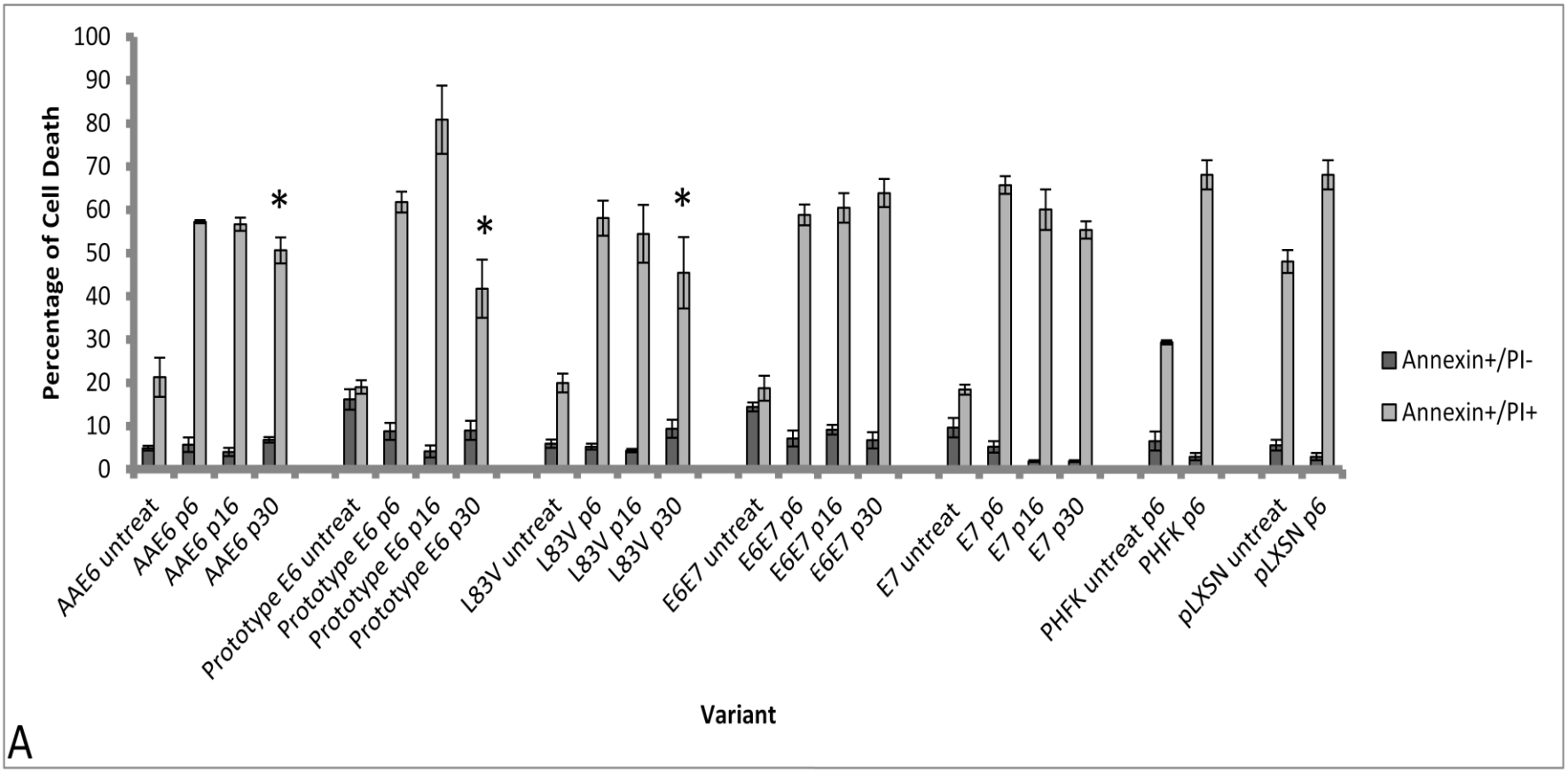
Figure 7. Relative expression of hTERT gene in primary human keratinocytes transduced with and without E6 variants. A real-time PCR using TaqMan gene assays for hTERT and HPRT1 was performed with the ABI-7500 system. It was observed that at passage 30 prototype E6 had significantly higher expression compared to L83V and AAE6 ($p < 0.00001$, two-way ANOVA, $n = 9$). HPRT1: Hypoxanthine phosphoribosyltransferase. An asterisk denotes significance.

3.2 The presence of the E6 oncoprotein variants facilitates PHFKs in overcoming anoikis, suggesting transformation has been achieved

3.2.1 Overcoming Detachment-Induced Apoptosis

One way to observe if cells have been transformed is to test the ability of the cells to overcome detachment-induced death, or anoikis. Cells can probably overcome this through expression of E6 which counteracts the pro-apoptotic effects of p53, therefore allowing the keratinocytes to survive (71). To assess the ability of the E6 variants to accomplish this, the cells were resuspended in media containing methylcellulose to recreate detachment conditions (1) after which annexin V-FITC flow cytometry was

completed. All E6 PHFK cultures showed significantly more anchorage-independent survival than PHFKs treated with semisolid medium ($p<0.00001$). There was a general trend of decreased cell death with increasing passage number beginning at passage 16 among the E6 variants as well as E7 suggesting that the cells were overcoming anoikis and were therefore in the beginning stages of becoming transformed (Fig. 8 a). In addition, in the absence of growth factors as evidenced during serum starved conditions and in the presence of basement membrane proteins which support anchorage, AAE6 PHFKs were still able to form colonies while this was drastically reduced in HeLa cells and little to no colonies were formed by the prototype E6 or L83V PHFKs (Fig. 8 b and c). This indicates that the AAE6 variant is more resilient and can survive nutrient poor conditions.


Percentage of Cell Death
100
90
80
70
60
50
40
30
20
10
0
*
*
*
AAE6 untreat
AAE6 p6
AAE6 p16
AAE6 p30
Prototype E6 untreat
Prototype E6 p6
Prototype E6 p16
Prototype E6 p30
L83V untreat
L83V p6
L83V p16
L83V p30
E6E7 untreat
E6E7 p6
E6E7 p16
E6E7 p30
E7 untreat
E7 p6
E7 p16
E7 p30
PHFK untreat p6
PHFK p6
pLXSN untreat
pLXSN p6
Variant
Annexin+/PI-
Annexin+/PI+
A

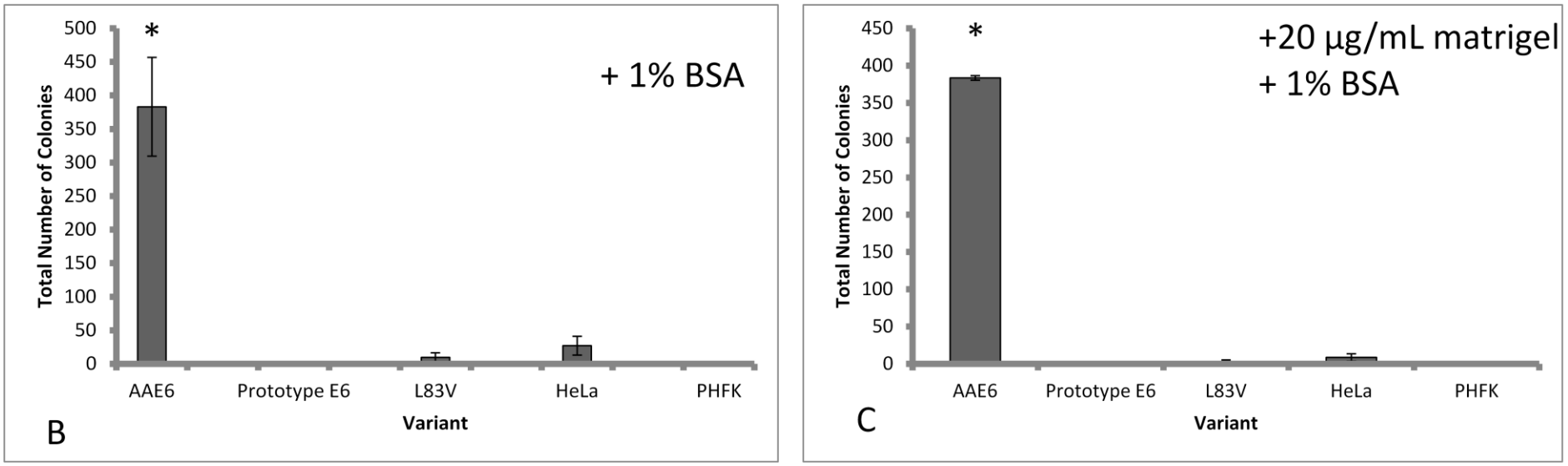
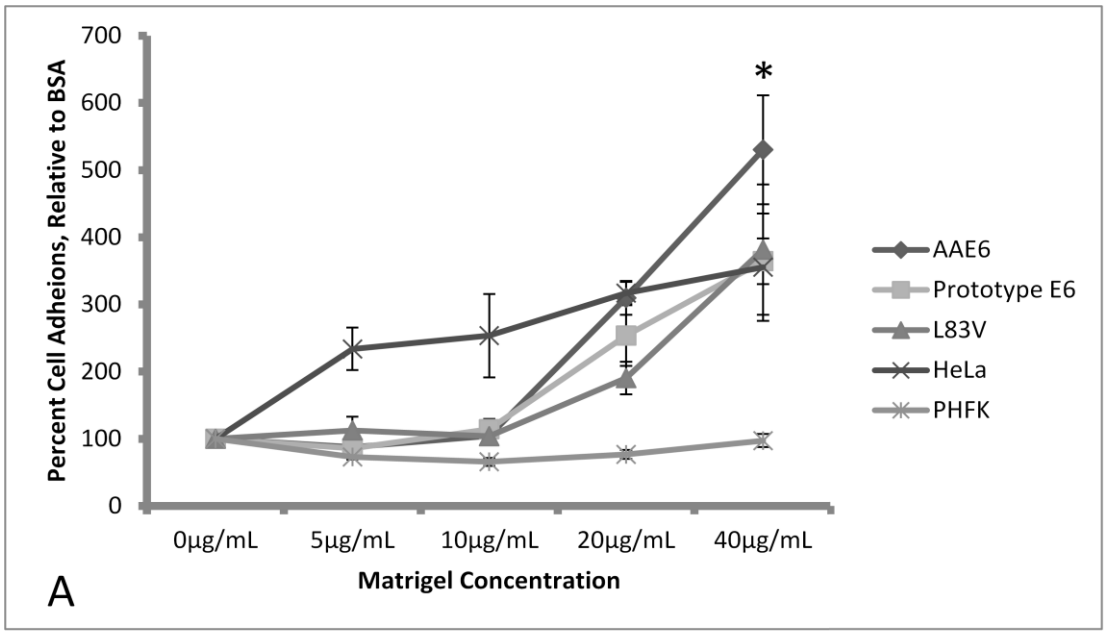


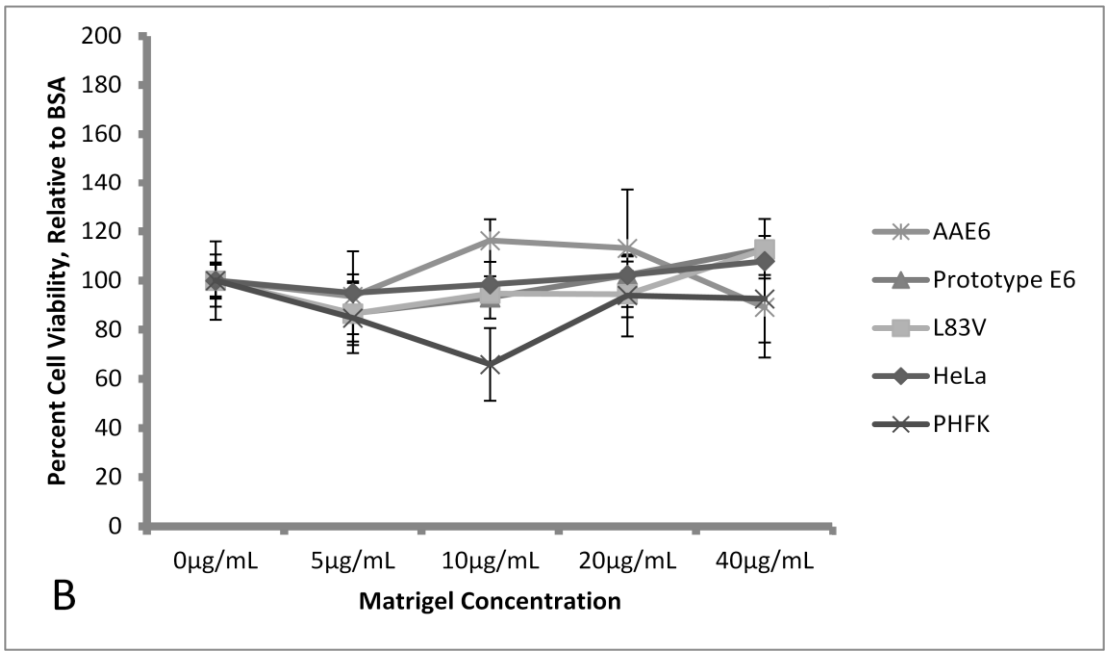
Figure 8. Transformation of primary human keratinocytes transduced with and without E6 variants. (A) Annexin V-FITC flow cytometry was performed to determine if the cells could overcome anoikis. It was observed that all E6 variants had significantly decreased anoikis compared to normal PHFKs ($p<0.00001$, two-way ANOVA, n=3). A transformation assay under serum starvation condition was performed in the absence (B) and presence (C) of anchorage supporting basement membrane proteins (Matrigel, BD Biosciences). It was observed that in the presence and absence of basement membrane protein the AAE6 variant formed significantly more colonies than prototype E6, L83V, HeLa and PHFK ($p < 0.05$, student „t" test, n = 3). An asterisk denotes significance.

3.3 Invasion and migration ability is increased in the presence of the E6 oncoprotein.

The initial step in the metastasis cascade is invasion which occurs when the primary tumour cells strongly attach to and invade through the epithelial layer into the stroma (21). The ability of primary tumour cells to strongly attach, interact and degrade the basement membrane proteins is a key process that initiates the metastasis cascade. To experimentally mimic these conditions *in vitro* a cell adhesion assay was initially performed to determine the adhesive capability of the E6 PHFKs which is an important survival mechanism of the cells to form heterogeneous cell subpopulations that can self-renew and be chemoresistant. It was observed that all E6 cultures showed a concentration dependent increase in adhesion starting from a concentration of 10 µg/mL matrigel (Fig. 9 a). Interestingly, it was observed that AAE6 had significantly higher adhesion capacity compared to prototype E6 and HeLa cells ($p<0.05$), while all the variants showed significantly increased adhesion at 40 µg/mL matrigel concentration compared to control PHFKs ($p<0.01$). It was observed that matrigel did not have a significant effect on the cell proliferation or its viability suggesting that the experimental conditions were conducive for cell growth (Fig. 9 b). To determine the migratory capability of these cells, a Boyden chamber assay was completed using the transduced PHFKs. Keratinocytes express collagenase-1 (matrix metalloproteinase 1), a collagen protein degrading enzyme necessary for their migration across type 1 collagen, another predominant basement membrane protein in the epithelial and endothelial surface (62). It was observed that AAE6 PHFKs migrated significantly more than prototype E6 and L83V PHFKs in the Boyden chamber ($p<0.05$) (Fig. 9 c). This trend was also observed in the presence of E7. HeLa cells had the highest migration capacity over all variants ($p<0.0001$). Interestingly, PHFKs have a higher migration ability compared to prototype E6, L83V, and E6E7. It is expected that the PHFKs have some migratory ability due to the expression of collagenase-1 so this may have been interrupted after infection with the oncogenes and may be reactivated later in carcinogenesis. One potential mechanism behind migration in these keratinocytes is the downregulation of E-cadherin, a cell-cell adhesion molecule. To investigate this, western blotting was performed on high passage keratinocytes. It was found that the AAE6 variant had lower expression of E-cadherin than the prototype E6

and L83V, and that HeLa cells had significantly lower E-cadherin expression than the prototype E6 ($P < 0.009$). The PHFKs also had downregulated E-cadherin expression (Fig. 10).





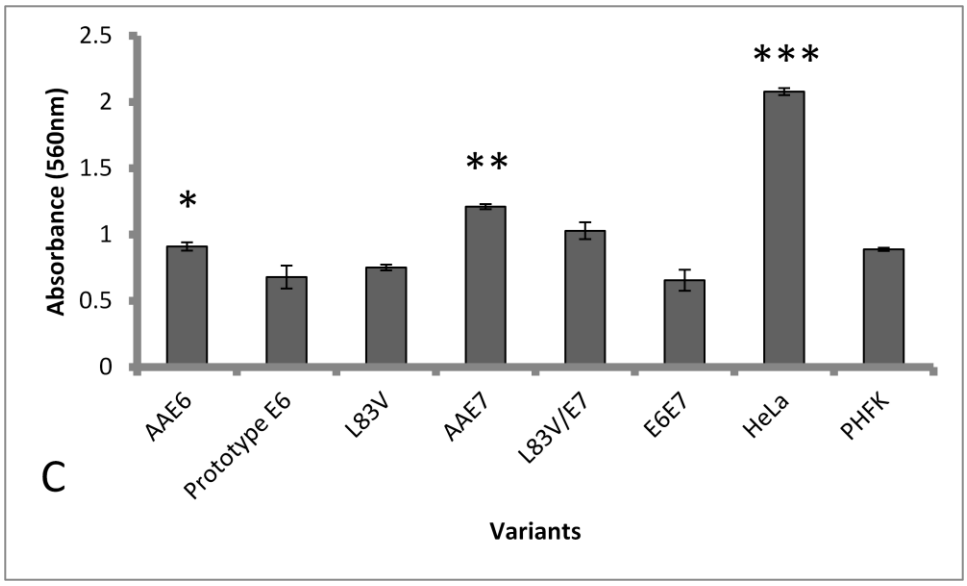


Figure 9. Metastatic potential of immortalized primary human keratinocytes. (A) Adhesiveness of the transduced PHFKs was tested using different concentrations of matrigel. We observed that between 10 – 40 µg/mL concentration, AAE6 had significantly increased adhesion capacity compared to prototype E6, HeLa and PHFKs ($p < 0.05$, student „t“ test , n = 4). Prototype E6 and L83V also had significantly higher adhesion capacity compared to PHFKs ($p < 0.01$, student „t“test, n =4). An asterisk denotes significance. (B) To determine whether the matrigel used in the analysis had any significant effect on the cell viability, the cells were treated with Resazurin (R&D systems) and incubated for 2 h and fluorescence intensity was measure as described in methods. We observed that there was no significant effect of matrigel in all the transduced PHFKs. (C) A Boyden Chamber assay using type I collagen coated Cytoselect 24 well Cell Haptotaxis assay was performed using high passage E6 variants. FBS (a minor chemoattractants for keratinocytes) was added to the bottom chamber. It was observed that the AAE6 variant had significantly higher migration capacity than prototype E6 and L83V. The AAE6 in the presence of E7 also had significantly higher migration capacity than the prototype E6E7 and L83V/E7 ($p < 0.05$, one-way ANOVA, n = 3). HeLa cells had the highest migration capacity over all variants ($p<0.0001$, one-way ANOVA, n=3). One and two asterisks denote statistical significance between the three variants while three asterisks denotes overall significance.

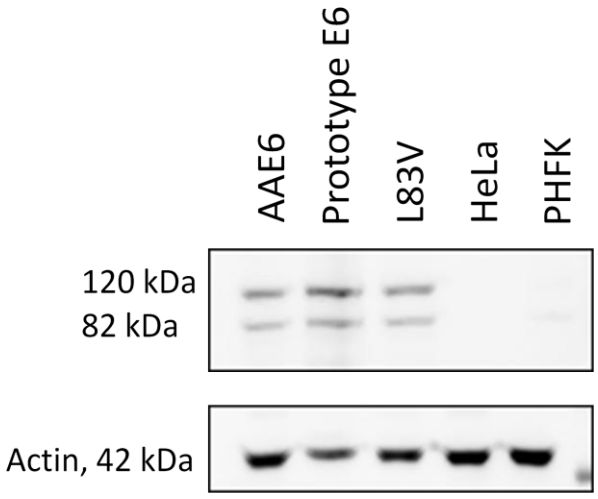


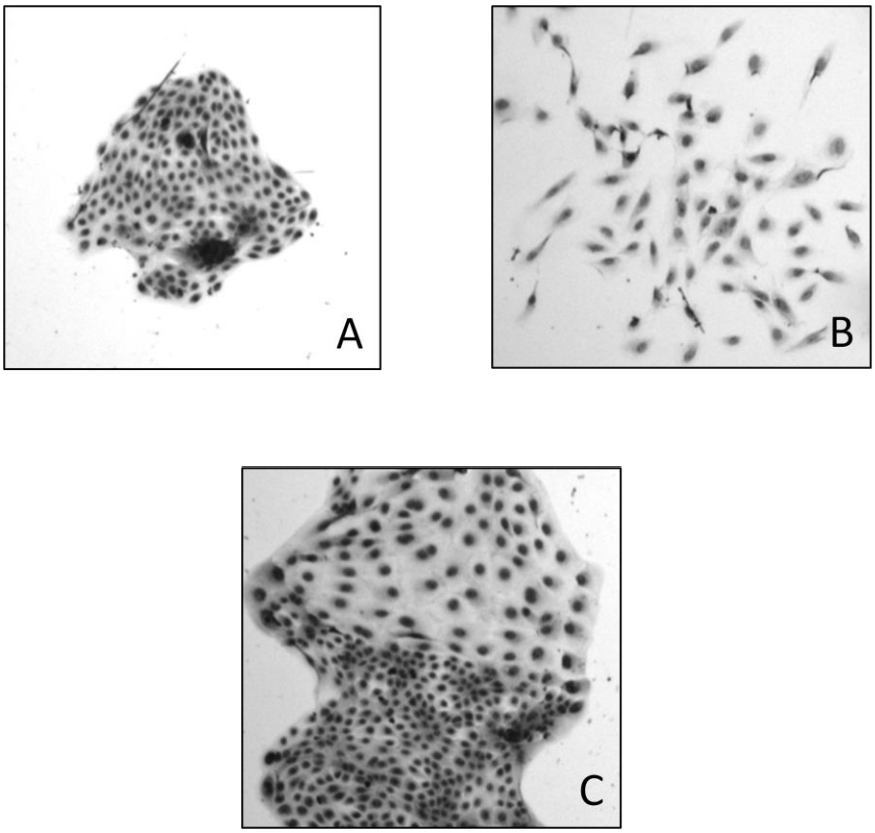
Figure 10. E-cadherin expression in the presence of different E6 oncogene variants. Western blot analysis on high passage-transduced PHFKs was performed. 120kDa and 82kDa signify the molecular weight of the mature protein and the fragmented protein, respectively. E-cadherin protein expression was found to be lower in the AAE6 variant than the prototype E6 and L83V, although this was not significant. Control HeLa cells and PHFKs both had completely downregulated E-cadherin, which was significant in HeLa cells compared to prototype E6 ($P < 0.009$, One-way Kruskal Wallis, n=3). kDa: kilodaltons.

3.4 The three E6 oncogene variants exhibit different resistance characteristics

3.4.1 Clonogenic assays clarify ability of E6 variants to survive unfavourable conditions

In order to observe any attributes of the variants that assist them in growth under unfavourable conditions, two types of clonogenic assays were performed. Usually, soft agar-based clonogenic assays are used to gather information about the ability of neoplastic cells to grow in a contact inhibited, exogenous growth factor depleted and anchorage independent environments, while the modified clonogenic assays that were used here provided anchorage dependent conditions that are free of exogenous growth factors and have extremely low seeding dilutions per square area of substratum (46). These conditions result in an environment mimicking that of one found during initial

tumour growth, and cause cells to group together in protective colonies to survive. Therefore, the presence of a subpopulation of transformed cells that can form closed colonies with defined edges and mixed colonies containing both scattered and closed colonies under these conditions is strongly characteristic of cells which have drug resistance potential and are capable of surviving in harsh environments in the body (46). Scattered colonies are a subpopulation of transformed cells having certain proliferative characteristics however they may be susceptible to drug or radiation therapy and may not survive as well in unfavourable conditions (46). Using a modified clonogenic assay, the resistance characteristics of the transduced PHFKs were indirectly determined and it was observed that colonies having resistance potential were only present in AAE6 and L83V populations (Fig. 11), suggesting that these variants had an increased potential to survive adverse conditions.



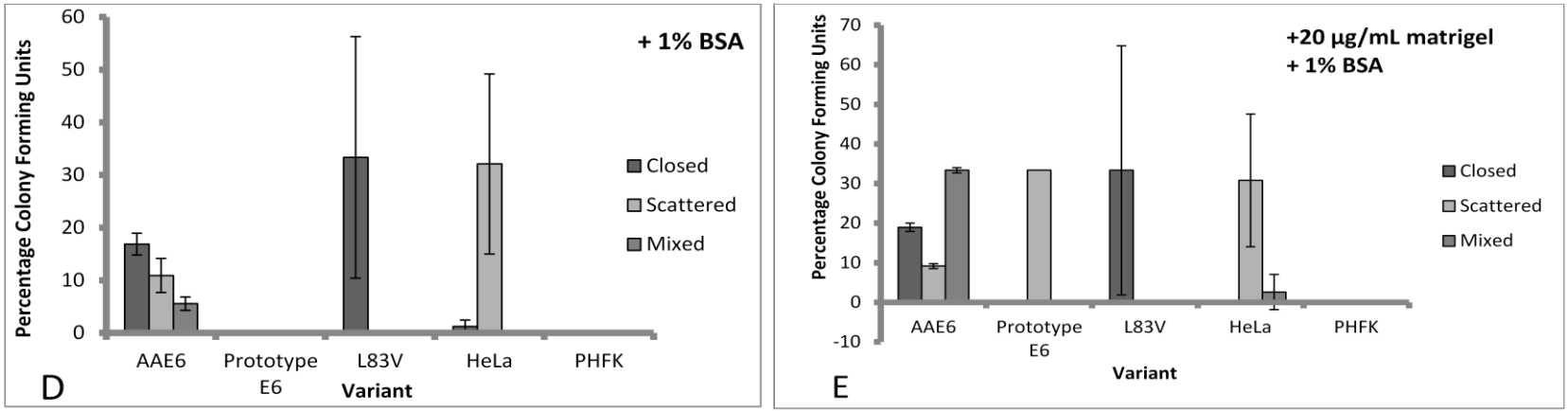


Figure 11. Types of colony forming units of PHFKs transduced with various E6 oncogene variants. A modified clonogenic assay as described by Franken *et al.* (25) was performed and high passage transduced PHFKs were grown for 11 days under serum starved conditions in the absence or presence of basement membrane proteins. We observed different kinds of colonies that the three E6 oncogene variants produced. (A) shows the morphology of a closed colony. (B) shows the morphology of a scattered colony. (C) shows the morphology of a mixed colony type that is comprised of both closed and scattered colonies. (D) We observed that in the absence of basement membrane protein, the AAE6 and L83V variants preferentially formed closed colonies while HeLa formed scattered colonies, and the prototype E6 along with normal PHFKs did not form any colonies. (E) In the presence of basement membrane proteins, the AAE6 variant preferentially formed mixed colonies while HeLa and the prototype E6 formed scattered colonies and the L83V variant formed closed colonies.

4.0 DISCUSSION

In an effort to examine the carcinogenic potential of three naturally occurring HPV 16 E6 gene products, a long-term cell culture study was performed using the E6 gene variants transduced into their natural keratinocyte host as an experimental model. The ability of E6 gene expression to immortalize and transform keratinocytes, and thereby increase the ability of the primary keratinocytes to invade and migrate into surrounding tissue was investigated. Until now, a similar detailed long term study has never been completed. Hence the results from this study fill a large knowledge gap within this field of study. Also, through this study a key scientific question about immortalization was addressed which began during the late 1980s, when E6 and E7 oncogenes were first reported to cooperate synergistically to extend primary human keratinocytes lifespan (32, 41, 56).

4.1 Extension of Lifespan of the Keratinocyte Model through Infection with E6 Oncogenes

It is imperative to clarify the role of the E6 oncogene in the cellular immortalization process as it is one of the initial steps in the molecular signalling cascade that may promote malignancies. Based on a literature analysis, a complete definition of immortalization in the keratinocyte model was composed. Appropriate experiments were then performed to validate its definition. According to published literature, before cells can reach the immortalized state, they must pass through the extension of lifespan stage (9). To observe this, PHFKs were transduced with the E6 gene variants or an empty vector (pLXSN) as a transduction control. The pLXSN-transduced PHFKs died before the normal PHFKs which was probably due to effects from the process of transduction. The keratinocytes containing the E6 gene variants were grown up to passage 30, an endpoint which was reached fastest by the AAE6 variant over the prototype E6 and L83V variants. This was expected, as earlier epidemiological findings have suggested that non-European variants such as AAE6 are more oncogenic than the E6 prototype based on increased prevalence in cervical cancer (5, 95). However, it was unexpected that the

prototype E6 reached passage 30 in a shorter amount of time than the L83V variant since this amino acid alteration is associated with higher oncogenic potential (2, 95). This could change once the cells reach an even higher passage as evidenced by results seen in a similar experiment completed by our group (data not published, manuscript submitted) in which a different primary keratinocyte donor was used and infected cells were grown until passage 60. In this donor, infection with the L83V E6 variant resulted in a shorter amount of time to reach passage 60 compared to the prototype E6. So if given enough time, the L83V variant in the current study may be able to overtake the prototype E6 variant. Keratinocytes infected with the E7 oncogene alone have been shown by one group (31) to sufficiently immortalize the keratinocytes but this characterization was based solely on growth to passage 30 and a visible crisis. In the current study, keratinocytes infected with E7 did in fact grow until passage 30 but their initial crisis was the longest out of any of the infected cells (about 450 hours, comparable to that of PHFKs), and nearing the experimental end-point, the culture exhibited two more very long crises (200 and 275 hours). This signifies that the culture was not very resilient and suggests that the cells may not have survived in culture if allowed to grow much longer.

4.2 Overcoming M1 and M2 through E6 Oncogene Expression

4.2.1 Functional E6 Oncogenes Allow Keratinocytes to Bypass M1

Under normal cellular conditions, DNA damage results in activated p53 which in turn activates p21. This activation is known to result in downregulation of cyclin-dependent kinases, consequently leading to G1 arrest (34). Once cells express the E6 protein and p53 function is abolished, there is no response to DNA damage, and therefore no cell cycle arrest (24, 34, 42). Through cell cycle analysis, it was shown that all three E6 variants could overcome actinomycin D-induced DNA damage and the resulting G1 arrest at least as early as passage 6, showing that the E6 oncogene in each of them was functional. It has also been shown that in the absence of p53, cells can bypass M1 (88). Therefore the presence of the functional E6 oncogene in the PHFKs suggests that the cells overcame M1. In cells that express the E7 protein, the interaction between pRb and E2F is blocked, leading to a release of E2F transcription factors (34, 77). These activate

various E2F-responsive genes that push the cells into S-phase (38), so it is expected that the presence of E7 alone should result in the keratinocytes overcoming G1 arrest, which was seen once at passage 30. However, because the E7 oncogene does not target p53 function, the presence of it may not be enough for the cells to overcome M1. In fact, Seavey *et al.* (70) found that E7 actually stabilizes p53, so it is expected that p53-induced apoptosis would override any mechanism by which E7 pushes cells past growth arrest. At this stage of immortalization, functioning E6 oncogenes can act as both functioning E6 and E7 oncogenes. Demers *et al.* (20) showed that functioning p53 can change the phosphorylation pattern of pRb to induce growth arrest in cells with damaged DNA. Therefore, if p53 function is destroyed by the E6 oncogene it can no longer alter the phosphorylation of pRb and cannot induce growth arrest.

4.2.2 Elevated hTERT Levels as Confirmation of Surpassing M2

An activation of hTERT is one of the defining factors of immortalization. It functions in rebuilding the ends of telomeres using its RNA template and reverse transcriptase enzyme. Therefore during cellular replication, telomeres are no longer constantly shortened, and the cells are not committed to apoptosis (14, 29, 30). It was observed that hTERT expression was elevated in all E6 oncogene variants at least as early as passage 6 and levels steadily increased as passage number increased. Activation of the hTERT gene has been shown to be caused by E6 expression through binding of c-Myc, another oncoprotein up regulated in many cancers (30, 44, 48, 54, 57, 63, 77, 82, 89), so it is expected that cells expressing E6 would show activated hTERT. PHFKs containing the E7 oncogene did not show any hTERT expression which leads us to the conclusion that the E7 oncogene is insufficient for immortalization of PHFKs.

This increase in hTERT expression is known to be directly associated with overcoming M2, a point during which telomeres are shortened to a critical length (9, 53, 66). Another method for overcoming M2 that works in conjuction with increased hTERT expression is through p53 degradation. Previous reports have shown that since the E6 oncoprotein aids in the ubiquitin dependent proteosomal degradation of p53 protein, it indirectly results in bypassing the M2 crisis stage (53, 66). Based on the results from this

study, it was confirmed that the E6 oncogene can in fact immortalize keratinocytes without E7. Moreover, the differences in ability between the gene variants to promote immortalization were also characterized and results showed that AAE6 was more proficient. These experiments were completed previously by our group (data not published, manuscript submitted) with PHFKs from another donor and the results are similar to the results seen here, meaning that the effects of the E6 oncogene on PHFKs are independent of donor-based lineage.

4.3 Transformation of PHFKs through E6 Oncogene Expression

4.3.1 Anoikis Resistance and Colony Formation as Indicators of Transformation

Along with immortalization, transformation is another important aspect of the carcinogenesis process that was explored in this study. It has been shown that one of the main causes of transformation is an up regulation of Notch1 signalling (65) which results in resistance to anoikis. Under anoikis-inducing conditions it was found that all E6 proteins showed increased resistance to anoikis with increasing passages. This is expected as E6 induces Notch 1 signalling (84) so it can be assumed that the cells are beginning to become transformed. However, compared to untreated controls, cell death was still significantly higher even in the E6E7 positive control. Perhaps to significantly decrease anoikis, transduced PHFKs must be entirely transformed and contain the complete HPV genome. It was observed that PHFKs containing only E7 also exhibited some decrease in cell death as passage number increased. This could be caused by its interaction with p600, a microtubule protein which has been implicated in deregulating anoikis in the presence of the Bovine papillomavirus E7 oncogene (19, 35, 52).

In this study it was found that AAE6-expressing PHFKs showed increased potential for transformation (compared to prototype E6 and L83V) through their ability to form colony forming units in both anchorage inhibiting and promoting conditions. These findings suggest that E6 gene expression is necessary for transformation *in vivo* and also may be important for promoting anchorage to the basement membrane *in vivo*. In order to test this clonogenic assays were performed in serum starved conditions in the presence

and absence of basement membrane proteins. The clonogenic assay is an indirect qualitative measure to determine the resiliency of the transduced PHFKs in adverse conditions. In such culture conditions, the transformed cells should exhibit characteristics that are necessary for EMT mediated cancer metastasis, as these types of cells should better survive minimalistic culture conditions (46). This could be attributed to the modulation of the Wnt/β-catenin signalling pathway (23, 80). For EMT mediated cancer metastasis, a sub-population of transformed cells will have to self-renew, be resistant to unfavourable growth conditions and be resistant to chemo or radio-therapy (46). It was found that when AAE6-expressing PHFKs were grown in serum starved nutrient media in absence of basement membrane protein substratum, they resisted apoptosis and produced closed cell colonies, suggesting they were capable of surviving inhospitable conditions in the human body. This is suggestive of an up-regulation of the Akt signalling pathway which is known to have anti-apoptotic functions (76). However, when serum starved AAE6-PHFKs were grown in the presence of basement membrane protein substratum they formed more mixed colonies that had both closed and scattered colony forming units suggesting an adaptable survival mechanism. Once infected keratinocytes form colonies, it has been shown that survival in nutrient-poor conditions precedes malignant transformation (61); therefore, the continued growth of AAE6 colonies under nutrient-poor conditions suggests these cells may be in the early stages of transformation. Moreover, AAE6-PHFKs had better adhesion capacity in the presence of basement membrane protein, suggesting that this variant has improved anchoring capability which might help increase its invasive potential. This concept is supported by the Boyden chamber assay results which found that AAE6 was able to migrate through type I collagen coated chamber filters significantly more than prototype E6 and L83V. Some groups have shown migration studies involving HPV16 and human umbilical vein endothelial cells (64), trophoblastic-like cells (10), cervical keratinocytes (81), or mutant keratinocytes (3)1, but the ability of different E6 proteins alone to cause migration and invasion in keratinocytes was not examined until now. Although there were marginal differences in migratory ability of the E6 variants, the presence of the E6 oncogene did not cause the PHFKs to migrate any more than they would normally. Yet for the AAE6 and L83V variants in the presence of E7, migration was increased and ability to migrate

was higher than that of the PHFKs as well as their counterparts containing only the E6 oncogene. This correlates with results by Charette *et al.* (13) which suggest that the E7 oncogene enhances migration in keratinocytes through the Akt signalling pathway. This effect was not seen in the prototype E6 variant in the presence of E7, suggesting that other pathways or cooperating factors are involved for European variants.

4.4 The E6 Oncogene Variants Activate Expression of Adhesion Molecules at Different Levels

During the metastasis stage, a major phenotypic change occurs involving an EMT that promotes an invasive and migratory phenotype. One important gene that is down-regulated during this phase is E-cadherin (85), whose down-regulation permits cells to release themselves from each other and prepare for invasion and metastasis (51). In the study by Matthews *et al.* (51), it was found that the E6 oncogene down-regulates E-cadherin expression, which might partially induce an EMT. However, our results show that E-cadherin protein expression is not completely downregulated by the E6 oncogene alone which suggests that in the case of HPV16, E7 may be necessary for optimal migration. This correlates with the Boyden chamber assay results which show that in the presence of E7, the AAE6 variant migrates more than its counterpart containing E6 alone. Also, for controls, the decreased level of E-cadherin protein expression directly correlates with an increase in migration. The same trend is seen between the E6 variants, with the AAE6 showing less E-cadherin protein expression compared to prototype E6 and L83V but migrating more. This signifies that E-cadherin protein downregulation may be one mechanism that induces migration in the keratinocytes. The downregulated E-cadherin expression in PHFKs may be due to the fact that they are primary cells from neonates with a mesenchymal phenotype due to retained embryogenesis pathways. The colony formation assays also showed no colony formation by PHFKs which could be related to this decrease in E-cadherin expression since cell-cell contacts are consequently decreased.

Another molecule involved in cellular adhesion is CAIX. CAIX is a metalloenzyme which is known to function in pH regulation and adhesion (60). Through western blotting, it was observed that CAIX protein expression is increased in all variants including HeLa cells, but not in PHFKs, and the AAE6 variant has the highest expression level over all variants. From the adhesion assay, it was observed that the AAE6 variant has increased adhesion capacity which may correlate with the observed increase in CAIX expression. The level of CAIX protein expression was also studied in the AAE6 and prototype E6 variants in the presence of E7 and it was found to be significantly increased in the AAE6/E7 variant over the prototype E6/E7 variant. CAIX is known to be an indicator of poor patient prognosis and aggressive tumours in cancers (45, 50) so this higher expression in the AAE6 variant may correlate with the increased prevalence of this variant in epidemiological studies. HeLa cells showed high CAIX protein expression and when this is accompanied by a decrease in E-cadherin protein expression scattered colonies can form. This was observed in the colony formation assay and is most likely caused by broken cell-cell contact and increased cellular adhesion.

CAIX can be modulated by both hypoxia inducible factor (HIF-1) activation and epidermal growth factor receptor (EGFR) (36, 47). Hypoxic tumours have been shown to be characteristically more aggressive, lead to a poor response to treatment, and lower survival rate (50, 90) due to the up-regulation of HIF-1. Along with increased HIF-1 expression in cervical cancer (7), multiple studies have found an up-regulation of EGFR in cervical cancers (8, 79), so up-regulation of CAIX through either pathway is quite plausible. It has been shown that CAIX activation up-regulates the Akt pathway (4, 47, 76) which leads to anti-apoptotic effects (59), and has been shown to induce cell cycle progression through pRb hyperphosphorylation and β-catenin signalling. This also links back to activation of the Wnt pathway (59).

One obvious experimental limitation of this study which could affect the observed *in vitro* results is the absence of immune cell factors which may interfere with E6 promoted cellular activity *in vivo*. This situation could not be accounted for in the cell culture studies. However, our group has extensively discussed the immune response to

HPV 16 infection and showed that interferon-kappa (IFN-κ), which initiates immune response post infection, is down-regulated by E6 expression (17). Another limitation of this study that could not be accounted for is the angiogenesis system and associated factors that occur *in vivo* that allow for systemic migration of the infected cells (85). Here, E7 might be required for the infected cells to survive the attacks of the immune system when they are in systemic blood circulation. However, these aspects were not within the scope of the current study which attempted to unravel the effects of E6 alone on PHFKs, the primary target of HPV 16 infection.

5.0 CONCLUSION

Through this study, it was demonstrated that certain naturally occurring E6 oncogenes that are found in cervical lesions are sufficient to immortalize keratinocytes, but only the AAE6 variant was able to transform keratinocytes and increase their metastatic potential. These findings support epidemiological studies showing that the incidence of AAE6 in cervical cancers is 20 times higher than other E6 variants or the prototype (5). Results from this study suggest that this variant is more likely to survive the metastatic cascade and possibly form secondary tumours thus contributing to increased oncogenic potential and mortality rates. The results begin to elucidate potential pathway activation which may affect the aggressiveness and prevalence of the different variants. It seems that CAIX up-regulation is an important factor in the increased oncogenic potential of the AAE6 variant.

Although we show the importance of the E6 oncogene, the necessity of the E7 gene does not go unrecognized, as many of these experiments showed that in the presence of E7, the E6 gene function was stabilized and optimal. Also, in the presence of E7 the AAE6 variant retained its enhanced oncogenic properties over the prototype E6. Clinically, our results suggest that the variable carcinogenic potential and resulting prevalence of these different HPV16 subtypes is primarily influenced by the E6 oncogene, and that the E7 oncogene may be necessary for enhancement of this E6 function to its full potential.

5.1 Future Directions

In light of these findings, the most important next step of this work would be to observe the effects of E6 oncogene expression alone *in vivo* to see the limits of what this oncogene can accomplish during the carcinogenic process. Another future step is to continue to unlock the pathways that are activated during carcinogenesis by these oncogenes and attempt to target them for therapy purposes. A look into HIF-1 or EGFR mediated pathways may be the next logical step. Proceeding with *in vivo* studies in wild-type and immunocompromised mice would assist in overcoming the limitations of this current study. The *in vitro* model is a controlled environment which can only give results suggestive of the *in vivo* environment so having an *in vivo* model would allow for the effects of angiogenesis pathways on the E6 oncogene function to be observed.

The ability to elucidate the power of a single oncogene, as well as observe the difference in power between oncogene variants would provide an extensive base knowledge for any future studies involving HPV and cervical cancer. This study may also clarify why the non-European variants are more prevalent than the prototype in cervical cancers (2, 5, 12, 83, 91, 92, 95). If it was observed that the E6 oncogene can control most of the major steps leading to invasive cervical cancer, then more focused therapy could be created to block the actual HPV infection from resulting in cancer, or it could be used as a druggable target for highly specific antibody or siRNA based therapy.

6.0 REFERENCES

1. **Adams JC, Watt FM.** 1989. Fibronectin inhibits the terminal differentiation of human keratinocytes. Nature. **340:** 307-309.
2. **Andersson S, Alemi M, Rylander E, Strand A, Larsson B, Sallstrom J, Wilander E.** 2000. Uneven distribution of HPV 16 E6 prototype and variant (L83V) oncoprotein in cervical neoplastic lesions. Br. J. Cancer. **83:** 307-310.

3. **Baudoin C, Fantin L, Meneguzzi G.** 2005. Proteolytic processing of the laminin α3 G domain mediates assembly of hemidesmosomes but has no role on keratinocyte migration. J. Invest. Dermatol. **125**: 883-888.
4. **Bedogni B, Welford SM, Cassarino DS, Nickoloff BJ, Giaccia AJ, Powell MB.** 2005. The hypoxic microenvironment of the skin contributes to Akt-mediated melanocyte transformation. Cancer Cell. **8:** 443-454.
5. **Berumen J, Ordonez RM, Lazcano E, Salmeron J, Galvan SC, Estrada RA, Yunes E, Garcia-Carranca A, Gonzalez-Lira G, Madrigal-de la Campa A.** 2001. Asian American variant of human papillomavirus 16 and risk for cervical cancer: a case-control study. J. Natl. Cancer. Inst. **93**: 1325-1330.
6. **Beutner KR, and Tyring S.** 1997. Human papillomavirus and human disease. Am. J. Med. **102:** 9-15.
7. **Birner P, Schindl M, Obermair A, Plank C, Breitenecker G, Oberhuber G.** 2000. Overexpression of hypoxia-inducible factor 1α is a marker for an unfavourable prognosis in early-stage invasive cervical cancer. Cancer Res. **60:** 4693-4696.
8. **Boiko IV, Mitchell MF, Hu W, Pandey DK, Mathevet P, Malpica A, Hittelman WN.** 1998. Epidermal growth factor receptor expression in cervical intraepithelial neoplasia and its modulation during an α-difluoromethylornithine chemoprevention trial. Clin. Cancer Res. **4:** 1383-1391.
9. **Bond JA, Haughton MF, Rowson JM, Smith PJ, Gire V, Wynford-Thomas D, Wyllie FS.** 1999. Control of replicative life span in human cells: barriers to clonal expansion intermediate between M1 senescence and M2 crisis. Mol. Cell. Biol. **19**: 3103-3114.
10. **Boulenouar S, Weyn C, Van Noppen M, Ali MM, Favre M, ODelvenne P, Bex F, Noël A, Englert Y, Fontaine V.** 2010. Effects of HPV-16 E5, E6 and E7 proteins on survival, adhesion, migration and invasion of trophoblastic cells. Carcinogenesis. **31**: 473-480.
11. **Brady C S, Duggan-Keen MF, Davidson JA, Varley JM, Stern PL.** 1999. Human papillomavirus type 16 E6 variants in cervical carcinoma: relationship to host genetic factors and clinical parameters. J. Gen. Virol. **80**: 3233–3240.

12. **Burk RD, Terai M, Gravitt PE, Brinton LA, Kurman RJ, Barnes WA, Greenberg MD, Hadjimichael OC, Fu L, McGowan L, Mortel R, Schwartz PE, Hildesheim A.** 2003. Distribution of human papillomavirus types 16 and 18 variants in squamous cell carcinomas and adenocarcinomas of the cervix. Cancer Res. **63:** 7215-7220.

13. **Charette ST, McCance DJ.** 2007. The E7 protein from human papillomavirus type 16 enhances keratinocyte migration in an Akt-dependent manner. Oncogene. **26:** 7386-7390.

14. **Colgin LM, Reddel RR.** 1999. Telomere maintenance mechanisms and cellular immortalization. Curr. Opin. Genetic.s Dev. **9**: 97-103.

15. **Counter CM, Avilion AA, LeFeuvre CE, Stewart NG, Greider CW, Harley CB, Bacchetti S.** 1992.Telomere shortening associated with chromosome instability is arrested in immortal cells with express telomere activity. EMBO J. **11**: 1921-1929.

16. **DeCarlo CA, Escott NG, Werner J, Robinson K, Lambert PF, Law RD, Zehbe I.** 2008. Gene expression analysis of interferon κ in laser capture microdissected cervical epithelium. Anal. Biochem. **381:** 59-66.

17. **DeCarlo CA, Rosa B, Jackson R, Niccoli S, Escott NG, Zehbe I.** 2012. Toll-like receptor transcriptome in the HPV-positive cervical cancer microenvironment. Clin. Dev. Immunol. **2012**: 1-9.

18. **DeFilippis RA, Goodwin EC, Wu L, DiMaio D.** 2003. Endogenous human papillomavirus E6 and E7 proteins differentially regulate proliferation, senescence, and apoptosis in HeLa cervical carcinoma cells. J. Virol. **77**: 1551-1563.

19. **DeMasi J, Chao MC, Kumar AS, Howley PM.** 2007. Bovine papillomavirus E7 oncoprotein inhibits anoikis. J. Virol. **81:** 9419-9425.

20. **Demers GW, Foster SA, Halbert CL, Galloway DA.** 1994. Growth arrest by induction of p53 in DNA damaged keratinocytes is bypassed by human papillomavirus 16 E7. Proc. Natl. Acad. Sci. USA. **91**: 4382-4386.

21. **Fidler IJ, Radinsky R.** 1989. Genetic control of cancer metastasis. J. Natl. Cancer Inst. **82**: 166-168.

22. **Flores ER, Allen-Hoffmann BL, Lee D, Sattler CA, Lambert PF.** 1999. Establishment of the human papillomavirus type 16 (HPV-16) life cycle in an immortalized human foreskin keratinocytes cell line. Virology. **262:** 344-354.

23. **Fodde R, Brabletz T.** 2007. Wnt/β-catenin signalling in cancer stemness and malignant behaviour. Curr. Opin. Cell Biol**. 19:** 150 -158**.**

24. **Foster SA, Demers GW, Etscheid BG, Galloway DA.** 1994. The ability of human papillomavirus E6 proteins to target p53 for degradation in vivo correlates with their ability to abrogate actinomycin D-induced growth arrest. J. Virol. **68**: 5698-5705.

25. **Franken NAP, Rodermond HM, Stap J, Haveman J, van Bree C.** 2006. Clonogenic assay of cells in vitro. Nat. Protocols. **1**:2315-2319.

26. **Frattini MG, Lim HB, Laimins LA.** 1996. *In vitro* synthesis of oncogenic human papillomavirus requires episomal genomes for differentiation-dependent late expression. Proc. Natl. Acad. Sci. USA. **93**: 3062-3067.

27. **Frazer IH.** 2004. Prevention of cervical cancer through papillomavirus vaccination. Nature. **4:** 46-54.

28. **Frisch SM, Francis H**. 1994. Disruption of epithelial cell-matrix interactions induces apoptosis. J. Cell. Biol. **124**: 620-626.

29. **Greider CW, Blackburn EH.** 1985. Identification of a specific telomere terminal transferase activity in tetrahymena extracts. Cell. **43**: 405-413.

30. **Hahn WC, Meyerson M.** 2001. Telomerase activation, cellular immortalization and cancer. Ann. Med. **33**: 123-129.

31. **Halbert CL, Demers GW, Galloway DA.** 1991. The E7 gene of human papillomavirus type 16 is sufficient for immortalization for human epithelial cells. J. Virol. **65**: 473-478.

32. **Hawley-Nelson P, Vousden KH, Hubbert NL, Lowy DR, Schiller JT.** 1989. HPV16 E6 and E7 proteins cooperate to immortalize human foreskin keratinocytes. EMBO J. **8**: 3905-3910.

33. **Herman L, Hubert P, Caberg J-H, Evrard B, Kedzia W, Boniver J, Delvenne P.** 2007. MIP3 alpha stimulates the migration of Langerhans cells in models of

human papillomavirus (HPV)-associated (pre)neoplastic epithelium. Cancer Immuno. Immunother. **56**: 1087-1096.

34. **Hickman ES, Bates S, Vousden KH.** 1997. Perturbation of the p53 response by human papillomavirus type 16 E7. J. Virol. **71**: 3710-3718.

35. **Huh KW, DeMasi J, Ogawa H, Nakatani Y, Howley PM, Munger K.** 2005. Association of the human papillomavirus type 16 E7 oncoprotein with the 600-kDa retinoblastoma protein-associated factor, p600. PNAS. **102:** 11492-11497.

36. **Ivanov S, Liao S, Ivanova A, Danilkovitch-Miagkova A, Tarasova N, Weirich G, Merrill MJ, Proescholdt MA, Oldfield EH, Lee J, Zavada J, Waheed A, Sly W, Lerman MI, Stanbridge EJ.** 2001. Expression of hypoxia-inducible cell-surface transmembrane carbonic anhydrases in human cancer. Am. J. Pathol. **158:** 905-919.

37. **Jeon S, Allen-Hoffmann BL, Lambert PF.** 1995. Integration of human papillomavirus type 16 into the human genome correlates with a selective growth advantage of cells. J. Virol. **1995:** 2989-2997.

38. **Jones DL, Munger K.** 1997. Analysis of the p53-mediated G1 growth arrest pathway in cells expressing the human papillomavirus type 16 E7 oncoprotein. J. Virol. **71**: 2905-2912.

39. **Jones DL, Thompson DA, Suh-Burgmann E, Grace M, Munger K.** 1999. Expression of the HPV E7 oncoprotein mimics but does not evoke a p53-dependent cellular DNA damage response pathway. Virol. **258**: 406-414.

40. **Kastan MB, Onyekwere O, Sidransky D. Vogelstein B, Craig RW.** 1991. Participation of p53 protein in the cellular response to DNA damage. Cancer Res. **51**: 6304-6311.

41. **Kaur P, McDougall JK, Cone R.** 1989. Immortalization of primary human epithelial cells by cloned cervical carcinoma DNA containing human papillomavirus type 16 E6/E7 open reading frames. J. Gen. Virol. **70**: 1261-1266.

42. **Kessis TD, Slebos RJ, Nelson WG, Kastan MB, Plunkett BS, Han SM, Lorincz AT, Hedrick L, Cho KR.** 1993. Human papillomavirus 16 E6 expression disrupts the p53-mediated cellular response to DNA damage. Proc. Natl. Acad. Sci. USA. **90**: 3988-3992.

43. **Klingelhutz AJ, Barber S, Smith PP, Dyer K, McDougall JK.** 1994. Restoration of telomeres in human papillomavirus-immortalized human anogenital epithelial cells. Mol. Cell. Biol. **14**: 961-969.

44. **Klingelhutz AJ, Foster SA, McDougall JK.** 1996. Telomerase activation by the E6 gene product of human papillomavirus type 16. Nature. **380**: 79-82.

45. **Kong CS, Narasimhan B, Cao H, Kwok S, Erickson JP, Koong A, Pourmand N, and Le Q.** 2009. The relationship between human papillomavirus status and other molecular prognostic markers in head and neck squamous cell carcinomas. Int. J Radiat. Oncol. Biol. Phys. **74:** 553–561.

46. **Kuhn NZ, Tuan RS.** 2010. Regulation of stemness and stem cell niche of mesenchymal stem cells: implications in tumorigenesis and metastasis. J. Cell. Physiol. **222:** 268-277.

47. **Li Y, Wang H, Tu C, Shiverick KT, Silverman DN, Frost SC.** 2011. Role of hypoxia and EGF on expression, activity, localization and phosphorylation of carbonic anhydrase IX in MDA-MB-231 breast cancer cells. Biochim. Biophys. Acta. **1813:** 159-167.

48. **Liu X, Dakic A, Zhang Y, Dai Y, Chen R, Schlegel R.** 2009. HPV E6 protein interacts physically and functionally with the cellular telomerase complex. Proc. Natl. Acad. Sci. USA. **106**: 18780-18785.

49. **Liu X, Disbrow GL, Yuan H, Tomaic V, Schlegel R.** 2007. Myc and human papillomavirus type 16 E7 genes cooperate to immortalize human keratinocytes. J. Virol. **81**: 12689-12695.

50. **Loncaster JA, Harris AL, Davidson SE, Logue JP, Hunter RD, Wycoff CC, Pastorek J, Ratcliffe PJ, Stratford IJ, and West CML.** 2001. Carbonic anhydrase (CA IX) expression, a potential new intrinsic marker of hypoxia: correlations with tumor oxygen measurements and prognosis in locally advanced carcinoma of the cervix. Cancer Res. **61:** 6394-6399.

51. **Matthews K, Leong CM, Baxter L, Inglis E, Yun K, Backstrom BT, Doorbar J, Hibma M.** 2003. Depletion of Langerhans cells in human papillomavirus type 16-infected skin is associated with the E6-mediated down regulation of E-cadherin. J. Virol. **77**: 8378-8385.

52. **McLaughlin-Drubin ME, Munger K.** 2009. The human papillomavirus E7 oncoprotein. Virology. **384:** 335-344.

53. **McMurray HR, McCance DJ.** 2004. Degradation of p53, not telomerase activation, by E6 is required for bypass of crisis and immortalization by human papillomavirus type 16 E6/E7. J. Virol. **78**: 5698-5706.

54. **McMurray HR, McCance DJ.** 2003. Humna papillomavirus type 16 E6 activates TERT gene transcription through induction of c-Myc and release of USF-mediated repression. J. Virol. **77**: 9852-9861.

55. **Morgenstern JP, Land H.** 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucl. Acids Res. **18:** 3587-3596.

56. **Munger K, Phelps WC, Bubb V, Howley PM, Schlegel R.** 1989. The E6 and E7 genes of human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. J. Virol. **63**: 4417-4421.

57. **Oh ST, Kyo S, Laimins LA.** 2001. Telomerase activation by human papillomavirus type 16 E6 protein: induction of human telomerase reverse transcriptase expression through Myc and GC-rich Sp1 binding sites. J. Virol. **75**: 5559-5566.

58. **Organization, W. H.** 2010. Accessed on 7 May 2012. Human papillomavirus and related cancers: summary report update. [Online.]

59. **Osaki M, Oshimura M, Ito H.** 2004. PI3K-Akt pathway: its functions and alterations in human cancer. Apoptosis. **9:** 667-676.

60. **Pastoreková S, and Závada J.** 2004. Carbonic anhydrase IX (CA IX) as a potential target for cancer therapy. J. Cancer Ther. **2:** 245-262.

61. **Pera MF, Gorman PA.** 1984. *In vitro* analysis of multistage epidermal carcinogenesis: development of indefinite renewal capacity and reduced growth factor requirements in colony forming keratinocytes precedes malignant transformation. Carcinogenesis. **5:** 671-682.

62. **Pilcher BK, Dumin J, Sudbeck BD, Krane SM, Welgus HG, Parks WC.** 1997. The activity of collagenase-1 is required for keratinocyte migration on a type 1 collagen matrix. J. Cell Biol. **137**: 1445-1457.

63. **Plug-DeMaggio AW, Sundsvold T, Wurscher MA, Koop JI, Klingelhutz AJ, McDougall JK.** 2004. Telomere erosion and chromosomal instability in cells expressing the HPV oncogene 16E6. Oncogene. **23**: 3561-3571.

64. **Raffaella R, Gioia D, De Andrea M, Cappello P, Giovarelli M, Marconi P, Manservigi R, Gariglio M, Landolfo S.** 2004. The interferon-inducible IFI16 gene inhibits tube mophogenesis and proliferation of primary, but not HPV 16 E6/E7-immortalized human endothelial cells. Exp. Cell Res. **293**: 331-345.

65. **Rangarajan A, Syal R, Selvarajah S, Chakrabarti O, Sarin A, Krishna S.** 2001. Activated Notch1 signaling cooperates with papillomavirus oncogenes in transformation and generates resistance to apoptosis on matrix withdrawal through PKB/Akt. Virol. **286**: 23-30.

66. **Rheinwald JG, Hahn WC, Ramsey MR, Wu JY, Guo Z, Tsao H, De Luca M, Catricalà C, O'Toole KM.** 2002. A two-stage $p16^{INK4A}$- and p53-dependent keratinocyte senescence mechanism that limits replicative potential independent of telomere status. Mol. Cell. Biol. **22**: 5157-5172.

67. **Richard C, Lanner C, Naryzhny SN, Sherman L, Lee H, Lambert PF, Zehbe I.** 2010. The immortalizing and transforming ability of two common human papillomavirus 16 E6 variants with different prevalences in cervical cancer. Oncogene. **29**: 3435-3445.

68. **Sang B and Barbosa MS.** 1992. Single amino acid substitutions in "low-risk" human papillomavirus (HPV) type 6 E7 protein enhance features characteristic of the "high-risk" HPV E7 oncoproteins. Proc. Natl. Acad. Sci. **89:** 8063-8067.

69. **Scheffner M, Takahashi T, Huibregtse JM, Minna JD, Howley PM.** 1992. Interaction of the human papillomavirus type 16 E6 oncoprotein with wild-type and mutant human p53 proteins. J. Virol. **66:** 5100-5105.

70. **Seavey SE, Holubar M, Saucedo LJ, Perry ME.** 1999. The E7 oncoprotein of human papillomavirus type 16 stabilizes p53 through a mechanism independent of p19 ARF. J. Virol. **73:** 7590-7598.

71. **Sedman SA, Barbosa MS, Vass WC,, Hubbert NL, Haas JA, Lowy DR, Schiller JT.** 1991. The full-length E6 protein of human papillomavirus type 16

has transforming and *trans*-activating activities and cooperates with E7 to immortalize keratinocytes in culture. J. Virol. **65**: 4860-4866.

72. **Semenza GL.** 2010. HIF-1: upstream and downstream of cancer metabolism. Curr. Opin. Genet. Dev. **20:** 1-10.

73. **Shay JW, Wright WE.** 2005. Senescence and immortalization: role of telomeres and telomerase. Carcinogenesis. **26**: 867-874.

74. **Shay JW, Wright WE, Brasiskyte D, Van der Haegen BA**. 1993. E6 of human papillomavirus type 16 can overcome the M1 stage of immortalization in human mammary epithelial cells but not in human fibroblasts. Oncogene. **8:** 1407-1413.

75. **Shay JW, Wright WE, Werbin H.** 1991. Defining the molecular mechanisms of human cell immortalization. Biochim. Biophys. Acta. **1072:** 1-7

76. **Stewart D, Edwards J, Richardson D, Jones L, Burke B, Waller D, Zeigler-Heitbrock L, Wardlaw A, O'Byrne K.** 2006. The inter-relationship of hypoxia and phospho-Akt in the pathobiology of malignant pleural mesothelioma. J. Clin. Oncol. **24**: 17057.

77. **Stöppler H, Stöppler MC, Johnson E, Simbunlan-Rosenthal CM, Smulson ME, Iyer S, Rosenthal DS, Schlegel R.** 1998. The E7 protein of human papillomavirus type 16 sensitizes primary human keratinocytes to apoptosis. Oncogene. **17**: 1207-1214.

78. **Talis AL, Huibregtse JM, Howley PM.** 1998. The role of E6AP in the regulation of p53 protein levels in human papillomavirus (HPV)-positive and HPV-negative cells. J. Biol. Chem. **273: 6439-6445.**

79. **Tervahauta A, Syrjanen S, and Syrjanen K.** 1994. Epidermal growth factor receptor, c-erbB-2 proto-oncogene and estrogen receptor expression in human papillomavirus lesions of the uterine cervix. Int. J. Gynecol. Pathol. **13:** 234-240.

80. **Üren A, Fallen S, Yuan H, Usubütün A, Küçükali T, Schlegel R, Toretsky JA.** 2005. Activation of the canonical Wnt pathway during genital keratinocyte transformation: a model for cervical cancer progression. Cancer Res. **65:** 6199-6206.

81. **VanDenBrule FA, Clausse N, Delvenne P, Franzen-Detrooz E, Castronovo.** 2000. Combined interferon-γ and tumour necrosis factor-α treatment differentially

affects adhesion and migration of keratinocyte-derived cells to laminin-1. Cell Adhesion Comm. **7**: 321-329.

82. **Veldman T, Horikawa I, Barrett JC, Schlegel R.** 2001. Transcriptional activation of the telomerase hTERT gene by human papillomavirus type 16 E6 oncoprotein. J. Virol. **75**: 4467-4472.

83. **Villa LL, Sichero L, Rahal P, Caballero O, Ferenczy A, Rohan T, Franco EL.** 2000. Molecular variants of human papillomavirus types 16 and 18 preferentially associated with cervical neoplasias.J. Gen. Virol. **81:** 2959-2968.

84. **Weijzen S, Zlobin A, Braid M, Miele L, Kast WM.** 2003. HPV16 E6 and E7 oncoproteins regulate Notch-1 expression and cooperate to induce transformation. J. Cell. Physiol. **194**: 356-362.

85. **Weinberg RA.** 2007. The Biology of Cancer. Garland Science. New York, NY

86. **Willey JC, Broussoud A, Sleemi A, Bennett WP, Cerutti P, Harris CC.** 1991. Immortalization of normal human bronchial epithelial cells by human papillomaviruses 16 or 18. Cancer Res. **51**: 5370-5377.

87. **Wright WE, Pereira-Smith OM, Shay JW.** 1989. Reversible cellular senescence: implications for immortalization of normal human diploid fibroblasts. Mol. Cell. Biol. **9**: 3088-3092.

88. **Wright WE, Shay JW.** 1992. The two-stage mechanism controlling cellular senescence and immortalization. Exp. Gerontol. **27**: 383-389.

89. **Wu K-J, Grandori C, Amacker M, Simon-Vermot N, Polack A, Lingner J, Dalla-Favera R.** 1999. Direct activation of *TERT* transcription by c-MYC. Nature Genet. **21**: 220-224.

90. **Wykoff CC, Beasley NJP, Watson PH, Turner KJ, Pastorek J, Sibtain A, Wilson GD, Turley H, Talks KL, Maxwell PH, Pugh CW, Ratcliffe PJ, Harris AL.** 2000. Hypoxia-inducible expression of tumor-associated carbonic anhydrases. Cancer Res. **60:** 7075-7083.

91. **Xi LF, Koutsky LA, Galloway DA, Kuypers J, Hughes JP, Wheeler CM, Holmes KK, Kiviat NB.** 1997. Genomic variation of human papillomavirus type 16 and risk for high grade cervical intraepithelial neoplasia. J. Natl. Cancer Inst. **89:** 796-802.

92. **Xi LF, Koutsky LA, Hidesheim A, Galloway DA, Wheeler CM, Winer RL, Ho J, Kiviat NB.** 2007. Risk for high-grade cervical intraepithelial neoplasia associated with variants of human papillomavirus types 16 and 18. Cancer Epidemiol. Biomarkers Prevent. **16:** 4-10.

93. **Yamada T, Manos MM, Peto J, Greer CE, Munoz N, Bosch FX, Wheeler CM.** 1997. Human papillomavirus type 16 sequence variation in cervical cancers: a worldwide perspective. J. Virol. **71:**2463-2472.

94. **Yasumoto S, Kunimura C, Kikuchi K, Tahara H, Ohji H, Yamamoto H, Ide T, Utakoji T.** 1996. Telomerase activity in normal human epithelial cells. Oncogene. **13:** 433-439.

95. **Zehbe I, Wilander E, Delius H, Tommasino M.** 1998. Human papillomavirus 16 E6 variants are more prevalent in invasive cervical carcinoma than the prototype. Cancer Res. **58**: 829-833.

96. **zur Hausen H.** 2000. Papillomavirus causing cancer: evasion from host-cell control in early events in carcinogenesis. J. Natl. Cancer Inst. **92:** 690-698.

97. **zur Hausen H, De Villiers E-M.** 1994. Human papilloma viruses. Annu. Rev. Microbiol. **48**: 427-447.