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Mouse Models for Papillomavirus-Mediated Phenotypes

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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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ΠΕΡΙΛΗΨΗ

Οι HPV έχουν συσχετιστεί με την ανάπτυξη της πλειονότητας των καρκίνων του τραχήλου της μήτρας, καθώς και με ένα υποσύνολο καρκίνου κεφαλής και λαιμού, καρκίνο του δέρματος και άλλων καρκίνων της γενετικής περιοχής. Στόχος μας ήταν να χρησιμοποιήσουμε μοντέλα ποντικιών που εκφράζουν τα γονιδια του ιού HPV για να μελετήσουμε τους μηχανισμούς μέσω των οποίων αυτοί οι ιοί συμβάλλουν στην καρκινογένεση.

Η καρκινογένεση πιστεύεται ότι προκαλείται σε μεγάλο βαθμό από τη συνεχή έκφραση των ιικών ογκογονιδίων Ε6 και Ε7. Αυτά τα ογκογονίδια δεν έχουν καμία γνωστή ενζυμική δραστικότητα και προάγουν καρκινογένεση επιδρώντας στις κυτταρικές διεργασίες του ξενιστή, συμπεριλαμβανομένης της ομοιόστασης των τελομερών. Η διατήρηση του μήκους των τελομερών πιστεύεται ότι διαδραματίζει σημαντικό ρόλο σε καρκίνους που προκαλούνται από τους ιούς HPV, καθώς τόσο η Ε6 όσο και η Ε7 εμπλέκονται στη ρύθμιση του μήκους των τελομερών μέσω της ενεργοποίησης της τελομεράσης και του ομόλογου ανασυνδιασμού των τελομερών αντιστοίχως. Παρόλο που αρκετές *in vitro* μελέτες δείχνουν ότι τα ογκογονίδια Ε6 και Ε7 εμπλέκονται στην τελομερική ρύθμιση, η συμμετοχή αυτών των ογκογονιδίων στην ομοιοστασία των τελομερών δεν έχει αποδειχθεί ποτέ *in vivo*. Χρησιμοποιήσαμε έτσι ζώα διαγονιδιακά για HPV16 Ε6 και Ε7 για να μελετήσουμε την αλληλεπίδραση των Ε6 και Ε7 ιικών ογκογόνιδίων με την ομοιόσταση των τελομερών. Συγκεκριμένα, εξετάσαμε εάν οι επιδράσεις των HPV16 Ε6 και Ε7 στα στρωματοποιημένα πλακώδη επιθήλια και στους πληθυσμούς των βλαστικών κυττάρων τους οφείλωνται στην ομοιόσταση των τελομερών. Έχουμε δείξει ότι το Terc δεν είναι απαραίτητο για τους περισσότερους από τους βραχυπρόθεσμους φαινοτύπους που προκαλούνται από ογκογονίδια του HPV16 στους ποντικούς. Επίσης, η Ε7 επαγώμενη μείωση των LRCs (Label Retaining Cells) επηρεάζεται από την απουσία του Terc, αλλά ο μηχανισμός με τον οποίο προκαλείται αυτός ο φαινότυπος δεν είναι καλά κατανοητός. Απαιτείται περαιτέρω εξέταση προκειμένου να εξεταστεί ο ρόλος του Terc σε μακροχρόνιους φαινοτύπους ή κατά τη διάρκεια φυσικών λοιμώξεων.

Παρόλο που τα διαγονιδιακά μοντέλα ήταν χρήσιμα για την κατανόηση του ρόλου των ογκογονιδίων σε σχέση με την αλληλεπίδραση του ιού με τον ιστό του ξενιστή και τα βλαστικά κύτταρα, δεν μπορούν να προσωμοιάσουν την πορεία της φυσικής λοίμωξης. Αυτό οφείλεται κυρίως στο γεγονός ότι οι ανθρώπινοι θηλωματοϊοί δεν μολύνουν παραγωγικά τα τρωκτικά. Πρόσφατα, απομονώθηκε και χαρακτηρίστηκε ένα στέλεχος ιού θηλώματος που μολύνει

ποντίκια (MmuPV1) και μπορεί να προκαλέσει θηλώματα *in vivo*.Ο MmuPV1 αποδείχθηκε ότι έχει πολλές ομοιότητες με τους HPVs του δέρματος. Για να κατανοήσουμε καλύτερα τα αποτελέσματα της ιογενούς μόλυνσης στα στρωματοποιημένα επιθήλια, χρησιμοποιήσαμε το γονιδίωμα του MmuPV1 για να μολύνουμε ποντίκια και πήραμε ιστούς από τα θηλώματα που παρουσιάστηκαν. Λάβαμε επίσης ιστούς από θηλώματα που αναπτύχθηκαν μετά από μόλυνση με τον ιό MmuPV1 σε ποντίκια. Εξετάσαμε τις οξείες επιδράσεις του ιού σε δείκτες επιδερμικών βλαστοκυττάρων και στη μορφολογία του επιθηλίου κατά τη διάρκεια της φυσικής μόλυνσης. Δείξαμε ότι τα K15, K14, CD34, p63 και Lrig1 υπερεκφράζονταν στους μολυσμένους με MmuPV1 ιστούς. Αυτή η υπερέκφραση συνοδεύτηκε από υπερπλασία του επιθηλίου που υποδεικνύεται με χρώση H&E. Αυτοί οι δείκτες έχουν προηγουμένως δειχθεί να υπερεκφράζονται σε διαγονιδιακούς ποντικούς που εκφράζουν HPV που μολύνουν είτε βλενογόνο είτε δέρμα. Πρόσφατες ενδείξεις που είναι σύμφωνες με τα ευρήματά μας υποδεικνύουν ότι αυτό το μοντέλο μπορεί να χρησιμεύσει ως βιολογικά σχετικό ζωικό μοντέλο για δερματικούς HPVs. Σχεδιάζουμε να χρησιμοποιήσουμε αυτό το μοντέλο ως εργαλείο για τον προσδιορισμό του τρόπου αλληλεπίδρασης της λοίμωξης από ιό HPV με τα βλαστοκύτταρα και την κυτταρική πλαστικότητα στο επιθήλιο κατά τη διάρκεια της μόλυνσης και της ογκογένεσης.

ABSTRACT

HPVs have been associated with the development of the majority of cervical cancers, a subset of head and neck cancers, skin cancer and other anogenital cancers. Our goal was to use mouse models of papillomavirus gene expression to study the mechanisms through which these viruses contribute to cancer.

HPV-mediated carcinogenesis is thought to be driven by the continuous expression of the viral oncogenes E6 and E7. These oncogenes have no known enzymatic activity and promote carcinogenesis by impinging on cellular processes, including telomere homeostasis Deregulation of telomere homeostasis has been observed in carcinogenesis as a mechanism of overcoming the telomere-shortening problem of the continuously proliferating cancer cells. Telomere maintenance has also been proposed to play an important role in HPV-driven cancers, as both E6 and E7 have been implicated in regulating telomere length by means of telomerase activation and alternative lengthening of telomeres (ALT) respectively. Even though several lines of evidence from *in vitro* studies show that E6 and E7 oncogenes are involved in telomeric regulation, the involvement of these oncogenes in telomere homeostasis has never been demonstrated *in vivo*. Thus, to probe potential in vivo roles of the viral oncogenes in telomere homeostasis we utilized animals transgenic for HPV16 E6 and E7 in order to study the interaction of the E6 and E7 viral oncogenes with telomere homeostasis. Specifically, we examined whether the short-term effects of HPV16 E6 and E7 on stratified epithelia and their stem cell populations are mediated through telomere homeostasis. Terc is the RNA subunit of telomerase and it is necessary for telomere elongation by telomerase. We showed that Terc is dispensable for most of the short-term HPV16 oncogene-mediated phenotypes in mice. Terc deficiency did not affect the E6 and E7-induced proliferation in basal and suprabasal layers of the epithelium, and the increase in keratin 15 (K15) expression in the hair follicle compared with the non-transgenic mice. Also, E6-mediated reduction of slowly cycling bulge cells (LRCs) was unaffected despite the absence of Terc. Surprisingly, E7-mediated reduction of LRCs was dependent on the presence of Terc, but the mechanism underlying this phenotype is not well understood. Further examination is needed in order to examine the role of Terc in longer-term phenotypes or during natural infection.

While transgenic models have been useful in teasing out the role of individual gene products, research geared toward understanding the interaction of the virus with its host tissue and stem cells was hindered by the lack of animal models that can recapitulate the course of

natural infection. This is mostly because papilloma virus infection is species-specific, and as a result, human papillomaviruses do not productively infect rodents. Recently, a papilloma virus strain that infects mice (MmuPV1) and can cause papillomas *in vivo* has been isolated and characterized. MmuPV1 was shown to have many similarities with cutaneus HPVs. To better understand the effects of viral infection on stratified epithelia, we used MmuPV1 genome to infect mice and obtained tissue from the papillomas that were developed. We also obtained tissues from papillomas developed after MmuPV1 infection on mice. We examined the acute effects of papillomavirus on epidermal stem cell markers and on the morphology of the epithelium within the course of natural infection. We showed that stem cell markers K15, K14, CD34, p63, and Lrig1 were upregulated in MmuPV1 infected tissues. These phenotypes mimic at least in part those previously observed in transgenic mice. This upregulation was accompanied with hyperplasia of the epithelium indicated by H&E staining. Additional recent evidence consistent with our findings indicates that this model can serve as a biologically relevant animal model for cutaneous HPVs. We plan to use this model as a tool to examine the interaction of papillomavirus infection with telomere homeostasis. Furthermore, we will use it to interrogate the interaction of papillomavirus infection with the stem cell compartment and cellular plasticity in the epithelium during infection and tumorigenesis.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

ALT	Alternative Lengthening of Telomers
BrdU	Bromodeoxyuridine
Cy3	Cyanine 3
CSCs	Cancer Stem Cells
DMEM	Dulbecco's Modified Eagle Medium
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
FITC	Fluorescein isothiocyanate
HPV	Human Papillomaviruses
HR	High Risk
HSPG	Heparan sulfate proteoglycan
KDM6	Lysine (K) – specific demethylase
Klf4	Kruppel-like factor 4
K14	Cytokeratin 14
K15	Cytokeratin 15
L1	Late protein 1
L2	Late protein 2
LR	Low Risk
LRCs	Label-retaining cells
MAPK	Mitogen-Activated Protein Kinase
MEFs	Mouse embryonic fibroblasts
MmuPV1	Mouse Papillomavirus
NOD/SCID	Nonobese diabetic/severe combined immunodeficiency
Oct4	Octamer-binding transcription factor 4
NTG	Non-transgenic
PAP	Papanikolaou
PBS	Phosphate Buffered Saline

PFA	Paraformaldehyde
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
pRb	Retinoblastoma
PV	Papillomaviruses
RT	Room Temperature
Terc	Telomerase RNA component
Tert	Telomerase Reverse transcriptase
Trap	Telomere Repeat A Protocol
TTL	Translation Termination Linker

CHAPTER 1: INTRODUCTION

1.1 HPV

1.1.1 HPV Epidemiology

Cervical cancer is the fourth most common cancer and cause of death among women worldwide. It ranks second among women 15-44 years old. Every year about 528,000 women are diagnosed with cervical cancer and 266,000 women die from this cancer (Bruni L and 2017., 2017). In sub-Saharan Africa, Mexico and North-West Latin America cervical cancer is the most common cancer among women (Figure 1).

HPVs are the main cause of cervical cancer, and they have been also associated with other anogenital cancers and a subset of head and neck cancer, as well as skin cancer. Overall, human papillomaviruses cause 5% of worldwide cancers (Asiaf et al., 2014, Haedicke and Iftner, 2013, McLaughlin-Drubin et al., 2012, Wang et al., 2012), and 70% of all cervical cancers are caused by HPV 16 and 18 (Bruni L and 2017., 2017).

Vulva cancer represents 4% of all gynecological cancers and is further subdivided in two subtypes with distinct profiles, namely the basaloid and keratinizing types. The basaloid vulva cancer has been associated with HPV detection. The keratinizing vulva carcinomas are the more commons lesions (>60%). They occur mostly in older women and usually they are not associated with HPV.

Vaginal cancer represents 2% of all gynaecologic cancers, and 90% of vaginal cancers are squamous cell carcinomas, which are mainly caused by HPV. HPV has also been associated in some cases with anal cancer (a rare cancer with incidence of 1 person per 100,000), whereas penile cancer, another rare form of malignancy, is also primarily caused by HPV, as more than 60% of precursor cancerous penile lesions are HPV DNA positive (de Martel et al., 2012).

HPV types 6 and 11 have also been associated with recurrent juvenile respiratory papillomatosis and genital warts (Lacey et al., 2006).

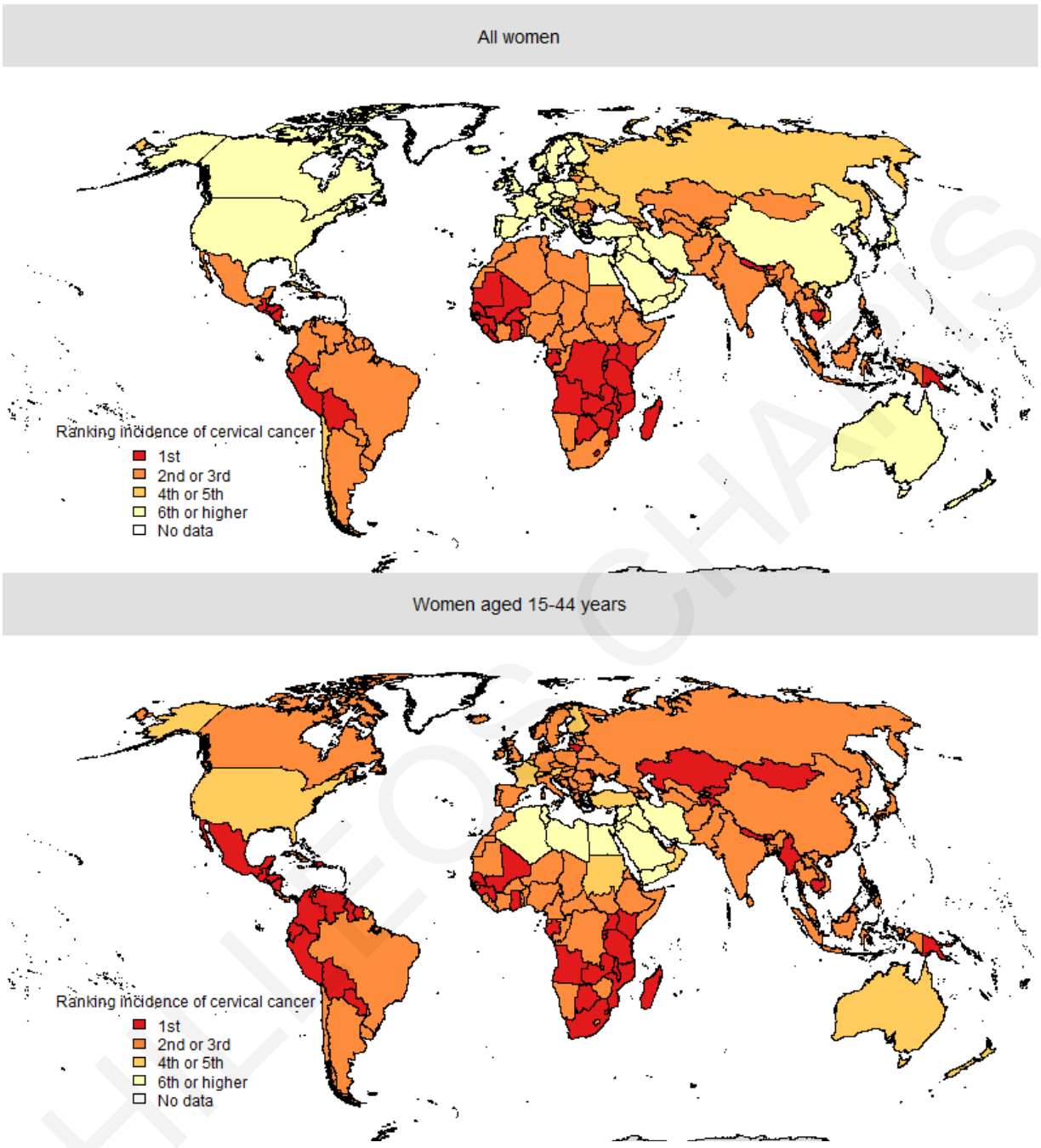


Figure 1: Worldwide ranking of cervical cancer incidence in 2012. The upper panel shows the ranking of cervical cancer incidence in women of all ages. The second panel shows the ranking of incidence in women aged 15-44 years old. Areas where cervical cancer incident is the most common are indicated in red, while orange indicates the areas where cervical cancer incidence ranks second and third. (Bruni L and 2017., 2017)

Head and neck squamous cell carcinoma (HNSCC) is a group of malignancies (oral cavity, throat, salivary glands, nasopharynx, oropharynx, hypopharynx, and larynx). These neoplasms arise from non-keratinizing epithelium. Worldwide, squamous cell carcinoma from this group collectively accounted for 932 000 new cases and about 379 000 deaths in 2015 (Fitzmaurice et al., 2017).

During the past four decades, the incidence of oropharyngeal squamous cell carcinoma (OPSCC) in young ages is steadily increasing in developed countries (Chaturvedi et al., 2011). This change on the epidemiological trends is a result of several societal factors. Oropharyngeal HNSCCs have increased due to trends in oral sexual practices, which in turn increased the incidents of infection with sexually transmitted viruses, such as HPV (D'Souza et al., 2009). In USA, every year more than 11,700 are diagnosed with OSPSCC and the incidence is higher in white men (Panwar et al., 2014). Currently there is no early detection method for HPV-associated OSPSCC.

Non-melanoma skin cancer (NMSC) is the most common cancer in the Caucasian population (Nindl et al., 2007). It has two most common types, basal cell carcinoma (75-80% of the cases) and squamous cell carcinoma (20 % of the cases) (Nindl et al., 2007). The main risk factor is ultraviolet (UV) radiation. Cutaneous HPV (mainly from genus Beta and Gamma) are suspected to be involved in the pathogenesis of this cancer (Tommasino, 2017, Nindl et al., 2007). The HPV 5 and HPV 8 types (genus Beta) were the first HPV identified in patients with epidermodysplasia verruciformis (EV) and were linked with NMSC (Orth, 2008, Orth et al., 1978).

As mentioned above, HPVs are the main cause of cervical cancer, the 4th most common cancer in women worldwide and 1st in low income countries. This is due to a scarcity of resources for cervical cancer screening programs, vaccinations and treatment plans in those countries (Forman et al., 2012).

The Papanikolaou smear (PAP smear) is an efficient method for early diagnosis of HPV infection (Tambouret, 2013). It can identify pre-cancerous conditions and cancer cells by tracing cell changes in the cervix. Pap smears should be carried out every 3 years in women between the ages of 21 to 65. The number of cervical cancer occurrence has decreased through the years due to early diagnosis (Moody and Laimins, 2010, Yugawa and Kiyono,

2009). Unfortunately, there are still many places in the world that do not have access to screening methods, or the national programmes do not have sufficient quality assurance during screening (Figure 2). HPV DNA test can specifically identify HPV genome and unveil cervical cancers or pre-cancer cells in women over 30 years old (American Cancer Society). HPV DNA test can also be used in younger women after abnormal results from Papanicolaou (PAP) smears.

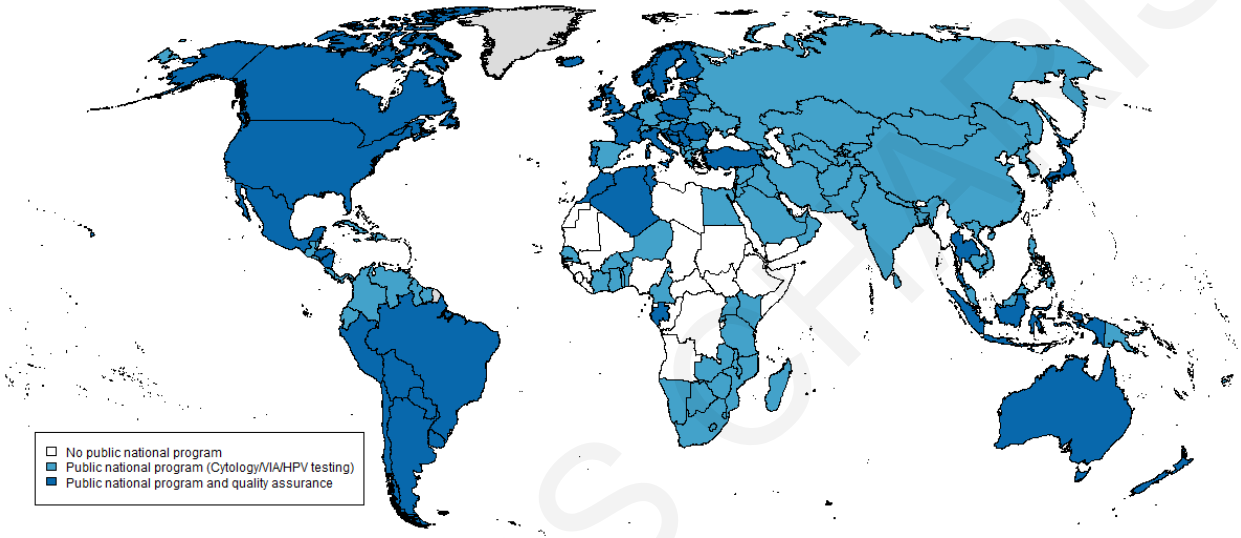


Figure 2: Availability of cervical cancer screening programmes worldwide (Bruni L, 2017). In America, Europe and Australia there are available national programmes for Cytology/VIA/HPV testing and also quality assurance at all levels of the screening process (from invitations to follow up if the screening is positive). If less than 50% of the regions in a country don't have a quality assurance plan, the country is categorized as not having one (light blue). Main countries in Africa don't even have a public national program for cervical cancer screening.

Vaccination was made available in 2006 and is the only preventative measure for some types of HPV (Bryan et al., 2016). Currently, prophylactic vaccination can protect against the most frequent viral strains that cause cervical cancer and genital warts (Yugawa and Kiyono, 2009). There are three vaccines, Gardasil (against HPV 16, 18, 6 ,11), Gardasil 9 (against HPV16, 18, 6, 11, 31, 33, 45, 52 and 58) and Cervarix (against HPV 16 and 18) (Bryan et al., 2016). With Gardasil 9 (approved by FDA in 2014) there is the potential to prevent ~90% of cervical cancer cases (Beavis and Levinson, 2016). Unfortunately, at least one third of the world does not have access to these vaccines (Figure 3).

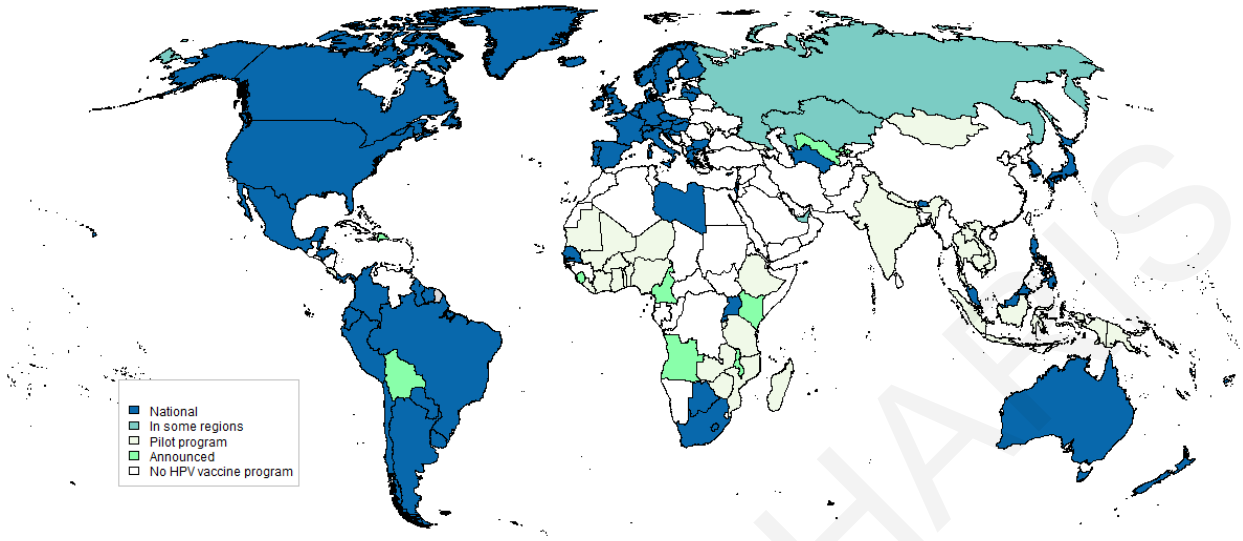


Figure 3: Status of vaccination programmes worldwide (Bruni L, 2017). In America, Europe and Australia national programmes are available for vaccination of the general population. In most places in Africa there are no available HPV vaccine programmes.

1.1.2. Papilloma Viruses Classification

The PV family consists of 29 genera (alpha to omega, and continue with a the prefix “dyo” for the genera exceeding the greek alphabet, starting from dyodelta to dyotheta), categorized based on the L1 (capsid protein; section 1.1.3.6) open reading frame (ORF) diversity (de Villiers et al., 2004, Bernard et al., 2010, de Villiers, 2013). In figure 4 are present 189 types of PVs divided to the 29 genera.

The HPV family is categorized in 5 genera (Alpha, Beta, Gamma, Mu and Nu) (Figure 4). Types sharing L1 sequence identity of 60% and up belong to the same genus. Types that belong to the same genus and share 60-70% L1 sequence identity are grouped together in the same species (de Villiers et al., 2004, Bernard et al., 2010, de Villiers, 2013). If two PVs share 71% to 89% of L1 sequence identity, they belong to the same type, and if the percentage is 90–98% they are subtypes. Those with similarity of more than 98% are considered variants (Harden and Munger, 2017). Currently more than 200 HPV types have

been identified based on the International Human Papillomavirus Reference Center (http://www.hpvcenter.se/html/refclones.html).

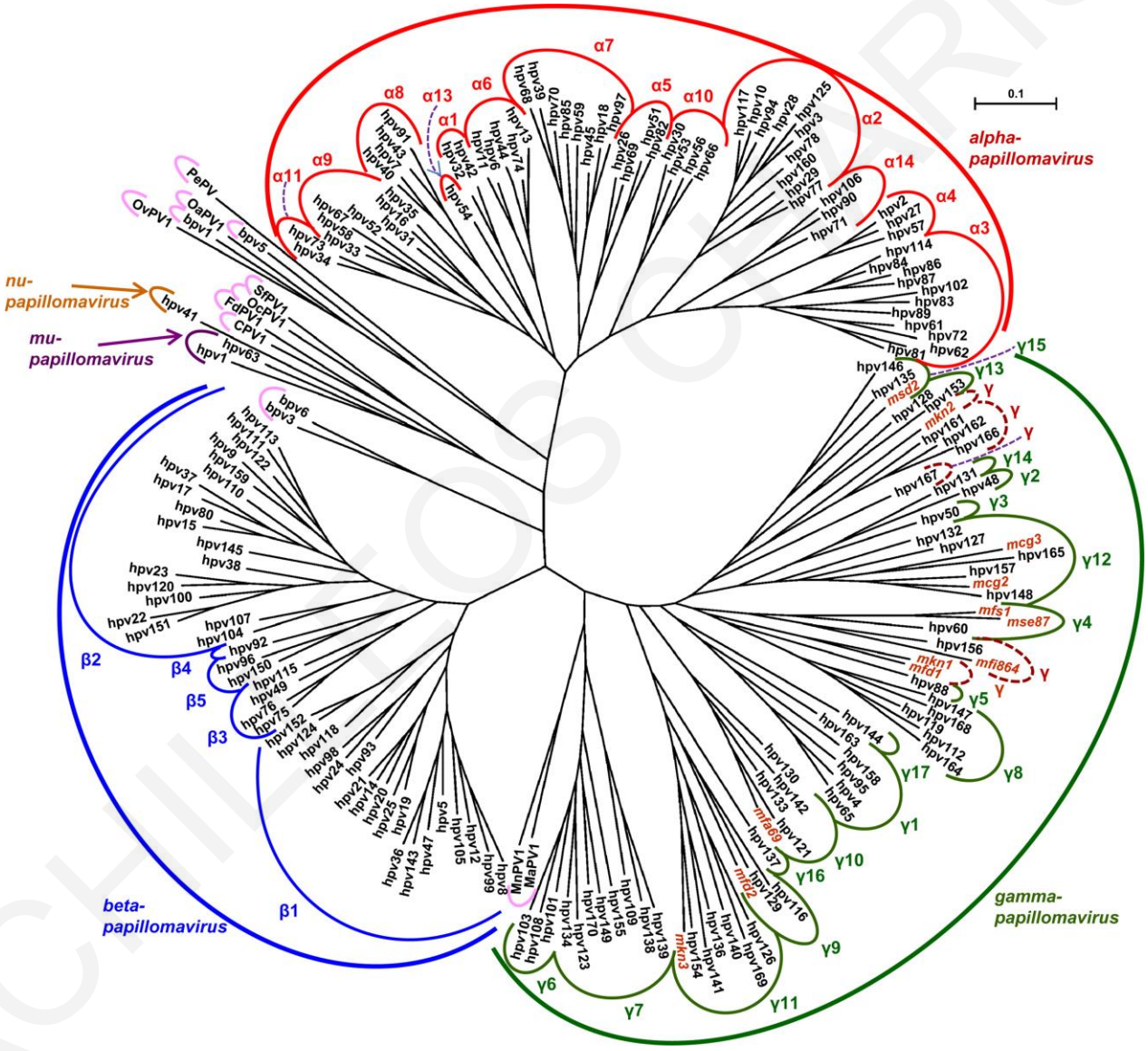


Figure 4: Phylogenetic tree of 170 human papillomaviruses types based on the L1 nucleotide sequences using the maximum likelihood method (de Villiers, 2013). In this phylogenetic tree, the 170 human papillomaviruses are divided into 5 genera.

HPVs are usually grouped as *cutaneous* or as *mucosal,* based on the tissue of origin (that they were isolated from) (de Villiers, 2013). The main HPV phylogenetic genera are Alpha (mucosally infective) and Beta (cutaneous infective). The Alpha genus is further divided in low-risk and high-risk viruses based on their ability to cause benign or malignant cancer lesions. HPV 16 and HPV 18 are the most studied high-risk viruses and their viral proteins E6 and E7 have been shown to be important viral oncogenes with major roles in carcinogenesis (Arbeit et al., 1993). The Beta genus has been associated with neoplastic precursors and with development of non-melanoma skin cancer (Dubina and Goldenberg, 2009, Nindl et al., 2007, Weissenborn et al., 2012). The most common type of HPV found on skin is HPV 5 (Beta Papillomavirus genus) (de Villiers et al., 2004). HPV 5 has been associated with skin cancers (in patients with *epidermodysplasia verruciformis)*. *Epidermodysplasia verruciformis* is an inherited autosomal recessive skin disorder characterized by flat wart-like lesions (Agrawal et al., 2013). These lesions can occur anywhere in the body after infection with some subtypes of HPV and can further develop to non-melanoma cutaneous carcinomas (Gul et al., 2007).

In 2011 a new mouse papillomavirus (MmuPV1), which naturally infects laboratory mice, was isolated and characterized. MmuPV1 is a member of the Pi (π) genus. MmuPV1 has more shared characteristics with cutaneous HPV types, such as Beta genus HPV. It has been shown that MmuPV1 E6 shares biological and biochemical activities with HPV8 E6 (Meyers et al., 2017a). This model is the only one, which recapitulates human infection in a live animal, since human specific HPVs do not infect laboratory mice. Models for studying the HPV cell cycle and carcinogenesis are discussed in section 1.4.

Experiments described throughout this dissertation mostly utilized the HPV 16 oncogenes, which were primarily expressed as transgenes in mice, whereas several additional experiments employed MmuPV1, as described in subsequent chapters. We utilized transgenic mice expressing the E6 and E7 oncoproteins of HPV 16. Using transgenic mice expressing these ongogenes under the keratin 14 promoter, we have been able to study the effect of HPV oncogenes on the stem cell compartment of the hair follicle (a model tissue) *in vivo*, which in turn enabled us to study the effects of E6 and E7 on the stem cell population in HPV-infected areas.

We also perform experiments utilizing the MmuPV1, that naturally infect mice, as we aim develop in our lab an *in vivo* model where infection can be recapitulated in a system with the genetic tools and versatility such as the laboratory mouse.

1.1.3 Papilloma Viruses Structure and Genome

Papillomaviruses (PVs) are small (55 nm in diameter), non-enveloped, DNA double-stranded viruses with a genome of about 8Kb (de Villiers et al., 2004) divided in three regions: the early and late regions, and the long control (or noncoding) region (LCR or NCR) (Figure 5) . These regions are separated by two polyadenylation (pA) sites (Doorbar, 2005). Eight viral proteins are usually encoded by eight open reading frames of the genome, however the number of viral proteins varies slightly depending on the HPV type. For example, in the Beta-HPV the E5 ORF is absent (with the exception of HPV 14) (Tommasino, 2014) . Due to the overlapping nature of the open reading frames encoding these proteins, alternative splicing is essential for gene expression.

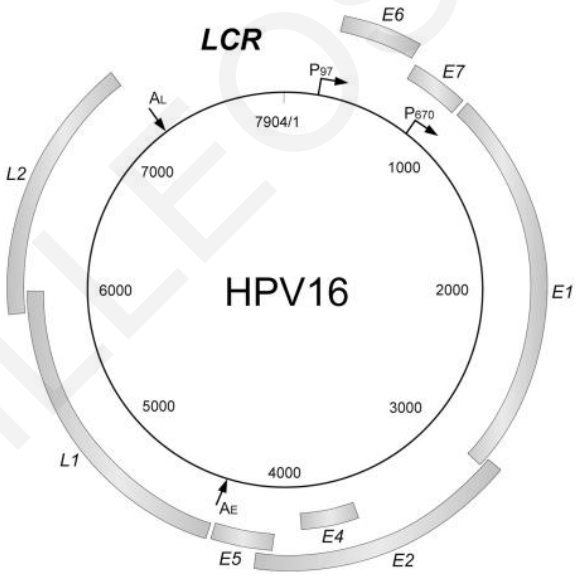


Figure 5: The HPV viral genome. HPV is about 8000bp with a circular double-stranded DNA that encodes 8 proteins. The major promoters and polyadenylation signals are indicated (P97, P670, AE, AL) (Kajitani et al., 2012)

The LCR covers about 10% of the virus genome and has no protein-coding function. It contains the origin of replication and transcription factor binding sites, promoter and enhancement elements (Thierry, 2009). There are four elements that are common in all PV types within the LCR: the polyadenylation site close to the 5' end of the LCR, several E2 protein binding sites, (number and position varies across types), a TATA box for the E6 gene promoter, and an E1 protein binding site (Thierry, 2009).

The late region covers about 40% of the virus genome and encodes for the L1 and L2 proteins that are the major and minor capsid proteins respectively (Buck et al., 2013, Kirnbauer et al., 1992). A papilloma virion comprises of 72 capsomeres, each of which is created through the assembly of 5 L1 proteins and 1 L2 protein.

The more well studied HPV types are the alpha high-risk HPV, which are mainly associated with cervical cancer. The early region of these viruses covers 50% of the virus genome, and encodes 6 proteins (E1, E2, E4, E5, E6 and E7). E1 and E2 are involved in virus transcription and replication. E1 mediates episomal DNA replication and E2 regulates viral copy number (Longworth and Laimins, 2004). E4 expressed during a productive infection is associated with cytokeratin filament collapse and virion release. E5, E6, and E7 are viral oncogenes whose expression induces cell immortalization and transformation (Longworth and Laimins, 2004). E5 downregulates surface MHC class I expression to dampen the immune response against infected cells and also activates EGF receptor to activate cell proliferation. E6 deregulates cell cycle control through p53 degradation, allowing the infected cells to escape apoptosis, and E7 promotes cell proliferation through inactivation of the Rb tumor suppressor. The function of E6 and E7 will be more extensively discussed later in subsequent sections.

Most of the early proteins are expressed at the early stage of an infectious cycle. E4 is expressed at a later point of the infection before the expression of the proteins from the late region (L1 and L2), which are expressed at the outermost epithelial layer (the granular Layer) (Moody and Laimins, 2010).

Many differences can be found among different types of HPVs (Klingelhutz and Roman, 2012) For example, there is a difference in anatomical tropism due to different gene expression controlled by the LCR (Mistry et al., 2007). HPV 16 is a high risk HPV that infects mucosal epithelium and is highly associated with cervical cancer, whereas HPV 5 infects skin

epithelial and is associated with skin cancer (Mistry et al., 2007). Another main difference is that Beta HPV genomes have a genome, which is shorter by about 300–500 nucleotides due specifically to a shorter LCR (Tommasino, 2017). Beta HPV types also lack the E5 gene (Tommasino, 2017). There are also differences between HPVs of the same genus. These differences are mainly manifested in the ability of these viruses to induce oncogenesis, and they are due to variations in the E6 and E7 oncogenes (Table 1).

Table 1: Functional differences of the E6 and E7 oncogenes between high risk and low risk alpha HPV (Doorbar et al., 2012)

	High-Risk Alpha	Low-Risk Alpha
E6	encodes E6* products	no E6* products
	binding and degradation of… •p53 •specific PDZ-domain proteins (e.g. Dlg, MAGI-1, Scribble)	weaker binding (no degradation) of… •p53 •no binding of PDZ-domain proteins
	interact with the 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… •Akt •Wnt •Notch •mTORC1	unknown
	telomerase activation	no activation
	c-myc activation	no activation
E7	binding and degradation of… •pRb •p107 •p130 binding (no degradation) of… •E2F1 •Cullin2 •HDAC	weaker binding (no degradation) of… •pRb •p107 •E2F1 binding 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 genome instability	no stimulation of instability
	suppression of STAT-1 function	no suppression
	immortalization and transformation functions	no such functions
	activation of signaling pathways… •Akt	unknown

1.1.3.1 HPV E1 and E2

Both E1 and E2 are regulatory elements of the virus. They have a major role in transcription and replication. The E1-E2 heterodimer complex binds at specific binding sites at the origins of replication of the early genes (Kurg, 2011). The E1-E2 heterodimer complex maintains the viral gene copy number in the host cell. The complex also enhances helicase binding on the origins (Stenlund, 2003) while repressing early gene transcription (Morshed et al., 2014, Tommasino, 2014). Inactivation of E2 leads to the overexpression of the early viral oncogenes (E6 and E7), which, in the case of high-risk HPVs, leads to carcinogenesis (Baker et al., 1987, Schwarz et al., 1985, Thierry, 2009).

1.1.3.2 HPV E4

E4 is expressed during the later stages of viral infection and is required for the completion of the productive cycle *in vivo (Doorbar, 2013a, Peh et al., 2004)*. It can be detected in the cytoplasm of upper epithelial cells. Its main role is the destabilization of cytokeratin filaments by binding on the keratins when E4 is phosphorylated. E4 binding on these filaments causes the disorganization of the protective layer formed by cytokeratins, and leads to the release of the encapsided virus out of the cells of the upper epithelial layer (Brown et al., 2006, Wang et al., 2004).

1.1.3.3 HPV E5

E5 is a small transmembrane protein expressed by some animal and human papillomaviruses (Venuti et al., 2011). The most studied E5 protein is that of BVP 1 and E5 of HPV 16. BVP 1 can induce benign neoplasia in cattle and horses (Lancaster and Olson, 1982, Carr et al., 2001) and has been used as an *in vitro* model of papillomavirus mediated transformation (Dvoretzky et al., 1980, Lowy et al., 1980). In fact, E5 is the major oncogene in BPV1 (DiMaio et al., 1986, Schiller et al., 1986, Yang et al., 1985). The primary target of BVP 1 E5 is the PDGF β receptor whereas the main target of the HPV 16 E5 is the EGF receptor (DiMaio and Petti, 2013). BVP 1 and HPV E5 can modulate the activity of cellular proteins. They can bind the vacuolar ATPase and they can also affect cell-cell communication and MHC class I expression (Figure 6) HPV 16 E5 can inhibit death receptor-mediated apoptosis

and it can also co-operate with E6 and E7 and immortalize human keratinocytes *in vitro (Stoppler et al., 1996).* During the productive stage of the viral life cycle, E5 expression can promote DNA synthesis in the normally quiescent, suprabasal (differentiated) cells (Genther et al., 2003). It also acts as an oncogene, as it activates the EGFR in a ligand-dependent manner (Crusius et al., 1998, Tomakidi et al., 2000). Using transgenic mice expressing the HPV 16 E5 protein under the promoter of keratin 14, Maoufer et al. showed that E5 acts as an oncogene and contributes to tumor formation during either a two-stage DMBA/TPA carcinogenesis assay (Maufort et al., 2007), or after treatment with exogenous estrogen mice (Maufort et al., 2010) .

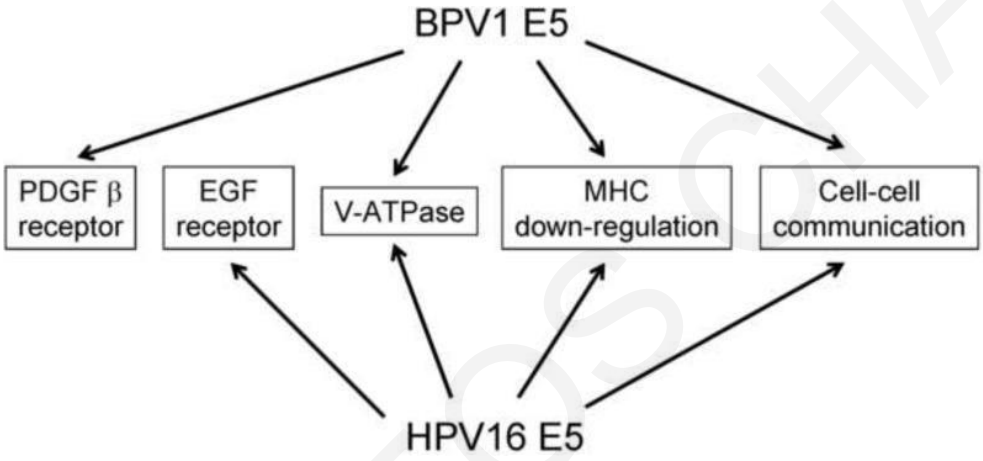


Figure 6: Main targets and activities of the E5 viral protein (DiMaio and Petti, 2013) during infection with BPV1 and HPV 16.

1.1.3.4 HPV E6

HPV E6 oncoprotein is a small protein, (HPV 16 E6 consists of 151 aminoacids) encoded by the early region of the virus. The basic structure feature of this protein is the existence of two atypical zinc fingers, joined by an interdomain linker; at the base of these fingers are two motifs that have two cysteines, which are conserved in all types of HPV (Figure 7) (Cai et al., 2013).

The primary target of E6 is the inactivation of the tumour suppressor protein p53 (Scheffner et al., 1990). E6 protein binds to the conserved motif of p53 LXXLL and subsequently binds with

the E6-associated protein (E6AP), which acts as a ubiquitin protein ligase and binds the central region of p53 and targets it for proteosomal degradation (Scheffner et al., 1993) (Figure 8). This in turn results in inhibition of apoptosis. E6 can also associate directly with p53, abrogating its transcription.

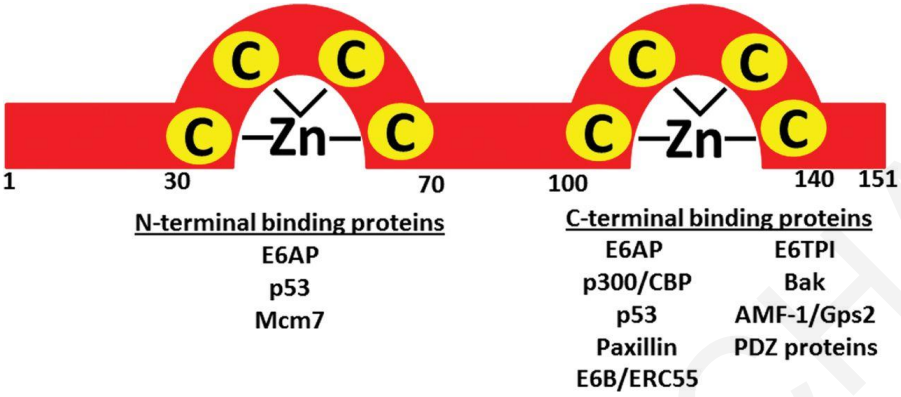


Figure 7: Structure of E6 oncoprotein with binding motifs for cellular partners (Miller et al., 2012). The two zinc-finger domains and all the n-terminal and c-terminal binding proteins identified are shown.

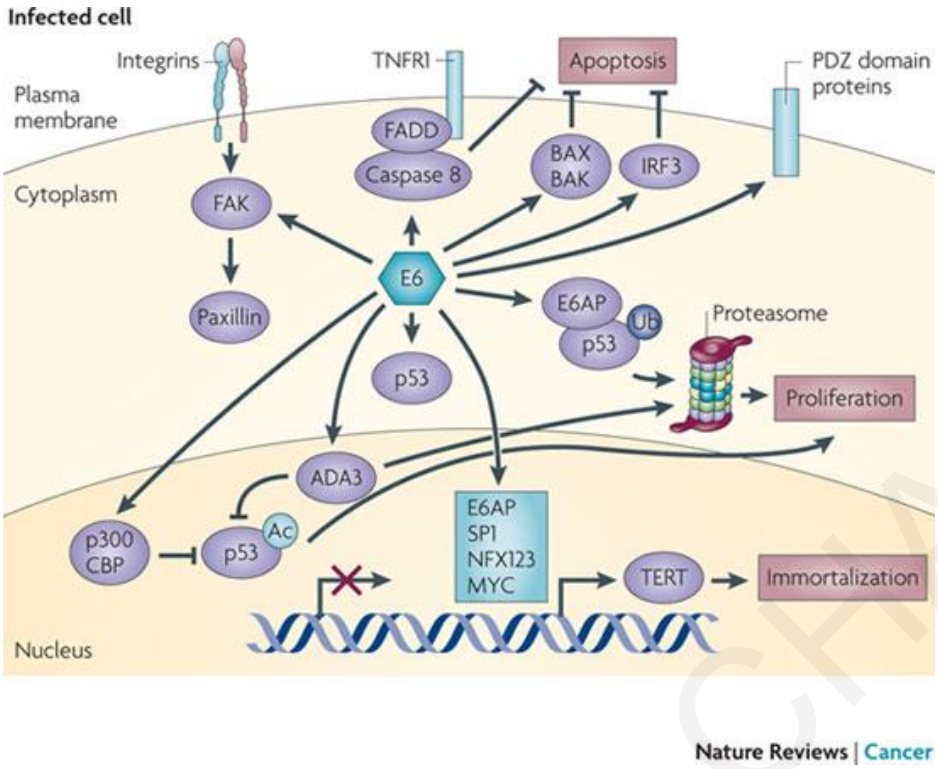


Figure 8: The E6 viral oncoprotein interacts with a diverse array of cellular targets, with functions in apoptosis, cytokeratin regulation, cell proliferation and telomere regulation (Moody and Laimins, 2010).

E6 also interferes with p53 functions independent of p53 binding and increases its instability through interactions with histone acetyltransferases p300, CREB binding domain and histone acetyltransferase ADA3. As a result the ability of these factors to acetylate p53 is inhibited, (Moody and Laimins, 2010), which in turn leads to inhibition of the transcriptional activity of p53 and deregulation of the activation of specific p53-dependent transcription factors (Patel et al., 1999).

E6 can also interfere with p53-independent apoptotic pathways. It can associate with Bak, a member of the Bcl-2 family. Bak acts through the formation of pores in the mitochondrial membrane in order to release Cytochrome-C from the mitochondria, which leads to the induction of caspase apoptotic cascades (Moody and Laimins, 2010).

Furthermore, E6 oncoprotein affects the telomerase activity by increasing the transcriptional levels of human telomerase reverse transcriptase (hTERT). This is an essential step to cell immortalization (Gewin et al., 2004, Moody and Laimins, 2010, Veldman et al., 2001, Veldman et al., 2003, Klingelhutz et al., 1996).

1.1.3.5 HPV E7

E7 oncoprotein is a small acidic polypeptide consisting of approximately 100 amino acids. The structure and function of the E7 oncoprotein has important similarities to the Adenovirus E1A protein, and the Sivian Virus 40 (SV40) large T antigen (LT). (Munger K. et al, 2001). E7 is divided into three main areas (Figure 9), the conserved domains CR1, CR2, CR3. CR2 encloses the LXCXE motif (shared by E1A and SV40 LT) that is required for the binding to the retinoblastoma tumor suppressor protein (pRb1) and its related proteins p107 and p130. All three proteins are master regulators of the cell cycle.

Inactivation of pRb leads to the release of the E2F transcription factors. Constitutive expression of E2F-responsive genes promotes entry into the S phase of the cell cycle resulting in DNA replication (Haedicke and Iftner, 2013, Hwang et al., 2002, Yugawa and Kiyono, 2009). Moreover, E7 affects the cell cycle by binding to CDK inhibitors p21 and p27 and activating cyclin E and cyclin A, which in turn activate CDK2 and permit G1 to S phase entry (Moody and Laimins, 2010, Gewin et al., 2004, Funk et al., 1997, Helt et al., 2002).

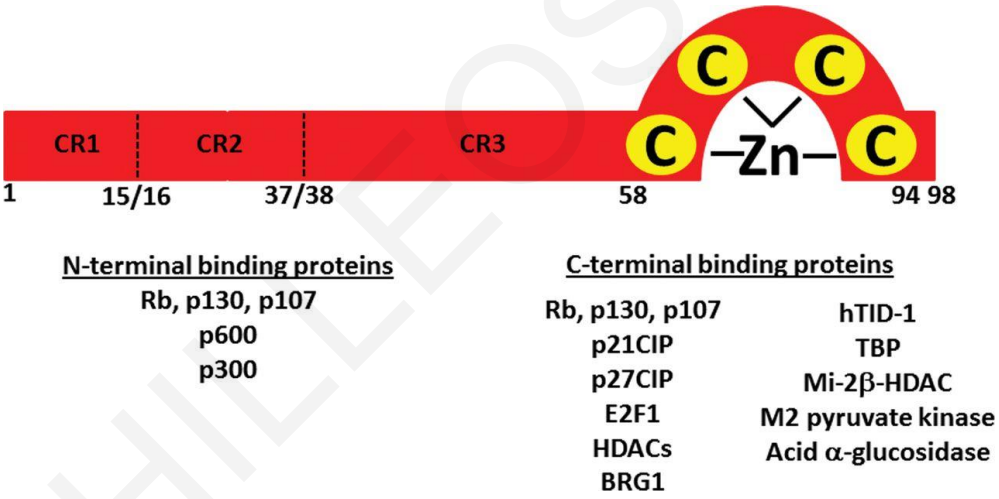


Figure 9: Structure of the E7 oncoprotein (Miller et al., 2012). In contrast to E6, E7 contains only one zinc-finger domain.

p130 acts during the G0/G1 transition whereas p107 acts in the G1/S transition as well as during the G2 phase. E2F4 and E2F5 normally form complexes with p107 or p130. p107 and p130 negatively regulate the activity of E2F family members (Songock et al., 2017). Degradation of p130 by E7 delays cell differentiation, allowing the virus to replicate its genome (Klingelhutz and Roman, 2012, Bodily et al., 2013) (Figure 10). It has also been

shown that degradation of p107 and p130 leads to increased transcription by RNA Polymerase III (Sutcliffe et al., 1999, Daly et al., 2005).

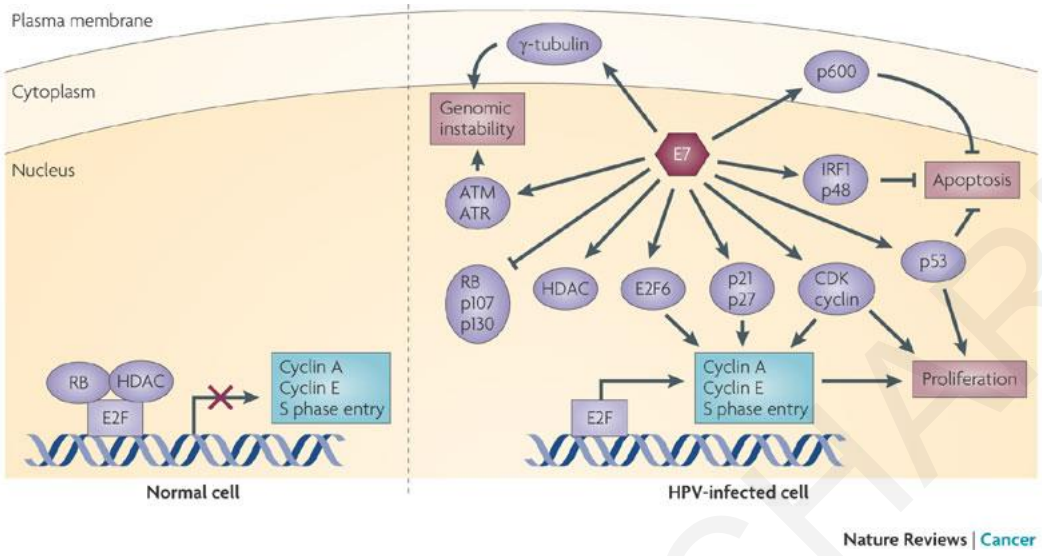


Figure 10: Schematic representation of E7 targets (Moody and Laimins, 2010).

1.1.3.6 L1 and L2

The L1 and L2 genes encode for the viral capsid proteins (McLaughlin-Drubin and Munger, 2009, Finnen et al., 2003). L1 is transcribed to 360 copies and L2 to 72 copies. These proteins are responsible for virion assembly and also have a major role in viral entry into the host cells (Buck et al., 2013, Kines et al., 2009). L1 is the major capsid protein and self-organizes into the capsid structure. While capsids can be formed using only the L1 proteins, no infectious virions can be produced since the viral genome cannot be encapsidated (Garcia-Vallve et al., 2005). L1-only capsids provide the basis for currently used HPV vaccine technologies (Schiller et al., 2012). L2 is the minor capsid protein and is necessary for viral DNA encapsidation. L1 can be used for phylogenetic analysis and categorization of the PV types into genera as it has a very conserved sequence (Tommasino, 2014).

1.1.4 Mode of Infection

1.1.4.1 HPV entry in the cells

HPV infects the cells in the basal layers of stratified squamous epithelia. Targeting of these particular cells in the epithelium is not random: they are the only actively dividing cells in the epithelial layer and HPV genome replication is tightly associated with the host-cell DNA replication.

The basal membrane contains heparan sulfate proteoglycans (HSPG). The L1 capsid protein attaches to the proteoglycans (Johnson et al., 2009, Joyce et al., 1999). This binding leads to the exposure of the L2 capsid protein N-terminus. This in turn enables furin to access the N-terminus and cleave it at the furin recognition site (Figure 11). The virion is then transferred to the cell membrane (Kines et al., 2009) and the virus enters the cell through endocytosis (Bousarghin et al., 2003).

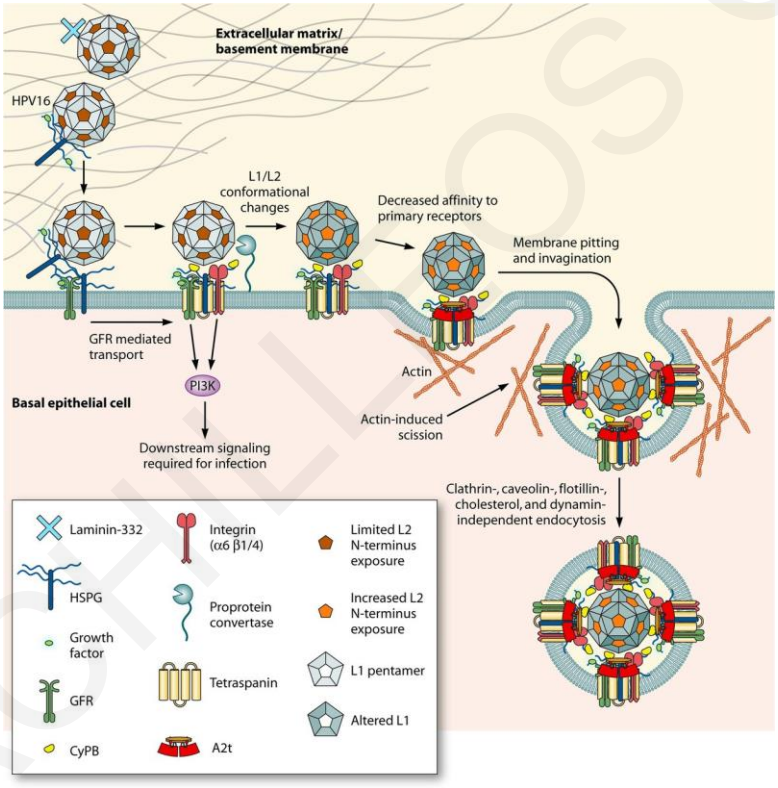


Figure 11: Viral entry into the host cell through endocytosis is facilitated by HSPG and Furin (Raff et al., 2013)

1.1.4.2 HPV Replication

Papilloma virus infects keratinocytes in the basal layer of the epithelium through microwounds. The infected basal cells continue their differentiation and migration to the epithelial surface. Meanwhile the virus continues to express its viral genes until mature virions are finally assembled and released from the uppermost epithelial layers (Figure 12).

After entry into the host cells, HPVs begin expressing the early proteins E1 and E2, which control viral replication from a circular episome in the nucleus (Doorbar et al., 2012, Egawa et al., 2012). The viral copy number is kept low (50-100 copies) and the number increases in the higher layers of the epithelium. E2 also acts as a repressor of several viral oncogenes and controls their expression (Doorbar et al., 2012, Blakaj et al., 2009, Doorbar, 2006). E6 and E7 viral oncoproteins interfere with cellular proteins and push the previously senescent suprabasal cells at the top epithelial layers into the S phase of cell cycle. While E7 binds to pRb and prevents its binding with transcription factor E2F, E6 binds to p53 and inhibits apoptosis (Klingelhutz and Roman, 2012). These events result in cell cycle entry and amplification of the HPV genome along with the host genome.

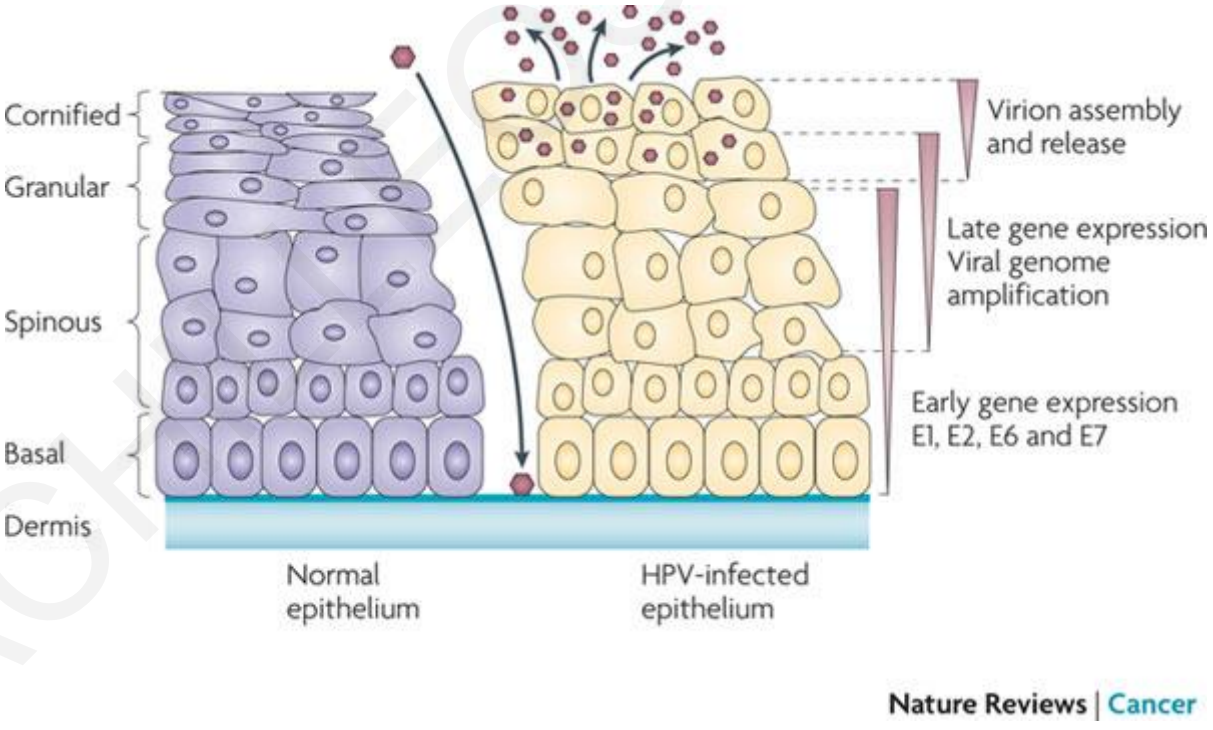


Figure 12: The HPV productive cycle (Moody and Laimins, 2010)

In the upper layers of the epithelium, transcription and translation of late viral genes L1 and L2 permits encapsidation of the virus, and subsequently the newly synthesized virions are released upon cell death. Newly released virions and can re-infect the tissue or spread to other hosts (Moody and Laimins, 2010)

1.1.5 Carcinogenesis

Persistent HPV infection can lead to cancer. Harald zur Hausen (awarded with the Nobel Prize in 2008) set a milestone for public health by discovering that cervical cancer can be caused by infection with high risk HPV types (Medicine, 2008).

Progression from an initial infection to carcinoma is very slow and needs years to decades to develop. This process is characterized by premalignant phases that can be identified with a Pap test (an examination that collects cervical cells which are then examined histologically for the presence of abnormal cells). Precursor lesions are categorized as cervical intraepithelial neoplasia (graded CIN1 to CIN3). The majority of CIN1 lesions (around 57%) regress spontaneously, an event that is probably mediated by cellular immunity (Ostor, 1993). From the remaining CIN1 lesions, about 11% to 20% progress to CIN2 (Doorbar, 2006). 43-55% of CIN2 lesions regress while 22% progress to CIN3 (Moore et al., 2007). As far as CIN3 lesions are concerned, 32% regress, and less than 12% eventually progress to cervical carcinoma.
The HPV genome is maintained in the host cell as an episome. Some times after persistent episomal replication of the virus, integration of the HPV genome into the host's genome may occur (Doorbar et al., 2012). There are indications that the sites of integration are not random but instead there are hotspots that favor integration, such as regions like the cytogenetic bands 3q28, 8q24.21 and 13q22.1 (Schmitz et al., 2012). Integration of the HPV genome at these sites may lead to the disruption of the E2 viral protein (Figure 13). E2 is a repressor of the expression of the viral oncogenes E6 and E7 and the disruption of E2 leads to overexpression of the oncogenes. As a result, host cells gain a growth advantage as they are "forced" by the oncogenes to proliferate and bypass apoptosis. They also exhibit genomic instability and they have a higher risk for immortalization and malignant transformation. Moreover, even though HPV infection triggers an inflammatory response, the virus manages to evade immunity and cause chronic inflammation along with the infection. This leads to production of reactive oxygen species (ROS) and cytokines in infected cells, and promotes carcinogenesis through DNA damage and genomic instability (Grivennikov et al., 2010).

There are also environmental co-factors that are implicating in HPV carcinogenesis. For example, UV radiation has been identified as a co-factor for skin carcinogenesis caused by cutaneous HPVs. It has been shown that cutaneous HPV predominate in areas that are sun-exposed (Accardi and Gheit, 2014). Another important co-factor in HPV carcinogenesis is estrogen levels (Elson et al., 2000).

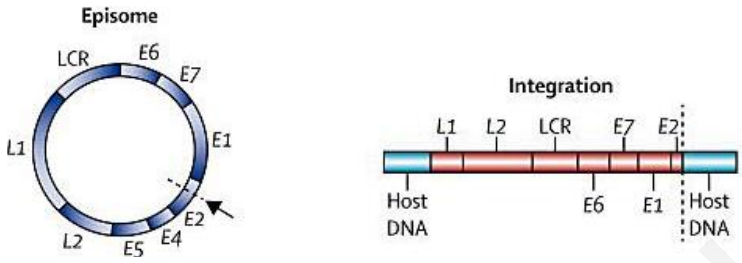


Figure 13: Integration of the viral episome into the host's DNA may lead to disruption of E2 viral protein. This event may lead to carcinogenesis due to increase and unregulated expression of E6 and E7 oncogenes. Adapted from (Woodman et al., 2007)

1.2 An HPV target: Telomerase and telomere homeostasis

HPV oncogenes have the ability to target cellular proteins such as pRb and p53, leading to increased proliferation and inhibition of apoptosis respectively. The increased number of divisions would normally come to an end due to shortening of the telomeres resulting in senescence (Harley, 1991, Masutomi et al., 2003). One of the ways in which HPV has been proposed to bypass this problem is by directly impinging on telomere homeostasis.

1.2.1 Structure and function of telomeres

Telomeres are repetitive nucleotide sequences at the end of the chromosomes (TTAGGG in vertebrates) that protect chromosomes from fusion, the DNA damage response mechanism and recombination. The name telomere derives from the Greek word telos (τέλος) that means "end" and the word meros (μέρος) that means "part". In humans the telomeric TTAGGG sequence is repeated about 2,500 times with the newborns having about 11 kb (Okuda et al., 2002) and older people less than 4kb (Arai et al., 2015). This reduction is due to the fact that telomeres cannot be fully replicated by DNA polymerase (end-replication problem), thus with

every cell division they get shorter and shorter. After a number of divisions, they reach a critically short length that leads to cellular senescence (Counter et al., 1992, Harley, 1991) (Figure 14). A study in 2007 showed that the critical short length of a telomere is 77bp in humans (Capper et al., 2007). If the telomeres get any shorter then fusion events in the end of the telomeres occur. Mice have a range in telomeric length between 20 to 150kb and the critical short length in mouse telomeres is 10kb (Zijlmans et al., 1997).

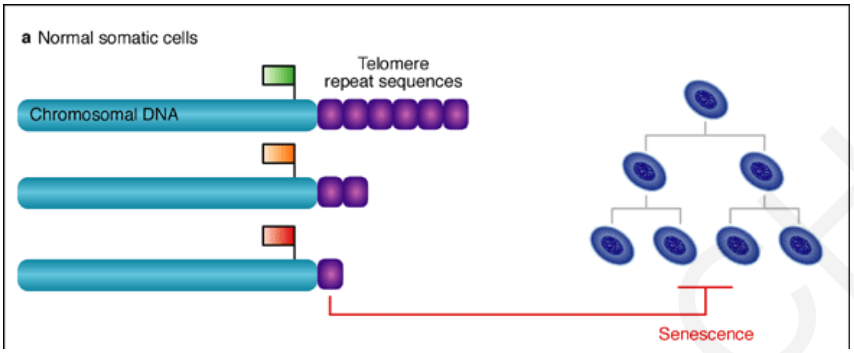


Figure 14: Telomeres shorten with every cell division until they reach a critical length that triggers senescence or apoptosis (Molecular Medicine Cambridge press 2002).

1.2.2 Structure and function of telomerase

Cells that undergo a large number of cell divisions, such as cancer cells, adult stem cells and embryonic stem cells have developed ways to avoid problems associated with telomere erosion. This is most frequently achieved via transcriptional activation of telomerase. Another way to maintain telomere length is the alternative lengthening of telomeres (ALT), a mechanism that is telomerase independent and is based on homologous recombination (HR) between the two telomere sister chromatids (Royle et al., 2009)

Telomerase is a special reverse transcriptase that can maintain telomere length (Greider and Blackburn, 1985) and is active in some cell types that have to undergo a large number of divisions, such as stem cells and cancer cells. Telomerase has additional roles such as preventing chromosomes from fusing (Blackburn et al., 2000, Kim et al., 2003, Zhu et al., 1999). Telomerase consists of three subunits, a) the Terc, an RNA subunit, b) Tert, a protein subunit and c) the protein dyskerin (Dkc1), which binds and stabilizes Terc.

TERC has a secondary core structure that is conserved among eukaryotes in general (Figure 15) and contains the TERT binding motifs but also include sequences and elements demonstrated to be critical for enzyme function (Lin et al., 2004).

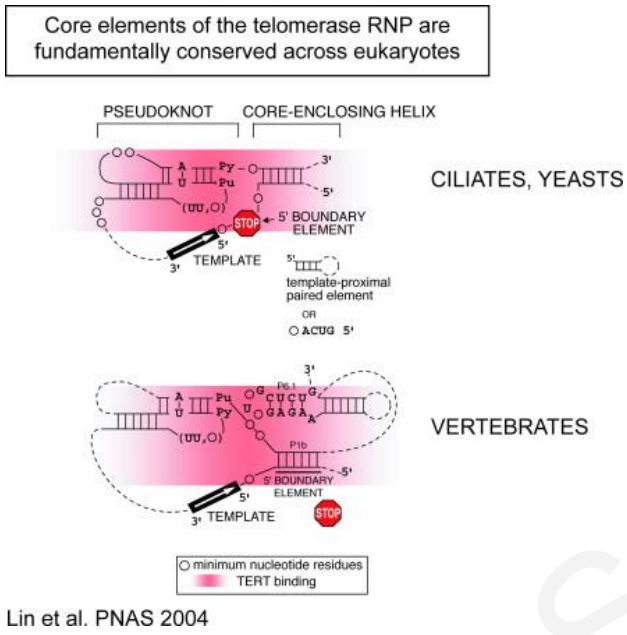


Figure 15: A universally conserved core in telomerase RNA.(Lin et al., 2004)

Various *in vitro* studies have established that activation of telomerase can lead to cell immortalization. It has been shown that human fibroblasts *in vitro* can be immortalized by transfection with a hTERT cDNA expressing vector (Bodnar et al., 1998). A study in 2012 has shown that hTERT is necessary but not sufficient to immortalize primary fibroblast from donkey, Burchelli's zebra and Grevy's zebra (equid species) (Vidale et al., 2012) due to incompatibility of the hTERT and equid TERC. This confirms that the immortalization of the fibroblast was due to telomeres elongation.

1.2.3 E6 and E7-driven cancers and telomere homeostasis

Telomerase is normally active in stem cells or in cancer cells in order to keep the telomeres to a normal length through subsequent rounds of cell division. *In vitro* studies have shown

that HPVs can maintain telomere length either through transcriptional induction of telomerase or through alternative lengthening of telomeres (ALT).

HPV-16 E6 oncoprotein increases telomerase activity via transcriptional and post-transcriptional mechanisms. It was shown to increase the transcriptional levels of human telomerase reverse transcriptase (hTERT). That is an essential step to cell immortalization (Liu et al., 2008b). This transcriptional activation of hTERT through E6 was shown to require its heterodimerization with E6AP (Galloway et al., 2005, James et al., 2006, Liu et al., 2009), or through interactions with other cellular proteins. For example E6 expression in keratinocytes reduces the amount of USF1 and USF2 that compete against c-MYc/Max for the binding sites on E-boxes in the proximal promoter of hTert (McMurray and McCance, 2003) resulting in enhancement of hTERT expression (Kyo et al., 2000). Furthermore, E6 oncoproteins interact directly with the nuclear transcription factor, X box binding protein 1-123 (NFX1-123) increasing the post-transcriptional levels of hTERT, by increasing its mRNA stability (Galloway et al., 2005). Telomerase activity can increase without any changes in the hTERT mRNA, but most likely with post-transcriptional modifications through the direct interaction of E6 with hTERT (Liu et al., 2009, Gewin et al., 2004, Klingelhutz et al., 1996). Kiyono et al. showed that the E7-induced inactivation of RB/p16 is necessary along with E6-induced activation of telomerase activity for immortalization of fibroblasts and keratinocytes in culture (Kiyono et al., 1998). It has been also demonstrated that E7 enhance the HPV E6 driven effect on telomerase activity (Liu et al., 2008b). Jeong et al show that re-expression of either E6 or E7 in Hela cells, after their removal, can increase hTERT (Jeong Seo et al., 2004).

Telomeres can also maintain their length through a telomerase independent way. This mechanism is termed alternative lengthening of telomeres (ALT). pRB has been shown to form a complex with Rad50 and RINT-1 that blocks the ALT mechanism. HPV E7 protein binds to pRb and causes its degradation, which results in the release of Rad50 (Zhang et al., 2012). Rad50 promotes ALT through the formation of the MRN complex. The E7 protein promotes ALT through the degradation of pRb and facilitates cell immortalization (Zhang et al., 2012) .

All the evidence presented here derives from *in vitro* models. The *in vivo effect of* telomeric lengthening on HPV-related carcinogenesis and stem cell mobilization is still unknown. It has been proposed that the multiple ways of targeting telomere homeostasis point to an

evolutionary adaptation, significant not only in the context of carcinogenesis but presumably during the viral lifecycle. A major barrier to further understand the importance of telomerase regulation in the context of HPV viral oncogene expression is the lack of studies performed *in vivo* in the context of physiological levels of oncogene expression. This study represents the first effort to examine the interaction of the HPV viral oncogenes with telomere homeostasis in the context of a living organism.

1.2.4 Telomere homeostasis in stem cells and in cancer

In order to study the *in vivo* roles of telomerase, mice lacking telomerase activity were previously generated (Blasco et al., 1997a, Blasco et al., 1997b). This inactivation can be achieved using deficient mice for TERT or TERC. Terc-deficient mice generated by Blasco and colleagues exhibit telomere shortening with every generation. (Blasco et al., 1997a, Blasco et al., 1997b). Terc-deficient mice with short telomeres have dysfunctional epidermal stem cells (these stem cells lose the ability to mobilize and regenerate the skin and the hair) (Flores et al., 2005). The Terc-deficient mice with very short telomeres, are resistant to multi-stage skin carcinogenesis (Gonzalez-Suarez et al., 2000), which clearly indicates the importance of normal telomeric length in carcinogenesis, as well as the vital role of telomerase in proliferating cells, including stem cells and cancer cells.

Gonzalez-Suarez (2001) has shown that K5Tert mice are about two-fold more susceptible to skin tumorigenesis than wild-type mice and exhibit an increased rate of wound healing. In subsequent studies, this group generated K5-Tert/Terc−/− mice and showed that increased tumorigenesis as well as faster wound healing (due to Tert overexpression), are dependent on Terc.

The mouse is a good animal model for studying telomere homeostasis as it can be engineered to have different levels of telomerase activity or be mutant for different telomere-binding proteins. Unfortunately, this model has some inherent limitations. Mus musculus has significantly longer telomeres compared to humans and mice with no detectable telomerase activity were able to survive for 6 generations. On the other hand, the threshold for critically short telomeres in mice is around 10kb (much longer than the respective threshold in humans – 77bp). In any case, these differences must always be considered when performing experiments related with telomere length maintenance in mice.

1.2.5 Other functions of telomerase

Tert reportedly is not involved exclusively in telomere lengthening, for example it has been reported to activate wnt signaling (Park et al., 2009) and also is involved in NF-κB pathway (Li and Tergaonkar, 2014, Ghosh et al., 2012) (Figure 16). Ghosh et al showed that telomerase binds to NF-κB p65 subunit and induces the IL-6, IL-8 and TNF-a cytokines, resulting to regulation of NF-κB-dependent gene expression. They also show that NF-κB can induce telomerase transcription, thus suggesting that this feed-forward regulation leads to coexistence of increased telomerase activity and chronic inflammation in human cancers (Ghosh et al., 2012). Ding et al showed that mutated hTERT that lacks reverse transcriptase activity, was able to activate NF-κB–mediated transcription and affect the expression of several NF-κB target genes the same way as the non-mutated hTERT (Ding et al., 2013).

Besides NF-κB pathway, telomerase is also involved in the Wnt/β-catenin pathway. Park et al using ChiP assay showed that in mouse intestine, TERT binds to promoters of Wnt target genes (cyclin D1 and c-Myc). They also show that in Xenopus embryos TERT or a catalytic inactive TERT binds to b-catenin and promotes anterior–posterior axis duplication (Park et al., 2009). Okamoto et al showed that TERT forms a complex with Brg1 and nucleostemin (NS) that can affect the Wnt/β-catenin pathway (Okamoto et al., 2011). A study from Blackburn's lab showed that in Hela and breast cancer cell lines there is no physical interaction among TERT and Brg1 (Brahma-related gene 1) or b-catenin (Listerman et al., 2014). Strong et al showed that in mice loss of TERT function in a physiological setting does not affect Wnt signaling (Strong et al., 2011). Thus, the telomerase role on Wnt signaling can differ due to different cell types, mouse background or experimental conditions (Li and Tergaonkar, 2014).

It has been also shown that TERT independently of telomere elongation activity, can activate the transcription of the vascular endothelial growth factor (VEFG) (Zhou et al., 2009), thus can promote angiogenesis during cancer progression.

The Terc subunit of telomerase in mammals reportedly is only involved in the canonical roles of telomerase (telomere elongation). Only a single study in zebrafish suggests non-canonical roles for the RNA subunit of telomerase (Alcaraz-Perez et al., 2014). In this study, Terc was shown to modulate expression levels of the transcription factors spi1 (myeloid regulator) and

gata1 (erythroid regulator), affecting the myeloid-erythroid fate (Alcaraz-Perez et al., 2014).

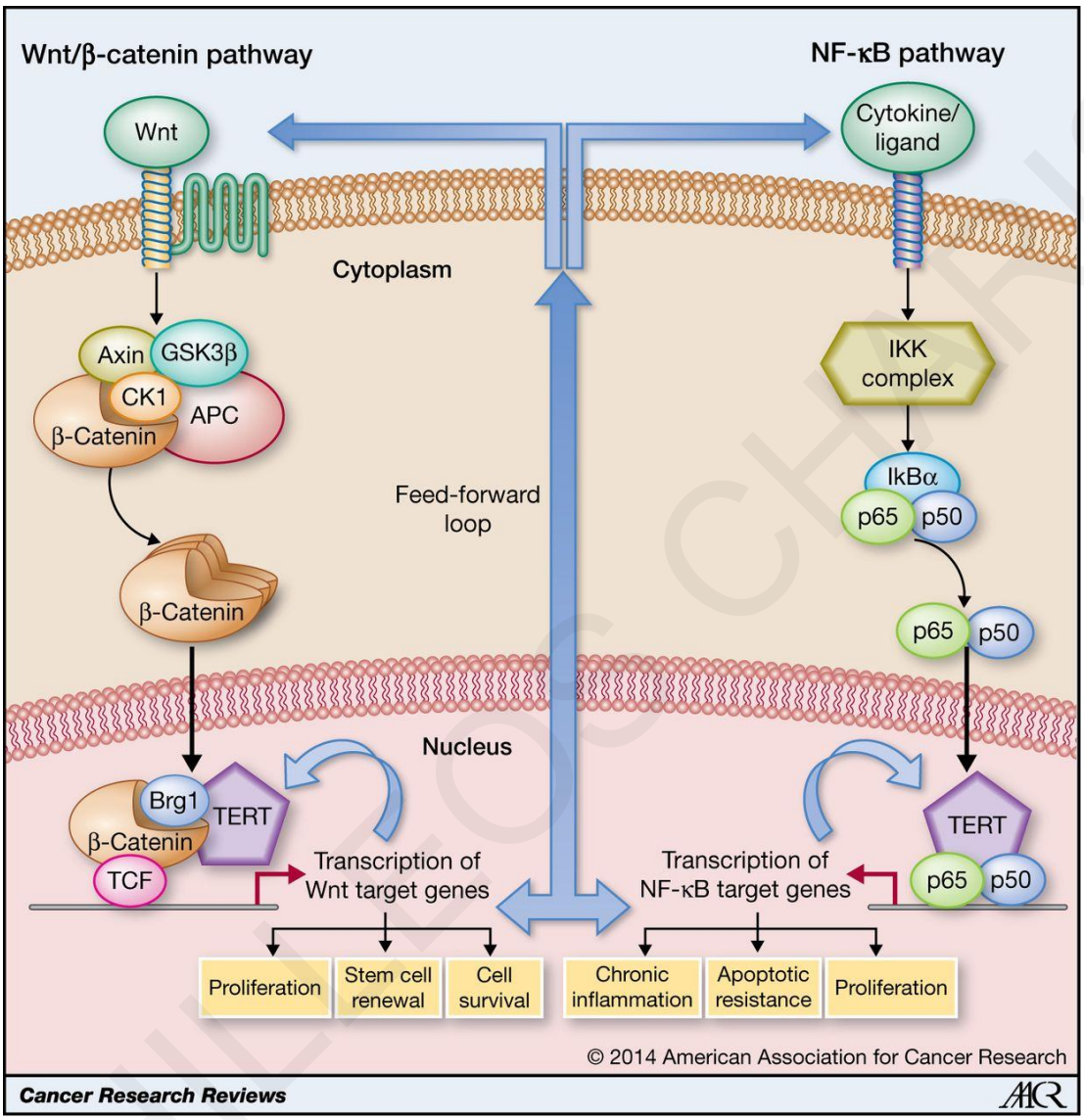


Figure 16: Schematic representation of the feed-forward regulation among TERT and Wnt/catenin pathway as well as NF-κB pathway during cancer progression (Li and Tergaonkar, 2014).

1.2.6 Telomere length as a regulator of genes

Telomeres can regulate genes over long distances through a mechanism called Telomere Position Effect (TPE) first described in Drosophila (Hazelrigg et al., 1984, Levis et al., 1985). It has been shown that telomeres can reach genes at 10 Mb away when they are long (Robin et al., 2014) (Figure 17). Shay et al suggested that the human *TERT* gene may autoregulate itself since it is located proximal to the telomere end of chromosome 5 (Shay and Wright, 2000). Kim et al. showed that long telomeres are indeed able to form a telomere –chromatin loop and suppress hTERT expression (Kim et al., 2016) and this mechanism is bypass during cancer progression or by aging as telomeres become shorter (Figure 18).

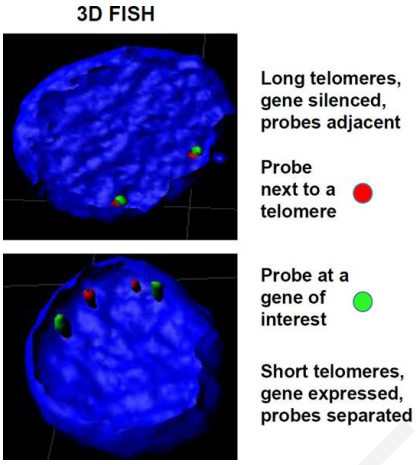


Figure 17: 3D FISH with a sub-telomeric probe and a distal gene of interest (Shay, 2016). Because of longer telomeric length probe signals are adjacent in younger cells, in contrast to older cells with shorter telomeres

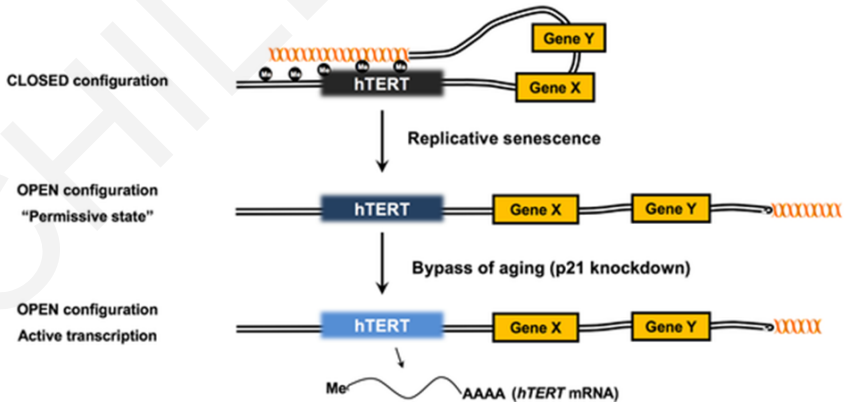


Figure 18: Schematic representation of telomere length dependent looping (Kim et al., 2016). hTERT is regulated by long telomeres (top), but telomere shortening abrogates regulation and allows

increased transcription of hTERT (bottom). Genes that are very close to telomeres (X and Y in the illustration) are not regulated by this mechanism.

1.3 Cancer, stemness and HPV

HPV gains access to the body through micro-wounds (or abrasions) and infects cells in the basal layers of stratified squamous epithelia. This layer includes committed cells as well as the tissue stem cells (Mascre et al., 2012). Current evidence suggests that the virus infects both committed cells, and stem cells. It has been suggested that viral infection of the stem cell tissue cells is more likely to lead to carcinogenesis than infection of committed cells (da Silva-Diz et al., 2013, Kranjec and Doorbar, 2016, Strati, 2017). HPV-mediated carcinogenesis is a decades-long process. Infection of a stem cell is thought to be one of the mechanisms in which the virus can maintain infection for long periods of time (Maglennon et al., 2011, Doorbar, 2013b).

Various studies suggested that stem cells play a crucial role in the tissue response to infection (Strati, 2017). Infection, as well as the creation of the micro-wounds, can trigger an inflammatory response that may also affect the stem cell population in the area and lead to tumorigenesis (Karin and Clevers, 2016, Michael et al., 2016). While transgenic animals have been critical tools to understand the changes in stem cells, which express viral oncogenes, they cannot adequately enlighten the changes occurring in tissue stem cells in the context of viral infection. The newly isolated murine papillomavirus infecting mice via a site of wounding could therefore help elucidate the role of inflammation on stem cell biology (Ingle et al., 2011a, Handisurya et al., 2013a, Uberoi et al., 2016a). Maglennon et al. showed that infection with rabbit oral papillomavirus (ROPV) persists in a latent state, and that the site of latency is a subset of basal epithelial cells (Maglennon et al., 2011), which the authors suggest they are epithelial stem cells. Committed cells have a shorter life in the tissue than stem cell cells. Thus, the length of time for which viral maintenance can occur cannot be sustained in a committed cell in stratified epithelia, which have a rapid turnover. The main site of high risk HPV infection is the cervix, and it has been hypothesized that the site of cancer origin within this tissue is the transformation zone (Lopez et al., 2012, Herfs et al., 2012, Herfs et al., 2013), an area that includes the reserve cells or the tissue stem cells (Elson et al., 2000, Herfs et al., 2012, Crum, 2000, Martens et al., 2007, Martens et al., 2009).

1.3.1 Skin as a model tissue for stem cell research

HPVs can infect cutaneous or mucosal stratified epithelia. The HR HPV types of genus alpha can lead to carcinogenesis of the genital and upper respiratory tracts after persistent infection. Cutaneous beta HPV types together with UV radiation can promote NMSC (non-melanoma skin cancer). HPV productive infection depends on the differentiation ability of the target tissue. Skin has been extensively used as a model tissue in HPV research as it is easily accessible and well-studied (Mascre et al., 2012). Given the lack of a well characterized stem cell population in the cervix, skin stem cells are currently the best described model for stratified epithelial tissue for both cutaneous and mucosal HPV types (Strati, 2017).

Different types of stem cells have been shown to be important during homeostasis and regeneration (Blanpain and Fuchs, 2014, Ito et al., 2005). Blanpain et al lineage traced 5 different stem cell progenitors in the hair follicle, in order to elucidate these processes (figure 19 A). In these experiments, stem cells isolated from the bulge were shown to be capable of regenerating the whole follicle (Figure 19 B).

The most well-studied stem cell population of the skin can be found in the hair follicle in an area called the bulge. These cells are slow-cycling, and when they divide asymmetrically, generating a stem cell and a progenitor cell. The progenitor cell can further divide or differentiate, in order to repair wounds in the tissue, whereas the stem cell produced ensures the stem cell population numbers in the tissue is replenished (Blanpain and Fuchs, 2014) (Figure 19 B).

There are several markers that can be used to detect and isolate stem cells in the hair follicle. CD34, a cell surface marker, is the best marker for the enrichment of hair follicle stem cells. The CD34+ population is subdivided based on α6 integrin expression (Blanpain et al., 2004). Keratin 15 (K15) is also a highly expressed marker in the bulge area and also co-localizes with CD34 expression (Cotsarelis, 2006, Liu et al., 2003, Lyle et al., 1998) .

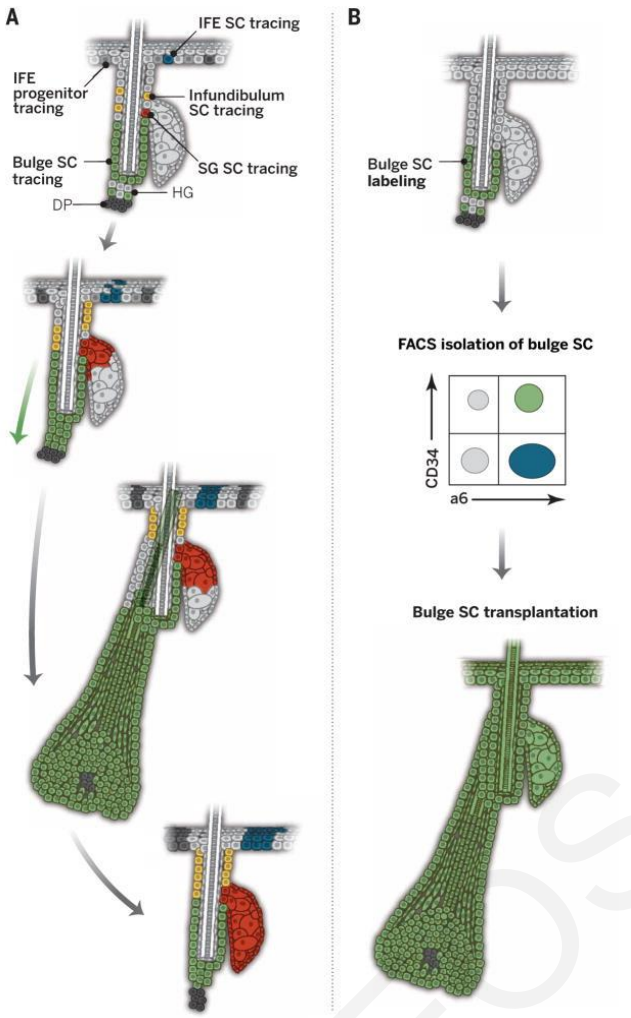


Figure 19: Hair follicle progenitor cells. A) Schematic illustration of the linage tracing of hair follicle progenitor cells in mice. B) Stem cells isolated from the bulge are capable of regenerating the whole hair follicle.(Blanpain and Fuchs, 2014)

The hair follicle model has been extensively used to study HPV infection and biology, as well as to study the implication of stem cells in carcinogenesis (da Silva-Diz et al., 2013, Michael et al., 2013a, Auewarakul et al., 1994, Lambert et al., 1993, Merrick et al., 1992, Lapouge et al., 2011). The hair follicle consists of the papilla, the matrix, the root sheath, the bulge and the sebaceous glands (Figure 20). Sebaceous glands, are responsible for the production of sebum, an oily substance that conditions the hair and the skin. The papilla is located in the base of the follicle and consists of connective tissue and capillary loops. The matrix is around

the papilla. The root sheath is composed of the internal and the external root sheath. These structures protect the hair shaft and help it form while growing.

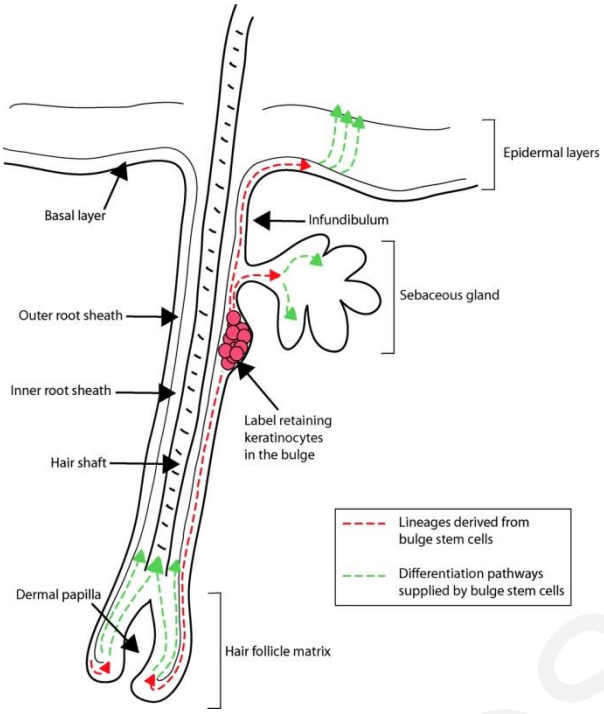


Figure 20: Schematic representation of hair follicle. The stem cells reside in the bulge and are thought to contribute to the lineages of the hair follicle, the sebaceous gland, and the epidermis (see red dashed lines). These are quiescent stem cells that can retain brdU. Transiently amplifying progeny of bulge stem cells in each of these regions differentiates as shown (see green dashed lines) (Alonso and Fuchs, 2003)

The hair follicle has distinct and regular cycles of involution and regeneration (Figure 21). Anagen is the growth phase. During this phase the cells are dividing rapidly, and the hair shaft is growing. After anagen is completed, the catagen phase follows. Catagen is a regressing phase. This phase lasts for a shorter period than anagen and at this stage the hair converts to a club hair (hair before shedding).

During catagen, the lower two-thirds of the hair follicle rapidly regress, mainly by apoptosis of the matrix, inner root sheath and outer root sheath keratinocytes, while bulge stem cells escape apoptosis.

After the club hair forms completely, the follicle enters the telogen phase. Telogen is a resting phase. In telogen, the hair follicles are very small and the layer of adipose tissue is thin. With each additional cycle telogen duration expands, and hair follicle cycling slows down with aging (Schneider et al., 2009). During telogen the dermal papilla get close to the bulge region

and allow direct interactions between stem cells and the papilla. This step is essential for the initiation of a new cycle and the anagen phase (Paus and Foitzik, 2004).

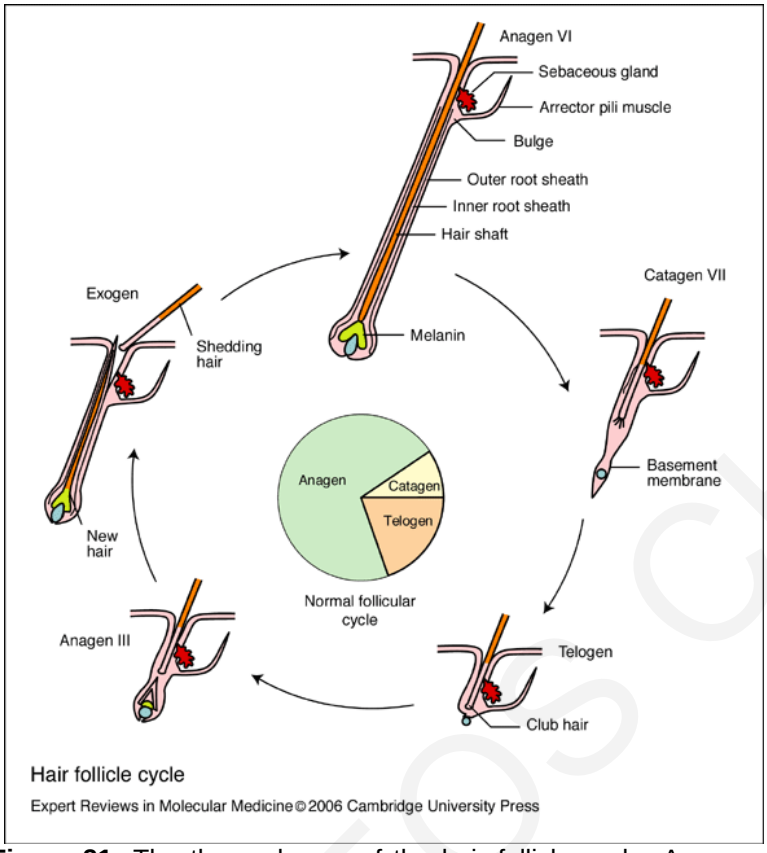


Figure 21: The three phases of the hair follicle cycle: Anagen (growth phase), catagen (regression phase) and telogen (resting phase) (Lu et al., 2006).

1.3.2 HPV and its role in stemness

Even though HPVs are well studied for their role in carcinogenesis and for the development of relevant vaccines, they have not been studied extensively for their link with stemness, and the effect of oncogene expression on stem cell characteristics *in vivo* is mainly unknown. There is evidence from studies in transgenic mice that mucosal (da Silva-Diz et al., 2013, Michael et al., 2013b) and cutaneous (Lanfredini et al., 2017) HPVs can affect stem cell dynamics during infection. Michael and others have shown that expression of early HPV16 oncogenes induces alterations in tissue stem cells causing them to express both typical stem cell markers (such as K15) (Michael et al., 2013b, da Silva-Diz et al., 2013) and atypical markers (such as p-cadherin) (da Silva-Diz et al., 2013). Loss of quiescence and increased

mobilization of stem cells was also observed (Michael et al., 2013b). In addition, it was previously shown that HPV-associated tumorigenesis is linked with non-quiescent stem cell populations in the skin (Jaks et al., 2008) and that HPV16-induced tumors in mice were derived from non-quiescent LGR5 positive cells. (da Silva-Diz et al., 2013). These results suggest that HPV infects the stem cell population causing an increase in their mobilization as well as other changes that make the tissue more susceptible to oncogenesis (Strati, 2017). Moreover, HPV oncogenes target cellular proteins that are implicated in stemness, such as pRb and p53 (direct targets of the E7 and E6 viral oncogenes respectively). pRb is known to control a pluripotency network of genes through the transcription factors sox-2 and oct-4 (Kareta et al., 2015) (Organista-Nava et al., 2016) (Brehm et al., 1997, Brehm et al., 1999). Furthermore, the expression of Nanog protein can be controlled by p53, a direct target of HPV E6 oncoprotein (Lin et al., 2005). A recent study showed that cancer stem-cell-like cells that were isolated from tumors positive for the HPV E6 oncoprotein expressed Oct4, Sox2, Nanog, and Lrig 1 (Tyagi et al., 2016). Another important pluripotency-related target is telomerase, which has been shown to be upregulated by the HPV E6 oncogene and to cause changes in the stem cell niche of the area (Flores et al., 2005, Liu et al., 2009, Sarin et al., 2005). Moreover, both high risk HPV (mucosal) and cutaneous HPVs, as well as newly isolated murine papillomaviruses can inhibit the Notch pathway inhibiting cell differentiation (Meyers et al., 2017b, Kranjec et al., 2017). Lastly, HPV E7 has a major role in the epigenetic reprogramming of the infected tissue as it can activate the transcription of two histone demethylases (KDM6A and KDM6B) (McLaughlin-Drubin et al., 2011).

The above studies suggest that infectious agents may directly target stem cells or transform committed cells to stem-cell-like cells with increase potential for carcinogenesis. Despite the obvious impact of HPV on stem cells of the host, it is still unclear how stem cell modulation by the virus affects the viral life cycle or cancer development. One of the aims of this study was to illuminate the extent to which the changes mediated on the stem cells by viral oncogenes are dependent on canonical telomere-lengthening.

1.4 Models of Papilloma Virus study

1.4.1 *In vitro*

HPV is the main cause of cervical cancer. The prevalence of HPV infection is high. The development of model systems that would facilitate the study of HPV infection *in vivo* is therefore extremely important, though challenging. Papillomaviruses are strictly species specific and thus human viruses cannot be studied in an animal host. HPV cannot be propagated in monolayer cell cultures, thus the *in vitro* system that is currently in use for HPV study is organotypic raft culture, where stratified and differentiated epithelial cells are generated (Frattini et al., 1997).

Organotypic raft culture systems provide a tissue culture system that recapitulates the full HPV life cycle, and the process of genome maintenance in the epithelial basal layer, to be modeled (Dollard et al., 1992, Meyers et al., 1992) (Figure 22). This model can be used to study the HPV life cycle (including virus production) (Dollard et al., 1992). It has been shown that raft cultures can exhibit characteristics similar to those of dysplastic lesions seen during an *in vivo* infection with HPV (Anacker and Moody, 2012). A limitation of this method is the need for primary cells in early passages. The viral genomes are usually transfected in primary keratinocytes and the efficiency of DNA transfection can vary among different batches of cells. Furthermore, the methods can be time consuming. While this has been ameliorated with the advent of CRISPR technologies, it is difficult to efficiently knockdown host proteins that are needed for basal cell proliferation (Chow, 2015). In addition, high titer of viruses and robust viral DNA amplification have not been reported in raft cultures of immortalized cells, compromising their use in mutagenic analyses (Chow, 2015). Recently a new study was published by Martin Sapp's lab that shows a high efficiency infection model for primary keratinocytes that allows the study of the complete viral lifecycle (Bienkowska-Haba et al., 2018).

There is also a complementary approach, using a monolayer culture with high-calcium medium or growth in suspension culture using medium containing methylcellulose, that can be used to examine some aspects of the HPV productive life cycle (Anacker and Moody, 2012). Methylcellulose suspension induces differentiation but fails to recapitulate the three dimensional structure of the epithelium which is a proxy for real-life infection.

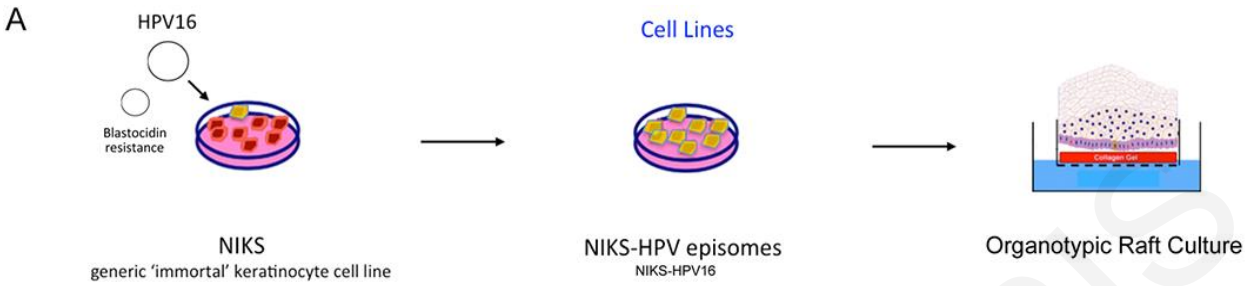


Figure 22: Schematic representation of the procedure for generating organotypic raft cultures (Doorbar, 2016). The basic procedure involves the co-transfection of papillomavirus genome along with a drug-resistance plasmid in keratinocytes. The keratinocytes used can be primary cells isolated from clinical biopsies, or an immortalized keratinocyte cell line, such as NIKS. After drug selection, transfected cells supporting viral DNA replication are expanded into cell lines, which can then be cultured at an air–liquid interface in organotypic raft culture to recreate the full differentiation-dependent HPV life cycle.

In addition to the expression of viral genes in keratinocytes, another approach is the study of isolated cells of HPV infected patients. A number of cell lines were isolated and are available for use: as w12, (contains episomal HPV16 genome), CIN-612 (with HPV31 episomes) (De Geest et al., 1993, Hummel et al., 1992, Stanley et al., 1989, Sterling et al., 1990).

The advantage of using these cell lines is that they originate from a natural *in vivo* infection. Those lines can support the full productive life cycle of their respective HPV genome when propagated in organotypic raft culture. These models are valuable tools to provide insights into the life-cycle organization of the PVs, especially if they are combined with analysis of clinical material, and with molecular studies that address the role viral protein proteins in the infected cell. However, the precise cellular origin of these cell lines and is not known, thus it is difficult to determine how different epithelial cells differentially regulate the viral gene expression (Doorbar, 2016)

1.4.2 In vivo models

There are many *in vitro* models but some aspects of papiloma virus biology such as carcinogenesis cannot be studied. In order to address those issues *in vivo* model are also widely used in the study of papilloma viruses.

1.4.2.1 Animal Papilloma virus infection models

Because papillomaviruses have stringent host tropism, human viruses do not successfully infect laboratory animals. Thus, a range of animal viruses have been used as models for papillomavirus studies.

Some of these models involve infection of animals with animal PVs that cause lesions (Brandsma, 1994). The first models were the bovine papillomavirus type 1 (BPV1) and cottontail rabbit papillomavirus (CRPV). While these models offered useful perspectives, neither of them is an ideal model system. Bovine papilloma virus 1 can cause cutaneous lesions but bovines are large animals and are inconvenient to handle. CRPV has a more easily manageable host (the cottontail rabbit) (Brandsma and Xiao, 1993, Brandsma et al., 1991, Brandsma et al., 1992, Zhang et al., 1999). However, cottontail rabbits are wild animals and do not like to be kept in captivity. For this reason, infection of domestic rabbits was preferred, and this model was used for the HPV vaccine design (Amella et al., 1994, Mejia et al., 2006).

One of the ways to study papillomaviruses using the more common and tangible laboratory model, the mouse, is xenotransplantation. Xenotransplantation approximate the *in vivo* infection but of course it depends on the availability of human tissue. This model requires the transplantation of human tissue or human tumor in mice either ectopically (at a different place than the origin of the tissue) or orthotopically (Hiroshima et al., 2015) (at the organ of origin in the mouse). The first studies used human skin implants that were infected with either HPV16, 11 or 1. Those implants were propagated under the kidney capsule of nude mice (Bonnez, 2005, Bonnez et al., 1998, Kreider et al., 1990, Stoler et al., 1990). This system allows successful production and amplification of large quantities of infectious HPV, which is required for reinfection and passage studies. Unfortunately, this model system is limited to

the study of established cancers, and it is not an appropriate tool for studying the life cycle of the virus.

In 2011 a new mouse papillomavirus (MmuPV1) that naturally infects mice was isolated and characterized (Ingle et al., 2011b). MmuPV1 belongs to the π genus of papilloma viruses with MaPV1, MmiPV, McPV2 and RnPV1 (de Villiers et al., 2004, Schulz et al., 2009) and has a genome of 7510bps with a 46.6mol% G+C content (Joh et al., 2011). It is organized like the other papilloma viruses in three regions (early region, late region and non-coding region) (Figure 23). MmuPV1 has seven open reading frames (E1, E2, E4, E6, E7, L1 and L2) (Joh et al., 2011) .

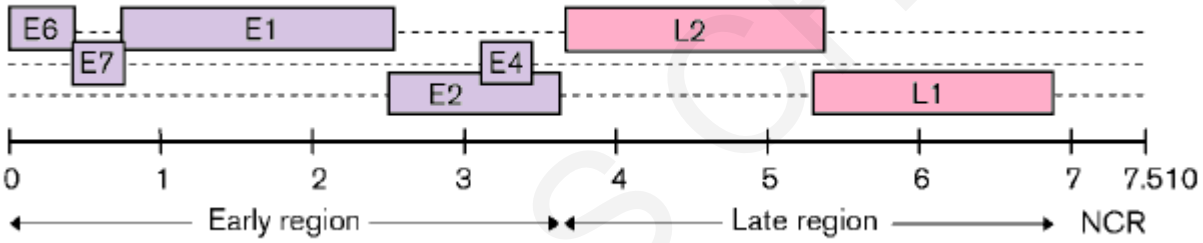


Figure 23: MmuPV1 genomic organization. MmuPV1 has an early region expressing its early genes (E1, E2, E4, E6, E7), a late region expressing its late genes (L1, L2) and a non-coding region (NCR) (Joh et al., 2011).

This new isolated virus can infect nude laboratory mice (NMRI-Foxn1nu/ Foxn1nu) causing papillomatosis at mucosal areas of the nose and mouth (Ingle et al., 2011b). It was shown that MmuPV1 shares some similarities with cutaneous HPV types such as same tissue specificity, and the development of warts (Meyers et al., 2017a). MmuPV1 also has the ability to suppress NOTCH and TGF-Beta signalling and cause increased proliferation.

On the other hand, there are also some differences. First of all they have low genetic homogeneity with HPV (49,8%) (Joh et al., 2011). L1, (considered to be the most conserved gene among different PVs, (de Villiers et al., 2004), is only 56.7% genetically similar between MmuPV1 and HPVs. The homology for E6 and E7 is even lower, at 44.7% and 39.9%, respectively (Joh et al., 2011). Another difference of MmuPV1 compared to alpha HPVs is that the ORF of E5 in MmuPV1 is omitted. Most rodent PVs have the Rb binding region on

the E7 oncoprotein (Fehrmann and Laimins, 2003), however in the MmuPV1 genome this Rb-binding site is located on the E6 oncoprotein (Joh et al., 2011). This was also observed in bottlenose dolphin PV types 1, 2 and 3 (TtPV1–3) and in Phocoena spinipinnis papillomavirus type 1 (PsPV1) (Joh et al., 2011, Rehtanz et al., 2006). These differences may occur as a result to host specificity.

As a secondary aim in this study we wanted to use the newly isolated murine papillomavirus (MmuPV1) and develop an effective PV infection animal model that would enable the study of PV interactions with epidermal stem cells in the context of a natural infection.

1.4.2.2 Transgenic mice expressing viral proteins

Infection of animal models with their respective papillomaviruses allow researchers to study the full viral lifecycle *in vivo*. However, these models cannot always be used to extrapolate conclusions for human papillomaviruses, as HPVs share low genetic homology with their animal counterparts and many processes of the viral lifecycle differ across different PVs. The generation of transgenic mice expressing viral gene products constitutively under the control of an exogenous promoter has been a critical breakthrough in the study of PV-mediated carcinogenesis (Brake and Lambert, 2005, Griep et al., 1998, Herber et al., 1996, Hilditch-Maguire et al., 1999, Riley et al., 2003, Schaeffer et al., 2004, Song et al., 1999, Lacey et al., 1986). The first group that created transgenic mice expressing PV proteins was Doug Hanahan et al. who generated mice expressing the BVP 1 genome (Lacey et al., 1986). In 1993 the first transgenic mice expressing HPV 16 oncoproteins E6 and E7 under the beta-actin promoter (Arbeit et al., 1993) were generated. The Lambert group has driven this work for the high risk HPV16 types using mice expressing the viral HPV16 E6/E7oncoproteins under the control of the K14 promoter, while Pfister's group developed transgenic mice expressing the early region of HPV-8. Further studies include the HPV20, HPV27 and HPV 38 transgenic models (Dong et al., 2005, Michel et al., 2006, Viarisio et al., 2011, Viarisio et al., 2018). However, transgenic mouse models have their limitations too. The viral genes are not controlled by an endogenous PV promoter. Furthermore, potential differences in the cellular targets of the papillomavirus genes between human and mouse may affect the interactions with viral genes and phenotypes observed. Such models cannot be used to evaluate several aspects of the viral life cycle including entry, replication etc.

The main part of this study is conducted using transgenic mice. Transgenic mice allow us to study the effect of the human oncoproteins in the context of a living organism. Moreover, using these transgenic animals we can take advantage of the large number of genetic tools available such as knockouts and other animal models. For related work in this dissertation, we utilized HPV 16 K14E6 and K14E7 mice bred with Terc-deficient mice. Resulting animals allowed us to examine the *in vivo* interplay of the HPV16 E6 and E7 oncogenes with telomerase, as well as whether the effects of these oncogenes on the stem cell populations are mediated through telomere homeostasis.

Chapter 2: EXAMINE THE INTERPLAY BETWEEN TELOMERE HOMEOSTASIS AND PAPILLOMAVIRUS ONCOGENES

This chapter is adapted from our published work in PLos One under the title “Terc is dispensable for most of the short-term HPV16 oncogene-mediated phenotypes in mice” (Achilleos et al., 2018).

Hypothesis

High risk HPVs have been associated with the development of the majority of cervical cancers, a subset of head and neck cancers, as well as other anogenital cancers. Carcinogenesis is thought to be mainly driven by the continuous expression of the viral oncogenes E6 and E7 (Goodwin et al. 2000, Jabbar et al. 2009). In parallel, the deregulation of telomere homeostasis has been observed in carcinogenesis as a mechanism of overcoming the telomere shortening problem of the continuously proliferating cancer cells. There is strong evidence for the effect that E6 and E7 oncogenes have on telomere homeostasis from *in vitro* models. As more groups begun to study this mechanism, it became apparent that HPVs extensively regulate telomere homeostasis, and this regulation may signal an evolutionary adaptation suggesting that telomere homeostasis is important in early infection and not necessarily only in carcinogenesis (Liu et al., 2009). Telomere homeostasis is a very important target for the virus, and it seems to have a vital role in viral lifecycle, stemness and carcinogenesis. The *in vivo effect of* telomere homeostasis on HPV related carcinogenesis and stem cell mobilization is still unknown but we believe that E6 and E7 may affect the telomere homeostasis *in vivo*.

Specific Aim

This study addresses the role of telomere homeostasis on the acute effects of the HPV16 oncogenes in stratified epithelia. This includes effects on the stem cell populations of such epithelia as previously reported (Michael et al. 2013). The skin was selected as the most suitable model tissue for this study, as several stem cell markers have already been identified and characterized in a distinct area known as the “bulge”, where quiescent stem cells reside.

2.2 Methodology

2.2.1 Mouse strains

We used transgenic mice that carry the K14E6/E7TTL or the K14E7/E6TTL constructs (Figure 24). These mice were generated in the lab of Dr. Paul Lambert (University of Wisconsin, Madison and they inbred in a pure FVB/N genetic background. They are identified by the name K14E6 and K14E7 (Herber et al., 1996; Song et al., 1999). Additionally, we used Terc-deficient mice (Terc is the the RNA template component of the telomerase enzyme) (Blasco, Lee et al. 1997a, Blasco, Lee et al. 1997b) that are commercially available from Jackson laboratories (http://jaxmice.jax.org/,stock no. 004132). These mice are inbred on a C57BL/6 genetic background. The first Terc-/- generation (G1) derived from heterozygous parents has been shown to have telomere lengths similar to wt mice (Terc+/+) (Blasco, Lee et al. 1997a, Blasco, Lee et al. 1997b). 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.

In order to study the interaction between telomerase and the HPV16 oncogenes we crossed Terc knockout mice, (lack of Terc eliminates detectable telomerase activity (Blasco et al., 1997) with transgenic mice expressing the E6 and E7 oncogene under the promoter of keratin 14 (Figure 25). Briefly, we crossed heterozygous *Terc*+/- mice with *K14E6* or *K14E7* mice. From this cross we generated *K14E6Terc*+/- and *K14E7Terc*+/- (F1 mice). F1 mice were then crossed with *Terc*+/- mice for the generation of G1 mice. The genotypes of interest of G1 mice are: *Terc*+/+, *Terc-/-, K14E6Terc*+/+, *K14E6Terc-/-, K14E7Terc*+/+, *K14E7Terc-/-.* The *Terc -/-* are deficient for the production of Terc. As Terc+/- mice were in a C57BL/6 genetic background and *K14E6* and *K14E7* were in an FVB/N pure background, the mice used in these experiments were in a mixed FVB-C57BL/6 background. All the genotypes were confirmed by means of PCR.

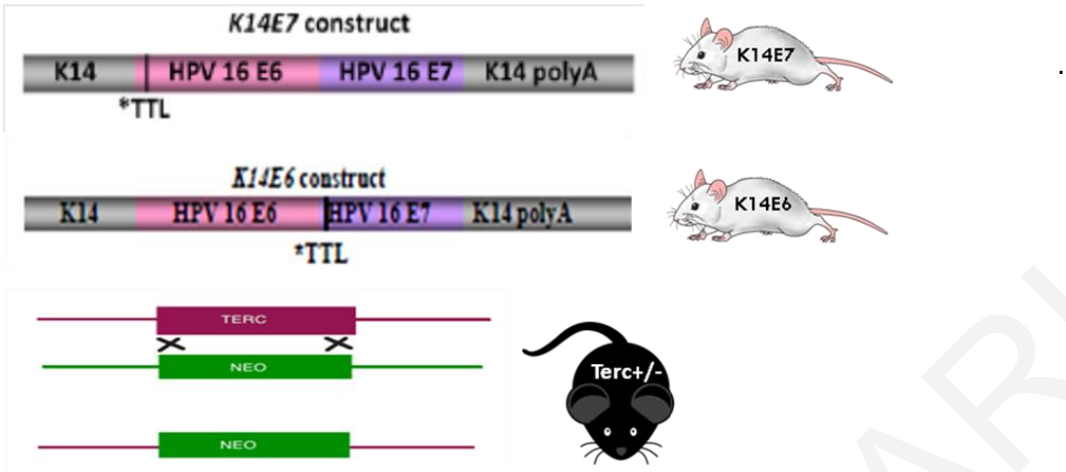


Figure 24: A) Schematic representation of the K14E7 and K14E6 construct. The K14E7 mice are expressing the HPV-16 E7 oncoprotein under the control of the Keratin 14 promoter (Song, Pitot et al. 1999). These mouse models express the E7 oncoprotein in the basal layer of the stratified squamous epithelia in an FVB/N pure genetic background , and were generated by the Lambert Lab (Song, Pitot et al. 1999)(Song et al., 1999). B) Schematic representation of the replacement of Terc with a neomycin cassette. The Terc-/- mouse is deficient for the production of the RNA template component of telomerase (Blasco, Lee et al. 1997a, Blasco, Lee et al. 1997b).

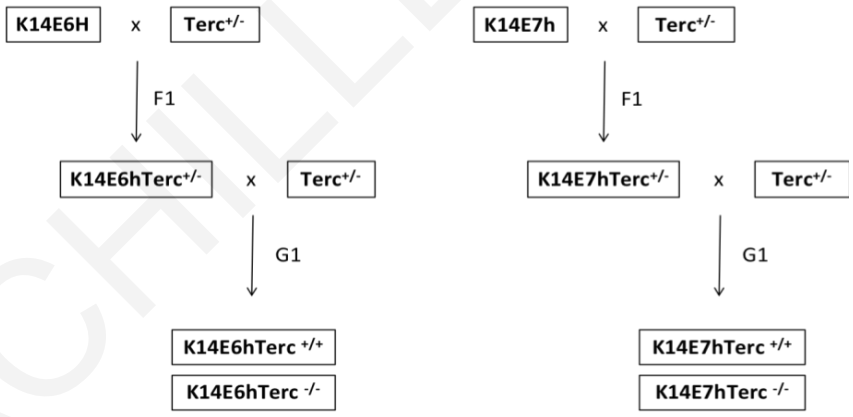


Figure 25: Crosses for the generation of G1 Terc-deficient mice. Left panel shows the crosses for the generation of K14E6Terc-/- mice and the right panel shows crosses for the generation of G1 *K14E7Terc-/-.* Heterogenous G1 terc mice generated by these crosses are omitted.

2.2.2 DNA extraction and Genotyping by PCR

The genotypes of the mice used in the experiments were confirmed by PCR. DNA was extracted from a piece of the mouse tail using the Extract-N-Amp Tissue kit as per manufacturer's instructions. 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 MgCl2, 200μM dNTPs, 0.4μM of each primer (Table 2) and 0.4 units of KapaTaq.

Table 2 : The primer sequences used in genotyping

Primer	Sequence 5' to 3'	Annealing T ^{o}C
709-1	GGC GGA TCC TTT TAT GCA CCA AAA GAG AAC TG	60
709-4	CCC GGA TCC TAC CTG CAG GAT CAG CCATG	60
E6TTL	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

2.2.3 BrdU assay

5-Bromo-2-deoxyuridine (BrdU) was administered intraperitoneally in mice at a final concentration of 50 mg/kg as first described previously (Bickenbach et al., 1986, Cotsarelis et al., 1990). BrdU incorporates in the DNA, and in every cell division the amount of BrdU is

divided to the two new daughter cells. In cells that undergo a large number of divisions, BrdU is undetectable after 60 days. The slowly cycling epidermal stem cells remain labeled and can be detected using a BrdU specific antibody (Figure 26).

For pulse chase experiments ten-day-old mice received an injection every 12 h for a total of four doses and they were subsequently euthanized 60 days after injections.

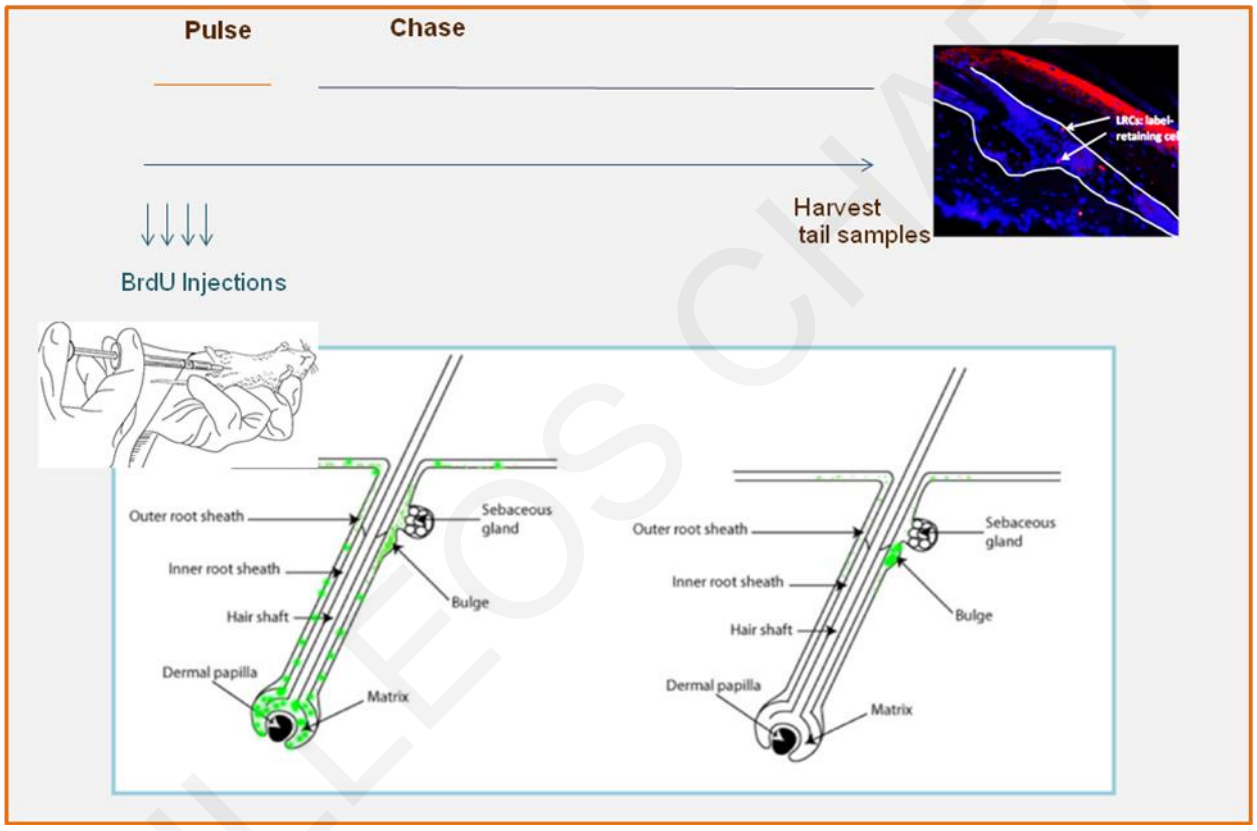


Figure 26: Schematic representation of the BrdU assay used in experiments described herein.

2.2.4 Q-FISH on Sections

Samples were de-paraffinized in xylene and rehydrated in a graded series of ethanol solutions. They were then treated with HCl/Pepsin for 10min at 37 ^{o}C. This step helps to digest away the cytoplasm on the slide and improves probe penetration. The samples were subsequently washed in PBS and fixed in 4% paraformaldehyde for 2 min. After a wash in

PBS they were dehydrated in a graded series of ethanol concentrations. The slides were air-dried and then the DNA was denaturated for 2 min at 80ºC and then hybridized for 2 hours with Cy3-conjugated peptide nucleic acid (PNA) probe in the dark at room temperature for 2h (Figure 27). Samples were then washed in wash solution (Final concentration: 70% formamide, 10mM tris, 0.1% BSA) twice for 15 minutes and three times for 5 minutes in TBS-Tween 0.08%. After washes, the slides were dehydrated in a graded series of ethanol and mounted with DAPI . Samples were analyzed on a Zeiss LSM 710 Axiovert confocal microscope using a 63× Plan-Neofluar 1.4 NA oil immersion objective lens. Images were analyzed with Axiovision 4.2 software and processed using TFL-Telo V2.

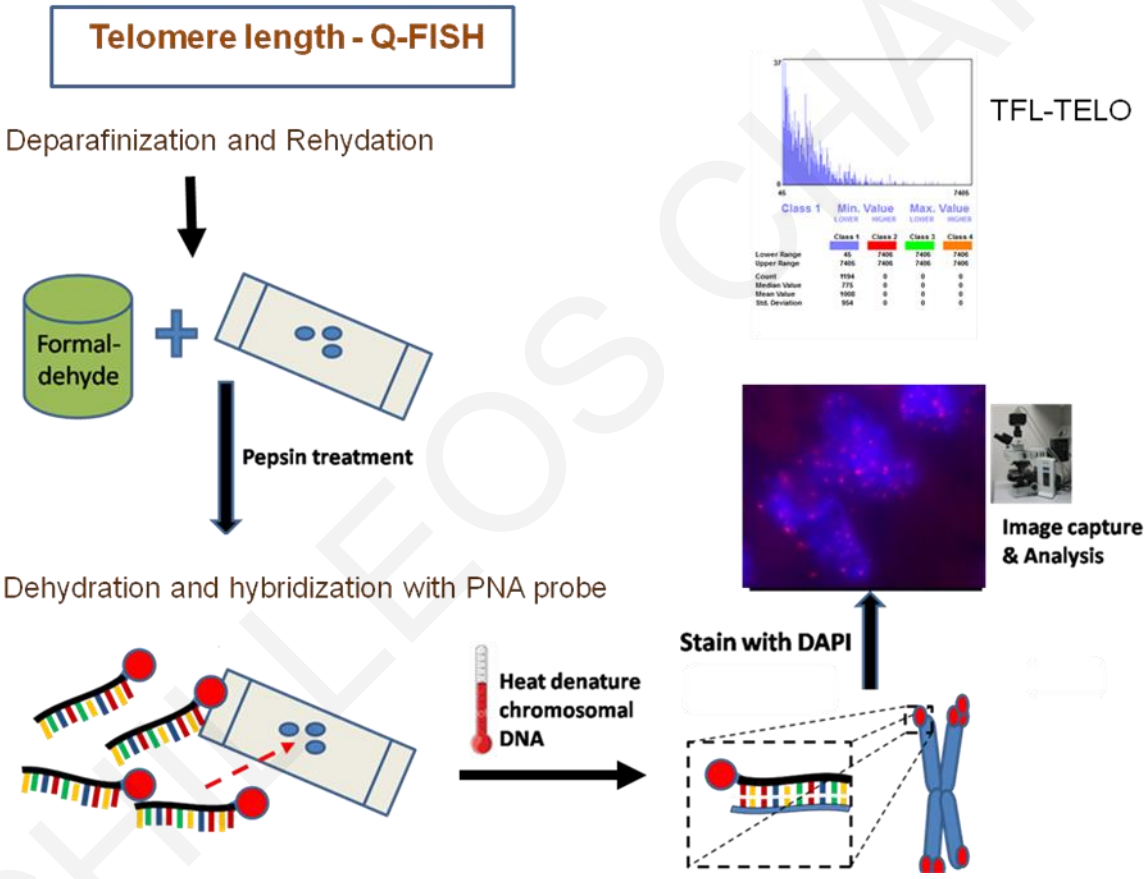


Figure 27: Schematic representation of the FISH workflow

2.2.5 TRAP assay

Trap assay was performed using the TRAPEZE® Telomerase Detection Kit (S7700-KIT; Millipore Company, Purchase, NY) (Figure 28). Briefly, fresh skin tissue was obtained and

floated with dermis side down in 0.25% trypsin at 4ºC O/N. Next day the dermis was peeled off and the epidermis was then homogenized while kept on ice and CHAPS lysis buffer was added (200ul per 10ug of tissue) and samples were incubated on ice for 30 minutes. Then, 160 µL of the supernatant was collected, and protein concentration was determined. A reaction mix containing TRAP buffer (20 mM Tris–HCl, pH 8.3, containing 1.5 mM $MgCl_2$, 63 mM KCl, 0.05% (v/v) Tween 20, 1 mM EDTA, and 0.01% BSA; TRAPeze telomerase detection kit), supplemented with dNTP mix, TS primer, TRAP primer mix, dH_2O, Taq polymerase at indicated concentrations was created. The mixture was incubated at 30 ºC for 30 min, and then PCR was performed (94 ºC for 30 s, 59 ºC for 30 s , 72 ºC for 1 min for 30 cycles and 72 ºC for 7 min in a thermocycler . PCR samples were run on a 10% (w/v) native-PAGE gel in 0.5× TBE for 1 h at 150 V. After electrophoresis, the gel was stained with ethidium bromide for 10 min at room temperature.

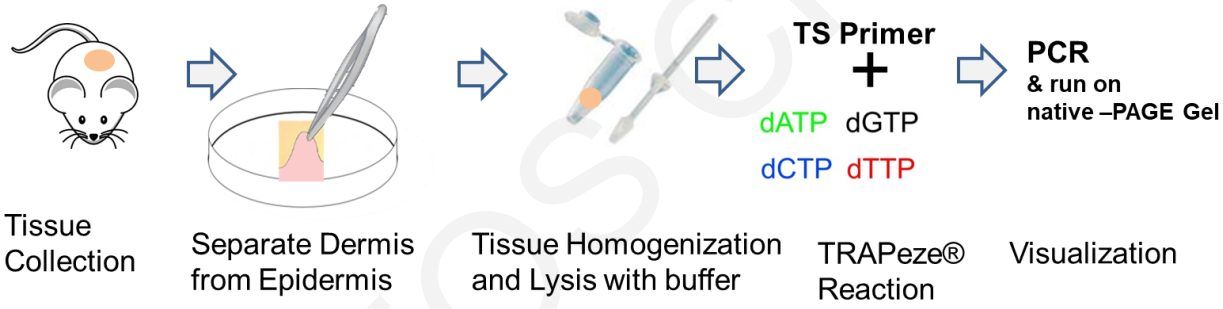


Figure 28: Schematic representation of the steps performed for the TRAP assay.

2.2.6 Immunohistochemistry

Mice were sacrificed in a CO_2 chamber, 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 min in 2 M HCl to enable antibody access to incorporated BrdU. Blocking and antibody incubations were variable and optimal for each different antibody used. Primary antibodies used include: 1:100 BrdU (Abcam), 1:500 K15 (SantaCruz), 1:100 PCNA (SantaCruz). Following primary antibody

incubation samples were washed in PBS. The following secondary antibodies were used: Cy3-streptavidin, biotin-rat and biotin -mouse all from Jackson ImmunoResearch and also Vectastain universal secondary (Vector laboratories).

2.2.7 Statistical tests

To determine statistical significance of the results in each experiment, 3 mice of each genotype were used and 75 hair follicles per genotype 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 $p \leq 0.05$.

2.3 Results

Infection with high risk HPV can induce carcinogenesis and this process has been linked to the ability of the virus to promote stem cell mobilization. Telomere homeostasis is known to affect both stemness and carcinogenesis. In this study we aim to examine the *in vivo* interplay of the HPV16 E6 and E7 oncogenes with telomerase and whether the effects of these oncogenes on the stem cell populations are mediated through telomere homeostasis.

2.3.1 Generation of mice expressing HPV16 oncogenes in the present or absence of Terc.

As described in section 2.2.1 mice expressing the E6 or E7 oncoproteins under the keratin 14 promoter, and mice lacking the terc component of the telomerase complex were crossed in order to generate mice expressing E6, E7 or neither of the oncogenes, in the presence or absence of the Terc component of telomerase. Mouse genotypes obtained through these crosses are listed in Table 3. Mixed FVB/C57BL/6 *Terc* +/+ mice were used as a control in order to eliminate differences attributed to the mixed background and not the deficient RNA component of the telomerase. All mouse genotypes were confirmed by PCR, using DNA extracted from a small piece of tail.

Table 3: Mice genotypes obtained by the crosses

Genotype	Transgene	Expected telomere elongation activity
K14NTG Terc+/+	None	Yes
K14E7 Terc+/+	E7	Yes
K14E6 Terc+/+	E6	Yes
K14NTG Terc-/-	None	No
K14E7 Terc-/-	E7	No
K14E6 Terc-/-	E6	No

2.3.2 Expression of the HPV16 E6 oncogene leads to increased telomerase activity *in vivo*

The HPV E6 oncoprotein has been reported to increase telomerase activity *in vitro*. To interrogate whether this also occurs *in vivo* we analyzed lysates from mouse tissues using a telomere repeat amplification protocol (TRAP). As expected, mice lacking the terc component of telomerase had no telomere elongation activity. Mice expressing the E6 oncoprotein showed a statistically significant increase in telomerase activity compared to NTG and E7 expressing mice (Figure 29). E6 oncoprotein is known to influence telomerase activity by increasing the transcriptional levels of TERT, and this interaction with Tert may lead to increased telomere elongation activity as well.

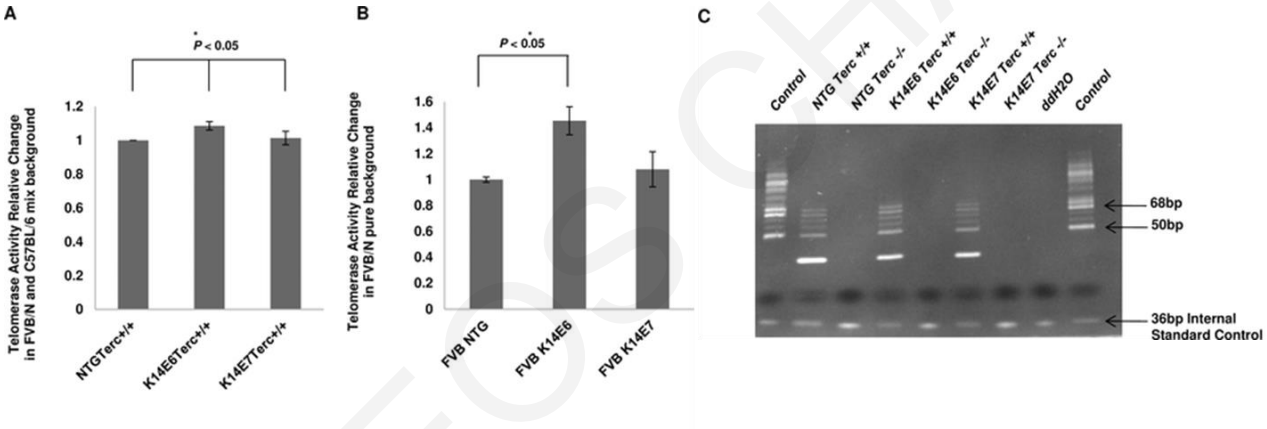


Figure 29: Expression of the HPV16 E6 oncogene increases telomerase activity *in vivo* (Achilleos et al., 2018). (A) and (B) The telomerase activity was determined using ImageJ software analysis and plotted for each genotype (columns); bars, SD. The experiments were done using three mice from each genotype and all statistical comparisons were performed using a two-sided Wilcoxon rank sum test. Statistical significance was observed between E6 and NTG or E7 mice. (NTG; Non Transgenic) (C) Representative native PAGE gel image of the telomerase products extracted from skin tissue and amplified using the Trap assay.

2.3.3 Telomeric length is unaffected by HPV16 oncogene expression *in vivo*

In order to examine the effect of HPV E6 and E7 oncogenes on telomere length, tissues from mice with defective Terc and wt Terc were subjected to Q-FISH and the average telomere length of telomeres from 75 nuclei were compared across all genotypes used (Figure 30). No statistically significant difference was observed among the different samples. Expression of the HPV16 E6 reduces the number of relatively quiescent cells detected at telogen even in the absence of telomere elongation activity. The telomerase holoenzyme is responsible for telomere elongation and is frequently found to be upregulated in cancers, presumably to maintain a sufficiently long telomeric length despite the constant rounds of cell division common in malignancy. Under normal conditions it is thought that telomerase only acts on critically short telomeres, thus the activation of telomerase in tumors is thought to be a late event in carcinogenesis. Thus, the absence of terc did not affect the size of the telomeres in any genotype.

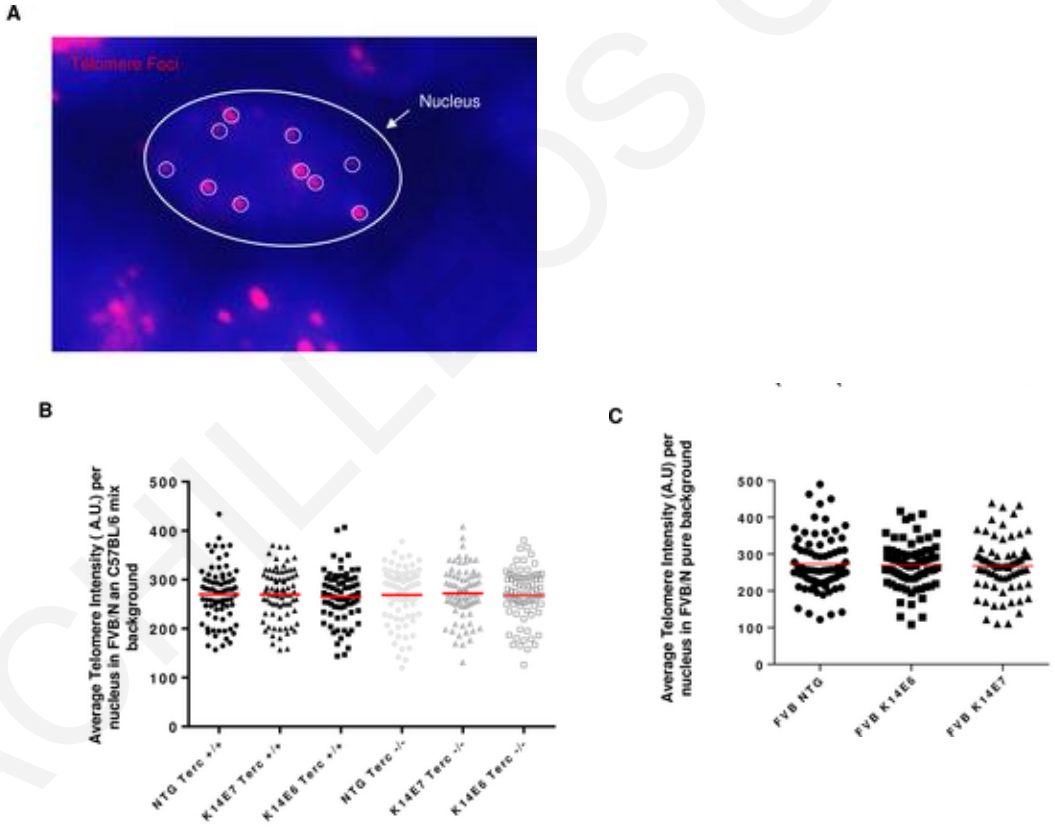


Figure 30: Expression of the HPV16 oncogenes does not affect telomeric length irrespective of the presence or absence of Terc (Achilleos et al., 2018). (A) Representative immunofluorescent image of telomeres (depicted by small white circles) using fluorescent in situ hybridization assay (FISH) on the

tail of 71 day-old mice. The nucleus is shown by an arrow. (B) and (C) The average length of the telomeres was determined by measuring the intensity of the telomeric foci using TFL-telo software in FVB/N and C57BL/6 mixed background (B) and FVB/N pure background (C). ~75 nuclei were counted for each genotype and the mean average was plotted for each genotype (columns); bars, SD. A.U; Arbitrary Units The experiments were done using three mice from each genotype and all statistical comparisons were performed using a two-sided Wilcoxon rank sum test.

2.3.4 Canonical roles of telomerase are dispensable for HPV16 oncogene-mediated increase in epithelial proliferation.

Proliferating cell nuclear antigen (PCNA) is a known molecular marker for DNA proliferation. It has been previously shown that the HPV16 E6 and E7 oncogenes cause an overexpression of PCNA in anagen hair follicles (Michael et al. 2013). It is not known whether impaired telomerase activity can affect proliferation in this E6 or E7 transgenic mice. Our results have verified that the presence of the oncogenes increases the PCNA staining of the epithelium as seen by an increase in the staining in both the basal as well as suprabasal layers of the epithelium (Figure 31). In addition, this effect is irrespective of the presence or absence of Terc, suggesting that the HPV-driven proliferation of the epithelium is not connected to the telomerase status of the epithelium. Whether the Tert component of the telomerase complex is involved in the HPV-driven proliferation is still unknown.

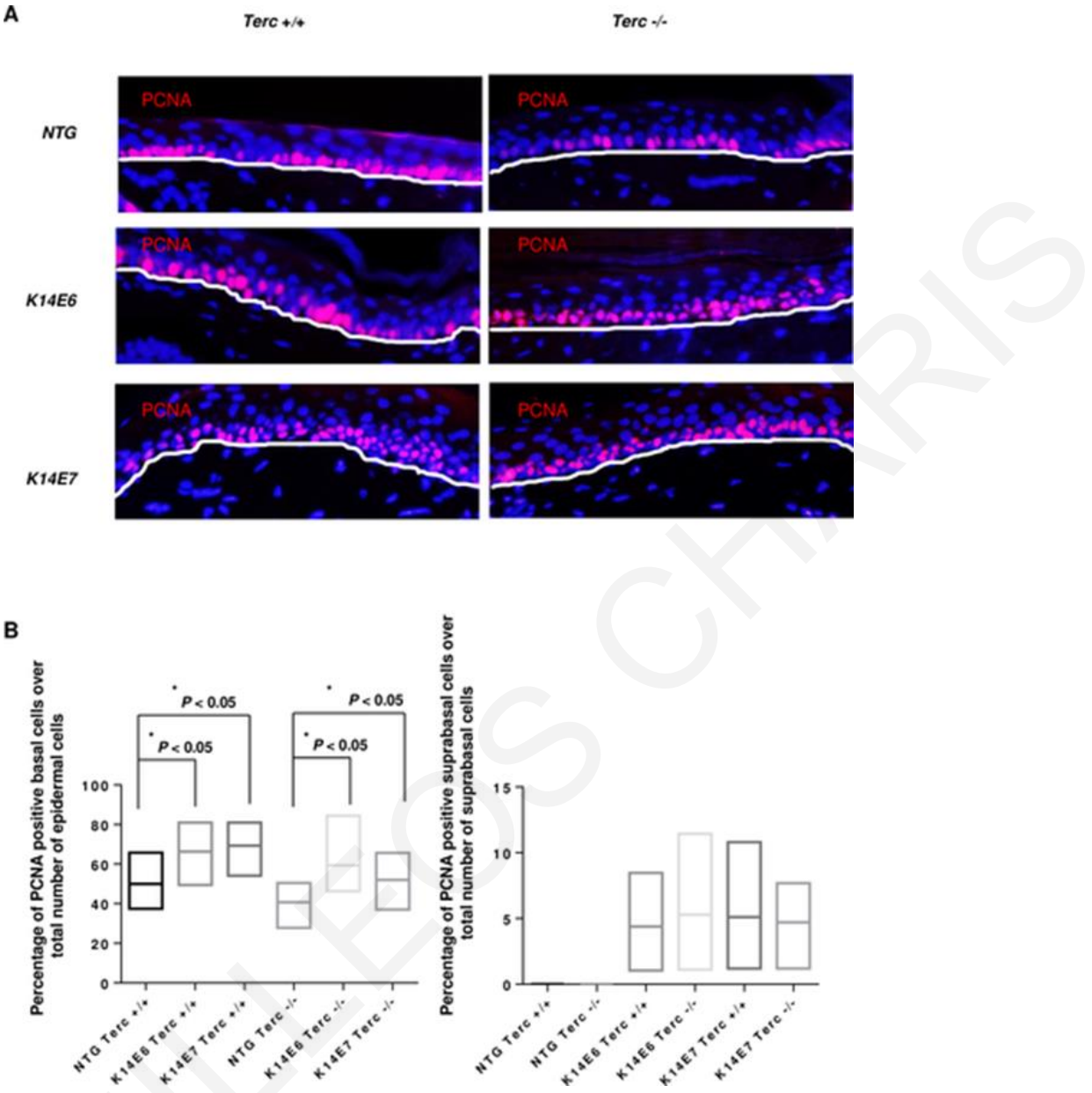


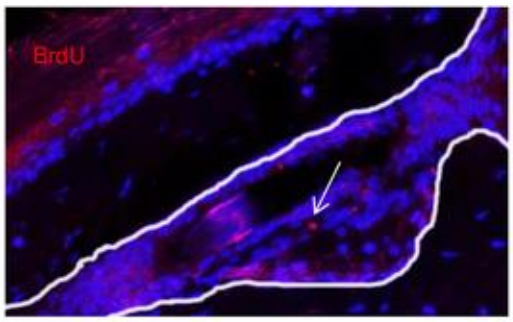
Figure 31: Expression of the HPV16 oncogenes increases the proliferative ability of the epithelium irrespective of the presence or absence of Terc (Achilleos et al., 2018). (A) Representative immunofluorescent images of the tail epithelium showing PCNA positive cells (red). Counterstaining was done with DAPI (blue). The white line indicates the basal membrane in each image. (B) The proliferative ability was determined by counting ~25 different regions of the basal (left) and suprabasal (right) layers. Mice were 71 days old. The positive cells were counted for each genotype and the percentage of positive cells over the total number of cells was plotted (columns); bars, SD. The experiments were done using three mice from each genotype and all statistical comparisons were performed using a two-sided Wilcoxon rank sum test. As expected, no positive PCNA cells were detected in the suprabasal layers of non-transgenic animals.

2.3.5 Terc is dispensable for E6 but not E7-mediated effects on hair follicle label-retaining cells

In this study, we wanted to examine whether the interplay between the viral oncogenes and telomere homeostasis is involved in stem cell modulation, previously reported by us and others. (da Silva-Diz et al., 2013, Michael et al., 2013a). In order to assess the effects of HPV 16 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), and the numbers of label-retaining cells (LRCs) at second telogen (resting phase of hair cycle) were compared in all genotypes (Figure 32). Both *K14E6Terc*+/+ and *K14E7Terc*+/+ mice showed a reduction in the number of LRCs. This result is consistent with previous studies, as well as with the increase in proliferation in the epithelium (indicated by increased PCNA expression) as already described above. Increased proliferation leads to increased cell divisions and reduced BrdU staining. The same result was also observed in a previous study (Michael et al. 2013) done in a pure FVB genetic background. We can therefore conclude that the reduced LRCs phenotype is consistent across genetic backgrounds.

The absence of Terc rescues the reduced LRC number phenotype in *K14E7Terc-/-* mice but not in *K14E6Terc-/-,* which still exhibits reduction of LRC numbers when compared to the non-transgenic terc-deficient mice. This result suggests that that the pathway(s) affected by E7 make LRCs more sensitive to the lack of functional telomerase.

A



B

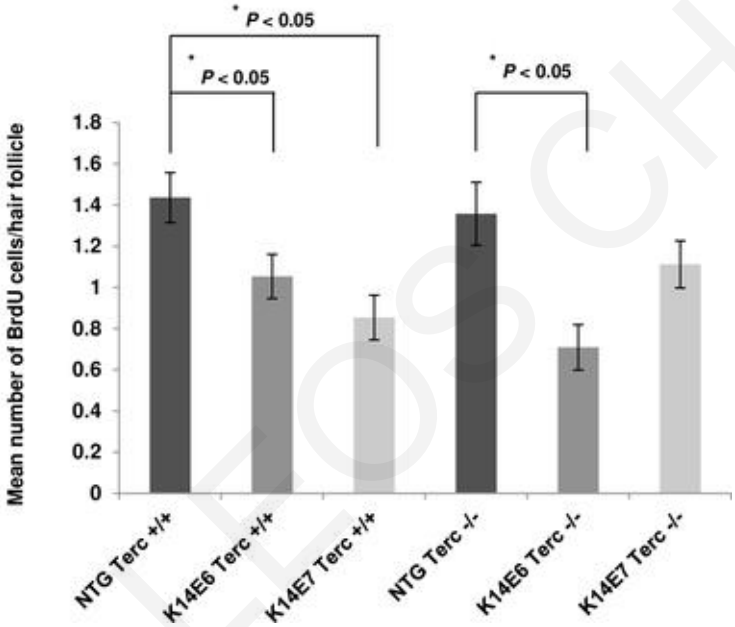


Figure 32: Terc is dispensable for E6 but not E7-mediated effects on hair follicle label- retaining cells (Achilleos et al., 2018). (A) Representative immunofluorescent image of a hair follicle showing BrdU positive cells (red). Counterstaining was done with DAPI (blue). The white line indicates the hair follicle. (B) LRCs were labelled using a BrdU pulse administered shortly after birth and chased until second telogen (71 days old). ~80 hair follicles were selected from at least 3 mice of each genotype (n = 3). The mean number of BrdU 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.

2.3.6 Terc is dispensable for E6-mediated expansion of stemness related markers

Keratin 15 was previously shown to be upregulated in the presence of E6 or E7. We examined whether this observation was dependent on the presence of normal telomere homeostasis. For this purpose, we measured K15-positive hair follicle cells in 3 mice (25 hair follicles each) and we found a statistically significant increase in K15 expression, in the E6 or E7 expressing mice in a Terc-deficient background comparing with the non-transgenic mice in the same background. These results indicate that the reduction of LRCs in mice expressing E6 or E7 is due to increased proliferation in the epithelium and aberrant mobilization and expansion of stem cells even in the absence of the Terc component. This result suggests that the telomere elongation activity of telomerase in G1 mice is not necessary for the mobilization and expansion of stem cells in the epithelium.

In the K14E6 or K14E7 mice, the keratin 14 promoter directs expression of the HPV16 oncogenes to the basal layer of stratified epithelia, including the bulge niche. K14 immunohistochemistry was performed on tissue samples from all genotypes (Figure 33) and the pattern of K14 expression was not disrupted by expression of the oncogenes. Keratin 10 and loricrin expression was also examined in all genotypes and found to be normal (Figure 33).

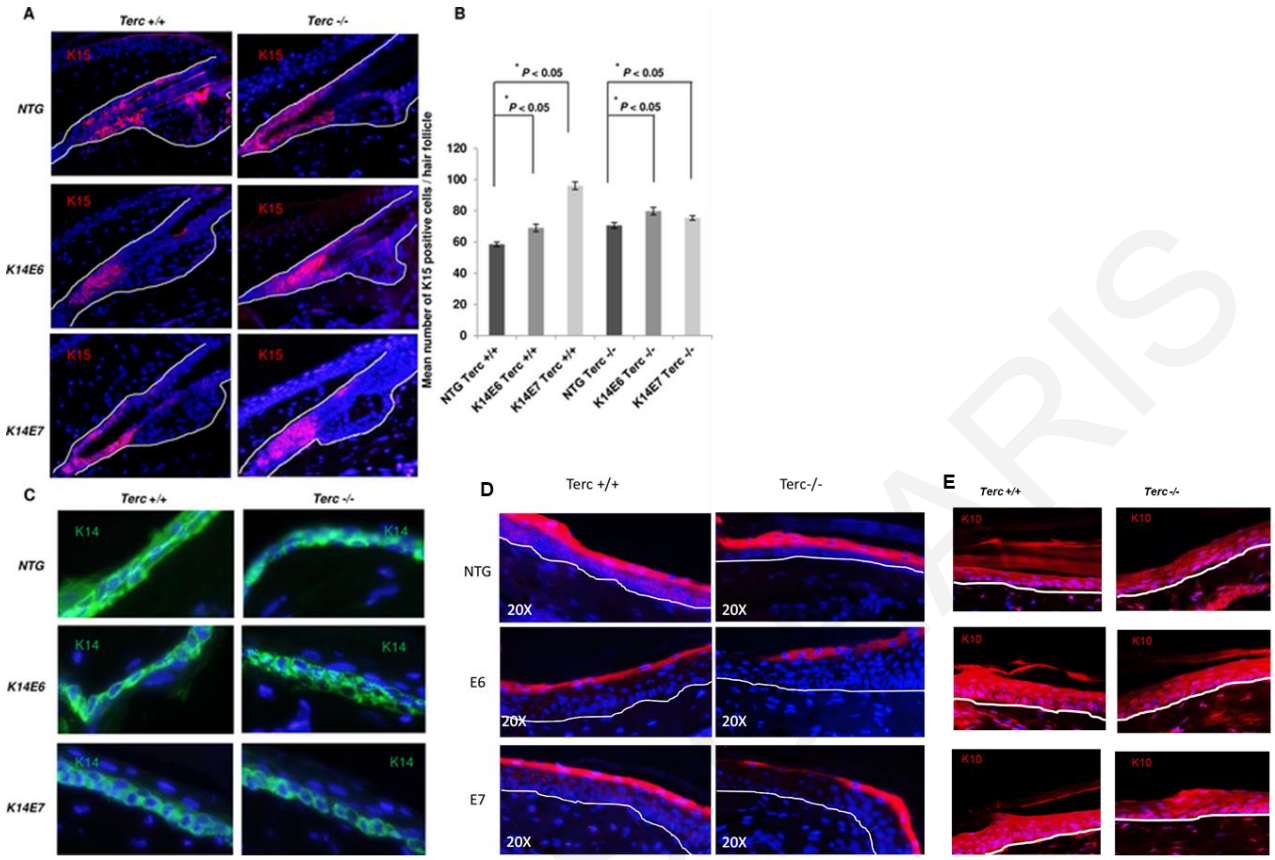


Figure 33: HPV16 oncogene expression causes an expansion of the bulge stemness marker K15. (A) Representative immunofluorescent images of the hair follicles showing K15 staining (red) using a K-15 specific antibody. Counterstaining was done with DAPI (blue). (B) ~80 hair follicles were selected from at least 3 mice of each genotype (n = 3). Mice were 71 days old. 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. (C) Representative immunofluorescent images from the tail epithelium showing K14 expression which correlates with the expression of the E6 and E7 viral oncogenes. (D) Representative immunofluorescence of Loricrin staining (red) in the epithelium of the genotypes examined. Counterstaining was done with DAPI (blue). (E) K10 is expressed in all genotypes. Representative immunofluorescence of Keratin 10 staining (red) in the epithelium of the genotypes examined. Counterstaining was done with DAPI (blue).

2.4. Discussion

The ability of HPVs to modulate telomere homeostasis has been argued to be critical to carcinogenesis and an evolutionary adaptation which may render cells more stem-like (Flores et al., 2005, Liu et al., 2009, Sarin et al., 2005). In this study we aimed to examine the *in vivo* interplay of the HPV16 E6 and E7 oncogenes with telomerase and whether the effects of these oncogenes on the stem cell populations are mediated through telomere homeostasis.

Firstly, we wanted to evaluate the effect of HPV oncogenes on telomeres length and telomerase activity in our *in vivo* model. Although tissue stem cells express low levels of telomerase, cancer cells seem to have constitutive telomerase activation. *In vitro* studies have shown that HPVs can maintain the host cells' telomere length either through transcriptional induction of telomerase by E6 or through ALT by E7.

In order to examine the effect of HPV E6 and E7 oncogenes on telomere length, tissue from mice from all genotypes (K14E6Terc-/-, K14E6Terc+/+, K14E7Terc-/-, K14E7Terc+/+, NTGTerc-/- and NTGTerc+/+) were obtained and subjected Q-FISH and an average telomere length in arbitrary units was assigned in each genotype. We found that all genotypes had telomeres with no statistical changes related to their length in the presence of the oncogenes. Thus, indicate that the rounds of cell division that were cause by the expression of the oncogenes were not sufficient in order to cause shorter telomeres.

In order to evaluate the effect of HPV oncogenes on telomerase activity in our new model, we used a telomere repeat amplification protocol (TRAP), which uses PCR to amplify the extension products of the telomerase enzyme. Fresh tissue from each genotype was obtained and subjected to TRAP assay. TRAP protocol was selected as it is a quite sensitive protocol and can detect as few as 0.01% positive cells (Ouellette et al., 2000).

As expected, mice lacking the Terc component of telomerase had no telomere elongation activity. We also observed that mice expressing the E6 oncoprotein showed a statistically significant increase in telomerase activity compared to NTG and E7 expressing mice (Figure 29). Increase in the enzymatic activity of telomerase caused by E6 oncogene is supported by many *in vitro* studies that showed the effect of E6 on the Tert component of the telomerase (Figure 29). *In vitro* studies showed that HPV-16 E6 oncoprotein affects the telomerase activity by increasing the transcriptional levels of human telomerase reverse transcriptase

(hTERT) (Galloway et al., 2005, James et al., 2006, Liu et al., 2009) and also by direct binding to telomeric DNA (Liu et al., 2009).

The increase in telomerase activity that we observe in this project was about 10% in the mixed background mice and 37% on the pure FVB mice. In previous studies, the effect of HPV 16 E6 oncoprotein was evaluated by *in vitro* studies in human primary cells or human cell lines (Klingelhutz et al., 1996, Liu et al., 2008a, Stoppler et al., 1997). These experiments were performed in transduced cells with retrovirus that contained the HPV E6 and/or E7 oncogenes and the increase in telomerase activity was much higher than what we observed. Transduction of the oncogenes results in much higher levels than normal infection in humans (Shai et al., 2007), so we believe that our model is more representative of the *in vivo* effects of the oncogenes in telomerase activity. In order to determine whether the low increase of telomerase activity observed here compared to the *in vitro* studies is due to the fact that we used human papillomavirus oncogenes in mice and not on human cells, we infected NOD SCID mice with the MmuPV1 genome and performed a TRAP assay on the papilloma that was formed. We observed a 10% increase in telomerase activity on the papilloma compared with tissue obtained from mock-infected mouse (data not shown). Another thing that also can explain the modest increase that we see compared to the *in vitro* studies is the fact that we performed TRAP assay on heterogenous tissues (epidermis) and not on a pure population of cells. In order to minimize the cell diversity within the epithelium tissue, we separated the dermis from the epidermis and used only the epidermis in our experiments. Of course the epidermis contains cells that are differently differentiated, which still results in differences in the levels of increase of telomerase activity in the tissue compared to a homogeneous population of cells in cell culture.

A limitation of using TRAP assays is the fact that TRAP evaluates the telomerase activity *with in vitro* conditions. The telomerase is isolated from the tissue and used *in vitro* in a PCR reaction. Using this method we miss other events that may occur *in vivo,* which may affect telomerase accessibility to the telomeres resulting in a different phenotype that the one observed *in vitro* through this assay. For example, changes in the Shelterin complex may occur *in vivo* and the results from TRAP assay differ from the *in vivo* phenotype. Sheltrin is a telomeric complex, that consists of six subunits; TRF1, TRF2, RAP1, POT1, TIN2 and TPP1. The main role of shelterin complex is to protect telomeric ends from fusion and from the DNA damage response. It has been proposed that Shelterin proteins play a role in stem cell and cancer biology where the tight regulation of telomeres homeostasis is important. Liu et al

showed that E6 interacst with active telomerase complexes and telomeric DNA sequences. In addition, using this method we cannot evaluate other extra-telomeric activities that may occur in the cell and affect stem cell biology. For example, Tert has been reported to activate wnt signaling (Park et al., 2009)

HPV oncogenes target cellular proteins that are implicated either in stem cell biology or in epigenetic reprogramming of the cells. An important pluripotency-related target is telomerase which has been shown to be upregulated by the HPV E6 oncogene and to cause changes in the stem cell niche of the area (Flores et al., 2005, Liu et al., 2009, Sarin et al., 2005). Previous studies showed that Terc-deficient mice with short telomeres had dysfunctional epidermal stem cells (stem cells lose the ability to mobilize and regenerate the skin and the hair) (Flores et al., 2005). In addition, these mice are resistant to multi-stage skin carcinogenesis (Gonzalez-Suarez et al., 2000). These results indicate the important role of telomerase in proliferating cells such as stem cells and cancer cells. Thus, we wanted to investigate whether the effects of HPV 16 oncogenes on the stem cell populations are mediated through telomere homeostasis.

Recent studies from our lab and others demonstrated that expression of the E6 and E7 oncoproteins causes stem cell mobilization in the epidermis, as seen through a reduction of label retaining cells (LRCs) and increased expression of K15, a marker of stem cells in the bulge. We examined these changes through a new model in order to gain further insights into the mechanism of modulation of telomere length by papillomaviruses. As previously mentioned, we crossed *K14E6* and *K14E7* transgenic mice with *Terc* +/- mice, and generated Terc-deficient mice expressing the viral oncogenes, as well as control mice. We focused on the quiescent epithelial stem cells that are found in the hair follicle bulge region in order to assess the effects of E6 and E7 expression *in vivo.* Skin *was* selected as it has a well-characterized stem cell population and is thought to provide a reservoir of latently infected cells that support the HPV life cycle. Using Brdu assays, we looked at the effect of viral oncogene expression on LRCs in the context of defective telomerase and demonstrated that the LRC reduction due to the presence of the HPV oncogenes is a consistent phenotype across genetic backgrounds other than FVB (Michael et al. 2013). In the context of defective TERC, which leads to elongation defective telomerase, E6-expressing mice showed a reduction in the number of LRCs. Even though the main role of telomerase is the elongation of telomeres, there is evidence showing 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). Thus, the reduction of LRCs in the presence of E6 was possibly enhanced by these extratelomeric activities and was able to overcome the fact that telomerase was unable to elongate telomeres.

On the other hand, in the context of defective TERC, E7 oncoprotein reduced the number of LRCs, but this reduction was not statistically significant. In NTG mice the mean BrdU positive cells was 1.4 cells. This number was reduce in *K14E7Terc*+/+ mice to 0.8 ($p<0.05$) and in *K14E7Terc-/-* mice to 1.1 cells ($p>0.05$). We are not aware of any effect of the E7 oncoprotein on other extra-telomeric activities of telomerase, thus we assume that the lack of telomere elongation activity by telomerase affects the E7 ability to significantly reduce LRC numbers.

Another important finding of this study was that the number of K15 positive cells increased in the presence of the oncogenes, even in the absence of the Terc component of telomerase. This expansion of the stem cell compartment was also demonstrated by Michael et al and da Silva-Diz et al. In this study, we further demonstrated that this increase is caused by the viral oncogenes even in the absence of active telomerase, indicating a telomerase-independent effect of the HPV 16 oncogenes. It's worth mentioning here that even though there was an increase in K15 expression in k14E7Terc-/- compared with NTGTerc-/- mice, there was also a statistical significant decrease in K15 expression in k14E7Terc-/- compared with K14E7+/+ mice. This result, along with the fact that in the context of defective Terc, E7 oncoprotein did not cause a significant reduction on the number of LRCs, suggest that E7-phenotypes are sensitive to the absence of Terc. Stem cells usually exhibit high telomerase levels, thus in the context of high stem cell mobilization (due to E7) the inability of telomerase for telomere lengthening may be vital, leading to stem cell "sensitization" to the lack of functional telomerase.

In addition to the effect of HPV oncogenes in the LRCs and K15 marker, we also wished to evaluate the effect of HPV oncogenes on the proliferation status of the tissue in the presence or absence of active telomerase. For this purpose, we checked for the presence of PCNA positive cells in the proliferative basal layer of the epidermis. PCNA is a general proliferation marker whose expression increases along with the severity of HPV-induced neoplasia and cancer progression (Branca et al., 2007). We found that both with and without active telomerase, PCNA-positive cells are increased in the basal layer of the epidermis in the presence of HPV oncogenes. We also evaluated changes in the suprabasal layer of the epithelium and only in the presence of the oncogenes we were able to find positive PCNA cells, even in the absence of telomerase. The increased proliferation that we observed may

lead to the increase of K15 positive cells when the oncogenes are expressed, as well as to the reduction of the LRCs in the presence of E6. These changes are independent of the telomere-elongation role of telomerase.

The Tert component of the telomerase has been shown to have other roles in the cell that are independent of telomere elongation. Using our model we were able to eliminate exclusively the telomere elongation role of the telomerase, as Terc does not have any other known roles in the cell. Conclusively, our results from this project suggest that even though Terc is necessary for the telomere elongation, it is dispensable for the E6-induced aberrant mobilization of the bulge stem cells. This was also suggested by Sarin et all, who showed that TERT overexpression causes proliferation of quiescent, multipotent stem cells in the hair follicle bulge region and this function of TERT does not require the telomerase RNA component (Sarin et al., 2005). Sarin at al, also showed that LRCs numbers were reduced, but the number of CD34+ cells in the bulge remained unaffected, indicating that stem cells divide but likely self-renew in order to maintain the CD34+ population.

As we evaluate our findings we need to have in mind that, inbred strains of mice (laboratory mice) have been selected for fast growth, big litter sizes and rapid maturation which may have discarded slow-aging genes including anti-cancer genes (Shay and Wright, 2001). Laboratory mice have very long telomeres and in case of generating mice without the TERT or TERC component, telomeres get shorter in later generations and one can observed aging phenotypes similar to humans (Sahin and Depinho, 2010).

Synopsis

In stem cells and cancer cells telomerase has an important role in keeping telomeres sufficiently long, in order to prevent the adverse consequences of dysfunctional telomeres on cell viability and chromosomal stability. HPV 16 E6 oncogene was shown to increase telomerase activity *in vitro*. We show that this increase in telomerase activity also occurs *in vivo*, in both mix and pure FVB background animals expressing the HPV E6 oncogene.

High-risk HPVs exhibit extensive regulation of telomere homeostasis, which strongly suggests that telomerase has a major role in the viral life cycle, carcinogenesis and “stemness” of the infected cells. Using Terc-deficient mice we show here that the Terc component of telomerase is dispensable for most of the acute HPV-16 E6 oncogene mediated phenotypes in mice.

Chapter 3: SET UP AND CHARACTERIZATION OF A MmuPV1 INFECTION MODEL.

Hypothesis

Recently our lab (Michael et al., 2013a) and others (da Silva-Diz et al., 2013) have shown that E6/E7 viral gene expression promotes mobilization of stem cells and expansion of stemness characteristics in the mouse epidermis. The molecular interactions underlying these observations are poorly understood and additional studies are necessary in order to define the effect of papillomavirus oncogene expression on the stem cell population, especially in the context of natural infection.

Research geared toward understanding the interaction of the virus with stem cells has been hindered by the lack of small and convenient animal models that can recapitulate the course of natural infection. This is mostly due to the fact that papilloma virus infection is species-specific and, as a result, human papillomaviruses do not productively infect rodents. For this reason, transgenic mice expressing the E6 and E7 viral oncoproteins have traditionally been used. Recently, a papilloma virus strain that infects mice (MmuPV1) was isolated and characterized (Ingle et al., 2011b, Joh et al., 2011). MmuPV1 and HPVs exhibit similar tissue tropisms, they both encode the E6 and E7 viral proteins, and they induce papillomas *in vivo*. MmuPV1 shares some similarities with cutaneous HPV types such as same tissue specificity and the development of warts (Meyers et al., 2017a), especially with the HPV-8, that can cause skin cancer. Meyers et al showed that both MmuPV E6 and HPV 8 E6 can bind to the transcriptional cofactors of NOTCH MAML1 and also to SMAD2/SMAD3 (transcriptional cofactors of TGF-Beta signaling) (Meyers et al., 2017a). In addition, MmuPV1 luck E5 protein something common with cutaneous HPVs. These observations, suggest that infection of mice with MmuPV1 could be a useful experimental system to model key aspects of cutaneous HPV infection, pathogenesis and carcinogenesis. This new model system, involving a mouse papillomavirus which naturally infects mice via a site of wounding, may shed light to this aspect of papillomavirus biology, as it closely mimics the conditions of real life infection (Ingle et al., 2011a, Handisurya et al., 2013a, Uberoi et al., 2016a). Recently, it was shown that MmuPV1 has also similarities with mucosal HPVs, as it can infect immunocompetent mice at mucosal sites and cause cancer (Cladel et al., 2017). Thus, it is important to determine

whether MmuPV1 affects the epidermal stem cells the same way that high risk HPV oncogenes (da Silva-Diz et al., 2013, Michael et al., 2013a) or *cutaneous* HPV do (Lanfredini et al., 2017).

Specific Aims

a) Use the newly isolated murine papillomavirus (MmuPV1) to set up an effective PV infection animal model that would enable the study of PV interactions with epidermal stem cells in the context of a natural infection.

b) Determine whether MmuPV1 affects the epidermal stem cells the same way, that high-risk HPV oncogenes or cutaneous HPV do.

3.2 Methodology

3.2.1 Methodology Workflow

MmuPV1 can infect mice using naked DNA genomes or papillomavirus virions introduced to epithelial tissues after microdermabrasion. To generate pseudovirions and encapsidated papilloma viruses (named virions for the rest of the chapter), as well as genomes we set up previously published protocols (Uberoi et al., 2016a, Handisurya et al., 2012, Handisurya et al., 2013b). Generation of pseudovirions was performed by co-transfection of 293FT cells with pCLucf and pMushell (Buck et al., 2005, Buck and Thompson, 2007) and purification using an optiprep gradient (Figure 34). Mice were infected by direct infection with the viral genome (the genome was isolated from the plasmid pMmuPV1 that encodes for the MmuPV1 genome) or virions (Figure 35). Skin infection was performed according to protocol described by the Schiller lab (Handisurya et al., 2012). Briefly, three days prior to the infection, the mouse hair was removed with a clipper and depilatory cream and the skin was slightly injured in the epidermis in an area of 2x1.5 cm, using a rotary tool (Dremel). Three days later, the viral genome was applied on the injured area and within four weeks, the first papillomas appeared. This was performed in NOD-SCID and CD1-Nude mice infected in our own lab. We also obtained tissue samples of *NMRI-FoxN1* $^{nu/nu}$ infected mice from Dr. Paul Lambert's lab at the University of Wisconsin. Tissue samples from all these strains were used to

evaluate the effects of virus infection on stem cells by examining stemness-associated markers and skin pathology.

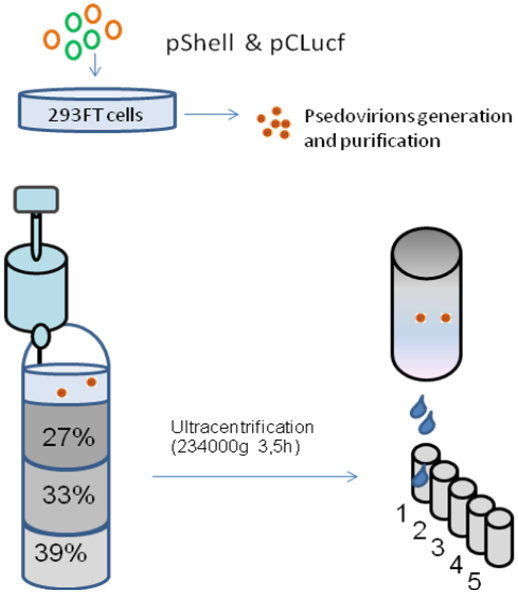


Figure 34: Schematic representation of the experimental protocol for pseudovirion production and isolation. 293FT cells were co-transfected with pCLucf and pMushell and pseudovirions were generated and purified using the protocol from Buck et al. 2004.

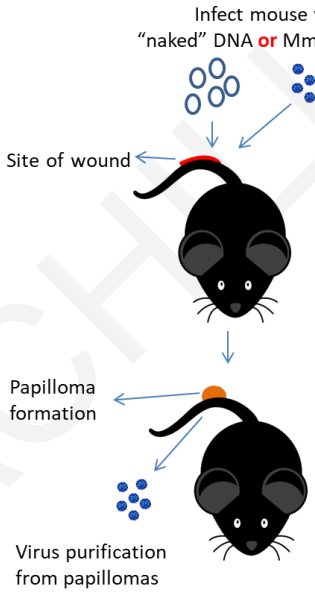


Figure 35: Schematic representation of MmuPV1 infection on SCID and Nude mice

2.2.7.1 Enzyme Digestion and Gel extraction

The MmuPV1 genome was excised from the vector using Xba I (# 1093A Takara) which cuts at the insertion sites (Figure 36). After enzyme digestion the sample was subjected to gel electrophoreses in 1% Agarose gel and ran at 120V for 1h. Two DNA bands of 7500bp and 2500bp were obtained. The 7500bp band was excised and gel extraction was performed using the Qiagen Gel extraction kit (#28704).

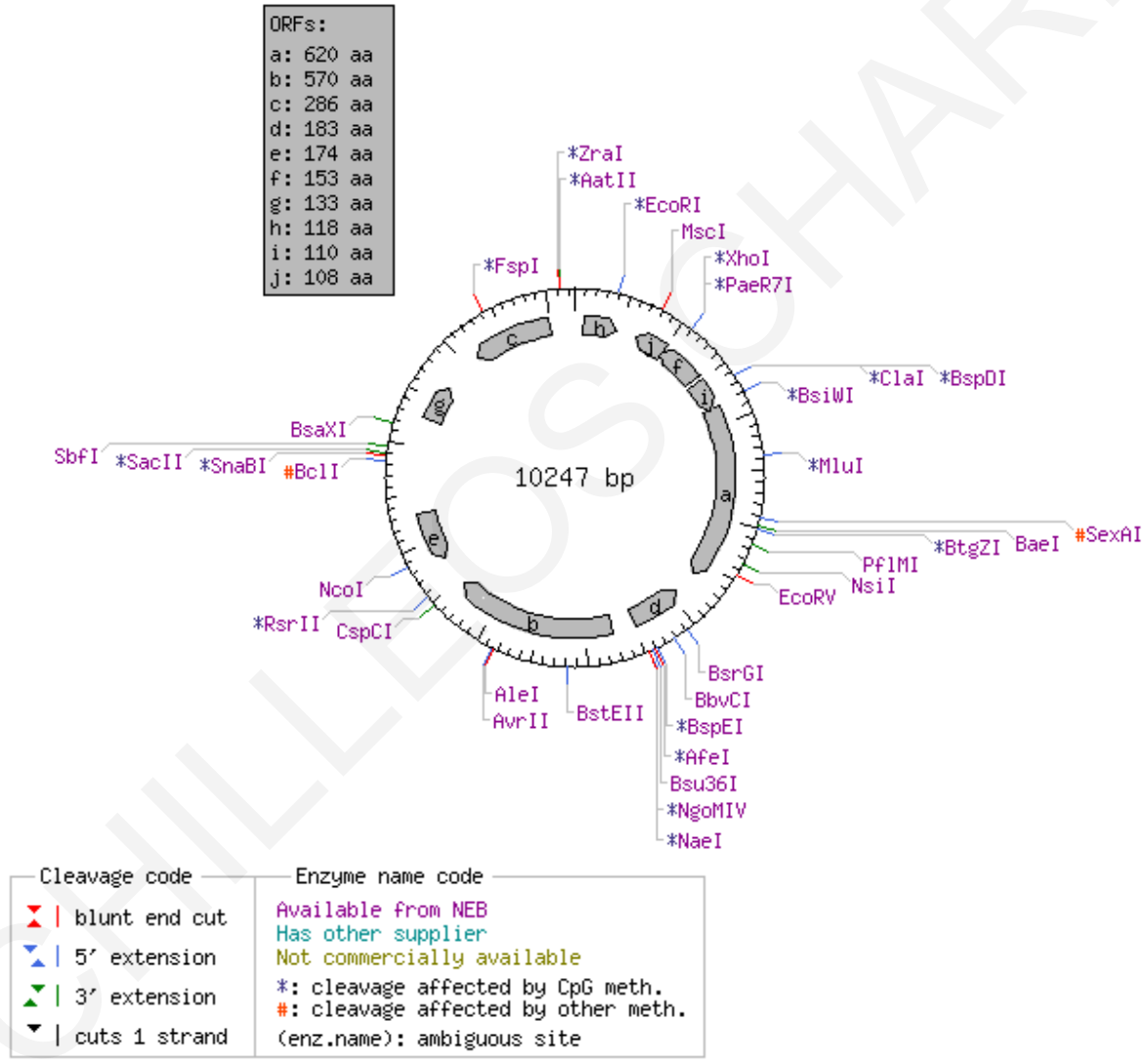


Figure 36: pMmuPV1 restriction enzymes map designed in NEBcutter (http://nc2.neb.com/NEBcutter2/).

2.2.7.2 Ligation

The isolated MmuPV1 genome needs to be re-ligated in order to be circularized again. Ligation was performed as previously described (Schowalter et al., 2011) . Briefly, 30ug of plasmid were re-ligated in a final volume of 9ml in an 1x Buffer and 6 µl of high concentration (2m U/ml) T4 DNA ligase (NEB). The sample was left at 16º C overnight. The next day 4.5 ml of 7.5 M ammonium acetate (Sigma cat# A2706) were added and mixed. Then 35 ml of 95% ethanol was added, mixed and incubated at 4ºC overnight. Then the sample was brought back to RT and centrifuged at ~5,000 x g (room temp) for 60 minutes. The pellet was washed with 10 ml of 70% ethanol, spun briefly. The ethanol was then removed, and the pellet was left to air dry. 50ul of TE was finally added to the pellet. The re-ligated genome was used for encapsidation to produce virions or to directly infect animals.

3.2.2 Infection with MmuPV1 genome

Infection of mouse skin was performed according to the protocol described by Handisurya (Handisurya et al., 2012). Briefly, three days prior to the infection, mouse hair was removed with a clipper and depilatory cream and the skin was slightly injured in the epidermis in an area of 2x1.5 cm, using a dremmel. Three days later, the virus genome was applied on the injured area (10mg of virus genome/ mouse). Approximately 4 weeks later, papillomas were visible on the face of the mice. This was performed in SCID mice and CD1- Nude mice. Nude mice were radiated with UVB with 300mJ/cm^2 at 365nm prior the infection in order to immune compromise mice (Uberoi et al., 2016b).

3.2.3 Cell Culture

293FT cells express enhanced SV40 large T antigen and were maintained and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 5% penicillin/streptomycin and 250ug/ml G418.

3.2.4 Transfection for Generation of Pseudovirions

For the generation of pseudovirions we co-transfected pCLucf (Figure 37) and pMushell (Figure 38) in 293FT cells and 3 days after transfection the cells were lysed in order to proceed with the purification of the psedovirions. Briefly, $3x10^6$ 293FTcells were plated in a volume of 10 ml complete growth medium per plate 18 – 24 hours before transfection (60 – 80% confluency). Cells were incubated overnight at 37ºC. The day of the transfection, X-tremeGENE HP DNA Transfection Reagent, DNA and diluent (Opti-MEM® I Reduced Serum Medium or serum-free medium) were allowed to warm to +15° C to +25° C. In a sterile tube containing 500ul of media we added 8 µg plasmid DNA in a molar ratio of 1: 1 = pCLucf plasmid : pShell plasmid. X-tremeGENE HP DNA Transfection Reagent was added in a 3:1 ratio of reagent to DNA. The mixture was added to the cells in a dropwise manner.

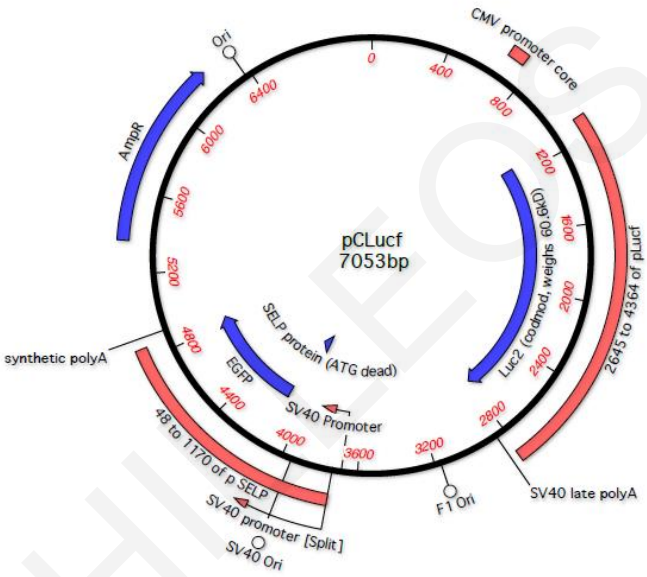


Figure 37: Map of the pCLucf (Addgene plasmid # 37328). This plasmid was donated to us by Dr. Schiller's lab (Johnson et al., 2009)

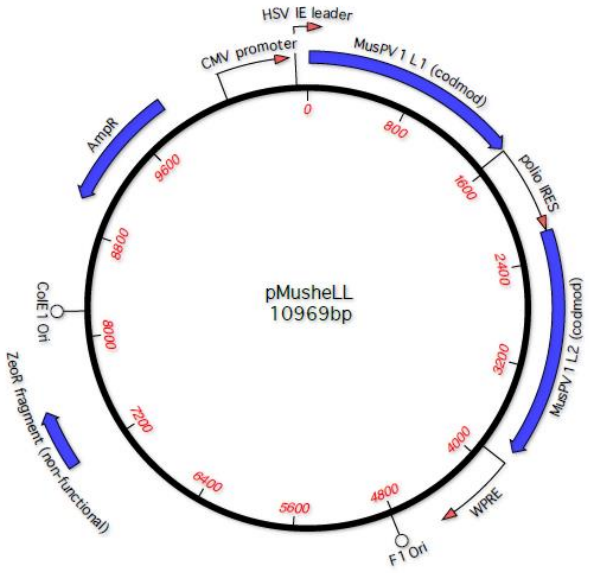


Figure 38: Map of pMushell (Addgene #47023). This plasmid encodes for Mouse PV L1 and L2 and is too large to self-package.

3.2.5 Pseudovirion Purification

For virus or psedovirion purification, cells were lysed using 10% Brij solution to a final concentration of 4% and $(NH4)_2SO_4$ ammonium sulfate was added to get final 25 mM ammonium sulfate and 1/1000th volume of RNase A/T1 cocktail. Then a gradient (called optiprep) was prepared. Using a long needle, the tube was filled with 3 different concentrations of optiprep (27%, 33% and 39%) and allowed to sit on the hood for 2,5 hours. Then on the top of the formed gradient, the sample containing the virus was added, followed by centrifugation at 230,000g for 3,5 h. The virus is not visible in the gradient after the centrifugation so using a needle a hole is created on the button of the tube and the fractions were collected dropwise (8 drops per tube) in siliconized tubes.

3.2.6 Pseudovirion Transduction

293T cells were plated in 24 well plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 5% penicillin/streptomycin. When the plate was 60% confluent 10ul of the selected fraction was added to the cells. Each fraction was added to a

designated well. 24 hours later the wells were evaluated for GFP expression and the fractions containing infective pseudovirions expressing GFP were selected.

3.2.7 Western Blot

293T cells were lysed in RIPA buffer, and protein concentration was determined with the Bio-Rad Protein assay using protein assay dye reagent concentrate (Bio-Rad, cat. #500-0006). Total lysates were separated on a 10 % SDS-PAGE gel for 1 h at 200 V. Proteins were subsequently transferred to a nitrocellulose membrane via semi-transfer for 25 min at 20 V, blocked with 5 % milk in TBS-Tween for 1 h at room temperature, and then incubated with the appropriate primary antibody at 4 °C overnight. Primary antibodies used were: Papiloma virus L1(#), and rabbit polyclonal to actin (Abcam, cat. #ab8226). Membranes were then incubated in blocking buffer plus anti-rabbit HRP-conjugated secondary antibody (Santa Cruz, cat. #SC2054) for 1h at room temperature and exposed using the LumiSensor™ chemiluminescent HRP substrate kit (GenScript, cat. #L00221V500)

3.2.8 Transfection on MEFs

The technique was performed in sterile conditions. 18ul of Xtreme 9 were added in 600ul empty DMEM and incubated at room temperature for 5 mins. 4ug of desired gene plasmid were mixed and added to the Xtreme9/DMEM mixture and incubated at room temperature for 30 mins. Meanwhile, MEFs were plated in a final concentration of 4.0 x 10^6 cells to a 100mm plate. The mixture was added to the cells drop-wise and incubate overnight at 37C, 5% CO_2.

3.2.9 IF on MEFS

Transfected MEFs were cultured on coverslips coated with poly-L-lysin. MEFs were fixed with 4% formaldehyde in PBS (stored at -20C) for 10 mins at RT. Then washed with x2 with PBS for 2-3 mins and permeabilised with 0.5% Triton-X in PBS for 10 mins at RT. The coverslips were moved to a flat plastic container wrapped with foil. The container had wet towels on the walls and parafilm on the bottom. 500ul PBG was used for blocking for 1h at RT. The 100ul of

primary antibody in PBG was added and samples were incubated overnight at 4C. Then cells were washed twice in PBS for 3 mins and incubated with secondary antibody. Coverslips were washed and a 20-30µl drop of embedding medium (50% plain - 50% with DAPI) was added and placed on microscope slides.

3.2.10 Immunohistochemistry

We obtained tissue from mice infected with MmuPV1 (infected in our own lab and also from Dr Lambert's group at the University of Wisconsin), as well as mouse tissue that was mock-infected. 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. Blocking and antibody incubations were variable and optimal for each different antibody used. Following primary antibody incubation samples were washed in PBS and stained with secondary antibodies. All images were acquired using a Zeiss Axio Observer.A1 microscope.

3.3 Results

3.3.1 Effective mouse infection with MmuPV1 genome

We were able to infect mice (CD1-Nude mice and SCID mice) with naked MmuPV1 genome. The mice developed papillomas one month after the infection. Even though the infection site was on the tail of the mouse, due to excessive grooming, its face was infected and developed papilomas (Figure 39A).

In order to verify that mouse tissue was indeed successfully infected on the infected tissues we performed fluorescent immunohistochemistry with antibody against the L1 capsid protein of the papilloma virus. As shown in Figure 39B, L1 was detected in MmuPV1-infected tissue.

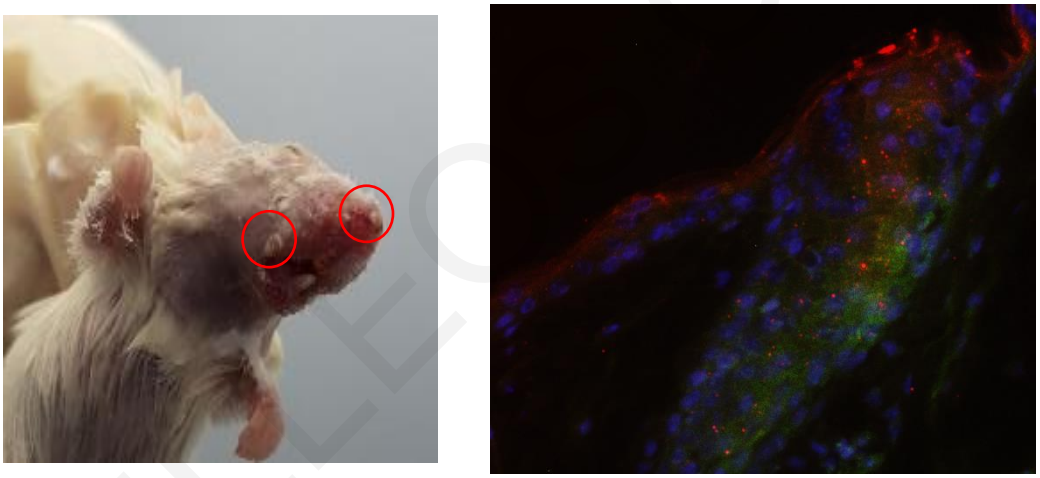


Figure 39: Successful infection of mice with naked MmuPV1 genome. A) Representative image of papillomas developed on SCID mice. The face of the mouse is shaved with depilatory cream. Papillomas are depicted with red circles. B) L1 (red) and K14 (green) staining on tissue sample of MmuPV1 infected mice. Nuclei were stained with DAPI (blue).

3.3.2 The effect of MmuPV1 infection on epidermal stem cells

Tissue samples of mice infected with the MmuPV1 genome were used to evaluate the effects of virus infection on stem cells by examining stemness-associated markers and skin pathology.

MmuPV1 has many similarities with cutaneous HPVs. Thus, the MmuPV1 mouse infection model can serve as a biologically relevant animal papillomavirus model for cutaneous HPVs and can be a useful tool in determining how cutaneous HPVs affect the stem cell population of the hair follicle. For this study we selected markers that are related with the morphology of the epidermis, or with stemness in general, or are specifically expressed by the stem cell population of the area and have been previously shown to be affected by cutaneous HPVs.

Type-I keratin K14 is the primary keratin of the keratinocytes of stratified squamous epithelia (Moll et al., 1982). K14 is extremely important for the physical stability of the epidermis and is a useful marker of epithelial morphology (Omary et al., 2004). Keratin 14 staining has been performed to visualize the epithelium and verify the hyperplasia caused by MmuPV1 infection. As expected, k14 expression was upregulated in MmuPV1 infected tissue (Figure 40A) and the epidermis was expanded.

As MmuPV1 has many similarities with the cutaneous HPVs, we wanted to also evaluate markers that have been shown to be affected in tissues infected with cutaneous HPVs. A recent study by Lanfredini showed that transgenic mice expressing HPV-8 (which belongs to the Beta-genus of PVs) cause an expansion of the Lrig1 positive and p63 positive keratinocytes population (Lanfredini et al., 2017) and not on the CD34+, LGR5+ and LGR6+ populations. Thus, we examined expression of the Lrig1 marker and p63, and we found them to be increased in the infected tissue compared to the uninfected (Figure 40B). Lrig1 marker is normally expressed only in the hair follicle and not the epidermis in the uninfected tissue but it was present in the infected epithelium. The transcription factor p63 is a homologue of the p53 tumor suppressor gene. p63 is involved in multiple functions during skin development and in adult stem cell regulation. It is involved in epithelial survival, germ cell surveillance, and neoplasia and several studies described p63 as a marker for basal squamous cells and subcolumnar reserve cells in cervical epithelium (Quade et al., 2001, Houghton and McCluggage, 2009). In addition, p63 and CK17 seem to be suitable markers for cervical stem cell identification and can therefore be used to identify HPV target cells (Martens et al., 2004).

P63 expression of the epithelium was increases. This result suggest that we have an expansion of the p63 expressing cells. These results suggest that MmuPV1 affects stemness markers that are associated with cutaneous HPVs.

Our lab and others, have also shown that sustained E6/E7 expression in the epithelial bulge stem cells causes upregulation of Keratin 15 (Michael et al., 2013a, da Silva-Diz et al., 2013). Keratin 15 (K15) is type I keratin protein. It is a minor component of the cytoskeleton and a stem cell marker in the bulge of hair follicles (Bose et al., 2013). We showed that K15 was upregulated on MmuPV1 infected tissue in respect with the uninfected tissue (Figure 40C) in all mice strains tested and present in the epithelium that normally is not present. These results are consistent with previous findings (Lanfredini et al., 2017, Michael et al., 2013a, da Silva-Diz et al., 2013) suggesting that MmuPV1 can serve as useful model in the study of the effect of papillomavirus on stemness markers. Moreover, cell surface marker CD34 was shown to be a specific marker of murine bulge keratinocytes (Trempus et al., 2003). CD34 was also upregulated on MmuPV1 infected tissue, as opposed to the uninfected control tissue. The upregulation of K15, Lrig1, p63 and CD34 indicates mobilization of stem cells from the bulge area and expansion of the cells expressing those markers upon infection with MmuPV1, and suggests that MmuPV1 infection can be a useful tool in the study of the acute effect of PV oncogenes in the context of a natural infection. These results were verified on papillomas obtained either from mice infected with MmuPV virions from Lambert's lab (two mice, one papilloma each) or Nude mice infected with naked MmuPV genome (3 mice with 3-5 papillomas each). p63 and CD34 staining was verified by two antibodies (p63: # ab53039 and # ab735, and CD34: # ab8158 and #VP-C345)

Oct-4 is a transcription factor involved in maintaining stem cell phenotype and pluripotency. Oct-4 expression in tumor cells correlates with cancer stem cell behavior, tumorigenic potential, and aggressive clinical features such as metastasis, disease progression, and reduced survival in bladder cancer (Atlasi et al., 2007, Chang et al., 2008) and breast cancer (Kim and Nam, 2011). We examined the expression of Oct-4 *in vitro,* using cervical cancer cell lines (Caski, Hela and C33A). Caski and Hela are positive for the presence of HPV. All cell lines expressed oct-4. We were interested to see whether a similar phenotype could be observed in tissue infected with MmuPV1. We performed immunohistochemistry on MmuPV1 infected and non-infected tissue and we observed positive oct-4 staining only in the infected tissue (Figure 40F). We evaluated two papillomas originated from two mice infected with MmuPV virions, using oct-4 antibodies from two different companies (abcam #ab19857 and

milipore #SCR077). The effect of Papillomavirus oncogenes on the expression of oct-4 is currently under examination by other members of our lab.

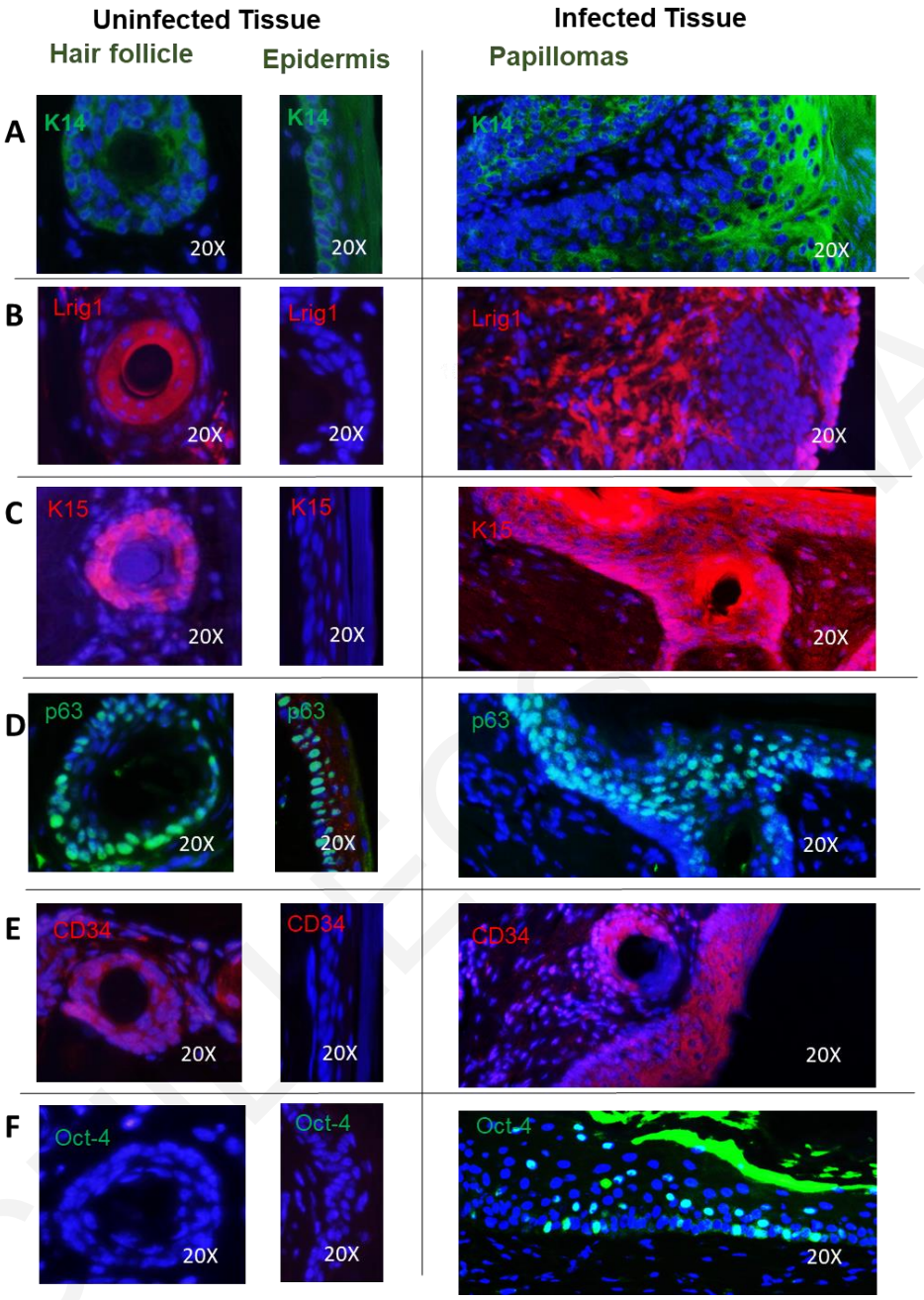


Figure 40: MmuPV1 infection leads to overexpression of stemness-related markers. A) Upregulation of K14 in MmuPV infected tissue.. Left panel: mock-infected tissue. Right panel:MmuPV1 infected tissue. Epidermis is grossly expanded in infected tissue and K14 expression correlates with this phenotype. K14 is shown in green and DAPI staining in blue. B) Upregulation of Lrig1 expression on the infected tissue. In the left panel we observe the uninfected tissue that has normal expression of Lrig1 and in the right panel we observe the infected tissue. Lrig1 is shown in red and DAPI staining in blue. C) Upregulation of K15 in MmuPV1 infected tissue. K15 is indicated with red and DAPI with blue. D) p63 staining is indicated with green. P63 positive cells are present in the whole MmuPV1 infected epithelium and not only in the basal layer as in the mock-infected epithelium. E) Upregulation of Cd34 in MmuPV1 infected tissue. CD34 is indicated with red and DAPI with blue. F) Oct-4 is expressed in the MmuPV1 infected tissue. Oct-4 is indicated with green.

We showed that K15, Lrig1, p63 and CD34 expressing cells were increased in tissue infected with MmuPV1 genome. In order to further validate these results, we also wished to infect mice with MmuPV1 virions. The initial step towards optimizing the MmuPv1 infection model with virions was the generation of pseudovirions. The pMushell and pCLucf plasmids (Figure 37 and Figure 38) were co-transfected in 293FT cells and the efficiency was about 80% (based on the GFP expression from pCLucf). The plasmid expression in the cells was verified by fluorescence microscopy for the pCLucf (Figure 41) that express GFP and with Western blot using an anti-L1 antibody that is expressed from the pMushell (Figure 41). The pseudovirus was purified as described in section 3.2.5, and the collected fractions were used to infect 293FT cells in order to detect the fractions containing the pseudovirions and check their infectivity. Fractions 16 to 19 contained infectious pseudovirions (Figure 42). These pseudovirions were selected for the optimization of the infection model.

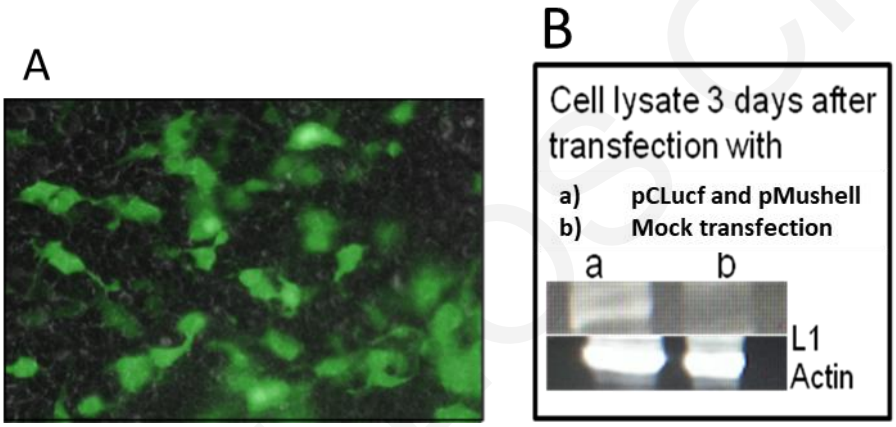


Figure 41: Transfection of 293FT cells for the generation of pseudovirions A) 293FT cells under fluorescent microscopy 3 days after transfection with pCLucf and pMushell. The green color indicates the GFP expression from pCLucf. B) Western blot using an anti-L1 antibody on 293FT lysate 3 days after transfection with a) pCLucf and pMushell or b) mock transfection. Actin was used as control.

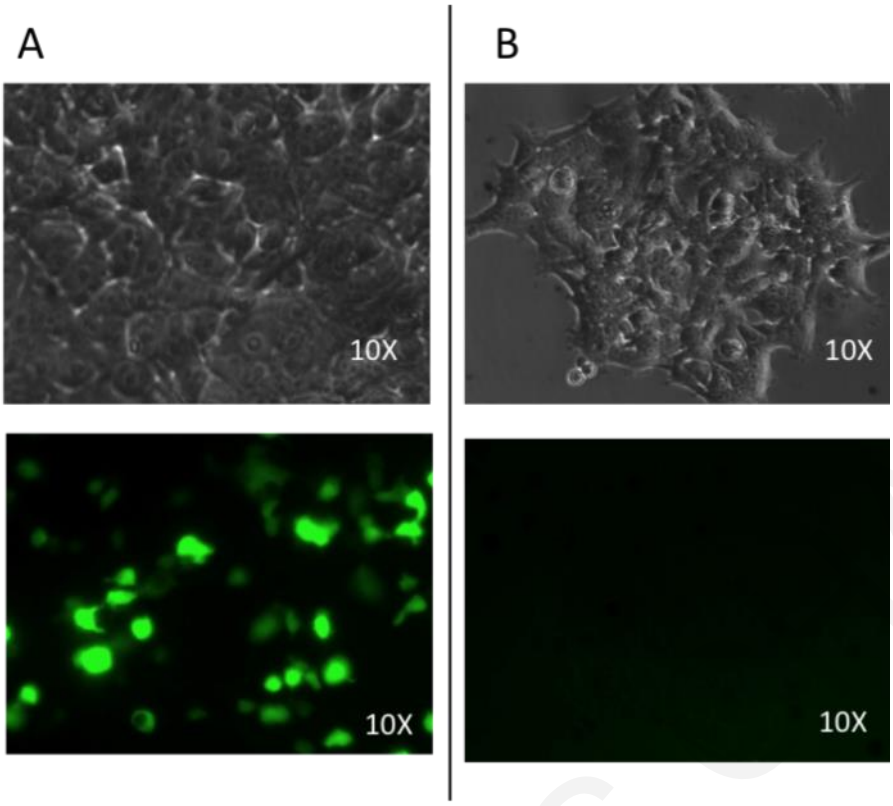


Figure 42: Pseudovirion transduction on 293T cells. 293T cells under phase-contrast and fluorescent microscopy (GFP expression is visible with green color) after infection with 10μl of a fraction containing pseudovirions (A) and a 10μl fraction without pseudovirions (B).

3.3.4 The Effect of MmuPV1 Transfection on MEFs

To complement our *in vivo* studies and initiate the *in vitro* characterization of MmuPV1, the plasmid containing the MmuPV1 genome was used to transfect primary cells. The target cells of MmuPV1 are primary keratinocytes. Obtaining primary keratinocytes is a time consuming procedure, as keratinocytes cannot be maintained for a long time in culture and their isolation requires newborn mice. Thus, we proceeded with transfection on MEFs, until we standardize the procedure and have a known timeframe for the time needed for MmuPV1 oncogenes to be expressed. In the future, this procedure will be repeated in primary mouse keratinocytes.

To determine whether transfection of viral sequences in primary cells leads to changes in proliferation, PCNA-specific IF was performed. The proliferating cell nuclear antigen (PCNA) is a 36 kDa molecular weight protein also known as cyclin. This protein has also been

identified as the polymerase-associated protein and is synthesized in early G1 and S phases of the cell cycle. Increased expression of PCNA is an indication of increased proliferation. We show here that MmuPV1 transfection on MEFs increases cell proliferation, as PCNA positive cells were increased by 16% in MmuPV1 transfected cells.

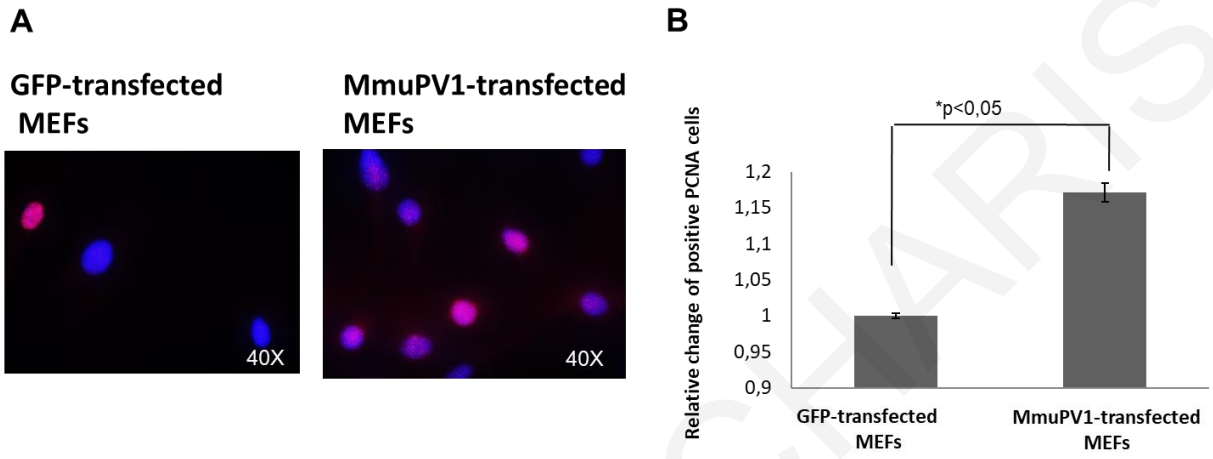


Figure 43: Transfection of cells with MmuPV1 leads to an increase in PCNA positive cells A) Representative images of immunofluorescence staining using PCNA antibody in MmuPV1 transfected MEFs (right image), as well as in GFP-transfected (control) cells (left image). PCNA positive cells are indicated with red. DAPI is indicated with blue. B) Graph shows the relative change of PCNA-positive cells among GFP-transfected MEFs (control cells) and MmuPV1 transfected MEFs. The experiments were done in triplicate (counting ≈1000 cells from each condition) and the statistical comparisons were performed using t-test.

3.3.5 Immune system infiltration on MmuPV1 infected tissue

Cancer and inflammation are usually linked through molecular events that activate oncogenes and inactivate tumor suppressor genes, causing transformation and neoplasia. Tumor-associated inflammation has also being shown to inhibit inflammatory components that usually contribute to suppression of tumor development (Balkwill and Mantovani, 2001, Mantovani et al., 2008), and in several cases immune cells with antitumor activity end up pro-tumorigenic. In addition, recent evidence suggests that inflammation may enable tumor invasion and metastasis (DeNardo et al., 2008) . HPV infection triggers an immune response that can lead to chronic inflammation (Amador-Molina et al., 2013, Danis et al., 2018). Thus, the immune system may be also implicated in HPV-associated cancers (Song et al., 2015). Using the new MmuPV1 infection model, where infection can be recapitulated in a system

with the genetic tools and versatility such as the laboratory mouse we may shed light in this process. We wanted to examine if the immune system had infiltrated in the infected tissue. Thus, it could be later on, further investigated the effect of immune response in PV life and effect on its cellular targets.

Using fluorescent immunohistochemistry, we examined the infected tissue for the presence of CD8 positive cells and macrophages (Figure 44). Populations of both of these cells were detected in the infected tissue, in contrast to the uninfected control. We also verified the expression of STAT-3 in the infected tissue (Figure 44). STAT3 has a vital role in regulation of innate and also adaptive immunity (Fu, 2006) and its expression is induce by many cytokines. It was shown that STAT 3 dificient mice fail to maintain antibody based immunity during viral infection, and their ability to generate T-follicular helper (Tfh) cells is impaired (McIlwain et al., 2015).

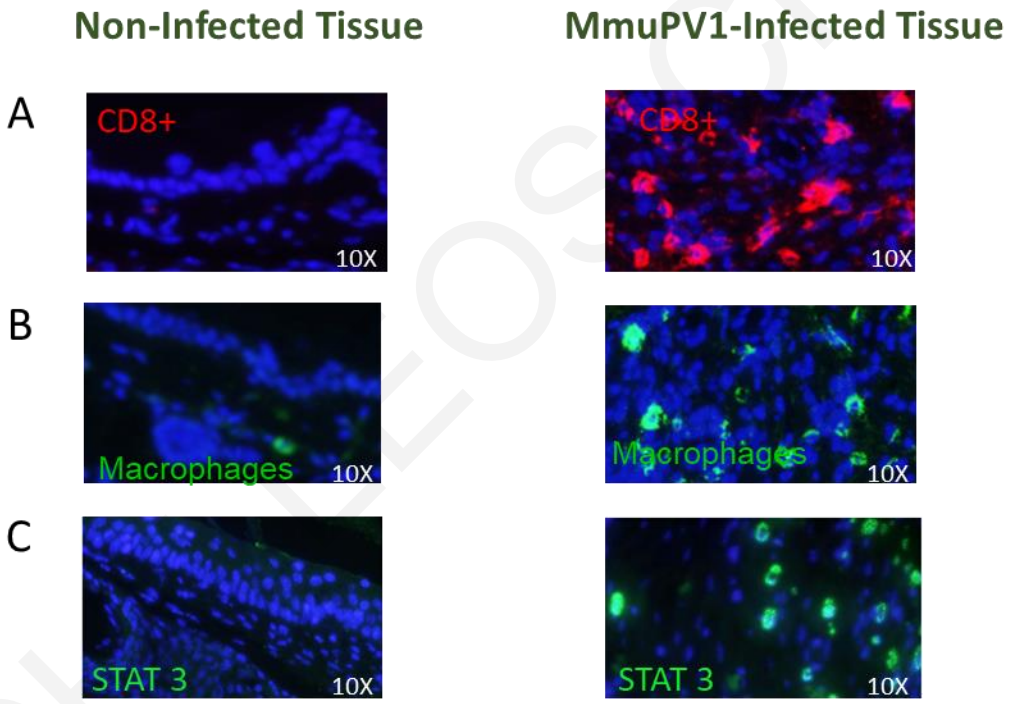


Figure 44: Immune system infiltration in MmuPV1 infected tissue. CD8 positive cells (A) and macrophages (B) are present in the infected tissue (right panel) and are absent from the non-infected tissue (left panel). Lymphocytes are indicated with red color and macrophages are indicated with green. Nuclei are visible using DAPI staining (blue). C) STAT3 has been detected only in the MmuPV1-infected tissue (right panel) and not in the uninfected tissue (left panel). STAT 3 staining is indicated with green. Nuclei are visible using DAPI staining (blue).

3.4 Discussion

In the 1970s, Harald zur Hausen was able to detect HPV in warts and cervical cancer, and isolated and cloned different strains of HPV. His research concluded that patients infected with high risk types of HPV (HPV types 16 and 18) are at an increased risk of developing cancer, a discovery that led to the Nobel Prize in 2008 (Medicine, 2008). Since then, papilloma viruses have been extensively studied and as a result, the research community has tried to develop the best model to study them. Currently there are various *in vitro* models that are able to support the full productive life cycle of HPV types, as well as a number of *in vitro* and *in vivo* models that can be used for studying the process of cancer progression and the role of the oncoproteins in this process. Unfortunately, the current models we use have various limitations due to the fact that HPV only replicates and completes its life cycle in humans, and cause cancers at epithelial sites that are not straightforward to model *in vivo.* An appropriate *in vivo* model is crucial for the study of the role of HPV gene products on neoplastic progression, immune regulation and virus entry. Recently the discovery of a papilloma virus that naturally infects mice (MmuPV1) opens up new opportunities for better understanding HPVs (Ingle et al., 2011b). This new murine papilloma virus shares many similarities with the cutaneous HPVs (Meyers et al., 2017a) and mucosal HPVs (Cladel et al., 2017). Cutaneous HPVs infect cutaneous epithelial and can even cause non-melanoma skin cancer (Ally et al., 2013, Harwood et al., 2000, Nindl et al., 2007). It was shown that MmuPV1 infection can lead to lesions that have malignant potential and can even progress to squamous cell carcinomas (SCCs) (Cladel et al., 2013). Moreover, Meyers et al. showed that HPV 8 E6 and MmuPV1 E6 have similar target proteins (MAML1 and SMAD2/3) (Meyers et al., 2017a). In addition, E5 is not present in MmuPV1, something that is also common with cutaneous HPVs. A recent study has shown that cutaneous HPVs can also affect the stem cell population of the epidermis (Lanfredini et al., 2017). We showed that MmuPV1 affects the Lrig1+ and p63+ stem cell population of the hair follicle in the same way as Beta genus HPVs (cutaneous) (Figure 40). On the other hand, Cladel et al showed that MmuPV1 can infect mucosal sites as well, by demonstrating infections in oral, vaginal, anal and penile tissues (Cladel et al., 2017), and that persistent infection can lead to cancer. In the present study, we show that MmuPV1 causes an increase in the K15+, CD34+ populations, something that was also observed in mice expressing HPV 16 oncogenes. Thus, the MmuPV1 mouse infection model can serve as a biologically relevant animal papillomavirus model for both cutaneous and mucosal HPVs and it can be a useful tool in determining how HPVs mechanistically

contribute to carcinogenesis. Moreover, this model will enable us to study the role of telomerase in vivo, as we can infect Terc deficient mice in a pure background and without time-consuming crosses. Moreover, we can infect Tert deficient mice and examine, along with telomere homeostasis, the extra-telomeric activities of telomerase in the context of papilloma virus infection.

In addition, the fact that it can naturally infect mice through microwounds will enable us to investigate the effects of the infection and inflammation in the process of carcinogenesis, virus cycle and stem cell regulation.

We also wanted to set up an *in vitro* model of infection, to complement the *in vivo* MmuPV1 findings. The first results from this effort show that MmuPV1 causes an increase in proliferation, as indicated by the increase in PCNA positive cells upon transfection with the MmuPV1 genome. This was an expected phenotype, as it was previously shown that HPV 16 E6 or E7 oncoproteins cause the same effect on the basal and suprabasal cells of the epidermis when expressed as transgenes in mice (Michael et al., 2013a). This result suggests that MmuPV1 may affect infected cells through the same pathways that HPVs do. The next step in the *in vitro* model is infection of primary keratinocytes with the MmuPV1 genome or mature virions. Primary keratinocytes are the target cells of the virus. This model will enable us to study the changes in gene expression mediated by MmuPV1 in primary keratinocytes. We are interested to examine how MmuPV1 affects keratinocyte colony formation, expression of stemness markers, proliferation rate, and apoptosis. In addition, we can infect different populations of keratinocytes that are sorted based on the expression of stem cell markers, in order to examine whether expression of specific markers is necessary for successful MmuPV1 infection and the effect that the expression of those marker has on the progression of infection. Moreover, the *in vitro* model will enable us to define mechanisms of MmuPV1 at the molecular and cellular level, using techniques such as RNAi.

3.4.1 The effect of MmuPV1 infection on the expression of stem cell markers *in vivo*

We wanted to evaluate whether the MmuPV infection model could serve as a useful tool for studying the effects of papillomavirus infection on the multipotent population of the hair follicle. We obtained tissues from Nude and SCID mice that were infected with MmuPV1 and

formed papillomas, as well as from control mice, in order to evaluate potential changes in stem cell markers.

Previous studies showed that MmuPV1 has many similarities with the β-type of PVs as it causes papillomas only in immunosuppressed mice (Meyers et al., 2017a). A recent study by Lanfredini showed that transgenic mice expressing HPV-8 exhibit expansion of Lrig1 positive keratinocytes, a marker of stem cells (Figure 45) and they propose that the b-genus PV infects the Lrig1 positive cells (Lanfredini et al., 2017). Lrig1 positive cells are a distinct multipotent stem cell population in mammalian epidermis, which resides in the hair follicle junctional zone in mice (Jensen and Watt, 2006). In addition to Lrig1, the group observed an expansion of the p63 marker, a marker that we also observed to be upregulated in transgenic mice expressing HPV-8 genes.

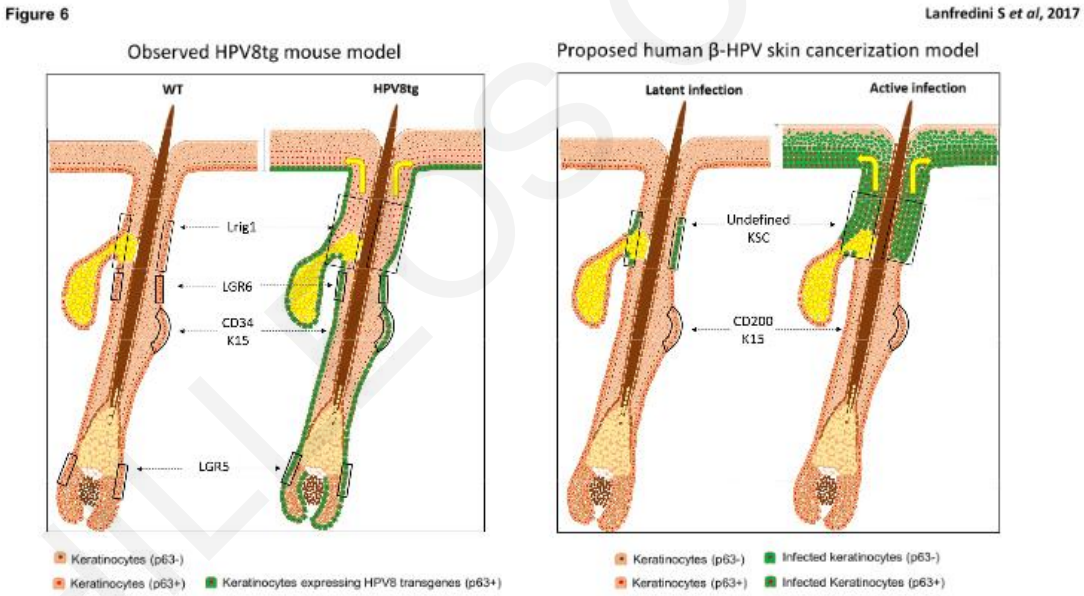


Figure 45: β -HPV Field Cancerization Model (Lanfredini S et al, 2017)

We also observed hyperplasia of the epithelium of the infected tissue (using H&E staining), as well as upregulation of Keratin 14. We also observed positive cd34+ and K15 cells in the epithelium of the infected mice that are not present on the uninfected tissue (usually restricted in stem cell niche eg. hair follicle). These results are very interesting, as we observed expression of stem cells markers in papillomavirus-infected tissues. Results were consistent

in all genetic backgrounds tested. Our next step is to use this MmuPV1 infection model in order to shed light on the effect that viral infection has on epidermal stem cells. We are planning to perform a brdU-pulse chase assay and evaluate potential changes in LRC numbers at different time points after infection with MmuPV1. In addition, we will examine if there are changes in additional markers such as Lgr5 and a6 intergrin in the infected tissues. Moreover, we wish to quantify the increase in the expression levels of the stemness markers seen in MmuPV1 infected tissue as previously discussed. As discussed in chapter 1.3.2, there are two main theories on the cells of origin of cancer stem cells. Stem cells that already have stemness characteristics or differentiated cells that transform to stem-cell like cells after the infection. We do not know whether this upregulation of the stem cell markers expression we observed is due to mobilization of tissue stem cells from the hair follicle that already expressed those markers, or whether the virus infection induces their expression in cells that normally do not express them. To conclusively answer this question we need a model for linage tracing the MmuPV1infected cells. Currently in the lab we are working toward the creation of this virus (Figure 46), which can be used in the *in vivo* model of infection and shed light to those questions. A plasmid containing the MmuPV1 genome and Lox P sites will be co-transfected with a cre recombinant plasmid in ROSA transgenic mice that have an innate Yellow Fluorescent Protein (YFP) marker that can be expressed in the presence of Cre. Using this system, we can trace the originally infected cells and, using time points, decipher whether the expansion of the stem cell compartment is started from stem cells or differentiated cells.

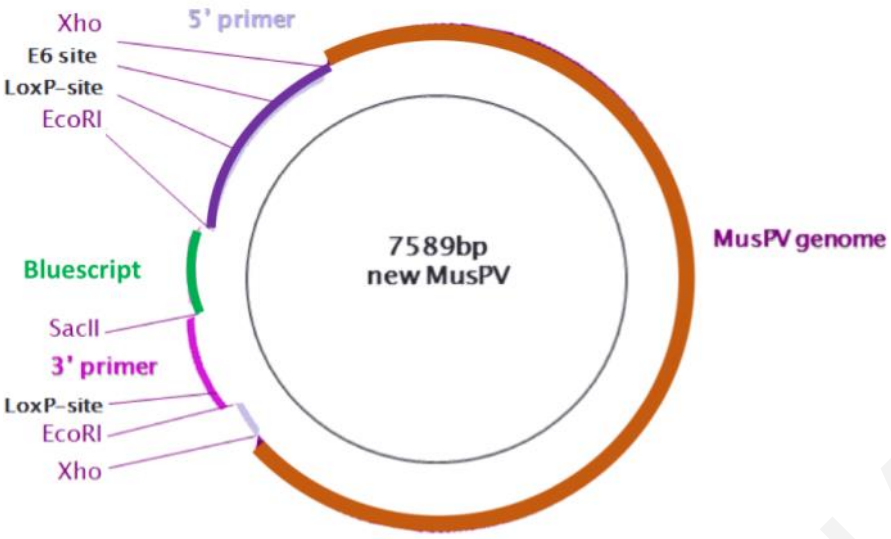


Figure 46: Construction of MmuPV1 vector with innate LoxP-sites. New plasmid with 7589bps contains the MmuPV1 genome, Bluescript package plasmid, 5' and 3' prime sequences. One LoxP-site is located on each prime sequence.

Another stemness marker that we are interested in is Oct-4. Oct-4 is a transcription factor involved in maintaining stem cell phenotype and pluripotency. Oct-4 is overexpressed in cervical cancer lesions (Li et al., 2015). It has been shown that Oct4 promotes de-differentiation of melanoma cells to CSC-like cells and that Oct-4-induced de-differentiation was associated with increased expression of endogenous Oct-4, Nanog and Klf4, and global gene expression changes that enriched for transcription factors promoting stemness. RNAi-mediated knockdown of Oct-4 in de-differentiated cells led to diminished CSC phenotypes suggesting that Oct-4 is a positive regulator of tumor de-differentiation (Kumar et al., 2012).We found that oct-4 was expressed in MmuPV1 infected tissue (we evaluated two mice, one tumor each) using oct4 antibodies from two different brands (abcam #ab19857 and milipore #SCR077). As oct-4 was also found to be expressed in cervical cancer cell lines that express the HPV 16 oncogenes (HeLA and Caski), we studied the expression of oct-4 in human cervical cancer microarrays in order to determine whether this is a consistent event during papilloma virus infection. We found that 78% of grade 2 Squamous cell carcinomas and about 60% of grade 1 and 3 squamous cell carcinomas had positive oct-4 expressing cells (data not shown).

3.4.2 Immune system infiltration in MmuPV1 infected tissues

Cancer and inflammation are usually linked through molecular events that activate oncogenes and inactivate tumor suppressor genes, causing transformation and neoplasia. Transformed cells in turn produce newly expressed or overexpressed transcription factors and other molecules involved in immunoregulation, thus inducing inflammation and creating an inflammatory microenvironment within and around the area where tumors develop. In these cases, tumorigenesis drives inflammation. Using immunohistochemistry in MmuPV1 infected mice we showed that natural killer cells and macrophages are present in the infected tissue. STAT -3 was also detectable in the infected tissue and not on the non infected tissue. The tissues used in this experiment were obtained a month after micro wounds were induced. Thus, more time points are needed after infection, in order to establish whether the presence of immune cells is the result of a response to wounding, or an indication of chronic inflammation caused by the presence of the virus in the epithelium. Moreover, infection with pseudovirus should be examined at the same time points in order to determine whether the response is triggered by the presence of the viral capsids, or by the expression of viral genes. Cladel et al., have recently show that MmuPV1 can infect immunocompetent mice at mucosal sites (Cladel et al., 2017). This suggests that MmuPV1 infection can take place under the influence of an intact immune system. Thus, this infection model can also be used as a tool to examine the additive effects of inflammation on epidermal stem cells at the site of infection, as well as the immunological response from the beginning of viral-host interaction.

SYNOPSIS

MmuPV1 is a useful model to study HPV infection, pathogenicity and carcinogenesis as it shares many similarities with cutaneous HPVs, especially HPV-8. We showed that MmuPV1 genome can lead to papillomas formation *in vivo* and also affect the stem cell population of the hair follicle the same way as cutaneous HPVs do. MmuPV1 can also affect markers that have been previously shown to be affected by mucosal HPVs (eg. keratin 15). Conclusively, MmuPV1 virus can manipulate either stem cells or non-stem cells in the tissue that it infects and cause an expansion of the stem cell compartment of the epithelium. It is critical to further understand the contribution of the papilloma virus oncogenes to stem cell identity as this can lead to better therapeutics.

Studies on MmuPV1 could reveal the characteristics that the host might acquire in the presence of MmuPV1 and consequently better treatments for genital or skin warts as well as aggressive skin cancer caused by HPV can be developed. Of course we always have in mind that as with all animal PVs, there is an evolution distance from human PVs and the interactions may differ at some points.

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CHAPTER 5: PUBLICATIONS

1. **Achilleos, C***., Michael, S*. & Strati, K. 2018. **Terc is dispensable for most of the short-term HPV16 oncogene-mediated phenotypes in mice**. PLoS One*,* 13**,** e0196604. . *=Equal contribution

2. Michael, S.*, **Achilleos, C.*,** Panayiotoy T*., Strati, K. **"Inflammation Shapes Stem cells and Stemness During Infection and Beyond.**" Front. Cell. and Developm. Biol. 2016 Nov 2;4:118. *=Equal contribution

3. Iacovides, D., Michael, S., **Achilleos, C.,** Strati, K. (2013). "**Shared mechanisms in stemness and carcinogenesis: lessons from oncogenic viruses.**" Front. Cell. Infect. Microbiol. *3,* doi: 10.3389/fcimb.2013.00066

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RESEARCH ARTICLE

Terc is dispensable for most of the short-term HPV16 oncogene-mediated phenotypes in mice

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Abstract

High-risk human papillomaviruses (HPVs) have been shown *in vitro* to impinge on telomere homeostasis in a number of ways. However, the *in vivo* interaction of viruses with the telomere homeostasis apparatus has not been previously explored. Since E6 and E7 are the main viral oncogenes and key for viral replication, we have explored here the short-term phenotypes of the genes in the context of defective telomere homeostasis. We examined the short-term phenotypes of E6 and E7 in a context where the Terc component of the telomerase holoenzyme was knocked out. We determined that Terc was dispensable for most oncogene-mediated phenotypes. Surprisingly, E7-mediated reduction of label retaining cells was found to be in part dependent on the presence of Terc. Under the conditions examined here, there appears to be no compelling evidence Terc is required for most short-term viral oncogene mediated phenotypes. Further studies will elucidate its role in longer-term phenotypes.

Introduction

Human papillomaviruses are considered to be important human pathogens and model systems of viral carcinogenesis. High risk HPVs have been associated with the development of the majority of cervical cancers, a subset of head and neck cancers, as well as other anogenital cancers. HPV-associated cancers are thought to account for >5% of the worldwide cancer burden [1].

In HPV-associated malignancies carcinogenesis is driven, in large part, by the continuous expression of the viral oncogenes E6 and E7 [2, 3]. These oncogenes have no known enzymatic actions but mediate their profound effects in infected cells by engaging important cellular proteins including the tumor suppressors p53 and pRb [4–7]. Interactions with cellular targets are critical determinants of viral lifecycle and are sometimes important in viral driven carcinogenesis, which is linked to persistent infection and often viral integration. There is credible evidence which suggests that telomerase reactivation and changes in telomere homeostasis are a contributing factor to the oncogenic effects of HPV.

Telomerase reactivation is observed in the overwhelming majority of cancers [8] and has been demonstrated to be the key mechanism, which counteracts cellular senescence and

apoptosis, brought on by telomere erosion in highly proliferative malignancies. In normal cells telomerase activity is high in embryonic and tissue stem cells but is gradually diminished during the normal differentiation process to low or undetectable levels [9, 10]. Reactivation during the oncogenic process, often during late stages, has been described to occur by means of transcriptional and/or translational upregulation of the enzymatic component of the telomerase holoenzyme Tert [11]. In a minority of cancers (10–15%) [12] telomeres are elongated via telomerase-independent Alternative Lengthening of Telomeres (ALT).

Intriguingly high-risk HPV oncogenes have been shown by *in vitro* studies to modulate telomere homeostasis in a variety of ways. Both E6 and E7 have been implicated in regulating telomere length by means of telomerase activation and ALT respectively. The HPV16 E6 oncoprotein affects telomerase activity by increasing the transcriptional levels of the human telomerase reverse transcriptase (hTERT) component of telomerase, an essential step for cell immortalization [13]. This can be achieved either by inducing the activation of the Tert promoter, via its interactions with the promoter's activator proteins (eg. Myc and NFX1-123) or repressor proteins (eg. NFX1-91), or by directly binding to the Tert protein [13–17] and telomeric repeats. In HeLa cells re-expression of either E6 or E7, after their removal, leads to increased hTERT [18]. Studies from Kiyono et al. showed that the E6-induced activation of telomerase activity requires the combined effect of the E7-induced inactivation of the Rb pathway for immortalization of both fibroblasts and keratinocytes in culture [19]. Other studies demonstrated that E7 can increase hTERT promoter-driven expression and augment telomerase activity driven by HPV E6 [20] and can immortalize keratinocytes by cooperating with Tert even when defective for telomere maintenance [21].

However, the multitude of ways in which the HPV oncogenes have been shown to modulate telomere homeostasis point to another intriguing possibility: that this is a phenomenon reflective of evolutionary adaptation of the virus [13]. This of course would suggest that telomere homeostasis is important not only during later stages of carcinogenesis but also during the viral lifecycle (and potentially contributing to earlier stages of carcinogenesis). For example such an alteration in telomere homeostasis, driving cells towards a more stem-like phenotype, could allow viral persistency a critical initial step for carcinogenesis.

This study addresses the role of telomere homeostasis on the effects of the HPV16 oncogenes on the stratified epithelia of the skin, as previously reported [22], using transgenic mice expressing E6 and E7 under the K14 promoter [23] as well as mice deficient for the expression of the telomerase RNA component [24]. We examined the *in vivo* interplay of the HPV16 E6 and E7 oncogenes with the telomerase complex and its effects on the short-term phenotypes caused by the viral oncogenes on target tissues. These phenotypes correlate both to events important to early carcinogenesis as well as to the viral lifecycle and shed light on the importance of the telomerase complex during viral replication and disease.

Materials and methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guidelines for the Protection of Laboratory Animals of the Republic of Cyprus. The animal facility is licensed by the Veterinary Services (Republic of Cyprus Ministry of Agriculture and Natural Resources), the government body in charge of approving and overseeing laboratory animal work in Cyprus (license number CY.EXP.105) and the protocol was approved by the same authority (License number CY/EXP/PR.L1/2013). The work described here does not involve procedures requiring anesthesia. Mice were sacrificed at the specified ages. We adhere to acceptable euthanasia guidelines using a CO_2 chamber.

Mice

The mouse strains used in the experiments were obtained from the crosses of K14E6/E7TTL referred to as K14E6, K14E7/E6TTL referred to K14E7 as previously described [23, 25] and Terc+/- mice [24]. The K14E6 and K14E7 mice were of a pure FVB/N genetic background and Terc+/- mice were on a pure C57BL/6 genetic background. All the genotypes were confirmed by means of PCR.

Generation of mice expressing the HPV16 oncogenes in the presence or absence of Terc

For assessing the role of the absence of Terc and thus of telomerase on the HPV-expressing epithelia, mice expressing the HPV16 oncogenes in the presence or absence of Terc were generated. 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. All the genotypes obtained from the crosses are listed on Table 1. The crossing scheme was consistent for all genotypes obtained. All mice genotypes were confirmed by means of PCR, using DNA extracted from tail clippings.

BrdU incorporation

5-Bromo-2-deoxyuridine (BrdU) was administered peritoneally in mice at a final concentration of 50 mg/kg as first described previously [26]. For pulse chase experiments ten-day-old mice received an injection every 12 h for a total of four doses. They were euthanized 60 days after injections.

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 of 10 μm thickness were obtained using 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 min in 2 M HCl. Blocking and antibody incubations were variable and optimal for each different antibody used. Primary antibodies used include: 1:100 BrdU (Abcam, ab6326, rat monoclonal), 1:500 K15 (SantaCruz, sc-47697, mouse monoclonal), 1:100 PCNA (SantaCruz,

Table 1. Experimental mice used.

Genotype	Transgene
NTG Terc+/+	None
K14E7 Terc+/+	E7
K14E6 Terc+/+	E6
K14NTG Terc-/-	None
K14E7 Terc-/-	E7
K14E6 Terc-/-	E6

The crossing scheme generated mice expressing telomerase (Terc+/+) and mice deficient for telomerase (Terc-/-). These mice were either non-transgenic for the viral oncogenes (NTG) or transgenic for the oncogenes (K14E6 and K14E7).

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sc-25280, mouse monoclonal). Following primary antibody incubation samples were washed in PBS. The following secondary antibodies were used: Cy3-streptavidin, biotin-rat and biotin -mouse all from Jackson ImmunoResearch and also Vectastain universal secondary (Vector laboratories, #016-160-084). All images were acquired using a Zeiss Axio Observer.A1 microscope. Quantification was performed in a blinded fashion.

Fluorescent in situ hybridization (FISH) assay

Samples were deparaffinised in xylene and rehydrated in a graded series of ethanol solutions. They were then treated with HCl/Pepsin for 10 min at 37˚C, washed in PBS and then fixed in 4% paraformaldehyde for 2 min, washed in PBS and dehydrated in a graded series of ethanol concentrations. The slides were air-dried and the DNA was denaturated for 2 min at 80˚C and then hybridized for 2 h with Cy3-conjugated peptide nucleic acid (PNA) probe in the dark at room temperature for 2 h. Samples were washed in wash solution (Final concentration: 70% formamide, 10mM Tris, 0.1% BSA) twice for 15 min and three times for 5 min in TBS-Tween 0.08%. After the washes, the slides were dehydrated in a graded series of ethanol solutions and mounted with DAPI. Samples were analyzed on a Zeiss LSM 710 Axiovert confocal microscope using a 63× Plan-Neofluar 1.4 NA oil immersion objective lens. Images were analyzed with Axiovision 4.2 software and processed using TFL-Telo V2.

Telomeric repeat amplification protocol (TRAP) assay

Trap assay was performed using the TRAPeze® Telomerase Detection Kit (S7700-KIT; Millipore). Briefly, fresh skin tissue was obtained and floated with the dermis side down in 0.25% trypsin at 4˚C O/N. On the next day, the dermis was peeled off and the epidermis was homogenized while kept on ice. CHAPS lysis buffer was added (200μl per 10μg of tissue) and samples were incubated on ice for 30 min. Then, 160μL of the supernatant was collected, and the protein concentration was determined. A reaction mix containing TRAP buffer (20 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 63 mM KCl, 0.05% (v/v) Tween-20, 1 mM EDTA, and 0.01% BSA; TRAPeze telomerase detection kit), supplemented with dNTP mix, TS primer, TRAP primer mix, dH_2O, Taq polymerase at indicated concentrations was prepared and incubated with the samples at 30˚C for 30 min. PCR was used for amplication (94˚C for 30 s, 59˚C for 30 s, 72˚C for 1 min for 30 cycles and 72˚C for 7 min in a thermocycler. PCR samples were run on a 10% (w/v) native-PAGE gel in 0.5XTBE for 1 h at 150 V. After electrophoresis, the gel was stained with ethidium bromide for 10 min at room temperature. The telomerase activity was estimated by measuring the intensity of the bands using ImageJ software.

Statistical tests

To determine the statistical significance between the genotypes in each experiment, 3 mice of each genotype were used and 60–80 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 $p \leq 0.05$.

Results

Expression of the HPV16 E6 oncogene leads to increased telomerase activity *in vivo*

To assess the levels of telomerase activity in mouse epithelia expressing the HPV16 oncogenes we performed TRAP assays. Using epithelial extracts from K14E6 and K14E7 mice we found

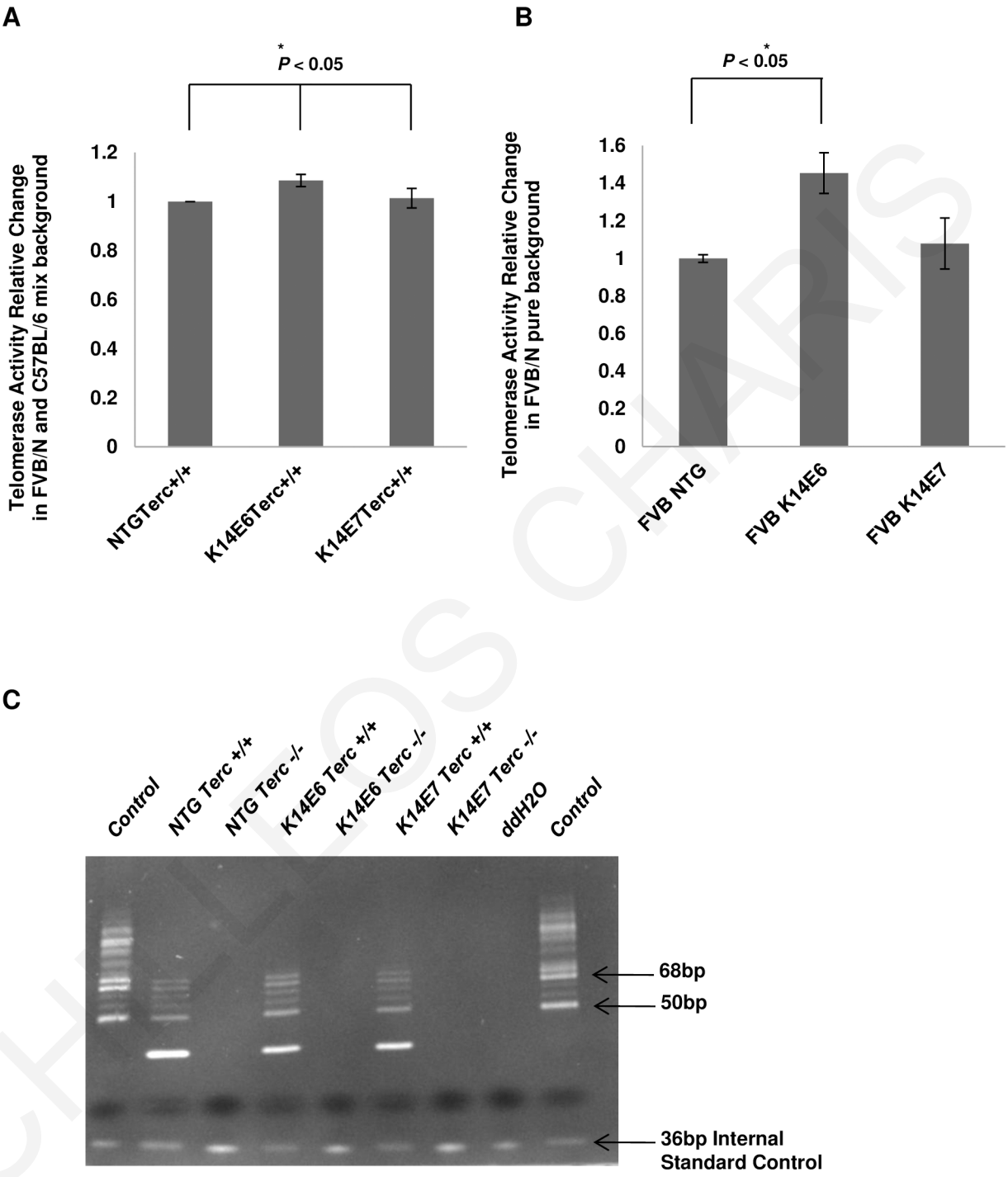

Fig 1. Expression of the HPV16 E6 oncogene increases telomerase activity *in vivo*. (A) and (B) The telomerase activity was determined using ImageJ software analysis and plotted for each genotype (columns); bars, SD. The experiments were done using three mice from each genotype and all statistical comparisons were performed using a two-sided Wilcoxon rank sum test. Statistical significance was observed between E6 and NTG or E7 mice. (NTG; Non Transgenic) (C) Representative native PAGE gel image of the telomerase products extracted from skin tissue and amplified using the Trap assay.

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that a low but statistically significant increase in telomerase activity was seen in epithelia for K14E6 animals, a result consistent in both mixed (Fig 1A) and pure (Fig 1B) genetic

backgrounds. This is the first time that such an increase is shown *in vivo* and this result is consistent with previously reported telomerase upregulation in human keratinocytes transduced with HPV16 oncogenes [27]. Thus, we sought to determine potential roles of telomerase in short-term phenotypes of transgenic animals.

To study the canonical-roles of telomerase we crossed animals transgenic for the HPV16 oncogenes with animals in which the RNA component of the telomerase holoenzyme (Terc) has been knocked-out. It has been previously shown that Terc knockout mice lack detectable telomerase activity [24]. To confirm the telomerase status of the mice genotypes used in our experiments, telomere repeat amplification protocol (TRAP) was used. Mice lacking the Terc component of telomerase showed no telomerase activity as expected (Fig 1C).

Telomeric length is unaffected by HPV16 oncogene expression *in vivo*

To examine the effect of the HPV16 E6 and E7 oncogene expression on telomere length, tissue from mice with defective and wild type Terc were subjected to Q-FISH (Fig 2A) and the average telomere length of telomeres from 75 nuclei was compared among all genotypes (Fig 2B). Results revealed no statistical differences among the different samples. Results were verified using mice on a pure FVB/N background (Fig 2C).

The telomerase holoenzyme is responsible for telomere elongation and under normal conditions preferentially acts on critically short telomeres slowing down their transition into senescence. It is frequently found to be upregulated in cancers, presumably to maintain a sufficiently long telomeric length despite the constant rounds of cell division common in malignancy. Thus, the activation of telomerase in tumors is thought to be a late event in carcinogenesis. These findings could explain why the absence of Terc and thus of telomerase in mice of sufficiently long telomeres did not affect the telomere length in any of the genotypes tested.

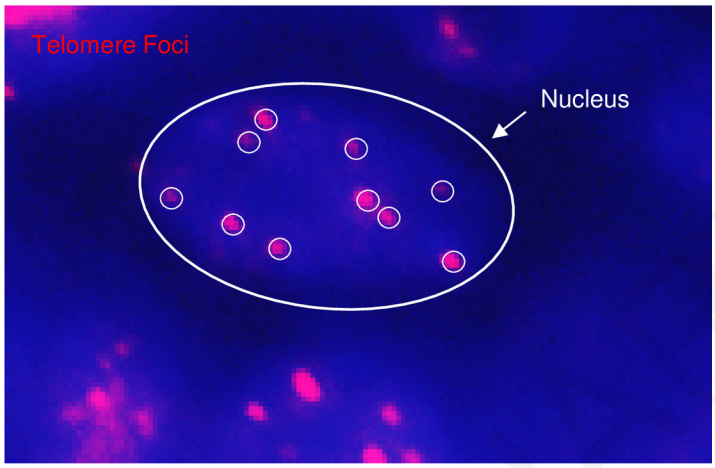
Canonical roles of telomerase are dispensable for HPV16 oncogene-mediated increase in epithelial proliferation

It has been previously shown that the HPV16 E6 and E7 oncogenes cause an increase in proliferation as marked by increased PCNA detection in anagen hair follicles [22]. Our results have verified previous findings, as an increase in PCNA positive cells in the mouse tail epithelium (Fig 3A), is seen in both basal (Fig 3B) and suprabasal (Fig 3C) layers. However, Terc was dispensable for these phenotypes suggesting that the HPV-driven proliferation of the epithelium is not connected to the telomerase status of the epithelium. Whether the Tert component of the telomerase complex is involved in the HPV-driven proliferation is still unknown.

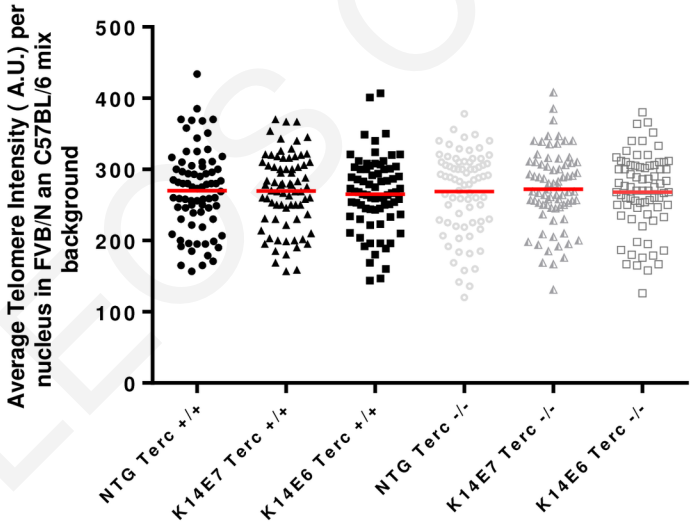
Terc is dispensable for E6 but not E7-mediated effects on hair follicle label-retaining cells

Our lab has previously shown that the HPV16 oncogenes can lead to a reduction in the numbers of label-retaining cells (LRCs) in hair follicles under resting conditions (telogen) attributed to increased proliferation [22]. We examined here the potential role of telomerase activity in these E6 and E7-mediated phenotypes. We performed BrdU pulse-chase assays as previously described [26, 28, 29], and the numbers of label-retaining cells (LRCs) at second telogen (resting phase of hair cycle) were compared in all the genotypes (Fig 4). In the presence of Terc, both E6 and E7 mice showed a reduction in the number of LRCs, a result that is consistent with the previous studies. This increase in proliferation leads to an increase in the number of cell divisions, mobilization of the quiescent cells and loss of BrdU staining.

A



B



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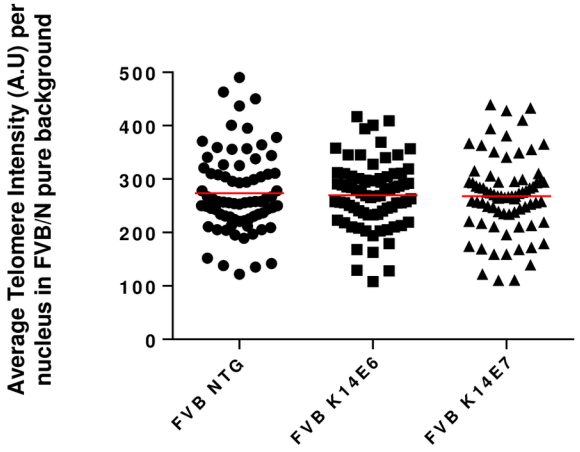

Fig 2. Expression of the HPV16 oncogenes does not affect telomeric length irrespective of the presence or absence of Terc. (A) Representative immunofluorescent image of telomeres (depicted by small white circles) using fluorescent in situ hybridisation assay (FISH) on the tail of 71 day-old mice. The nucleus is shown by an arrow. (B) and (C) The average length of the telomeres was determined by measuring the intensity of the telomeric foci using TFL-telo software in FVB/N and C57BL/6 mixed background (B) and FVB/N pure background (C). ~75 nuclei were counted for each genotype and the mean average was plotted for each genotype (columns); bars, SD. A.U; Arbitrary Units The experiments were done using three mice from each genotype and all statistical comparisons were performed using a two-sided Wilcoxon rank sum test.

https://doi.org/10.1371/journal.pone.0196604.g002

In the absence of Terc however, there is a rescue in the reduced LRC number phenotype of E7 mice but not of E6, which exhibit a statistically significant reduction of LRCs compared to the non-transgenic Terc deficient mice. E6 and E7 mediate their effects on the hair follicle LRCs via separate pathways [22]. We speculate that the pathway(s) perturbed by E7 render LRCs more sensitive to the lack of functional telomerase.

Terc is dispensable for HPV-mediated expansion of stemness related markers

Concurrent with aberrant stem cell mobilization, we have previously reported an expansion of keratin 15 (K15), an endogenous marker of bulge stem cells. Our results showed that the expression of K15 was expanded in both E6 and E7 expressing mice in regions not known to contain stemness markers. This significant increase and expansion was also seen in E6 and E7 expressing mice in a Terc deficient background when compared with the non-transgenic mice of the same background (Fig 5A and 5B). These results indicate that the reduction of LRCs in mice expressing E6 or E7 is due to an increase in the proliferation of the epithelium and an aberrant mobilization and expansion of stem cells even in the absence of the Terc component. Our results also show that the pattern of other differentiation markers such as K14 expression is consistent in all the genotypes obtained and irrespective of the mice mixed background (Fig 5C).

This suggests that the telomere elongation activity of telomerase in mice with sufficiently long telomeres, is not necessary for the mobilization and expansion of stem cells in the epithelium. The role of Tert in this process still needs to be examined in order to evaluate the necessity of the non-telomeric functions of telomerase in those processes.

Discussion

The high-risk HPVs have been extensively reported to modulate telomere homeostasis, most notably via impinging on the transcription and translation of the telomerase enzyme [13–17]. It has been proposed that the level of regulation seen, particularly by the E6 oncoprotein may point to a way in which E6 alters the "stemness" of infected cells with potential effect on the viral lifecycle and carcinogenesis [13]. To our knowledge, ours is the only study which attempts to decipher the consequences stemming from the interplay of telomere homeostasis and the HPV16 oncogenes *in vivo*.

Our results indicate that telomerase activity is indeed increased when HPV16 E6 is expressed in stratified epithelia. This is not seen when HPV16 E7 is expressed. These findings are consistent with results seen in human keratinocytes transduced with E6 or E7 expressing retroviruses. In human cells the increase in telomerase activity has previously been shown to be mediated via transcriptional and translational pathways: binding to the Tert promoter or by direct binding to the Tert protein [13–17] leading to an increased telomere elongation activity.

Contrary to what is observed in those systems, the expression of E6 and E7 in our transgenic mice does not lead to an elongation of telomeres, as quantified by a sensitive telomere-

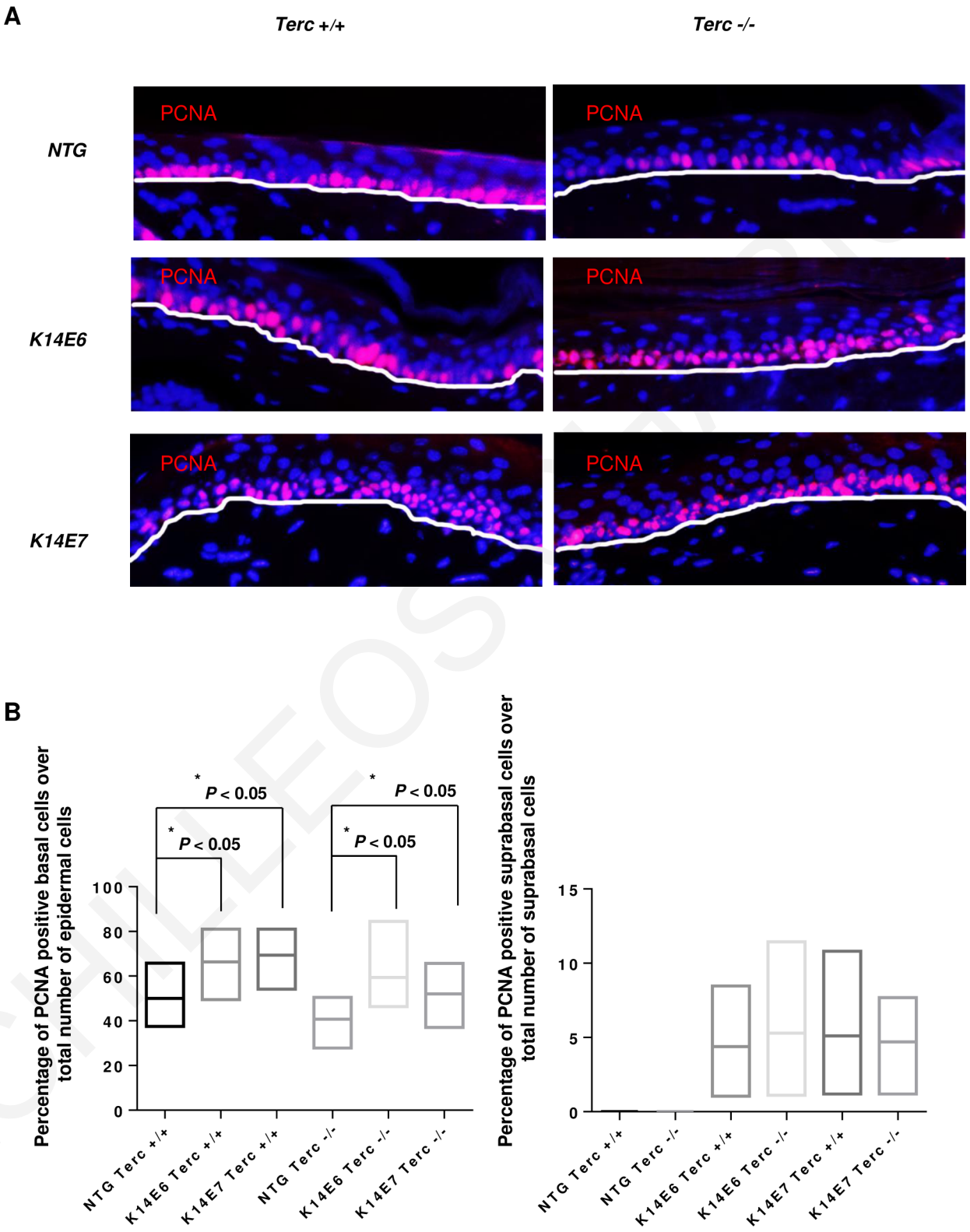

Fig 3. Expression of the HPV16 oncogenes increases the proliferative ability of the epithelium irrespective of the presence or absence of Terc. (A) Representative immunofluorescent images of the tail epithelium showing PCNA positive cells (red). Counterstaining was done with DAPI (blue). The white line indicates the basal membrane in each image. (B) The proliferative ability was determined by counting ~25 different regions of the basal (left) and suprabasal (right) layers. Mice were 71 days old. The positive cells were counted for each genotype and the

percentage of positive cells over the total number of cells was plotted (columns); bars, SD. The experiments were done using three mice from each genotype and all statistical comparisons were performed using a two-sided Wilcoxon rank sum test. As expected, no positive PCNA cells were detected in the suprabasal layers of non-transgenic animals.

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FISH assay. A likely factor for the discrepancy between previous results and those observed here may be the different levels of oncogene expression. In our system there is a lower expression of E6 and E7, as opposed to the retroviral systems, which is in line with those encountered during human infection [30] and correlates the *in vivo* physiological effects of the oncogenes. While mouse telomeres are longer than those of humans, telomerase preferentially elongates short telomeres and the threshold for what constitutes a short/unprotected telomere also varies accordingly between mouse and human. In physiological contexts (mouse or human), when telomeres are sufficiently long it is not unexpected to see lack of telomere elongation for long periods of time despite elevated levels of telomerase activity [31]. While we cannot exclude the possibility that our results could be attributed to differences between human and mouse telomeres, the mouse has been an incredibly useful model in elucidating the role of telomere homeostasis *in vivo* [24, 32–36].

A number of short-term phenotypes have been previously attributed to the expression of the HPV16 oncogenes *in vivo*, including increased proliferation and aberrant stem cell homeostasis [22, 37]. To determine the contribution of a functional telomerase enzyme in such short-term phenotypes we crossed animals transgenic for the HPV16 oncogenes to animals in which Terc, the RNA component of telomerase, in not expressed. This has previously been shown to reliably ablate the assembly of a functional telomerase enzyme and lack of telomere elongation activity is corroborated by our results. Our system therefore, enables the first study addressing the role of telomerase and HPV16 oncogene interaction in the short-term *in vivo* phenotypes, which may correlate with important aspects of the viral lifecyle [38] and carcinogenesis, where the viral oncogene expression is still relatively low.

We determined that the presence of a functional telomerase enzyme was dispensable for most short-term phenotypes examined. It is conceivable that if elevated telomerase activity (as confirmed in the presence of E6 oncogene expression) has a role in the viral lifecycle, it may relate to aspects of viral replication or maintenance which are not directly probed in our system. The same would be true, in the case of effects which require the synergistic action of the two oncogenes [39]. Potential roles in carcinogenesis may exist in later steps during that process.

Surprisingly, the ability of E7 to contribute to LRC reduction was found to be in part dependent on the presence of Terc and functional telomerase assembly. Since stem cells are often characterized by increased telomerase activity we propose that in a context where there is increased stem cell mobilization (as has been previously shown for E7) the inability to elongate telomeres may be of increased importance. Increased mobilization of stem cells may "sensitize" them to a lack of functional telomerase.

There also exists controversial evidence for non-canonical functions of components of the telomerase enzyme, in particular Tert [40]. Since such non-canonical effects of the enzyme have been heavily debated we chose to focus our studies on the canonical effects, the main and evolutionarily conserved function of the enzyme. Thus, we performed our studies using a mouse in which Terc has been eliminated. Extratelomeric effects have not been reported for terc in mammalian systems or in tissue types relevant to the HPV replication [41]. Further studies would need to be performed to examine potential non-canonical role of Tert expression in the context of HPV oncogene expression *in vivo*.

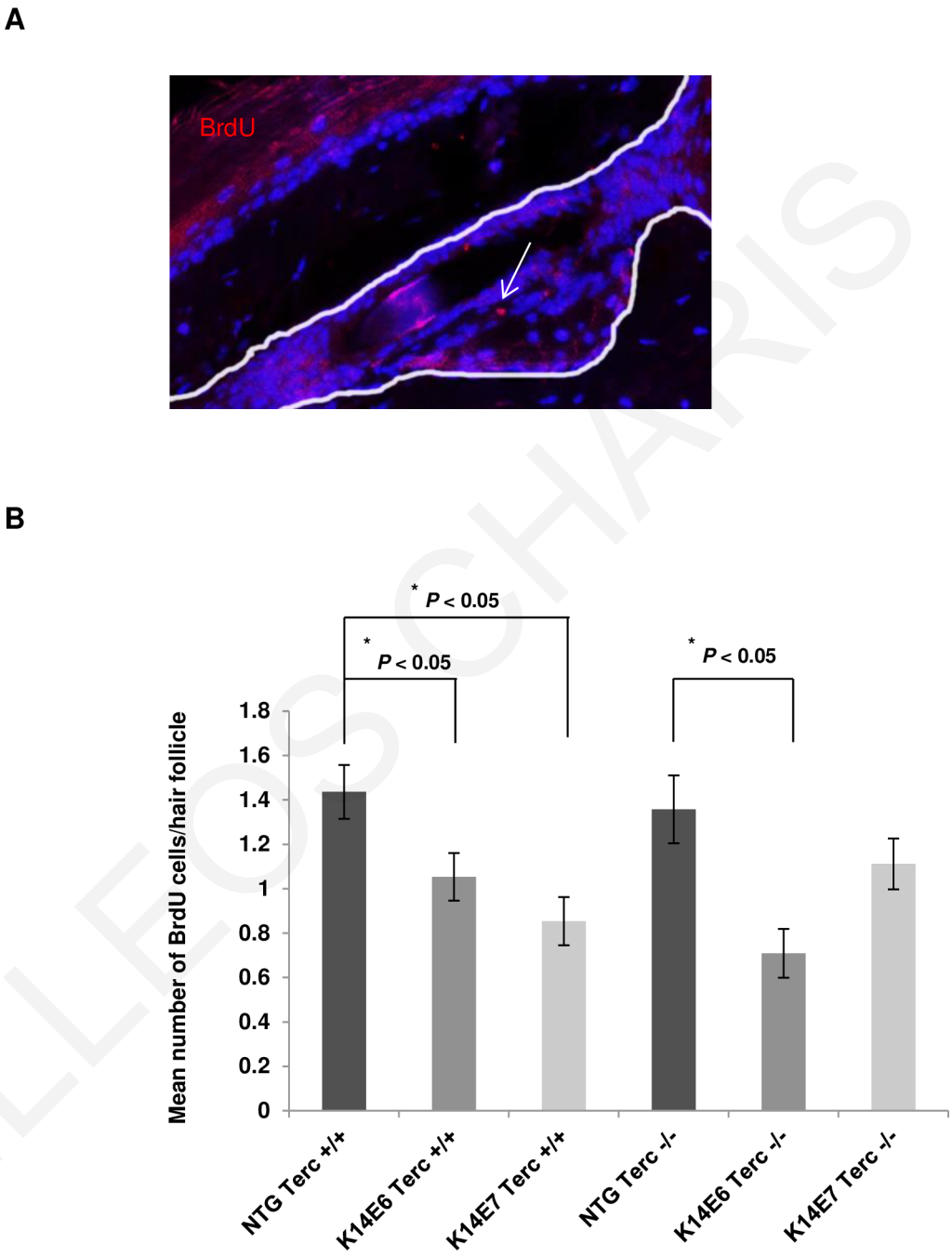

Fig 4. Terc is dispensable for E6 but not E7-mediated effects on hair follicle label- retaining cells. (A) Representative immunofluorescent image of a hair follicle showing BrdU positive cells (red). Counterstaining was done with DAPI (blue). The white line indicates the hair follicle. (B) LRCs were labelled using a BrdU pulse administered shortly after birth and chased until second telogen (71 days old). ~80 hair follicles were selected from at least 3 mice of each genotype (n = 3). The mean number of BrdU 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.

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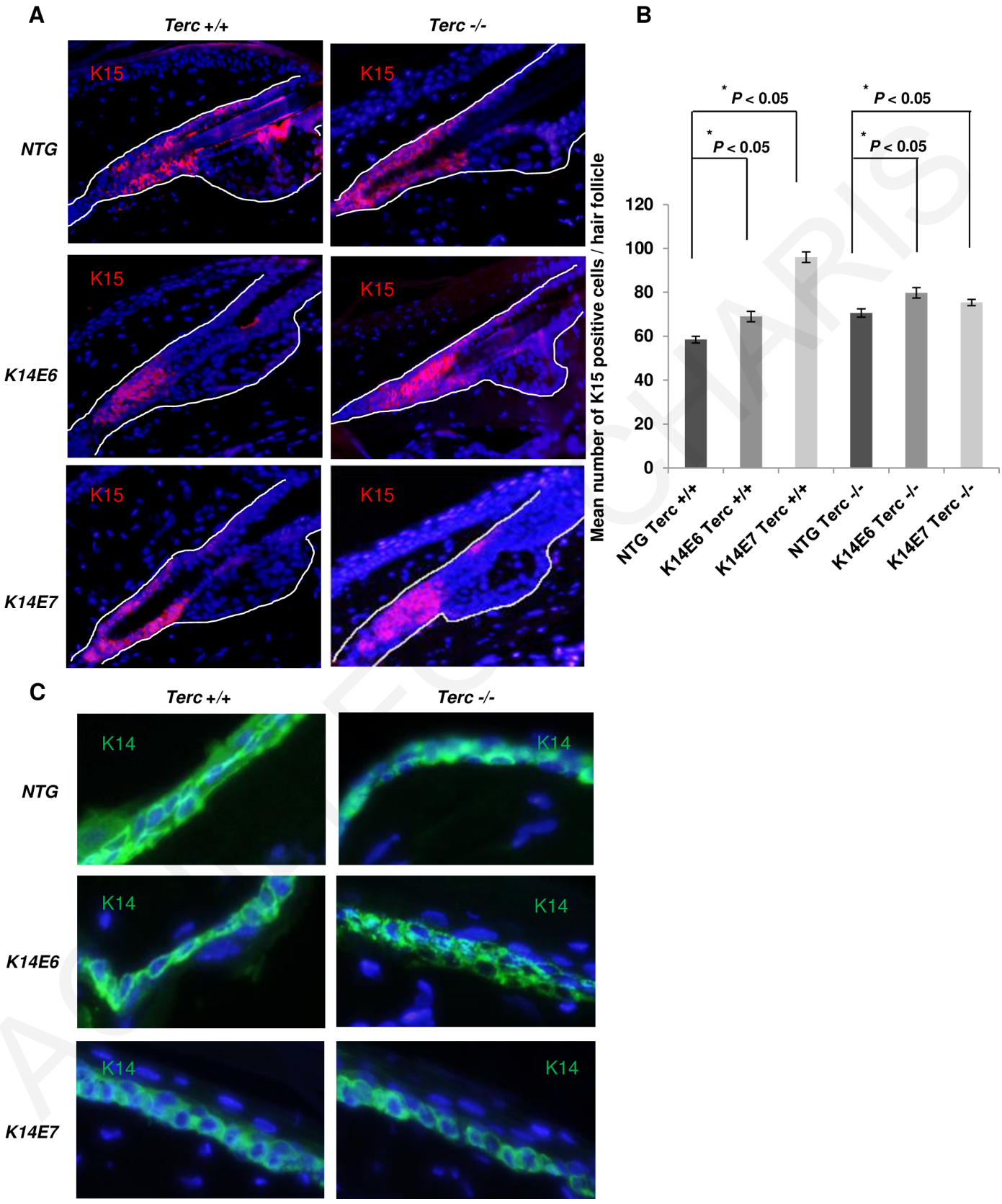
A
Terc +/+
Terc -/-
NTG
K14E6
K14E7
K15
B
Mean number of K15 positive cells / hair follicle
* P < 0.05
* P < 0.05
* P < 0.05
* P < 0.05
120
100
80
60
40
20
0
NTG Terc +/+
K14E6 Terc +/+
K14E7 Terc +/+
NTG Terc -/-
K14E6 Terc -/-
K14E7 Terc -/-
C
Terc +/+
Terc -/-
NTG
K14E6
K14E7
K14

Fig 5. HPV16 oncogene expression causes an expansion of the bulge stemness marker K15. (A) Representative immunofluorescent images of the hair follicles showing K15 staining (red) using a K-15 specific antibody. Counterstaining was done with DAPI (blue). (B) ~80 hair follicles were selected from at least 3 mice of each genotype (n = 3). Mice were 71 days old. 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. (C) Representative immunofluorescent images from the tail epithelium showing K14 expression which correlates with the expression of the E6 and E7 viral oncogenes.

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Formal analysis: Charis Achilleos, Stella Michael.

Funding acquisition: Katerina Strati.

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Methodology: Charis Achilleos, Stella Michael.

Project administration: Katerina Strati.

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Inflammation Shapes Stem Cells and Stemness during Infection and Beyond

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The outcome of an inflammatory incident can hang in the balance between restoring health and tissue integrity on the one hand, and promoting aberrant tissue homeostasis and adverse outcomes on the other. Both microbial-related and sterile inflammation is a complex response characterized by a range of innate immune cell types, which produce and respond to cytokine mediators and other inflammatory signals. In turn, cells native to the tissue in question can sense these mediators and respond by migrating, proliferating and regenerating the tissue. In this review we will discuss how the specific outcomes of inflammatory incidents are affected by the direct regulation of stem cells and cellular plasticity. While less well appreciated than the effects of inflammatory signals on immune cells and other differentiated cells, the effects are crucial in understanding inflammation and appropriately managing therapeutic interventions.

Keywords: inflammation, stem cells, stemness, stem cells and sterile inflammation, stem cells and microbes

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INTRODUCTION

Inflammation has a well-established role in the defense of organisms against microbial invasion. The presence of commensal and pathogenic microbes, both intracellular and extracellular, is usually detected by receptors residing mostly in the surface of innate immune cells known as pattern recognition receptors (PRRs). These receptors are the first line of the surveillance system which ultimately recognizes pathogen associated molecular patterns (PAMPs) and triggers the inflammatory response. Acute and chronic inflammation in a number of diseases associated with pathogens and the interplay between infection and inflammation is of paramount importance to clinical outcomes (Apidianakis and Ferrandon, 2014).

While inflammation is a defense against pathogens it can also be triggered during processes unrelated to microbial insult. This process, termed sterile inflammation, is typically linked to chemical or physical triggers. Inflammatory cells present at the site of the damage recognize danger-associated molecular patterns (DAMPs) and secrete molecules which prime the tissue restoration via the proliferation of quiescent adult stem cells (Nagaoka et al., 2000; Koh and DiPietro, 2011; Petrie et al., 2014; Kizil et al., 2015). Sterile inflammation can have profound effects on tissue homeostasis and repair, for example during wound healing, or during the onset and initiation of inflammatory diseases.

We summarize here evidence for the direct crosstalk between the inflammatory response and stem cells both in cases of microbial and sterile induced inflammation (**Figure 1**). Inflammation is emerging as an important regulator of stem cells and plays an intricate role in health and disease.

REGULATION OF STEM CELLS IN RESPONSE TO MICROBIAL MOTIFS

Recently PRRs were shown to be expressed in the surface of tissue stem cells suggesting that there is the potential for direct effects of PAMPs on stem cell behavior (Boiko and Borghesi, 2012). For example, hematopoietic stem cells express toll-like receptors (TLRs) whose activation leads to the differentiation of myeloid progenitors intomonocytes and macrophages immune cells (Nagai et al., 2006) as seen in the presence of the vaccinia virus in the bone marrow (Singh et al., 2008). Stem cells of solid tissues, more prominently the gut, have also been shown to express PRRs integrating inflammation to immune clearance and subsequent tissue regeneration. Intestinal stem cells (ISCs) expressing TLR4 show increased proliferation and expansion of the stem cell population in the intestinal epithelium (Santaolalla et al., 2013). ISCs have also been shown to express Nod2, a general sensor for peptidoglycan (Girardin et al., 2003). The constitutive expression of Nod2, in ISCs, provides protection against stress (Nigro et al., 2014). The ability of gut stem cells to respond

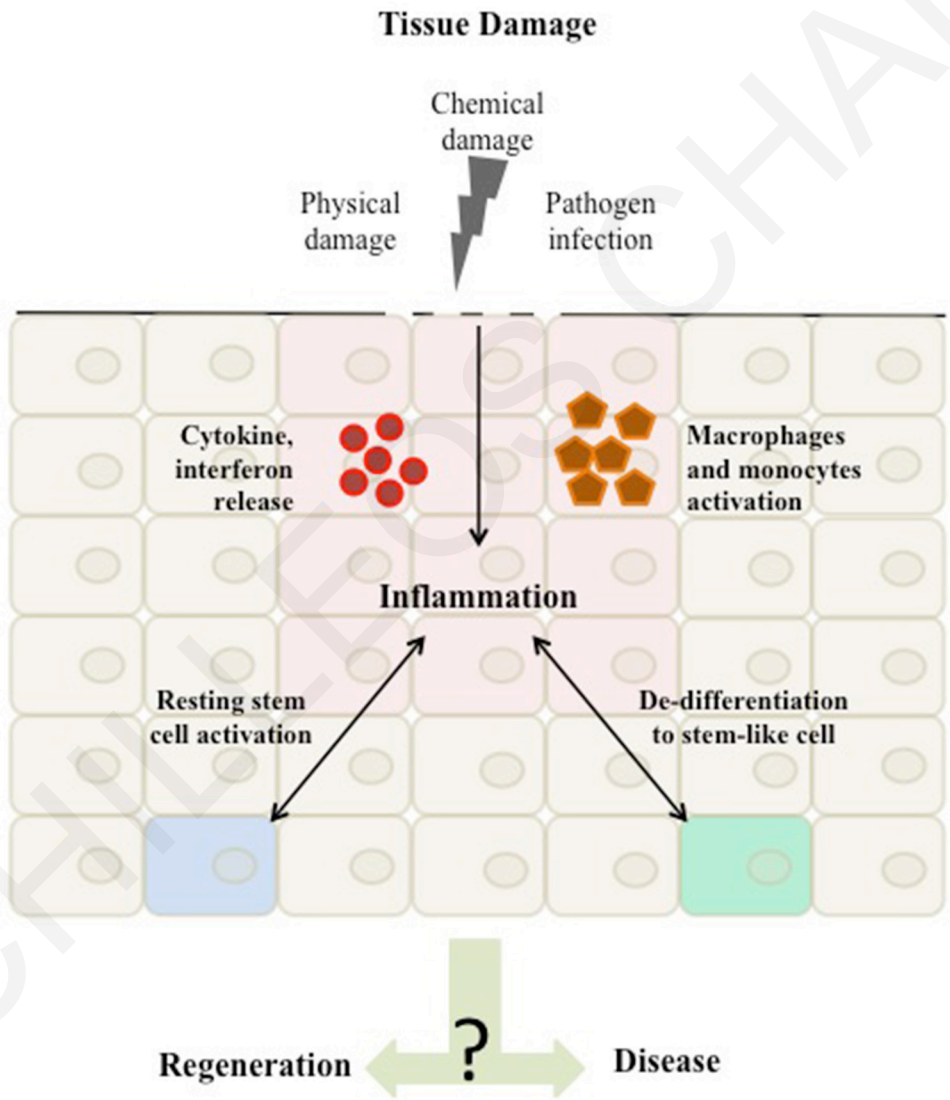

FIGURE 1 | Tissue damage can arise as a result of physical damage, chemical damage or pathogen infection. Once the damage is detected a homeostatic inflammatory response is activated to regenerate the tissue. This inflammation is characterized by the activation of immune cells, such as macrophages and monocytes and the release of inflammatory mediators, such as cytokines and interferons to the site of damage. These mediators in turn can affect native tissue cells to respond by migrating and proliferating resulting in tissue repair. Evidence suggest that the inflammatory response acts as a regulator of tissue stemness either by directly affecting tissue stem cells or by shifting differentiated cells toward a stem-like cell character. The balance in this inflammatory response and its mediated stemness is a critical driver of either maintaining tissue integrity or promoting aberrant homeostasis and disease.

directly to patterns, such as LPS (via TLR4) and peptidoglycan (via Nod2), may underlie mechanisms of tissue response not only to pathogenic bacteria but also commensals and is likely important to general tissue homeostasis via the interaction with intestinal microbiota. The findings in mammalian systems are corroborated by extensive literature in other experimental systems, such as Drosophila (Panayidou and Apidianakis, 2013).

Once an inflammatory program has already been initiated the production of cytokines, interferons etc. by local immune populations can further impact the behavior of stem cells. In the gut, innate lymphoid cells produce interleukin-22, a potent survival factor, which can directly act on ISCs promoting growth and epithelial regeneration (Lindemans et al., 2015). Chronic HBV infection also stimulates release of interleukin-22 by inflammatory cells, inducing proliferation of liver stem cells (Feng et al., 2012). In addition, the mediator of inflammation TNF-α is activated as a result of brain inflammation in neural stem cells seen in conditions of trauma, multiple sclerosis and pathogen infections. TNF-α activation ultimately brings about proliferation of the neural stem cells (Widera et al., 2006). Neural stem cells in the hippocampus have also been shown by *in vivo* studies to proliferate upon the presence of bacterial enterotoxins (Wolf et al., 2009). In the urinary tract, upon *E. coli* infection, the uroepithelial stem cells are activated for epithelial renewal in response to the inflammatory response (Mysorekar et al., 2009).

The presence of pathogenic burden in the hematopoietic system rapidly depletes immune cells stimulating intermediate blood progenitors to maintain blood cell balance (Hawkins et al., 2006). Inflammation-induced myelopoiesis, due to pathogen presence, results in the release of interleukin-27 causing activation and differentiation of hematopoietic stem cells (HSCs) (Furusawa et al., 2016). Chronic infection, as seen in the presence of the *Mycobacterium avium,* results in the activation of quiescent HSCs through the release of the inflammatory mediator interferon-γ (Baldridge et al., 2010).

Inflammation is proposed to promote tissue recovery via its effects on differentiated cells to regenerate the tissue (Karin and Clevers, 2016). In some cases the differentiated cells de-differentiate in response to inflammation, acquiring stem-like characteristics and increased cellular plasticity. In support of this idea, the induction of immunity was found to be required for efficient nuclear reprogramming *in vitro* (Lee et al., 2012).

Tissue reprogramming is achieved through the upregulation of growth factors and cytokines in the inflammatory microenvironment (Grivennikov et al., 2010). This could be attributed to changes in the expression of specific genes/pathways which shift a differentiated cell closer toward a stem cell character. Alternatively, the effects could impart tissue stem cells or progenitors with increased/altered capabilities.

There are a number of examples to support the idea that inflammation caused by infections leads to tissue regeneration and/or cellular stemness. Such a response has been observed in viral infections of HBV and HCV where inflammation in the liver induced expression of stemness markers (Karakasiliotis and Mavromara, 2015). Specifically, the secretion of interleukin 6 (IL6) by the inflammatory cells during HBV infection regulates the expression of Oct4 and Nanog pluripotency factors (Chang et al., 2015). Furthermore, the hypoxia factor HIF-1α produced in the HCV virally-infected cells confers an epithelial-mesenchymal transition (EMT) character (Wilson et al., 2012). It is important to note that in this case, the EMT is accompanied by enhanced viral replication.

In fact, inflammation-mediated changes on the differentiation status of the tissue are a factor in the pathology which accompanies disease. Persistent induction of stemness in the infected tissue in the presence of chronic inflammation, as seen in infections of the gut, can likely contribute to carcinogenesis (Apidianakis and Ferrandon, 2014; Kuo et al., 2016). However, emerging concept suggests that it may also be beneficial to the pathogen. Several groups have shown that for some infectious agents it can play a role during their replication (as in the case of HBV and HCV), their dissemination, and protection (Masaki et al., 2013; Nigro et al., 2014; Karakasiliotis and Mavromara, 2015). A more prominent 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 then migrate to the mesenchyme where they re-differentiate to mesenchyme tissue allowing for expansion of the infection (Masaki et al., 2013). The innate immune response has been shown to precede this reprogramming (Masaki et al., 2014). 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.

The inflammatory response is important to the host organism as a protective mechanism against pathogen invasion as well as tissue regeneration through the induction of stemness. In some cases however, through inflammation, pathogens are able to escape immune surveillance for their protection and dissemination with possible consequences to their lifecycle and replicative potential, as we have seen in the cases of the HBV and HCV viruses or of bacteria in the intestine or the nervous system.

REGULATION OF STEM CELLS DURING TISSUE (RE)GENERATION

Sterile inflammation has also been shown to lead to profound changes in the differentiation status of the tissue. An example is the regenerative process which takes place in response to wound healing. Wound healing requires an ordered sequence of events ranging from acute inflammation, tissue organization, and remodeling (Gurtner et al., 2008; Karin and Clevers, 2016). However, the period of tissue repair varies between the extent of the damage and the site of the damaged tissue (Meyer et al., 1992; Gordon et al., 2003; Pitsouli et al., 2009).

Tissue repair following an insult restores health in the tissue and preserves the state of homeostasis. Regeneration in the tissue is achieved via the priming of resident slow-cycling stem cells to adopt a proliferative state and yield transit-amplifying cells which will differentiate to restore tissue architecture. Sterile inflammation plays an important role in this process in

ways which are likely distinct to those seen during infection (Bezbradica et al., 2016).

The intestine can regenerate very rapidly. Tissue restoration is mediated via neutrophil infiltration at the site of the damage. These are responsible for JNK activation and the priming and proliferation of slow–cycling ISCs (Karin and Clevers, 2016). Work from Riehl et al. (2000) further supported the role of inflammatory signals, as administration of inflammatory cytokines and growth factors following radiation exposure rescued damaged epithelium via the proliferation and differentiation of intestinal stem cells (Riehl et al., 2000). However, homeostasis is sustained via the activation of developmental and inflammatory pathways which activate dormant ISCs. For instance, JNK signaling pathway is responsible for the secretion of IL6 which activates the JAK-STAT pathways leading to ISC proliferation (Jiang et al., 2009; Liu et al., 2010; Kuhn et al., 2014).

In the case of the skin epithelium, which also encounters frequent damage, it is proposed that keratinocytes trigger the inflammatory phase of the wound healing by secreting molecules like IL6 and TNFα (Wang et al., 2004; Ryser et al., 2014; Rittié, 2016). These inflammatory molecules along with other developmental mediators, such as extracellular matrix components (Kurbet et al., 2016) signal to the niche of slow-cycling adult stem cells in order to train them toward proliferation and ultimate differentiation (Cotsarelis et al., 1990; Taylor et al., 2000).

Another organ that has a fast-paced regeneration with the ability to recover its mass even after substantial loss is the liver (Michalopoulos, 2007). While it remains controversial whether the adult liver depends on a stem cell population during homeostatic conditions, various stem cell or progenitor populations have been described to participate in its regeneration following injury (Yimlamai et al., 2014; Wang et al., 2015). Soon after partial hepatectomy, the inflammatory cytokines TNF-α and IL6 are upregulated at the site of regeneration guiding hepatocytes to enter mitosis and restore lost tissue. Beyond the involvement of unipotent mature hepatocytes, which assist in the repair process of the liver particularly in response to acute inflammation, liver progenitor cells are also present to mediate liver repair. This is typically the case in the context of chronic inflammation, when mature hepatocytes have reached their replicative limit (Viebahn and Yeoh, 2008; Español-Suñer et al., 2012). While some controversy exists with regard to the exact characteristics of liver progenitors (Dollé et al., 2010), they respond to inflammatory signals following injury to generate differentiated cells essential for liver regeneration.

Our understanding of the effects of inflammatory signaling on stem cells, stems mostly from model tissue systems, such as the intestine, skin and liver. However, the inflammatory signaling to resident tissue stem cells is corroborated as crucial to regeneration in more poorly understood tissues and non-mammalian model systems as well (Apidianakis and Rahme, 2011). In mice, the recently described dclk1 progenitors have been shown to respond to inflammation during pancreatic regeneration (Westphalen et al., 2016). There is also extensive work implicating crosstalk between neural stem cells and inflammation in mammals and zebrafish (Kizil et al., 2015). Specifically, during zebrafish brain regeneration, inflammation is the trigger which initiates neural stem cell proliferation (Kyritsis et al., 2012).

While inflammation can prime stem cell responses it is becoming increasingly clear that in certain contexts stem cells possess immunomodulatory potential. Mesenchymal stem cells and neural stem cells are the two cell types most often ascribed with immunomodulatory potential (Kizil et al., 2015; Le Blanc and Davies, 2015). In both these examples stem cells have been shown to dampen or alter inflammation with beneficial outcomes in inflammation-associated disease. Despite the fact that the mechanisms are still poorly understood there is justified excitement for the potential application of these properties in therapeutics.

The crosstalk between sterile inflammation and stem cell plasticity within a tissue during the wound healing response is a critical step in the regenerative process. Priming stem cells that reside in the circulation, in addition to the stem cells of the regenerating tissue, may also contribute to this process. HSCs are likely directed to liver and skin following physical damage (Rennert et al., 2012). It was further suggested that inflammatory cytokines and growth factors released due to tissue injuries can stimulate a signaling wave toward bone marrow-residing stem cells to enter the circulation and inhabit the injured site. These bone-marrow stem cells and locally residing tissue stem cells hold the capacity of tissue regeneration. Perhaps more surprisingly, sterile inflammatory signaling, such as that initiated by IFNγ and TLR4, plays a role not only in the regeneration of adult tissue but is a well-conserved regulator in their production during development (Li et al., 2014; He et al., 2015). These findings certainly reframe currently held ideas about the evolutionary function of inflammation.

INFLAMMATORY SIGNALING DURING DISEASE

In some cases inflammation, particularly where it is chronic, can lead to the development of disease. The continuous and often aberrant response of stem cells as a result of this signaling has been shown to play an important part in this process. Intestinal stem cells express TLR4 the activation of which can lead to ER-stress, a trigger for stem cell apoptosis during necrotizing enterocolitis (Afrazi et al., 2014). In Barrett's esophagus $Lgr5^{+}$ gastric cardia stem cells can migrate in response to the inflammatory signaling and are the likely source of the metaplastic and dysplastic cells observed in the course of the disease (Quante et al., 2012).

The disease most commonly associated with inflammation is of course cancer. Multiple studies have produced substantial evidence suggesting that cancer and inflammation are in many cases connected, interdependent biological processes (Coussens and Werb, 2002; Balkwill et al., 2005; Karin, 2006). This is true in cases where the cancer is associated with microbial or viral causes, but also in cases where no pathogen is directly linked. For example, in addition to their mutagenic effects, carcinogens in tobacco smoke cause damage and chronic inflammation to the lungs and increase the risk of cancer

development (Punturieri et al., 2009). Autoimmunity is also associated with increased tumor development. The chance of developing colitis-associated cancer or lymphoma is increased in people suffering from inflammatory bowel or celiac disease, respectively (Kraus and Arber, 2009; Waldner and Neurath, 2009).

The important bidirectional link between inflammation and stem cells has direct implications on cancer development. Studies have suggested that HSC recruitment and differentiation is directly linked to increased inflammation. For example, CD34+ progenitor cells are recruited to sites with increased inflammation, probably using the same adhesion and chemokine receptors used for stem cell homing to the bone marrow (PSGL-1, CXCL12, α4β1 integrin, CD44, and others) (Blanchet and McNagny, 2009). Inflammatory mediators seem to have a vital role in inducing expression of stemness-related genes. The expression of stemness-related genes in cancer is likely linked to the generation and evolution of the compartment of cells able to regenerate tumor diversity, the cancer stem cells (CSCs) (Kuo et al., 2016; Uthaya Kumar et al., 2016). Upregulation of OCT4 has been shown to contribute to tumor cell migration and resistance to cancer therapeutics (Ma et al., 2011; Chang et al., 2015, 2016; Bhatt et al., 2016).

While inflammation is one of the stimuli suggested to initiate such transcriptional changes, once CSCs form, evidence suggests that they can serve to further amplify inflammatory signaling. Chemoresistant CSCs were found to express proinflammatory gene signatures, mainly due to the sustained activation of NF-kB and interferon-stimulated regulatory element (ISRE)-dependent pathways. Notably, tumor-associated macrophages in this environment protect tumor cells from chemotherapeutic agents by promoting and enhancing the tumor growth properties of CSCs (Jinushi et al., 2011). A proinflammatory signature has also been exhibited by leukemia stem cells which can promote chemoresistance by means of metabolic adaptation (Ye et al., 2016). Thus, in some cases, tumors respond to chemotherapy by altering the immunological profile of the microenvironment in part due to the direct action of CSCs, thus further enabling tumor growth (Jinushi et al., 2011; Jinushi, 2014).

In more rare cases recruited circulating stem cells, subjected to chronic inflammation in a tissue, have been proposed to act as cancer-initiating cells themselves. Houghton et al. (2004), used a mouse model to demonstrate an extremely important connection between chronic inflammation, hematopoietic stem-cell recruitment and cancer development at the site of inflammation (Houghton et al., 2004). In this study, infection by *Helicobacter pylori* in the mouse caused the recruitment and subsequent engraftment of bone marrow derived stem cells (BMDC) into the stem cell compartment of the gastric mucosa. Within their new, inflamed niche, these engrafted stem cells accumulated mutations, and eventually gave rise to gastric tumors. This study showcased a direct connection between chronic inflammation, stem cell recruitment and increased cancer development (Houghton et al., 2004).

CONCLUSION

Inflammatory signaling promotes cellular responses with critical ramifications during infection, tissue generation/regeneration, cancer and other diseases. We summarize here work which demonstrates that stem cells can respond to and participate in inflammatory cascades in a direct manner. They express receptors which detect PAMPs and DAMPs, the initial triggers of the inflammatory response. They are able to mobilize and proliferate in response to inflammation in addition to producing cytokines which further amplify the response. Accumulating evidence suggests that in cases where a pathogen is involved, the changes in stemness mediated by inflammation also have a profound influence on the lifecycle of the pathogen. This is an area which merits further research. Understanding the crosstalk between stem cells and inflammation is an important piece of the puzzle which refines our understanding of the evolutionary roles of inflammation. Furthermore, it provides indispensable tools in our quest to harness knowledge into useful therapeutics.

AUTHOR CONTRIBUTIONS

SM, CA, and TP performed literature searches, and co-wrote the review. These three authors contributed equally. KS conceived the mini review topic, performed literature searches, and co-wrote the review.

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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.

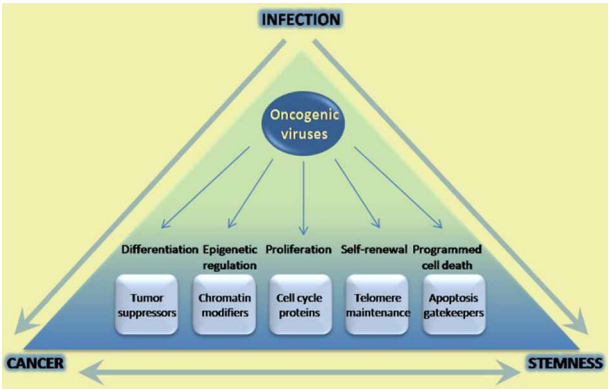

FIGURE 1 | Infection with oncogenic viruses highlights parallels in cancer and stem cell biology. Oncogenic viruses modulate a variety of cellular pathways with parallel roles in the carcinogenic process and stem cell homeostasis. The parallels between these two processes have been extensively documented, 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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