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DEPARTMENT OF BIOLOGICAL SCIENCES

INVESTIGATING THE ROLE OF THE HPV16 ONCOGENES IN MODULATING STEM CELL BIOLOGY

DOCTOR OF PHILOSOPHY DISSERTATION

STYLIANI MICHAEL

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INVESTIGATING THE ROLE OF THE HPV16 ONCOGENES IN MODULATING STEM CELL BIOLOGY

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The present Doctoral Dissertation was submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy at the ***Department of Biological Sciences*** *and was approved on the 9th May 2016 by the members of the* ***Examination Committee****.*

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The present doctoral dissertation was submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy of the University of Cyprus. It is a product of original work of my own, unless otherwise mentioned through references, notes or any other statements.

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This work is dedicated to

My beloved parents Koulla and Michalis for their love, support and for making me who I am today. This PhD is a reward for all your sacrifices and I hope it makes you proud for being able to give your children the education you always dreamt of.

My precious little brother Neophytos, for being one of the most valuable people in my life.

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Love you all.
Dr. Stela Michael

ΠΕΡΙΛΗΨΗ

Τα καρκινικά κύτταρα τα οποία χαρακτηρίζονται από τη βλαστική τους ικανότητα και τα οποία οφείλονται για την έναρξη της καρκινογένεσης, σχηματίζονται είτε απευθείας από τα βλαστικά κύτταρα των ιστών είτε από τον επαναπρογραμματισμό (αποδιαφοροποίηση) των διαφοροποιημένων κυττάρων. Οι μολυσματικοί παράγοντες που σχετίζονται με τον καρκίνο, όπως οι ιοί, θεωρείται ότι προάγουν την καρκινογένεση μέσω της στόχευσης των βλαστοκυττάρων ή των πρόγονων κυττάρων στους ιστούς τους οποίους μολύνουν. Τέτοια στόχευση έχει πιθανώς επιπτώσεις και στον κύκλο ζωής του ιού, για παράδειγμα στην ικανότητα του να παραμένει στους ιστούς για μεγαλύτερο χρονικό διάστημα.

Ο ιός του HPV16 μολύνει στρωματοποιημένα πλακώδη επιθήλια, όπως αυτά που βρίσκονται στο δέρμα και στη γεννητική οδό αποκτώντας πρόσβαση στη βασική στοιβάδα του επιθηλίου. Σε αυτή τη στοιβάδα τα κύτταρα υφίστανται πολλαπλασιασμό, τον οποίο χρησιμοποιεί ο ιός για την εξάπλωση του, και πιστεύεται ότι είναι η περιοχή στην οποία βρίσκονται τα βλαστοκύτταρα του ιστού. Στον τράχηλο, την πιο συχνή εστία μόλυνσης του HPV16, ο καρκίνος προκύπτει συχνά από τη μεταβατική ζώνη την προτεινόμενη περιοχή των βλαστοκυττάρων (Elson et al., 2000). Ενώ ο ιός θεωρείται ότι μολύνει βλαστικά κύτταρα καθώς επίσης και διαφοροποιημένα κύτταρα της βασικής στιβάδας δεν είναι γνωστό αν οι καρκίνοι του HPV προκύπτουν συγκεκριμένα από τον ένα ή και τους δύο αυτούς πληθυσμούς. Υποθέσαμε ότι ο HPV16 μπορεί να ρυθμίσει τη συμπεριφορά των υφιστάμενων βλαστοκυττάρων όπως επίσης και να συμβάλει στην ανάπτυξη νέων βλαστικών πληθυσμών μέσω της έκφρασης των ιικών ογκογονιδίων E6 και E7.

Για την καλύτερη κατανόηση του ρόλου των E6 και E7 στη ρύθμιση των βλαστοκυττάρων των ιστών *in vivo,* χρησιμοποιήσαμε ως μοντέλα διαγονιδιακά E6 και E7 ποντίκια. Έχουμε δείξει ότι η έκφραση των ογκογονιδίων κινητοποιεί τα αδρανή βλαστοκύτταρα στο θύλακα της τρίχας. Επιπλέον, η έκφραση των ογκογονιδίων προκαλεί επέκταση των κυττάρων με βλαστική ικανότητα σε περιοχές που χαρακτηρίζονται συνήθως από απουσία βλαστοκυττάρων. Για την ταυτοποίηση ενός κυτταρικού εταίρου των E6 και E7, μέσω του οποίου ασκούν τις επιδράσεις τους, μελετήθηκε η *in vivo* αλληλεπίδραση τους με την τελομεράση. Η E6 επαγόμενη μείωση στα LRCs βρέθηκε να είναι ανεξάρτητη της τελομεράσης. Εν τω μεταξύ, η E7 επαγόμενη μείωση στα LRCs μπορεί να επηρεάζεται από την ομοιόσταση των τελομερών.

Επιπρόσθετα, ερευνήθηκε εάν τα ογκογονίδια του HPV16 εμπλέκονται στην ανάπτυξη βλαστοκυττάρων από διαφοροποιημένους κυτταρικούς πληθυσμούς. Η έκφραση των ογκογονιδίων κατά τη διάρκεια του επαναπρογραμματισμού των διαφοροποιημένων κυττάρων σε πολυδύναμα βλαστοκύτταρα (iPS) ενισχύει τη διαδικασία με την αύξηση του αριθμού των αποικιών. Η άμεση συμβολή τους στη διαδικασία ήταν εμφανής από την αντικατάσταση ενός από τους μεταγραφικούς παράγοντες που απαιτείται για την αποτελεσματική διαμόρφωση iPS αποικιών.

Η εργασία μας δείχνει ότι τα ογκογονίδια HPV16 μπορούν να τροποποιήσουν άμεσα τη συμπεριφορά των βλαστοκυττάρων των ιστών, με τρόπους που μπορούν να τα καταστήσουν πιο επιρρεπή σε περαιτέρω προσβολή από ογκογονικά ερεθίσματα. Επίσης, προωθούν την ανάπτυξη βλαστοκυττάρων από διαφοροποιημένα κύτταρα. Αυτές οι συνέπειες των ιικών ογκογονιδίων μπορεί να είναι ζωτικής σημασίας για την κατανόηση της μακροζωίας των HPV λοιμώξεων σε ανθρώπους, καθώς και για την ενδεχόμενη ανάπτυξη της καρκινογένεσης.

ABSTRACT

Cancer initiating cells with stem-cell like character are speculated to form either directly from tissue stem cells or from the reprogramming (dedifferentiation) of differentiated cells. Infectious agents associated with cancer such as viruses, have been proposed to promote carcinogenesis in part via the targeting of stem cells or stem-cell like cells. Such targeting presumably has implications for the viral life cycle as well, for example on the ability of the virus to persist in tissues for longer time periods.

HPV16 infects stratified squamous epithelia, such as those lining the skin and the anogenital tract as the virus gains access to the basal proliferative layer of such epithelia, where tissue stem cells are thought to reside. In the cervix, the most common site of HPV16 infection, cancer frequently arises from the transition zone, an area that is the proposed stem cell niche of the tissue (Elson et al., 2000). While the virus is thought to infect stem cells as well as committed cells of the basal layer it is not known whether HPV-positive cancers arise specifically from one or both of those populations. We hypothesized that HPV16 can regulate the behavior of existing stem cells as well as contribute to the development of stem-cell like populations through the expression of the main viral oncogenes E6 and E7.

To better understand the role of E6 and E7 in regulating tissue stem cells *in vivo* we used transgenic animal models. In mice transgenic for the HPV16 oncogenes we have shown that quiescent hair follicle stem cells aberrantly mobilize in response to oncogene expression. Furthermore, oncogene expression induces expansion of stemness markers to areas where stem cells do not normally reside. To identify a cellular partner of E6 and E7, through which these effects are mediated, their *in vivo* interaction with telomerase was investigated. E6 induced reduction in label-retaining cells was found to be telomerase independent. Meanwhile E7 induced label-retaining cell reduction may be affected by telomere homeostasis.

We also investigated whether the HPV16 oncogenes are involved in the development of cells with stem-cell like characteristics from differentiated cell populations. The expression of the oncogenes during the reprogramming of differentiated cells to induced pluripotent stem cells (iPS) enhanced the process by increasing colony numbers. Their direct contribution in the process was evident by the replacement of one of the transcription factors required for efficient iPS formation.

Our work shows that the HPV16 oncogenes can directly modify the behavior of tissue stem cells in ways that may render them more susceptible to further oncogenic insult. They

also promote the development of stem-cell like characteristics in differentiated cells. These consequences of the viral oncogenes may be crucial to understanding the longevity for HPV infections in humans as well as the eventual development of carcinogenesis.

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ABBREVIATIONS

ATP	Adenosine Triphosphate
BrdU	Bromodeoxyuridine
Cdk4	Cyclin dependent kinase 4
Cy3	Cyanine dye 3
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DNMT	DNA Methyltransferase
dNTPs	Deoxyribose Nucleoside Triphosphate
E1	Early protein 1
E2	Early protein 2
E4	Early protein 4
E5	Early protein 5
E6	Early protein 6
E6AP	E6 – Associated Protein
E7	Early protein 7
EDTA	Ethylenediaminetetraacetic acid
FADD	Fas-Associated protein with Death Domain
FITC	Fluorescein isothiocyanate
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
HDM	Histone Demethylase
HMT	Histone Methyltransferase
HPV	Human Papillomaviruses
IARC	International Agency for Research on Cancer
iPS	Induced pluripotent stem cells
K14	Cytokeratin 14
K15	Cytokeratin 15
KDM6	Lysine (K) – specific demethylase
Klf4	Kruppel-like factor 4
L1	Late protein 1
L2	Late protein 2
LRCs	Label Retaining Cells
MEFs	Mouse Embryonic Fibroblasts

MEM	Minimum Essential Medium
Nfatc1	Nuclear factor of activated T-cells
NOD/SCID	Nonobese diabetic/severe combined immunodeficiency
NTG	Non-transgenic
Oct4	Octamer-binding transcription factor 4
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
pRb	Retinoblastoma
RNA	Ribonucleic acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
P/S	Penicillin/Streptomycin
PV	Papillomaviruses
RT	Room Temperature
Sox2	Sex determining region Y – box 2
Terc	Telomerase RNA component
Tert	Telomerase reverse transcriptase
TTL	Translation Termination Linker

CHAPTER 1

INTRODUCTION

INTRODUCTION

Some infectious agents can "hijack" the host cellular machinery for the agent's replication. In some cases infections can lead to cancer, and estimates have shown that the worldwide risk of cancer from infections is around 16%, ranging from 7% in developed countries to 23% in less developed countries (de Martel et al., 2012). Carcinogenic viruses unlike other viruses with acute infection profiles do not kill their host cell. Often, such viruses have evolved to evade the immune suppression of the host and ensure long-term and persistent infection. This persistency allows for viruses to interfere with the host's cellular pathways, either by inhibiting tumor suppressors or by activating oncogenes. Thus cell proliferation increases, further genetic damage accumulates leading to immortalization and the eventual development of cancer. Carcinogenesis is sometimes associated with events that deregulate the expression of certain viral genes (eg. HPV, Merkel cell) (Chang and Moore, 2012; Collins et al., 2009).

Viruses were initially believed to be causative agents for cancer only in animals. Their ability to cause cancer in humans was first discovered in 1964 when viral particles from the Epstein-Barr virus (EBV) were found in cultures of Burkitt's lymphoma (Epstein et al., 1964), followed by the discovery of viral particles in hepatitis B from the hepatitis B virus (HBV) (Dane et al., 1970). This created an interest in identifying the causal relationship between viruses and cancer and this was possible by the breakthrough isolation of the human papillomavirus (HPV) from human cervical cancer specimens in 1983 (Durst et al., 1983).

HPV belongs to a group of more than 200 viruses with diverse tissue tropisms and disease outcomes. The infections of HPVs can be asymptomatic or they can show clinical manifestations from benign lesions and warts, to malignant lesions and carcinomas. They are one of the most common sexually transmitted infections accounting for 5% of the worldwide cancer cases as stated in the IARC monograph (IARC, 2007). The most prevalent HPV cancer is cervical cancer in which HPV is considered a necessary factor for its development. HPV viral sequences have been identified in more than 99% of the cases (Walboomers et al., 1999). Other cancers in which HPV is implicated, include head and neck and non-melanoma skin cancers (Gillison et al., 2000; Herrero et al., 2003; Harwood et al., 1999). Although advances in the field of HPV virology have been made in the last decade, including the preventative HPV vaccines, cervical cancer caused by HPV still remains one of the most common cancer types worldwide (Table 1) and of great interest to virologists.

Our work- focuses on one of the 200 HPV viruses, the high risk HPV16 and its effect on -stem cell biology. HPV16 is the type most frequently encountered in cervical cancers and HPV-positive head and neck cancers (Jabbar et al., 2010; Walboomers et al., 1999).

Table 1: The 2012 worldwide estimated Incidence and mortality for cervical cancer by the World Health Organization (WHO).

Estimated numbers (thousands)	Cases	Deaths
World	528	266
More developed regions	83	36
Less developed regions	445	230
WHO Africa region (AFRO)	92	57
WHO Americas region (PAHO)	83	36
WHO East Mediterranean region (EMRO)	15	8
WHO Europe region (EURO)	67	28
WHO South-East Asia region (SEARO)	175	94
WHO Western Pacific region (WPRO)	94	43
IARC membership (24 countries)	206	103
United States of America	13	7
China	62	30
India	123	67
European Union (EU-28)	34	13

1.1 HPV

1.1.1 CLASSIFICATION, STRUCTURE AND GENOME

Human papillomaviruses (HPVs) are DNA viruses, members of the Papillomaviridae family. There are more than 200 HPV types identified so far which have been divided into the five HPV genera (Alpha, Beta, Gamma, Delta, Nu and Mu) based on their L1 sequence homologies. They are epitheliotropic establishing infections in stratified epithelia such as those lining the skin, anogenital tract, and the oropharynx (IARC, 2007). The most well studied genera is the Alpha whose types are divided into cutaneous or mucosal based on the epithelia which they target. The cutaneous types cause common warts while the mucosal are further clinically classified into two major categories based on their oncogenic potential: “low-risk” and “high-risk” (de Villiers et al., 2004). The low risk HPVs, such as HPV6 and 11 cause benign warts while high risk HPVs, such as HPV16 and 18, lead to the formation of malignant lesions and carcinomas.

The HPVs are small circular viruses with an icosahedral capsid and a double stranded genome of 8000bp. Their genome consists of numerous open reading frames (ORFs), all transcribed from a single DNA strand, divided in 3 parts: a region consisting of early (E) expressed genes (E1, E2, E4, E5, E6, E7), a region consisting of late (L) expressed genes (L1, L2) and a non-coding upstream regulatory region (URR) consisting of regulatory elements such as the promoter (Figure 1) (Stanley, 2012). HPVs do not encode enzymes that are required for DNA replication so they depend on the host DNA replication machinery to ensure viral genome replication.

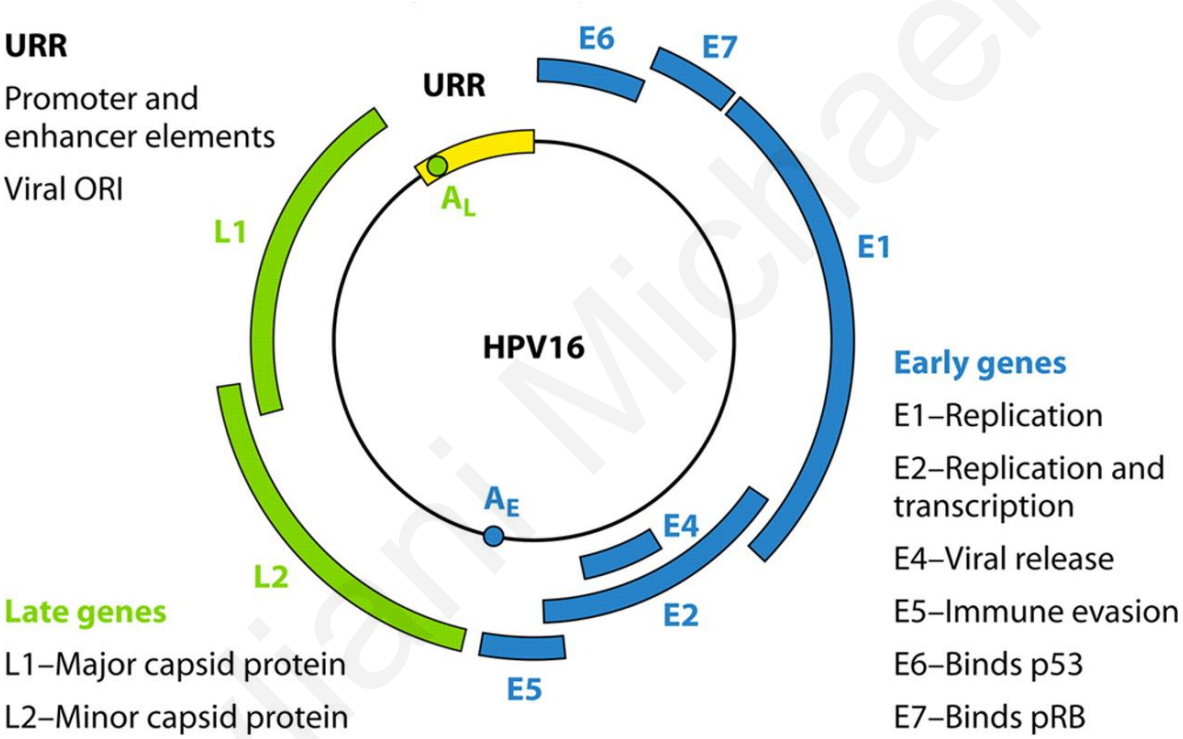

Figure 1: The HPV genome. (Stanley, 2012)
HPV consists of theupstream regulatory region (URR), the early expressed gene region (blue) and the late expressed genes region (green).

1.1.1.1 E1 and E2

E1 and E2 are both actively regulating the virus' transcription and replication. On the origins of replication of the early genes there are specific E1 and E2 binding sites (E1BS and E2BS) on which E1 and E2 form a heterodimer complex (Figure 2) (Kurg, 2011). The complex binds with high affinity repressing early gene transcription (Morshed et al., 2014; Tommasino, 2014) and maintaining a constant viral gene copy number in the cells. E2 acts

as the loading protein providing sequence specificity and enhancing E1 ATPase and helicase binding on the origins (Stenlund, 2003). In the presence of ATP hydrolysis E2 is displaced from the complex allowing E1 to form a hexamer able to unwind the DNA and recruit replication factors for replication initiation. E2 therefore controls viral gene expression and is responsible for the transforming abilities of HPV as its inactivation can lead to the overexpression of the viral oncogenes and the development of lesions.

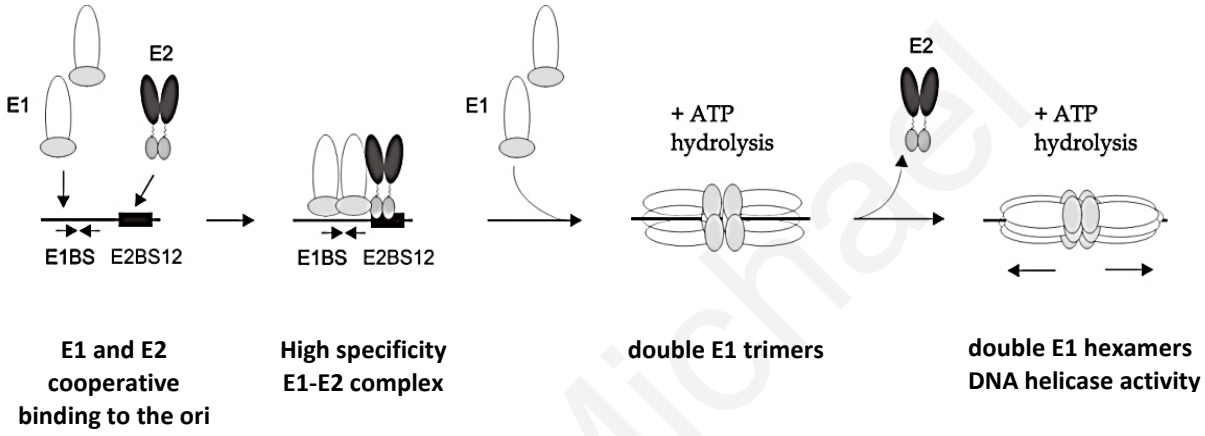

Figure 2: The binding of the E1 and E2 proteins on the origins of replication. (Kurg, 2011)
E1 and E2 binding sites (E1BS, E2BS) in the origins of replication of the early genes allow E1 and E2 heterodimer complex formation. E2 displacement by ATP hydrolysis allows E1 hexamer formation able to unwind the DNA.

1.1.1.2 E4

E4, although an early protein, was found to accumulate at the onset of viral amplification during the later stages of viral infection. Accumulation was evident when cells progress through the final stages of the cell cycle before destined for differentiation. Its expression is mainly detected in the cytoplasm of differentiated keratinocytes in upper epithelial cells so it has been suggested as a biomarker of active viral infection and disease staging. E4 is phosphorylated during the cell cycle and binds keratins with high affinity destabilizing the cytokeratin network of the cells and the cornified cell envelope of the terminally differentiated epithelial cells that acts as a protective barrier (Brown et al., 2006; Wang et al., 2004). Since HPV does not lyse the cells for its release it is thought that this disorganization allows for protein sequestration and accelerated viral particle transfer into the terminally differentiated cells and release from the upper epithelial layer (Brown et al., 2006). Its precise role in the viral cycle has not been identified yet but it has been shown to

be required for the completion of the productive cycle *in vivo* (Doorbar, 2013; Peh et al., 2004).

1.1.1.3 E5

The E5 gene is only expressed by the carcinogenic alpha papillomavirus type, which indicates that it is not essential for the life cycle of the virus but rather gives it an advantage for undergoing transformation (Venuti et al., 2011). It is known to have immune evasion activities by inhibiting death receptor-mediated apoptosis (Venuti et al., 2011) as well as transforming abilities by cooperating with E6 and E7 *in vitro* to immortalize human keratinocytes (Stoppler et al., 1996). It can act as an oncogene having a subtle role during the productive stage of the viral life cycle by reprogramming quiescent suprabasal differentiated cells to support DNA synthesis (Genther et al., 2003). In the skin, it has been shown to be involved in the malignant progression of carcinogenesis by increasing mitogenic signals of growth factors such as with the epidermal growth factor receptor (EGFR) in these differentiated layers and the MAPK pathway (Genther Williams et al., 2005). In the cervix it has been shown to cause cervical cancer after prolonged treatment with estrogen. Transgenic mice that express E5, E6 and E7 show increased numbers of tumors as well as increased disease severity (Maufort et al., 2010).

1.1.1.4 E6 and E7

E6 and E7 are considered two of the most important HPV genes in either low or high risk HPVs because although they lack enzymatic activity, they can associate with a broad range of cellular proteins (Figures 4 and 5) (Moody and Laimins, 2010). This is because in their sequences, both low and high risk E6 and E7 proteins, contain binding motifs that allow recognition, binding and inactivation of cellular targets (Figure 3) (Barbosa et al., 1989; McLaughlin-Drubin and Münger, 2009). Although they can affect common pathways, some differences that can be found in the motifs of the low and high risk E6 and E7 proteins, are responsible for their ability to exert different effects on their targets (Table 2) (Doorbar et al., 2012) and for rendering the high risk E6 and E7 proteins the major viral oncogenes required for the initiation and maintenance of cancer (Jabbar et al., 2012).

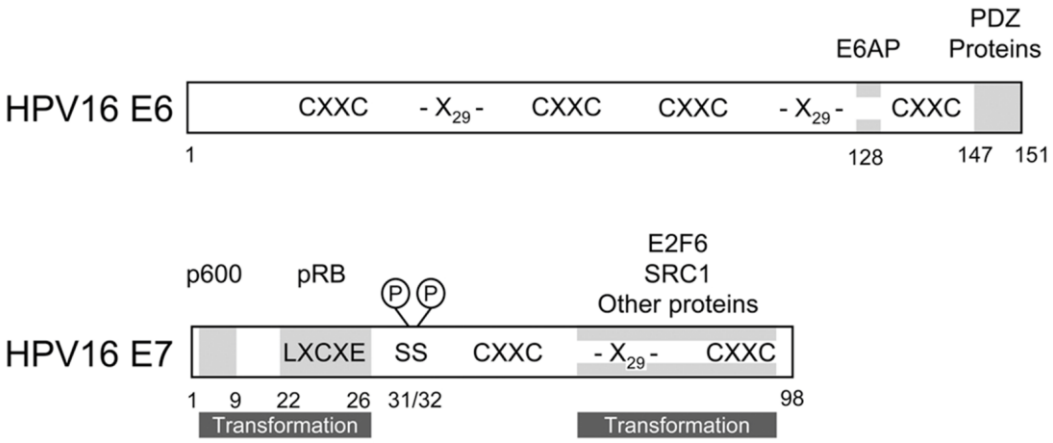

Figure 3: The HPV16 E6 and E7 binding motifs. Adapted from (McLaughlin-Drubin and Münger, 2009)
Important biochemical motifs through which E6 and E7 engage with cellular binding partners are indicated.

Both low and high risk E6 proteins bind cellular targets (Figure 4) by recognizing and binding their leucine rich peptides (LXXLL) such as the ubiquitin ligase E6AP. The complex formed by the high risk E6, unlike the complex of the low-risk E6 (Figure 4), binds p53 with high affinity which is subsequently ubiquitinated and targeted for proteasomal degradation (Cooper et al., 2003). This removes the anti-proliferative and pro-apoptotic effects of p53 allowing for subsequent cell cycle progression (Scheffner et al., 1990; Scheffner et al., 1993). To abrogate the activation of p53-responsive genes by p53 acetylation, high risk E6 interacts with the histone acetyltransferases p300 and ADA3 which act as transcriptional activators on the promoters of genes involved in cell cycle regulation and differentiation. In addition to inhibiting p53 and its targets, the high risk E6/E6AP complex was found to target the transcriptional repressor of Tert NFX1-19 through its direct binding by the complex, its ubiquitination and degradation (Gewin et al., 2004). This results in induction of Tert transcription and telomerase activation that lead to cell immortalization. Moreover, high risk E6 allows for evasion of apoptosis by interfering with members of both the intrinsic and the extrinsic apoptotic cascades. Examples include the interactions of E6 with members of the tumor necrosis factor (TNF) pathway (Filippova et al., 2004; Filippova et al., 2007; Tungteakkhun et al., 2008) and the degradation of pro-apoptotic proteins such as Bax (Magal et al., 2005). In addition both low and high risk E6 control the antiviral immune response by interacting with members of the interferon response (IRF) such as IRF3 transactivator (Ronco et al., 1998).

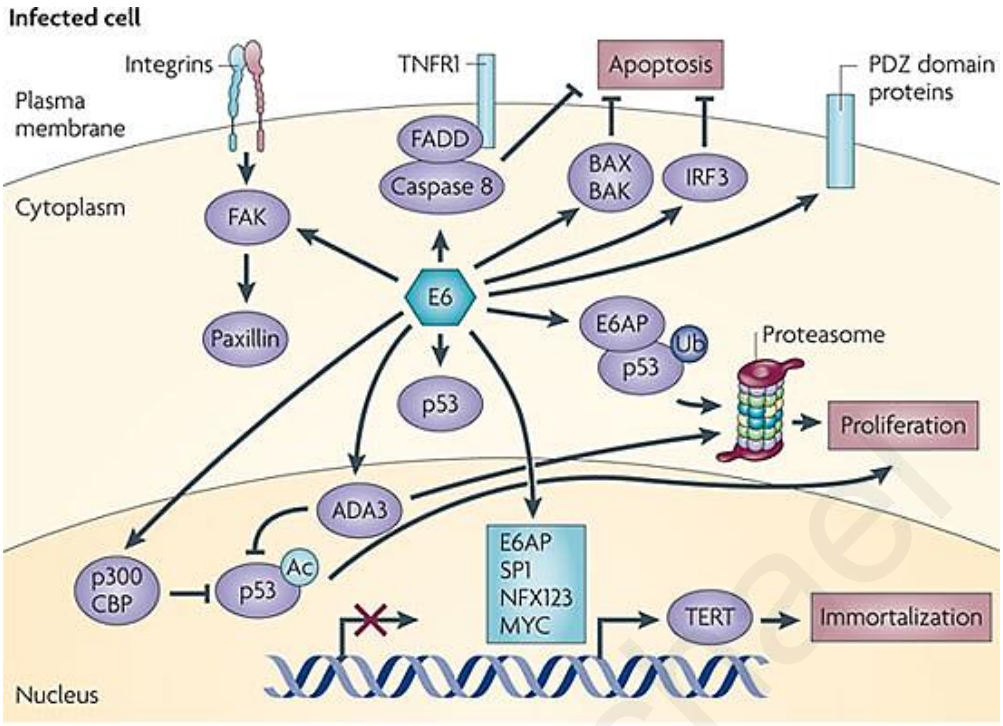

Figure 4: High risk E6 targets. (Moody and Laimins, 2010)
High risk E6 proteins bind both cytoplasmic and nuclear proteins interfering with signaling pathways and gene transcription causing changes in cellular proliferation, immortalization and apoptosis.

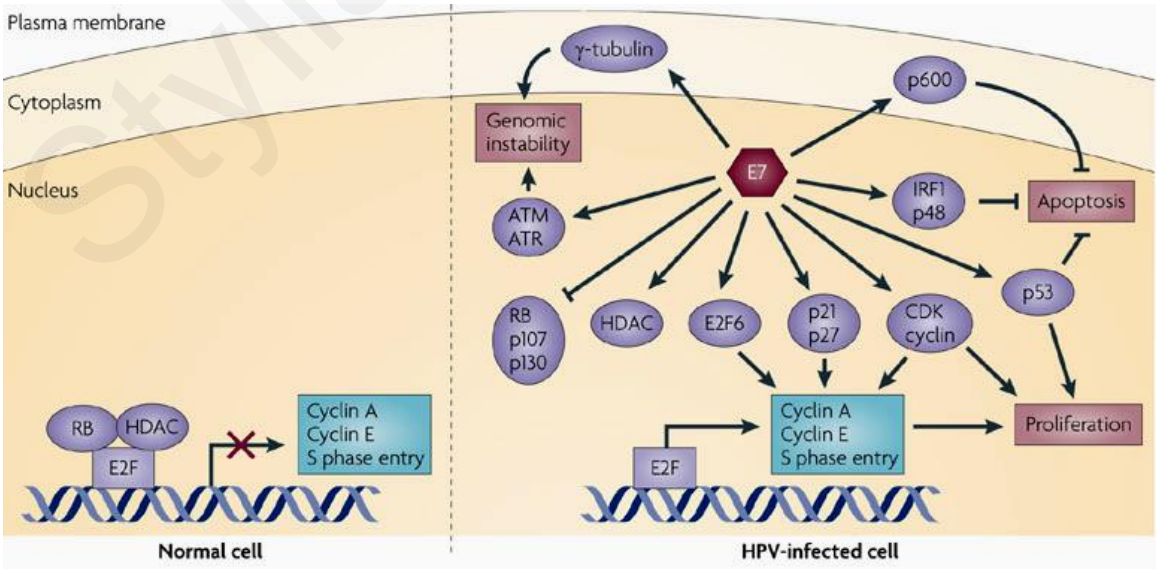

Figure 5: High risk E7 targets. (Moody and Laimins, 2010)
High risk E7 proteins bind both cytoplasmic but mostly nuclear proteins interfering with the cell cycle regulation and gene transcription causing changes in cellular proliferation, genomic stability and apoptosis.

Table 2: Differences in the functions of the high and low risk E6 and E7 proteins. Adjusted from (Doorbar et al., 2012)

	High risk Alpha	**Low risk Alpha**
E6	Binds and degrades p53	Weakly binds p53, no degradation
	Binds and degrades PDZ-domain proteins	No binding to PDZ-domain proteins
	Interaction with E6AP ubiquitin ligase, inhibition of p53 transactivation and acetylation	
	Inhibition of apoptosis	Unknown
	Bypass of growth arrest following DNA damage	Normal growth arrest following DNA damage
	Inhibition of keratinocyte differentiation	Unknown
	Inhibition of interferon response	Weaker inhibition of interferon response
	Activation of signaling pathways	Unknown
	Telomerase activation	No activation
	c-myc activation	No activation

	High risk Alpha	**Low risk Alpha**
E7	Binding and degradation of: • pRb • p107 • p139	Weaker binding (no degradation) of: • pRb • p107 • E2F1
	Binding (no degradation) of E2F1	Binding (no degradation) of p130
	Binding of regulatory proteins including E2F6, p600, HAT, PP2A Induction of cell cycle entry and DNA synthesis Role in genome amplification	
	Induction of genomic instability	No stimulation of instability
	Immortalization and transformation	No such functions
	Activation of signaling pathways	Unknown

Both low and high risk E7 proteins interact through their pRb-binding motif with the retinoblastoma family of pocket proteins which include pRb, p107 and p130 (Figure 5) (Moody and Laimins, 2010). These proteins negatively regulate E2F family members' activity, through their direct interaction, halting cell cycle progression. High risk E7, unlike the low risk, binds to pRb with higher affinity destabilizing it, which is then targeted for proteasomal degradation along with its associated family members p107 and p130. These changes result in the removal of the inhibitory effect of the Rb family proteins on the transcription of E2F responsive genes (Boyer et al., 1996; Chellappan et al., 1992) causing aberrant E2F-mediated transcription. In addition, high risk E7 targets the cell cycle directly by activating cyclins and cdks or by inactivating p21 and p27 cyclin/cdk inhibitors that are responsible for cell cycle arrest during differentiation. As a result of these changes there is uncontrolled G1/S transition and continuous cell replication, a phenotype seen in differentiating epithelial cells upon viral infection (Cheng et al., 1995). To evade the recognition of these changes and the antiviral response high risk E7 was found to inhibit interferon proteins (Barnard et al., 2000) as well as inhibit p53 indirectly through binding its activators (Avvakumov et al., 2003). In addition, both low and high risk E7 proteins can repress the transcriptional activity of p53 (Massimi and Banks, 1997).

The high risk E6 and E7 oncoproteins are almost invariably expressed and co-regulated in all HPV-associated cancers. Although they contribute differently to carcinogenesis, by affecting different cellular targets, they were found to act synergistically in the formation of cervical, skin as well as head and neck cancers (Jabbar et al., 2010; Song et al., 2000; Riley et al., 2003). In experimental systems, E6 and E7 have also been shown to be essential to tumor maintenance and as such represent important therapeutic targets.

In transgenic animal models, the oncogenic potency of each oncoprotein differs based on the tissue of expression, with E7 being more potent in the formation of carcinomas in the cervix and head and neck regions and E6 being more potent in the skin (Jabbar et al., 2009; Strati and Lambert, 2007; Song et al., 2000). This is likely due to the differential expression and importance of their targets in each epithelium. A model of their co-operative action states that the aberrant cell cycle entry by E7 is mediated via its interaction with pRb, activates senescence and apoptotic responses in the cell. The escape of the apoptotic barrier of the cell, activated as a result of E7's action, is mediated by E6 via its interaction with p53. The interaction of viral proteins with pRb or p53 is not only restricted to HPVs, but it is evident in other DNA viruses such as the oncolytic Adenovirus, the *Simian Virus 40* (SV40) and the Hepatitis B virus (HBV) (Lavia et al., 2003; Mesri et al., 2014). Such viruses have evolved strategies to avoid clearance by the

host's gatekeeper functions in order to ensure their replication and propagation in their host. E1A adenovirus protein, the large T antigen of the SV40 as well as high risk E7 share the binding motif with which they bind the Rb family proteins and exert their effects on the cell cycle (Dahiya et al., 2000; Chellappan et al., 1992). In addition E1B adenovirus protein, the large T antigen of the SV40, HBV DNA and high risk E6 bind p53 tumor suppressor (Yew and Berk, 1992; Lane and Crawford, 1979; Qu et al., 2009). Nevertheless, it should be noted that experimental evidence suggests that interactions of E6 and E7 beyond those with p53 and pRb also contribute to carcinogenesis (Strati and Lambert, 2007; Shai et al., 2007).

1.1.1.5 L1 and L2

L1 and L2 encode for the structural capsids required for viral assembly and release (McLaughlin-Drubin and Münger, 2009) as well as viral entry to the host (Kines et al., 2009). L1 is the major capsid protein and is responsible for the initial binding of the virus with the host (Buck et al., 2013). It has a conserved sequence and thus it has been used to categorise the hpv types into genera (Tommasino, 2014). L2 is the minor capsid protein and aids during the binding of the viral capsid to the host and its cellular entry (Kines et al., 2009).

1.1.2 MODE OF INFECTION

1.1.2.1 Normal Productive Cycle

HPVs show tropism for cutaneous and mucosal epithelia (Longworth and Laimins, 2004). Infections specifically arise in stratified squamous epithelia such as those lining the skin, the anogenital tract and oral epithelia. The infections occur through wounds or abrasions and are thought to arise through infection of cells within the basal layer of squamous epithelia (Figure 6) (Woodman et al., 2007), which is the layer of cells that makes direct contact with the basement membrane and constitutes the proliferative compartment of these epithelia (Kines et al., 2009). In this basal compartment both stem cells and committed basal cells exist which act as a reservoir for the upper layers. In the cervix the infections are thought to occur in an area called the transformation zone, where both columnar and squamous cells co-exist and it is the area that contains both the tissue's reserve cells and also basal cells. The most accepted theory for the HPV infection is that all

cell types can be infected but it is still not known if HPV targets one of the two cell types preferentially.

The viral capsid protein L1 contacts the heparan sulfate proteoglycans (HSPG) on the basement membrane which induces conformational changes on the capsid and exposure of the L2 N-terminus. This allows for cleavage of a furin recognition site on L2 leading to its further exposure. Exposed L2 is then able to bind to a non-HSPG receptor found on the cells that migrated towards the basement membrane during the wound healing response (Figure 7) (Woodman et al., 2007; Day and Schiller, 2009). The virus then enters the cells via endocytosis either clathrin dependent with HPV16 or caveolae dependent with HPV31 (Bousarghin et al., 2003). The presence of both L1 and L2 in the virion is required for delivering the viral genome in the nucleus and this delivery is thought to be aided by the wound healing response that induces cell division. In the nucleus E2 is recruited to the sites of replication in order to control the replication of the virus. In healthy cells, the presence of E2 induces the recruitment of E2 repressor proteins such as p53 which bind E2 inhibiting its action (McBride, 2008).

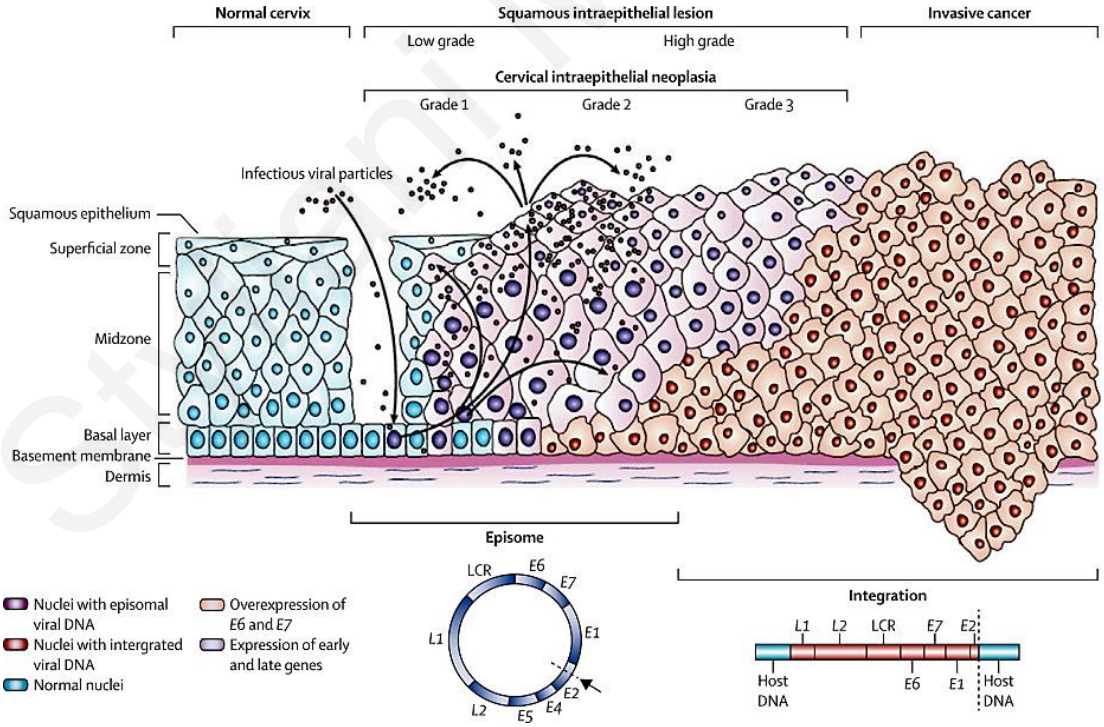

Figure 6: HPV infection on stratified squamous epithelium. (Woodman et al., 2007)
Comparison of normal and HPV-infected epithelium leading to neoplasia or cancer. In neoplasia HPV remains as an episome expressing its genes in the epithelium until release of infectious viral particles occurs in upper epithelial layers. In cancer HPV integrates allowing for overexpression of E6 and E7 throughout the epithelium.

Despite this inhibition, following infection of the basal cells the virus can still undergo three types of replication (Figure 8) (Ozbun, 2007). The first involves the establishment of the virus as an autonomously replicating episome where the viral genes are maintained at low copy numbers (50-100) and is independent of the cell cycle. The second type is responsible for the persistent viral infection by initiating a limited genome amplification in mitotically active basal cells, synchronized with the host chromosomes, maintaining constant viral copy numbers. Both types of replication use the host's replication machinery and thus occur in the cycling basal cells where proliferation is active (McBride, 2008). In both cases, the E2 protein's action on E1 is inhibited by the presence of the repressor proteins such as p53 controlling the expression of the viral genes (Stubenrauch et al., 1998). E6 expression can remove the inhibitory action of p53 on E2, allowing for viral gene replication. In turn, E2 can downregulate the expression of E6 and E7 and this regulatory loop keeps viral replication at a balance. The third type of replication results in an ordered vegetative amplification of the virus and requires infected cells that are committed for differentiation. When a basal cell divides, its daughter cell loses contact with the basement membrane and moves to the suprabasal layer. Once the virus enters the suprabasal layer, the differentiation-dependent promoter of the virus gets activated resulting in the upregulation of the viral proteins E1 and E2. Subsequently, there is high expression of the E6 and E7 proteins required for the post-mitotic cells to re-enter S-phase. This is due to E7 that inhibits pRb and allows for transcription of E2F responsive genes required for cell cycle entry, so that the viral genome is further amplified using the host's replication machinery before viral synthesis (Doorbar et al., 2012). At high expression levels, E1 and E2 can induce G2 arrest for sustained viral genome synthesis (McBride, 2008). Normally these "faulty" cells would have been detected and eliminated by p53 but the presence of E6 prevents their removal by inhibiting the action of p53 due to its degradation.

Once in the upper layers, the late proteins L1 and L2 are expressed for genome encapsidation and viral packaging. The virus matures and expresses its antigens in the uppermost surface, limiting the chances to be recognized by the host's immune system. L1 forms disulphide bonds in the upper epithelial layers and with the help of E4 that disrupts the keratin network allows release from the dying keratinocytes (Wang et al., 2004).

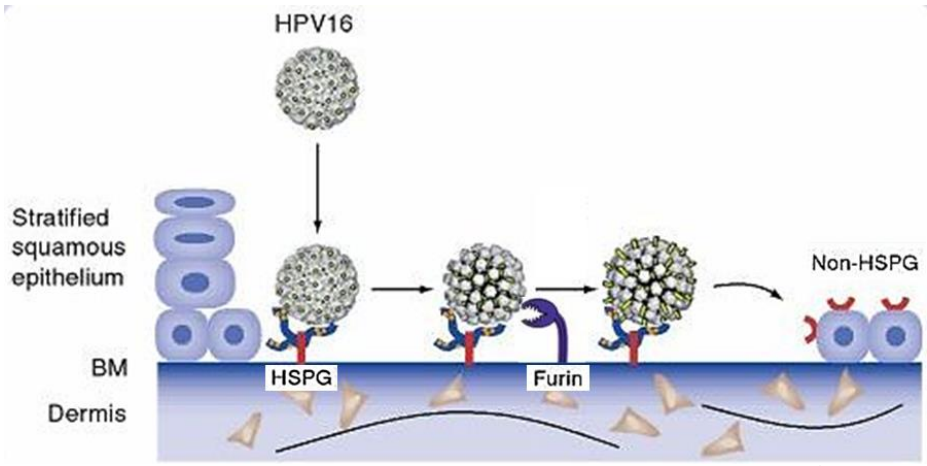

Figure 7: HPV binding on the basement membrane. Adjusted from (Day and Schiller, 2009) HPV L1 binds to HSPG receptors on the membrane inducing conformational changes and exposure of the L2 N-terminus. Furin cleaves L2 further exposing it which allows subsequent binding to cellular non-HSPG receptors through which the virions enter the cells by clathrin-mediated endocytosis.

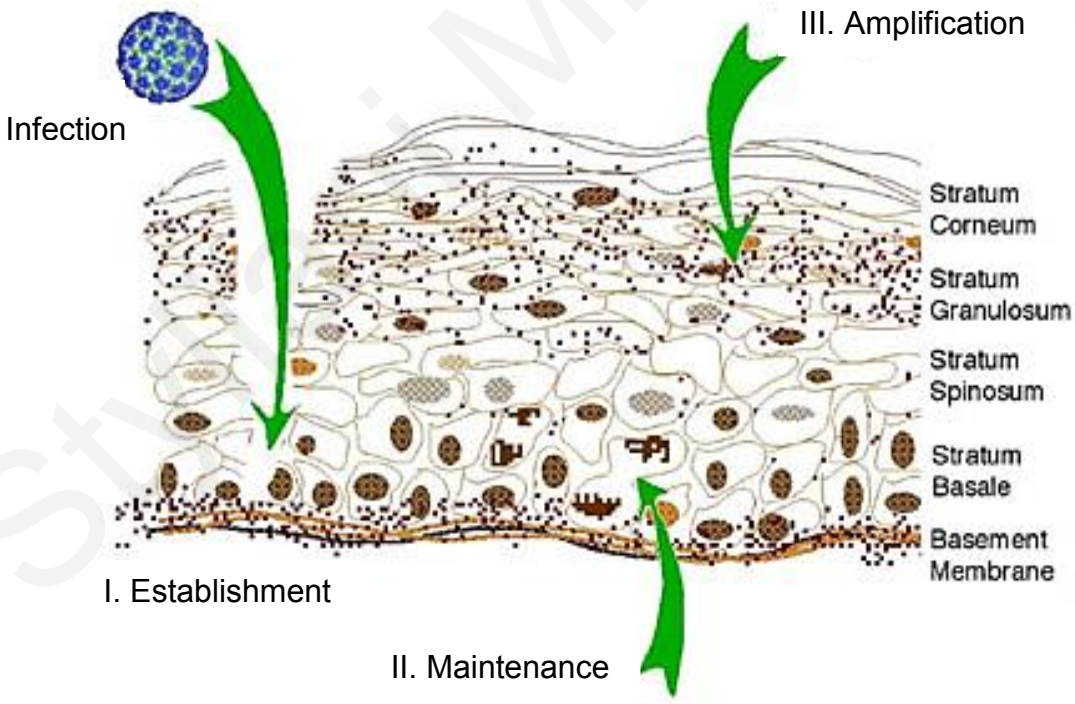

Figure 8: The three types of replication of the HPV. (Ozbun, 2007)
Following infection, the virus is first established as an autonomously replicating episome with minimal viral expression. Secondly, the virus is maintained in the tissue by undergoing genome amplification along with the host's chromosomes. Thirdly, the virus undergoes vegetative amplification inducing cell cycle entry in differentiated cells, independent of the host replication, allowing expansion throughout the epithelial layers.

In this normal productive life cycle, the expression of the viral genes such as E6 and E7 is tightly regulated in the suprabasal cells and only occurs in cells that will eventually be lost from the epithelium. This ordered expression of the genes and the production of infectious virions without cell lysis, results in infections being at their majority (more than 90%) asymptomatic or producing benign lesions that eventually regress (Hariri et al., 2011). This is achieved through clearance by the host's immune system usually in the first 6 months following viral entry in both low and high risk types (Figure 9) (Molano et al., 2003; Mesri et al., 2014). However, there are some cases where infection is latent and cleared after about 5 years and in this case the virus needs to overcome the barrier of being recognized and cleared by the host immune system. The mechanisms to do this are first of all that infection in the basal cells occurs away from the dermal immune cells. In addition, the viral replication does not cause cell lysis so viral particle release occurs without triggering any invasion signals (Stanley et al., 2007). Finally, the oncogenes downregulate pathogen recognition receptors (PRRs) of the innate immune response such as the Toll-like receptors (TLRs), cytokines such as interferons (IFNs) that link innate and adaptive immunity (Karim et al., 2011; Hibma, 2012; Grabowska and Riemer, 2012; IARC, 2007) and also cell surface molecules that present antigens to the T-cells (Ashrafi et al., 2002).

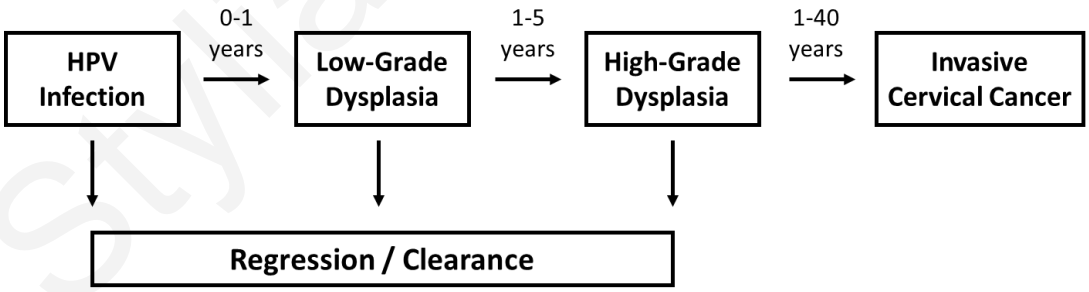

Figure 9: The timeline of HPV infections. Adjusted from (Mesri et al., 2014)
Infection can initiate low-grade dysplasia within the first year which can proceed to high grade dysplasia. All three stages can be cleared by the immune system. In the case of persistent infection the high grade dysplasia proceed to invasive cervical cancers.
*The 1 year timeline for invasive cancer development is rarer than the higher end of that range.

1.1.2.2 HPV and Cancer Development

As in most cases infections are usually cleared out it is obvious that the infection of the virus and its productive life cycle are necessary but not sufficient to cause carcinogenesis. Evidence indicates that significant factors that aid the oncogenic transformation of a tissue are chronic infection and its resulting long-term inflammation (Grivennikov et al., 2010; Fernandes et al., 2015). The differences seen in the susceptibility of individuals to undergo infections, to sustain persistent infections or progress to invasive cancers point to the fact that carcinogenesis is a combination of influences from both the internal and the external cellular environment (Castellsagué and Muñoz, 2003).

In the case of the high risk HPV16 and cervical cancer, external cofactors that increase susceptibility to carcinogenesis have been well established and include parity, oral-contraception usage and smoking. High parity increases the risk of HPV positive women to progress to carcinomas (Castellsagué and Muñoz, 2003) probably due to the changes in the hormonal levels, caused by pregnancies, which affect the immunity against HPV and its progression (Sethi et al., 1998). In addition, high parity maintains the transformation zone of the exocervix, where cervical cancers are thought to arise, providing the ground to HPV for easy access (Autier et al., 1996). Usage of oral contraceptives gives a dose-relationship response with increasing years of use as the hormones they contain, such as estrogen and their receptors, have been shown to synergize with the virus for inducing squamous carcinomas (Moreno et al., 2002; Son et al., 2014; Arbeit et al., 1996). In bitransgenic E6E7 mice, the development of cervical cancers was shown to be dependent on the persistent exposure of the tissue to estrogen (Arbeit et al., 1996). The mechanism by which oral contraceptives contribute to carcinogenesis is suggested to be the presence of estrogen that stimulates the transcription of the viral oncogenes. Increasing exposure to smoking has also been linked as a cofactor of carcinogenesis as it significantly increases the risk of progression to malignancy as seen in HPV16 immortalized human endocervical cells. The chemical carcinogens in tobacco may induce DNA damage in the cells (Castellsagué and Muñoz, 2003) or may reduce the effect of the local immune response (Poppe et al., 1995). Women who have been smoking were shown to maintain longer infections compared to the non-smokers (Giuliano et al., 2002) while on the other hand, smoking cessation was shown to result in lesion size reduction (Szarewski et al., 1996).

Together with these external cofactors, the ability of HPV16 infection to lead to the development of a number of cancers including cervical, anal, as well as head and neck cancers, lies in its potential to persist in the tissue. This persistence allows infected cells to

undergo cycles of proliferation and with the loss of the p53 repair abilities to accumulate genetic alterations required for oncogenic transformation. Infection induces an inflammatory response as part of the normal host defence mechanism for pathogen elimination. However, pathogens such as HPV16 can evade immunity and persist in the tissue along with long-term inflammation. Long-term inflammation was found to have a role in the induction as well as promotion of carcinogenesis (Figure 10) (Fernandes et al., 2015; Grivennikov et al., 2010). At the initial stages, the production of agents such as reactive oxygen species as well as cytokines in the cells, make them prone to induction of DNA damage and genomic instability. The role of chronic inflammation in the promotion of carcinogenesis comes from the release of growth factors and cytokines from the infected cells, which results in transcription of genes involved in survival and proliferation. These induce a positive feedback cascade where more immune cells are attracted expanding the compartment making it more susceptible to transformation (Grivennikov et al., 2010).

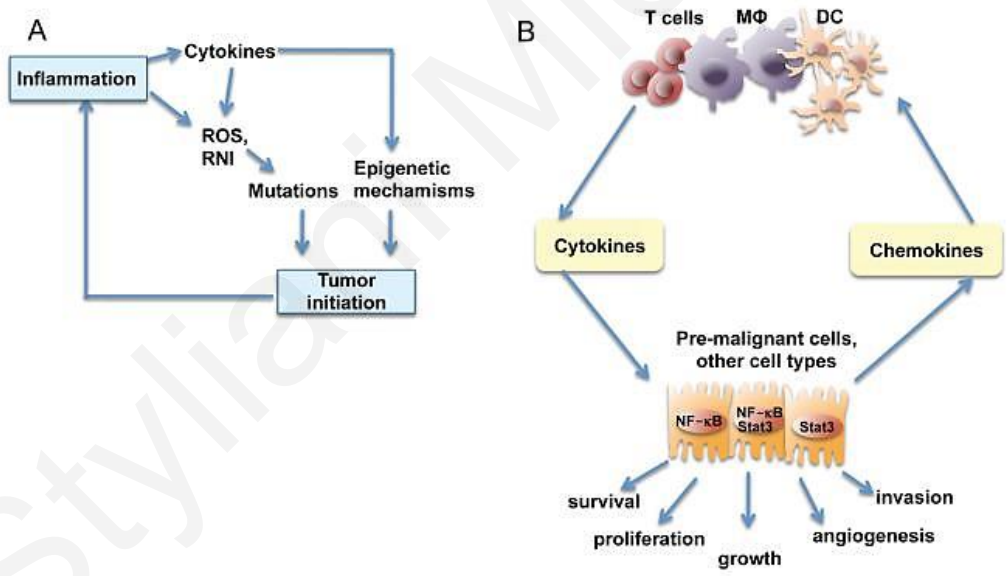

Figure 10: The role of chronic inflammation in HPV induced carcinogenesis. (Grivennikov et al., 2010)
(A) The release of cytokines and the production of ROS leads to increase in the epigenetic changes making cells prone to genomic instability aiding tumor initiation. (B) The release of cytokines from chronically infected cells induces the activation of transcription of genes involved in the survival, proliferation, growth, angiogenesis and invasion of the cells. In turn, these changes induce a positive response where more inflammation is observed and the compartment increases.

The ability of the cofactors to increase the vulnerability of the individual is established however, it is not sufficient for oncogenic transformation. Transformation is initiated towards the development of cancerous lesions when the virus integrates in the host genome a step that results in viral life cycle deregulation. The deregulation causes not only suprabasal host cells to re-enter S-phase but can also induce aberrant proliferation of the basal infected cells resulting in high viral expression throughout the epithelium (Figure 6) (Woodman et al., 2007). The integration occurs near fragile chromosome sites, an event that causes the deletion of viral genes (Smith et al., 1992; Thorland et al., 2003). In cases where the insertion deletes the E2's ORF, the expression of E2 is disrupted an event that results in deregulated expression of E6 and E7, a hallmark of cervical cancer, that gives cells a selective growth advantage a critical step for progression into cancer (Baker et al., 1987; Kurg, 2011). These two most important papillomaviral oncogenes, E6 and E7, have been shown not only to be necessary for the viral life cycle but also for the development as well as the persistence of HPV-associated cancers (Goodwin and DiMaio, 2000; Jabbar et al., 2009). The reason for that is that despite their two well characterized targets pRb and p53, E6 and E7 are implicated in the deregulation of a number of pathways affecting vital cellular processes (Wallace and Galloway, 2015).

To begin with, a phenotype seen in HPV expressing cells required for their transformation, is their extended life span and immortalization. This has been shown by *in vitro* studies where expression of the two oncogenes causes immortalization of different cell types including fibroblasts and keratinocytes (Yamamoto et al., 2003; Halbert et al., 1991; Shiga et al., 1997; Münger et al., 1989). Continuous replications of the HPV expressing cells would normally lead to shortening of the ends of chromosomes, a mechanism preventing the uncontrolled proliferation of somatic cells. However, HPV immortalizes cells through the ability of E6 to upregulate transcription of telomerase both *in vitro* and *in vivo* (Klingelhutz et al., 1996; Veldman et al., 2003) or through the ability of E7 to induce alternative lengthening of telomeres (ALT) both leading to telomere maintenance (Spardy et al., 2008).

The ability of the oncogenes to deregulate both the pRb and the p53 pathways is not the only way the virus uses to cause loss of control of cell cycle regulation and aberrant proliferation. Loss of cell cycle control is reinforced by disrupting gene expression through chromatin remodeling by affecting both transcriptional repressors as well as activators of histones (histone deacetylases/HDACs, histone methyltransferases/HMTs, histone acetyltransferases/HATs and histone demethylases/HDMs). In addition, it affects gene expression directly by impinging on the epigenetic regulation of DNA through DNA

methyltransferases (DNMTs). Examples from studies on HPV16 include the inhibition of binding of the repressive HDACs to the HIF-1α angiogenesis factor by E7 and the transcription factor Oct4 by both E6 and E7 (Bodily et al., 2011; Liu et al., 2012). E6 also interacts with HMTs inhibiting their activity on p53 responsive elements controlling p53 action (Hsu et al., 2012). Interaction of E7 with E2F6 can in part inhibit its association with polycomb group complexes, which are not only crucial to cell fate decisions but can also bind histone 3 (McLaughlin-Drubin et al., 2008). This binding is inhibited by induction of the histone demethylase KDM6 in E7 expressing cells in which is responsible for removing suppressive methyl marks (McLaughlin-Drubin et al., 2011). E7 also binds pCAF HAT, an activator of p53, inhibiting its action (Avvakumov et al., 2003). In addition the epigenetic enzyme DNA methyltransferase 1 (DNMT1), known to control the epigenetic silencing of tumor suppressors by methylation, was found upregulated in cells isolated from cervical cancers. This upregulation was mediated by E6, as knockdowns of p53 exhibited decreased DNMT1 levels, a result that adds to the findings that hypermethylation of tumor suppressors is a common event in carcinogenesis (Kanai and Hirohashi, 2007; Au Yeung et al., 2010).

This increased proliferative capacity of the cells leads to the accumulation of DNA damage and thus genomic instability, factors that are crucial for the transformation of the cells and the development of cancer (Duensing and Munger, 2004; Pett et al., 2004). The ease by which immortalization of the cells occurs, has been reported to be inversely proportional to the chromosomal aberrations (Schutze et al., 2016). It has been long hypothesized that multipolar mitoses and aneuploidy is a phenotype associated with malignant tumors with HPV malignancies not being an exception (Boveri, 2008). Studies have shown that the forced entry into the cell cycle induces double strand breaks (DSBs) in cells expressing the HPV16 oncogenes (Winder et al., 2007). Normal cell mitosis is not affected in E7 expressing cells, in which centrosome defects and asymmetric spindle formation were detected, leading to cell aneuploidy a phenotype that worsens when the oncogenes are co-expressed (Duensing et al., 2000; Duensing et al., 2001). In addition to cells expressing the viral oncogenes, the centrosome abnormalities were detected in the stratified epithelia of mice transgenic for either E6 or E7 (Riley et al., 2003). Apart from centrosome defects, the oncogenes induce chromosomal abnormalities such as gains and losses on chromosomes (Hashida and Yasumoto, 1991), as well as anaphase bridges seen by the joining of unprotected and damaged telomeres during the separation of sister chromatids (Duensing and Munger, 2002) leading to changes in the number and structure of chromosomes.

Such events normally activate the DNA damage response in the host cell and abnormalities get eliminated either by correcting the defect or by leading the cell to programmed death. However, the oncogenes have established ways to evade the apoptotic response in a number of ways allowing the further accumulation of mutations. Apart from p53, E6 can block the extrinsic apoptotic cascade of Fas by interfering with receptors and ligands as well as degrading the apoptotic proteins procaspase-8 and FADD adaptor molecule in undifferentiated cells (Tungteakkhun et al., 2008; Filippova et al., 2007; Filippova et al., 2004). It also controls the induction of low level caspase activation seen in differentiated cells by E7 that subsequently increases anti-apoptotic factors enabling a balance between the response and the survival of the cells (Grabowska and Riemer, 2012; Moody et al., 2007). In addition, E6 can block the intrinsic apoptotic cascade by reducing both the mRNA and protein stability of pro-apoptotic proteins such as Bax (Magal et al., 2005). Moreover, E7 escapes DNA damage checkpoints by proteolytic cleavage of claspin, a regulator that arrests cells in G2 upon recognition of DNA damage (Spardy et al., 2009).

1.2 CANCER, STEMNESS AND HPV

The current view in carcinogenesis is that all cancers are propagated and maintained due to the existence of a cancer stem cell population, whose origin has not been conclusively defined yet. While HPV cervical cancers are thought to arise from an area in the cervix where stem cells reside the notion that infected cervical stem cells are those initiating carcinogenesis is highly controversial. Due to the critical implications understanding cancer initiation in a context where infection precedes carcinogenesis by several years and often decades, we became interested in understanding the link between HPV-related cancers and stemness.

1.2.1 Cancer and Stemness

For cancer to initiate, a number of cumulative molecular changes such as the activation of an oncogene, the inactivation of a tumor suppressor or genomic instability, are required. These molecular changes can be the result of spontaneous mutations or oncogenic infections and result in the transformation of cells that undergo expansion in the tissue replacing normal cells and creating a "cancerized field" as firstly proposed by Slaughter in 1953 (Slaughter et al., 1953). In this "cancerized field" the tumor initiating cells are responsible for the tumorigenic transformation of the tissue. The idea of this field was proposed based on the observations that: i) normal tissue adjacent to the tumor was found to carry molecular changes, ii) multiple primary tumors could be formed and iii) following surgery and removal of the tumor the site was able to reform tumors. It has been long hypothesized that cancers propagate due to the existence of a subpopulation in the field, the "cancer stem cells" (CSCs) that like stem cells can self-renew and differentiate into multiple cell types having the ability to regenerate the tumor (Swanson et al., 2016). Long-standing observations have noted a number of parallels linking the behaviour and characteristics of stem cells with CSCs, for example their characteristic quiescence when compared to other cells in a tissue/tumor (Simple et al., 2015).

The existence of CSCs was first established in leukemia and the first evidence came from the research of Bonnet and Dick in 1997, who identified a subpopulation of leukemia cells that when transplanted in NOD/SCID mice resembled the functionality forming tumors (Bonnet and Dick, 1997). Investigations to identify stem cell populations from solid tumors are ongoing. Evidence from brain tumors showed that cells exhibited clonogenic and sphere forming capacities, characteristic of stem cells, while they could differentiate in culture resembling the tumor (Singhal et al., 2016). In addition, breast cancer cells isolated

from humans were grown in mice and only a small subset of these cells could be serially passaged each time forming new tumors (Al-Hajj et al., 2003).

The origin of these cancer stem cells to identify the tumor initiating cell is an active research debate with the existence of two prevailing theories:

The first theory claims that adult tissue stem cells are the cells of origin for some cancers. Their long lifespan within the tissue and their frequent proliferation especially in tissues of high turnover, such as the skin, allows for the accumulation of genetic damage a pre-requisite for the development of cancer. There is a growing body of literature that supports the notion that infectious agents perturb stem cell homeostasis in target tissues (da Silva-Diz et al., 2013; López et al., 2012; Pitsouli et al., 2009). For example, uropathogenic *E.coli* have been shown to lead to mobilization of urogenital stem and progenitor cells, a key feature for their pathogenic effects *in vivo*. Although, it has not been shown yet, infection of stem cells by oncogenic viruses may give viruses the opportunity for latent infection and persistent replication. Furthermore, the implications of viral oncogene expression may be different in a stem cell versus those following the infection of a differentiated cell.

The second theory supports the notion that infection occurs in a differentiated cell within the tissue and the cell undergoes reprogramming into a stem-like cell. In the case of virally induced cancers, this reprogramming could also impact the viral lifecycle in addition to setting the platform for carcinogenesis. For example, in the case of HPV it could be linked to the long latency of the virus, in addition to carcinogenesis (Lapouge et al., 2011; Friedmann-Morvinski et al., 2012). Other pathogens have been shown to reprogram infected tissues with profound impact on the lifecycle outcome. A striking example is the intracellular leprosy bacterium which induces the convertion of lineage-commited cells to stem-cell like cells, and suggests that reprogramming infected differentiated cells aids the dissemination of the bacteria from an initial infected site (Masaki et al., 2013). This has not been conclusively shown in the case cancer-causing pathogens.

In the case of pathogen-associated cancers inflammation is also a relevant factor to consider. Apart from being a normal defence mechanism for eliminating infected cells, it was also found to play a major role in carcinogenesis when persistent (Panayidou and Apidianakis, 2013). The inflammation induced by pathogens has been found to be involved in tissue regeneration by reprogramming epithelia. This reprogramming is achieved through the upregulation of growth factors and cytokines in the inflammatory microenvironment which gives cells a stem-cell like phenotype (Grivennikov et al., 2010).

Such a response has been observed in viral infections of HBV and HCV where inflammation induced expression of stemness markers (Karakasiliotis and Mavromara, 2015). In the case of HPV there is induction of immunity, as the virus itself is an infectious agent, but also induction of an inflammatory response as infection occurs through an open wound. In addition, the persistent infection of HPV causes chronic inflammation. This could be an additional contributing factor for the reprogramming of the epithelia during viral infection.

Moreover, oncogenic viruses have been implicated in the promotion of stemness and the generation of CSCs by deregulating factors crucial in the biology of stem cells such as Nanog and Oct4 (Zhang et al., 2009; Iacovides et al., 2013) but also tumour suppressors such as p53 and pRb that have been shown to act as roadblocks in the reprogramming of cells to pluripotency regulating stemness (Conklin and Sage, 2009; Bonizzi et al., 2012).

In support of the idea that the normal differentiation status of tissues is often deregulated or regressed into a more stem-like state during carcinogenesis, is the ability to affect a number of cellular proteins. These include the transcription factors Klf4, Sox2 and Oct4 widely used in the reprogramming of somatic cells to induced pluripotent stem cells. Klf4 transcription factor has tissue specific roles acting either as an oncogene or as a tumor suppressor. In the normal skin epithelium, it is found expressed in differentiated cells acting as a tumor suppressor (Li et al., 2012b) but it is also found in the stem cells of the hair follicle contributing to the skin regeneration during wound healing (Li et al., 2012a). In carcinogenesis Klf4 switches to an oncogene used by viruses to complete their infection and whose persistent infection is a marker of poor prognosis (Nawandar et al., 2015; Tai et al., 2011). Sox2 transcription factor is not normally expressed in the skin epithelium (Lesko et al., 2013) but was upregulated in melanoma cells required for their self-renewal capacity and tumorigenicity (Santini et al., 2014). In addition its expression was found in both mouse and human skin squamous cell carcinomas, in tumor initiating cells, and was required for their growth and tumor maintenance (Siegle et al., 2014; Boumahdi et al., 2014). The transcriptional programme of the tumor initiating cells was different from normal skin epithelial stem cells, evidence pointing towards the reprogramming of differentiated cells by aberrant expression of stemness factors. Oct4 embryonic transcription factor is absent from normal somatic tissues but re-expressed in human tumors (Monk and Holding, 2001; Tai et al., 2005).

In addition to stem cell-related factors being involved in carcinogenesis, we are starting to understand that proteins important in carcinogenesis are important in cellular reprogramming. For example,, the cell cycle regulator cyclin D1 responsible for activating

its downstream kinases that phosphorylate pRb and allow cell cycle progression, was found upregulated in a number of cancers (Decker et al., 2002; Hosokawa and Arnold, 1998; Cheung et al., 2001; Rousseau et al., 2001), but also was proven to reprogram differentiated epidermal cells (Zhao et al., 2016). To add to this, what seems to be an important stemness factor is telomerase expression, which has been reported in the presence of oncogenic viruses (Liu et al. 2008). Although tissue stem cells express low levels of telomerase, cancer cells seem to have constitutive telomerase activation another difference adding to the reprogramming idea (Shay and Wright, 2011).

In a very recent study, in an attempt to identify the events that initiate melanoma formation, Kaufman et al. 2016, used a zebrafish melanoma model and an *in vivo* reporter expressed in progenitor and melanoma cells. They identified that melanoma initiates from a single differentiated cell that acquires progenitor identity reinitiating an embryonic signature and gene program (Kaufman et al., 2016). This is to our knowledge the first study that conclusively demonstrated via lineage tracing that an intermediate multipotent/stem-like state can precede the formation of tumors from differentiated cells.

1.2.2 HPV and Its Role in Stemness

In the case of HPV it is generally believed that it can target either stem cells or committed cells in the basal compartment that it infects. However, it is still not clear whether one of the two infected cell types is more frequently the cell of origin for the cancer stem cell population.

In the cervix, the most common site of HPV16 infection, cancers have been long hypothesized to arise from the transformation zone an unstable area found where columnar epithelia transition into squamous epithelia (Elson et al., 2000). In this zone the tissue's reserve or stem cells are thought to reside giving rise to the basal cells of the squamous epithelia. It has been suggested that the high frequency of cancers in this area is due to the ease by which the cells can be targeted compared to the protected permanent squamous epithelia (Doorbar, 2006). Recent evidence also implicates the cells at the squamocolumnar junction adjacent to the transformation zone as the cells from where tumorigenesis arises (Herfs et al., 2012).

Although the HPV oncogenes have been well studied for their effect in proliferation, differentiation and carcinogenesis there is a scarcity of publications addressing their interplay with tissue stem cells. A limited body of evidence supports the notion that papillomaviruses can infect cells with stem-like characteristics (Schmitt et al., 1996) but

the consequences of viral oncogene expression in tissue stem cells *in vivo* are largely unknown. In a rabbit papillomavirus model it has been shown that the virus maintains persistent infection in the tissue stem cells which could explain the long latency time between infection and carcinogenesis (Maglennon et al., 2011). In addition, in mice expressing the entire HPV16 genome cancers in the skin epithelium arose from a population of stem cells in the hair follicle (da Silva-Diz et al., 2013). It is therefore evident that stem cells are a potential direct target of infectious agents and, in accordance with the studies indicating that some cancers derive from tissue stem cells, this may have an important role in carcinogenesis. However, it is still not clear whether HPV, or other similar cancer causing viruses, directly affect the development, function, or plasticity of tissue stem cells. It is also not clear whether such effect has an eventual impact in the viral life cycle or cancer development.

In support of the idea that HPV affects stemness in the epithelia it is known that both E6 and E7 have the ability to modulate the function of cellular targets implicated in stem cell biology. This ability is seen by the "reprogramming" of the cells either at the cellular or the transcriptional level, as seen by their behavior in the tissues they colonize. One group of these targets are the cell cycle regulators pRb and cyclin D1. pRb whose downregulation is an extremely vital step for viral carcinogenesis was shown to directly affect the stem cell niche (Ruiz et al., 2004). In conditional knockouts for pRb, a parallel to the degradation seen by E7 expression, the LRCs of the skin are shown to be decreased. Cyclin D1, a protein upregulated in response to the viral oncogenes and which is required for the viral transformation (Almadori et al., 2002; Al Moustafa et al., 2004), was found to reprogram differentiated epithelial cells to a stem-like state (Zhao et al., 2016). Another pluripotency-related target is telomerase, whose upregulation by E6 expression in the stem cell niche leads to quantitative and functional changes in this compartment (Flores et al., 2005; Liu et al., 2009; Sarin et al., 2005). Moreover, the changes in the levels or function of chromatin modifiers, either repressors or activators, leads to epigenetic reprogramming of the cells allowing for aberrant transcription, cell cycle progression and proliferation. An important example is the activation of the transcription factor Oct4, a factor used as marker of undifferentiated cells. The activation is achieved through the removal of the repressive HDAC by the HPV oncogenes (Liu et al., 2012) or by the direct binding on Oct4 (Brehm et al., 1999) both leading to its overexpression in HPV positive cells (Liu et al., 2011) mimicking stem cell activity. Therefore, since HPV can target molecules and affect pathways which play a role in stem cell biology, it can be hypothesized that the HPV oncogenes can impinge on stem cell homeostasis. In a study that supports our hypothesis,

HPV8 expression increased the numbers of keratinocytes with stem-cell like properties (Hufbauer et al., 2013).

1.2.2.1 The Interplay between HPV and Its Stem Cell Targets Telomeres & Telomerase

One of the characterized interactions of the HPV16 oncogenes is telomerase and telomere homeostasis. Telomeres are the capping nucleoprotein structures protecting the ends of chromosomes from fusions, recombinations and the cellular DNA damage surveillance systems. They cannot be replicated by DNA polymerase due to the 'end replication problem' so in most somatic cells telomeric repeats are lost with consecutive rounds of replication slowly reaching a critical telomeric length which is thought to be limiting to the cellular life span. This telomere shortening is therefore the mechanism to restrict unlimited proliferation in somatic cells which after dividing a few times they head towards senescence or apoptosis. However, in certain cell types such as stem cells, immune cells or the highly proliferating germ line cells, which need to undergo constant rounds of cell division, attenuation of telomere shortening is crucial. Telomerase is the enzyme responsible for adding the telomeric repeats at the end of chromosomes in those cells. It consists of two subunits: Tert, the reverse transcriptase enzyme and Terc, the RNA component that acts as a template for the extension of the telomeres (Figure 11). It has been shown that the important limiting factor for the activity of telomerase is the level of Tert, as the essential Terc component is expressed in all the tissues (Akincilar et al., 2016).

Telomerase is not expressed or expressed at low levels in normal somatic cells when they need to undergo proliferation in which case the telomerase activity is tightly regulated (Blackburn, 2005). It is also important to note that while telomerase is activated in stem cells, it is only able to slow the shortening of their telomeres but not stop it (Shay and Wright, 2005). However during cellular reprogramming of differentiated cells to iPS telomerase re-activation is crucial to the telomere "rejuvenation" observed in the reprogrammed cells (Marion et al., 2009).

Due to the important role of telomeres and telomerase in cell biology, continued investigation of molecular pathways involved in the deregulation of telomere homeostasis is a topic of constant investigation. Such a deregulation is observed during carcinogenesis, as cancer cells have evolved mechanisms to overcome the telomere shortening problem either by upregulating telomerase or by the alternative lengthening of the telomeres (ALT) achieved by homologous recombination. This allows cancer cells to undergo unlimited

proliferation for sustaining tumor growth and progression. Studies have shown that telomerase deficient mice are resistant to tumorigenesis (Gonzalez-Suarez et al., 2000). Furthermore, telomerase has additional roles from that of maintaining telomere length such as regulating the Wnt pathway (Park et al., 2009). In addition, the overexpression of Tert was associated with neoplasia and cancers in mice (Artandi et al., 2002), in which the Terc component was required (Cayuela et al., 2005), as well as mobilization and proliferation of hair follicle stem cells independent of Terc (Sarin et al., 2005).

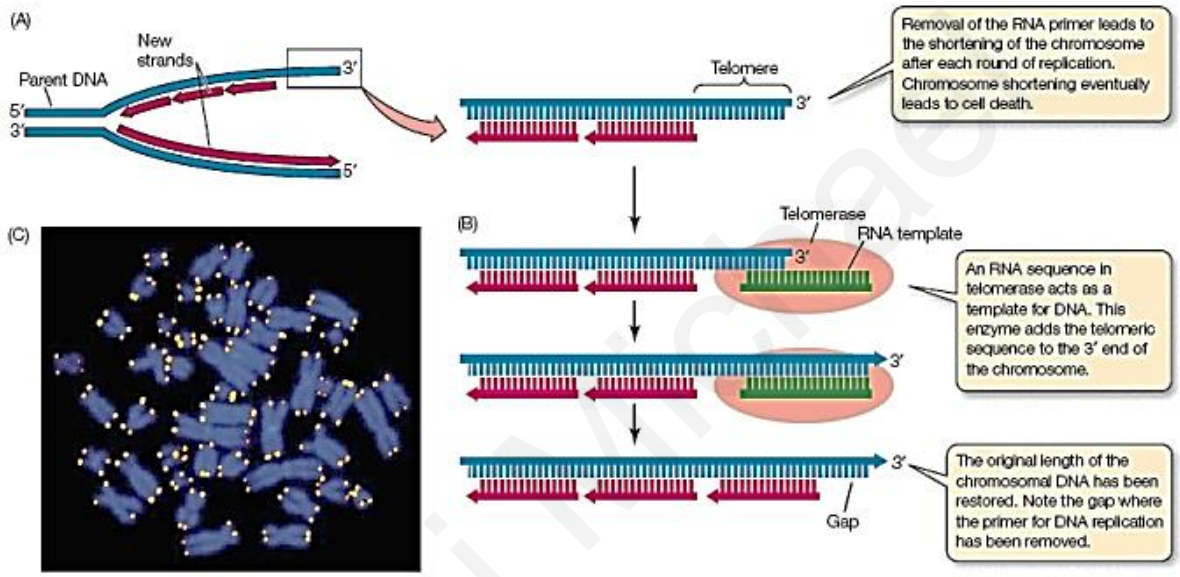

Figure 11: Telomerase and telomeres (Jordan, 2008)
A) The 'end replication problem' where the 3' end of a lagging strand cannot be replicated leading to telomere shortening. B) Telomerase elongating the telomeres using its RNA component as template. C) Representative immunofluorescence image showing chromosomes (blue) and their telomeres (yellow).

Telomere maintenance has also been proposed to play an important role in human papillomavirus (HPV) driven cancers however that role is not well understood. Most of the evidence implicating telomeres and telomerase in HPV-driven cancers has been generated *in vitro*, and in some cases conflicting data have been obtained. The *in vitro* experiments are very useful in identifying interactions of the viral oncogenes but are severely limited in terms of identifying which of those interactions have an active participation in carcinogenesis. But so far they have shown us that both E6 and E7 have been implicated in regulating telomere length by means of telomerase activation and ALT respectively. Specifically E6 was found to upregulate telomerase, a critical step for HPV cell immortalization (Liu et al., 2008a), either by inducing the activation of the Tert promoter via its interactions with the promoter's activator proteins (eg. Myc and NFX1-123),

repressor proteins (eg.NFX1-91) or by directly binding to the Tert protein (Veldman et al., 2003; Liu et al., 2005; Liu et al., 2009; Klingelhutz et al., 1996; Gewin et al., 2004). In addition, E7 was found to increase the E6-induced promoter activity of Tert, to maintain telomere length in the absence of both E6 and telomerase as well as immortalize keratinocytes by cooperating with Tert even when defective for telomere maintenance (Liu et al., 2008a; Stoppler et al., 1997; Miller et al., 2013). However, the real *in vivo* contribution of telomerase activation and telomeric lengthening to HPV related carcinogenesis is unknown.

1.2.2.2 Skin as a Model of HPV and Stem Cell Activity

To be able to assess the consequences of viral oncogene expression in tissue stem cells we need well-characterized markers that would enable their successful detection as well as functional assays for tissue stem cells. This has been a clear challenge for the stem cells of the cervix which haven't been characterized yet and cannot be used as a model of viral infection. For this purpose, the skin can be used as an alternative model for stratified epithelia as its stem cell compartments have been extensively characterized and various populations with "stemness characteristics" have been described. The best understood stem cell population of the skin can be found in the hair follicle in a specialized niche called the "bulge" (Figure 12). This is a quiescent stem cell population with a slow-cycling nature and has the ability to retain its label. The nucleotide pulse-chase technique has been extensively used for its detection which led to its identification as a multipotent stem cell population. Evidence for its multipotency comes from the fact that it contributes not only to hair follicle regeneration *in vivo*, but also to interfollicular epidermal skin healing after wounding (Ito et al., 2005; Morris et al., 2004; Taylor et al., 2000; Tumbar et al., 2004; Zhang et al., 2009).

Contributing to the choice of using the skin as a model, is the fact that the bulge stem cell population can be used to study the functionality of stem cells in response to different conditions. These cells take part in the highly ordered process of homeostasis which consists of cycles of hair follicle growth and regression. In these cycles, following follicle regression the bulge stem cells enter a resting phase, referred to as telogen, in which they are quiescent and show no activity. Following telogen the cells have the ability to mobilize during the growth phase, referred to as anagen, and contribute to the regeneration of the hair follicle. This stem cell model has been extensively used to study not only HPV infection and biology but also the implication of stem cells in carcinogenesis (Auewarakul

et al., 1994; Lambert et al., 1993; Merrick et al., 1992). It should be noted that several lines of evidence implicate the bulge stem cells specifically, as the cell of origin in squamous cell carcinomas (Lapouge et al., 2011; da Silva-Diz et al., 2013).

Therefore, to elucidate the role of viral oncogenes in modulating the behavior of quiescent tissue stem cells, transgenic animals expressing the viral oncogenes E6 and E7 of HPV16 in stratified squamous epithelia of the skin have been used, to assess the effects of these oncogenes on the stem cell populations.

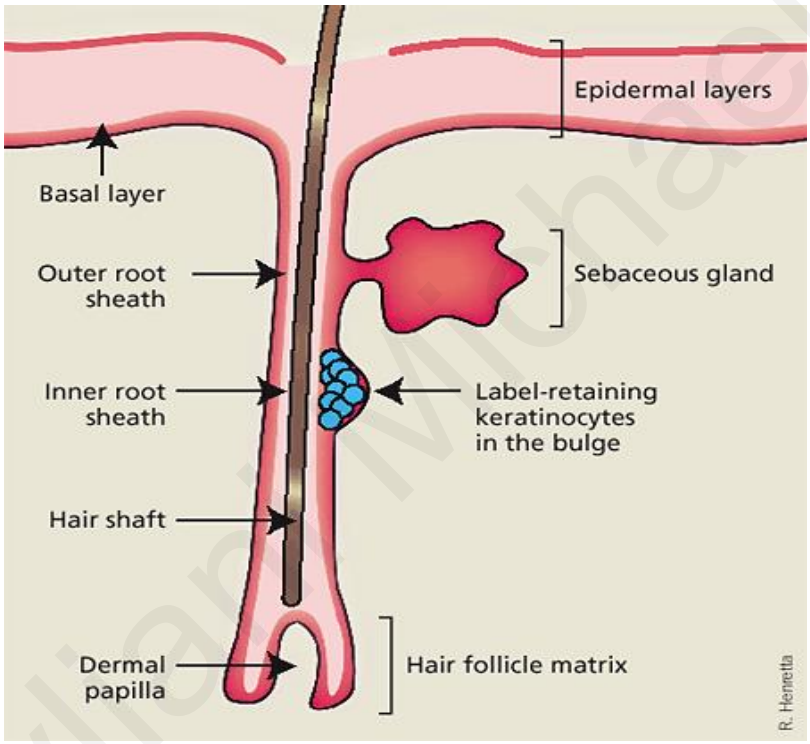

Figure 12: **Skin hair follicle** (Khavari, 2004).
The hair follicle, an extension of the epidermis with its label retaining population (blue) in the bulge region.

1.2.2.3 Reprogramming as an *In Vitro* Model of HPV and Cellular Plasticity

Despite the use of these *in vivo* models, it is very hard to perform studies for the evaluation of the effects of the oncogenes on cellular pluripotency and plasticity *in vivo* and thus they need to be assessed *in vitro*. The challenges of assessing the contribution of either E6 or E7 towards a developmentally multipotent population have been mostly due to the lack of manageable assays. Ideally such an assay should contribute to an assessment of whether the expression of E6 and E7 can contribute to the "developmental regression" of cells or alternatively, whether cells expressing E6 and E7 are more amenable to this sort of

process. In this sense, the reprogramming technology used to derive induced pluripotent stem cells (iPS) from differentiated cells, is an attractive candidate (Figure 13) (Takahashi and Yamanaka, 2006; Yamanaka and Blau, 2010).

Initially, this technology has been thus far thought of as a technology serving in the derivation of embryonic stem cell-like cells for validation and improvement of future cell replacement therapeutics, drug discovery and testing (Bellin et al., 2012). However, a more immediate use of cellular reprogramming in disease modelling became routine and changed the attitude about the unidirectionality of differentiation and its implication in diseases including cancer. For example, in pancreatic cancer due to the lack of early stage progression models the iPS technology was used to convert advanced stage cancer cells to early stage cancer cells in order to recapitulate the events during progression (Kim et al., 2013).

It has been demonstrated that cell populations developmentally closer to a pluripotent state such as multipotent tissue stem cells are more amenable to reprogramming (Kim et al., 2009), a discovery in favour of the idea that infections could arise in the tissue stem cells. Thus we feel that the reprogramming assay can effectively be used as an *in vitro* assay to assess the developmental plasticity of cell populations to move towards a pluripotent state when the oncogenes of HPV16 are expressed.

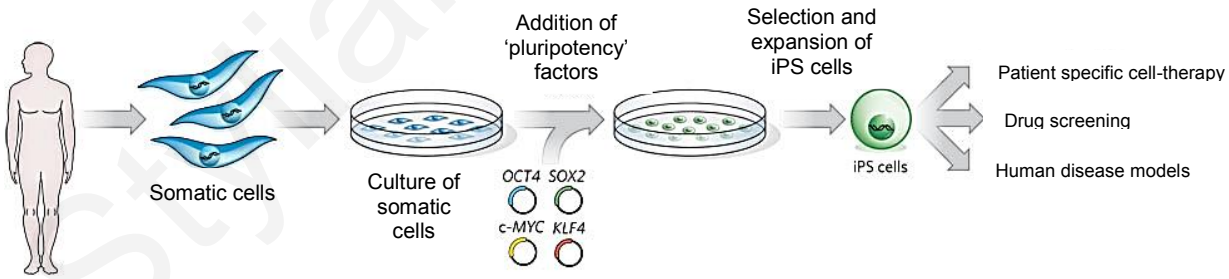

Figure 13: The Yamanaka iPS technology and its applications (Yamanaka and Blau, 2010)
Differentiated somatic cells are transduced in culture with retroviruses expressing transcription factors. Cells are reprogrammed to form iPS cells which can be used in therapeutics, drug validation and screening and disease modeling.

1.3 HYPOTHESIS AND SIGNIFICANCE

1.3.1 Aim 1

1.3.1.1 The effect of the HPV16 oncogenes on the stem cells *in vivo*

There are a few studies indicating that stem cells or the immediate progeny of stem cells are the initial targets of eventual carcinogenesis in both the skin and cervical epithelia (da Silva-Diz et al., 2013; Lapouge et al., 2011; López et al., 2012). We hypothesized, that HPV infection directly targets stem cell populations in these epithelia interfering with their normal homeostasis and plasticity. We examined the effects of E6 and E7 HPV16 oncogenes in these populations for providing the first *in vivo* conclusive demonstration on the effects of HPV oncogenes specifically on infected stem cells.

1.3.1.2 The effect of telomerase absence on the changes in the stem cells induced by HPV16 oncogenes *in vivo*

The ability of the oncogenes to affect targets implicated in stem cell biology prompted us to investigate one of its well-known interactions, telomerase. Our goal was the identification of a potential target through which the oncogenes may mediate their effects in these stem cell compartments. These results would provide the link between HPV16 induced carcinogenesis, stem cells and telomere homeostasis.

1.3.2 Aim 2

1.3.2.1 The effect of the HPV16 oncogenes on the induction of pluripotency *in vitro*

The emerging evidence that differentiated cells regress developmentally to a more stem-cell-like state during carcinogenesis, indicate the possibility that HPV16 can also infect the tissue non-stem cells interfering with their cellular plasticity. For our second aim we studied the effects of viral gene expression in the developmental potential of a cell, using the iPS technology, to determine whether HPVs can reprogram cells *in vitro* and whether this reprogramming may be related to ensuing carcinogenesis.

CHAPTER 2
METHODOLOGY

2.1 *IN VIVO* EXPERIMENTS

2.1.1 Mouse strains

For this study, we used mice on a pure FVB/N inbred genetic background that were generated in the lab of Dr. Paul Lambert (University of Wisconsin, Madison). The mice were engineered to carry either the K14E6/E7TTL or the K14E7/E6TTL constructs (Figure 14) and they were referred to as K14E6 and K14E7 respectively, as previously described (Herber et al., 1996; Song et al., 1999). In addition, we used mice on a C57BL/6 genetic background commercially available from Jackson laboratories (http://jaxmice.jax.org/, stock no. 004132). These were deficient in the production of Terc, the RNA template component of the telomerase enzyme.

All mice were housed at the University of Cyprus, in accordance with regulations and protocols approved by the Department of Veterinary Services, Cyprus Ministry of Agriculture.

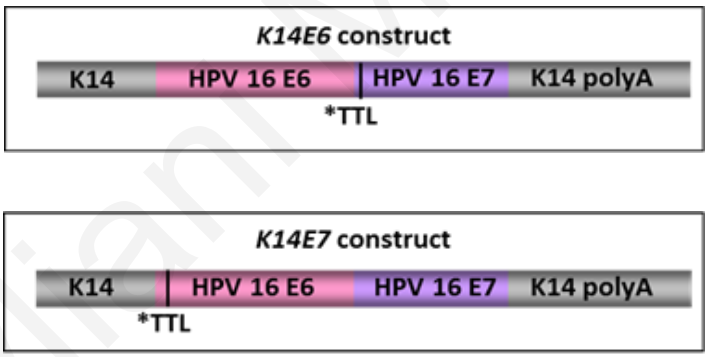

Figure 14: Schematic of the transgene constructs used for the generation of transgenic mice
The constructs utilize the keratin 14 (K14) promoter that drives expression of the genes to the basal layer of stratified squamous epithelia. Each construct is comprised of a Translation Termination Linker (TTL) in either the E6 (bottom) or the E7 (top) ORF that interrupts the expression of E6 or E7 respectively.

2.1.2 Generation of transgenic mice expressing the HPV16 oncogenes

For studying the effect of the oncogene expression we required mice of the following 4 genotypes: non-transgenic (NTG), K14E6, K14E7 and K14E6E7 (Figure 15). For the generation of bitransgenic mice, K14E6 were crossed with K14E7. All the experimental mice used were in a heterozygous state.

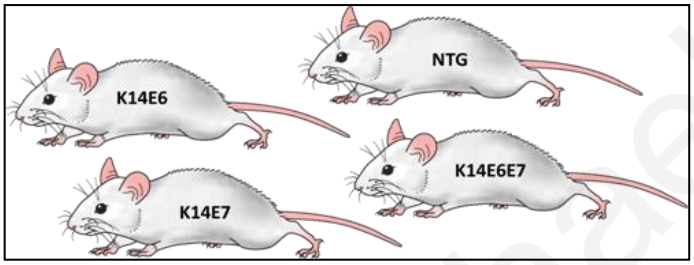

Figure 15: The genotypes of the FVB mice used in the experiments
The mice used in the experiment of the first aim include non-transgenic (NTG), E6 expressing (K14E6), E7 expressing (K14E7) and bitransgenic for E6 and E7 (K14E6E7).

2.1.3 Generation of Terc knockout mice

For studying the contribution of telomerase on the oncogene behavior we used Terc knockout mice that are deficient for the production of Terc, the RNA template component of telomerase. It has been shown that lack of Terc eliminates detectable telomerase activity (Blasco et al., 1997). Heterozygous $Terc^{+/-}$ mice, were crossed with K14E6 or K14E7 mice to generate the F1 $K14E6Terc^{+/-}$ and $K14E7Terc^{+/-}$. These F1 mice were subsequently crossed with $Terc^{+/-}$ to generate the first generation (G1) of mice deficient for the production of Terc (Figure 16). From this cross, the genotypes used were: $Terc^{+/+}$, $Terc^{-/-}$, $K14E6Terc^{+/+}$, $K14E6Terc^{-/-}$, $K14E7Terc^{+/+}$, $K14E7Terc^{-/-}$.

The mice used in these experiments were on a mixed FVB-C57BL/6 background so in order to ensure less variability siblings were used as appropriate controls.

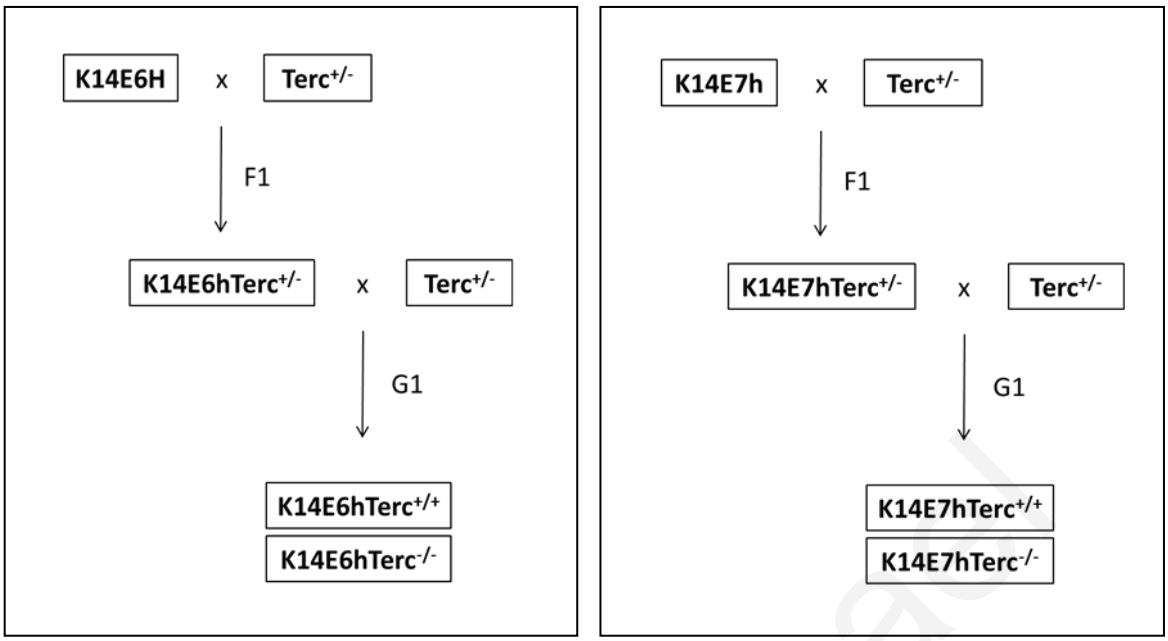

Figure 16: Crossing scheme for the generation of G1 Terc knockout mice
K14E6H (left panel) and K14E7h (right panel) mice were crossed with $Terc^{+/-}$ mice to generate the required F1 genotypes $K16E6hTerc^{+/-}$ and $K14E7hTerc^{+/-}$ respectively. The F1 were subsequently crossed with $Terc^{+/-}$ to generate the required G1 genotypes $K16E6hTerc^{+/+}$, $K16E6hTerc^{-/-}$, $K16E7hTerc^{+/+}$and $K16E7hTerc^{-/-}$ used in the experiments.

2.1.4 DNA extraction and Genotyping

The genotypes of the mice used in the experiments were confirmed by means of PCR screening. A small piece of the mouse tail was cut and genomic DNA was extracted using Extract-N-Amp Tissue kit as per manufacturer's instructions. Extraction solution (Sigma) and Tissue Preparation Solution (Sigma) were mixed at a ratio of 4:1, added to the tail and incubated for 15mins at 37ºC and then for 3mins at 95ºC. Neutralization Solution (Sigma) was then added to the mixture followed by vortex and pulse-spin. The extracted DNA was then amplified by PCR reactions using the KAPATaq (KapaBiosystems) standard PCR protocol. Reactions were performed in 20µl final volume containing 1x buffer with $MgCl_2$, 200µM dNTPs, 0.4µM of each primer and 0.4 units of KapaTaq.

Conditions and primers used in PCR:

Initial denaturation	95ºC	x 3 min
Denaturation	95ºC	x 30 sec
Primer Annealing	see table 3	x 30 sec
Primer Extension	72ºC	x 1 min
Hold	15ºC	x ∞

Table 3: The primer sequences used in genotyping

Primer Name	Sequence 5' to 3'	Annealing TºC
709-1	GGC GGA TCC TTT TAT GCA CCA AAA GAG AAC TG	60
709-4	CCC GGA TCC TAC CTG CAG GAT CAG CCA TG	60
E6TTLtop	GCT TAG TTA ACT AAT GCA AAC	60
E7TTL	AGC CTT AGT TAA CTA ACA TTA C	60
Oligo 2	GCA TGA TTA CAG CTG GGT TTC TCT ACG	60
p53-1	TAT ACT CAG AGC CGG CCT	60
p53-2	ACA GCG TGG TGG TAC CTT AT	60
p53-3	TCC TCG TGC TTT ACG GTA TC	60
Terc W – F	CTC GGC ACC TAA CCC TGA T	54
Terc W – R	CGC TGA CGT TTG TTT TTG AG	54
Terc M – F	CTT GGG TGG AGA GGC TAT TC	54
Terc M – R	AGG TGA GAT GAC AGG AGA TC	54

2.1.5 BrdU incorporation, anagen induction and hair shaving

5-bromo-2-deoxyuridine (BrdU) was administered intraperitoneally in mice at a final concentration of 50mg/kg as first described previously (Bickenbach et al., 1986; Cotsarelis et al., 1990). For pulse chase experiments ten-day-old mice received an injection every 12 hours for a total of four doses and they were euthanized 60 days after injections. For induction of anagen in age-matched mice, at day 60 after BrdU injection, hair was shaved and mice were treated every 48 hours with TPA (20nmol in acetone) for a total of four doses.

2.1.6 Tissue processing

Mice were sacrificed, and tissues obtained were fixed in 4% paraformaldehyde (PFA) in PBS overnight at 4ºC. PFA was removed by rinsing with PBS and the samples were dehydrated in a graded series of ethanol concentrations (70%, 85%,100%) and xylene before they were embedded in TissuePrep® paraffin wax (Fischer Scientific).Sections were obtained at 5-10μm thickness on a microtome, added on slides and left overnight to dry at room temperature.

2.1.7 H&E staining

Tissue samples on slides were deparaffinised in xylene and dehydrated by treating twice with 100% ethanol followed by a graded series of methanol solutions (100%, 75% and 30%). After being washed with ddH2O slides were incubated in haematoxylin solution (Scharlau) for 5 mins, rinsed in running tap water for 5 mins, followed by treatment with 70% ethanol/0.5% (v/v) HCl for 10 sec and rinsing in running tap water for 10 sec. Samples were then incubated in 0.5% (w/v) Eosin solution (Fischer) for 4 mins and rinsed in ddH2O. Rehydration was performed with methanol solutions (30%, 75%, 90% and 100%), ethanol and xylene. Mounting medium was used to secure the coverslips.

2.1.8 Immunohistochemistry

Tissue samples on slides were deparaffinised in xylene and rehydrated in a graded series of ethanol solutions (100%, 95%, 70% and 50%). Antigen retrieval was done in a microwave using 10mM citrate buffer pH=6.0 (2.941g tri-sodium citrate in 1L dH2O) and for BrdU immunohistochemistry, samples were also incubated for 20mins in 2M HCl. For preventing non-specific antibody binding, blocking in 5% horse serum was performed following by antibody incubations. Between primary and secondary antibody incubations samples were washed in PBS. Conditions for blocking, primary and secondary antibody incubations were variable and optimal for each different antibody used (tables 4 and 5).

Table 4: Blocking and primary antibody conditions

Primary Antibody	**Source**	**Blocking**	**Antibody Dilution & Incubation**
Keratin 14 (K14)	Covance (PRB-155P)	1hr	1:1000 in 5% serum for 1hr
BrdU	Abcam (ab6326)	2hrs	1:25 in 5% serum overnight 4^{o}C
Keratin 15 (K15)	SantaCruz (sc-47697)	30mins	1:500 in 1xPBS for 1hr
Proliferating Cell Nuclear Antigen (PCNA)	SantaCruz (sc-25280)	30mins	1:100 in 5% serum for 1hr
Nfatc1	SantaCruz (sc-7294)	30mins	1:10 in PBS for 1hr
Cdk4	SantaCruz (sc-260)	30mins	1:200 in PBS for 1hr

Table 5: Secondary antibody conditions

Secondary Antibody	Source	Dilution in 5% horse serum
FITC-rabbit	Jackson ImmunoResearch (711-095-152)	1:25
Cy3-mouse	Jackson ImmunoResearch (715-165-150)	1:100
Cy3-rat	Jackson ImmunoResearch (712-165-150)	1:100
Cy3-streptavidin	Jackson ImmunoResearch (016-160-0840)	1:100
Biotin-rat	Jackson ImmunoResearch (712-065-150)	1:100
Vectastain universal	Vector Laboratories (PK-6200)	1:100

Following the last antibody incubation samples were washed in PBS and mounting medium with DAPI (Dako) was used when securing the coverslips. Images were then acquired using a ZeissAxio Observer.A1 microscope. Quantification was performed in a blinded fashion.

2.2 *IN VITRO* EXPERIMENTS

Table 6: Cell lines

Name	Description
MEFs	Mouse embryonic fibroblasts
293T	Human embryonic kidney cells
HeLa	HPV18 positive human cervical carcinoma cells
Caski	HPV16 positive human cervical carcinoma cells
C33A	HPV negative human cervical carcinoma cells

Table 7: Media compositions

Name	Composition
DMEM	Dulbecco's Modified Eagle Medium (GIBCO), 10% Fetal Bovine Serum (GIBCO), 1% Penicillin/Streptomycin (GIBCO)
iPS	Dulbecco's Modified Eagle Medium, 1% Penicillin/Streptomycin, 1% β-mercaptoethanol (GIBCO), 15% Knockout Serum Replacement (GIBCO), 1% non-essential amino acids (GIBCO), 0.1% Lif (Millipore)
RPMI	RPMI 1640 Medium, 10% Fetal Bovine Serum (GIBCO), 1% Penicillin/Streptomycin (GIBCO)
MEM	Minimum Essential Medium (MEM), 10% Fetal Bovine Serum (GIBCO), 1% Penicillin/Streptomycin (GIBCO)

2.2.1 Isolation of Mouse Embryonic Fibroblasts (MEFs)

Keratinocytes are the host-cells for natural HPV infection but our studies were initiated using mouse embryonic fibroblasts (MEFs), a cell type much more amenable to isolation and *in vitro* culture. C57/BL6 non transgenic mice were used at 13.5 days post conception. The embryos were harvested from the mouse uterus and placed in a falcon containing fresh Phosphate Buffered Saline (PBS), pH 7.4 (GIBCO), and Penicillin-Streptomycin (P/S) antibiotics. The embryos were transferred in a 10cm dish with PBS and P/S and all the membranes were removed to allow embryo isolation. The embryo's head was cut below the eye and visible viscera such as the liver were removed. The embryo was then transferred to a fresh 10cm dish containing 0.05% trypsin-EDTA 1X (GIBCO) in PBS and

was chopped with a razor blade. The dish was incubated in a 37ºC incubator for 20mins and the cells were pipetted up and down until a single-cell suspension was obtained which was also incubated for another 20mins. DMEM media (table 7) was added in the dish mixed well and the cells were then transferred to T75 bottles until confluent.

2.2.2 Retroviral vector transduction for cell reprogramming

293T cells were transfected with 4μg retroviral E6, E7 or E6E7 expressing, Sox2, Klf4 or Oct4 expressing or empty pLXSN control vectors (table 8) together with the packaging pCL-ECO vector using empty DMEM and Xtreme9 transfection reagent (Roche). The mixture was incubated for 20mins and added dropwise on plated 293T cells at a density of $4x10^6$ cells/10cm dish. The cells were incubated overnight in a 37ºC/5% CO_2 incubator. The next day 293T cell media was replaced with fresh DMEM while MEFs were plated in 6-well plates at a density of $1x10^5$cells/well. For the next two days supernatant containing packaged viruses was collected twice a day from the 293T cells and filtered through a 0.45μm filter. Polybrene (Millipore) was added at a concentration of 1:1000 and the supernatant was added to the MEFs (1ml of each vector to each well). On the last day, MEF media was replaced by iPS media (table 7), which was changed every other day until colonies form. Around day 15 post-transduction colonies were grown at the expected size, fixed and stained.

Table 8: Plasmid vectors used in tissue culture

Gene/Insert name	Plasmid name	Plasmid #	Source
pLXSN			
pLXSN16E6	HPV16 E6	52395	Addgene
pLXSN16E7	HPV16 E7	52396	Addgene
pLXSN16E6E7	HPV16 E6E7	52394	Addgene
SRY-box containing gene 2 (Sox2)	pMXs-Sox2	13367	Addgene
Kruppel-like factor 4 (gut) (Klf4)	pMXs-Klf4	13370	Addgene
POU domain, class 5, transcription factor 1 (Oct4)	pMXs-Oct3/4	13366	Addgene
gag/pol/env	pCL-Eco	12371	Addgene

2.2.3 Alkaline phosphatase staining

Transduced MEFs or keratinocytes were left in culture for about 15 days post-transduction to grow iPS colonies. When colonies formed, the medium was aspirated and cells were fixed with 4% PFH for 1-2mins. The fixative was removed and the cells were rinsed with TBST (20mM Tris-HCl, pH 7.4, 0.05% Tween-20). TBST was aspirated and the alkaline phosphatase staining was performed (Millipore). Fast Red Violet solution was mixed with Naphthol AS-BI phosphate solution and water in a 2:1:1 ratio and enough mixture was added to cover the cells. The cells were incubated in the dark until colonies stained red/lilac. The mixture was aspirated and the colonies were left to dry.

2.2.4 RNA isolation, cDNA synthesis and PCR

RNA was isolated from cells in culture using Qiazol as per manufacturer's instructions (Qiagen) under RNAse free conditions. Cells grown in 10cm dishes were lysed by the addition of 1ml Qiazol, disrupted using cell scrapers and collected in eppendorf tubes. 0.2ml chloroform was added, tubes were shaken vigorously by hand for 15secs and centrifuged at 12000g for 10mins at 4^{o}C. The aqueous phase in the tube was transferred to a fresh tube into which 0.5ml isopropanol was added to precipitate the RNA. The tubes were mixed by repeated inversion and vortexing, incubated on ice for 5mins and centrifuged at 13000g for 10mins at 4^{o}C. The supernatant was removed and RNA was washed with 0.5ml of 75% ethanol by inverting the tubes and centrifuging at 13000g for 1min. All the supernatant was removed, the pellet was air dried and dissolved in 50µl UltraPure™ DNase/RNase-Free Distilled Water (GIBCO). The concentration of the RNA was measured using a NanoDrop 2000 Spectrophotometer (Thermo Scientific) and 300ng RNA from each sample was converted into cDNA using the iScript™ cDNA Synthesis Kit (BIO RAD) as per manufacturer's instructions. cDNA was then amplified by PCR reactions using the KAPATaq (KapaBiosystems) standard PCR protocol. Reactions were performed in 20µl final volume containing 1x buffer with $MgCl_2$, 200µM dNTPs, 0.4µM of each primer and 0.4 units of KapaTaq.

Conditions and primers used in PCR:

Initial denaturation	95°C	x 3 min
Denaturation	95°C	x 30 sec
Primer Annealing	see table 9	x 30 sec
Primer Extension	72°C	x 1 min
Hold	15°C	x ∞

Table 9: The primer sequences used in RT-PCR

Primer Name	**Sequence 5' to 3'**	Annealing T°C
E. Sox2 F mm	TAG AGC TAG ACT CCG GGC GAT GA	60
E. Sox2 R mm	TTG CCT TAA ACA AGA CCA CGA AA	60
E. Oct4 F mm	TCT TTC CAC CAG GCC CCC GGC TC	60
E. Oct4 R mm	TGC GGG CGG ACA TGG GGA GAT CC	60
E. Klf4 F mm	GCG AAC TCA CAC AGG CGA GAA ACC	60
E. Klf4 R mm	TCG CTT CCT CTT CCT CCG ACA CA	60
T. Sox2 F mm	ATG GGC TCT GTG GTC AAG TC	53
T. Sox2 R mm	CCC TCC CAA TTC CCT TGT AT	53
T. Oct4 F mm	CCA ATC AGC TTG GGC TAG AG	53
T. Oct4 R mm	CTG GGA AAG GTG TCC CTG TA	53
T. Klf4 F mm	CTG AAC AGC AGG GAC TGT CA	53
T. Klf4 R mm	GTG TGG GTG GCT GTT CTT TT	53
Actin F mm	GAC GGC CAG GTC ATC ACT AT	53
Actin R mm	AAG GAA GGC TGG AAA AGA GC	53
Sox2 F hs	CAC AAC TCG GAG ATC AGC AA	53
Sox2 R hs	GTT CAT GTG CGC GTA ACT GT	53
Oct4 F hs	GAA GGA TGT GGT CCG AGT GT	53
Oct4 R hs	GTG AAG TGA GGG CTC CCA TA	53
Klf4 F hs	CCC ACA CAG GTG AGA AAC CT	53
Klf4 R hs	TTC TGG CAG TGT GGG TCA TA	53

* E.= endogenous T.= Transgene mm = mus musculus hs = homo sapiens

2.3 Statistical tests

To determine the statistical significance in each experiment, either 3 mice of each genotype were used or the experiment was performed in triplicates. For the IHC experiments, around 75 hair follicles were counted. Statistical analysis was done using the "Mstat" software (version 5.5.3, McArdle Laboratory for Cancer Research, University of Wisconsin-Madison [http://mcardle.oncology.wisc.edu/mstat/]). Results were compared using a Wilcoxon rank-sum test. For all statistical tests differences were considered statistically significant at $p \leq 0.05$.

CHAPTER 3

RESULTS

3.1 RESULTS OF AIM 1

3.1.1 The effect of the HPV16 oncogenes on the stem cells *in vivo* adapted from(Michael et al., 2013))

3.1.1.1 Expression of HPV16 Oncogenes Reduces Number of Relatively Quiescent Cells Detected at Telogen

The transgenic K14E6 and K14E7 mice have been previously generated and extensively characterized (Herber et al., 1996; Song et al., 1999). They are under the control of the keratin 14 promoter which directs expression of the HPV16 oncogenes to the basal layer of stratified epithelia, including the bulge niche (Figure 17) (Arbeit et al., 1994). In order to assess the effects of E6 and E7 expression in quiescent bulge stem cells, BrdU pulse-chase assays were performed as previously described (Bickenbach et al., 1986; Cotsarelis et al., 1990; Morris and Potten, 1999), and the numbers of label-retaining cells (LRCs) at second telogen (resting phase of hair cycle) were compared in wild type animals and animals transgenic for either one or both of the oncogenes (Figure 18A). The numbers of LRCs were significantly reduced in mice expressing either one of the two, or both viral oncogenes suggesting either an overall reduction in the numbers of this particular stem cell type or enhanced proliferation, which could lead to a more rapid label loss.

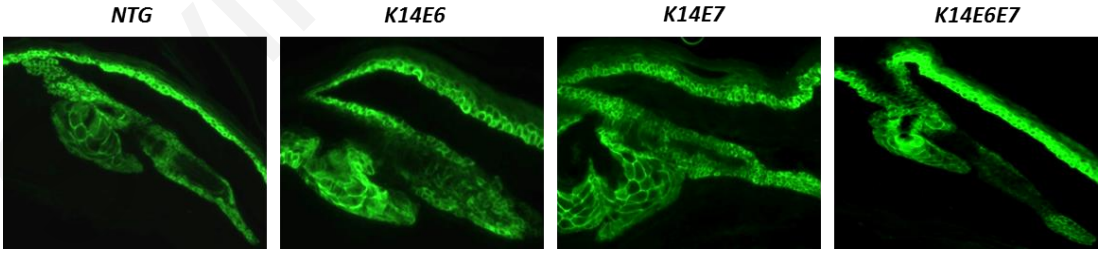

Figure 17: The K14 promoter drives expression of the HPV16 oncogenes to the basal layer of the skin and its appendages.
Representative images from all the genotypes examined showing the pattern of K14 expression which correlates with the expression of the HPV16 oncogenes in the basal layer of the skin and its appendages. K14 positive cells were detected by immunofluorescence using a K14-specific antibody.

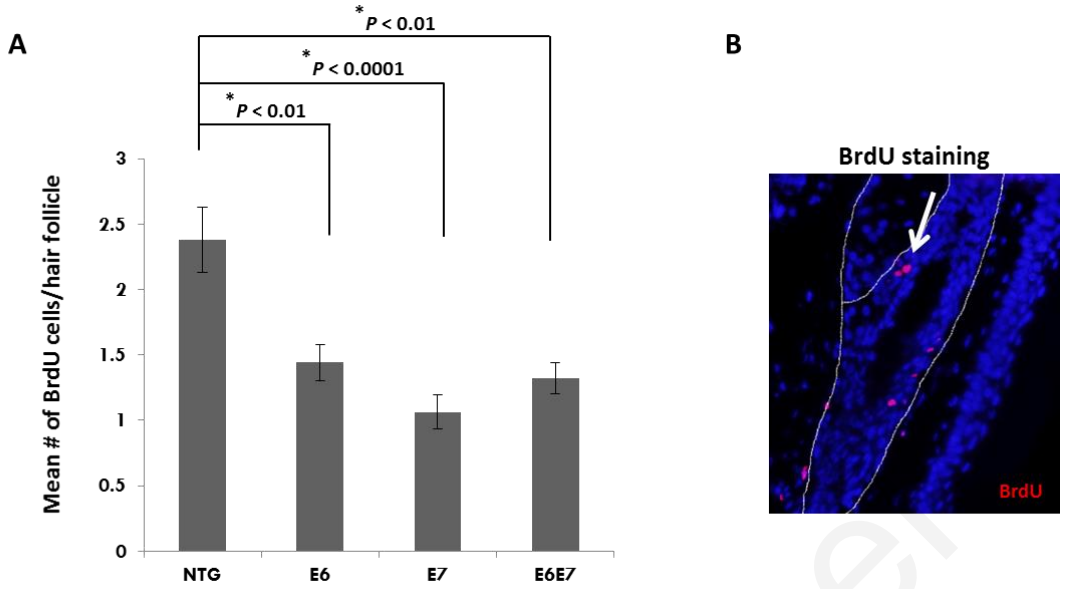

Figure 18: Expression of HPV oncogenes leads to reduced detection of LRCs in hair follicle bulge at telogen.
(A) LRCs were labelled using a BrdU pulse administered shortly after birth and chased until second telogen. At least 3 mice of each genotype (n=3), NTG, K14E6, K14E7 and K14E6E7 mice, were selected and hair follicle bulge regions were quantified. The mean number of BrdUrd positive cells per hair follicle was plotted for each genotype (columns); bars, SD. All statistical comparisons were performed using a two-sided Wilcoxon rank sum test.
(B) Representative immunofluorescence figure of a hair follicle showing BrdU positive cells (red-white arrow). Counterstaining was done with DAPI (blue).

3.1.1.2 Stem Cells expressing HPV16 oncogenes have increased mobilization upon acute anagen induction.

In order to define the cause of the reduction, the proliferation potential of stem cells expressing viral oncogenes was further investigated by acute induction of anagen. A pulse-chase protocol was performed as above and anagen was induced by repeated TPA administration prior to harvesting. Successful anagen induction was validated by the characteristic hair follicle elongation in all genotypes examined (Figures 19A and 18B). LRC mobilization was monitored as a function of BrdU label loss by means of BrdU-specific immunohistochemistry. In animals expressing E6, E7 or both oncogenes mobilization of stem cells was more pronounced compared to that seen in wild type animals (Figure 20). This result suggests that tissue stem cells expressing E6 or E7 are more poised to proliferate, explaining in part the reduced numbers of LRCs detected in telogen conditions (Figure 18).

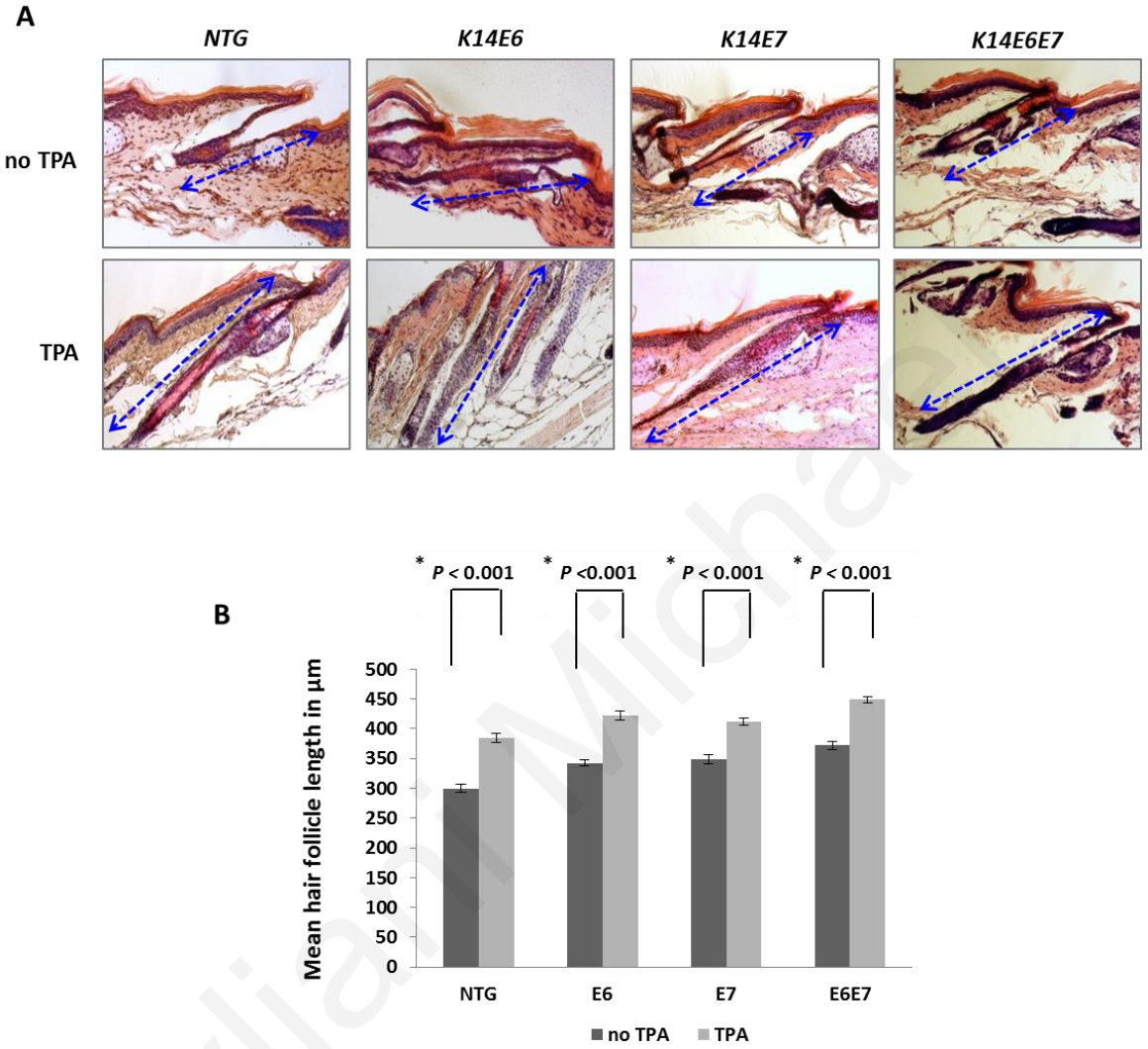

Figure 19: TPA successfully induces anagen in all the mice.
(A) In order to induce anagen in mice where LRCs were labelled, TPA was applied on mice every 48 hours for four times. H&E staining was performed on all tail hair follicles. Hair follicle length was quantified to verify effective anagen induction.
(B) At least 3 mice of each genotype (n=3), NTG, K14E6, K14E7 and K14E6E7 mice, were selected and hair follicle lengths were quantified. The mean hair follicle length in µm was plotted for each genotype (columns); bars, SD. All statistical comparisons were performed using a two-sided Wilcoxon rank sum test.

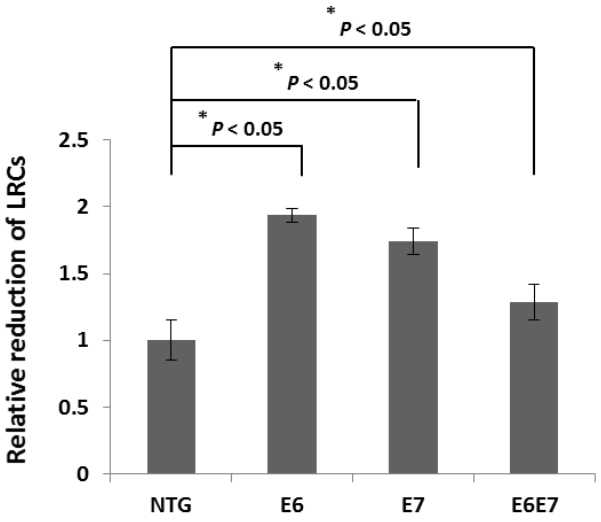

Figure 20: Expression of HPV oncogenes leads to more rapid mobilization of LRCs in response to acute anagen induction.
In order to track the mobilization of LRCs in response to acute anagen induction, the relative reduction of LRCs was tracked per genotype. At least 3 mice of each genotype (n=3) at anagen and telogen, NTG, K14E6, K14E7 and K14E6E7 mice, were selected and hair follicle bulge regions were quantified. The mean number of BrdUrd positive cells per hair follicle was plotted for each genotype (columns); bars, SD. All statistical comparisons were performed using a two-sided Wilcoxon rank sum test.

3.1.1.3 Combined expression of E6E7 gives a robust proliferative ability in hair follicles.

To investigate the LRC's mobilization outcome on proliferation, hair re-growth was monitored. The backs of transgenic and control mice were shaved at day 65, previously characterized to correspond to the end of second telogen. Hair re-growth was monitored for up to 8 days post-shaving and was enhanced in mice expressing both E6 and E7 (Figure 21). Complete hair re-growth, consistent with increased tissue stem cell activity in those animals was only evident in mice bitransgenic for both E6 and E7. Also consistent with that result a greater increase in PCNA staining in anagen hair follicles from the bitransgenic animals was seen (Figures 22A and 22B).

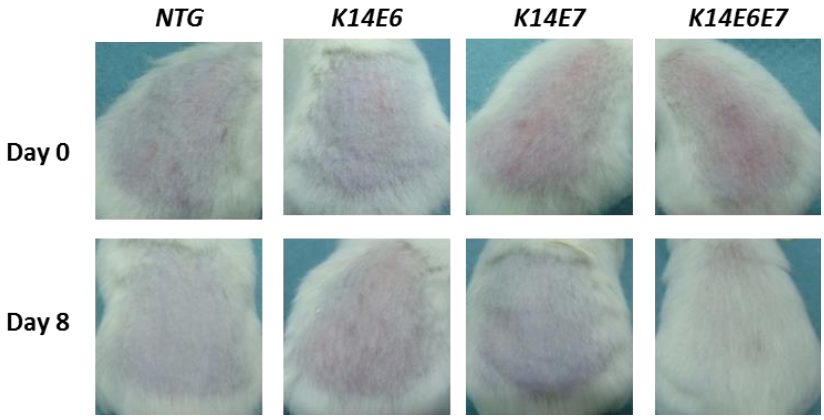

Figure 21: Mice expressing the oncogenes show increased hair growth.
Backs of telogen mice of all genotypes were shaved and pictures were taken at days 0 and 8 after shaving.

3.1.1.4 HPV16 oncogene expression does not lead to an overall reduction of other bulge stem cell markers.

In order to eliminate the possibility that reduced LRC numbers (Figure 18) are indicative of aberrant reduction in the number of stem cells, endogenous markers of bulge stem cells, such as the expression of K15 was tested (Liu et al., 2003). The results showed an increase in the numbers of K15 positive cells and thus expansion of the K15 layer (Figures 23A and 23B). Thus, the reduction in LRC numbers is not consistent with an overall decrease in stem cell markers. On the contrary, an aberrant expansion in the K15 compartment is detected, suggesting that an increased ability to proliferate might not be the only change induced in stem cells by E6 and E7 expression.

3.1.1.5 The Nfatc1 pathway is perturbed upon viral oncogene expression.

In order to test whether the quiescence of bulge stem cells is affected in oncogene expression, the nuclear localization of the Nuclear factor of activated T-cells (Nfatc1), shown to be the gatekeeper of bulge stem cell quiescence, was tested in both telogen and anagen conditions (Horsley et al., 2008). No significant changes were observed in nuclear localization in conditions of follicle growth when the oncogenes were expressed (Figure 24B). However, under resting conditions the expression of E6 and E6E7 led to a significant reduction in the numbers of stem cells with nuclear Nfatc1 (Figure 24A). Furthermore, the expression of Cdk4, a downstream target of Nfatc1 and also a cell cycle gene implicated in the pRb pathway was tested showing an increase in both telogen and anagen conditions when the oncogenes are expressed (Figures 25A and 25B).

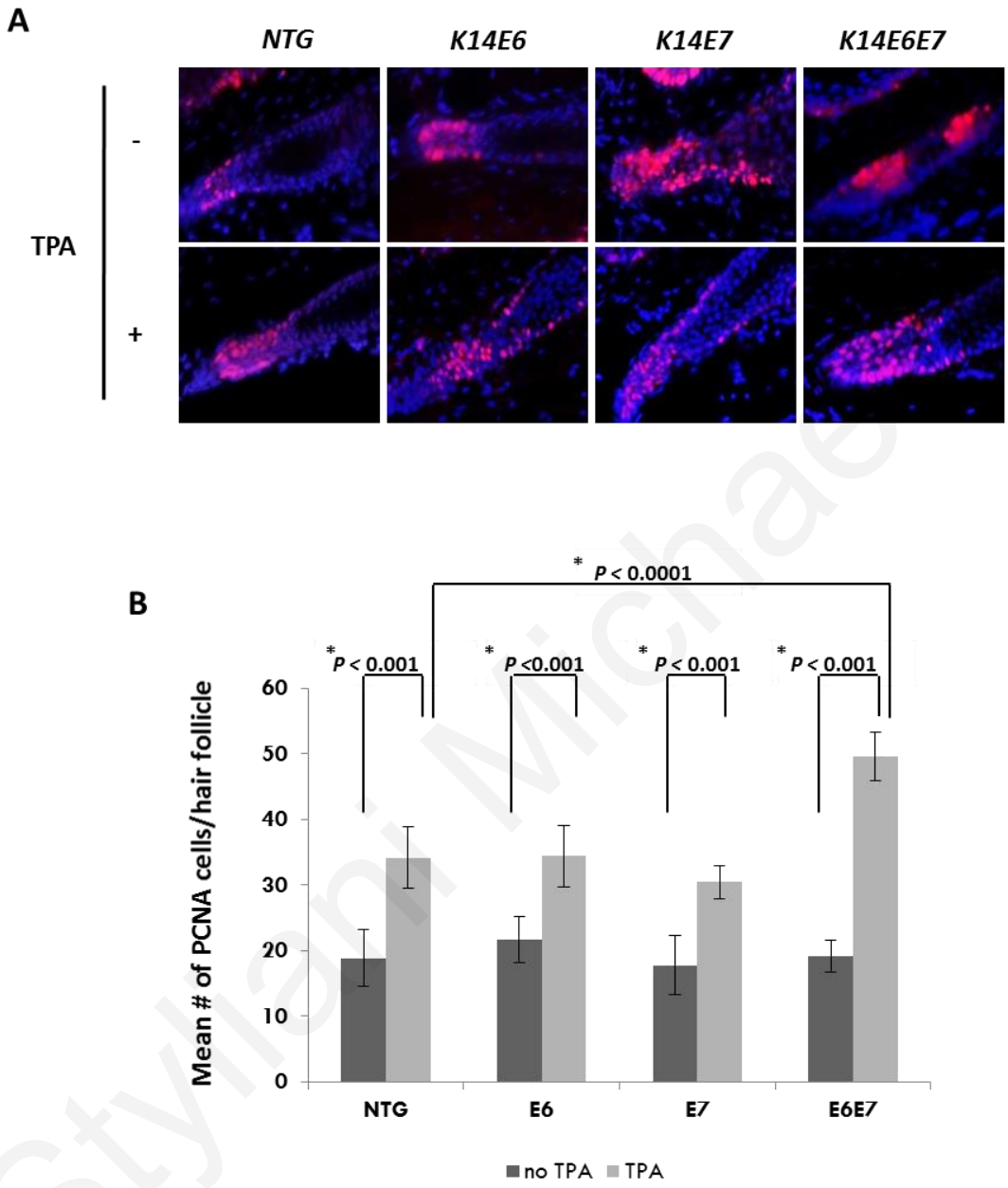

Figure 22: Increased hair growth correlates with increased mobilization of LRCs in mice expressing E6 and E7.

(A) At least 3 mice of each genotype (n=3), NTG, K14E6, K14E7 and K14E6E7 mice, were selected and hair follicles were quantified. The mean number of PCNA positive cells at the base of each hair follicle was plotted for each genotype (columns); bars, SD. All statistical comparisons were performed using a two-sided Wilcoxon rank sum test.

(B) Representative immunofluorescent figures of hair follicles showing PCNA positive cells (red). Counterstaining was done with DAPI (blue).

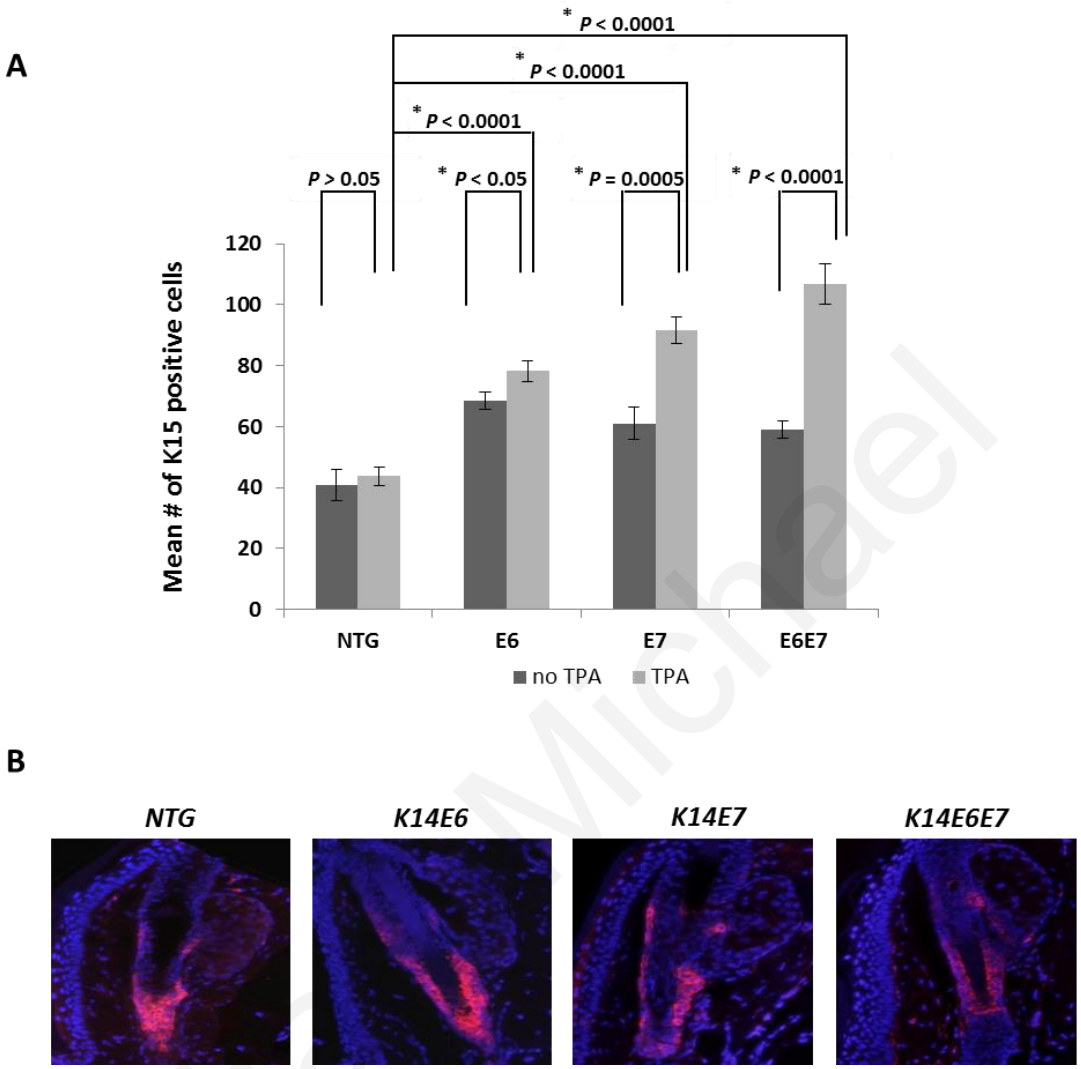

Figure 23: Other markers of bulge stem cells are not reduced in response to HPV oncogene expression.

(A) Immunofluorescence was performed using a K15-specific antibody. At least 3 mice of each genotype (n=3), NTG, K14E6, K14E7 and K14E6E7 mice, were selected and hair follicles were quantified. The mean number of K15 positive cells of each hair follicle was plotted for each genotype (columns); bars, SD. All statistical comparisons were performed using a two-sided Wilcoxon rank sum test.

(B) Representative immunofluorescence of K15 staining (red) in the hair follicles of the genotypes examined. Counterstaining was done with DAPI (blue).

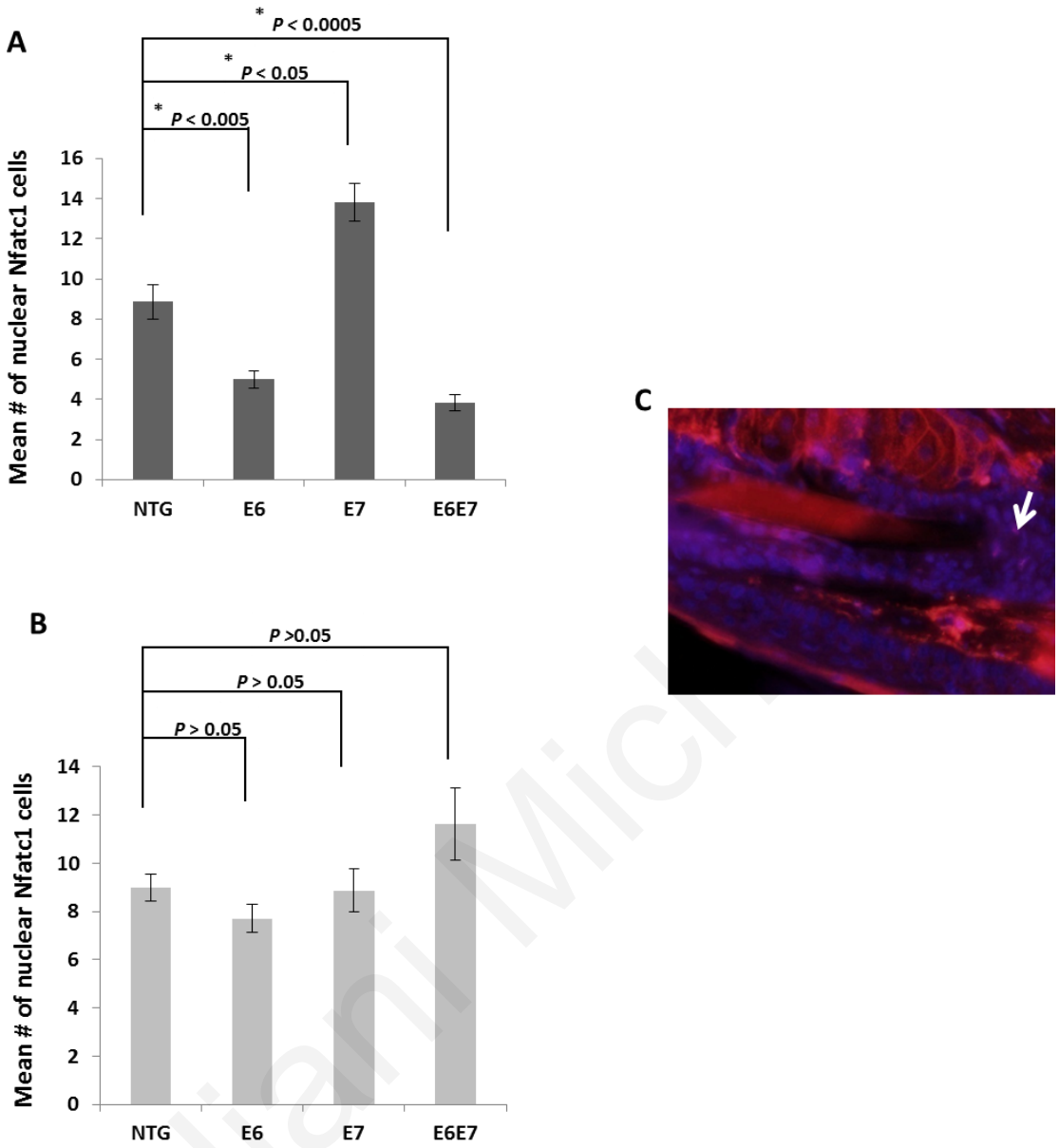

Figure 24: The quiescence of the bulge stem cell population is affected upstream of the Nfatc1 pathway by oncogene expression.

(A-B) At least 3 mice of each genotype (n=3), NTG, K14E6, K14E7 and K14E6E7 mice, were selected and hair follicles were quantified. The mean number of positive cells of each hair follicle in (A) telogen and (B) anagen was plotted for each genotype (columns); bars, SD. All statistical comparisons were performed using a two-sided Wilcoxon rank sum test.

(C) Representative immunofluorescense staining of nuclear Nfatc1 positive cells is depicted by arrows. Counterstaining was done with DAPI (blue).

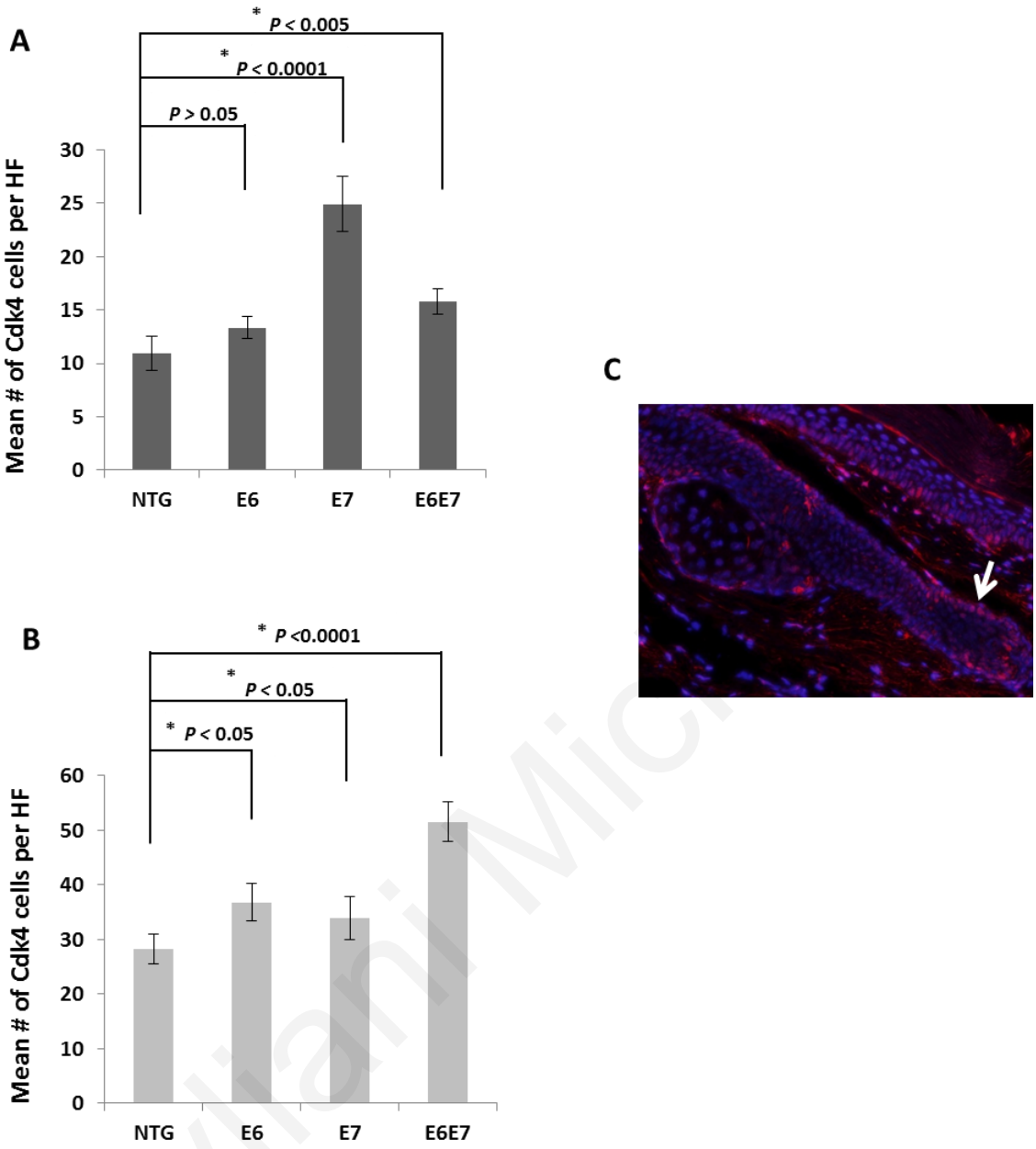

Figure 25: The quiescence of the bulge stem cell population is affected downstream of the Nfatc1 pathway by oncogene expression.

(A-B) At least 3 mice of each genotype (n=3), NTG, K14E6, K14E7 and K14E6E7 mice, were selected and hair follicles were quantified. The mean number of positive cells of each hair follicle in (A) telogen and (B) anagen was plotted for each genotype (columns); bars, SD. All statistical comparisons were performed using a two-sided Wilcoxon rank sum test.

(C) Representative immunofluorescense staining of Cdk4 positive cells is depicted by arrows. Counterstaining was done with DAPI (blue).

3.1.2 The effect of telomerase absence on the changes in the stem cells induced by HPV16 oncogenes *in vivo*

3.1.2.1 HPV16 induced LRC reduction is a phenotype consistent across genetic backgrounds other than FVB.

To define telomerase as a potential target through which the oncogenes exert their effect on the stem cells, K14E6Terc$^{+/-}$ and K14E7Terc$^{+/-}$ mice were crossed with Terc$^{+/-}$ mice to generate Terc knockouts. These are deficient for the RNA component of telomerase and show no detectable telomerase activity. Before examining how the absence of telomerase affects the oncogenes, any differences that could be attributed to the mixed FVB/C57BL/6 background of the new mice were eliminated. The BrdU pulse chase assay was repeated and the numbers of label-retaining cells (LRCs) at second telogen were compared in wild type animals and animals transgenic for the oncogenes. It was shown that the presence of the oncogenes significantly reduces the numbers of the LRCs despite the mixed background (Figure 26).

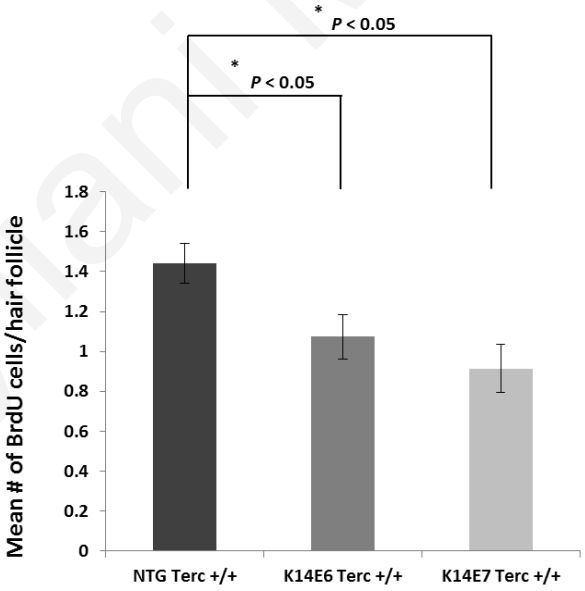

Figure 26: Expression of HPV oncogenes leads to reduced detection of LRCs in telogen hair follicle bulge in mice of mixed background.
LRCs were labelled using a BrdU pulse administered shortly after birth and chased until second telogen. At least 3 mice of each genotype (n=3) were selected and hair follicle bulge regions were quantified. The mean number of BrdUrd positive cells per hair follicle was plotted for each genotype (columns); bars, SD. All statistical comparisons were performed using a two-sided Wilcoxon rank sum test.

3.1.2.2 The absence of telomerase rescues the reduced LRC number phenotype seen in E7 expressing mice.

To assess the effect of telomerase absence on the reduction of stem cells seen by the oncogene expression, the BrdU pulse chase assays were performed on K14E6Terc$^{-/-}$ and K14E7Terc$^{-/-}$ mice. The phenotype observed during the expression of E6 is unaffected by the absence of telomerase as there was still a significant reduction in the number of LRCs. However, the expression of E7 seems to be affected by the absence of telomerase (Figure 27). In this context (K14E7Terc$^{-/-}$) the number of the LRCs was not significantly less than that observed in the non-transgenic control. This suggests that either telomerase activity or the Terc component of telomerase could act as a potential target through which E7 exerts its effects on the stem cells.

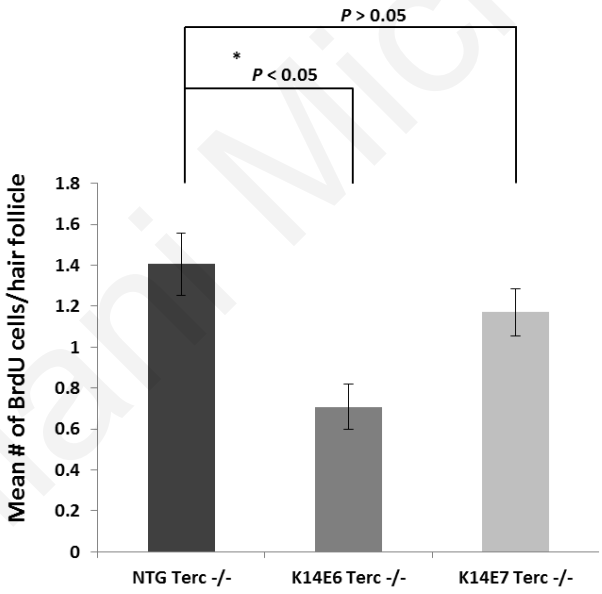

Figure 27: LRC number reduction is rescued in E7 mice in the absence of telomerase.
LRCs were labelled using a BrdU pulse administered shortly after birth and chased until second telogen. At least 3 mice of each genotype (n=3) were selected and hair follicle bulge regions were quantified. The mean number of BrdUrd positive cells per hair follicle was plotted for each genotype (columns); bars, SD. All statistical comparisons were performed using a two-sided Wilcoxon rank sum test.

3.1.1.8 Testing the expression of other markers of bulge stem cells in telomerase sufficient and deficient HPV expressing mice (work in progress)

To assess how the absence of telomerase affects the expansion of the stem cell compartment, previously seen by the oncogene expression, we tested the expression of the stem cell marker K15 in the mice. The results so far show an increase in the numbers of K15 positive cells and thus expansion of the K15 layer in both Terc sufficient and deficient mice (Figure 28). The results however could not be statistically compared due to small sample size.

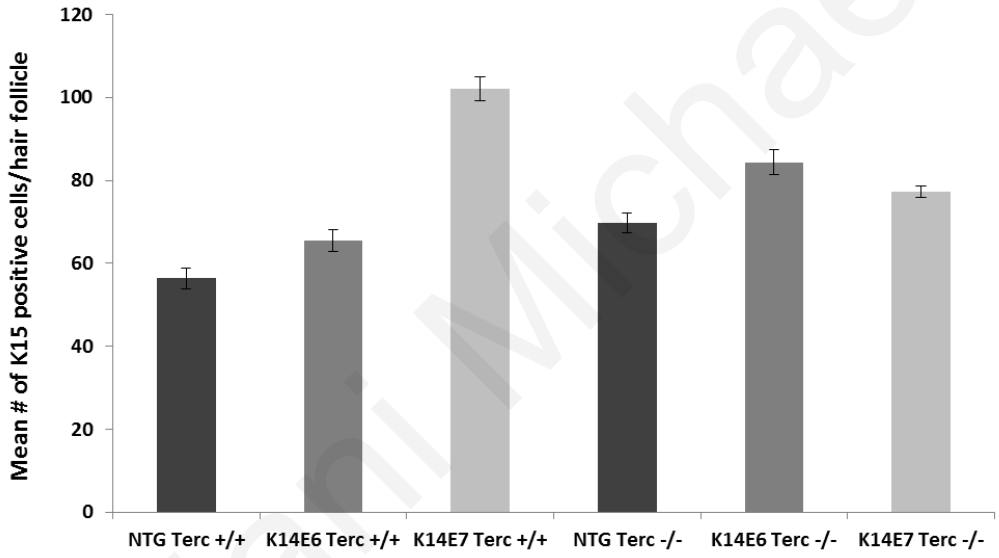

Figure 28: The K15 expression in the hair follicles of Terc mice
Immunofluorescence was performed using a K15-specific antibody. The mean number of K15 positive cells per hair follicle was plotted for each genotype. Statistical comparisons have not been made yet due to the small sample size.

3.1.1.8 Testing the proliferation status of telomerase sufficient and deficient HPV expressing mice (work in progress)

To assess how the absence of telomerase affects the proliferation of the tissue we are testing the expression of the proliferation marker PCNA in the basal layer of the epidermis. The results so far show an increase in the numbers of PCNA positive cells and thus increased proliferation in Terc sufficient mice (Figure 29). In the absence of telomerase activity proliferation in E6 expressing mice seems to be unaffected however it seems to be

affected in the E7 expressing mice. The results however could not be statistically compared due to small sample size.

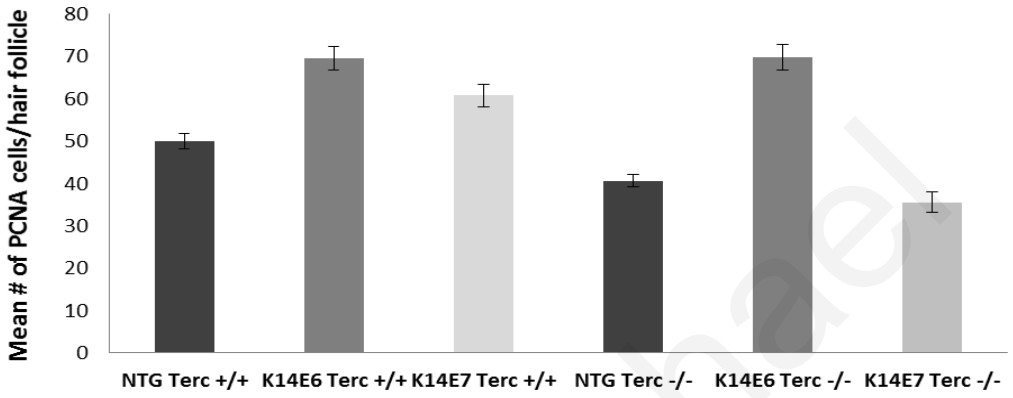

Figure 29: The PCNA expression in the epidermis basal layer of Terc mice
Immunofluorescence was performed using a PCNA-specific antibody. The mean number of PCNA positive cells in the basal layer of the epidermis was plotted for each genotype. Statistical comparisons have not been made yet due to the small sample size.

3.2 RESULTS OF AIM 2

3.2.1 The effect of the HPV16 oncogenes on pluripotency *in vitro*

3.2.1.1 HPV16 Oncogene Expression Increases Reprogramming Efficiency.

To assess the contribution of the oncogene expression on "stemness", the Yamanaka reprogramming experiment was used and the efficiency of the process in the presence of the oncogenes was tested. The classical cocktail of transcription factors, Sox2-Klf4-Oct4, was introduced in MEFs together with either pLXSN, E6, E7 or E6E7 vectors and the successful reprogramming was confirmed by the appearance of round and shiny iPS colonies (Figure 30). It has been shown that in cells expressing either one or both oncogenes there is a statistically significant increase in the percentage of iPS positive colonies (Figure 31). This result suggests that either the oncogenes increase the proliferative capacity of the colonies or they are directly involved in the reprogramming process.

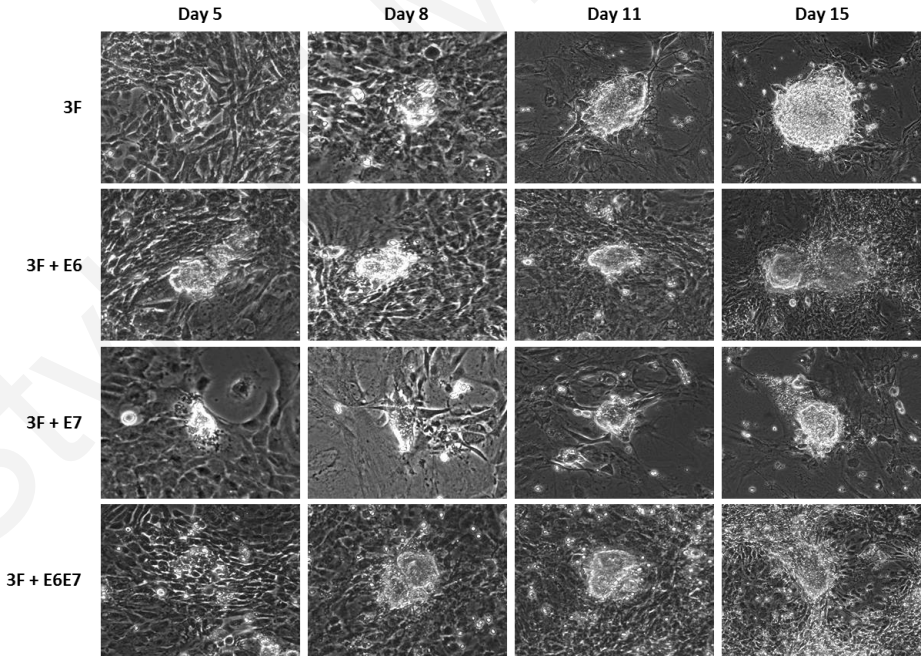

Figure 30: MEFs are successfully reprogrammed and form iPS colonies in the absence or presence of the HPV16 oncogenes.
MEFs were transduced with the 3 reprogramming factors (Sox2, Klf4 and Oct4) together with empty pLXSN vector, E6, E7 or E6E7 expressing vectors. The formation of the iPS colonies was followed and recorded.

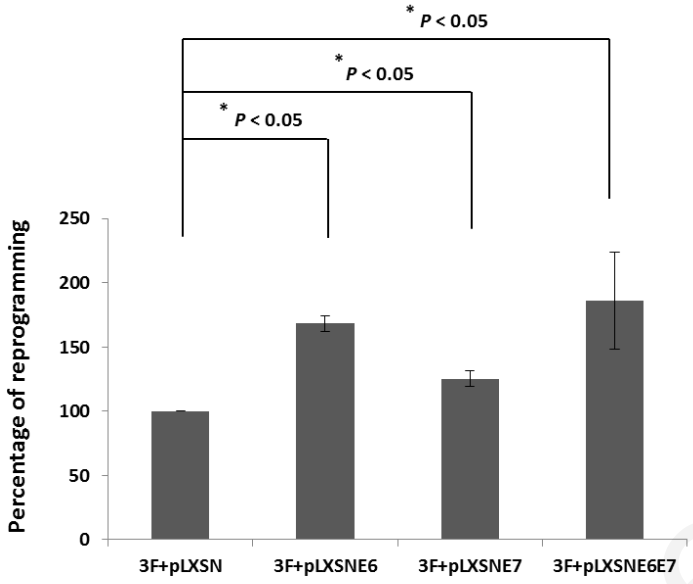

Figure 31: HPV16 oncogene expression increases reprogramming efficiency.
MEFs were transduced with the 3 reprogramming factors (Sox2, Klf4 and Oct4) together with empty pLXSN vector, E6, E7 or E6E7 expressing vectors. Each experiment was performed in triplicates for 3 times and the total number of iPS positive colonies were identified by alkaline phosphatase staining counted and plotted (columns); bars, SD. All statistical comparisons were performed using a two-sided Wilcoxon rank sum test.

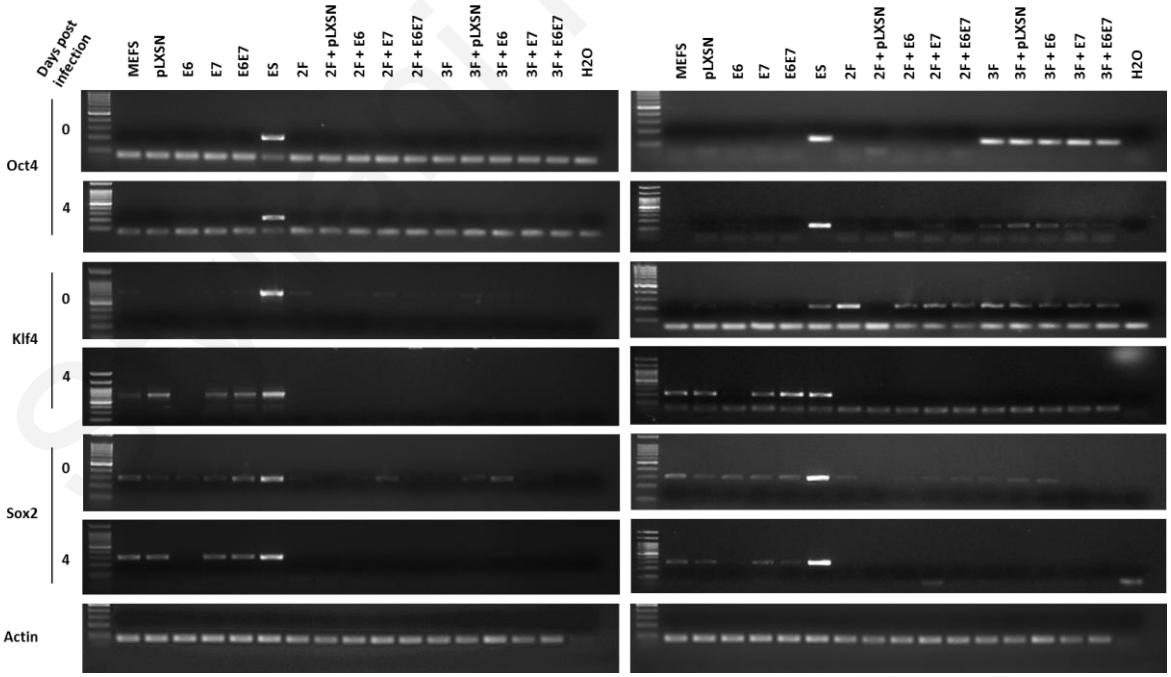

Figure 32: HPV16 oncogene expression does not cause significant changes in the expression of the transcription factors during the initial stages of reprogramming.
The endogenous (left) and total (right) expression of the transcription factors Sox2, Oct4 and Klf4 0 and 4 days post-transduction was tested in transduced MEFs by RNA isolation and RT-PCR.

3.2.1.2 The HPV16 oncogene expression does not cause any significant changes in the expression of the transcription factors during the initial stages of reprogramming.

To check if the expression of the transcription factors changes by the presence of the oncogenes during the initial stages of the reprogramming process, RNA was isolated from transduced MEFs at 0 and 4 days post-transduction and analyzed by RT-PCR. Both the total and the endogenous expression of the transcription factors was tested and results showed that no dramatic changes in their levels are observed during the initial stages of the reprogramming process (Figure 32).

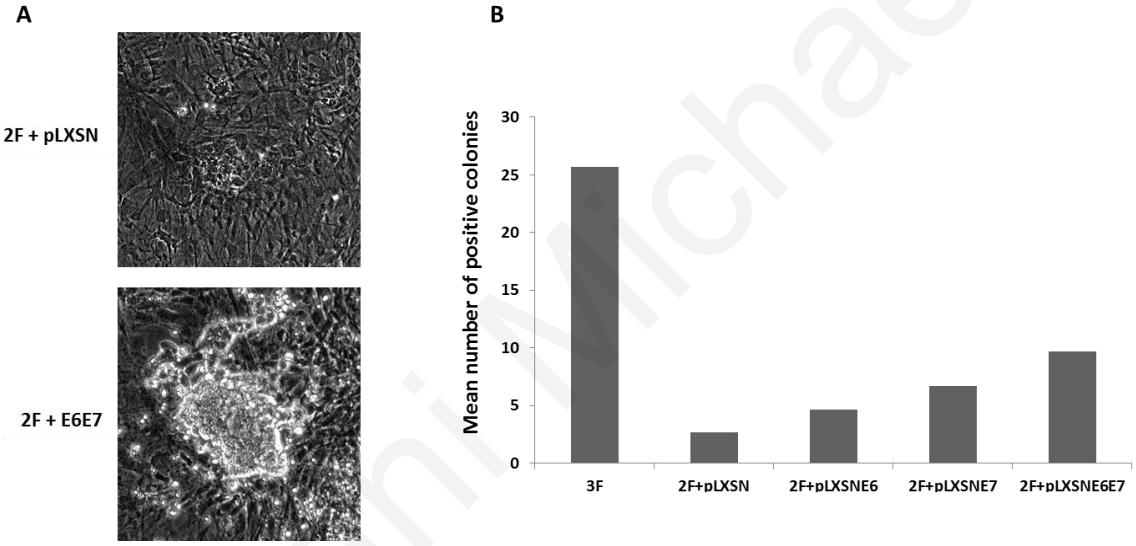

Figure 33: HPV16 oncogene expression reprograms cells in the absence of Oct4.
MEFs were transduced with the 3 reprogramming factors (Sox2, Klf4) together with empty pLXSN vector, E6, E7 or E6E7 expressing vectors. Each experiment was performed in triplicates for 3 times and the total number of iPS positive colonies were identified by alkaline phosphatase staining counted and plotted (columns); bars, SD. All statistical comparisons were performed using a two-sided Wilcoxon rank sum test.

3.2.1.3 The HPV16 oncogenes can reprogram cells in the absence of Oct4

To investigate the involvement of the oncogenes in the reprogramming process, their ability to reprogram cells to iPS cells in the absence of Oct4 was tested. The experiment was repeated as above but this time Oct4 was omitted from the cocktail of transcription factors. It was found that in the presence of both oncogenes, the cells can be reprogrammed and form colonies that resemble the characteristic structure of colonies formed by the three transcription factors (Figure 33).

3.2.1.4 The expression of HPV affects the expression of the transcription factors in human cervical carcinoma cell lines.

To examine how the expression of HPV affects the reprogramming transcription factors Sox2, Oct4 and Klf4 we tested their expression by RT-PCR in the human cervical carcinoma cell lines HeLa (HPV18), Caski (HPV16) and C33A (HPV negative). From the results we observe that while Sox2 is not expressed in the presence of the virus, Oct4 and Klf4 are slightly upregulated (Figure 34).

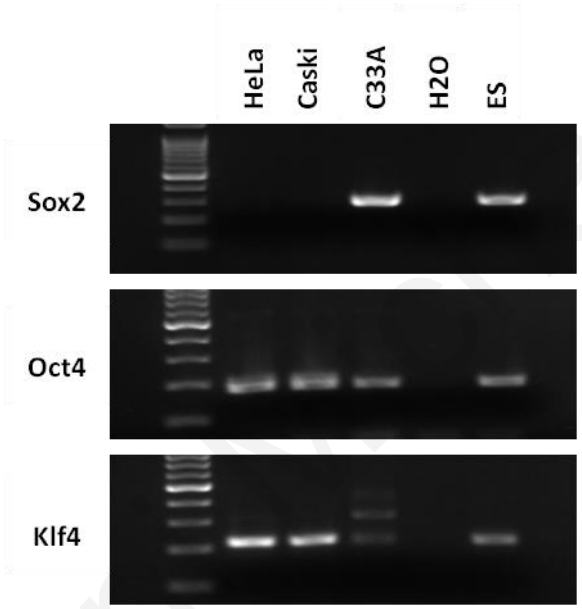

Figure 34: The expression of the reprogramming transcription factors in human cervical carcinoma cell lines.
RNA was collected from the cervical carcinoma cell lines HeLa, Caski and C33A, converted to cDNA and amplified by RT-PCR using Klf4, Oct4 and Sox2 specific primers.

CHAPTER 4

DISCUSSION

DISCUSSION

Cancers have long been hypothesized to contain cancer stem cells, a subpopulation that has the characteristics of self-renewal and multipotency capable of full tumor regeneration. The existence of the two theories for the emergence of cancer stem cells has been a topic of constant debate and investigation: Cancer stem cells have been proposed to derive either from tissue stem cells or de-differentiated committed cells. Matters are further complicated in the case of infectious cancers. Infectious agents, such as bacteria and viruses, have been implicated either in the perturbation of stem cell homeostasis or in the reprogramming of differentiated cells into stem-like cells. In the case of HPV-related cancers, there is no concrete conclusion in support of either theory.

4.1.1 The effect of the HPV16 oncogenes on the stem cells *in vivo*

Tissue stem cells have been implicated as the cells-of-origin in several cancer types. In the case of cervical cancers, they have been proposed to derive from multipotent cervical progenitors either because of differential infection or infection outcome in these types of cells. Despite the sustained interest around this topic very little work addressing the expression of viral oncogenes in tissue stem cells has been done, mostly due to lack of understanding of what constitutes a true cervical multipotent cell and the lack of characterization of this population in the cervix.

We have chosen the quiescent epithelial stem cells found in the hair follicle bulge region to assess the effects of E6 and E7 expression *in vivo* because this stem cell population is well-characterized, and is thought to provide a reservoir of latently infected cells that support the HPV life cycle. In this study, we observed that expression of E6 and E7, the main viral oncogenes of HPV16, can compromise the relative quiescence of epidermal stem cells and lead the hair follicles to precocious anagen entry as seen by the reduction in LRC numbers in both telogen and acute anagen conditions. This is true for both oncogenes and more consistently noted in bitransgenic animals where both oncogenes are co-expressed, as would be the case in the context of a natural infection. This demonstration of disruption specifically of quiescent tissue stem cell homeostasis *in vivo* represents a novel phenotype associated with the expression of these oncogenes.

To verify that a reduction in LRCs is not indicative of a reduction in tissue stem cells overall, we characterized the expression of other stem cell markers. Expression of other

stem cell markers such as K15 is not reduced in the presence of the oncogenes diminishing the possibility that the results seen are the result of reduced stem cell numbers. On the contrary there was an increase in the K15 positive cells in the presence of the oncogenes, which correlates with an expansion in the stem cell compartment of the hair follicle. Another study focusing on the effects of the expression of the HPV16 genes in non-quiescent populations of the hair follicle confirmed our findings (da Silva-Diz et al., 2013). In this study the K14-HPV16 mice, which contained the entire HPV16 early region, are models of the HPV-induced squamous cell carcinoma pathology. Their findings also showed an expansion of the K15 compartment and specifically identified that Lgr5 positive cells from the expanded region, contribute to HPV induced tumor development in the epithelium. The Lgr5 positive cells are the long-lived progeny of the activated bulge cells and are involved in the regeneration of new hair follicles and the maintenance of all cell lineages (Jaks et al., 2008). We also tested the expression of other proposed stem cell markers such as Lgr5 and Lgr6 (Jaks et al., 2008; Snippert et al., 2010), by immunofluorescence but our results were inconclusive largely due to the lack of high-specificity antibodies for these proteins.

In an attempt to explain the reduction in the LRC numbers, the quiescence status of the hair follicle was examined by testing the nuclear localization of the quiescence marker Nfatc1. Inactive Nfatc1 can be found residing in the cytoplasm and when active it translocates to the nucleus where it represses the transcription of its downstream target Cdk4. Cdk4 is a cell cycle regulatory gene required for G1 to S phase progression for example through pRb phosphorylation and removal of its inhibition from E2F transcription (Figure 35). Previous studies have shown that Nfatc1 is preferentially expressed by hair follicle stem cells and coincides with the LRCs following chase experiments. Its expression persists in all phases of the hair cycle maintaining quiescence of the cells even in conditions of growth (Horsley et al., 2008). Our results showed that although the quiescence of the hair follicle is unaffected by the presence of the oncogenes in conditions of hair growth, it can be compromised in resting conditions in the presence of E6 expression. This suggests that the Nfatc1 pathway could be a possible direct or indirect target of the oncogene. The expression of its downstream target Cdk4 increased in both the resting telogen and anagen conditions indicative of an increase in cell cycle entry and thus aberrant proliferation. The lack of exact correlation between the Nfatc1 and Cdk4 results in the two transgenic animals may suggest that E6 and E7 are affecting cellular quiescence via independent ways. For example it is more likely, that at least in resting conditions E7 is affecting factors downstream of Nfatc1.

Since the expression of the oncogenes results in aberrant cell cycle entry in both conditions of telogen and anagen we wanted to see the effect of this on the proliferation status of the hair follicle. For this we monitored how oncogenes affect both hair growth and the expression of the proliferation marker PCNA. The presence of both oncogenes resulted in faster complete hair regrowth and increased numbers of PCNA positive cells results that are consistent with the aberrant cell cycle entry and the increased tissue stem cell activity leading to reduced LRC numbers. PCNA is a general proliferation marker whose expression increases along with severity of HPV neoplasia and progression to cancer (Branca et al., 2007). However, its expression also in normal epithelia inhibited its use as a predictive and/or prognostic marker. Therefore, the fact that the proliferation phenotype is only observed in bitransgenic E6E7 mice could require the additive milder effects of the expression of both oncogenes. Nevertheless it should be noted that during natural infection as well as carcinogenesis, both oncogenes are invariably expressed. Thus the results seen in bitransgenic animals are likely to be reflective of infection and cancer more so than those in animals bearing only one of the two transgenes.

Conclusively, our work shows that the proliferation defects seen *in vivo* apply specifically to the quiescent label retaining cells and can be attributed to the viral oncogenes. The quiescence of the stem cell population acts as a tumor suppressor in squamous tumors and during telogen (quiescent phase) this quiescence is dominant over oncogenic mutations (White et al., 2014). Therefore, the ability of the virus to modulate stem cells even during their quiescence could be crucial to their ability to contribute to carcinogenesis since these types of cells were shown to be the cells of origin in epithelial cancers. The enhanced ability of stem cells expressing HPV oncogenes could improve the ability of those cells to repopulate wounded tissue, a prerequisite for HPV infection, at the expense of non-infected cells.

Furthermore, the aberrant expansion of other markers of stem cells supports the development of an aberrant stem cell compartment, in the presence of HPV oncogenes, capable of enhanced proliferation. This type of aberrant compartment was found to arise from a progeny of activated bulge stem cells that under normal homeostasis are responsible for hair follicle regeneration and maintenance (Jaks et al., 2008). In the presence of the oncogenes this progeny of multipotent cells expands and migrates to the epidermis and contributes to the neoplastic lesions and the epidermal tumors formed (Figure 36).

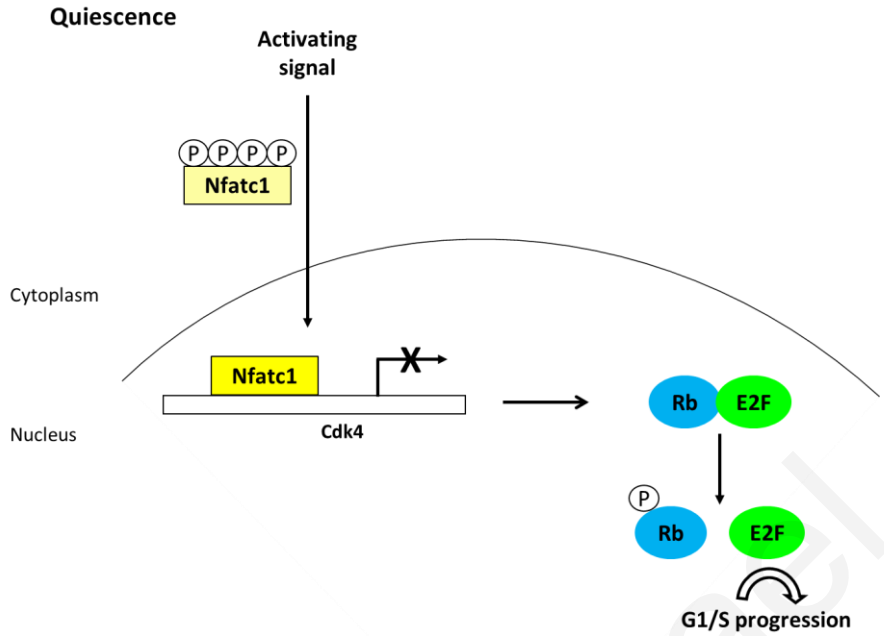

Figure 35: The Nfatc1 and Cdk4 pathway in the hair follicle
Inactive Nfatc1 is located in the cytoplasm and upon activation it translocates to the nucleus. There it inhibits the transcription of its target Cdk4 which is involved in cell cycle regulation. Cdk4 inhibition prevents the release of pRb from E2F, resulting in stalked cell cycle progression.

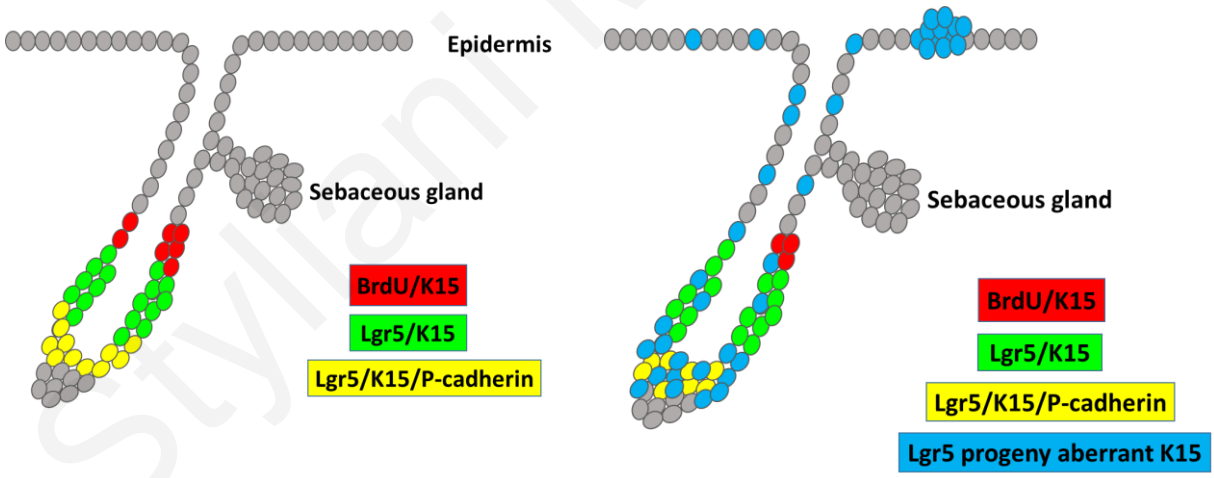

Figure 36: Schematic representation of the effects of HPV16 on the stem cell compartments of the hair follicle.
This diagram represents a summary of the work done by Michael et al. 2013 and da Silva-Diz et al. 2013. Under normal homeostasis (left) quiescent BrdU positive bulge cells (red) can be activated to proliferate and give rise to Lrg5 positive populations (yellow and green) and the K15 layer (green). The expression of the HPV16 oncogenes (right) in the skin epithelium induces aberrant bulge stem cell mobilization and a reduction in their numbers (red). At the same time it induces an expansion of the K15 layer and the creation of an aberrant K15 positive cell population (blue) derived from the Lgr5 cells. This population has the ability to migrate to the epithelium and contribute to the formation of tumors.

4.1.2 The effect of telomerase absence on the changes in the stem cells induced by HPV16 oncogenes *in vivo*

The relevant cellular targets of E6 and E7 that are associated with this phenotype are not known, however, both E6 and E7 have the ability to modulate the function of cellular targets implicated in tissue stem cell biology. These include telomerase, pRb, E2F6 and polycomb group complexes and also histone demethylases. In order to identify a potential molecular target or pathway affected in the presence of the viral oncogenes we decided to study one of the characterized interactions of the oncogenes and that is telomerase.

In the skin stem cells, telomerase is expressed at higher levels than those found in differentiated cells, but it is still not sufficient to prevent telomere shortening as a result of ageing. Terc knockout mice, deficient in one of the components of telomerase, have been previously shown to lack detectable telomerase activity. In the first generation (G1), the mice maintain their telomere length while in the third generation (G3) the mice have short telomeres. These mice can therefore be used to investigate independently the role of telomerase absence and telomere length in the stem cell compartment of the hair follicle. It has been previously shown that the absence of telomerase impairs tissue stem cell mobilization and proliferation, a phenotype more profound when telomeres are short in G3 mice (Flores et al., 2005).

While it is generally acknowledged that the majority of the effects of telomerase are accounted by its ability to elongate telomeres, more controversial evidence points to some telomere-independent effects of telomerase. For example the overexpression of the Tert component of telomerase, induced activation of the stem cells in the hair follicle and entry into anagen, independently of its role in telomere synthesis (Sarin et al., 2005).

For our work the Terc knockout mice were crossed with the K14E6 and K14E7 mice, in order to study how the absence of telomerase affects the phenotypes mediated by the oncogenes on the stem cell compartment. We demonstrated that LRC reduction is a phenotype consistent across genetic backgrounds other than FVB. In the context of telomerase absence, due to Terc absence, we have shown that E6's ability to reduce the LRC numbers remains unaffected. On the other hand, E7's ability to affect the numbers of the LRC population is impaired. In addition, we are currently investigating how the stem cell compartment or the proliferation status of the tissue are affected in Terc sufficient and deficient mice expressing the oncogenes. So far, we see increased numbers in K15 positive cells and thus expansion of the stem cell compartment in either the presence or absence of

the Terc component, consistent with our previous finding. For the proliferation status of the tissue we checked the proliferative basal layer of the epidermis and so far we found that proliferation increases by the expression of the oncogenes when telomerase is present. In the absence of telomerase the increase in proliferation by E6 remains unaffected however, E7's proliferative ability is reduced which could be linked to the observation seen in the LRCs.

There are no previous studies in which the effect of the oncogenes on the Terc component of telomerase is investigated. Most of the research performed focused on the relationship between the oncogenes and the Tert component of telomerase. For example, it has been shown that E6 induces telomerase activity by activating the Tert component of telomerase. This was done either by direct binding to its promoter or indirectly by interacting with its activator or repressor proteins. The E7 oncogene on the other hand, does not bind or activate Tert transcription *in vivo*, however it augments the E6-induced Tert promoter activity (Liu et al., 2008b).

The studies on Tert, revealed new roles in the cell independent of its ability to elongate telomeres. For example, Tert has been proposed to play a role in cell survival and inhibition of apoptosis in cancer (Cao et al., 2002) and induction of stemness in glioma cells (Beck et al., 2011). Terc on the other hand, has not been implicated in having extra-telomeric effects in mice. Therefore, using our model we can only make conclusions regarding the effect of telomerase activity on the phenotypes involving the ability of the telomerase holoenzyme to elongate telomeres.

Conclusively, our results so far indicate that telomerase, although a direct target of the E6 with roles in cell immortalization and carcinogenesis, is dispensable for the E6-induced aberrant mobilization of the bulge stem cells. On the other hand, the absence of telomerase activity or of the Terc component itself, or the defective response to telomere attrition, may play a role in the ability of E7 to induce stem cell mobilization by a mechanism still unknown.

In the future it will be worth examining the role of telomere length on the effects of the oncogenes and whether short telomeres are protective against HPV-induced tumorigenesis. This will be important as the defect on the stem cell compartment was more profound when telomeres were short. Not only that but it was also shown that in the absence of telomerase and presence of short telomeres, the abrogation of the protective p53, restored stem cell mobilization induced in response to mitogenic stimuli (Flores and Blasco, 2009). To examine if the oncogenes can overcome the defect of the short telomeres and whether this is aided by their ability to affect proteins such as p53, G3 mice will be crossed with

K14E6 and K14E7 mice. Finally, to investigate if the effects of E6 and E7 on the LRC population are through the Tert component of telomerase using its extra-telomeric roles, Tert deficient mice can be used.

4.2.1 The effect of the HPV16 oncogenes on pluripotency *in vitro*

The second theory in the origin of cancer stem cells is that differentiated cells can in some cases be the target of infectious agents, which interfere with their differentiation status and their plasticity. Infection has been previously shown to profoundly impact the differentiation status of the tissue in ways that affect infection outcome (Masaki et al., 2013).

The impact of infectious agents on the differentiation status of the tissue, is not exclusive to carcinogenesis, but plays a role during their dissemination and protection. For example, the leprosy bacterium infects preferentially Schwann cells of the nervous system and induces their reprogramming into stem-like cells. The infected stem-like cells will then migrate to the mesenchyme where they re-differentiate to mesenchyme tissue allowing for expansion of the infection (Masaki et al., 2013). For efficient dissemination, the cells need to evade the host immunity and they do so by inducing an inflammatory response achieved through the release of factors from the stem-like cells. This subsequently recruits macrophages that form granulomas able to bypass immunity and migrate. Inflammation is also observed in the gut where the microbiota release compounds that stimulate the expression of an innate immune sensor, Nod2. The constitutive expression of Nod2, in intestinal stem cells, provides protection against stress (Nigro et al., 2014). Therefore immunity and inflammation, in some cases of infection, allows for the dissemination as well as survival of the infectious agent in the host tissue.

The well-established roles of inflammation in a tissue were the elimination of host pathogens and the repair of the tissue. In a recent review, the role of inflammation in tissue regeneration has also been discussed (Karin and Clevers, 2016). Inflammation is proposed to activate not only stem cells but also differentiated cells to proliferate, migrate and regenerate the tissue. The differentiated cells in response to inflammation will de-differentiate acquiring stem-like characteristics. In support of this idea, is the fact that the induction of immunity was found to be required for efficient nuclear reprogramming (Lee et al., 2012). In the case of HPV16, the virus not only infects through a wound, which induces an immune response, but also establishes long-term inflammation along with its

persistent infection. Therefore, it was hypothesized that HPV16 is able to perturb the normal differentiation status of the cells and induce their de-differentiation.

For this part of our work, we chose the reprogramming assay of Yamanaka in which differentiated cells are converted to induced pluripotent stem cells (iPS), as a model to study how the oncogene expression interferes with the reversed differentiation process that the cells undergo. We have observed that the presence of the oncogenes increases the number of iPS colonies formed. This observation suggests that either the oncogenes increase the proliferation potential of the cells and thus the rate at which reprogramming occurs or that they have a direct involvement in the process.

Reprogramming of unsorted MEFs in the absence of one of the transcription factors has been previously shown to disrupt their normal homeostasis. Subsequently, it induces a transient state with some expression of stem cell markers, but it fails to fully reprogram cells into iPS (Nemajerova et al., 2012). Therefore, we decided to attempt reprogramming in the absence of Oct4 transcription factor as an experiment to investigate the direct involvement of the oncogenes in the process. A recent paper using the viral E1A protein, demonstrated replacement of the Sox2 factor and successful two-factor iPS cell formation (Marthaler et al., 2016). We have observed that in the absence of Oct4, the presence of both oncogenes can reprogram differentiated cells and form colonies that resemble the morphology of the iPS colonies. However, the efficiency at which the reprogramming of the two factors occurs, is lower compared to the three factors. It was previously shown that E7 binds to and activates the promoter of Oct4 (Brehm et al., 1999), a possible mechanism through which the oncogenes reprogram differentiated cells even if exogenous Oct4 is emitted from the process.

In an attempt to check whether this can be attributed to a change in the gene expression levels in the cells we tested the transcription factors Sox2, Oct4 or Klf4. The expression was tested by RT-PCR in either MEFs that express the oncogenes or in MEFs undergoing reprogramming with two or three transcription factors. Our initial results include days 0 and 4 post-infection where no significant changes in the presence of the oncogenes were detected. In agreement with previous studies, the silencing of the exogenous retroviral factors occurs as early as 4 days after their expression (Yamanaka and Blau, 2010) while changes in the endogenous expression of the transcription factors seems to be a later event in the process when cells have successfully reprogrammed.

The inability to detect clear and consistent changes in the expression levels of the cells could be explained by the heterogeneity of the MEF population. This is attributed first of all to the fact that MEFs are isolated from embryonic tissue that shows both phenotypic

and developmental heterogeneity (Singhal et al., 2016). It was previously shown that sorting of cells based on their surface markers can result in the isolation of a population that is more prone to two factor differentiation (Nemajerova et al., 2012). Heterogeneity is also created during the infection of the cells with the transcription factors as not all MEFs manage to receive all three factors. In addition, since reprogramming is considered to be a stochastic event it only occurs in a small fraction of the cells. In the future we could perform experiments using a single vector, which encodes all reprogramming factors, in cells that allow selection of eg. Oct4-GFP. This will allow us to select a more homogeneous pool of cells where a larger subset of the cells are on their way to reprogramming thus obtaining more definitive mechanistic answers. Furthermore we will be able to perform more representative time-course experiments assessing the transcriptional changes at stages of reprogramming later than day 4.

The expression of the transcription factors was also tested in the HPV positive cervical cancer cell lines HeLa and Caski and in the non-HPV cervical cancer cell line C33A. The Oct4 marker of pluripotency, although not normally expressed in somatic cells is found overexpressed in cancers. The same applies to Klf4, which although not expressed in differentiated cells, it is expressed in cancer cells acting as an oncogene. These observations are in agreement with our results where both Oct4 and Klf4 are expressed in the cervical cancer cell lines in which the presence of HPV enhances this effect. On the other hand, Sox2's expression was only apparent in the non-HPV cervical cell line. Sox2, a transcription factor required for the reprogramming process, is not found expressed in normal epithelium. Its overexpression in a number of cancers such as skin, head and neck and cervical cancer, was observed in tumor initiating cells correlating with initiation and growth of the tumor and premalignant regions (Siegle et al., 2014; Schröck et al., 2014). However, this Sox2 amplification was not seen in HPV induced head and neck cancers or in cervical cancers with poor prognosis, a result that supports our finding (Schröck et al., 2014; Kim et al., 2015).

Conclusively, our *in vitro* work shows that the expression of the viral oncogenes in differentiated cells can enhance their reprogramming to stem-like cells and suggests for their direct contribution in this process. While there are several reports of viral oncogenes directly or indirectly upregulating pluripotency-related genes (Iacovides et al., 2013), our finding provides the first direct evidence that contribution to functional reprogramming can be induced by an oncogenic virus. The mechanisms however, by which the viral contribution takes place, have not been elucidated yet. The reprogramming of somatic cells can be observed at different stages: epigenetically by altering the expression or activity of

histone modifiers or transcriptionally by binding directly to the promoter sequences of cells altering their expression. In the case of the HPV16 oncogenes, the reported epigenetic and transcriptional changes that the infected cells undergo, could explain this induction of pluripotency by the virus.

Alkaline phosphatase expression correlates with undifferentiated cells but it is not a true marker for pluripotency (Štefková et al., 2015). Therefore in future experiments, the functional capacity of the iPS colonies formed in the two factor experiment need be examined. Their stem-cell functionality could be investigated by checking their ability to generate functional differentiated cells or by their ability to form teratomas when injected in nude mice. In addition, the ability of the oncogenes to reprogram keratinocytes, the cells naturally infected by the virus, could provide evidence for the changes in the expression levels induced by the oncogenes during the process. For elucidating the role of the known interactions of the oncogenes in reprogramming, E6 and E7 plasmids defective in their ability to bind p53 and pRb respectively, will be used.

Furthermore, characterization of cells reprogrammed by including E6 or E7 may give insights related to the potential carcinogenicity of these cells. The overexpression or downregulation of known targets of the oncogenes, crucial to their oncogenic potential, could be investigated. These could provide information as to the changes induced by the oncogenes in the infected cells *in vivo*.

SYNOPSIS

Taking everything into account, we can conclude that the virus has the potential to manipulate either stem cells or non-stem cells in the tissue that it infects. Whichever type of cell it is infected, the frequency with which this occurs or whether is of importance to the viral life cycle or carcinogenesis, it is still not known.

HPV-oncogene expressing cancers in mouse skin carcinogenesis models haven't been reported yet to be generated from basal keratinocytes, but are rather generated from cells in the hair follicle that are closer to a stem-like character. This does not exclude the possibility that in human cancers both types of cells (stem and non-stem) can contribute to the formation of tumor initiating cells. It is likely that the virus requires a stem-cell character to ensure latent and persistent infection in the tissue. This ability of the virus may increase the tissue's genetic instability and allows acquisition of changes required for the promotion of malignancy. The cells with the stem-cell character will constitute the cancer stem cell population responsible for the growth and regeneration of the tumor. Our improved understanding of how viral oncogenes contribute to stem cell identity in cancers is critical. Both the targeting of the HPV oncogenes, as well as the targeting of cancer stem cells are thought to be the key to improved therapeutics. (Gu et al., 2011).

CHAPTER 5

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The HPV16 oncogenes cause aberrant stem cell mobilization

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ABSTRACT

Human Papilloma Virus related epithelial cancers have been speculated to derive from virus-infected tissue stem cells. Stem cells also are thought to provide a reservoir of latently infected cells that can persist for long periods. In this study we have examined the effects of HPV16 E6 and E7 oncogenes on multipotent epithelial stem cells, using *in vivo* systems. Our results show that expression of HPV16 oncogenes reduces the number of bulge label-retaining cells within hair follicles at telogen suggesting aberrant mobilization, a result supported by increased mobilization upon acute anagen induction. Importantly the loss of relative quiescence, a hallmark feature of stem cells, occurs in the absence of a reduction in other stem cell markers. This points to an atypical stem cell compartment in the context of E6 and E7 expression. We hypothesize that this aberrant compartment may have important roles in the viral life cycle and/or ensuing carcinogenesis.

Introduction

A growing body of literature supports the notion that infectious agents perturb stem cell homeostasis in target tissues (Pitsouli et al., 2009). For pathogens that complete their life cycle inside the cell, such as viruses, changes in tissue stem cell homeostasis could have profound effects on infection outcome. This host–pathogen interaction is particularly interesting in cases where infection is associated with carcinogenesis, where aberrant stem cell homeostasis could be related to ensuing carcinogenesis. Cervical carcinomas have long been hypothesized to arise from cervical stem cells, but there is a scarcity of publications addressing the interplay of the viral oncogenes with tissue stem cells.

Human papillomaviral infections specifically arise in stratified squamous epithelia such as those lining the skin, the anogenital tract and oral epithelia and, in the context of high risk HPV infections, leads to the development of a number of cancers including cervical, anal, as well as head and neck cancers. The two most important papillomaviral oncogenes, E6 and E7, not only are necessary for the viral life cycle but also for the development as well as the persistence of HPV-associated cancers (Goodwin and DiMaio, 2000; Jabbar et al., 2009). Papilloma virus infections are thought to arise through infection of cells within the basal layer of stratified squamous epithelia, which is the layer of cells that makes direct contact with the basement membrane and constitutes the proliferative compartment of these epithelia (Kines et al., 2009). A limited body of evidence supports the notion that papillomaviruses infect cells with stem-like characteristics (Schmitt et al., 1996) but the consequences of viral oncogene expression in tissue stem cells *in vivo* are largely unknown. Furthermore, it has been proposed that tissue stem cells are the sites of persistent infection by the virus (Maglennon et al., 2011). In the cervix, the most common site of HPV16 infection, cancers are thought to arise from the transition zone between glandular and squamous epithelia where stem-like cells are thought to reside. It is therefore evident that stem cells are a potential direct target of infectious agents and, in accordance with studies indicating that some cancers derive from tissue stem cells, this may have an important role in carcinogenesis. However, it is still not clear whether HPV, or other similar cancer causing viruses, directly affects the development, function, or plasticity of tissue stem cells.

A clear challenge in assessing the consequences of viral oncogene expression in cervical tissue stem cells is the lack of well-characterized markers that would enable their successful detection as well as functional assays for tissue stem cells. An alternative model, which has been extensively used to study HPV infection and biology, is the skin (Auewarakul et al., 1994; Lambert et al., 1993; Merrick et al., 1992). The stem cell compartments of the mouse skin have been extensively characterized and various populations with "stemness characteristics" have now been described. The best understood stem cell population lies in the skin hair follicle, in a niche dubbed the "bulge". This is a population that, like many other tissue stem cells, is relatively quiescent and the identification of its slow-cycling nature and label retaining ability dates back to 1990 (Cotsarelis et al., 1990). The nucleotide pulse-chase technique has been extensively used for the detection of the bulge population and led to its identification as a stem cell population and its contribution not only to hair follicle regeneration *in vivo*, but also to interfollicular epidermal skin healing after wounding (*i.e.* this is a multipotent tissue stem

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cell compartment) (Ito et al., 2005; Morris et al., 2004; Tumbar et al., 2004; Zhang et al., 2009). In addition to the pulse-chase technique, a marker used to genetically target bulge cells for lineage-tracing analysis is cytokeratin 15 (K15), which allows for labeling of the bulge and secondary hair germ regions (Liu et al., 2003; Lyle et al., 1998; Morris et al., 2004).

Contributing to the choice of using the skin tissue as a model is also the fact that it undergoes homeostasis resulting in the highly ordered process of hair follicle growth, which consists of cycles of growth and regression. In these cycles, a subset of bulge stem cells have been shown to mobilize out of their niche during the growth phase, known as anagen or stem cell mobilization, migrate to lower regions of the hair bulb where they proliferate and differentiate contributing to the formation of new hair (Blanpain and Fuchs, 2006). This stem cell model has been widely used to determine the implication of stem cells in carcinogenesis and it should be noted that several lines of evidence implicate the bulge stem cells specifically, as the cells of origin in squamous cell carcinomas (Lapouge et al., 2011).

To elucidate the role of viral oncogenes in modulating the behavior of quiescent tissue stem cells, we used transgenic animals expressing the viral oncogenes E6 and E7 of HPV16 in stratified squamous epithelia. Focusing specifically in the hair follicle bulge stem cells we describe here that, while the overall numbers of bulge stem cells do not appear to be reduced upon E6 and E7 expression, their ability to remain quiescent and thus retain label, is compromised, and their ability to proliferate upon anagenic stimuli is enhanced. A potential modulation of tissue stem cell mobilization may have important implications on infection outcome, including eventual carcinogenesis.

Results

Expression of the HPV16 oncogenes reduces the number of relatively quiescent cells detected at telogen

K14E6 and K14E7 mice have been previously generated and extensively characterized (Herber et al., 1996; Song et al., 1999). The keratin 14 promoter directs expression of the HPV16 oncogenes to the basal layer of stratified epithelia, including the bulge niche (Arbeit et al., 1994). In order to assess the effects of E6 and E7 expression in quiescent bulge stem cells, BrdU pulse-chase assays were performed as previously described (Cotsarelis et al., 1990; Bickenbach et al., 1986; Morris and Potten, 1999), and the numbers of label-retaining cells (LRCs) at second telogen (resting phase of hair cycle) were compared in wild type animals and animals transgenic for either one or both of the oncogenes (Fig. 1A). The numbers of LRCs were significantly reduced in mice expressing either one of the two, or both viral oncogenes suggesting either an overall reduction in the numbers of this particular stem cell type or enhanced proliferation, which could lead to a more rapid label loss.

Stem cells expressing the HPV16 oncogenes have increased mobilization upon acute anagen induction

In order to further investigate the proliferation potential of stem cells expressing viral oncogenes, anagen was acutely induced. A pulse-chase protocol was performed as above and anagen was induced by repeated TPA administration prior to harvesting. Successful anagen induction was validated by the characteristic hair follicle elongation in all genotypes examined (Fig. 2A and B). Hair elongation was also observed in follicles expressing the HPV16 oncogenes under resting conditions. LRC mobilization was monitored as a function of BrdU label loss by means of BrdU-specific immunohistochemistry. In animals expressing E6, E7 or both oncogenes mobilization of stem cells was more pronounced compared to that seen in wild type animals (Fig. 2C) suggesting that HPV oncogene expression can lead to a higher level of basal proliferation, or even precocious anagen even in the absence of external anagenic stimuli. This result suggests that tissue stem cells expressing E6 or E7 are more poised to proliferate, explaining in part the reduced numbers of LRCs detected in telogen conditions (Fig. 1).

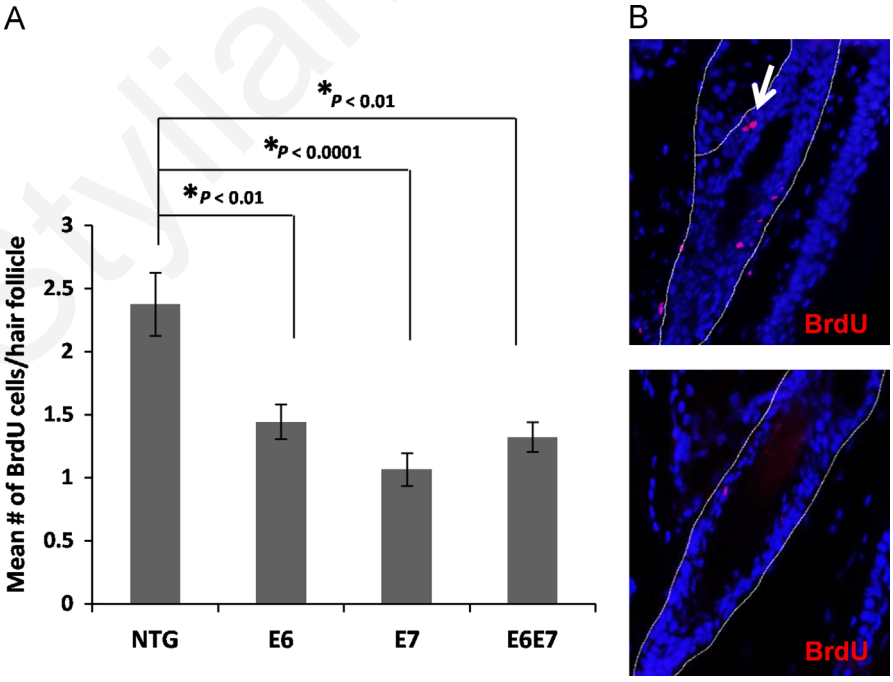

Fig. 1. Expression of the HPV16 oncogenes leads to reduced detection of LRCs in hair follicle bulge at telogen. (A) LRCs were labeled using a BrdU pulse administered shortly after birth and chased until second telogen. ~50 Hair follicles were selected from at least 3 mice of each genotype, NTG, E6, E7 and E6E7 mice. The mean number of BrdUrd positive cells per hair follicle bulge was quantified and plotted for each genotype (columns); bars, SD. All statistical comparisons were performed using a two-sided Wilcoxon rank sum test. **(B)** Representative immunofluorescent figures of hair follicles showing BrdU positive cells (red–white arrow). Counterstaining was done with DAPI (blue).

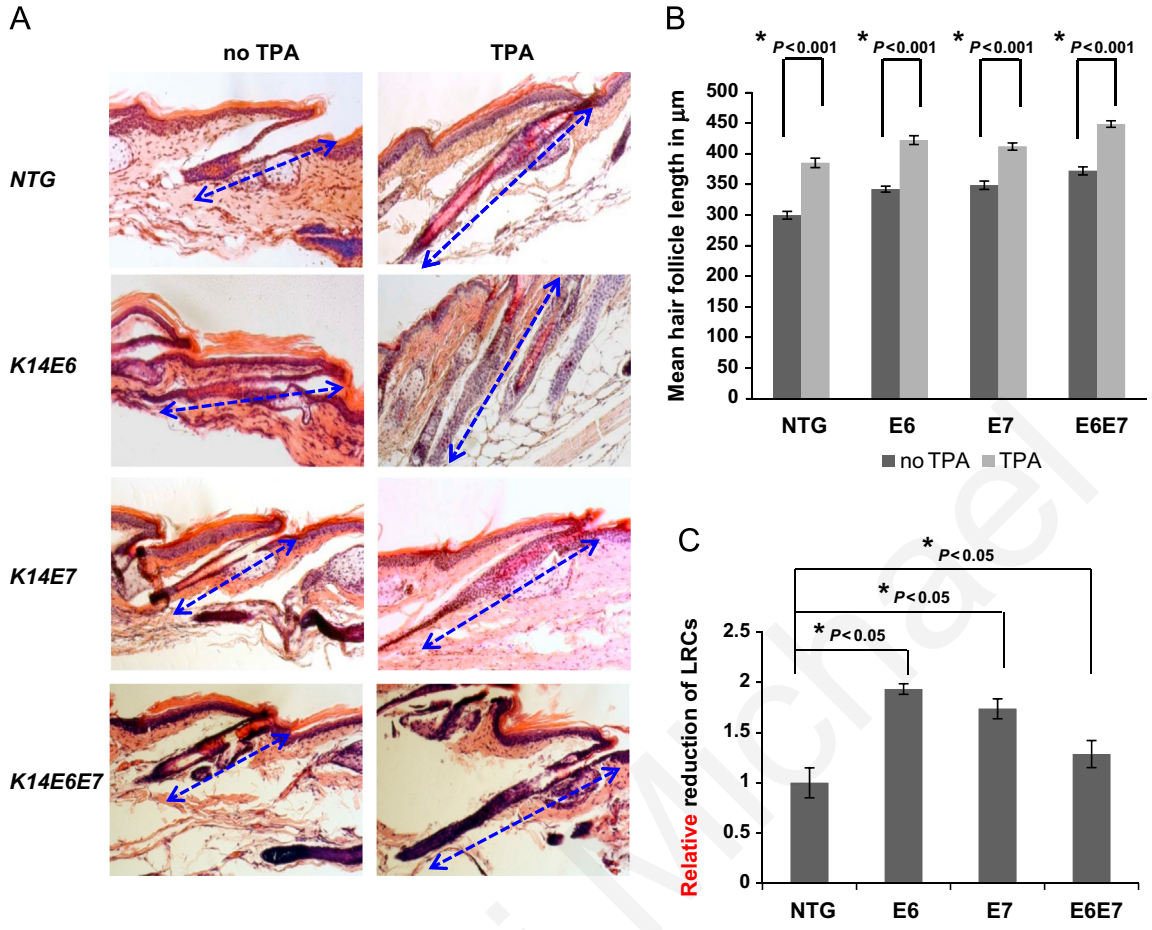

Fig. 2. Expression of the HPV16 oncogenes leads to more rapid mobilization of LRCs in response to acute anagen induction. (A) In order to induce anagen in mice where LRCs were labeled, TPA was applied on mice every 48 h for four times. H&E staining was performed on all tail hair follicles. Hair follicle length was quantified to verify effective anagen induction. **(B)** ~70 Hair follicles were selected from at least 3 mice of each genotype, NTG, E6, E7 and E6E7 mice. The mean hair follicle length in μm was measured and plotted for each genotype (columns); bars, SD. All statistical comparisons were performed using a two-sided Wilcoxon rank sum test. Statistical significance was also observed between NTG and transgenic mice under resting (no TPA) conditions. **(C)** In order to track the mobilization of LRCs in response to acute anagen induction, the percentage reduction of LRCs was tracked per genotype. At least 3 mice of each genotype at anagen (TPA) and telogen (no TPA), NTG, E6, E7 and E6E7 mice, were selected and hair follicle bulge regions were quantified. The relative reduction of BrdUrd positive cells per hair follicle bulge was plotted for each genotype (columns); bars, SD. All statistical comparisons were performed using a two-sided Wilcoxon rank sum test. Statistical significance was also observed between E6 and E6E7 mice.

Combined expression of the HPV16 E6 and E7 oncogenes gives a robust proliferative ability in hair follicles

To investigate LRC mobilization outcome, hair re-growth was monitored. The backs of transgenic and control mice were shaved at day 65, previously characterized to correspond to the end of second telogen. Hair re-growth was monitored for up to 8 days post-shaving and was enhanced in mice expressing both E6 and E7 (Fig. 3A). Complete hair re-growth, consistent with increased tissue stem cell activity in those animals was only evident in mice bitransgenic for both E6 and E7. Also consistent with that result a greater increase in PCNA staining in anagen hair follicles from the bitransgenic animals was seen (Fig. 3B and C).

HPV16 oncogene expression does not lead to an overall reduction of other bulge stem cell markers

In order to eliminate the possibility that reduced LRC numbers (Fig. 1) are indicative of aberrant reduction in numbers of stem cells, endogenous markers of bulge stem cells, such as the expression of K15 was tested (Liu et al., 2003). The results showed an increase in the numbers of K15 positive cells and thus expansion of the K15 layer in both conditions of telogen and anagen (Fig. 4A and B). Thus, the reduction in LRC numbers is not consistent with an overall decrease in stem cell markers. On the contrary, an aberrant expansion in the K15 compartment is detected, suggesting that an increased ability to proliferate might not be the only change induced in stem cells by E6 and E7 expression.

The Nfatc1 pathway is perturbed upon viral oncogene expression

In order to test whether the quiescence of bulge stem cells is affected by oncogene expression, the nuclear localization of the Nuclear factor of activated T-cells (Nfatc1), shown to be the gate-keeper of bulge stem cell quiescence, was tested in both telogen and anagen conditions (Horsley et al., 2008). No significant changes were observed in nuclear localization in conditions of follicle growth when the oncogenes were expressed (Fig. 5B). However, under resting conditions the expression of E6 or both E6 and E7 led to a significant reduction in the numbers of stem cells with nuclear Nfatc1 (expression of E7 alone did not have the same result, in fact it led to a significant increase of nuclear Nfatc1) (Fig. 5A). Furthermore, the expression of Cdk4, a downstream target of Nfatc1 and also a cell cycle gene implicated in the Rb pathway was tested showing an increase in both telogen and anagen conditions when the oncogenes are expressed (Fig. 5C and D).

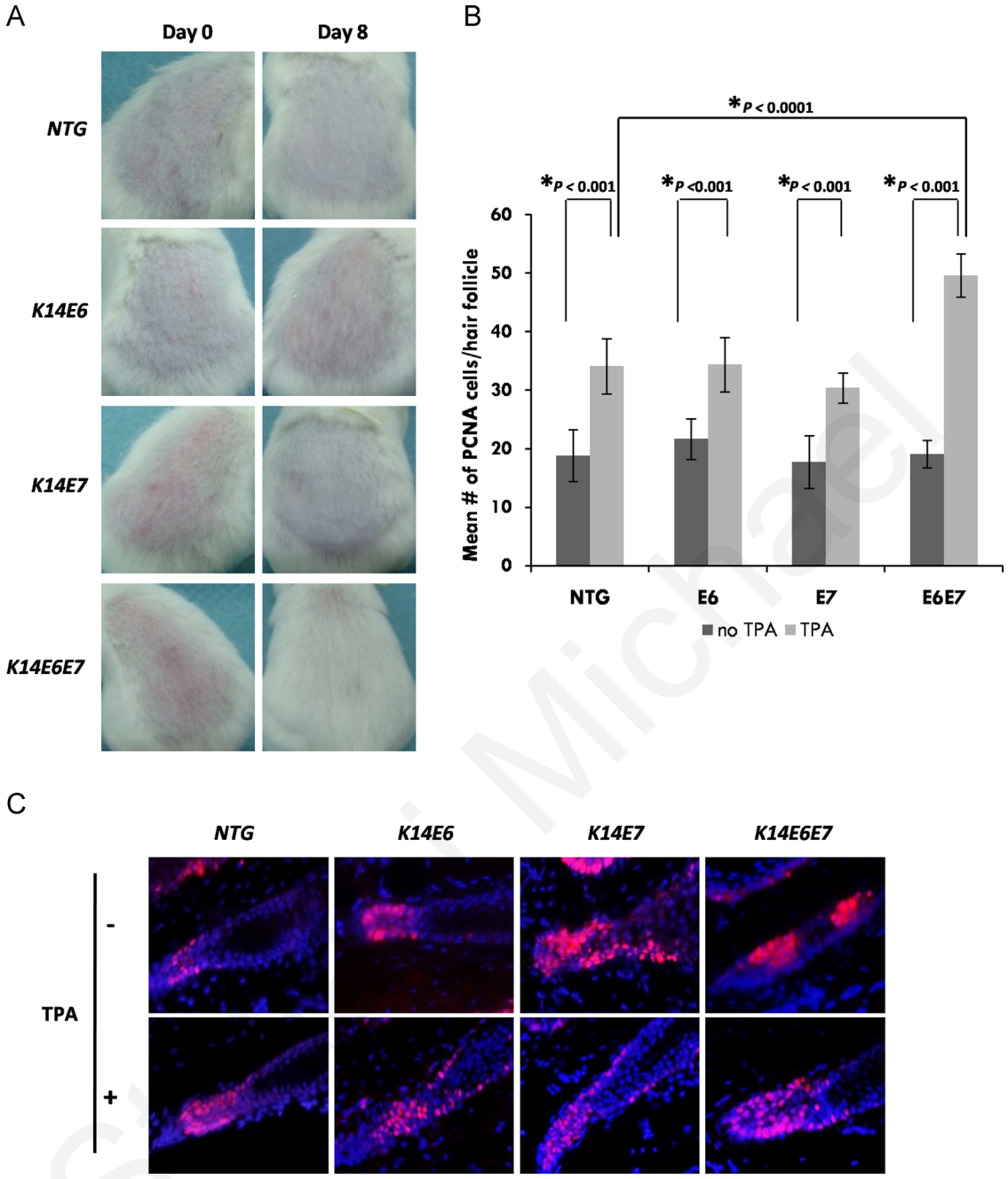

Fig. 3. Increased mobilization of LRCs correlates with increased hair growth in mice expressing the HPV16 E6 and E7 oncogenes. (A) Backs of telogen mice of all genotypes were shaved and pictures were taken at days 0 and 8 after shaving. **(B)** ~50 Hair follicles were selected from at least 3 mice of each genotype, NTG, E6, E7 and E6E7 mice. The mean number of PCNA positive cells at the base of each hair follicle was quantified and plotted for each genotype (columns); bars, SD. All statistical comparisons were performed using a two-sided Wilcoxon rank sum test. **(C)** Representative immunofluorescent figures of hair follicles showing PCNA positive cells (red). Counterstaining was done with DAPI (blue).

Discussion

Tissue stem cells have been implicated as the cells of origin in several cancer types. In the case of cervical cancers, they have been proposed to derive from multipotent cervical progenitors because tissue stem cells provide a reservoir of latently infected cells that support the viral life cycle (Maglennon et al., 2011), or because of differential infection or infection outcome in these types of cells. Despite the sustained interest around this topic *i.e.* the cell of origin in cancers, very little work addressing the expression of viral oncogenes in tissue stem cells has been done, mostly due to lack of understanding of what constitutes a true cervical multipotent cell, even though the prevailing dogma suggests that these reside in the so-called transformation zone. We have chosen the quiescent epithelial stem cells found in the hair follicle bulge region to assess the effects of E6 and E7 expression *in vivo* because this stem cell population is well-characterized. We propose that the expression of viral oncogenes in tissue stem cells promotes aberrant mobilization consistent with an improved ability of these cells to repopulate the tissue and possible roles in cancer initiation.

Two recent publications propose an alternate hypothesis to tissue stem cells as the cells of origin in cancers. These authors suggest that specific cell subpopulations of embryonic origin are

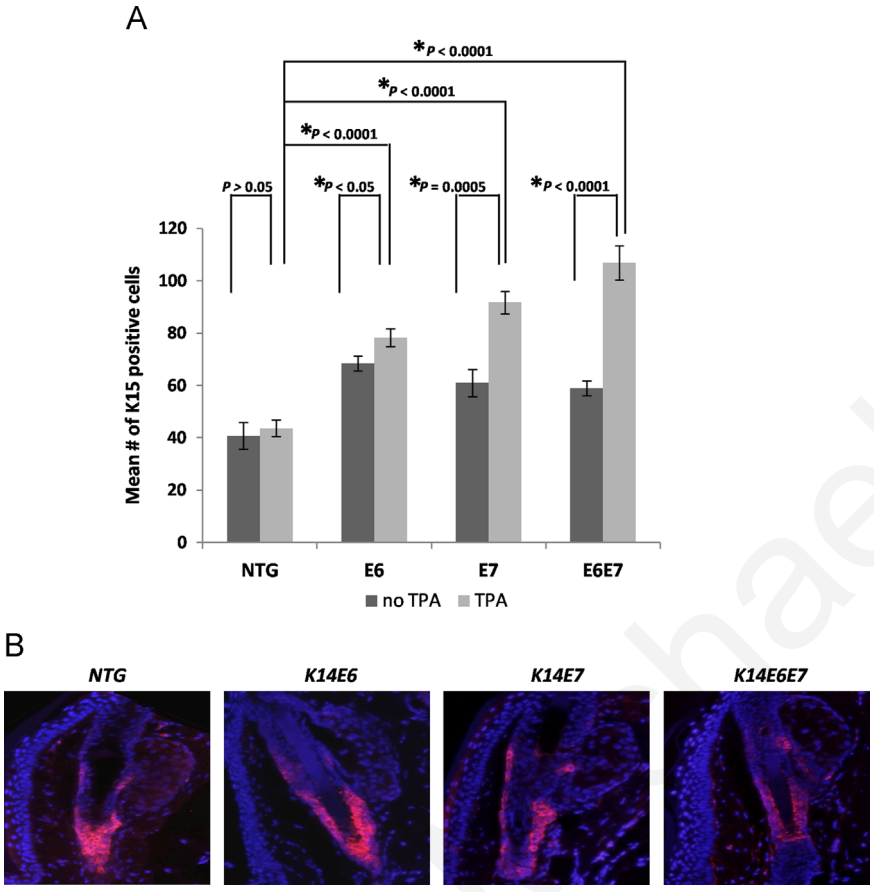

Fig. 4. Other markers of bulge stem cells are not reduced in response to HPV16 oncogene expression. (A) Immunofluorescence was performed using a K15-specific antibody. ~50 Hair follicles were selected from at least 3 mice of each genotype, NTG, E6, E7 and E6E7 mice. The mean number of K15 positive cells of each hair follicle was quantified and plotted for each genotype (columns); bars, SD. All statistical comparisons were performed using a two-sided Wilcoxon rank sum test. Statistical significance was also observed between the NTG and the transgenic mice (no TPA) as well as between the various transgenics (TPA-treated). **(B)** Representative immunofluorescence of K15 staining (red) in the hair follicles of the genotypes examined. Counterstaining was done with DAPI (blue).

the precursors of some premalignant, and consequently malignant lesions including some cervical malignancies (Herfs et al., 2012; Wang et al., 2011). This is due, at least in part, to the ability of these cells to outcompete neighboring populations in situations of tissue insult such as HPV infection. Of course, the proposed mechanism, and the traditionally held view that at least some cancers may be derived from the transformation zone are not mutually exclusive. The exact role of these squamocolumnar populations during normal tissue homeostasis is not known, nor is it known whether this anatomic location harbors cells with stem-like characteristics. In either case, our results support the notion that specific cell subpopulations which express the HPV16 oncogenes are more poised to repopulate the tissue, consistent with the cell-competition theory proposed.

In this study, we observed that expression of E6 and E7, the main viral oncogenes of HPV16, can compromise the relative quiescence of epidermal stem cells and lead hair follicles to precocious anagen entry as seen by the reduction in LRC numbers in both telogen and acute anagen conditions (Figs. 1 and 2C). This demonstration of disruption specifically of quiescent tissue stem cell homeostasis *in vivo* represents a novel phenotype associated with the expression of these oncogenes. Another recent study focusing on the effects of all HPV16 genes in non-quiescent populations of the hair follicle confirmed our findings (da Silva-Diz et al., in press). While the reduction in LRCs is a direct measure of proliferation in relatively quiescent populations which tracks the loss of label, staining for PCNA correlates to a great extent but not fully. The reason for this is that PCNA staining reflects the cumulative proliferation in the hair follicle including that derived from other populations.

To further verify that a reduction in LRCs is not indicative of a reduction in tissue stem cells overall, we characterized the expression of other stem cell markers. Expression of other stem cell markers such as K15 is not reduced in the presence of the oncogenes diminishing the possibility that the results seen are the result of reduced stem cell numbers (Fig. 4A). In contrast, there is an expansion of the K15 layer in both telogen and anagen conditions which might indicate the expansion of an aberrant population which shares some but not all stem cell characteristics. The expression of other proposed stem cell markers such as Lgr5 and Lgr6 (Jaks et al., 2008; Snippert et al., 2010), were tested by immunofluorescence but results were inconclusive.

In an attempt to explain the reduction in the LRC numbers, the nuclear localization of the quiescence marker Nfatc1 was also tested (Fig. 5). The results showed that the quiescence of the follicle cells can be compromised in resting conditions when E6 is expressed suggesting that the Nfatc1 pathway could be a possible direct or indirect target of the oncogene. Surprisingly, the number of Nfatc1 positive cells in hair follicles expressing E7 only was higher, despite all other evidence contrary to quiescence. Furthermore, in some cases (Fig. 5B and D) we observe an uncoupling of the expected relationship between the detection of nuclear Nfatc1 and its downstream target CDK4. This leads us to the conclusion that while under normal conditions anagen induction is marked by loss of nuclear Nfatc1, forced anagen induction need not occur by direct action on Nfatc1 but may in fact be marked by

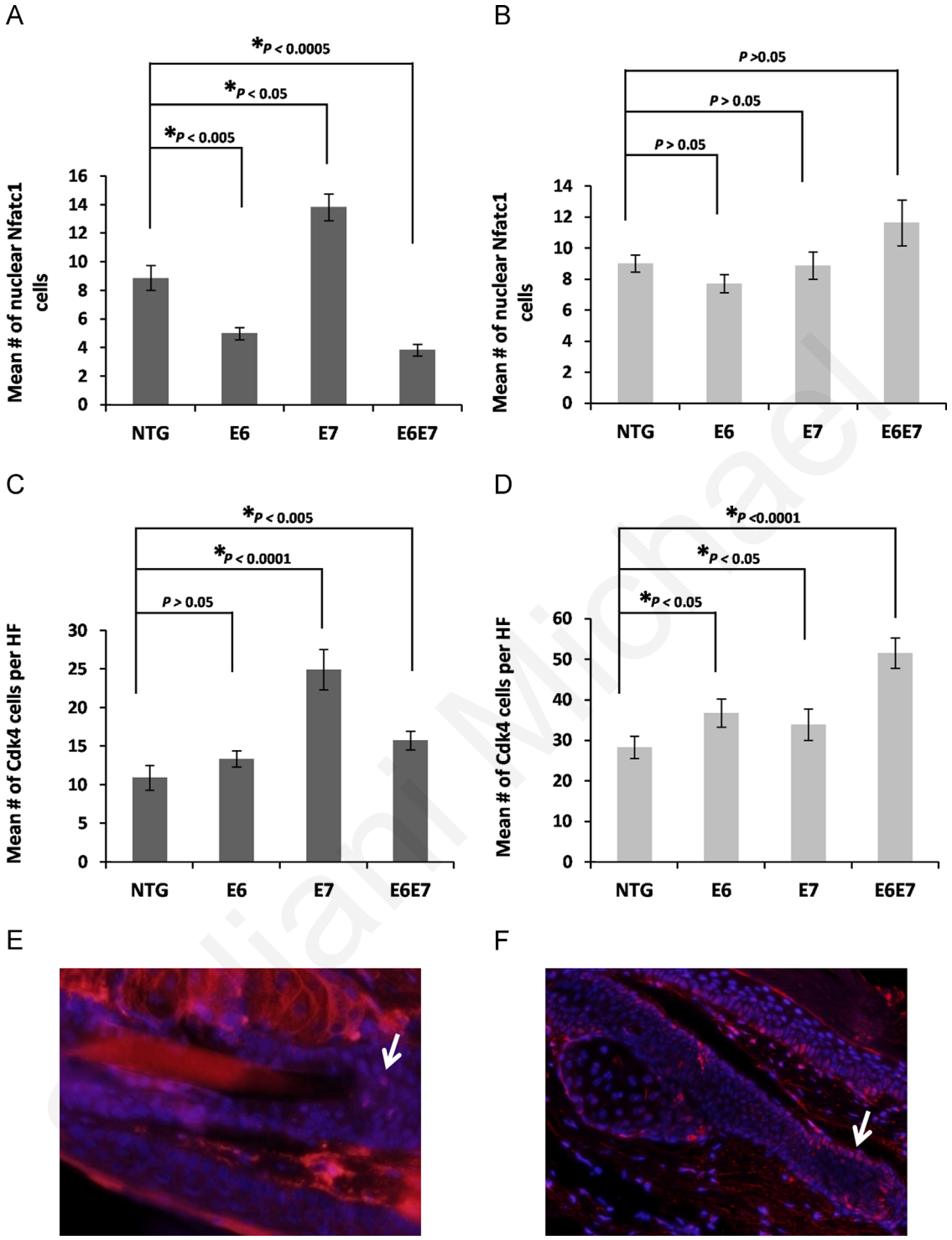

Fig. 5. The quiescence of the bulge stem cell population is affected upstream as well as downstream of the Nfatc1 pathway by HPV16 oncogene expression. (A–D) ~50 Hair follicles were selected from at least 3 mice of each genotype, NTG, E6, E7 and E6E7 mice. The mean number of Nfatc1 **(A,B)** and Cdk4 **(C,D)** positive cells of each hair follicle in telogen (no TPA) **(A,C)** and anagen (TPA) **(B,D)** conditions was quantified and plotted for each genotype (columns); bars, SD. All statistical comparisons were performed using a two-sided Wilcoxon rank sum test. **(E,F)** Representative immunofluorescense staining of (E) nuclear Nfatc1 and (F) Cdk4 positive cells is depicted by arrows. Counterstaining was done with DAPI (blue).

perturbation of the pathway downstream of it. In those cases, CDK4 is actually a more reliable marker of the balance between proliferation and quiescence. The expression of Cdk4, a downstream target of Nfatc1 and also a cell cycle regulatory gene involved in Rb homeostasis, increased both in the resting telogen and anagen conditions when E7 or both oncogenes are expressed (when E6 only is expressed the increase is statistically significant only in anagenic conditions) (Fig. 5C and D). The lack of exact correlation between the Nfatc1 and Cdk4 results in the E6 and E7 transgenic animals may suggest that E6 and E7 are affecting cellular quiescence in independent ways. For example it is more likely, that at least in resting conditions E7 is affecting factors

downstream of Nfatc1 including Cdk4 which could be targeted directly (Fig. 5A and C). The increase in Nfatc1 positive cells in E7-expressing hair follicles may represent a yet-to be described compensation mechanism.

Our results indicate that relative quiescence and K15 are independent determinants of stemness and appear to be differentially regulated by the HPV oncogenes. We speculate that E6 and E7 may lead to cellular reprogramming giving rise to an aberrant stem-like population that is not relatively quiescent but expresses some markers of stemness such as K15. Future studies may shed some light on this interesting possibility.

Our work shows that the proliferation defects seen apply specifically to the quiescent label retaining cells and can be attributed to the viral oncogenes. Moreover, both oncogenes seem to be contributing to this phenotype, consistent with an important role for both in the context of carcinogenesis. Furthermore, the aberrant expansion of other markers of stem cells supports the development of an aberrant stem cell compartment, in the presence of HPV oncogenes, capable of enhanced proliferation.

The relevant cellular targets of E6 and E7 that are associated with this phenotype are not known, however, both E6 and E7 have the ability to modulate the function of cellular targets implicated in tissue stem cell biology. These include telomerase, pRb, E2F6 and polycomb group complexes and also histone demethylases. E6 can upregulate telomerase activity by intricate regulation at the transcriptional and posttranscriptional level (Liu et al., 2009). It has been shown that increased telomerase expression in the progenitor cell niche leads to quantitative and functional changes in this compartment (Flores et al., 2005; Sarin et al., 2005). The same applies for E7, whose prominent target pRb has also been shown to directly affect the progenitor cell niche (Ruiz et al., 2004). In conditional knockouts for *RB*, a parallel to the degradation seen by E7 expression, the LRCs of the skin are shown to be decreased. Furthermore, E7 has been shown to interact with E2F6 and in part inhibit its association with polycomb group complexes, which are crucial to cell fate decisions (McLaughlin-Drubin et al., 2008). These complexes can also be inhibited from binding to histone 3 by induction of histone demethylases KDM6A and KDM6B in E7 expressing cells (McLaughlin-Drubin et al., 2011).

To conclude, this study reveals the ability of the virus to modulate stem cells which could be crucial to their ability to contribute to carcinogenesis since these types of cells were shown to be the cells of origin in epithelial cancers. The enhanced ability of stem cells expressing HPV oncogenes to proliferate could put them in a better position to repopulate wounded tissue at the expense of non-infected cells. Continuous repopulation of tissue by cells expressing the oncogenes may be linked to ensuing carcinogenesis. Further studies to identify the exact molecular targets and pathways that are affected in the presence of the viral oncogenes could aid in the better understanding of stem cell homeostasis in the context of viral infection.

Materials and methods

Mice

Mouse strains were generated in the lab of Dr. Paul Lambert (University of Wisconsin, Madison). The mice used in the experiments were kept on a pure FVB/N inbred genetic background and were K14E6/E7TTL referred to as K14E6 or E6 and K14E7/E6TTL referred to K14E7 or E7 as previously described (Herber et al., 1996; Song et al., 1999). For the generation of bitransgenic mice K14E6 were crossed with K14E7 and the genotypes were confirmed by means of PCR. All the experimental mice used were in a heterozygous state. Mice were housed at the University of Cyprus, in accordance with regulations and protocols approved by the Cyprus Ministry of Agriculture.

BrdU incorporation, anagen induction and hair shaving

5-Bromo-2-deoxyuridine (BrdU) was administered peritoneally in mice at a final concentration of 50 mg/kg as first described previously (Bickenbach et al., 1986; Cotsarelis et al., 1990). For pulse chase experiments ten-day-old mice received an injection every 12 h for a total of four doses and they were euthanised 60 days after injections. For induction of anagen in age-matched mice, at day 60 after BrdU injection, hair was shaved and mice were treated every 48 h with TPA (20 nmol in acetone) for a total of four doses.

Immunohistochemistry

Mice were sacrificed, and tissues obtained were fixed in 4% paraformaldehyde overnight at 4 °C. Dehydration of the samples was performed in a graded series of ethanol concentrations and xylene before they were embedded in paraffin wax. Sections were obtained at 10 μm thickness on a microtome and left overnight to dry at room temperature. Samples were deparaffinised in xylene and rehydrated in a graded series of ethanol solutions. Antigen retrieval was done in a microwave using 10 mM citrate buffer and for BrdU immunohistochemistry, samples were also incubated for 20 mins in 2 M HCl. Blocking and antibody incubations were variable and optimal for each different antibody used. Primary antibodies used include: BrdU (Abcam), K15 (SantaCruz), Nfatc1 (SantaCruz), PCNA (SantaCruz). Following primary antibody incubation samples were washed in PBS. The following secondary antibodies were used: FITC-rabbit, Cy3-rat, Cy3-mouse, Cy3-streptavidin and biotin-rat all from Jackson ImmunoResearch and also Vectastain universal secondary (Vector laboratories). All images were acquired using a Zeiss Axio Observer.A1 microscope. Quantification was performed in a blinded fashion.

Statistical tests

To determine the statistical significance between the genotypes in each experiment, 3 mice of each genotype were used and 50–70 hair follicles were counted. Statistical analysis was done using "Mstat" software (version 5.5.3, McArdle Laboratory for Cancer Research, University of Wisconsin–Madison [http://mcardle.oncology.wisc.edu/mstat/]). Results were compared using a Wilcoxon rank sum test. For all statistical tests differences were considered statistically significant at $p \leq 0.05$.

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Shared mechanisms in stemness and carcinogenesis: lessons from oncogenic viruses

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A rise in technologies for epigenetic reprogramming of cells to pluripotency, highlights the potential of understanding and manipulating cellular plasticity in unprecedented ways. Increasing evidence points to shared mechanisms between cellular reprogramming and the carcinogenic process, with the emerging possibility to harness these parallels in future therapeutics. In this review, we present a synopsis of recent work from oncogenic viruses which contributes to this body of knowledge, establishing a nexus between infection, cancer, and stemness.

Keywords: cancer, stemness, reprogramming, HBV, HCV, HPV, EBV, KSHV

INTRODUCTION

Long-standing observations have noted a number of parallels between the homeostasis of cancer cells and that of stem cells. A complicated picture includes the involvement of tissue stem cells as the cells-of-origin for some cancers, a stem cell compartment thought to maintain most tumors [commonly known as cancer stem cells (CSCs)], as well as more recent concepts of differentiated cells being reprogrammed back to pluripotency during the carcinogenic process (Lapouge et al., 2011; Friedmann-Morvinski et al., 2012). Several publications have shown that classic tumor suppressors such as p53 and pRb have emerging roles in the regulation of stemness (Conklin and Sage, 2009; Bonizzi et al., 2012). In addition to that, genes generally known for their key roles in stem cell biology, for example Nanog, appear to be deregulated in a number of cancers (Zhang et al., 2012; Lu et al., 2013). In the cutting edge field of reprogramming cells to pluripotency, key players in tumor suppression have been implicated in crucial roadblocks to the reprogramming process. While there is still a lot to be understood, it has been proposed that understanding the complicated relationship between stemness and cancer may hold the key to more successful future therapies; for example targeting cancer stem cells may reduce the possibility of future cancer recurrence.

Virally-induced cancers, thought to account for about 20% of the global cancer incidence, have long been studied to enable better understanding of the clinical manifestation of the disease as well as for their value as models of carcinogenesis overall (Farrell, 2002). Such cancers are attributed mainly to Hepatitis B Virus (HBV), Hepatitis C Virus (HCV), Human Papilloma Virus (HPV), Epstein-Barr Virus (EBV), Kaposi's Sarcoma-associated Herpes virus (KSHV), Human T-cell Leukemia Virus-1 (HTLV-1), and more recently, Merkel Cell Polyoma Virus (MCPyV) (Samanta et al., 2003; Bonilla Guerrero and Roberts, 2005; Bajaj et al., 2007; Schiffman et al., 2007; Saha et al., 2010; Jeong et al., 2012; Amber et al., 2013; Cook et al., 2013). These viruses encode proteins shown to impinge on various cellular processes including cell cycle regulation, apoptosis, cell signaling, transcriptional regulation, and epigenetic regulation, resulting in carcinogenesis (Saha et al., 2010). We present here evidence which implicates oncogenic viruses in the regulation of pluripotency at various levels. We argue that virus-associated cancers can serve as models to understand the general link between cancer and stemness, as well as the distinct role that infection plays in these cases. It should be noted that other types of infectious agents, most notably the leprosy bacterium and Helicobacter pylori, have also been shown to modulate stemness-associated processes and pathways in host cells, raising the possibility that strategies involving the manipulation of cellular stemness may serve as evolutionary advantages to pathogens (Fujii et al., 2012; Wegner, 2013). Here, we review the available evidence for regulation of stemness by oncogenic viruses with particular emphasis on results coming from *in vivo* model systems. We also propose key questions that remain to be addressed.

INTERACTION OF ONCOGENIC VIRUSES WITH TISSUE STEM CELLS

Tissue stem cells and committed tissue progenitor cells destined for terminal differentiation are target cells of several oncogenic viruses. While no known oncogenic virus displays exclusive tropism for such specific cell populations, infection of either a stem or progenitor population may provide the opportunity of a longer-lived cellular reservoir for viral replication. In addition, infection of these cells might in some cases enable viruses to evade the immune system, since tissue progenitor/stem cells might be

immune privileged (Di Trapani et al., 2013), even though this notion is still controversial (Tseng et al., 2010).

Gammaherpesviruses, including KSHV and its murine cousin MHV68 and EBV infect primarily resting mature B cells. However, these cells are short-lived and non-proliferating, which points to the possibility that herpesviruses may also be able to infect a progenitor, stem cell-like population of B cells, which normally gives rise to mature B cells, in order to ensure continuous viral genome propagation and viral latency maintenance. Indeed, there is some evidence that both human and murine gammaherpesviruses infect hematopoetic progenitor cells. KSHV has been detected in immature hematopoetic cells in the bone marrow of transplant recipients (Luppi et al., 2000; Lapouge et al., 2011) and in hematopoietic progenitor cells in Kaposi's sarcoma patients (Henry et al., 1999; Friedmann-Morvinski et al., 2012), whereas MHV68 was detected in immature splenic B cells in the mouse (Marques et al., 2003; Collins et al., 2009). Moreover, KSHV-infected human hematopoietic progenitor stem cells gave rise to KSHV-infected mature human B-cells and monocytes when transplanted in NOD/SCID mice (Wu et al., 2006). Coleman et al. examined developing B cell infection by MHV68, a model for gammaherpesviruses, in a fully immunocompetent mouse host. They showed that this virus establishes long-term latency in immature B cells in the bone marrow as well as in transitional B cells in the spleen (Coleman et al., 2010). Since these self-renewing stem cell populations of developing B cells give rise to mature resting B cells, the authors speculate that infection of these cell populations by herpesviruses might play a key role in the maintenance of lifelong infection in the host.

Even though the direct involvement of Human Cytomegalovirus (HCMV) in tumor initiation is still not well-documented, a variety of malignancies have been associated with HCMV infections and persistence but the association is more widely accepted for malignant gliomas (Harkins et al., 2002; Samanta et al., 2003; Soderberg-Naucler, 2006; Michaelis et al., 2009). In normal brain tissue, HCMV appears to primarily target cells in the subventricular zone (SVZ) of the brain (Perlman and Argyle, 1992; Fritschy et al., 1996; Odeberg et al., 2007), which is the source of local stem cells and progenitor cells within this organ (Seri et al., 2006). Differentiation of neural precursors into mature neurons seems to reduce susceptibility to HCMV infection (Lokensgard et al., 1999; Cheeran et al., 2005) and activation of PDGFR alpha (essential to the self-renewal potential of neural stem cells) (Kofman et al., 2011) by HCMV is necessary for successful infection (Soroceanu et al., 2008). These results further support the possibility that the primary cell reservoir for HCMV, at least in the brain, is the stem cell compartment (Dziurzynski et al., 2012), and that infection of HCMV of this cell population might be a way for the virus to successfully establish lifelong latency in the host.

HPVs are strongly associated with a number of malignancies, most notably cervical carcinoma (CC). Several studies have proposed the existence of multiple HPV target cells within the host epithelium. There is increasing support for the hypothesis that stem cells of the transformation zone (TZ) of the cervical epithelium are the primary site of persistent HPV infection (Lopez et al., 2012). Given the anatomical observation that a lot of cervical cancers are derived from the TZ, a connection between infection of tissue stem cells and eventual carcinogenesis has been proposed. The long latency period between infection with HPV and development of cervical dysplasias supports the hypothesis that these cells can be targets of HPV infection and serve as a vehicle for long-term established viral latency in the cervix. Using laser capture microdissection in a rabbit oral papillomavirus (ROPV) model system, Maglennon et al. (2011) showed that ROPV indeed persists in a latent state, even after immune-mediated regression of induced papillomas, and that the site of latency is a subset of basal epithelial cells which the authors propose are the epithelial stem cells. It should be noted that expression of papillomavirus genes in stem cells has been shown to modulate their behavior *in vivo* and may be associated with ensuing carcinogenesis. In a study using mice transgenic for the HPV16 oncogenes our group showed that expression of viral oncogenes in label-retaining epithelial stem cells caused aberrant mobilization (Michael et al., 2013). In a related study, using animals expressing the entire HPV16 viral genome in all basal cells of stratified epithelia, skin cancers were shown to derive from tissue stem cells (da Silva-Diz et al., 2013).

VIRUSES GIVING RISE TO CANCER STEM CELLS

CSCs are cells within a tumor that possess stem cell properties, namely the ability to self-renew and give rise to progeny destined for differentiation to regenerate tumor cell diversity. Though genetic changes or oncogenic infection of an undifferentiated cell is usually thought to give rise to tumor initiating cells, tumors have been shown to originate from differentiated cells as well (Friedmann-Morvinski et al., 2012). It has been suggested that cellular reprogramming mediated by oncogenic viruses may promote the formation of tumor initiating cells or CSCs. The term "tumor initiating cells," strictly referring to the initial cells from which a tumorigenic transformation occurs, is used interchangeably in most cases, describing the ability of CSCs to fully regenerate, or "reinitiate" the tumor.

Several reports have implicated oncogenic viruses in the generation of CSCs. Arzumanyan et al. recently showed that HBV might induce initiation of hepatocellular carcinomas (HCC) by activating cellular factors that promote stemness (Arzumanyan et al., 2011). HBV encoded X antigen (HBVx), important in the viral life cycle as well as carcinogenesis, was shown to activate stemness associated factors Oct-4, Nanog, Klf4, beta catenin, and EpCAM *in vitro*. In addition, this protein was shown to induce cell migration, sphere formation, and growth in soft agar, all phenotypic characteristics of CSCs. These results were confirmed in liver biopsies obtained from HCC patients, since the above stemness associated markers were observed in the majority of HBV associated HCCs (Arzumanyan et al., 2011). Interestingly, microarray data from HBV-associated HCC showed that miR-181, recently found to contribute to tumorigenesis (Agami, 2010), was over-expressed in hematopoietic stem cells (HSCs) and CSCs, and was also found to be upregulated in HBx-expressing cells and HBx-positive liver biopsies (Arzumanyan et al., 2011) suggesting that this micro-RNA might be involved in stemness or CSCs induction and maintenance in HBV-associated HCCs.

The HCV has also been implicated in induction of CSCs. Machida et al. isolated tumor initiating stem-like cells from transgenic mice expressing HCV core, as well as from patients with HCC, and showed that the Tlr4-Nanog pathway was upregulated in these cells and was necessary for their tumorigenic properties (Machida et al., 2009, 2012). Nanog, a stem/progenitor cell marker was further shown to be upregulated through activation of the TLR4 pathway by NS5A, a non-structural protein encoded by HCV (Machida et al., 2012). Furthermore, a study by Ali et al. showed that infection of cultured hepatic cells with an HCV subgenomic replicon resulted in acquisition of CSC characteristics, including expression of Lgr5, c-myc, and DCAMKL-1 (Ali et al., 2011). A DCAMKL-1 enriched cell population was subsequently shown to form tumors with expression of proteins associated with metastatic potential in athymic nude mice. Importantly, removing the HCV replicon from these cells dramatically reduced expression of the stem cell-associated markers. The results correlated well with analysis of liver biopsies from HCV-infected patients, further highlighting the possibility that HCV promotes a CSC-like phenotype *in vivo*.

Several studies have suggested the possibility that EBV might exert its tumorigenic properties at least in part by giving rise to CSCs within the infected tissue. In an important study, Kong et al. investigated the role of EBV LMP2A protein in CSC modulation in nasopharyngeal carcinoma (NPC) cells, and showed that expression of this protein induced cell invasion and epithelial-mesenchymal transformation (EMT) (Kong et al., 2010). Overexpression of LMP2A was found to enrich stem cell like cells within the NPC tumor cell population, and increased the number of cells that were capable of re-establishing tumors in nude mice (Kong et al., 2010). These results were subsequently confirmed in NPC patient biopsies, further suggesting that a possible mechanism of tumorigenesis in EBV-infected tissues is the modulation of the tissue stem cell compartment and the induction of tumor initiating cancer stem cells. A subsequent study showed that, similar to LMP2A, EBV encoded LMP1 latent membrane protein also stimulated EMT, induced a CSC/CPC-like phenotype and enhanced the self-renewal potential in nasopharyngeal epithelial cell lines, further supporting EBV involvement in modulation of cellular plasticity and induction of CSC cellular phenotypes (Kondo et al., 2011). This notion is also highlighted by a more recent study (Lun et al., 2012), which showed up-regulation of multiple stem cell markers in an EBV-positive NPC cell line with increased tumorigenic potential and high resistance to chemotherapy. Finally, a recent study by Port et al. demonstrated that NPC is frequently associated with deregulation of the Hedgehog (HH) pathway, a pathway that is associated with stem cell maintenance. In an *in vitro* model of NPC, the authors showed that EBV activates the HH pathway through induction of the SHH ligand, which leads to increased expression of stemness-associated genes and induction of stem cell phenotypes in these cells (Port et al., 2013).

The long length of papillomavirus infection usually preceding malignant pathologies has been proposed to relate to latency of viral infection in tissue stem cells. Infected tissue stem cells may serve as tumor initiating or CSC in HPV-induced CCs. In support of this hypothesis, a study showed that the invasive and metastatic potential of cervical squamous cell carcinoma (CSCC) was correlated with cancer stem cell-associated genes, and supported the idea that high-risk HPV might induce CSC phenotypes in the TZ of the cervical epithelium (Liu et al., 2010). In addition, expression of HPV E6 and E7 viral oncogenes was shown to induce epigenetic reprogramming in human keratinocytes, through modulation of chromatin structure and global methylation/acetylation events involving cellular factors that have significant role in tumorigenesis and stemness. For example, Hyland et al. showed that E6/E7-expressing primary human foreskin keratinocytes have elevated levels of the EZH2 methyltransferase and the KDM6A demethylase, which results in a reduction of global H3K27 trimethylation and upregulation of downstream targeted HOX genes (Hyland et al., 2011). Reduction in trimethylation of H3K27 associated with elevated EZH2 was also demonstrated in high-grade squamous cervical intraepithelial lesions. In a related study, McLaughlin et al. demonstrated that repressive H3K27 trimethylation was reduced in HPV-positive cervical lesions, and that this was a result of E7-mediated induction of KDM6A and KDM6B demethylases, which subsequently lead to significantly higher expression of homeobox genes (McLaughlin-Drubin et al., 2011). These findings support the possibility that HPV-induced epigenetic reprogramming is important in viral oncogenesis, and further highlight the commonalities between stemness and carcinogenesis, at least in the context of the oncogenic virus life cycle. Further research is needed to fully understand whether HPV-associated cancers are related to cellular reprogramming of infected tissue stem cells or more differentiated cells. The impact of such reprogramming on the viral life cycle also remains unknown.

PATHWAYS TARGETED BY ONCOGENIC VIRUSES ARE ASSOCIATED WITH STEMNESS

A number of reports have shown that classic tumor suppressors and their pathways, notably p53 and pRb, which are long known to be targets of oncogenic viruses (Felsani et al., 2006; Levine, 2009), have important roles in modulation of stemness.

The p53/ARF pathway is a well-established stemness repressor and cells in which this pathway is inactivated can be more efficiently reprogrammed to pluripotency (Hanna et al., 2009; Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009; Marion et al., 2009; Utikal et al., 2009). p53 was also recently found to induce miR-34a and miR-145, which negatively regulate stemness-associated factors (Xu et al., 2009; Jain et al., 2012). More recently, two separate reports further highlighted the importance of p53 in stem cell biology. Chiche et al. showed that somatic loss of p53 resulted in higher numbers of stem/progenitor cells in mammary epithelium (Chiche et al., 2013). Sato et al. reported that p53 activation promoted proteosome-dependent degradation of Nanog and differentiation of glioma stem cells (Sato et al., 2013). It is therefore possible that p53 inactivation, a common strategy of oncogenic viruses, may contribute positively to the viral life cycle in a way additional to the proposed viral escape of apoptosis of infected cells.

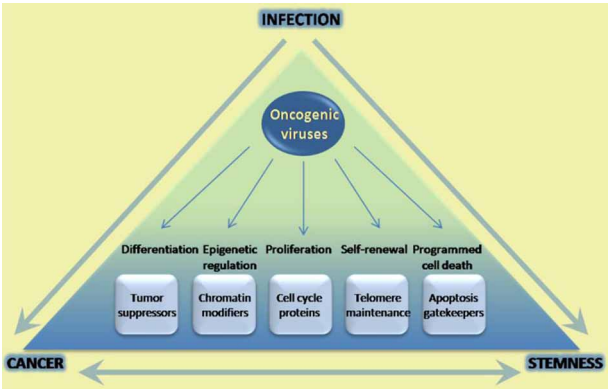

FIGURE 1 | Infection with oncogenic viruses highlights parallels in cancer and stem cell biology. Oncogenic viruses modulate a variety of cellular pathways with parallel roles in the carcinogenic process and stem cell homeostasis. The parallels between these two processes have been extensively documented, and increasingly well-understood in terms of being able to reprogram cell state. However little has been done in the way of uncovering potential roles of these pathways in infection success. Increasing understanding of common pathways modulated may yield better tools to prevent and treat infection, as well as ensuing carcinogenesis.

The retinoblastoma tumor suppressor (pRb) is another major target of oncogenic viruses, since inhibition of Rb liberates the E2F transcription factor, which stimulates entry of the cell into the cell cycle, thus favoring viral replication. Increasing evidence has implicated this pathway in stemness modulation, initially in plants (Ebel et al., 2004; Wildwater et al., 2005) and subsequently in animals (Liu et al., 2009). Accumulating evidence reinforces the role of pRb in stem cell homeostasis (Conklin and Sage, 2009). The pRb pathway was shown to have a critical role as a roadblock in the reprogramming of human fibroblasts to iPSCs, as well as cell fate determination, as elegantly shown by Calo et al. (2010). Conceivably then, like p53 inactivation, the inactivation of pRb could promote cellular plasticity and stemness, which in turn would confer an ideal niche for virus persistence and latency.

There is mounting evidence supporting the recently suggested notion that tumor suppressor pathways, traditionally key targets of oncogenic viruses, might play a significant role in cellular plasticity and modulation of stemness. Even cellular factors activated by genetic events in virally-induced cancers such as c-myc in Burkitt's lymphoma, have well-described involvement in cancer as well as stemness (Dang, 2012; Buganim et al., 2013). Therefore, it is also not surprising that factors traditionally involved in stemness and cellular plasticity are increasingly being identified as targets of oncogenic viruses. Indeed, HCV, HBV, and EBV have been shown to regulate a number of pluripotency and stem cell-associated factors (Ruf et al., 1999; Machida et al., 2009; Ali et al., 2011; Lun et al., 2012). In addition, telomerase activation and telomere maintenance are important in both cancer and stemness, and it is therefore not surprising that oncogenic viruses evolved to regulate these processes. Most, if not all, tumor viruses, including the oncogenic retrovirus HTLV-1, induce transcriptional activation of telomerase (Kuhlmann et al., 2007; Bellon and Nicot, 2008), and EBV and HPV are also known to regulate telomerase post-transcriptionally.

DISCUSSION

Oncogenic viruses cause cancer after long-term infection of their natural niche. These viruses interfere with signaling pathways that are important in a number of major cellular processes including cell proliferation and cell division, apoptosis, and cell differentiation. Accumulating evidence suggests that oncogenic viruses may also manipulate cellular stemness in various ways. Stem cells or progenitor cells are targets of infection and normal cell homeostasis is disrupted as a result. Moreover, pathways that are traditionally associated with self-renewal and lineage-commitment have been shown to be transcriptionally regulated by viral oncoproteins. Regulation of such pathways, and of oncogenic pathways now understood to play key roles in stemness, may lead to cellular reprogramming. Whether regulation of stemness is necessary for ensuing carcinogenesis, or whether it has any impact on the viral life cycle, has not been conclusively addressed. However, it is conceivable that infection of tissue stem cells might positively affect the viral life cycle, especially in terms of establishing a successful chronic infection (**Figure 1**). It should also be noted that regulation of innate immunity and inflammation, also known to be linked to carcinogenesis, is now beginning to be linked to stemness as well (e.g., TLR4-Nanog, TLR3) (Machida et al., 2009; Lee et al., 2012). Additional studies are necessary in order to fully investigate this notion, especially in the context of *in vivo* infection models. As we continue to explore the parallels between cellular stemness and the carcinogenic process, oncogenic viruses continue to serve as excellent paradigms with plenty to teach.

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