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Study of the Papillomavirus Genome Replication and Segregation

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Institute of Technology, Faculty of Science and Technology, University of Tartu, Estonia

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LIST OF ORIGINAL PUBLICATIONS

I **Geimanen, J**.; Isok-Paas, H.; Pipitch, R.; Salk, K.; Laos, T.; Orav, M.; Reinson, T.; Ustav, M. Jr.; Ustav, M.; Ustav, E. (2011). Development of a cellular assay system to study the genome replication of high- and low-risk mucosal and cutaneous human papillomaviruses. Journal of Virology, 85(7), 3315–3329.

II Sankovski, Eve; Männik, Andres; **Geimanen, Jelizaveta**; Ustav, Ene; Ustav, Mart (2014). Mapping of Beta-Papillomavirus HPV5 Transcription and Characterization of Viral Genome Replication Function. Journal of Virology, 88(2), 961–973.

III Silla, T.; Hääl, I.; **Geimanen, J**.; Janikson, K.; Abroi, A.; Ustav, E.; Ustav, M. (2005). Episomal maintenance of plasmids with hybrid origins in mouse cells. Journal of Virology, 79 (24), 15277−15288.

IV Marit Orav, Liisi Henno, Helen Isok-Paas, **Jelizaveta Geimanen**, Mart Ustav, Ene Ustav (2013) Recombination-Dependent Oligomerization of Human Papillomavirus Genomes upon Transient DNA Replication. Journal of Virology, 87 (22), 12051–12068

V Marit Orav, **Jelizaveta Geimanen**, Eva-Maria Sepp, Liisi Henno, Ene Ustav, and Mart Ustav (2015). Initial amplification of the HPV18 genome proceeds via two distinct replication mechanisms. Scientific Reports 5: 15952. doi:10.1038/srep15952.

My contributions to the listed articles are as follows:

Ref. **I** I performed all experiments, exept the cytokeratin level control in confluent HPV-containing U2OS cells, RT-PCR analysis of viral mRNA levels of amplification of the 18#1.13 cell line, and generation and replication analysis of truncated or E1, E2 mutant genomes. I analyzed the data and wrote the paper with the contribution of other co-authors.

Ref. **II** I performed HPV5 and HPV8 subcloning and participated in the analysis using 1D and 2D gel electrophoresis.

Ref. **III** I constructed hybrid replicons, designed and performed the flow cytometry experiments.

Ref. **IV** I participated in the performance of the experiments in U2OS cells related to the restriction analysis of HPV18 genomic oligomers, the analysis of topoisomerase I treatment on HPV18wt and HPV18E8^{-} genomic oligomers, the 2D N/N analysis of the uncut and nicked HPV18wt genomes, the 2D N/N analysis of transient DNA replication of HPV18 URR minicircle DNA in the presence of E1/E2 expression vectors or the mcHPV18E8^{-}; I participated in interpretation of the data.

Ref. **V** I designed and performed all the experiments, exept the 3dim gel electrophoresis, interpreted the results, paticipated in writing the paper with the contribution of other co-authors.

Inventions: Method and a kit for identifying compounds capable of inhibiting human papilloma virus replication; Owner: Icosagen Cell Factory OÜ; Autors: Mart Ustav, Ene Ustav, **Jelizaveta Geimanen**, Regina Pipitš, Helen Isok-Paas, Tormi Reinson, Mart Ustav, Triin Laos, Marit Orav, Anu Remm, Kristiina Salk, Andres Männik; Priority Number: PCT/EE2010/000010; Priority date: 19.05.2010.

Vectors, cell lines and their use in obtaining extended episomal maintenance replication of hybrid plasmids and expression of gene products; Owners: Cell Factory OÜ; Authors: Toomas Silla, Ingrid Tagen, **Jelizaveta Geimanen**, Kadri Janikson, Aare Abroi, Ene Ustav, Mart Ustav, Tiiu Mandel; European/EPO Patent No: EP1851319.

LIST OF ABBREVIATIONS

1D	–	one-dimensional
1N	–	unit lengh
2D	–	two-dimensional
2N	–	twice unit length
3D	–	three-dimensional
AFM	–	atomic force microscopy
AGE	–	agarose gel electrophoresis
ATM	–	ataxia-telangiectasia mutated
ATP	–	adenosine triphosphate
ATR	–	ATM and Rad3-related
ATRIP	–	ATR interacting protein
BIR	–	break-induced replication
BPV1	–	bovine papillomavirus type 1
Brd4	–	bromodomain protein 4
BS	–	binding site
C-terminal	–	carboxyl- terminal
CCC	–	covalently closed circle
COPV	–	canine oral papillomavirus
CRPV	–	cottontail rabbit papillomavirus
D-loop	–	displacement loop
DDR	–	DNA damage response
DNA-PK	–	DNA-dependent protein kinase
DNA-PKcs	–	DNA-PK comprises a large catalytic subunit
DSBR	–	double stranded break repair
dsDNA	–	double-stranded DNA
E	–	early region
E. coli	–	*Escherichia coli*
E1BS	–	E1 protein binding site
E2BS	–	E2 protein binding site
EBV	–	Epstein-Barr virus
EM	–	electron microscopy
EtBr	–	ethidium bromide
EV	–	epidermodysplasia verruciformis
FISH	–	Fluorescence in situ hybridization
FR	–	family of repeats
GFP	–	Green Fluorescent Protein
G418	–	geneticin
HHV8	–	human herpesvirus 8
HJ	–	Holliday junction
HPV	–	human papillomavirus
HR	–	homologous recombination
K10	–	cytokeratin-10 (CK-10) or keratin-10
kbp	–	kilobasepair

L	–	late region
LCR	–	long control region
LMW	–	low molecular weight
LT	–	large T antigen
MME	–	minichromosome maintenance element
MO	–	minimal origin
MRN	–	MRE11-Rad50-Nbs1 complex
N-terminal	–	amino- terminal
N/A	–	neutral/alkaline
N/N	–	neutral/neutral
N/N/A	–	neutral/neutral/alkaline
NHEJ	–	non-homologous end-joining
OC	–	open circular
ORF	–	open reading frame
PCNA	–	proliferating cell nuclear antigen
PDX	–	patient-derived xenograft
PHK	–	primary human keratinocyte
pRb	–	retinoblastoma protein
PV	–	papillomavirus
PyV	–	polyomavirus
RCR	–	rolling circle replication
rDNA	–	ribosomal DNA
RDR	–	recombination-dependent replication
RFC	–	replication factor C
RI	–	replication intermediate
RPA	–	replication protein A
SC	–	subcutaneous
SCC	–	squamous cell carcinoma
SCID	–	severe combined immunodeficiency
SDSA	–	synthesis-dependent strand annealing
Sp1	–	specificity protein 1
ssDNA	–	single-stranded DNA
SV40	–	Simian Virus 40
TFIID	–	transcription factor II D
URR	–	upstream regulatory region
UV	–	ultraviolet
VLP	–	virus-like particle
wt	–	wild type

1. INTRODUCTION

Papillomavirus infections are common in both people and animals. Generally, these viruses may result in asymptomatic infections and can cause benign tumors as condylomas and papillomas in mucous membranes or warts and skin papillomas. However, in the case of persistent infections, these viruses may induce malignant tumors at various body sites. For a long time, papillomaviruses were considered as harmless viruses, but a discovery made by German virologist Harald zur Hausen put it them on par with pathogens responsible for deadly diseases. In 2008, the Nobel Prize in Physiology and Medicine was awarded to zur Hausen for his papillomavirus research. He was able to demonstrate that human papillomaviruses (HPVs) can cause cervical cancers – the second-most-common cancers in women worldwide. HPV DNA replicates in the epithelial tissue, which is mostly composed of differentiating keratinocytes. HPV genomic DNA replication occurs in three clearly distinguishable phases, making the study and control of the virus complicated, because it is difficult to mimic all three stages of the virus infection cycle in the regular tissue culture cells under laboratory conditions.

The first part of my thesis gives a general overview of papillomaviruses, their infection cycles and replication phases. Model systems widely used for the study of HPV biological functions will also be reviewed, and the topic of how recombination could be involved into HPV genome replication will be introduced.

The research part of my thesis will focus on using the human osteosarcoma cell line U2OS as a model system for studying the specific stages of genome replication of several types of HPV (Ref. **I** and **II**). We have demonstrated that head-to-tail oligomers (HPV DNA that contain several copies of the viral genome) are formed during HPV DNA replication (Ref. **I** and **IV**) and that two different mechanisms are involved in the HPV genome replication (Ref. **IV** and **V**), shedding new light on the events that occur during the different phases of papillomavirus DNA replication. Finally, I included an analysis of the papillomavirus and the Epstein-Barr virus's segregation functions. Our data demonstrated that BPV1 E2 protein and minichromosome maintenance element (MME), or EBNA1 and FR dependent stable maintenance functions, are necessary for the stable episomal multicopy nuclear replication of the plasmids with hybrid replication origin in different types of dividing cells (Ref. **III**).

2. LITERATURE REVIEW

2.1. Introduction to Papillomaviruses

Papillomaviruses (PVs) are a diverse group of viruses that infect keratinocytes of the epithelia, which serve as a barrier between the environment and internal organs of host. HPVs may have asymptomatic infections and do not cause clear clinical signs. They are usually eliminated from the epithelium by the adaptive immune system (Antonsson et al., 2000, Antonsson et al., 2003). Humans are often simultaneously colonized by several types of HPVs that cause subclinical infections in the skin and mucosa. Thereby, HPVs could be considered as a component of the normal skin microbiota that persists throughout people's lives. However, certain HPV infections of the epithelia can have clinical manifestations ranging from hyperkeratotic lesions (small benign tumors, known as papillomas or warts) to malignant growth (e.g., cervical or anal cancers) (de Villiers et al., 2004, Clifford et al., 2003). HPVs account for more than 30% of all infection-associated cancers in humans (Zur Hausen, 2009).

Currently, there are two commonly used HPV vaccines on the market. Both of them consist of recombinant purified, refolded structural L1 proteins, which form HPV type-specific virus-like particles (VLPs). None of the vaccines contain live attenuated viruses. The Gardasil and Cervarix vaccines are targeted against HPV6, HPV11, HPV16, HPV18 and HPV16, HPV18. HPV vaccines are preventive; they do not eliminate existing infection and have no therapeutic effect in diseases caused by HPV. Recently, a nine-valent vaccine was approved by FDA (Gardasil 9) (clinicaltrials.gov). The viral E6 and E7 oncoproteins are suitable targets for therapeutic vaccination. A multivalent HPV therapeutic vaccine candidate against six HPVs was reported (genticel.com); however, this vaccine is only in preclinical studies, so there is an urgent medical need for drugs that suppress HPV DNA replication.

The nucleotide sequence of the capsid protein L1 gene is used to classify papillomaviruses because it appears to be the most conserved from coding sequences of these viruses. If the sequence coding for L1 differs from that of known viruses by at least 10%, the virus is classified as a new subtype (Bernard et al., 2010, Bravo and Félez-Sánchez, 2015). The family of Papillomaviridae has a long evolutionary history. PV DNA can be detected in a wide range of vertebrate species, including birds (Tachezy et al., 2002, Terai et al., 2002, Van Doorslaer et al., 2009) and reptiles (Bravo et al., 2010, Lange et al., 2011). Based on the structure of their skin, the most suitable hosts for these viruses are mammals (de Villiers et al., 2004, Bravo et al., 2010). According to recent information, the family Papillomaviridae contains 205 different PV types, which can be clustered into five genera: Alpha-, Beta-, Gamma-, Mu- and Nu- (α, β, γ, μ, ν) papillomaviruses (Bzhalava et al., 2015, Kocjan et al., 2015, www.hpvcenter.se). Most HPVs belong to α, β, or γ- papillomaviruses (de Villiers et al., 2004). Many mechanisms have governed the evolution of

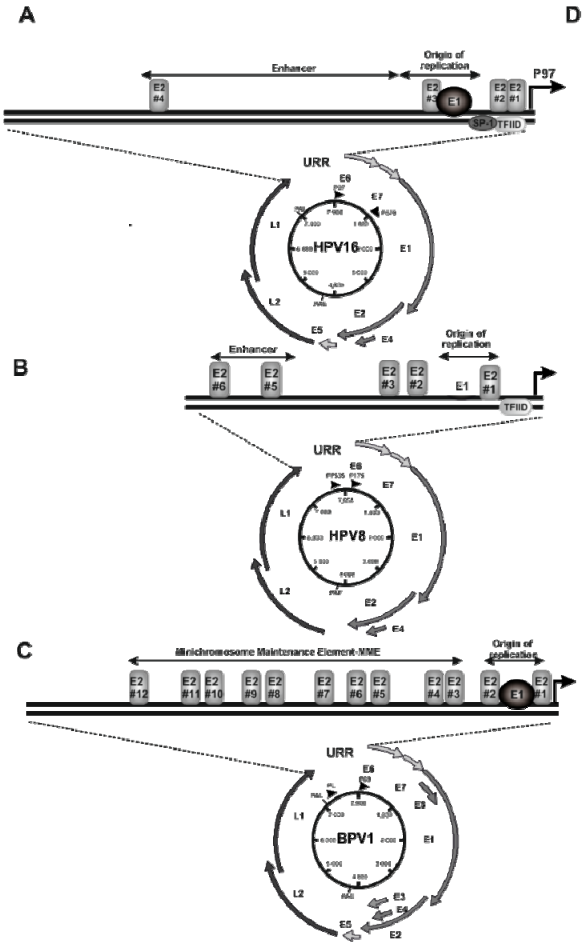
papillomaviruses, including recombination between the viral and host genomes (Gottschling et al., 2007, Lazarczyk et al., 2009).

HPVs are divided into cutaneous and mucosal types; the latter group (α-HPVs) is subdivided into high-risk and low-risk subgroups, based on the capacity of the viruses to cause malignant progression of the lesions. Low-risk HPVs such as HPV6 and HPV11 cause genital warts. Induction of cervical cancers are associated with the high-risk HPVs (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82); three HPVs are classified as probable high-risk types (26, 53, and 66) (Muñoz et al., 2003). HPV16 infection causes not only cervical cancer but also vaginal, vulvar, penile and anal cancers (Al Moustafa et al., 2014, Muñoz et al., 2006). A significant fraction of head and neck cancers are also caused by HPV16 and HPV18 (Betiol et al., 2013, Adams et al., 2014). β-HPVs have been associated with squamous cell carcinomas (SCC) of the skin (non-melanoma skin cancers) in immunocompromised and immunosuppressed individuals with the rare hereditary disease *Epidermodysplasia Verruciformis* (EV). HPV5 and HPV8 DNA have been detected in 90% of cutaneous SCCs in EV cases, and HPV5 DNA has been detected in primary tumors and metastases, suggesting a prominent role for HPV5 in SCC (Dell'Oste et al., 2009, Doorbar et al., 2012). β-HPV DNA has also been detected in non-melanoma skin cancers of immunocompetent non-EV patients (Boxman et al., 1997, Harwood and Proby, 2002, Purdie et al., 2005, Akgül et al., 2006, Nindl et al., 2007). The other PV genera, γ, μ and ν, comprise cutaneous HPVs that are usually linked to the development of cutaneous papillomas and warts (de Villiers et al., 2004).

2.2. Papillomavirus virion and genome organization

Papillomavirus virions lack a lipid envelope and are relatively small, approximately 55 nm in diameter (Doorbar, 2006, Favre et al., 1997). The PV genome is composed of ~8 kbp of double-stranded DNA (dsDNA) packed into nucleosomes by the histones. The DNA genome contains three major regions: a noncoding upstream regulatory region (URR), also-called a long control region (LCR), and two coding regions: an early region (E) and a late region (L) (Fig. 1).

The URR region comprises viral and cellular transcription factors binding sites (Fig. 1) and is responsible for the control of PV gene expression (Doorbar, 2007, Zheng and Baker, 2006, Bernard and Apt, 1994). β-HPVs have a shorter URR and different organization of regulatory sequences compared to α-HPVs and other genera (Reh and Pfister, 1990, Muller and Demeret, 2012). The URR of β-HPV contains a less easily identifiable binding site for the E1 replication protein. In addition, it has been shown that regulation mechanisms of the early promoter of α-HPV and β-HPV are different (Reh and Pfister, 1990, Horn et al., 1993, May et al., 1994, Akgül et al., 2006).



D

Locus	Function
URR	• Harbours transcription factor-binding sites • Controls gene expression
E6	• Promotes cellular immortalization by degradation of p53 • Modifies cell adhesion and differentiation by degradation of TAp63 and p73
E7	• Promotes pRb degradation, permitting cell progression to S-phase of cell cycle • Induces chromosomal instability
E5	• Induces cell proliferation • Contributes to evasion of apoptosis • Downregulates MHC expression
E1	• DNA helicase/ ATPase activity • Initiation of viral DNA replication
E2	• Coactivator of viral DNA replication • Main viral transcriptional regulator • Transcription repressor of HPV E6 and E7 • Regulates cell cycle and apoptosis • Interacts with chromatin for segregation of viral genome
E4	• Binds cytoskeletal proteins and distrupts cytoskeletal structure of the G2 arrested cell
L2	• Minor capsid protein • Recruits L1 protein for virus assembly
L1	• Major capsid protein

Figure 1. *(A), (B) and (C)* Schematic representations of circular dsDNA genomes of high-risk α-HPV16, β-HPV8, and bovine papillomavirus type 1(BPV1), showing the location of the ORFs for early and late proteins and the URR region which contains the origin of replication, consisting of E1BS (E1 binding site) and E2BSs (E2 binding sites). The position of early and late promoters along with early and late polyadenylation sites are indicated. The minichromosome maintenance element (MME) of BPV1 is depicted in *C*. Figure *A* is adapted from (Kajitani et al., 2012). Figure *B* is adapted from (Lazarczyk et al., 2009). Figure *C* is adapted from (Skiadopoulos and McBride, 1998). Summary table *(D)* gives an overview of the basic functions of PV proteins and was adapted from (Bravo and Félez-Sánchez, 2015).

The early coding region contains open reading frames (ORFs) for PV proteins E6, E7, E1, E2, E4 and E5 (E5 is not present in β-HPVs) (Lazarczyk et al., 2009). Early HPV proteins have regulatory functions and are responsible for oncogenic transformation of the host cells in the case of high-risk virus types.

PV E1 protein belongs to the group of viral initiator-proteins. One well-studied representative of this group is SV40 (Simian virus 40, family *Polyomaviridae*) large T-antigen (LT), which has certain similarities to E1 (Lambert, 1991). There is much in common in the manner in which polyomaviruses and papillomaviruses use the cellular DNA replication mechanisms to replicate their viral genomes. Many research groups that study papillomaviruses DNA replication use the knowledge obtained from SV40 DNA replication to study the same aspects of papillomaviruses (Trowbridge et al., 1999, Simmons et al., 1998, Hu et al., 2006, Archambault and Melendy, 2013).

E2 protein is a transcriptional regulator of viral gene expression and a viral replication protein. It also provides a chromatin attachment (tethering) function, which helps maintain a stable copy number of viral episomes in dividing cells. We and others have shown that the truncated version of E2 protein, E8^E2, acts as a negative regulator of E2 and functions as a repressor of transcription and replication in β-HPVs (Sankovski et al., 2014). The same phenomenon has also been shown by others in α-HPVs (Stubenrauch et al., 2000, Kurg et al., 2010, Zobel et al., 2003).

E6 binds the tumor suppressor protein p53 and promotes its ubiquitination and degradation. Protein E7 binds to several cellular tumor suppressor proteins, including the retinoblastoma protein (pRb), to modulate cell-cycle regulation. E6 and E7 activity promotes uncontrolled cell division (Zheng and Baker, 2006).

Late proteins of HPVs (L1 and L2) form the viral capsid and mediate packaging of viral DNA into the virions (Buck et al., 2008). Other important functions of HPV proteins are presented in the table that is included in Fig. 1D.

2.3. Phases of the papillomavirus infection cycle

The infection cycle (also known as the life cycle or replication cycle) of HPV is tightly linked to the differentiation program of the infected host cells, known as keratinocytes. Normal keratinocytes continuously stratify and differentiate, renewing the epithelium every two to three weeks. The cutaneous or mucosal stratified epithelium is separated from underlying tissues by the basement membrane. Just above the basement membrane is the basal layer, which contains the only cells in the epithelium that can divide. When the cell divides, one of the daughter cells moves up to the suprabasal layer where the differentiation is initiated. This cell stops dividing and continues moving upward, replenishing the overlying cell layers that are lost because of desquamation (Kaur and Li, 2000).

The PV virions enter basal keratinocytes through microwounds, targeting quickly dividing, transiently amplifying cells and/or slowly dividing stem cells

in the basal layer or in the hair follicles (Quint et al., 2015, Egawa et al., 2015, Schmitt et al., 1996). The receptors required for PV entry into cells are currently undefined. Possibly, PV entry requires the initial binding to heparan sulfate on the surface of basal cells; this initial binding is likely followed by association with a secondary receptor to allow virus entry (Conway and Meyers, 2009, Kines et al., 2009, Horvath et al., 2010, Schäfer et al., 2015).

After virus entry into the host cell via endocytosis, L1 protein dissociates from the viral DNA and the genomic DNA is imported into the host cell nucleus (Surviladze et al., 2015, Bousarghin et al., 2003). Initial amplification of the HPV DNA of up to 50–300 copies per cell occurs (Garner-Hamrick and Fisher, 2002, Maglennon et al., 2011). This relatively small number of HPV DNA copies per cell is likely sufficient for the virus to express enough early gene products while avoiding damage to the infected cell and triggering an immune response against the virus and infected cells (Evans et al., 2003, McKinney et al., 2015). Additionally, the low level of expression of early genes and the small number of HPV DNA copies might be caused by the presence of the cellular proteins inside the undifferentiated cells, which negatively regulate viral gene expression and may compete with viral proteins (E1 and E2) for binding to the viral origin of replication.

Next, the papillomavirus genome establishes stable maintenance (latent infection) in the basal cells of the differentiating epithelium. Long-lasting latent infection is most likely connected with maintenance and replication of the PV genome in long-lived stem cells in this layer.

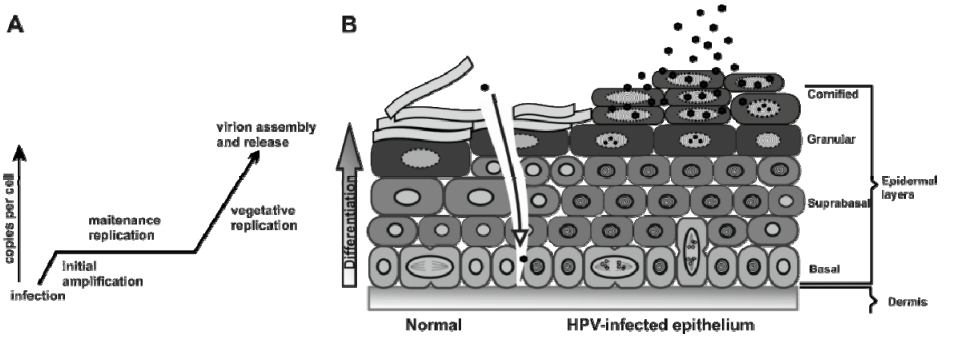


Figure 2. *(A)* Changes in viral DNA copy number during the different phases of PV replication. *(B)* Normal and HPV-infected epithelia. Upon infection, the viral genomes are established in the host cell nucleus as low-copy episomes amplified with the help of viral replication proteins. Next, the viral genomes replicate in synchrony with cellular DNA replication (the maintenance replication period). Differentiation of HPV-positive cells induces the productive phase of the viral infection cycle, allowing vegetative amplification of the viral genome. In the late phase, L1 and L2 proteins are expressed and encapsidate newly synthesized viral genomes; virions are shed from the uppermost layers of the epithelium. Figure adapted from (Fisher, 2015).

At the productive stage of the virus infection cycle, the second amplification replication (also known as late or vegetative amplification) of the viral genome takes place. This occurs in the uppermost layer of the terminally differentiated keratinocytes and is associated with the synthesis of viral capsid proteins and assembly of viral particles. Finally, infectious PV particles are released from the remains of the host cell (Fig. 2B).

For this study, it is crucial to understand the processes involving HPV DNA in infected cells. The various stages of replication of the virus genome will be reviewed next.

2.3.1. Initial amplification replication of HPV DNA

The replication of PV DNA is initiated at the origin of genome replication that binds virus-encoded replication factors: E1 and the full-length form of E2 proteins (Del Vecchio et al., 1992, Ustav et al., 1993, Ustav and Stenlund, 1991). All other replication enzymes and proteins are derived from the host cells (Remm et al., 1992, Ustav et al., 1991). The host DNA-replication machinery includes DNA polymerase α-primase, DNA polymerase δ, RPA (replication protein A – the single-stranded DNA binding protein), PCNA (proliferating cell nuclear antigen), RFC (the replication factor C), topoisomerases and cyclin E/cdk2 (Chow and Broker, 2013, Kurg, 2011).

E2 dimers bind to specific recognition sequences, short palindrome with consensus 5'-ACCGNNNNCGGT-3', called E2 binding sites (E2BSs). These elements are located in several positions in the PV genome (Steger and Corbach, 1997, McBride et al., 1991) (Fig. 1A–C). The BPV1 genome contains 17 E2 binding sites, 12 of which are located in the URR (Li et al., 1989) (Fig. 1C). For both α-HPV and β-HPV, the number of E2 binding sites is restricted to four in the URR, but the location and distribution relative to the viral promoters vary between α-HPV and β-HPV (Rogers et al., 2011). In the URR of α-HPVs, three sites (E2BS #1 to #3) are confined to the proximal region of the promoters (Doorbar, 2006) (Fig. 1A). The fourth site, E2BS #4, is located far from the others, upstream of the transcriptional enhancer (Demeret et al., 1997). At low concentrations, the E2 protein attaches to the most distal promoter, E2BS #4, which the strongest affinity; this binding activates the expression of early genes. When the level of E2 concentrations rise, the appropriate conditions for transient replication of HPV genome are created.

When the levels of E2 protein become high, early gene expression is repressed. This is caused by the overlap of the E2BSs with the binding sites of transcription factors TFIID and Sp1; therefore, the binding of E2 interferes with the binding of these transcription factors at the promoter (Demeret et al., 1997) (Fig. 1A). This regulation of viral gene expression may contribute to HPV DNA copy number control in undifferentiated cells.

Binding of the E2 to the E2BSs, located adjacent to the E1 recognition sequence (E1BS), helps load E1 onto the origin (Berg and Stenlund, 1997). E1

alone binds weakly to the origin sequences; thus, complex formation with E2 is required (Sedman and Stenlund, 1995, Sedman et al., 1997). In the next step, E2 dissociates from the complex, allowing the ATP-dependent assembly of E1 into double hexameric rings (Sanders and Stenlund, 1998, Titolo et al., 1999). The E1 hexameric complex has DNA helicase activity, which can unwind the DNA and initiate DNA replication (Schuck and Stenlund, 2005, Sedman and Stenlund, 1998). The E1 helicase also recruits host replication factors to the viral origin of replication (Skiadopoulos and McBride, 1998).

Transient HPV DNA replication occurs in a bidirectional manner and leads to the formation of structures that resemble the Greek letter θ (*theta*) (Flores and Lambert, 1997). The *theta* model of replication was first described by the biochemist John Cairns (CAIRNS, 1963). This replication mode is also a common for other small circular dsDNA viruses such as SV40. According to the *theta* scheme, replication of circular DNA genome begins at the origin of replication. DNA unwinding occurs and then two replication forks (RFs) assemble and progress in opposite directions. Expansion occurs along the circular genome until RFs converge opposite of the origin. Next, daughter molecules are separated (Fig. 3). In each fork, one strand (the leading strand) is synthesized continuously in the direction of the movement of the RF. The other strand (the lagging strand) is synthesized discontinuously in the opposite direction in the form of Okazaki fragments, which are later connected by ligations.

Theta replication converts the parental circular dsDNA molecule into two daughter molecules, each of which has one strand from the parental DNA molecule and a second newly synthesized strand (semiconservative synthesis).

In addition to bidirectional replication, with two replicating forks moving away from the origin in opposite directions, there is a unidirectional mode of replication, in which one fork moves away from the other, which remains fixed at the origin of replication (Lilly and Camps, 2015, McKinney and Oliveira, 2013).

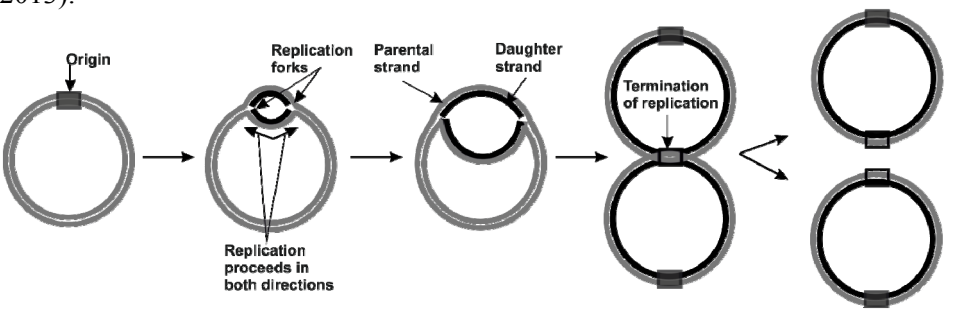


Figure 3. ***Theta* replication** is a type of replication common in *E. coli (Escherichia coli)* and other organisms possessing circular DNA.

Determination of the mechanisms of HPV genome replication at the transient replication stage in the novel model system based on U2OS cells was one of the main topics of this thesis.

2.3.2. Stable maintenance of HPV genome replication

Papillomaviruses may establish persistent infection in epithelial tissue. During the maintenance phase of replication, the viral genome is replicating in S phase in synchrony with the host genome DNA. The extrachromosomal viral genomes are tethered to the host genome by E2 protein during the mitosis, which provides the segregation function for the HPV DNA in dividing cells.

The repressor protein E8^E2 could play an important role at this stage; expression of this protein could contribute to switching from the transient replication stage to persistent infection (McKinney et al., 2015, Stubenrauch et al., 2000).

Replication during the maintenance phase can occur in an ordered way, e.g., each episome replicates once per cell cycle, or randomly, whereby some episomes replicate several times and others do not replicate at all. The mechanism of preference depends on the E1 levels in the host cell (Kadaja et al., 2009, Piirsoo et al., 1996, Hoffmann et al., 2006). As demonstrated by the examples of HPV16 and HPV31, viral DNA can replicate randomly or once per S phase, depending on the cell lines used. Thus, in the W12 cells, which are derived from a cervical lesion, HPV16 DNA replicates only once per S phase, but in another spontaneously immortalized human keratinocyte cell line, NIKS, HPV16 DNA replicates randomly (Hoffmann et al., 2006). However, these data should be further evaluated because the physical status of HPV16 DNA in the W12 clone used was not analyzed in that study. Integrated viral DNA, as demonstrated for HPV16 in W12 cells by Coleman and colleagues (Pett et al., 2006), must replicate once per cell cycle.

During the maintenance phase of replication, it is important for HPV to retain extrachromosomal genomes within dividing cells. Some viruses integrate their genome into the host genome, which promotes viral genome replication and segregation along with cellular DNA. For PVs, integration into the host genome terminates the viral infection cycle. Therefore, similar to the genomes of the Epstein-Barr virus (EBV) and human herpes virus 8 (HHV8), the genomes of PVs are usually not integrated into the host cell chromosome. During the viral latent phase, these viruses use an efficient mechanism that allows nearly equal distribution of episomes among daughter cells during host-cell division by attaching the viral genome to the cell chromosomes using specific viral proteins (PVs-E2; EBV-EBNA1; HHV8-LANA1) and their special binding sites in the viral genome (You, 2010).

However, the segregation functions of different types of PVs are different. All PVs distribute their genomic DNA through attachment to mitotic host chromosomes during cell division. The most-studied segregation mechanism is that of BPV1. This DNA attachment is mediated by E2 protein: its C-terminal DNA-binding domain is bound to viral DNA through the E2BSs at the URR, and the N-terminal transcription activation domain of the E2 is bound to the cellular chromatin (Kurg, 2011, Silla et al., 2005, Abroi et al., 2004). E2 is the only BPV1-encoded protein that is necessary and sufficient for chromatin

tethering of the plasmids, containing BPV1 URR via the E2 binding sites (Bastien and McBride, 2000, Ilves et al., 1999). The cellular receptor for BPV1 E2 on mitotic chromosomes has been suggested as bromodomain protein 4 (Brd4) (You et al., 2004).

Segregation mechanisms of α- and β-HPVs have not been studied to a similar extent as BPV1. It is known that α-HPV E2 interacts weakly with Brd4. Additionally, it is assumed that various HPV E2 proteins may attach the viral genomes to host chromosomes via different cellular targets (McPhillips et al., 2006, Oliveira et al., 2006, Donaldson et al., 2007). Unlike E2 of BPV1, transcriptional activation and the Brd4-interacting domain of E2 of β-HPVs (e.g., HPV8) are not required for chromosome binding. In contrast to the dispersed and random pattern of binding to host chromosomes observed for E2 of BPV1, E2 of HPV8 targets the short arms of acrocentric chromosomes. Furthermore, E2 of HPV8 interacts with the repeated ribosomal DNA units (rDNA) found in the same location. Accordingly, HPV8 E2-mediated genome tethering could occur using a Brd4-independent mechanism through novel interactions with specific regions of mitotic chromosomes (Poddar et al., 2009, Sekhar et al., 2010). Thus, HPV E2 proteins operatively bind with E2BSs and potential cellular proteins to interact with mitotic chromatin during segregation.

2.3.3. Vegetative HPV genome replication

When HPV infected basal cells divide, viral genomes are distributed between the daughter cells. One of these daughter cells detaches from the basal layer and moves towards the *stratum granulosum*. As soon as epithelial cells detach from basal layer, the cell cycle and division stop. Thus, because necessary cellular DNA polymerases and replication factors are produced only in mitotically active cells, HPVs should induce marked alterations in the biological activity of keratinocytes, affecting their differentiation. At this stage, HPV E6 and E7 play an important role. These proteins influence the cellular checkpoint mechanisms that ensure that the cell cycle is completed properly. E7 binds to retinoblastoma family members and promotes their degradation (Rashid et al., 2015), which induces unscheduled re-entry into the S phase. E6 prevents the induction of apoptosis in response to unscheduled S-phase entry through degradation of p53 (Mantovani and Banks, 2001, Sherman et al., 1997). Thus, overexpression of E6 and E7 can initiate neoplastic progression and, *in vitro*, immortalize primary human keratinocytes (PHKs), allowing the differentiating keratinocytes to begin uncontrolled proliferation and therefore supporting amplification of the viral genomes (vegetative phase of DNA replication) (Ganti et al., 2015, DeFilippis et al., 2003).

The exact mechanism of PV genome vegetative amplification has not been described fully; however, there is some evidence that the mode of replication is different from that seen at the maintenance phase, i.e., replication might shift from the bidirectional *theta* model (Auborn et al., 1994) to another model – pre-

sumably the rolling-circle replication (RCR) model (Flores and Lambert, 1997, Burnett et al., 1989, Dasgupta et al., 1992). Additionally, some recent studies of HPV DNA replication show that differentiation-dependent genome amplification activates the DNA-damage response (DDR) and repair-related pathways (Moody and Laimins, 2009, Fradet-Turcotte et al., 2011). This effect suggests that viral genomes might be amplified in differentiated cells by repair-related replication (Sakakibara et al., 2013). As a result, the viral DNA copy number amplifies from hundreds to thousands of copies per cell (Bedell et al., 1991).

The late proteins L1 and L2 are produced in cells that reach the upper layers of the epithelium, where virion assembly takes place (Doorbar, 2006, Schwartz, 2000).

The infection cycle of HPVs is closely connected with the differentiation program of the host cells – i.e., keratinocytes – and infectious HPV virions can only be cultivated in special organotypic raft cultures containing a population of cells at various developmental stages, mimicking skin.

2.4. Model systems to study the infection cycles of HPVs

To create therapeutic antiviral drugs, it is important to understand the molecular mechanisms of HPV DNA replication; therefore, there is a great need for model systems that can mimic papillomavirus infection in humans. The following section will give an overview of the model systems and techniques used to study various aspects of differentiation-dependent HPV genome replication at every step of the virus infection cycle. Several model systems are widely used. Because HPV cannot be propagated in the conventional way in monolayer cell cultures, other systems are used. Some use differentiating human keratinocytes to model productive infection in organotypic raft culture or through xenotransplantation. These methods mimic *in vivo* infection but are technically complex and depend on the availability of human tissues. Animal models are also used because they can present with lesions similar to those found in humans.

2.4.1. Epithelial cells

HPVs infect the mucosal or cutaneous epithelium. For that reason, PV research tends to focus on epithelial cell differentiation. Some cell types are able to divide *in vitro* only a few times; others can persist for up to a hundred cell generations or indefinitely. Based on these differences, cultured epithelial cells can be divided into primary keratinocyte cell cultures and immortalized epithelial cell lines.

Primary cell cultures are cells that have been taken from a human or animal tissue (such as the kidney or liver) and transferred into culture conditions. Most of these cells are capable of limited growth *in vitro* for no more than five to ten division cycles. They are widely used in laboratory studies and for vaccine production.

Immortalized epithelial cells can divide indefinitely *in vitro*. Such "immortal" cell lines usually originate from tumors or generate after introduction of mutations that make cells immortal. Often, because numerous successive transformation steps take place during long cultivation, these immortal cells lose their similarity to the cells of origin. The most common indicator of such changes is the cells' indefinite life span.

Immortalized epithelial cell lines can be obtained from normal and tumor tissues of animals and humans. Immortalized epithelial cell lines derived from cervical carcinomas, such as SiHa (HPV16 positive), HeLa (HPV18), CIN-612 (HPV31), CaSki (HPV16) and NIKS (HPV-negative), along with spontaneously immortalized human dermal keratinocytes (Adler et al., 1997, Allen-Hoffmann et al., 2000), are widely used in experimental virology. The great advantage of immortalized epithelial cell lines compared to primary cell cultures is that they can be propagated for an indefinitely long time by passaging cells at certain intervals.

Some immortalized cell lines were adapted to grow in suspension culture. Such cultures are particularly useful in biochemical studies of viral DNA replication and in commercial vaccine production. Primary human keratinocyte cultures derived from foreskin circumcisions are most frequently used to develop organotypic cultures (see below) (Henken et al., 2011, Blanton et al., 1991).

Immortalized epithelial cell lines were used in early studies of animal papillomaviruses. The mouse fibroblast cell line C127 transfected with the BPV1 genome can support episomal viral DNA replication with a constant copy number (Law et al., 1981). These cells yielded the first data on PV replication cycles and have been used to study basic mechanisms and regulation of viral gene transcription and viral DNA replication (Berg and Stenlund, 1997, Neary and DiMaio, 1989).

The HPV16-containing W12 model cell line of cervical squamous carcinoma has become a tool for studies of HPV replication cycle. At early passages in monolayer cultures, cells maintain HPV16 episomes at 100 to 200 copies per cell (Stanley et al., 1989). The expression of E2 efficiently suppresses the transcription of E6 and E7 (Jeon et al., 1995, Romanczuk and Howley, 1992, Bechtold et al., 2003, Dall et al., 2008). Integration can occur at any time during episome maintenance. However, spontaneous loss of episomes occurs during long cultivation periods, resulting in cells containing only integrated HPV16 (Pett et al., 2006). In cells containing integrated viral DNA the unregulated expression of the viral oncogenes E6 and E7 occurs because of the loss of E2, which represses viral transcription because the E2 ORFs are often disrupted during integration (Jeon et al., 1995, Romanczuk and Howley, 1992, Stanley et al., 2007).

Not long ago, it was believed that most cutaneous β-HPVs have no transforming properties. However, a group of researchers has demonstrated that HPV38 has *in vitro* transforming activity (Caldeira et al., 2003). That group has characterized several transforming properties of three cutaneous β-HPVs (10, 20, and 38) and presented compelling evidence that E7 of HPV38 – but not of

HPV10 and HPV20 – induces loss of cell cycle control. The functions of HPV38 E6 and E7 appear to be more similar to those of the high-risk α-HPV oncoproteins. These data suggest a role for HPV38 infection in skin carcinogenesis.

The use of epithelial cells has made an enormous contribution to our understanding of the DNA replication of papillomaviruses and the paths that lead to cervical cancer. However, the full life cycle of the virus, which includes infection, DNA replication and persistence of the viral genome as an episome, cannot be investigated in this model, because this system is not capable of progeny virus production.

2.4.1.1. Induction of differentiation of epithelial cells

In the epidermis, keratinocyte proliferation is restricted to the basal level. The cells eventually differentiate as they move up through the suprabasal layers (Fig. 2B). The differentiation of keratinocytes has been studied in models that include orthotopic and primary keratinocyte cultures. To simplify culture procedures, many laboratories use monolayered primary cultures of keratinocytes. Most keratinocyte cell cultures can be directed to differentiation by placing them into suspension in methylcellulose (Green, 1977). Both high calcium concentration (Boyce and Ham, 1983, Berghard et al., 1990, Li et al., 1996) and cell confluency (Poumay and Pittelkow, 1995, Minner and Poumay, 2009) induce stratification and terminal differentiation of epithelial cells. These methods have been used in experiments with epithelial cells containing HPV genomes (Longworth and Laimins, 2004, Wilson and Laimins, 2005, Hong et al., 2011, Hebner et al., 2007, Donaldson et al., 2012). The advantage of these systems is the simplicity of the method and the reduced duration of the experiment. Therefore, these techniques make it possible to study the interactions of host proteins with the virus and to determine those proteins' role in the viral life cycle. This method might also prove useful for the study of chemical inhibitors affecting cell proliferation and screening for inhibitors of HPV DNA replication. Nevertheless, artificial differentiation of epithelial cells imposes certain limitations. Although late viral DNA amplification does occur, the progeny virus particles are not produced efficiently. Thus, some aspects connected with the viral production phase cannot be investigated in these models.

2.4.2. Organotypic epithelial raft cultures

Organotypic epithelial raft culture is one of the most promising *in vitro* models and is often used for the study of HPVs (Chow, 2015). Initially, organotypic epithelial raft culture was developed by dermatologists as a rapid method to obtain epithelial tissue for burn patients (Asselineau and Prunieras, 1984). Organotypic epithelial raft culture is the only *in vitro* system that can support full epithelial differentiation and enable reproduction of the entire life cycle of HPVs, from infection to the production of progeny virions (Dollard et al., 1992,

Cheng et al., 1995). However, for studying the HPV life cycle, this method is both time-consuming and expensive. First, collagen plugs are grown and epithelial cells sown onto them. When the epithelial cells reach confluence, the submerged monolayer is transferred to a metal support grid and maintained at an air-liquid interface. Over the next two to three weeks, the cells stratify and differentiate, forming a full-thickness epithelium that produces differentiation-specific cytokeratins (Ozbun and Patterson, 2014, Chow, 2015, Anacker and Moody, 2012). This technique can be used with primary human keratinocytes and established epithelial cell lines; it can easily be adapted for use with epithelial tissues from normal or diseased biopsies (Grassmann et al., 1996, Henken et al., 2011, Dollard et al., 1992, Bedell et al., 1991).

This technique was first used by Bedell et al. (Bedell et al., 1991) with the CIN-612 cell line, which was derived from a low-grade cervical lesion. They found that cells contained episomal DNA copies of HPV31 subtype. At the air-liquid interface of *in vitro* raft cultures, this cell line differentiated in a way that histologically resembled a low-grade cervical lesion *in vivo*. The amplification of HPV31 genomes in the upper layer of the stratified epithelium was similar to that found in productive HPV infections *in vivo*. Unfortunately, although transcripts from the late region of HPV31 were also detected in stratified raft cultures, no capsid protein synthesis was found (Bedell et al., 1991). However, when Dollard et al. applied condylomas containing HPV11 DNA to raft cultures, the epithelial cells underwent terminal differentiation and the full virus infection cycle took place, including virion assembly (Dollard et al., 1992). A later group of researchers from the University of New Mexico School of Medicine used the raft system to generate large yields of viral-DNA-containing particles of HPV31b. They developed a biological homogenization system for virion extraction from raft epithelial tissues and described HPV virion production in the raft tissue-culture system from naturally infected cells (Ozbun, 2002a, Ozbun, 2002b). After the creation of a raft culture from naturally infected cells, publications have described the use of the electroporation-based system to create cell lines capable of stably maintaining episomal HPV18 and HPV45 genomes and HPV virion production (Meyers et al., 1997, McLaughlin-Drubin et al., 2003). Despite the inability of low-risk HPVs to immortalize normal keratinocytes, organotypic epithelial rafts can be used for studies of their infection cycles (Thomas et al., 2001).

Until recently, it was believed that most cutaneous β-HPVs have no transforming properties. However, a group of researchers have demonstrated that HPV38 has *in vitro* transforming activity (Caldeira et al., 2003). They have characterized several transforming properties of three cutaneous β-HPVs (10, 20, and 38) and presented compelling evidence that E7 of HPV38 – but not of HPV10 and HPV20 – induces loss of cell cycle control. The functions of HPV38 E6 and E7 appear to be more similar to those of the high-risk α-HPV oncoproteins. These data suggest a role for HPV38 infection in skin carcinogenesis.

2.4.3. Animal models used to study HPVs

A direct connection between PVs and cancer was first demonstrated in rabbits (cottontail rabbit papillomavirus, or CRPV), cattle (BPV) and dogs (canine oral papillomavirus, or COPV) at the end of the last century (Brandsma et al., 1991, Brandsma, 1994, Sundberg et al., 1986, Amtmann et al., 1984, Kreider and Bartlett, 1981). For many years, however, it was not known that some HPVs (HPV16 and HPV18) can be carcinogenic to humans. Experimental studies with animal papillomaviruses were a critical factor in this understanding. Animal papillomas were invaluable material in the investigation of viral biology, in the relationship between the virus and the host, in the immune response to the virus, and in the development of the first anti-papillomavirus vaccines (Campo and Roden, 2010). Animal PVs have been studied both as agents of disease in animals and as models for understanding HPV infection. In addition to the study of PV infection in animals, *in vitro* studies with animal PV proteins have contributed greatly to the understanding of the mechanisms of cell transformation.

2.4.3.1. BPV as a model system

BPV1 has been an excellent model for the study of papillomaviruses; despite differences in genome organization and tissue tropism, it is also a useful model for HPVs. BPV1 E2 was the first PV factor to be identified as a transcriptional regulator (Spalholz et al., 1985, Yang et al., 1985). Moreover, BPV1 studies have shown the involvement of E2 in viral DNA replication (Ustav et al., 1991, Ustav and Stenlund, 1991, Ustav et al., 1993, Stenlund, 2003). BPV1's association with mitotic chromosomes has been suggested as a mechanism for the segregation/partitioning of viral genomes between daughter cells (Abroi et al., 2004, Lehman and Botchan, 1998, Ilves et al., 1999, Skiadopoulos and McBride, 1998).

It has been shown that the early region of the BPV1 genome is sufficient for mouse cell transformation and that the virus genome is maintained as an extrachromosomal multicopy nuclear element, without the generation of viral particles (Lowy et al., 1980, Sarver et al., 1981, Uemura et al., 1992, Van Craenenbroeck et al., 2000). The early region is successfully used in the cloning vectors (Pavlakis and Hamer, 1983, Sambrook et al., 1985). The BPV1-based vector system enjoys the benefit of a long-term episomal persistence in the cells that express E1 and E2 proteins. These vectors contain a BPV1 minimal origin (MO) and the minichromosome maintenance element (MME), which consists of at least two E2 binding sites (Ilves et al., 1999, Ustav et al., 1993, Skiadopoulos and McBride, 1998).

The optimal vector system that can be used in gene therapy should provide conditions under which the gene of interest would remain active at physiologically relevant levels for a long time period. The episomal vectors should have a high transfection efficiency and not integrate into the host cells (integration can modify the vector DNA or the DNA of the host cell or even cause oncogenic transformation). Because each virus-vector system has their strengths

and weaknesses, an effort has been made to create various hybrid vectors, designed to combine features of a variety of viral systems (Roberts and Weintraub, 1986). Such vectors are very useful in transient protein production. The hybrid episomal vectors can also be used during gene therapy to obtain a high level of expression of the gene of interest in the dividing cells over a long period of time; however, successful use of such vectors in humans remains to be demonstrated.

Animal papillomavirus research was invaluable in understanding the nature of HPVs. Advances in molecular and cell-culture techniques have enabled scientists to shift their efforts from animal to human PVs.

2.4.3.2. Transgenic mice

Transgenic animals have been one of the most rapidly developing tools for papillomavirus cancer-related studies over the last 20 years. Many biomedical researchers have been focused on modeling human diseases both to study the pathogenesis and to search for new therapies. Transgenic models also enable easy detection of the effects of carcinogenic and other chemical agents.

An animal model system can be used to mimic physiological processes that are also taking place in humans. One area in medicine and biology that makes the most use of transgenic mice models is oncology. The first transgenic mice with skin cancer were obtained using BPV1 (Lacey et al., 1986, Hanahan et al., 1989, Sippola-Thiele et al., 1989, Lindgren et al., 1989). Numerous laboratories have also succeeded in engineering transgenic mice that carry the entire early region of HPV1 (Tinsley et al., 1992) or the E6 and E7 genes of HPV16 (Kondoh et al., 1991, Arbeit et al., 1993, Lambert et al., 1993).

As mentioned above, the links between skin cancer and the transforming activities of β-HPVs have not yet been fully characterized (Caldeira et al., 2003). It is also believed that some cutaneous β-HPVs effectively inhibit apoptosis in response to UV (ultraviolet) damage by impairing DNA repair through E6 activity (Jackson and Storey, 2000, Akgül et al., 2005, Leverrier et al., 2007). Studies performed on transgenic mice have confirmed the role of β-HPVs such as HPV8 and HPV38 in the development of UV-induced skin cancer (Viarisio et al., 2011, Hufbauer et al., 2011).

In addition to viral proteins E6 and E7, a major influence on the development of HPV-caused cervical cancer is estrogen (Schneider et al., 1982, Swaneck and Fishman, 1988, Chung and Lambert, 2009, Auborn et al., 1991, Chung et al., 2008). The use of transgenic mice also helped shed light on estrogen's effect on the development of cervical cancer.

2.4.3.3. Xenograft model systems

Mouse models of cancer are frequently used to search for new anticancers. Currently, patient-derived xenografts (PDXs) and cell-line xenograft models are used.

Cell-line xenograft models – i.e., human tumor cells that have been extensively passaged *in vitro* – are injected into immunodeficient mice, resulting in *in vivo* tumor growth. Because of the simplicity and the relatively low cost of the technique, it is widely used. Another advantage is the wide range of available tumor types – i.e., explants (cell lines and tumor tissues of patients cultured *in vitro*). Although xenograft preclinical trials have been used to develop most of the recently used cancer drugs, this approach may be of uncertain value in predicting clinical efficacy in human patients (Voskoglou-Nomikos et al., 2003).

Patient-derived xenografts are based on tumor tissue transplanted from a patient and directly injected into severe combined-immune-deficiency mice (SCID) (Clarke, 1996). Next, the tumor is transplanted from one mouse to another. Tumors can be implanted either heterotopically or orthotopically (Siolas and Hannon, 2013). Orthotopic transplantation more closely mimics human tumors than do models in which tumors have been obtained after multiple passages in mice (Rubio-Viqueira and Hidalgo, 2009).

2.5. The DNA-damage response during the papillomavirus replication cycle

2.5.1. A short overview of DDR pathways

Although DNA is relatively stable, many factors that can damage DNA are known, including internal factors such as replication stress, reactive metabolic intermediates and exogenous factors (e.g., chemicals, radioactivity and UV radiation) (Lindahl and Barnes, 2000, McKinney et al., 2015, Weitzman and Weitzman, 2014, Nakamura et al., 2014). Molecular mechanisms of the DNA-damage response (DDR) include a network of dynamically interacting proteins and protein complexes, such as the MRE11-Rad50-Nbs1 complex (MRN) and the ATR-ATRIP complex formed by binding ATRIP to replication protein A (RPA). These complexes detect DNA damage and signal the need to stop the cell cycle while either eliminating damage to or inducing the death of damaged cells (Weitzman and Weitzman, 2014). The MRN and ATRIP complexes, which detect a wide range of DNA damage, recruit ataxia-telangiectasia mutated (ATM) and ATM and Rad3-related (ATR) serine/threonine protein kinases, which play a main role in these repair pathways (Lee, 2007). Both of those kinases act (in part) by controlling cell-cycle checkpoints at the G1, S and G2 phases (Turnell and Grand, 2012).

ATR has broader functions in organizing the response to stalled replication forks and exposing single-stranded regions of DNA (Harper and Elledge, 2007, Cimprich and Cortez, 2008). The molecular mechanisms of ATR activation by a single-stranded DNA are fairly well known (Figure 4B) (Fanning et al., 2006, Cortez et al., 2001, Zhou and Elledge, 2000, Kumagai et al., 2006, Ohashi et al., 2014, Lin et al., 2012, Shigechi et al., 2012). DNA repair is not the only role of

the DDR, which is also involved in cell-cycle regulation, arresting cell division until DNA repair is completed (McKinney et al., 2015, Stracker et al., 2009).

Unlike single-strand breaks, dsDNA breaks are uncommon; however, they are one of the most serious types of DNA damage. The consequences of ATM activation are still debated. dsDNA breaks are bound by the MRN complex, which forms a bridge to the DNA gap and activates the ATM signaling cascade (Uziel et al., 2003, Bencokova et al., 2009, Moody and Laimins, 2009, Nikitin and Luftig, 2011) (Figure 4A). ATM promotes the repair of double-stranded breaks through homologous recombination (HR) (Noon et al., 2010).

DNA-dependent protein kinase (DNA-PK) plays a central role in double-stranded break repair (DSBR), regulating non-homologous end-joining (NHEJ) (Burma and Chen, 2004) (Figure 4C).

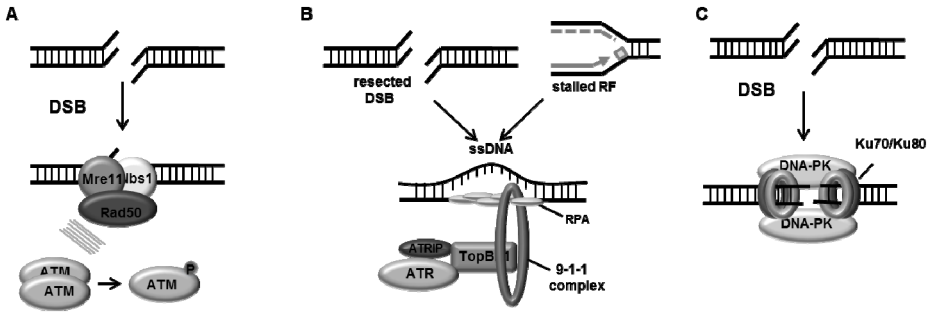


Figure 4. DNA-damage response activation. (A) *Activation of ATM-based signaling pathway.* DSBRs are sensed by the MRN (Mre11-Rad50-Nbs1) complex, which attracts ATM and probably contributes to its activation. It is not well established how ATM is converted from its inactive to active conformation; one possibility is that it changes from an inactive dimer to an active monomeric form. (B) *ATR activation.* ATR is activated by ssDNA (single-stranded DNA), which can be derived either from resected DSB (left) or from stalled replication forks (right). RPA-coated ssDNA loads the ATRIP-ATR complex and the 9-1-1 complex (consisting of Rad9, Hus1 and Rad1), which brings together ATR and its allosteric activator TopBP1 (topoisomerase-binding protein 1 (C) *Activation of DNA-PK-dependent pathways.* DNA-PK comprises a large catalytic subunit (DNA-PKcs) and two regulatory subunits (Ku70 and Ku86). The Ku complex recognizes and binds to DSBs and then recruits and stabilizes the interaction of DNA-PKcs with damaged DNA. Two DNA-PKcs molecules, working in concert, tether DNA ends together in a synaptic complex and recruit the DNA ligase IV–XRCC4 complex to rejoin the broken DNA ends. Figure adapted from (Lopez-Contreras, 2012).

Over the last 20 years, many efforts have been made to characterize the relationship between viral infection and DDR. Because infection with many viruses leads to DDR activation, the relationship between viral infection and DDR is of particular and increasing scientific interest (Turnell and Grand, 2012, Weitzman et al., 2010, Nikitin and Luftig, 2011, Weitzman and Weitzman, 2014).

DDR can be activated as a result of unusual structures of the viral DNA (linear or circular extrachromosomal molecules), unscheduled viral DNA syn-

thesis, viral protein expression, or abnormal proliferation of infected cells. Some early studies revealed that viral DNA is recognized by the host cell as damaged DNA, possibly in an antiviral response directly targeting infection. Viruses, in turn, employ a complicated series of mechanisms to inhibit the activation of the cellular damage-response pathways. However, it remains unclear whether the activation of DDR is a by product of infection or a result of viral activity. The question of how viruses either avoid the cellular response or selectively activate DDR paths beneficial for them is a subject of significant interest (Edwards et al., 2013, Weitzman et al., 2010, Nikitin and Luftig, 2011). Although the role of DDR in the virus infection cycle is well studied for some DNA viruses, the relationship between HPV and DDR should be examined more closely (Turnell and Grand, 2012, Blackford et al., 2008, Baker et al., 2007, Cheng et al., 2011, Forrester et al., 2011, Orazio et al., 2011, Shirata et al., 2005).

2.5.2. The activation of DDR during HPV genome replication

The expression of HPV E1, E2, E6 and E7 proteins in undifferentiated primary keratinocytes and differentiated suprabasal epithelial cells leads to activation of the host ATR and ATM-dependent DDR signaling pathways. Moody and Laimins have shown that upon keratinocyte differentiation and viral episome amplification, the ATM kinase self-phosphorylates. Interestingly, the inhibition of ATM prevented episome amplification upon differentiation, but not the maintenance of HPV episomes in undifferentiated keratinocytes (Moody and Laimins, 2009). In other studies, it has been shown that over-expression of HPV E1 induced cell-cycle arrest and DDR activation in undifferentiated keratinocytes in the absence of viral DNA (Fradet-Turcotte et al., 2011, Sakakibara et al., 2011, Reinson et al., 2013).

E1 expression is increased during the differentiation phase of epithelial cells, resulting in elevated replication of viral DNA. The HPV early protein E7 binds to ATM and promotes Chk2-regulated, caspase-dependent activation of the HPV E1 protein (Moody and Laimins, 2009). We have previously demonstrated that high-level expression of E1 from a heterologous vector system initiates replication from integrated HPV origins by multiple times in a single S phase (Kadaja et al., 2007). This replication can disrupt cell-cycle control in differentiating cells. Thus, repeated replication of HPV DNA and activation of the full ATM response becomes possible.

HPV replication in differentiated cells occurs in nuclear replication foci (Sakakibara et al., 2013, Moody and Laimins, 2009, Turnell and Grand, 2012). Nuclear replication foci are so-called “replication factories,” i.e., specific regions in the nucleus that contain the components required for the synthesis of viral DNA. In these foci, the cellular DNA-damage response is induced and many markers of the ATM/ATR pathways are recruited. As mentioned above, the overexpression of E1 replication protein is sufficient to activate DDR; E1 protein was shown to co-localize in the cellular replication foci with DDR

components (Sakakibara et al., 2011, Fradet-Turcotte et al., 2011, Reinson et al., 2013). It is likely that the virus mimics cellular DNA replication, including DNA-damage events, resulting in foci formation, attracting many DNA replication factors and repairing the damage. Thus, using DDR pathways, HPV can take advantage of cellular capabilities and resources for its own DNA replication.

Another factor in DDR activation is changes in viral DNA amounts and structures in differentiated cells (Nikitin and Luftig, 2011, Sakakibara et al., 2013). In the stable maintenance phase of the viral infection cycle, papillomavirus genomes might replicate in a bidirectional *theta* mode. However, the increasing number of viral episomes can cause ATM activation, which is important for choosing the mode of replication (Moody and Laimins, 2009). Robust viral DNA replication through *theta* intermediates might generate nicks in the viral DNA, which may facilitate the switch from the *theta* mechanism to rolling-circle replication (Flores and Lambert, 1997, Kusumoto-Matsuo et al., 2011, Auborn et al., 1994). BPV1 can also transition from bidirectional replication to unidirectional rolling-circle replication (Dasgupta et al., 1992, Burnett et al., 1989).

Efficient replication is linked to a homologous recombination-related process referred to as recombination-dependent replication (RDR) (Asai et al., 1994, Kuzminov, 1999, Kogoma, 1996). Furthermore, we will show both that HPV genomes may use a unique recombination-related replication mechanism for multiplication of the viral DNA and that HPV genomic hetero-oligomers are formed through an RDR, not rolling-circle replication (RCR) (Orav et al., 2013). It remains unclear how the transition from *theta* mode to recombination-dependent replication occurs and how papillomaviruses use host recombination factors to recruit, engage, and manipulate viral DNA replication at various stages of the virus infection cycle.

2.5.3. Pathways of dsDNA-breaks repair

dsDNA breaks can be repaired either through homologous recombination (HR) or non-homologous end joining (NHEJ). NHEJ is the pathway in which two broken DSBs are sealed directly, without the need for homologous templates. DNA end ligation without preservation of the correct reading frame results in the loss of genetic information; for that reason, NHEJ can be prone to errors. In yeast, HR is the primary choice when repairing DSBs, whereas mammalian cells rely on NHEJ despite the higher probably of genomic instability. Nevertheless, NHEJ can occur in any stage of the cell cycle, which makes it simpler and faster than HR (Kee and D'Andrea, 2010).

The high accuracy of HR pathways is provided by the exchange of DNA sequences between two similar or identical DNA strands. After a double-stranded break occurs, sections of ssDNA around the 5' ends of the break of one strand are excised in a process called resection. In the strand-invasion step that

follows, an overhanging 3’ end of the other strand is now formed as a result of a resection at one side of the DNA fragment. The ssDNA-binding protein RPA binds to 3’ overhangs and is later replaced by Rad51. Rad51, together with several other proteins, forms a filament. The Rad51 nucleoprotein filament searches for homologous sequences and mediates the formation of a displacement loop (D-loop). A DNA polymerase then extends the invading 3’ and the D-loop structure is changed into an X-shaped structure called a Holliday junction (HJ). The 3′ strand of the second end serves as a primer for repair synthesis. (Fig. 5) (Krejci et al., 2012).

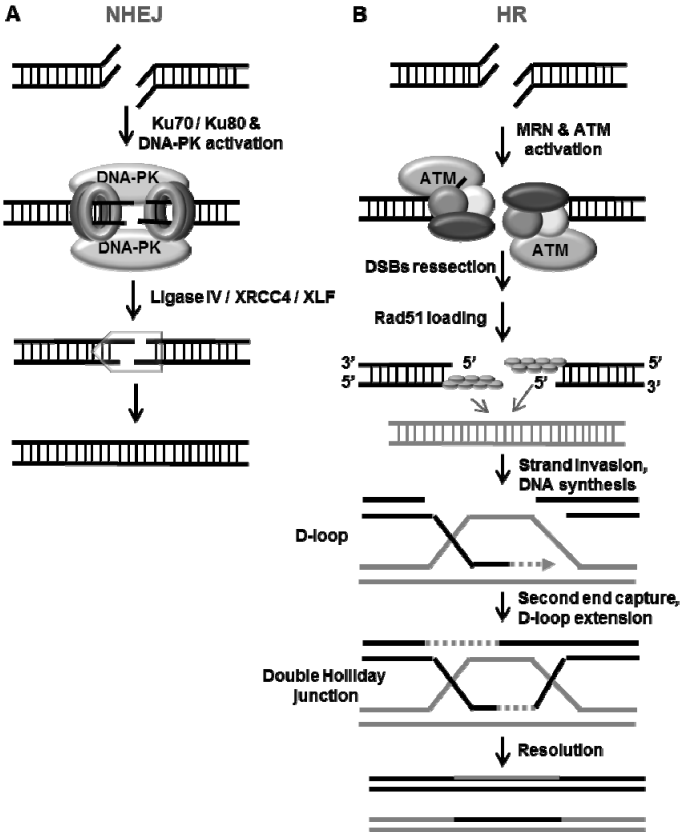


Figure 5. DSB-repair mechanisms. (A) *NHEJ*. DSBs are sensed by the ring-shaped heterodimer Ku70/Ku80, which then stabilizes the two DNA ends and recruits DNA-PK. Next, DNA-PK phosphorylates and activates the NHEJ effector complex (ligase IV/XRC44/XLF), which religates the broken DNA. (B) *HR*. The ATM kinase is recruited to a DSB via an interaction with the MRN complex. Once at the break, ATM becomes activated and phosphorylates multiple substrates. The DSBs that are resected form ssDNA strands. These ssDNA regions attract Rad51 and other associated proteins. The Rad51-coated nucleoprotein filaments then invade the undamaged sister strands, forming HJ structures. Figure adapted from (Lopez-Contreras, 2012).

The synthesis of D-loop intermediates can be classified into one of several pathways: synthesis-dependent strand annealing (SDSA), double-strand break repair (DSBR), or break-induced replication (BIR) (Stafa et al., 2014). Because possible replication modes of the HPV genome are a topic of this paper, we will consider the aspects of break-induced replication in more detail. BIR refers to recombination-dependent DNA synthesis initiated from one end of a DNA double-strand break; it can extend for more than 100 kb. The mechanisms that limit DNA synthesis during gene-conversion repair but promote extensive longer DNA synthesis in BIR remain unknown. Previous studies have shown that BIR can occur through either conservative or semiconservative DNA synthesis (Donnianni and Symington, 2013). Because a homologous DNA sequence is required for proper DNA repair, HR is restricted to the S and G2 phases of the cell cycle, when homologous sister chromatids are present to provide a template.

Figure 5 describes the two primary models of DSB repair, HR and NHEJ, both of which require multiple-step reactions.

As previously noted, HPV DNA replication in differentiated cells occurs in so-called nuclear replication foci in which the MRN components are colocalized (Moody and Laimins, 2009, Reinson et al., 2013). Gillespie et al. have investigated the potential relationship between HPV genome replication and cellular repair pathways through HR (Gillespie et al., 2012). This group of scientists examined the levels of the cellular proteins involved in HR, including Rad51, BRCA1, pRPA, S33, concluding that they are also recruited to HPV replication foci, thus indicating the possibility that HR could be directly involved in HPV-genome amplification.

3. AIMS OF THE STUDY

The diagnosis and treatment of diseases associated with HPV infections attract the attention of various experts because of the high level of infections by these pathogens in developing countries and the virus's ability to transform epithelial cells, causing malignant pathology. Cervical and other HPV-dependent cancers are the result of a long chain of pathologic changes that can take place over many years of persistent HPV infection. There are no specific antiviral drugs against papillomavirus infections; therapeutic vaccines to treat persistent HPV infection are still under development. The goal of therapy should be the elimination of clinical and subclinical forms of HPV infection.

There is a great need for model systems that can mimic papillomavirus infection in human tissues. To create new antiviral drugs, it is important to understand the molecular mechanisms of HPV DNA replication. The lack of a simple cellular assay system capable of supporting replication of multiple HPVs and mimicking the different stages of the viral infection cycle is a serious challenge for this research.

The general objective of the research presented in this thesis was to generate and validate cellular assay systems, thus enabling the study of different replication phases of the papillomavirus genomes.

The specific objectives of this research were as follows:

1. To develop and use a fast, efficient, reliable and cost-effective cellular system based on the human osteosarcoma cell line U2OS and to demonstrate that this system is capable of supporting all three stages of the HPV genome replication cycle;
2. To study cutaneous and mucosal HPVs (both low-risk and high-risk) using the U2OS cell-line-based system and to analyze and characterize the physical state of HPV genomic DNA during various replication stages in those cells;
3. To analyze the replication intermediates of the HPV genome using one-dimensional (1D), two-dimensional (2D) and three-dimensional (3D) agarose-gel electrophoresis (AGE) methods to identify the replication mechanisms, concentrating mostly on the first, transient replication phase in the U2OS cell-line-based model system;
4. To demonstrate that the segregation functions of various DNA viruses are principally interchangeable; to develop hybrid replication origins based on BPV1 and PyV, on the one hand, and EBV and PyV, on the other hand, thus allowing stable replication;
5. To demonstrate that hybrid origins provide stable multicopy nuclear replication functions to the therapeutic protein production vectors in the cells, which express all necessary viral *trans*-factors – LT (PyV) and E2 (BPV1) or EBNA1 (EBV) and can be used in the production of therapeutic proteins.

4. MATERIALS AND METHODS

The materials and methods used during this study are described in detail in various research articles and/or their supporting materials. However, some explanations that aid in understanding this work will be presented below.

Because one of the main purposes of my work was to develop a model system for HPV DNA replication studies that is based on the human osteosarcoma cell line U2OS, this system will be discussed in more detail.

The human osteosarcoma U2OS cell line was derived in 1964 from a moderately differentiated sarcoma of the tibia of a 15-year-old girl. It was one of the first cell lines generated and is frequently used in various areas of biomedical research. Two tumor-suppressor genes, p53 and pRb, are functional in U2OS cells (Wesierska-Gadek and Schmid, 2005, Isfort et al., 1995).

Although osteosarcoma cell lines are not the natural host of HPV, U2OS cells support all replication stages of HPV genomes. We simulated the transient, stable, and vegetative stages of HPV replication by transfecting the U2OS cells with plasmids containing HPV genomes. In Ref. **I**, HPV genomes were excised from bacterial vectors pBR322 (HPV18, HPV6b and HPV5), pUC19 (HPV16 and HPV11) or pUC9 (HPV8) and religated. In Ref **IV** and **V**, the technology for producing circular HPV genomes was changed. The minicircle parental plasmid pMC-HPV18 was constructed following insertion of the HPV18 genome into pMC.BESPX the vector. Minicircle (plasmid lacking bacterial sequences) production was performed in the *E. coli* strain ZYCY10P3S2T based on the previously published protocol (Kay et al., 2010). DNAs almost perfectly corresponding to HPV18 genomes were then purified from *E. coli* as supercoiled minicircles and used to transfect U2OS cells.

The papillomavirus infection cycle is closely related to the differentiation of the host cells. This step is supported by the fact that the vegetative amplification of viral DNA and the transcription of late genes occur only in differentiated suprabasal epithelial cells. In a keratinocyte culture that is dense or grown in a Methocell semi-solid medium, amplification of the viral genome is triggered as a result of the initiation of differentiation. To study HPV in the vegetative-amplification stage, we used a simple system mimicking the differentiation of the epithelium. Subclones were grown without splitting for approximately two weeks, and fresh medium was added every few days. Proliferation slows under these conditions and the culture becomes dense over the next two weeks. However, the U2OS cells did not die. Because these cells harbored functional HPV genomes, it became possible to imitate all DNA replication steps. It should be mentioned, however, that the system remains limited because of the lack of expression of late proteins; moreover, there was no virion production. However, this limitation increases laboratory safety while handling the cells because there is no risk of infection.

We used 2D or 3D AGE to analyze the DNA replication products in Refs **I**, **IV** and **V**. During the first dimension of 2D neutral/neutral (N/N) gel electrophoresis, molecules are separated primarily according to their size (low agarose

percentage, low voltage and no ethidium bromide (EtBr) added). The strip of the agarose gel containing separated DNA is then cut out and oriented perpendicular to the first dimension; a new gel with high agarose concentration is then poured around it. The second dimension is run at a high voltage in the presence of EtBr. Under these conditions, the molecules are separated according to their topology (conformation).

The first dimension of 2D neutral/alkaline (N/A) gel electrophoresis is similar to the 2D N/N. Before the second dimension of electrophoresis, the gel was treated under alkaline denaturing conditions to separate the newly synthesized leading-strand DNA from the parental strand proportional to the length of the single-stranded chain.

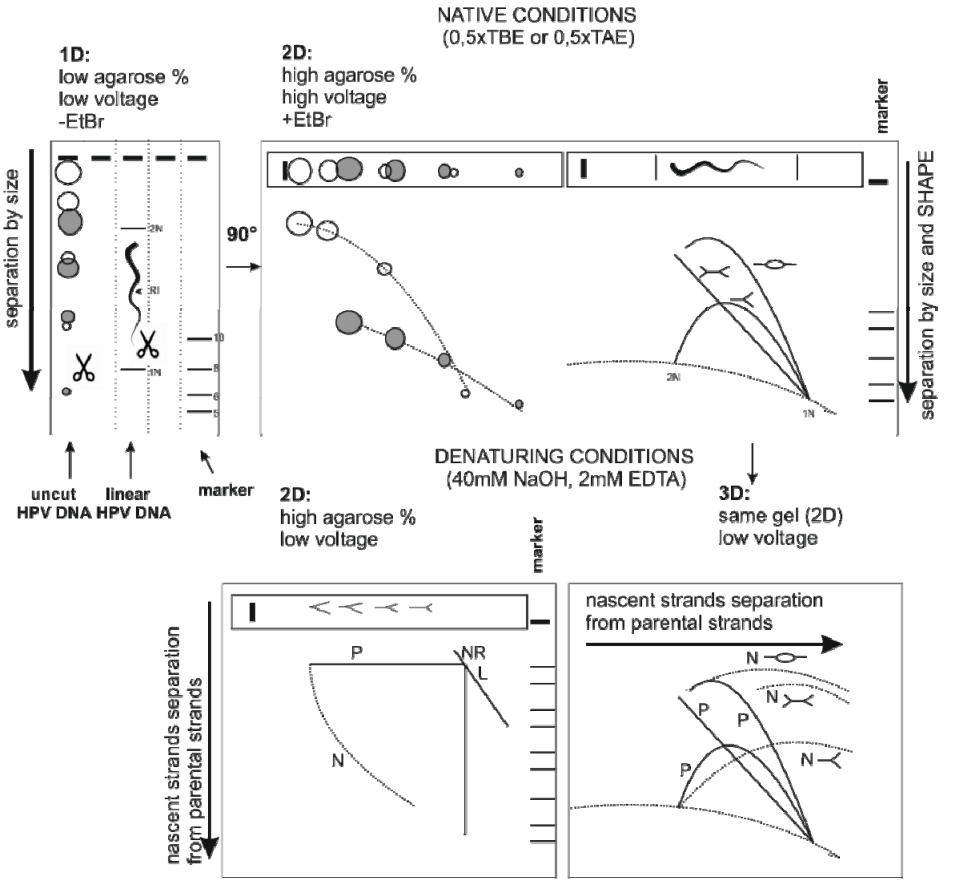


Figure 6. Schematic representation of the principles of 2D N/N, 2D N/A and 3D N/N/A AGE (N, nascent strand; P, parental strand; L, linear; NR, non-replicating DNA; EtBr, ethidium bromide; 1N, starting spot of the position (unit length); 2N, position at which the 1N molecules that were almost completely replicated would be expected to migrate (twice unit length); RI, replication intermediates). For more information, see Ref. **V**, Materials and Methods.

The first and the second dimensions of the 3D N/N/A gel electrophoresis were run in a manner that was identical to the 2D N/N analysis. The third dimension allows for the separation of nascent strands from the parental strands following their separation by size and topology in the first two dimensions (Fig. 6).

The first dimension of 2D neutral/alkaline (N/A) gel electrophoresis is similar to the 2D N/N. Before the second dimension of electrophoresis, the gel was treated under alkaline denaturing conditions to separate the newly synthesized leading-strand DNA from the parental strand proportional to the length of the single-stranded chain.

The first and the second dimensions of the 3D N/N/A gel electrophoresis were run in a manner that was identical to the 2D N/N analysis. The third dimension allows for the separation of nascent strands from the parental strands following their separation by size and topology in the first two dimensions (Fig. 6).

The engineering of the hybrid vector system based on BPV1 and PyV replicons, on the one hand, and EBV and PyV replicons, on the other hand, was one of the goals of this work. The mouse fibroblast cell lines COP5, which constitutively express mouse polyomavirus T-antigens, were modified by inserting the cassettes for expression of the BPV1 E2 and EBV EBNA1 proteins (Ref. **III**).

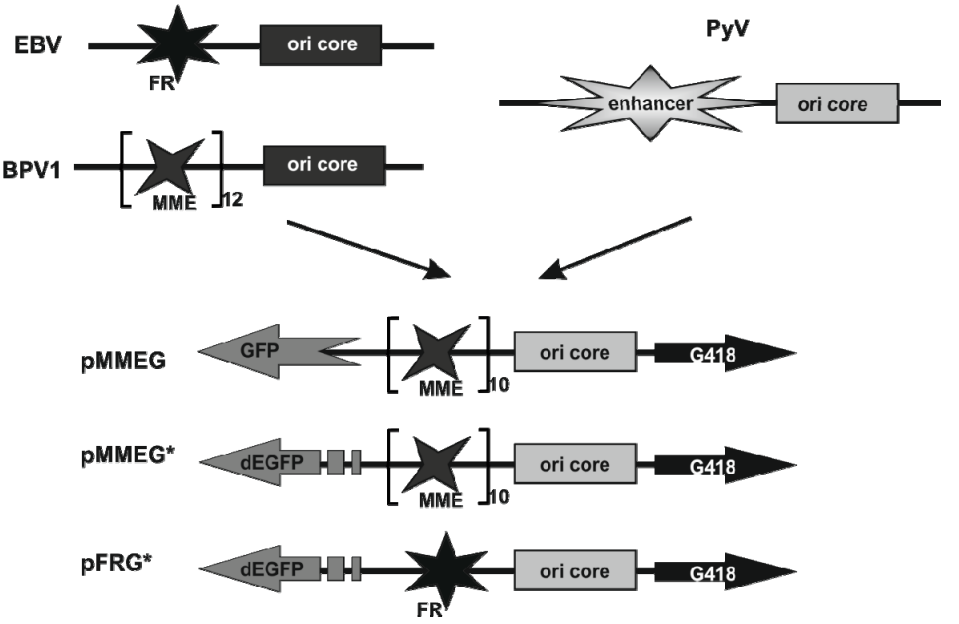


Figure 7. Schematic representation of the construction of PyV and BPV1/EBV hybrid origin. Constructs in which the PyV enhancer element is replaced by 10 E2 binding sites (MME) or the family of repeats element (FR), eukaryotic selection cassett (G418) and green fluorescent protein expression cassette (GFP or dEGFP) are shown.

The hybrid vector systems were designed by combining different viral *cis*-sequences. We replaced the PyV enhancer, which is part of the polyomavirus replication origin, with enhancer element from the BPV1 using the E2 protein-dependent MME element (Fig 7). Alternatively, we used the Epstein-Barr virus EBNA1-dependent FR (family of repeats) element.

We designed hybrid origin constructs (pMMEG, pMMEG* and pFRG*) that contain the expression cassette for the selection marker geneticin (G418). This is required for a long-term expression under selection of any gene of interest. We also inserted green fluorescent proteins (either long-half-life EGFP or short-half-life d1EGFP) into the expression cassette, thus making it possible to test and follow the behavior of the hybrid origin construct inside the cells over time, for example, by using fluorescence-activated cell sorter analysis (FACS).

5. RESULTS AND DISCUSSION

5.1. Model system to study the episomal maintenance of plasmids with hybrid origins (Ref. III)

Genomes of papillomaviruses and the Epstein-Barr virus are maintained in the host cells as extrachromosomal plasmids. The stable maintenance of these viruses' genomes depends on the viral *cis*-elements and *trans*-factors. Although a minimal origin (MO) is sufficient for transient BPV1 replication, stable extrachromosomal maintenance also requires the minichromosome maintenance element (MME), which contains multiple E2 binding sites. Thus, MO and MME are *cis*-elements essential for BPV1-stable DNA replication. E1 and E2 are the only *trans* factors that are absolutely essential for papillomavirus DNA replication. An attachment of the papillomavirus genome to the host mitotic chromosomes occurs via E2, which binds both to the E2 binding sites in MME and to receptor protein(s) on the chromosomes. This would be a useful property for plasmid vectors designed for the long-term expression of proteins of interest.

That said, vector systems based on the BPV1 platform have some disadvantages. One of the reasons that the BPV1-based vector systems were not widely used is that it is very hard to achieve the initial high levels of amplification of such vectors in transfected cells. Another reason is that the papillomavirus replication protein E1 can cause dsDNA breaks, thereby activating a cellular response to DNA damage (Reinson et al., 2013, Van Craenenbroeck et al., 2000), which is a disadvantage for vector systems. Therefore, we tried to solve these issues by generating the hybrid replication origin.

For the hybrid system, the mouse polyomavirus (PyV) replication origin and a virus-encoded replication factor, large T antigen were selected. PyV is a lytic virus and PyV origin-containing plasmids replicate very rapidly during productive infection. Therefore, PyV-based vector systems can efficiently replicate the foreign DNA in permissive cells. There was no need to use wt-origin (enhancer and minimal origin) *in cis* and Large-T helicase *in trans* during the vector system development, because they usually tend to overreplicate and disrupt the establishment of stable maintenance. Therefore, the enhancer of polyomavirus replication origin was removed and only the minimal origin that is able to initiate replication in the presence of LT was used. Furthermore, the wt enhancer of PyV was replaced with either an artificial enhancer element from the BPV1 or an FR-element from the EBV (Materials and Methods, Fig. 7).

The plasmids pMMEG, pMMEG* and pFRG* (Ref. **III**, Fig. 6A; Materials and Methods, Fig. 7) with hybrid origins were transfected into the COP5E2/Puro or COP5EBNA1/Puro cells, which stably express the PyV LT, BPV1 E2 and EBV EBNA1 proteins. These cells were able to support the efficient transient replication of hybrid origin plasmids. Because the vectors expressed enhanced GFP (EGFP), the replication was detected using the Southern blot method and flow cytometry. Transfected cells were grown in continuous culture in the presence or absence of Geneticin for up to 96 days.

The percentage of the cells expressing GFP was measured every second day throughout the experiment.

The percentage of GFP-expressing cells under selective conditions rapidly reached its maximum level (approximately 90%) and was stable during the passaging of cells for more than three months. The percentage of cells expressing destabilized enhanced GFP (d1EGFP) under selective conditions was somewhat lower, in the range of 40 to 70% (Ref. **III**, Fig. 6B, C and D). To test the hybrid system in the absence of selective pressure, a medium without antibiotic was used. As expected, under these conditions the percentage of EGFP and d1EGFP positive cells started to decrease. Over 55 days, the percentage of EGFP-containing cells dropped precipitously, from 90% to 1% in the case of pMMEG (from 64% to 2.4% in 37 days in the case of pMMEG* and from 40% to 1% in 30 days in the case of pFRG*) (Ref. **III**, Fig. 6B, C and D). When G418 selection was restored, the percentage of EGFP and dEGFP-positive cells was also restored to their previous high level over 10 days (Ref. **III**, Fig. 6B, C and D).

Thus, under nonselective conditions, the percentage of the reporter protein-positive cells began to decrease. The reasons for the loss of fluorescence may be different, for example, inactivation of the promoter used to drive the expression of EGFP. To find out the reasons for the loss of fluorescence, both plasmid DNA content in cells and FACS were analyzed. After removing the selection and taking FACS measurements every second day, total DNA was extracted. Total DNA analysis shows an increased amount of cellular DNA that is coherent with the growth of the culture (Ref. **III**, Fig. 7). Because this coincided with decreased EGFP fluorescence, it was concluded that in these cells, the EGFP-expressing plasmids were not integrated, but resided as episomes. The addition of selection pressure contributes to episomal rescue. The fact that the number of episome (EGFP) positive cells under nonselective conditions is gradually reducing in the cell population might indicate that the episomes are not distributed evenly into the daughter cells, thus leading to the gradual reduction of the percentage of plasmid-positive cells.

To analyze this process, the rate of loss was calculated for each plasmid. To characterize the kinetics of loss, a first-order reaction $(\lambda) = (-1/t)(\ln Nt/N0)$ was used, where $N0$ is the percentage of fluorescent cells at the beginning of the experiment and Nt is the percentage of luminous cells after t generations. The rate of loss during one cell generation of plasmids containing hybrid origin was approximately 6% (pMMEG, pMMEG*) and 13% (pFRG*) (Ref. **III**, table 1); this was much lower than for control plasmids (pEGFP-C1 and pd1EGFP-N1) that did not contain hybrid origin of replication, at –22–33%. These data suggest this replicating system is suitable for use even without selection.

This study also revealed that EBNA1/FR elements and E2/MMEs confer comparable segregation/partitioning functions on the plasmids containing hybrid PyV origin in the analyzed cell models.

5.2. U2OS cell line as an *in vitro* model system to study various stages of HPV DNA replication (Ref. I and II)

As reviewed in Section 2.4, many different model systems are used to study papillomaviruses. Each assay system has strengths and weaknesses; by efficiently combining them, we can obtain a complete picture of the nature of the virus infection cycle. HPVs are strictly epitheliotropic and are generally active in keratinocytes. This term is often restricted to cells derived from squamous epithelia or, even more specifically, to cells from epidermis that express keratin (columnar and simple epithelia). At each stage of their differentiation, keratinocytes express specific keratins (e.g., keratin 1, keratin 5, keratin 10 and keratin 14), along with other differentiation-specific markers such as involucrin, loricrin, transglutaminase, filaggrin and caspase 14 (Del Bino et al., 2004, Ekanayake-Mudiyanselage et al., 1998, Tu et al., 2012).

Because the replication modes of HPV genomes are host cell differentiation-dependent, for a long time there were no efficient, easy-to-use cell systems for studies of HPV DNA replication. Artificial skin models such as raft cultures can be used to study the spatial distribution of HPV late functions within a differentiated epithelium, but they are time-consuming, not very reliable and prone to variability. We have developed and examined the utility of an alternative simplified system for the rapid analysis of early and late stages of HPV DNA replication.

Although appropriate cell culture systems supporting HPV DNA replication are limited, the replication of papillomavirus-origin-containing plasmids could be easily reconstituted in many cell types of various species expressing the viral replication proteins E1 and E2 from heterologous expression vectors. Such results suggest that the regulated expression of the viral replication proteins from the HPV genomes could restrict HPV genome replication to certain cell types.

We analyzed the ability of several immortalized or transformed human cell lines (C33A, 293, HaCaT, HeLa, SiHa, U2OS) to support the E1- and E2-dependent replication of HPV-origin-containing plasmids. Although most of the human cell lines analyzed supported this type of DNA replication, one cell line, U2OS, was remarkably more efficient than others. We observed that U2OS efficiently supports the replication of HPV-origin-containing plasmids, even at low concentrations of viral replication proteins. Next, the recircularized full-length HPV genomes were introduced into these cells by electroporation, and we were able to demonstrate efficient viral DNA replication in U2OS cells in transient assays.

The goal of this part of the work was to demonstrate the suitability of the U2OS cells for studying the replication of genomes of various HPV types. U2OS is derived from a moderately differentiated osteosarcoma, has an adherent epithelial morphology and carries the wild-type pRb and p53 genes. The main advantage of the U2OS cellular assay system over keratinocyte-based systems is that, as we learned, U2OS can support replication of not only α-HPV

(high-risk HPV16, HPV18 and low-risk HPV6b, HPV11) genomes but also β-HPV (HPV5, HPV8) genomes.

We have been able to show that this cellular assay system has a unique capacity to support the transient, stable and late amplification of HPV genomes (Ref. **I**). Moreover, compared to organotypic raft cultures or xenograft models, this system is simple, easy and safe to use. Thus, it can be applied in conditions that are suitable for automated industrial use, such as developing vaccines and antiviral drugs or screening HPV-replication inhibitors.

Another important point of this model system is that the transcription maps of HPV18, HPV5 and HPV11 genomes replicating in U2OS cells (Toots et al., 2014, Sankovski et al., 2014, Isok-Paas et al., 2015) are similar, if not identical, to the transcription maps obtained from HPV-infected human keratinocyte raft-culture systems (Wang et al., 2011, Chow et al., 1987). These observations confirm that the gene-expression profiles of different high- and low-risk α-HPVs and of β-HPVs in U2OS cells are similar to HPV gene expression patterns in their natural environment (undifferentiated keratinocytes). Therefore, we believe that U2OS system can be used to study the fundamental properties and functions of HPVs.

5.2.1. Initial HPV genome amplification in the human U2OS cell line

The early-gene products E1 and E2 are necessary for the initiation of HPV- and BPV1-genome replication either in transformed cells or in primary keratinocytes (Hubert and Laimins, 2002, Lace et al., 2008, Ustav and Stenlund, 1991, Chiang et al., 1992, Frattini and Laimins, 1994, Sverdrup and Khan, 1994). Even without feeder cells, the U2OS cell line efficiently supports the replication of HPV URR-containing plasmids given that viral replication proteins are additionally expressed from independent expression vectors. Moreover, U2OS cells can support viral genome replication without the need for the cotransfection of plasmids used for E1 and E2 expression, indicating that sufficient expression levels of E1 and E2 are achieved directly from the viral genome. Cutaneous high-risk α-HPVs (HPV16, HPV18), low-risk α-HPVs (HPV6b, HPV11) and mucosal β-HPVs (HPV5, HPV8) were chosen for analysis. By transfecting U2OS cells with different recircularized DNAs representing HPV genomes, we demonstrated efficient replication (Ref. **I**, Fig. 1A, B, C, D). These results suggest that all of the analyzed HPV types can trigger the expression of viral replication factors from viral promoters at a level sufficient to initiate replication of the viral genome in U2OS cells.

Additionally, these observations provided us with an opportunity to study various aspects of the HPV life cycle, including the transcription and segregation functions of HPV genomes using U2OS monolayer cultures. Importantly, for the first time, U2OS cells provided us an opportunity to study the behavior of mucosal β-HPV genomes in monolayer cultures.

To confirm that α-HPV genome replication is completely dependent on the expression of viral E1 and E2 and that these proteins are essential for the initiation of viral genome replication in U2OS cells, we generated a set of mutant genomes (described in Ref. **I**, Materials and Methods, and Ref. **I**, Fig. 3H, line 1 for scheme). More specifically, we created a subgenomic construct of HPV18 lacking the genes that encode the L1 and L2 proteins. Because the viral origin and the ORFs of early proteins are intact, this genome truncation does not affect transient DNA replication (Ref. **I**, Fig. 1E, lanes 1, 2). We also generated several mutant genomes of HPV18 (wt or subgenome context), which are defective for expression of the E1 or E2. Defects in the E1 ORF (Ref. **I**, Materials and Methods, mutations in the *OliI* and *BcuI* restriction sites) and E2 ORF (Ref. **I**, Materials and Methods, mutations in the *StuI* site) eliminated the capability of the mutated HPV18 subgenome to replicate in U2OS cells (Ref. **I**, Fig. 1E, lanes 9 and 10, 15 and 16, and 5 and 6, respectively). However, when we cotransfected the E1- and E2-defective recircularized genomes/subgenomes with the HPV18 E1 or E2 expression vectors, respectively (Kadaja et al., 2007), replication was restored (Ref. **I**, Fig. 1E, lanes 7 and 8, 13 and 14, 19 and 20, and 25 and 26). In addition, replication competence could be restored by cotransfecting the E1-defective genomes with E2-defective ones, thus demonstrating the complementary functions of both replication proteins (Ref. **I**, Fig. 1E, lanes 11 and 12, 17 and 18, and 23 and 24).

Similar experiments were conducted with the β-HPV5 genome. E1- and E2-defective mutant genomes, which are unable to replicate, were generated (Ref. **II**, Fig. 1A, lanes 4–9). Again, because of the complementation of the genetic defects, cotransfection of these defective genomes resulted in the restored replication capability of both mutant genomes (Ref. **II**, Fig. 1A, lanes 10–12). Taken together, these experiments indicate not only that E1 and E2 are both necessary for initiating the replication of the mucosal and cutaneous HPV genomes in U2OS cells but also that for both of these HPV groups, the two defective viral genomes can complement each other's defects.

5.2.2. Stable replication of the HPV18 genome in U2OS cells

Both our success in the initial replication of six types of HPV genomes in the U2OS cell line and the fact that the replication signal increases over time suggested that HPV genomes remained in these cells and underwent stable replication. To analyze the stable episomal maintenance stage, we cotransfected HPV18, –16, –11, –6b, –5 and –8 genomes with the plasmid containing a selection marker. Next, a subset of the cells was maintained without the addition of G418 to the medium, and another subset was grown in the presence of antibiotic. Three weeks later, geneticin-selected cells were separated and subcloned. Additionally, the geneticin-selected cell pools and cells grown without antibiotic were analyzed using low-molecular-weight (LMW) cellular DNA extracts (Ref. **I**, Fig. 2A). A Southern blot analysis showed that all of the

samples contained HPV genomes at comparable levels. These data indicate both that U2OS cells support the stable replication of all types of analyzed HPV genomes and that HPV-transfected cells were efficient at maintaining viral DNA even under nonselective conditions (Ref. **I**, Fig. 2A, lanes 1, 3, 5, 7).

The cell pools, which were grown under antibiotic pressure, were subcloned (from the single-cell colonies) and cultivated. Total DNA was extracted from every HPV-type cell sample and analyzed. As indicated by the Southern blot (Ref. **I**, Fig. 2C), the viral-DNA copy number in different cell clones ranges from low to high. Interestingly, the variance in the HPV DNA copy number was much greater in α-HPV-positive subclones than in β-HPV-positive subclones.

The next stage of the stable HPV DNA replication study was to assess the long-term, stable persistence of HPV genomes in U2OS cells. HPV-positive subclones were regularly passaged for at least two months. Every week, total cellular DNA samples were taken and analyzed by Southern blotting (Ref. **I**, Fig. 2D). This analysis revealed that majority of HPV subclones was stable in U2OS cells. In particular, high-risk HPV18 subclones such as 18#1.13 and 18#1.4 maintained HPV genome extrachromosomally and only some subclones (such as 18#1.10) showed HPV18 DNA integration into the host genome. The high-risk HPV16 subclones also maintained HPV16 DNA stably, with the exception of subclone 16#3.3, which demonstrated a loss of episomal copy numbers over time. The low-risk α-HPVs and β-HPV subclones showed a lower rate of establishing the maintenance of viral genomes compared to high-risk α-HPVs. Thus, we observed not only a rapid loss of the viral genome in some low-risk HPV subclones (Ref. **I**, Fig. 2D, 6b#41) but also integration (Ref. **I**, Fig. 2D, 11#3.13).

5.2.3. HPV-genome amplification in confluent cell cultures

As mentioned above, the infection cycle of HPV is tightly linked to the differentiation program of keratinocytes. The final phase of the HPV replication cycle is vegetative amplification, in which the viral genome is actively replicated and the HPV DNA copy number increases multiple times. In natural infection, capsid proteins (L1, L2) are synthesized during the vegetative phase to enable virion assembly. However, in high-risk HPV-positive keratinocytes in monolayer cultures (see 2.4.1.1), various methods used to induce differentiation have managed to achieve HPV-genome amplification only; no late proteins synthesis can be detected.

By keeping the cells under confluent conditions, we decelerated the cell division and imitated the start of vegetative amplification in HPV-positive U2OS subclones to analyze the induction of HPV-genome amplification. Subclones from various HPV types were seeded onto plates at fixed subconfluent densities and grown for 12 days without splitting; the media were exchanged in every two days (Ref. **I**, Fig. 3C and G). Measurement of the viral DNA copy number at various point in time revealed that the copy number increased over

time, imitating the start of vegetative amplification. Quantification performed for the HPV18 subclone 18#1.13 showed an approximately 10-fold increase in the amount of HPV DNA per cell in the confluent culture at day 12 (Ref. **I**, Fig. 3D). Amplification of the DNA of other HPV subtypes was also achieved under the confluent culture conditions (Ref. **I**, Fig. 3G). Perhaps, in mimicking the conditions of cell differentiation, we have created conditions for the viral genome that allowed a second amplification of viral DNA. The cell-growth graph (Ref. **I**, Fig. 3A) demonstrates that after reaching confluence, cell division slows down, which is probably a prerequisite for the virus to generate more viral DNA copies per cell.

One additional method to confirm HPV DNA amplification in confluent culture conditions is FISH (fluorescence in situ hybridization) analysis, which was performed on interphase cells. Samples of HPV18 subclone 18#1.13 were collected from subconfluent and confluent conditions. HPV genomes localized into a few fixed spots in the nuclei of proliferating cells. Maintenance of the cells under confluent culture conditions induced the increase in the amount of HPV DNA at these sites (Ref. I, Fig. 4), thus confirming the results obtained from the Southern blot analysis.

Amplification of the DNA of low-risk HPVs was not as efficient, and only one out of six analyzed β-HPV subclones, 5#23, demonstrated a slight tendency to amplify viral DNA in confluent cell culture (Ref. **I**, Fig. 3G 5#23; Ref. **II**, Fig. 1C, D). Additionally, it was found that the HPV5 late promoter was activated in confluent U2OS cells; however, we were unable to identify late polyadenylation cleavage sites for the late transcripts (Sankovski et al., 2014).

5.3. Concatemerization of HPV genomes (Ref. I, IV and V)

Using the U2OS cell line as a cellular assay system to study the DNA replication of α-HPV low- and high-risk and β-HPVs, we have shown, that these cells efficiently support the replication of various HPV genomes during initial transient amplification, stable replication and late amplification, which was reminiscent of vegetative replication.

Next, we explored the physical state of HPV DNA during various replication stages in U2OS cells. We used 1D analysis of uncut DNA fractions from HPV18-transfected cells (Ref. **I**, Fig. 5A, Ref. **IV**, Fig. 1C) and uncut DNA from HPV18 subclone 18#1.13 (Ref. **I**, Fig. 5B). An analysis of low-molecular-weight HPV DNA showed not only that replication signals accumulated over time but also that dimeric and higher oligomeric forms of HPV genome appeared at later points.

Moreover, these oligomers are efficiently maintained in proliferating HPV-positive U2OS cell lines during multiple passages (Ref. **I**, Fig. 5A, lane 2–3, 4–7 and Ref. **I**, Fig. 5B, lane 2–6). The role of the oligomeric state of DNA in the HPV life cycle is unknown. HPV18 oligomeric genomes might have an advantage in maintenance over monomeric genomes. We assume that oligo-

merization could be a viral mechanism to assemble a set of maintenance, segregation, and partitioning functions to achieve an advantage by retaining the maximal number of DNA copies during the stable maintenance phase in the condition under which the expression levels of viral replication proteins E1 and E2 remain low.

5.3.1. HPV genomic oligomers are extrachromosomal concatemeric molecules containing the viral genome in a head-to-tail orientation

Next, we used the U2OS cell-based assay system to characterize oligomeric forms of HPV genomes. First, we applied restriction analysis (Ref. **I**, Fig. 5C and 5D and Ref. **IV**, Fig. 2A) and digested HPV DNA from an HPV18-positive subclone 18#1.13 with three different linearizing enzymes. Oligomeric forms present in the undigested DNA (Ref. **I**, Fig. 5C and 5D, lane 1, 2, 6, 7) were cut into single-unit-length linear fragments with all three linearizing endonucleases (Ref. **I**, Fig. 5C and 5D, lane 3–5, 8–10; Ref. **IV**, Fig. 2A, lane 1–4). This simple experiment demonstrates that the multimeric genomes are head-to-tail concatemers (the multiple copies of the HPV DNA sequences are not randomly oriented within the concatemer but instead are all oriented in the head-to-tail form).

Second, to support the previous conclusion, a partial digestion analysis was performed. As expected, the results showed that the oligomeric HPV genomes were organized as head-to-tail episomal concatemers (Ref. **IV**, Fig. 2B). We also found that oligomerization does not depend on the topology of the input DNA (Ref. **IV**, Fig. 1A and B), nor does it require any other viral factors in addition to the replication proteins E1 and E2 (Ref. **I**, Fig. 1E; Ref. **IV**, Fig. 3B). We have also shown the oligomerization of HPV18 and HPV11 genomes in the SiHa, HeLa and C33A cell lines, all of which were derived from cervical carcinomas (Ref. **IV**, Fig. 7).

The DNA of HPV in patient samples is often present as genome multimers (Adachi et al., 1996, Cullen et al., 1991, Kalantari et al., 2001, Kristiansen et al., 1994, Lehn et al., 1988). We also demonstrated the presence of oligomeric HPV genomes in clinical samples obtained from HPV-infected patients. (Ref. **IV**, Fig. 8). These data suggest that HPV genome oligomerization is common for all *in vivo* HPV infections. The existence of oligomeric episomal HPV genomes has also been previously demonstrated for HPV16 in the W12 cell line (Alazawi et al., 2002) and for HPV31 in the CIN612 cell line (Hong and Laimins, 2013).

These results indicate that oligomerization is a natural process in HPV-genome replication; it does not occur only in U2OS cells. Accordingly, we can conclude that oligomerization during the HPV infection cycle in our model system mimics the oligomerization process during HPV infection in keratinocytes.

5.3.2. HPV replication-dependent genome oligomers arise through homologous recombination

Head-to-tail oligomeric molecules may be a product of various mechanisms associated with DNA replication. First, oligomers are generated through RCR that uses a single molecule as a template. RCR can generate long concatemeric linear molecules that consist of the same type of monomeric units. Thus, RCR products are obligatory homo-oligomers, and hetero-oligomers cannot be obtained in this manner (Fig. 8A). The second possible mechanism, RDR, takes place between two separate molecules (Fig. 8B). The head-to-tail oligomeric molecules generated through this process therefore might be not only homo-oligomers but also hetero-oligomers.

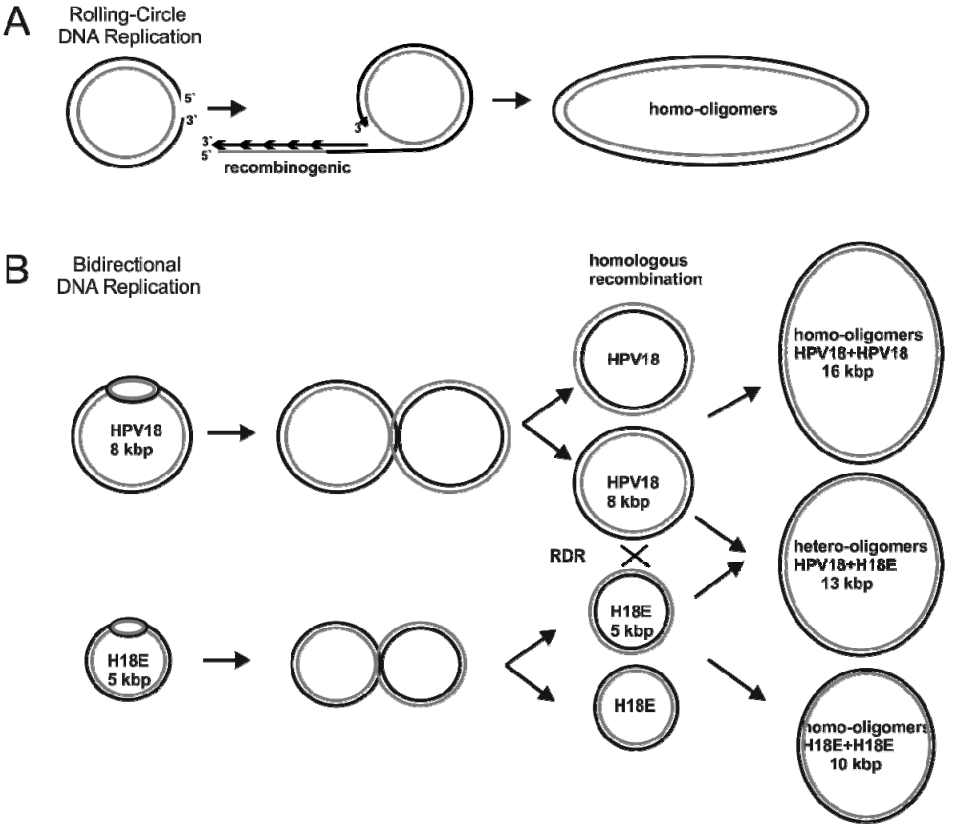


Figure 8. A schematic representation of the formation of homo- and hetero-oligomers. The formation of homo-oligomers can be obtained from rolling-circle replication (A) and the bidirectional *theta*-replication (B); hetero-oligomeric molecules can be only a product of homologous recombination. RDR refers to recombination dependent replication.

Cotransfection of U2OS cells with two different replicating HPV plasmids (8-kbp wtHPV18 genomic and 5.4-kbp HPV18E subgenomic construct) allowed us to demonstrate that during HPV DNA replication, hetero-oligomers can be generated (Ref. **IV**, Fig. 5B, lines 5–8; Fig. 8). Hetero-oligomers can also be obtained through the cotransfection of an HPV18 genome with a URR-containing plasmid (Ref. **IV**, Fig. 4E).

Homologous RDR is the only replication mode that can create oligomers between different homologous but not identical HPV molecules. Based on these results, we suggest that observed DNA oligomers may be produced through this process.

5.4. Identification of replication intermediates arising during the initial HPV genome amplification (Ref. I and V)

5.4.1. Topology of HPV genomes in U2OS cells

To examine the topological forms of HPV DNA replication intermediates (RI) in U2OS cells more thoroughly, we used neutral-neutral two-dimensional agarose gel electrophoresis (AGE). Analysis of the uncut forms of DNA RIs using 2D AGE facilitates the separation of linear, open circular (OC), and covalently closed circular (CCC) molecules (Ref. **I**, Fig. 5F-H). These figures represent the RI patterns of undigested HPV18 18#1.13 DNA both from the stable assay (Ref. **I**, Fig. 5F) and from vegetative amplification (Ref. **I**, Fig. 5H). As expected, we detected signals that are interpretable not only as the oligomeric forms of the genome but also as signals from the monomeric episomes in both the CCC and OC forms.

The development of a U2OS-based transient replication assay has enabled us to study early HPV DNA replication events in a time-dependent manner. To trace the appearance of oligomeric forms of HPV18 genomes over time, U2OS cells were transfected with HPV18wt minicircles. The results of 2D AGE (Ref. **V**, Fig. 1A) confirmed the data obtained through 1D analysis indicating that oligomers become more prevalent over time (Ref. **I**, Fig. 5A).

Recently, it has been shown that the HPV18E8^{-} mutant does not produce the E8^E2 repressor protein, resulting in a much stronger replication signal at earlier time points (Kurg et al., 2010, Reinson et al., 2013). Nevertheless, we could not detect qualitative differences in replication patterns between HPV18wt and HPV18E8^{-} genomes by 1D and 2D analysis (Ref. **V**, Fig. 1A and C). In addition to the signals from linear, OC and CCC molecules detected for uncut samples from HPV18wt transfected cells, signals characteristic of bidirectional *theta*-replication intermediates are clearly visible for similarly treated samples from HPV18E8^{-} genome transfected cells (Ref. **V**, Fig. 1C, marked with a bold arrows).

The works of Martín-Parras, Belanger, Cohen and others demonstrate that RCR intermediates form a so-called eyebrow-shaped signal during 2D N/N gel

electrophoresis (Cohen et al., 2005, Martín-Parras et al., 1998, Belanger et al., 1996). However, we cannot detect that type of classical long RCR signal in our experiments (Ref. **V**, Fig. 1C). Instead, some related shorter RIs could be seen as horizontal lines connecting several structures; although their appearance becomes more intensive in a time-dependent manner and seen even in the wtHPV18 case, the exact nature of these molecules is unclear.

5.4.2. Two different populations of molecules arise during the HPV genome transient replication

As demonstrated in chapter 5.1, all three phases of HPV DNA replication can be studied using the U2OS model system. Transient HPV DNA replication closely mimics that occurring in the early phase of the HPV infection cycle, thus representing the initial amplification phase of viral DNA replication. The episomal DNA extracted from HPV18E8^{-}-transfected U2OS cells three days post-transfection (Ref. **V**, Fig. 3A) and five days post-transfection (data not shown) was linearized with either *BglI, Bpu1102I, XmaJI* or *PsyI* and analyzed via 2D N/N AGE (Materials and Methods, Fig. 6, native conditions). We chose restriction endonucleases that cleave the plasmid only once at positions distributed throughout the HPV genome (Ref. **V**, Fig. 3C).

Digestion of circular HPV molecules with *BglI* or *Bpu1102I* (corresponding recognition sites are located close to HPV origin) produces a diagonal arc initiating near the 1N spot of unreplicated or replicated HPV18 DNA. This diagonal line represents RIs, with two opposite-direction converging replication forks (RFs) (usually toward the termination site located approximately 180° from ori) arranged such that cleavage with these enzymes opens the bubble (Ref. **V**, Fig. 3A, *BglI* and *Bpu1102I,* marked with a black arrow). In the analysis of the results of restrictions on *XmaJI* and *PsyI*, enzymes that cut opposite the origin, the appearance of specific arcs indicates the presence of "bubble" intermediates in these fragments (Ref. **V**, Fig. 3A, *XmaJI* and *PsyI,* marked with a black arrow).

There was also an accumulation of molecules with high molecular mass and branched topology in every case (Ref. **V**, Fig. 3A, marked with a black arrowhead). Because these molecules migrate at the end of the signal representing the dY intermediates, they may represent nearly fully replicated, late *theta* intermediates; however, the exact nature of these linked molecules remains unclear.

Despite the use of different restriction enzymes, one pattern, in addition to that of the one interpreted as corresponding to *theta* RIs, did not change (Ref. **V**, Fig. 2A and 3A, marked with a white arrows) and became more intense over time (Ref. **V**, Fig. 2A, marked with a white arrows). These novel signals represent molecules that could not arise during the bidirectional *theta*-replication of monomeric HPV genomes.

The analysis of the uncut samples isolated from U2OS cells transfected with HPV18E8^{-} minicircles indicated that bidirectional *theta*-replication might

also be initiated from dimeric HPV genomes (Ref. **V**, Fig. 1C, marked with a white arrow). The bidirectional *theta*-replication of dimeric HPV genomes with only one active origin of replication was expected, after *BglI* digestion, to result in simple Y-shape RIs (in principle, similar to Ref. **V**, Fig. 2B, simple Y intermediates). However, the obtained migration pattern of these novel molecules did not resemble the migration pattern characteristic of simple Y molecules (Ref. **V**, Fig. 2A to 2B). The migration pattern of these novel molecules with regard to the migration pattern of *theta* RIs did not change upon alteration of the conditions used for the 2D N/N AGE (Ref. **V**, Supplementary Fig. 1S). Because the novel RI does not have common features and characteristics with the intermediates of bidirectional replication, we suggest that a second mechanism is involved in the replication of the HPV18 genome.

In our 1D study, we demonstrated that the formation of oligomers of HPV genomes increases over time (Ref. **I**, Fig 5A, Ref. IV, Fig 1C). To prove that the new, supposedly second mode of HPV DNA replication is associated with the formation of oligomers, we purified and linearized HPV DNA from U2OS cells at late time points 144, 168, 192, 216 and 240 hours (data not shown). It was observed that the pattern of *theta*-replication became less prominent or completely disappeared at later time points and novel or secondary RI became much more prominent. This indicates that the switch into the next mode of HPV DNA replication could be taken place at later time points in the transient replication assay. A similar switch has been described by Flores and Lambert for differentiation-dependent HPV16 and HPV31 genome replication (Flores and Lambert, 1997), but they interpret their data as *theta*-replication being replaced by RCR. This may be because those authors only analyzed the RI from small fragments of the genome; thus, their results may have been misleading. In contrast, because we conducted 2D AGE with the whole genome, we did clearly observe that our second replication intermediates could not originate from RCR (Ref. **V**, Fig. 3A).

To exclude the possibility that the second mechanism of replication of the HPV18 genome could occur only in U2OS cells, we repeated experiments in HaCaT cells (spontaneously immortalized human keratinocyte cell line) that have been widely used for studies of skin biology and differentiation. HaCaT is an HPV-negative cell line that can support the replication of the HPV18E8^{-} genome, albeit with much lower efficiency than U2OS cells (Ref. **V**, Fig. 7). Nevertheless, the analysis of RIs in HaCaT cells revealed the presence both of dY molecules representing the intermediates of bidirectional *theta*-replication (Ref. **V**, Fig. 7, marked with a black arrow) and of the intermediates of the second replication mechanism (Ref. **V**, Fig. 7, marked with white arrows). These results indicate that analogous replication intermediates are not specific to U2OS cells.

We also conducted a restriction analysis of viral DNA isolated from HPV-positive subclones of U2OS cells at the amplification stage. HPV18 subclone 18#1.13 was seeded on plates at fixed subconfluent densities and grown for 12 days without splitting while media were exchanged every two days. Next, the

DNA was extracted and linearized with numerous restriction endonucleases that cleave the HPV genome only once at positions distributed throughout the HPV sequence, and the RIs were analyzed on 2D gels (data not shown). As a result, we obtained RI patterns similar to those seen during the initial amplification of the HPV genome. Although the *theta*-replication pattern could not be seen, the second replication mechanism was clearly dominant, indicating that at the later time points of HPV DNA replication, the second mechanism, most likely homologous RDR, is more prevalent.

5.4.3. Analysis of nascent strands during the initial amplification of HPV genomes

In addition to bidirectional *theta*-replication intermediates, we can see signals of unknown origin on images obtained from 2D analysis. These new RIs retained the same migration pattern in all experiments regardless of which type of restriction enzyme was used (Ref. **V**, Fig. 3A, marked with a white arrows). To better understand the nature of these replication signals, we used the 3D N/N/A AGE method (Ref. **V**, Fig. 3B). Using this method, it is possible to separate nascent and parental strands after the second dimension (Fig. 6; Ref. **V**, Fig. 3D). As a result of this experiment, we found that in the secondary replication mechanism, the chains were separated, confirming that the previously observed molecules are indeed intermediates of DNA replication. The nascent strands of the second RIs form a single arc containing molecules that are continuously increasing in length (up to the length of the parental strands). According to the schematic image (Ref. **V**, Fig. 3D), the migration pattern of nascent strands originating from this second replication somewhat resembles the migration pattern of simple Y molecules. However, as we see from Ref. **V**, Fig. 3A (marked with a white arrows), the arcs of these molecules from the second replication do not begin at the 1N molecule, thus rendering uncertain the conclusion that the molecules are Y-shaped. In other words, the secondary replication arcs move somehow unexpectedly.

To ensure a better understanding of the mechanisms of HPV DNA replication, we used the neutral/alkaline (N/A) 2-dimensional method. In this assay, the alkaline second dimension allows separation of newly synthesized leading-strand DNA from the parental strand.

For this analysis, we first digested isolated LMW DNA with *XmaJI* or *BglI*. The restriction fragments obtained using *XmaJI* contain an origin of replication approximately in the middle (bubble RI). Alternatively, in the case of digestion with *BglI*, *theta* RIs appeared in the form of dY intermediates (Ref. **V**, Fig. 4D). Nascent-strand-migration models originating from different RI types during the 2D N/A gel electrophoresis are depicted in Ref. **V**, Fig. 4A (Nawotka and Huberman, 1988). To facilitate the interpretation of data obtained by N/A analysis, the second dimension of N/A AGE was performed in parallel with the second dimension in N/N neutral conditions. The results were aligned based on the migration progressions in the 1D analysis (Ref. **V**, Fig. 4B and C).

As we can clearly see, the N/A analysis of samples isolated from U2OS cells that were transfected with HPV18E8^{-} minicircle DNA digested with linearizing restriction enzymes revealed the presence of bidirectional *theta*-replication intermediates (Ref. **V**, Fig. 4B and C, marked with a black arrows). In the case of cleavage with *BglI*, *theta*-replication resulted in dY or converging fork intermediates and *XmaJI* digestion resulted in bubble intermediates. These data are coherent both with the results obtained using N/N-analysis and with the scheme depicting the migration patterns formed by nascent strands originating from *theta*-replication during 2D N/A gel electrophoresis (Ref. **V**, Fig. 4A).

Simultaneously, another arc of the nascent strands is present (Ref. **V**, Fig. 4B and C, marked with a white arrows, N/A). This arc is a continuous Y or asymmetric dY-shape (Ref. **V**, Fig 4A) that eventually reaches the size of the parental strands, suggesting unidirectional replication.

However, the intermediates of the novel HPV-replication mechanism yielded a signal that was initially (closer to 1N) similar to the arc of nascent strands originating from the replication-bubble intermediates (Ref. **V**, Fig. 4A). As an additional confirmation, during the analysis of the *XmaJI* digestion, the signals representing nascent strands originating from bidirectional *theta*-replication and the second replication mechanism partially overlapped, which is a strong indication of shared characteristics (Ref. **V**, Fig. 4C, N/A, marked by black and white arrows).

The fact that RIs do not originate from the 1N point might indicate the presence of a diffuse initial signal consisting of simple Ys, dYs and bubbles; however, when the replication intermediates increase in size, they more closely resemble the Y structures. The secondary replication mechanism might also be a mechanism of recombination initiated by the invasion of a resectioned ssDNA end that originates from dsDNA breaks in the HPV18 genome into an intact homologous HPV molecule. The invasion of the resectioned ssDNA end results in the creation of D-loop structures and eventually the assembly of a unidirectional replication fork. During bidirectional *theta*-replication, nicks can occur either in parental strands or in collapsed replication forks, which may disrupt bidirectional replication, thus enabling the switch to the unidirectional mode of replication. Unidirectional replication could then occur in either direction.

5.4.4. The novel replication mechanism can initiate unidirectional replication forks in both directions

To determine the polarity of replication forks initiated by different replication mechanisms, we resorted to *in gelo* digestion analysis. Using this type of analysis, it is possible to determine whether the replication forks are moving via *theta*-replication or other mechanisms.

At the beginning of bidirectional *theta*-replication, two replicative forks are formed. These forks begin to move in opposite directions from the origin until they meet in the region of termination. As shown in Ref. **V**, Fig. 6D, the repli-

cation forks of the HPV18E8⁻ genome are moving from the *BglI* restriction site to the termination area located between the *PsyI* and *XmaJI* restriction sites. The migration structures of these replication forks, following *in gelo* digestion, are demonstrated in Ref. **V**, Fig. 6C (Brewer et al., 1992, Gerhold et al., 2014, Ivessa, 2013). As is clearly seen from the *in gelo* analysis (Ref. **V**, Fig. 6A and B), in the *XmaJI-BglI* and *BglI-PsyI* fragments, the replication forks emerge bidirectionally (Ref. **V**, Fig. 6A and B, marked by black and red arrows). Replication forks moving in the opposite direction also appear in both fragments (Ref. **V**, Fig. 6A and B, marked by a white arrow).

We cannot exclude the possibility that these signals result from the bidirectional *theta*-replication of dimeric HPV18 molecules with only one active replication origin, as shown in diagram Ref. **V**, Fig. 6E. However, in the case of dimeric molecules replicating via *theta*-replication, the ratio of replication forks of the *XmaJI-BglI* and *BglI-PsyI* fragments in opposite directions would be equal. Our results indicated otherwise, although we did not quantify the signals (quantification is difficult for 2D gels and various hybridization probes were used to analyze the *BglI-PsyI* and *BglI-XmaJI* fragments). In the *BglI-PsyI* fragment, the replication fork arising through bidirectional *theta*-replication is much more prevalent than the fork traveling in the opposite direction. In the *BglI-XmaJI* fragment, the replication forks with opposing polarity appear to be of equal prevalence. These results indicate that although secondary unidirectional replication can be initiated in either direction, replication forks traveling clockwise seem to be preferred, at least at this time point.

5.4.5. 2D neutral/neutral agarose gel electrophoresis analysis of subgenomic HPV18E8⁻ fragments

We also conducted a distinct restriction analysis of samples isolated from U2OS cells transfected with HPV18E8⁻ minicircle using combinations of two linearizing enzymes (*Cfr10I* and *Bpu1102I*, *Bpu1102*I and *PsyI, PsyI* and *XmaJI, XmaJI* and *Cfr10I*). In this way, the HPV18E8⁻ genome could be divided into two fragments of approximately 2 kbp and 6 kbp across the genome. These fragments were analyzed by 2D N/N gel electrophoresis, and four hybridization probes that recognized different genome regions were used (Ref. **V**, Fig. 5).

As expected, we saw evidence of *theta*-replication intermediates in all of the smaller ca. 2 kbp genome fragments. All known interpretable structures were present: bubble, Y, dY, some of X arcs or termination arcs (Ref. **V**, Fig. 5A and B, marked with black arrows).

All of the large, ca. 6 kbp fragments yielded similar secondary replication intermediate patterns, which did not originate from 1N. The large branched molecules at the highest tip of *theta* signals, which likely represent late *theta* intermediates (incomplete, linked sister molecules), accumulated over time.

Surprisingly, physical linkage of the joined molecules was detected along the entire length of the genome by the 2D method. Joined molecules seem to be

formed during DNA replication, and newly replicated sister molecules most likely remain linked. The existence of homologous-recombination-linked pathways for the replication HPV genomes and the formation of joined molecules can be resolved by analyzing the intermediates *via* atomic force microscopy (AFM) and electron microscopy (EM).

Our study demonstrates that a novel replication mechanism is involved in HPV replication. However, we have not been able to visualize the replication intermediates of these replication modes using EM or AFM; thus, we can only speculate about the nature of these undefined replication intermediates. Therefore, we cannot distinguish *theta*-replication from the second mechanism of replication to accurately characterize the later process. It is clear both that initial papillomavirus DNA replication starts with *theta*-replication and that *theta*-replication is dependent on E1 and E2. We do not know whether the novel replication mechanism is dependent on these proteins. However, during initial virus DNA replication, not only monomeric but also oligomeric genomes are generated, and homologous recombination is clearly involved.

6. CONCLUSIONS

1. Plasmids with hybrid origins, composed of a BPV1 E2 protein-dependent MME and mouse polyomavirus minimal origin were constructed. The replication enhancer and the stable maintenance functions were observed in the presence of the mouse polyomavirus large-T-antigen and the BPV1 E2 protein. The same functions can be provided by Epstein-Barr virus EBNA1 protein when Family of Repeats (FR) was used instead of MME of BPV1. In the mouse fibroblast cell line COP5E2/Puro expressing polyomavirus large-T-antigen and BPV1 E2 protein, the reporter-protein encoding plasmids harboring hybrid origins were stably replicating and maintained for a long time; the episomal status of hybrid origin containing DNAs was monitored for more than 3 months. The described chimeric system makes it possible to express a gene of interest for an extended time period in these cells.
2. We have demonstrated that HPV genomes replicate efficiently in the human osteosarcoma cell line U2OS, which is capable of supporting the genome replication of many different subtypes (α-HPVs of high-risk HPV18 and HPV16; low-risk HPV6b and HPV11 and β-HPVs of HPV5 and HPV8). U2OS cells capable of supporting the maintenance of the HPV genomes provide a useful model system in which the mechanism and regulation of viral DNA replication during various phases of the viral life cycle, including initial amplification (transient replication), stable maintenance and second amplification (vegetative replication), can be analyzed.
3. We show the increasing prevalence of oligomeric HPV genomes in U2OS cells transfected with HPV DNA during the initial establishment stages, in stable maintenance and in the vegetative phase. We characterized these oligomers as episomal head-to-tail concatemeric molecules. HPV DNA obtained from patient samples has also been found to be oligomeric. We can conclude that HPV genome oligomerization is common for HPV infections *in vivo*. Homologous recombination-dependent replication might be involved in this process.
4. Analysis of the replication intermediates arising during HPV18 genome replication showed that two types of replication intermediates arise during the transient amplification replication of HPV genomes. The intermediates are characteristic both of *theta*-replication and of a yet-unknown secondary replication mechanism. We propose that the second replication mechanism might involve homologous recombination.

SUMMARY IN ESTONIAN

Papilloomiviiruse genoomi replikatsiooni ja säilumise uurimine

2008. aastal jagati meditsiini Nobeli preemia kahele suuri avastusi teinud teadlasele, kellest üks oli Harald zur Hausen. Saksa viroloog zur Hausen tõestas, et inimese papilloomiviirused (HPVd) ei põhjusta ainult kahjutuid soolatüükaid, vaid nende nakkus inimesel võib viia emakakaelavähi tekkeni. Emakakaelavähk on naiste hulgas maailmas sageduselt teine onkoloogiline haigus.

Papilloomiviirused on ümbriseta ja kaheahelalise DNA genoomiga viirused, mis on rangelt liigispetsiifilised. Siiski on inimest võimelised nakatama enam kui sada erinevat HPV tüüpi. Neist omakorda 18 tüüpi peetakse kõrge riskiga (kasvajaid tekitavateks) viirusteks. Kõige levinumad kõrge riskiga tüübid on HPV16 ja HPV18, mille vältimiseks on arendatud ka vaktsiinid. HPV vastane vaktsineerimine vähendab haigestumise tõenäosust vähemalt 70% võrra, kuid täielikku kaitset see paraku pakkuda ei suuda. Lisaks on olemasolevad vaktsiinid mõeldud ainult haiguste ennetamiseks ja on seega kasutud viirusega juba nakatunud inimestele. Paraku ei ole veel välja töötatud ravimeid, mis oleksid võimelised papilloomiviiruse infektsiooni inimese organismist kõrvaldama.

HPV on kõrge koespetsiifilisusega viirus ja nakatab epiteeli peamist rakutüüpi – keratinotsüüte. Nakatamiseks peab viirus sisenema naha epiteeli basaalkihi rakkudesse või genitaalide ja teiste organite limaskestade vastavatesse rakkudesse. Kuna HPV elustükkel sõltub täielikult keratinotsüütide diferentseerumisest, raskendab selle viiruse uurimist asjaolu, et uurimistöös kasutatavates rakulistes mudelsüsteemides on keratinotsüütide diferentseerumise käivitamine väga keerukas.

Antud töös kirjeldatakse inimese sääreluu kasvajast algatatud U2OS rakuliinil põhinevat rakulist mudelsüsteemi, mida saab kasutada HPV genoomide replikatsiooni uurimiseks. Loodud mudelsüsteem on võimeline toetama erinevat tüüpi HPVde DNA replikatsiooni nii nagu see toimub viiruse loomuliku elutsükli eri etappidel. Samuti võimaldab U2OS rakuliinil põhinev mudelsüsteem võrdlevalt uurida nii limaskesta sptesiifilisi kõrge riski (HPV16, HPV18), kui ka madala riski (HPV6b, HPV11) ning naha papilloomiviiruseid (HPV5, HPV8), mille uurimine teistes rakulistes mudelsüsteemides on seni olnud üsna keerukas.

Uudse mudelsüsteemi abil oleme suutnud iseloomustada HPV DNA molekulide erinevaid vorme, mis moodustuvad replikatsioonistükli eri etappidel. Viiruse genoomidega transfekteeritud U2OS rakuliinist ja patsientide koeproovidest pärineva viiruse DNA uurimisel selgus, et HPV genoom on võimeline moodustama oligomeere – DNA molekule, mis sisaldavad mitut viiruse genoomi koopiat. Sellised multimeersed molekulid on tuvastatavad igas viiruse elutsükli etapis.

Samuti näitasime, et HPV DNA replikatsiooni on kaasatud vähemalt kaks erinevat mehhanismi. Ühte neist on varasemalt juba kirjeldatud kui võimalikku papilloomiviiruste päriliku materjali paljundamise meetod – selleks on kahe-

suunaline *theta*-replikatsioon. U2OS rakuliinil põhineva mudelsüsteemi abil tuvastasime aga veel teise, ühesuunalise replikatsioonimehhanismi, mis oletatavalt põhineb homoloogse rekombinatsiooni kasutamisel ja mille roll HPV elutsüklis on veel ebaselge. Selleks, et täpsemalt mõista uudse kirjeldatud mehhanismi iseärasusi, plaanime tulevikus kasutada elektron- või aatomjõumikroskoopiat.

Lisaks ülaltoodule näidati käesolevas töös, et kõikide vajalike viiruseliste *trans*-faktorite juuresolekul on heteroloogsed originid, mis sisaldavad veise papilloomiviirusest (BPV1) või ka Epstein-Barr viirusest (EBV) pärinevaid segregatsioonielemente, võimelised tagama neid sisaldavate plasmiidide stabiilse säilumise.

REFERENCES

Abroi, A., Ilves, I., Kivi, S. and Ustav, M. (2004) 'Analysis of chromatin attachment and partitioning functions of bovine papillomavirus type 1 E2 protein', *J Virol,* 78(4), pp. 2100–13.

Adachi, A., Kiyono, T., Hayashi, Y., Ohashi, M. and Ishibashi, M. (1996) 'Detection of human papillomavirus (HPV) type 47 DNA in malignant lesions from epidermodysplasia verruciformis by protocols for precise typing of related HPV DNAs', *J Clin Microbiol,* 34(2), pp. 369–75.

Adams, A. K., Wise-Draper, T. M. and Wells, S. I. (2014) 'Human papillomavirus induced transformation in cervical and head and neck cancers', *Cancers (Basel),* 6(3), pp. 1793–820.

Adler, K., Erickson, T. and Bobrow, M. (1997) 'High sensitivity detection of HPV-16 in SiHa and CaSki cells utilizing FISH enhanced by TSA', *Histochem Cell Biol,* 108(4–5), pp. 321–4.

Akgül, B., Cürten, M., Haigis, H., Rogosz, I. and Pfister, H. (2006) 'Interferon regulatory factor 5.2 acts as a transcription repressor of Epidermodysplasia verruciformis-associated human papillomaviruses', *Arch Virol,* 151(12), pp. 2461–73.

Akgül, B., Lemme, W., García-Escudero, R., Storey, A. and Pfister, H. J. (2005) 'UV-B irradiation stimulates the promoter activity of the high-risk, cutaneous human papillomavirus 5 and 8 in primary keratinocytes', *Arch Virol,* 150(1), pp. 145–51.

Al Moustafa, A. E., Al-Awadhi, R., Missaoui, N., Adam, I., Durusoy, R., Ghabreau, L., Akil, N., Ahmed, H. G., Yasmeen, A. and Alsbeih, G. (2014) 'Human papillomaviruses-related cancers. Presence and prevention strategies in the Middle east and north African regions', *Hum Vaccin Immunother,* 10(7), pp. 1812–21.

Alazawi, W., Pett, M., Arch, B., Scott, L., Freeman, T., Stanley, M. A. and Coleman, N. (2002) 'Changes in cervical keratinocyte gene expression associated with integration of human papillomavirus 16', *Cancer Research,* 62(23), pp. 6959–6965.

Allen-Hoffmann, B. L., Schlosser, S. J., Ivarie, C. A., Sattler, C. A., Meisner, L. F. and O'Connor, S. L. (2000) 'Normal growth and differentiation in a spontaneously immortalized near-diploid human keratinocyte cell line, NIKS', *J Invest Dermatol,* 114(3), pp. 444–55.

Amtmann, E., Volm, M. and Wayss, K. (1984) 'Tumour induction in the rodent Mastomys natalensis by activation of endogenous papilloma virus genomes', *Nature,* 308(5956), pp. 291–2.

Anacker, D. and Moody, C. (2012) 'Generation of organotypic raft cultures from primary human keratinocytes', *J Vis Exp,* (60).

Antonsson, A., Erfurt, C., Hazard, K., Holmgren, V., Simon, M., Kataoka, A., Hossain, S., Håkangård, C. and Hansson, B. G. (2003) 'Prevalence and type spectrum of human papillomaviruses in healthy skin samples collected in three continents', *J Gen Virol,* 84(Pt 7), pp. 1881–6.

Antonsson, A., Forslund, O., Ekberg, H., Sterner, G. and Hansson, B. G. (2000) 'The ubiquity and impressive genomic diversity of human skin papillomaviruses suggest a commensalic nature of these viruses', *J Virol,* 74(24), pp. 11636–41.

Arbeit, J. M., Münger, K., Howley, P. M. and Hanahan, D. (1993) 'Neuroepithelial carcinomas in mice transgenic with human papillomavirus type 16 E6/E7 ORFs', *Am J Pathol,* 142(4), pp. 1187–97.

Archambault, J. and Melendy, T. (2013) 'Targeting human papillomavirus genome replication for antiviral drug discovery', *Antivir Ther,* 18(3), pp. 271–83.

Asai, T., Bates, D. B. and Kogoma, T. (1994) 'DNA replication triggered by double-stranded breaks in E. coli: dependence on homologous recombination functions', *Cell,* 78(6), pp. 1051–61.

Asselineau, D. and Prunieras, M. (1984) 'Reconstruction of 'simplified' skin: control of fabrication', *Br J Dermatol,* 111 Suppl 27, pp. 219–22.

Auborn, K. J., Little, R. D., Platt, T. H., Vaccariello, M. A. and Schildkraut, C. L. (1994) 'Replicative intermediates of human papillomavirus type 11 in laryngeal papillomas: site of replication initiation and direction of replication', *Proc Natl Acad Sci U S A,* 91(15), pp. 7340–4.

Auborn, K. J., Woodworth, C., DiPaolo, J. A. and Bradlow, H. L. (1991) 'The interaction between HPV infection and estrogen metabolism in cervical carcinogenesis', *Int J Cancer,* 49(6), pp. 867–9.

Baker, A., Rohleder, K. J., Hanakahi, L. A. and Ketner, G. (2007) 'Adenovirus E4 34k and E1b 55k oncoproteins target host DNA ligase IV for proteasomal degradation', *J Virol,* 81(13), pp. 7034–40.

Bastien, N. and McBride, A. A. (2000) 'Interaction of the papillomavirus E2 protein with mitotic chromosomes', *Virology,* 270(1), pp. 124–34.

Bechtold, V., Beard, P. and Raj, K. (2003) 'Human papillomavirus type 16 E2 protein has no effect on transcription from episomal viral DNA', *J Virol,* 77(3), pp. 2021–8.

Bedell, M. A., Hudson, J. B., Golub, T. R., Turyk, M. E., Hosken, M., Wilbanks, G. D. and Laimins, L. A. (1991) 'Amplification of human papillomavirus genomes in vitro is dependent on epithelial differentiation', *J Virol,* 65(5), pp. 2254–60.

Belanger, K. G., Mirzayan, C., Kreuzer, H. E., Alberts, B. M. and Kreuzer, K. N. (1996) 'Two-dimensional gel analysis of rolling circle replication in the presence and absence of bacteriophage T4 primase', *Nucleic Acids Res,* 24(11), pp. 2166–75.

Bencokova, Z., Kaufmann, M. R., Pires, I. M., Lecane, P. S., Giaccia, A. J. and Hammond, E. M. (2009) 'ATM activation and signaling under hypoxic conditions', *Mol Cell Biol,* 29(2), pp. 526–37.

Berg, M. and Stenlund, A. (1997) 'Functional interactions between papillomavirus E1 and E2 proteins', *J Virol,* 71(5), pp. 3853–63.

Berghard, A., Gradin, K. and Toftgård, R. (1990) 'Serum and extracellular calcium modulate induction of cytochrome P-450IA1 in human keratinocytes', *J Biol Chem,* 265(34), pp. 21086–90.

Bernard, H. U. and Apt, D. (1994) 'Transcriptional control and cell type specificity of HPV gene expression', *Arch Dermatol,* 130(2), pp. 210–5.

Bernard, H. U., Burk, R. D., Chen, Z., van Doorslaer, K., zur Hausen, H. and de Villiers, E. M. (2010) 'Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments', *Virology,* 401(1), pp. 70–9.

Betiol, J., Villa, L. L. and Sichero, L. (2013) 'Impact of HPV infection on the development of head and neck cancer', *Braz J Med Biol Res,* 46(3), pp. 217–26.

Blackford, A. N., Bruton, R. K., Dirlik, O., Stewart, G. S., Taylor, A. M., Dobner, T., Grand, R. J. and Turnell, A. S. (2008) 'A role for E1B-AP5 in ATR signaling pathways during adenovirus infection', *J Virol,* 82(15), pp. 7640–52.

Blanton, R. A., Perez-Reyes, N., Merrick, D. T. and McDougall, J. K. (1991) 'Epithelial cells immortalized by human papillomaviruses have premalignant characteristics in organotypic culture', *Am J Pathol,* 138(3), pp. 673–85.

Bousarghin, L., Touzé, A., Sizaret, P. Y. and Coursaget, P. (2003) 'Human papillomavirus types 16, 31, and 58 use different endocytosis pathways to enter cells', *J Virol,* 77(6), pp. 3846–50.

Boxman, I. L., Berkhout, R. J., Mulder, L. H., Wolkers, M. C., Bouwes Bavinck, J. N., Vermeer, B. J. and ter Schegget, J. (1997) 'Detection of human papillomavirus DNA in plucked hairs from renal transplant recipients and healthy volunteers', *J Invest Dermatol,* 108(5), pp. 712–5.

Boyce, S. T. and Ham, R. G. (1983) 'Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture', *J Invest Dermatol,* 81(1 Suppl), pp. 33s–40s.

Brandsma, J. L. (1994) 'Animal models of human-papillomavirus-associated oncogenesis', *Intervirology,* 37(3–4), pp. 189–200.

Brandsma, J. L., Yang, Z. H., Barthold, S. W. and Johnson, E. A. (1991) 'Use of a rapid, efficient inoculation method to induce papillomas by cottontail rabbit papillomavirus DNA shows that the E7 gene is required', *Proc Natl Acad Sci U S A,* 88(11), pp. 4816–20.

Bravo, I. G., de Sanjosé, S. and Gottschling, M. (2010) 'The clinical importance of understanding the evolution of papillomaviruses', *Trends Microbiol,* 18(10), pp. 432–8.

Bravo, I. G. and Félez-Sánchez, M. (2015) 'Papillomaviruses: Viral evolution, cancer and evolutionary medicine', *Evol Med Public Health,* 2015(1), pp. 32–51.

Brewer, B. J., Lockshon, D. and Fangman, W. L. (1992) 'The arrest of replication forks in the rDNA of yeast occurs independently of transcription', *Cell,* 71(2), pp. 267–76.

Buck, C. B., Cheng, N., Thompson, C. D., Lowy, D. R., Steven, A. C., Schiller, J. T. and Trus, B. L. (2008) 'Arrangement of L2 within the papillomavirus capsid', *J Virol,* 82(11), pp. 5190–7.

Burma, S. and Chen, D. J. (2004) 'Role of DNA-PK in the cellular response to DNA double-strand breaks', *DNA Repair (Amst),* 3(8–9), pp. 909–18.

Burnett, S., Zabielski, J., Moreno-Lopez, J. and Pettersson, U. (1989) 'Evidence for multiple vegetative DNA replication origins and alternative replication mechanisms of bovine papillomavirus type 1', *J Mol Biol,* 206(1), pp. 239–44.

Bzhalava, D., Eklund, C. and Dillner, J. (2015) 'International standardization and classification of human papillomavirus types', *Virology,* 476, pp. 341–4.

CAIRNS, J. (1963) 'The bacterial chromosome and its manner of replication as seen by autoradiography', *J Mol Biol,* 6, pp. 208–13.

Caldeira, S., Zehbe, I., Accardi, R., Malanchi, I., Dong, W., Giarrè, M., de Villiers, E. M., Filotico, R., Boukamp, P. and Tommasino, M. (2003) 'The E6 and E7 proteins of the cutaneous human papillomavirus type 38 display transforming properties', *J Virol,* 77(3), pp. 2195–206.

Campo, M. S. and Roden, R. B. (2010) 'Papillomavirus prophylactic vaccines: established successes, new approaches', *J Virol,* 84(3), pp. 1214–20.

Cheng, C. Y., Gilson, T., Dallaire, F., Ketner, G., Branton, P. E. and Blanchette, P. (2011) 'The E4orf6/E1B55K E3 ubiquitin ligase complexes of human adenoviruses exhibit heterogeneity in composition and substrate specificity', *J Virol,* 85(2), pp. 765–75.

Cheng, S., Schmidt-Grimminger, D. C., Murant, T., Broker, T. R. and Chow, L. T. (1995) 'Differentiation-dependent up-regulation of the human papillomavirus E7

gene reactivates cellular DNA replication in suprabasal differentiated keratinocytes', *Genes Dev,* 9(19), pp. 2335–49.
Chiang, C. M., Ustav, M., Stenlund, A., Ho, T. F., Broker, T. R. and Chow, L. T. (1992) 'Viral E1 and E2 proteins support replication of homologous and heterologous papillomaviral origins', *Proc Natl Acad Sci U S A,* 89(13), pp. 5799–803.
Chow, L. T. (2015) 'Model systems to study the life cycle of human papillomaviruses and HPV-associated cancers', *Virol Sin,* 30(2), pp. 92–100.
Chow, L. T. and Broker, T. R. (2013) 'Human papillomavirus infections: warts or cancer?', *Cold Spring Harb Perspect Biol,* 5(7).
Chow, L. T., Nasseri, M., Wolinsky, S. M. and Broker, T. R. (1987) 'Human papillomavirus types 6 and 11 mRNAs from genital condylomata acuminata', *J Virol,* 61(8), pp. 2581–8.
Chung, S. H. and Lambert, P. F. (2009) 'Prevention and treatment of cervical cancer in mice using estrogen receptor antagonists', *Proc Natl Acad Sci U S A,* 106(46), pp. 19467–72.
Chung, S. H., Wiedmeyer, K., Shai, A., Korach, K. S. and Lambert, P. F. (2008) 'Requirement for estrogen receptor alpha in a mouse model for human papillomavirus-associated cervical cancer', *Cancer Res,* 68(23), pp. 9928–34.
Cimprich, K. A. and Cortez, D. (2008) 'ATR: an essential regulator of genome integrity', *Nat Rev Mol Cell Biol,* 9(8), pp. 616–27.
Clarke, R. (1996) 'Human breast cancer cell line xenografts as models of breast cancer. The immunobiologies of recipient mice and the characteristics of several tumorigenic cell lines', *Breast Cancer Res Treat,* 39(1), pp. 69–86.
Clifford, G. M., Smith, J. S., Plummer, M., Muñoz, N. and Franceschi, S. (2003) 'Human papillomavirus types in invasive cervical cancer worldwide: a meta–analysis', *Br J Cancer,* 88(1), pp. 63–73.
clinicaltrials.gov First received: July 21, 2009. Updated: December 29, 2015. A study of V503 in preadolescents and adolescents.
Cohen, S., Agmon, N., Yacobi, K., Mislovati, M. and Segal, D. (2005) 'Evidence for rolling circle replication of tandem genes in Drosophila', *Nucleic Acids Res,* 33(14), pp. 4519–26.
Conway, M. J. and Meyers, C. (2009) 'Replication and assembly of human papillomaviruses', *J Dent Res,* 88(4), pp. 307–17.
Cortez, D., Guntuku, S., Qin, J. and Elledge, S. J. (2001) 'ATR and ATRIP: partners in checkpoint signaling', *Science,* 294(5547), pp. 1713–6.
Cullen, A. P., Reid, R., Campion, M. and Lörincz, A. T. (1991) 'Analysis of the physical state of different human papillomavirus DNAs in intraepithelial and invasive cervical neoplasm', *J Virol,* 65(2), pp. 606–12.
Dall, K. L., Scarpini, C. G., Roberts, I., Winder, D. M., Stanley, M. A., Muralidhar, B., Herdman, M. T., Pett, M. R. and Coleman, N. (2008) 'Characterization of naturally occurring HPV16 integration sites isolated from cervical keratinocytes under noncompetitive conditions', *Cancer Res,* 68(20), pp. 8249–59.
Dasgupta, S., Zabielski, J., Simonsson, M. and Burnett, S. (1992) 'Rolling-circle replication of a high-copy BPV-1 plasmid', *J Mol Biol,* 228(1), pp. 1–6.
de Villiers, E. M., Fauquet, C., Broker, T. R., Bernard, H. U. and zur Hausen, H. (2004) 'Classification of papillomaviruses', *Virology,* 324(1), pp. 17–27.
DeFilippis, R. A., Goodwin, E. C., Wu, L. and DiMaio, D. (2003) 'Endogenous human papillomavirus E6 and E7 proteins differentially regulate proliferation, senescence, and apoptosis in HeLa cervical carcinoma cells', *J Virol,* 77(2), pp. 1551–63.

Del Bino, S., Vioux, C., Rossio-Pasquier, P., Jomard, A., Demarchez, M., Asselineau, D. and Bernerd, F. (2004) 'Ultraviolet B induces hyperproliferation and modification of epidermal differentiation in normal human skin grafted on to nude mice', *Br J Dermatol,* 150(4), pp. 658–67.

Del Vecchio, A. M., Romanczuk, H., Howley, P. M. and Baker, C. C. (1992) 'Transient replication of human papillomavirus DNAs', *J Virol,* 66(10), pp. 5949–58.

Dell'Oste, V., Azzimonti, B., De Andrea, M., Mondini, M., Zavattaro, E., Leigheb, G., Weissenborn, S. J., Pfister, H., Michael, K. M., Waterboer, T., Pawlita, M., Amantea, A., Landolfo, S. and Gariglio, M. (2009) 'High beta-HPV DNA loads and strong seroreactivity are present in epidermodysplasia verruciformis', *J Invest Dermatol,* 129(4), pp. 1026–34.

Demeret, C., Desaintes, C., Yaniv, M. and Thierry, F. (1997) 'Different mechanisms contribute to the E2-mediated transcriptional repression of human papillomavirus type 18 viral oncogenes', *J Virol,* 71(12), pp. 9343–9.

Dollard, S. C., Wilson, J. L., Demeter, L. M., Bonnez, W., Reichman, R. C., Broker, T. R. and Chow, L. T. (1992) 'Production of human papillomavirus and modulation of the infectious program in epithelial raft cultures. OFF', *Genes Dev,* 6(7), pp. 1131–42.

Donaldson, M. M., Boner, W. and Morgan, I. M. (2007) 'TopBP1 regulates human papillomavirus type 16 E2 interaction with chromatin', *J Virol,* 81(8), pp. 4338–42.

Donaldson, M. M., Mackintosh, L. J., Bodily, J. M., Dornan, E. S., Laimins, L. A. and Morgan, I. M. (2012) 'An interaction between human papillomavirus 16 E2 and TopBP1 is required for optimum viral DNA replication and episomal genome establishment', *J Virol,* 86(23), pp. 12806–15.

Donnianni, R. A. and Symington, L. S. (2013) 'Break-induced replication occurs by conservative DNA synthesis', *Proc Natl Acad Sci U S A,* 110(33), pp. 13475–80.

Doorbar, J. (2006) 'Molecular biology of human papillomavirus infection and cervical cancer', *Clin Sci (Lond),* 110(5), pp. 525–41.

Doorbar, J. (2007) 'Papillomavirus life cycle organization and biomarker selection', *Dis Markers,* 23(4), pp. 297–313.

Doorbar, J., Quint, W., Banks, L., Bravo, I. G., Stoler, M., Broker, T. R. and Stanley, M. A. (2012) 'The biology and life-cycle of human papillomaviruses', *Vaccine,* 30 Suppl 5, pp. F55–70.

Edwards, T. G., Helmus, M. J., Koeller, K., Bashkin, J. K. and Fisher, C. (2013) 'Human papillomavirus episome stability is reduced by aphidicolin and controlled by DNA damage response pathways', *J Virol,* 87(7), pp. 3979–89.

Egawa, N., Egawa, K., Griffin, H. and Doorbar, J. (2015) 'Human Papillomaviruses; Epithelial Tropisms, and the Development of Neoplasia', *Viruses,* 7(7), pp. 3863–90.

Ekanayake-Mudiyanselage, S., Aschauer, H., Schmook, F. P., Jensen, J. M., Meingassner, J. G. and Proksch, E. (1998) 'Expression of epidermal keratins and the cornified envelope protein involucrin is influenced by permeability barrier disruption', *J Invest Dermatol,* 111(3), pp. 517–23.

Evans, M. F., Aliesky, H. A. and Cooper, K. (2003) 'Optimization of biotinyl-tyramide-based in situ hybridization for sensitive background-free applications on formalin-fixed, paraffin-embedded tissue specimens', *BMC Clin Pathol,* 3(1), pp. 2.

Fanning, E., Klimovich, V. and Nager, A. R. (2006) 'A dynamic model for replication protein A (RPA) function in DNA processing pathways', *Nucleic Acids Res,* 34(15), pp. 4126–37.

Favre, M., Ramoz, N. and Orth, G. (1997) 'Human papillomaviruses: general features', *Clin Dermatol,* 15(2), pp. 181–98.

Fisher, C. (2015) 'Recent Insights into the Control of Human Papillomavirus (HPV) Genome Stability, Loss, and Degradation', *J Clin Med,* 4(2), pp. 204–30.

Flores, E. R. and Lambert, P. F. (1997) 'Evidence for a switch in the mode of human papillomavirus type 16 DNA replication during the viral life cycle', *Journal Of Virology,* 71(10), pp. 7167–7179.

Forrester, N. A., Sedgwick, G. G., Thomas, A., Blackford, A. N., Speiseder, T., Dobner, T., Byrd, P. J., Stewart, G. S., Turnell, A. S. and Grand, R. J. (2011) 'Serotype-specific inactivation of the cellular DNA damage response during adenovirus infection', *J Virol,* 85(5), pp. 2201–11.

Fradet-Turcotte, A., Bergeron-Labrecque, F., Moody, C. A., Lehoux, M., Laimins, L. A. and Archambault, J. (2011) 'Nuclear accumulation of the papillomavirus E1 helicase blocks S-phase progression and triggers an ATM-dependent DNA damage response', *J Virol,* 85(17), pp. 8996–9012.

Frattini, M. G. and Laimins, L. A. (1994) 'The role of the E1 and E2 proteins in the replication of human papillomavirus type 31b', *Virology,* 204(2), pp. 799–804.

Ganti, K., Broniarczyk, J., Manoubi, W., Massimi, P., Mittal, S., Pim, D., Szalmas, A., Thatte, J., Thomas, M., Tomaić, V. and Banks, L. (2015) 'The Human Papillomavirus E6 PDZ Binding Motif: From Life Cycle to Malignancy', *Viruses,* 7(7), pp. 3530–51.

Garner-Hamrick, P. A. and Fisher, C. (2002) 'HPV episomal copy number closely correlates with cell size in keratinocyte monolayer cultures', *Virology,* 301(2), pp. 334–41.

genticel.com GTL002 (Multivalent HPV).

Gerhold, J. M., Sedman, T., Visacka, K., Slezakova, J., Tomaska, L., Nosek, J. and Sedman, J. (2014) 'Replication intermediates of the linear mitochondrial DNA of Candida parapsilosis suggest a common recombination based mechanism for yeast mitochondria', *J Biol Chem,* 289(33), pp. 22659–70.

Gillespie, K. A., Mehta, K. P., Laimins, L. A. and Moody, C. A. (2012) 'Human papillomaviruses recruit cellular DNA repair and homologous recombination factors to viral replication centers', *J Virol,* 86(17), pp. 9520–6.

Gottschling, M., Stamatakis, A., Nindl, I., Stockfleth, E., Alonso, A. and Bravo, I. G. (2007) 'Multiple evolutionary mechanisms drive papillomavirus diversification', *Mol Biol Evol,* 24(5), pp. 1242–58.

Grassmann, K., Rapp, B., Maschek, H., Petry, K. U. and Iftner, T. (1996) 'Identification of a differentiation-inducible promoter in the E7 open reading frame of human papillomavirus type 16 (HPV-16) in raft cultures of a new cell line containing high copy numbers of episomal HPV-16 DNA', *J Virol,* 70(4), pp. 2339–49.

Green, H. (1977) 'Terminal differentiation of cultured human epidermal cells', *Cell,* 11(2), pp. 405–16.

Hanahan, D., Wetzel, E., Skowronski, J., Sippola-Thiele, M., Lindgren, V. and Howley, P. (1989) 'Tumorigenic latency and separable stages during fibrosarcoma development in transgenic mice carrying papillomavirus genomes', *Princess Takamatsu Symp,* 20, pp. 289–96.

Harper, J. W. and Elledge, S. J. (2007) 'The DNA damage response: ten years after', *Mol Cell,* 28(5), pp. 739–45.

Harwood, C. A. and Proby, C. M. (2002) 'Human papillomaviruses and non-melanoma skin cancer', *Curr Opin Infect Dis,* 15(2), pp. 101–14.

Hebner, C., Beglin, M. and Laimins, L. A. (2007) 'Human papillomavirus E6 proteins mediate resistance to interferon-induced growth arrest through inhibition of p53 acetylation', *J Virol,* 81(23), pp. 12740–7.

Henken, F. E., Banerjee, N. S., Snijders, P. J., Meijer, C. J., De-Castro Arce, J., Rösl, F., Broker, T. R., Chow, L. T. and Steenbergen, R. D. (2011) 'PIK3CA-mediated PI3-kinase signalling is essential for HPV-induced transformation in vitro', *Mol Cancer,* 10, pp. 71.

Hoffmann, R., Hirt, B., Bechtold, V., Beard, P. and Raj, K. (2006) 'Different modes of human papillomavirus DNA replication during maintenance', *J Virol,* 80(9), pp. 4431–9.

Hong, S. and Laimins, L. A. (2013) 'The JAK-STAT transcriptional regulator, STAT-5, activates the ATM DNA damage pathway to induce HPV 31 genome amplification upon epithelial differentiation', *Plos Pathogens,* 9(4), pp. e1003295–e1003295.

Hong, S., Mehta, K. P. and Laimins, L. A. (2011) 'Suppression of STAT-1 expression by human papillomaviruses is necessary for differentiation-dependent genome amplification and plasmid maintenance', *J Virol,* 85(18), pp. 9486–94.

Horn, S., Pfister, H. and Fuchs, P. G. (1993) 'Constitutive transcriptional activator of Epidermodysplasia verruciformis-associated human papillomavirus 8', *Virology,* 196(2), pp. 674–81.

Horvath, C. A., Boulet, G. A., Renoux, V. M., Delvenne, P. O. and Bogers, J. P. (2010) 'Mechanisms of cell entry by human papillomaviruses: an overview', *Virol J,* 7, pp. 11.

Hu, Y., Clower, R. V. and Melendy, T. (2006) 'Cellular topoisomerase I modulates origin binding by bovine papillomavirus type 1 E1', *J Virol,* 80(9), pp. 4363–71.

Hubert, W. G. and Laimins, L. A. (2002) 'Human papillomavirus type 31 replication modes during the early phases of the viral life cycle depend on transcriptional and posttranscriptional regulation of E1 and E2 expression', *J Virol,* 76(5), pp. 2263–73.

Hufbauer, M., Lazić, D., Reinartz, M., Akgül, B., Pfister, H. and Weissenborn, S. J. (2011) 'Skin tumor formation in human papillomavirus 8 transgenic mice is associated with a deregulation of oncogenic miRNAs and their tumor suppressive targets', *J Dermatol Sci,* 64(1), pp. 7–15.

Ilves, I., Kivi, S. and Ustav, M. (1999) 'Long-term episomal maintenance of bovine papillomavirus type 1 plasmids is determined by attachment to host chromosomes, which Is mediated by the viral E2 protein and its binding sites', *J Virol,* 73(5), pp. 4404–12.

Isfort, R. J., Cody, D. B., Lovell, G. and Doersen, C. J. (1995) 'Analysis of oncogenes, tumor suppressor genes, autocrine growth-factor production, and differentiation state of human osteosarcoma cell lines', *Mol Carcinog,* 14(3), pp. 170–8.

Isok-Paas, H., Männik, A., Ustav, E. and Ustav, M. (2015) 'The transcription map of HPV11 in U2OS cells adequately reflects the initial and stable replication phases of the viral genome', *Virol J,* 12, pp. 59.

Ivessa, A. S. (2013) 'Directionality of replication fork movement determined by two-dimensional native-native DNA agarose gel electrophoresis', *Methods Mol Biol,* 1054, pp. 83–103.

Jackson, S. and Storey, A. (2000) 'E6 proteins from diverse cutaneous HPV types inhibit apoptosis in response to UV damage', *Oncogene,* 19(4), pp. 592–8.

Jeon, S., Allen-Hoffmann, B. L. and Lambert, P. F. (1995) 'Integration of human papillomavirus type 16 into the human genome correlates with a selective growth advantage of cells', *J Virol,* 69(5), pp. 2989–97.

Kadaja, M., Silla, T., Ustav, E. and Ustav, M. (2009) 'Papillomavirus DNA replication – from initiation to genomic instability', *Virology,* 384(2), pp. 360–8.

Kadaja, M., Sumerina, A., Verst, T., Ojarand, M., Ustav, E. and Ustav, M. (2007) 'Genomic instability of the host cell induced by the human papillomavirus replication machinery', *EMBO J,* 26(8), pp. 2180–91.

Kajitani, N., Satsuka, A., Kawate, A. and Sakai, H. (2012) 'Productive Lifecycle of Human Papillomaviruses that Depends Upon Squamous Epithelial Differentiation', *Front Microbiol,* 3, pp. 152.

Kalantari, M., Blennow, E., Hagmar, B. and Johansson, B. (2001) 'Physical state of HPV16 and chromosomal mapping of the integrated form in cervical carcinomas', *Diagn Mol Pathol,* 10(1), pp. 46–54.

Kaur, P. and Li, A. (2000) 'Adhesive properties of human basal epidermal cells: an analysis of keratinocyte stem cells, transit amplifying cells, and postmitotic differentiating cells', *J Invest Dermatol,* 114(3), pp. 413–20.

Kay, M. A., He, C. Y. and Chen, Z. Y. (2010) 'A robust system for production of minicircle DNA vectors', *Nature Biotechnology,* 28(12), pp. 1287–1289.

Kee, Y. and D'Andrea, A. D. (2010) 'Expanded roles of the Fanconi anemia pathway in preserving genomic stability', *Genes Dev,* 24(16), pp. 1680–94.

Kines, R. C., Thompson, C. D., Lowy, D. R., Schiller, J. T. and Day, P. M. (2009) 'The initial steps leading to papillomavirus infection occur on the basement membrane prior to cell surface binding', *Proc Natl Acad Sci U S A,* 106(48), pp. 20458–63.

Kocjan, B. J., Bzhalava, D., Forslund, O., Dillner, J. and Poljak, M. (2015) 'Molecular methods for identification and characterization of novel papillomaviruses', *Clin Microbiol Infect,* 21(9), pp. 808–16.

Kogoma, T. (1996) 'Recombination by replication', *Cell,* 85(5), pp. 625–7.

Kondoh, G., Murata, Y., Aozasa, K., Yutsudo, M. and Hakura, A. (1991) 'Very high incidence of germ cell tumorigenesis (seminomagenesis) in human papillomavirus type 16 transgenic mice', *J Virol,* 65(6), pp. 3335–9.

Kreider, J. W. and Bartlett, G. L. (1981) 'The Shope papilloma-carcinoma complex of rabbits: a model system of neoplastic progression and spontaneous regression', *Adv Cancer Res,* 35, pp. 81–110.

Krejci, L., Altmannova, V., Spirek, M. and Zhao, X. (2012) 'Homologous recombination and its regulation', *Nucleic Acids Res,* 40(13), pp. 5795–818.

Kristiansen, E., Jenkins, A. and Holm, R. (1994) 'Coexistence of episomal and integrated HPV16 DNA in squamous cell carcinoma of the cervix', *J Clin Pathol,* 47(3), pp. 253–6.

Kumagai, A., Lee, J., Yoo, H. Y. and Dunphy, W. G. (2006) 'TopBP1 activates the ATR-ATRIP complex', *Cell,* 124(5), pp. 943–55.

Kurg, R. 2011. The Role of E2 Proteins in Papillomavirus DNA Replication. DNA Replication-Current Advances: InTech, Chapters published

Kurg, R., Uusen, P., Võsa, L. and Ustav, M. (2010) 'Human papillomavirus E2 protein with single activation domain initiates HPV18 genome replication, but is not sufficient for long-term maintenance of virus genome', *Virology,* 408(2), pp. 159–66.

Kusumoto-Matsuo, R., Kanda, T. and Kukimoto, I. (2011) 'Rolling circle replication of human papillomavirus type 16 DNA in epithelial cell extracts', *Genes Cells,* 16(1), pp. 23–33.

Kuzminov, A. (1999) 'Recombinational repair of DNA damage in Escherichia coli and bacteriophage lambda', *Microbiol Mol Biol Rev,* 63(4), pp. 751–813, table of contents.

Lace, M. J., Anson, J. R., Turek, L. P. and Haugen, T. H. (2008) 'Functional mapping of the human papillomavirus type 16 E1 cistron', *J Virol,* 82(21), pp. 10724–34.

Lacey, M., Alpert, S. and Hanahan, D. (1986) 'Bovine papillomavirus genome elicits skin tumours in transgenic mice', *Nature,* 322(6080), pp. 609–12.

Lambert, P. F. (1991) 'Papillomavirus DNA replication', *J Virol,* 65(7), pp. 3417–20.

Lambert, P. F., Pan, H., Pitot, H. C., Liem, A., Jackson, M. and Griep, A. E. (1993) 'Epidermal cancer associated with expression of human papillomavirus type 16 E6 and E7 oncogenes in the skin of transgenic mice', *Proc Natl Acad Sci U S A,* 90(12), pp. 5583–7.

Lange, C. E., Favrot, C., Ackermann, M., Gull, J., Vetsch, E. and Tobler, K. (2011) 'Novel snake papillomavirus does not cluster with other non-mammalian papillomaviruses', *Virol J,* 8, pp. 436.

Law, M. F., Lowy, D. R., Dvoretzky, I. and Howley, P. M. (1981) 'Mouse cells transformed by bovine papillomavirus contain only extrachromosomal viral DNA sequences', *Proc Natl Acad Sci U S A,* 78(5), pp. 2727–31.

Lazarczyk, M., Cassonnet, P., Pons, C., Jacob, Y. and Favre, M. (2009) 'The EVER proteins as a natural barrier against papillomaviruses: a new insight into the pathogenesis of human papillomavirus infections', *Microbiol Mol Biol Rev,* 73(2), pp. 348–70.

Lee, J. S. (2007) 'Functional link between DNA damage responses and transcriptional regulation by ATM in response to a histone deacetylase inhibitor TSA', *Cancer Res Treat,* 39(3), pp. 116–24.

Lehman, C. W. and Botchan, M. R. (1998) 'Segregation of viral plasmids depends on tethering to chromosomes and is regulated by phosphorylation', *Proc Natl Acad Sci U S A,* 95(8), pp. 4338–43.

Lehn, H., Villa, L. L., Marziona, F., Hilgarth, M., Hillemans, H. G. and Sauer, G. (1988) 'Physical state and biological activity of human papillomavirus genomes in precancerous lesions of the female genital tract', *J Gen Virol,* 69 (Pt 1), pp. 187–96.

Leverrier, S., Bergamaschi, D., Ghali, L., Ola, A., Warnes, G., Akgül, B., Blight, K., García-Escudero, R., Penna, A., Eddaoudi, A. and Storey, A. (2007) 'Role of HPV E6 proteins in preventing UVB-induced release of pro-apoptotic factors from the mitochondria', *Apoptosis,* 12(3), pp. 549–60.

Li, L., Tennenbaum, T. and Yuspa, S. H. (1996) 'Suspension-induced murine keratinocyte differentiation is mediated by calcium', *J Invest Dermatol,* 106(2), pp. 254–60.

Li, R., Knight, J., Bream, G., Stenlund, A. and Botchan, M. (1989) 'Specific recognition nucleotides and their DNA context determine the affinity of E2 protein for 17 binding sites in the BPV-1 genome', *Genes Dev,* 3(4), pp. 510–26.

Lilly, J. and Camps, M. (2015) 'Mechanisms of Theta Plasmid Replication', *Microbiol Spectr,* 3(1), pp. PLAS-0029-2014.

Lin, S. J., Wardlaw, C. P., Morishita, T., Miyabe, I., Chahwan, C., Caspari, T., Schmidt, U., Carr, A. M. and Garcia, V. (2012) 'The Rad4(TopBP1) ATR-activation domain

functions in G1/S phase in a chromatin-dependent manner', *PLoS Genet,* 8(6), pp. e1002801.
Lindahl, T. and Barnes, D. E. (2000) 'Repair of endogenous DNA damage', *Cold Spring Harb Symp Quant Biol,* 65, pp. 127–33.
Lindgren, V., Sippola-Thiele, M., Skowronski, J., Wetzel, E., Howley, P. M. and Hanahan, D. (1989) 'Specific chromosomal abnormalities characterize fibrosarcomas of bovine papillomavirus type 1 transgenic mice', *Proc Natl Acad Sci U S A,* 86(13), pp. 5025–9.
Longworth, M. S. and Laimins, L. A. (2004) 'The binding of histone deacetylases and the integrity of zinc finger-like motifs of the E7 protein are essential for the life cycle of human papillomavirus type 31', *J Virol,* 78(7), pp. 3533–41.
Lopez-Contreras (2012) 'Signalling DNA Damage', *Protein Phosphorylation in Human Health*.
Lowy, D. R., Dvoretzky, I., Shober, R., Law, M. F., Engel, L. and Howley, P. M. (1980) 'In vitro tumorigenic transformation by a defined sub-genomic fragment of bovine papilloma virus DNA', *Nature,* 287(5777), pp. 72–4.
Maglennon, G. A., McIntosh, P. and Doorbar, J. (2011) 'Persistence of viral DNA in the epithelial basal layer suggests a model for papillomavirus latency following immune regression', *Virology,* 414(2), pp. 153–63.
Mantovani, F. and Banks, L. (2001) 'The human papillomavirus E6 protein and its contribution to malignant progression', *Oncogene,* 20(54), pp. 7874–87.
Martín-Parras, L., Lucas, I., Martínez-Robles, M. L., Hernández, P., Krimer, D. B., Hyrien, O. and Schvartzman, J. B. (1998) 'Topological complexity of different populations of pBR322 as visualized by two-dimensional agarose gel electrophoresis', *Nucleic Acids Res,* 26(14), pp. 3424–32.
May, M., Grassmann, K., Pfister, H. and Fuchs, P. G. (1994) 'Transcriptional silencer of the human papillomavirus type 8 late promoter interacts alternatively with the viral trans activator E2 or with a cellular factor', *J Virol,* 68(6), pp. 3612–9.
McBride, A. A., Romanczuk, H. and Howley, P. M. (1991) 'The papillomavirus E2 regulatory proteins', *J Biol Chem,* 266(28), pp. 18411–4.
McKinney, C. C., Hussmann, K. L. and McBride, A. A. (2015) 'The Role of the DNA Damage Response throughout the Papillomavirus Life Cycle', *Viruses,* 7(5), pp. 2450–69.
McKinney, E. A. and Oliveira, M. T. (2013) 'Replicating animal mitochondrial DNA', *Genet Mol Biol,* 36(3), pp. 308–15.
McLaughlin-Drubin, M. E., Wilson, S., Mullikin, B., Suzich, J. and Meyers, C. (2003) 'Human papillomavirus type 45 propagation, infection, and neutralization', *Virology,* 312(1), pp. 1–7.
McPhillips, M. G., Oliveira, J. G., Spindler, J. E., Mitra, R. and McBride, A. A. (2006) 'Brd4 is required for e2-mediated transcriptional activation but not genome partitioning of all papillomaviruses', *J Virol,* 80(19), pp. 9530–43.
Meyers, C., Mayer, T. J. and Ozbun, M. A. (1997) 'Synthesis of infectious human papillomavirus type 18 in differentiating epithelium transfected with viral DNA', *J Virol,* 71(10), pp. 7381–6.
Minner, F. and Poumay, Y. (2009) 'Candidate housekeeping genes require evaluation before their selection for studies of human epidermal keratinocytes', *J Invest Dermatol,* 129(3), pp. 770–3.

Moody, C. A. and Laimins, L. A. (2009) 'Human papillomaviruses activate the ATM DNA damage pathway for viral genome amplification upon differentiation', *PLoS Pathog,* 5(10), pp. e1000605.

Muller, M. and Demeret, C. (2012) 'The HPV E2-Host Protein-Protein Interactions: A Complex Hijacking of the Cellular Network', *Open Virol J,* 6, pp. 173–89.

Muñoz, N., Bosch, F. X., De Sanjosé, S., Castellsagué, X., Herrero, R., Shah, K. V., Snijders, P. J. F. and Meijer, C. J. L. M. (2003) 'Epidemiologic classification of human papillomavirus types associated with cervical cancer', *New England Journal of Medicine,* 348(6), pp. 518–527.

Muñoz, N., Castellsagué, X., de González, A. B. and Gissmann, L. (2006) 'Chapter 1: HPV in the etiology of human cancer', *Vaccine,* 24 Suppl 3, pp. S3/1–10.

Nakamura, J., Mutlu, E., Sharma, V., Collins, L., Bodnar, W., Yu, R., Lai, Y., Moeller, B., Lu, K. and Swenberg, J. (2014) 'The endogenous exposome', *DNA Repair (Amst),* 19, pp. 3–13.

Nawotka, K. A. and Huberman, J. A. (1988) 'Two-dimensional gel electrophoretic method for mapping DNA replicons', *Molecular and Cellular Biology,* 8(4), pp. 1408–1413.

Neary, K. and DiMaio, D. (1989) 'Open reading frames E6 and E7 of bovine papillomavirus type 1 are both required for full transformation of mouse C127 cells', *J Virol,* 63(1), pp. 259–66.

Nikitin, P. A. and Luftig, M. A. (2011) 'At a crossroads: human DNA tumor viruses and the host DNA damage response', *Future Virol,* 6(7), pp. 813–830.

Nindl, I., Gottschling, M. and Stockfleth, E. (2007) 'Human papillomaviruses and non-melanoma skin cancer: basic virology and clinical manifestations', *Dis Markers,* 23(4), pp. 247–59.

Noon, A. T., Shibata, A., Rief, N., Löbrich, M., Stewart, G. S., Jeggo, P. A. and Goodarzi, A. A. (2010) '53BP1-dependent robust localized KAP-1 phosphorylation is essential for heterochromatic DNA double-strand break repair', *Nat Cell Biol,* 12(2), pp. 177–84.

Ohashi, E., Takeishi, Y., Ueda, S. and Tsurimoto, T. (2014) 'Interaction between Rad9-Hus1-Rad1 and TopBP1 activates ATR-ATRIP and promotes TopBP1 recruitment to sites of UV-damage', *DNA Repair (Amst),* 21, pp. 1–11.

Oliveira, J. G., Colf, L. A. and McBride, A. A. (2006) 'Variations in the association of papillomavirus E2 proteins with mitotic chromosomes', *Proc Natl Acad Sci U S A,* 103(4), pp. 1047–52.

Orav, M., Henno, L., Isok-Paas, H., Geimanen, J., Ustav, M. and Ustav, E. (2013) 'Recombination-Dependent Oligomerization of Human Papillomavirus Genomes upon Transient DNA Replication', *Journal of Virology,* 87(22), pp. 12051–12068.

Orazio, N. I., Naeger, C. M., Karlseder, J. and Weitzman, M. D. (2011) 'The adenovirus E1b55K/E4orf6 complex induces degradation of the Bloom helicase during infection', *J Virol,* 85(4), pp. 1887–92.

Ozbun, M. A. (2002a) 'Human papillomavirus type 31b infection of human keratinocytes and the onset of early transcription', *J Virol,* 76(22), pp. 11291–300.

Ozbun, M. A. (2002b) 'Infectious human papillomavirus type 31b: purification and infection of an immortalized human keratinocyte cell line', *J Gen Virol,* 83(Pt 11), pp. 2753–63.

Ozbun, M. A. and Patterson, N. A. (2014) 'Using organotypic (raft) epithelial tissue cultures for the biosynthesis and isolation of infectious human papillomaviruses', *Curr Protoc Microbiol,* 34, pp. 14B.3.1–14B.3.18.

Pavlakis, G. N. and Hamer, D. H. (1983) 'Regulation of a metallothionein-growth hormone hybrid gene in bovine papilloma virus', *Proc Natl Acad Sci U S A,* 80(2), pp. 397–401.

Pett, M. R., Herdman, M. T., Palmer, R. D., Yeo, G. S., Shivji, M. K., Stanley, M. A. and Coleman, N. (2006) 'Selection of cervical keratinocytes containing integrated HPV16 associates with episome loss and an endogenous antiviral response', *Proc Natl Acad Sci U S A,* 103(10), pp. 3822–7.

Piirsoo, M., Ustav, E., Mandel, T., Stenlund, A. and Ustav, M. (1996) 'Cis and trans requirements for stable episomal maintenance of the BPV-1 replicator', *EMBO J,* 15(1), pp. 1–11.

Poddar, A., Reed, S. C., McPhillips, M. G., Spindler, J. E. and McBride, A. A. (2009) 'The human papillomavirus type 8 E2 tethering protein targets the ribosomal DNA loci of host mitotic chromosomes', *J Virol,* 83(2), pp. 640–50.

Poumay, Y. and Pittelkow, M. R. (1995) 'Cell density and culture factors regulate keratinocyte commitment to differentiation and expression of suprabasal K1/K10 keratins', *J Invest Dermatol,* 104(2), pp. 271–6.

Purdie, K. J., Surentheran, T., Sterling, J. C., Bell, L., McGregor, J. M., Proby, C. M., Harwood, C. A. and Breuer, J. (2005) 'Human papillomavirus gene expression in cutaneous squamous cell carcinomas from immunosuppressed and immunocompetent individuals', *J Invest Dermatol,* 125(1), pp. 98–107.

Quint, K. D., Genders, R. E., de Koning, M. N., Borgogna, C., Gariglio, M., Bouwes Bavinck, J. N., Doorbar, J. and Feltkamp, M. C. (2015) 'Human Beta-papillomavirus infection and keratinocyte carcinomas', *J Pathol,* 235(2), pp. 342–54.

Rashid, N. N., Rothan, H. A. and Yusoff, M. S. (2015) 'The association of mammalian DREAM complex and HPV16 E7 proteins', *Am J Cancer Res,* 5(12), pp. 3525–33.

Reh, H. and Pfister, H. (1990) 'Human papillomavirus type 8 contains cis-active positive and negative transcriptional control sequences', *J Gen Virol,* 71 (Pt 10), pp. 2457–62.

Reinson, T., Toots, M., Kadaja, M., Pipitch, R., Allik, M., Ustav, E. and Ustav, M. (2013) 'Engagement of the ATR-dependent DNA damage response at the human papillomavirus 18 replication centers during the initial amplification', *J Virol,* 87(2), pp. 951–64.

Remm, M., Brain, R. and Jenkins, J. R. (1992) 'The E2 binding sites determine the efficiency of replication for the origin of human papillomavirus type 18', *Nucleic Acids Res,* 20(22), pp. 6015–21.

Roberts, J. M. and Weintraub, H. (1986) 'Negative control of DNA replication in composite SV40-bovine papilloma virus plasmids', *Cell,* 46(5), pp. 741–52.

Rogers, A., Waltke, M. and Angeletti, P. C. (2011) 'Evolutionary variation of papillomavirus E2 protein and E2 binding sites', *Virol J,* 8, pp. 379.

Romanczuk, H. and Howley, P. M. (1992) 'Disruption of either the E1 or the E2 regulatory gene of human papillomavirus type 16 increases viral immortalization capacity', *Proc Natl Acad Sci U S A,* 89(7), pp. 3159–63.

Rubio-Viqueira, B. and Hidalgo, M. (2009) 'Direct in vivo xenograft tumor model for predicting chemotherapeutic drug response in cancer patients', *Clin Pharmacol Ther,* 85(2), pp. 217–21.

Sakakibara, N., Chen, D. and McBride, A. A. (2013) 'Papillomaviruses use recombination-dependent replication to vegetatively amplify their genomes in differentiated cells', *PLoS Pathog,* 9(7), pp. e1003321.

Sakakibara, N., Mitra, R. and McBride, A. A. (2011) 'The papillomavirus E1 helicase activates a cellular DNA damage response in viral replication foci', *J Virol,* 85(17), pp. 8981–95.

Sambrook, J., Rodgers, L., White, J. and Gething, M. J. (1985) 'Lines of BPV-transformed murine cells that constitutively express influenza virus hemagglutinin', *EMBO J,* 4(1), pp. 91–103.

Sanders, C. M. and Stenlund, A. (1998) 'Recruitment and loading of the E1 initiator protein: an ATP-dependent process catalysed by a transcription factor', *EMBO J,* 17(23), pp. 7044–55.

Sankovski, E., Männik, A., Geimanen, J., Ustav, E. and Ustav, M. (2014) 'Mapping of betapapillomavirus human papillomavirus 5 transcription and characterization of viral-genome replication function', *J Virol,* 88(2), pp. 961–73.

Sarver, N., Gruss, P., Law, M. F., Khoury, G. and Howley, P. M. (1981) 'Bovine papilloma virus deoxyribonucleic acid: a novel eucaryotic cloning vector', *Mol Cell Biol,* 1(6), pp. 486–96.

Schmitt, A., Rochat, A., Zeltner, R., Borenstein, L., Barrandon, Y., Wettstein, F. O. and Iftner, T. (1996) 'The primary target cells of the high-risk cottontail rabbit papillomavirus colocalize with hair follicle stem cells', *J Virol,* 70(3), pp. 1912–22.

Schneider, J., Kinne, D., Fracchia, A., Pierce, V., Anderson, K. E., Bradlow, H. L. and Fishman, J. (1982) 'Abnormal oxidative metabolism of estradiol in women with breast cancer', *Proc Natl Acad Sci U S A,* 79(9), pp. 3047–51.

Schuck, S. and Stenlund, A. (2005) 'Assembly of a double hexameric helicase', *Mol Cell,* 20(3), pp. 377–89.

Schwartz, S. (2000) 'Regulation of human papillomavirus late gene expression', *Ups J Med Sci,* 105(3), pp. 171–92.

Schäfer, G., Blumenthal, M. J. and Katz, A. A. (2015) 'Interaction of human tumor viruses with host cell surface receptors and cell entry', *Viruses,* 7(5), pp. 2592–617.

Sedman, J. and Stenlund, A. (1995) 'Co-operative interaction between the initiator E1 and the transcriptional activator E2 is required for replicator specific DNA replication of bovine papillomavirus in vivo and in vitro', *EMBO J,* 14(24), pp. 6218–28.

Sedman, J. and Stenlund, A. (1998) 'The papillomavirus E1 protein forms a DNA-dependent hexameric complex with ATPase and DNA helicase activities', *J Virol,* 72(8), pp. 6893–7.

Sedman, T., Sedman, J. and Stenlund, A. (1997) 'Binding of the E1 and E2 proteins to the origin of replication of bovine papillomavirus', *J Virol,* 71(4), pp. 2887–96.

Sekhar, V., Reed, S. C. and McBride, A. A. (2010) 'Interaction of the betapapillomavirus E2 tethering protein with mitotic chromosomes', *J Virol,* 84(1), pp. 543–57.

Sherman, L., Jackman, A., Itzhaki, H., Stöppler, M. C., Koval, D. and Schlegel, R. (1997) 'Inhibition of serum- and calcium-induced differentiation of human keratinocytes by HPV16 E6 oncoprotein: role of p53 inactivation', *Virology,* 237(2), pp. 296–306.

Shigechi, T., Tomida, J., Sato, K., Kobayashi, M., Eykelenboom, J. K., Pessina, F., Zhang, Y., Uchida, E., Ishiai, M., Lowndes, N. F., Yamamoto, K., Kurumizaka, H., Maehara, Y. and Takata, M. (2012) 'ATR-ATRIP kinase complex triggers activation of the Fanconi anemia DNA repair pathway', *Cancer Res,* 72(5), pp. 1149–56.

Shirata, N., Kudoh, A., Daikoku, T., Tatsumi, Y., Fujita, M., Kiyono, T., Sugaya, Y., Isomura, H., Ishizaki, K. and Tsurumi, T. (2005) 'Activation of ataxia

telangiectasia-mutated DNA damage checkpoint signal transduction elicited by herpes simplex virus infection', *J Biol Chem,* 280(34), pp. 30336–41.
Silla, T., Hääl, I., Geimanen, J., Janikson, K., Abroi, A., Ustav, E. and Ustav, M. (2005) 'Episomal maintenance of plasmids with hybrid origins in mouse cells', *J Virol,* 79(24), pp. 15277–88.
Simmons, D. T., Trowbridge, P. W. and Roy, R. (1998) 'Topoisomerase I stimulates SV40 T antigen-mediated DNA replication and inhibits T antigen's ability to unwind DNA at nonorigin sites', *Virology,* 242(2), pp. 435–43.
Siolas, D. and Hannon, G. J. (2013) 'Patient-derived tumor xenografts: transforming clinical samples into mouse models', *Cancer Res,* 73(17), pp. 5315–9.
Sippola-Thiele, M., Hanahan, D. and Howley, P. M. (1989) 'Cell-heritable stages of tumor progression in transgenic mice harboring the bovine papillomavirus type 1 genome', *Mol Cell Biol,* 9(3), pp. 925–34.
Skiadopoulos, M. H. and McBride, A. A. (1998) 'Bovine papillomavirus type 1 genomes and the E2 transactivator protein are closely associated with mitotic chromatin', *J Virol,* 72(3), pp. 2079–88.
Spalholz, B. A., Yang, Y. C. and Howley, P. M. (1985) 'Transactivation of a bovine papilloma virus transcriptional regulatory element by the E2 gene product', *Cell,* 42(1), pp. 183–91.
Stafa, A., Donnianni, R. A., Timashev, L. A., Lam, A. F. and Symington, L. S. (2014) 'Template switching during break-induced replication is promoted by the Mph1 helicase in Saccharomyces cerevisiae', *Genetics,* 196(4), pp. 1017–28.
Stanley, M. A., Browne, H. M., Appleby, M. and Minson, A. C. (1989) 'Properties of a non-tumorigenic human cervical keratinocyte cell line', *Int J Cancer,* 43(4), pp. 672–6.
Stanley, M. A., Pett, M. R. and Coleman, N. (2007) 'HPV: from infection to cancer', *Biochem Soc Trans,* 35(Pt 6), pp. 1456–60.
Steger, G. and Corbach, S. (1997) 'Dose-dependent regulation of the early promoter of human papillomavirus type 18 by the viral E2 protein', *J Virol,* 71(1), pp. 50–8.
Stenlund, A. (2003) 'E1 initiator DNA binding specificity is unmasked by selective inhibition of non-specific DNA binding', *EMBO J,* 22(4), pp. 954–63.
Stracker, T. H., Usui, T. and Petrini, J. H. (2009) 'Taking the time to make important decisions: the checkpoint effector kinases Chk1 and Chk2 and the DNA damage response', *DNA Repair (Amst),* 8(9), pp. 1047–54.
Stubenrauch, F., Hummel, M., Iftner, T. and Laimins, L. A. (2000) 'The E8–E2C protein, a negative regulator of viral transcription and replication, is required for extrachromosomal maintenance of human papillomavirus type 31 in keratinocytes', *Journal of Virology,* 74(3), pp. 1178–1186.
Sundberg, J. P., O'Banion, M. K., Schmidt-Didier, E. and Reichmann, M. E. (1986) 'Cloning and characterization of a canine oral papillomavirus', *Am J Vet Res,* 47(5), pp. 1142–4.
Surviladze, Z., Sterkand, R. T. and Ozbun, M. A. (2015) 'Interaction of human papillomavirus type 16 particles with heparan sulfate and syndecan-1 molecules in the keratinocyte extracellular matrix plays an active role in infection', *J Gen Virol,* 96(8), pp. 2232–41.
Sverdrup, F. and Khan, S. A. (1994) 'Replication of human papillomavirus (HPV) DNAs supported by the HPV type 18 E1 and E2 proteins', *J Virol,* 68(1), pp. 505–9.
Swaneck, G. E. and Fishman, J. (1988) 'Covalent binding of the endogenous estrogen 16 alpha-hydroxyestrone to estradiol receptor in human breast cancer cells:

characterization and intranuclear localization', *Proc Natl Acad Sci U S A,* 85(21), pp. 7831–5.

Tachezy, R., Rector, A., Havelkova, M., Wollants, E., Fiten, P., Opdenakker, G., Jenson, B., Sundberg, J. and Van Ranst, M. (2002) 'Avian papillomaviruses: the parrot Psittacus erithacus papillomavirus (PePV) genome has a unique organization of the early protein region and is phylogenetically related to the chaffinch papillomavirus', *BMC Microbiol,* 2, pp. 19.

Terai, M., DeSalle, R. and Burk, R. D. (2002) 'Lack of canonical E6 and E7 open reading frames in bird papillomaviruses: Fringilla coelebs papillomavirus and Psittacus erithacus timneh papillomavirus', *J Virol,* 76(19), pp. 10020–3.

Thomas, J. T., Oh, S. T., Terhune, S. S. and Laimins, L. A. (2001) 'Cellular changes induced by low-risk human papillomavirus type 11 in keratinocytes that stably maintain viral episomes', *J Virol,* 75(16), pp. 7564–71.

Tinsley, J. M., Fisher, C. and Searle, P. F. (1992) 'Abnormalities of epidermal differentiation associated with expression of the human papillomavirus type 1 early region in transgenic mice', *J Gen Virol,* 73 (Pt 5), pp. 1251–60.

Titolo, S., Pelletier, A., Sauvé, F., Brault, K., Wardrop, E., White, P. W., Amin, A., Cordingley, M. G. and Archambault, J. (1999) 'Role of the ATP-binding domain of the human papillomavirus type 11 E1 helicase in E2-dependent binding to the origin', *J Virol,* 73(7), pp. 5282–93.

Toots, M., Männik, A., Kivi, G., Ustav, M. and Ustav, E. (2014) 'The transcription map of human papillomavirus type 18 during genome replication in U2OS cells', *PLoS One,* 9(12), pp. e116151.

Trowbridge, P. W., Roy, R. and Simmons, D. T. (1999) 'Human topoisomerase I promotes initiation of simian virus 40 DNA replication in vitro', *Mol Cell Biol,* 19(3), pp. 1686–94.

Tu, C. L., Crumrine, D. A., Man, M. Q., Chang, W., Elalieh, H., You, M., Elias, P. M. and Bikle, D. D. (2012) 'Ablation of the calcium-sensing receptor in keratinocytes impairs epidermal differentiation and barrier function', *J Invest Dermatol,* 132(10), pp. 2350–9.

Turnell, A. S. and Grand, R. J. (2012) 'DNA viruses and the cellular DNA-damage response', *J Gen Virol,* 93(Pt 10), pp. 2076–97.

Uemura, N., Ozawa, K., Tojo, A., Takahashi, K., Okano, A., Karasuyama, H., Tani, K. and Asano, S. (1992) 'Acquisition of interleukin-3 independence in FDC-P2 cells after transfection with the activated c-H-ras gene using a bovine papillomavirus-based plasmid vector', *Blood,* 80(12), pp. 3198–204.

Ustav, E., Ustav, M., Szymanski, P. and Stenlund, A. (1993) 'The bovine papillomavirus origin of replication requires a binding site for the E2 transcriptional activator', *Proc Natl Acad Sci U S A,* 90(3), pp. 898–902.

Ustav, M. and Stenlund, A. (1991) 'Transient replication of BPV-1 requires two viral polypeptides encoded by the E1 and E2 open reading frames', *EMBO J,* 10(2), pp. 449–57.

Ustav, M., Ustav, E., Szymanski, P. and Stenlund, A. (1991) 'Identification of the origin of replication of bovine papillomavirus and characterization of the viral origin recognition factor E1', *EMBO J,* 10(13), pp. 4321–9.

Uziel, T., Lerenthal, Y., Moyal, L., Andegeko, Y., Mittelman, L. and Shiloh, Y. (2003) 'Requirement of the MRN complex for ATM activation by DNA damage', *EMBO J,* 22(20), pp. 5612–21.

Van Craenenbroeck, K., Vanhoenacker, P. and Haegeman, G. (2000) 'Episomal vectors for gene expression in mammalian cells', *Eur J Biochem,* 267(18), pp. 5665–78.

Van Doorslaer, K., Sidi, A. O., Zanier, K., Rybin, V., Deryckère, F., Rector, A., Burk, R. D., Lienau, E. K., van Ranst, M. and Travé, G. (2009) 'Identification of unusual E6 and E7 proteins within avian papillomaviruses: cellular localization, biophysical characterization, and phylogenetic analysis', *J Virol,* 83(17), pp. 8759–70.

Viarisio, D., Mueller-Decker, K., Kloz, U., Aengeneyndt, B., Kopp-Schneider, A., Gröne, H. J., Gheit, T., Flechtenmacher, C., Gissmann, L. and Tommasino, M. (2011) 'E6 and E7 from beta HPV38 cooperate with ultraviolet light in the development of actinic keratosis-like lesions and squamous cell carcinoma in mice', *PLoS Pathog,* 7(7), pp. e1002125.

Voskoglou-Nomikos, T., Pater, J. L. and Seymour, L. (2003) 'Clinical predictive value of the in vitro cell line, human xenograft, and mouse allograft preclinical cancer models', *Clin Cancer Res,* 9(11), pp. 4227–39.

Wang, X., Zheng, Z. M., Meyers, C., Wang, H. K. and Chow, L. T. (2011) 'Construction of a full transcription map of human papillomavirus type 18 during productive viral infection', *Journal of Virology,* 85(16), pp. 8080–8092.

Weitzman, M. D., Lilley, C. E. and Chaurushiya, M. S. (2010) 'Genomes in conflict: maintaining genome integrity during virus infection', *Annu Rev Microbiol,* 64, pp. 61–81.

Weitzman, M. D. and Weitzman, J. B. (2014) 'What's the damage? The impact of pathogens on pathways that maintain host genome integrity', *Cell Host Microbe,* 15(3), pp. 283–94.

Wesierska-Gadek, J. and Schmid, G. (2005) 'The subcellular distribution of the p53 tumour suppressor, and organismal ageing', *Cell Mol Biol Lett,* 10(3), pp. 439–53.

Wilson, R. and Laimins, L. A. (2005) 'Differentiation of HPV-containing cells using organotypic "raft" culture or methylcellulose', *Methods Mol Med,* 119, pp. 157–69.

www.hpvcenter.se International Human Papillomavirus Reference Center

Yang, Y. C., Spalholz, B. A., Rabson, M. S. and Howley, P. M. (1985) 'Dissociation of transforming and trans-activation functions for bovine papillomavirus type 1', *Nature,* 318(6046), pp. 575–7.

You, J. (2010) 'Papillomavirus interaction with cellular chromatin', *Biochim Biophys Acta,* 1799(3–4), pp. 192–9.

You, J., Croyle, J. L., Nishimura, A., Ozato, K. and Howley, P. M. (2004) 'Interaction of the bovine papillomavirus E2 protein with Brd4 tethers the viral DNA to host mitotic chromosomes', *Cell,* 117(3), pp. 349–60.

Zheng, Z. M. and Baker, C. C. (2006) 'Papillomavirus genome structure, expression, and post-transcriptional regulation', *Front Biosci,* 11, pp. 2286–302.

Zhou, B. B. and Elledge, S. J. (2000) 'The DNA damage response: putting checkpoints in perspective', *Nature,* 408(6811), pp. 433–9.

Zobel, T., Iftner, T. and Stubenrauch, F. (2003) 'The papillomavirus E8–E2C protein represses DNA replication from extrachromosomal origins', *Mol Cell Biol,* 23(22), pp. 8352–62.

Zur Hausen, H. (2009) 'The search for infectious causes of human cancers: where and why', *Virology,* 392(1), pp. 1–10.

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3. Marit Orav, Liisi Henno, Helen Isok-Paas, Jelizaveta Geimanen, Mart Ustav, Ene Ustav (2013) Recombination-Dependent Oligomerization of Human Papillomavirus Genomes upon Transient DNA Replication. *Journal of Virology*, 87 (22), 12051–12068
4. Sankovski, Eve; Männik, Andres; Geimanen, Jelizaveta; Ustav, Ene; Ustav, Mart (2014). Mapping of Beta-Papillomavirus HPV5 Transcription and Characterization of Viral Genome Replication Function. Journal of Virology, 88(2), 961–973.
5. Marit Orav, Jelizaveta Geimanen, Eva-Maria Sepp, Liisi Henno, Ene Ustav, and Mart Ustav (2015). Initial amplification of the HPV18 genome proceeds via two distinct replication mechanisms. Scientific reports 5: 15952. doi:10.1038/srep15952.

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2. Geimanen, J.; Isok-Paas, H.; Pipitch, R.; Salk, K.; Laos, T.; Orav, M.; Reinson, T.; Ustav, M. Jr.; Ustav, M.; Ustav, E. (2011). Development of a cellular assay system to study the genome replication of high- and low-risk mucosal and cutaneous human papillomaviruses. Journal of Virology, 85(7), 3315–3329.
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4. Sankovski, Eve; Männik, Andres; Geimanen, Jelizaveta; Ustav, Ene; Ustav, Mart (2014). Mapping of Beta-Papillomavirus HPV5 Transcription and Characterization of Viral Genome Replication Function. Journal of Virology, 88(2), 961–973.
5. Marit Orav, Jelizaveta Geimanen, Eva-Maria Sepp, Liisi Henno, Ene Ustav, and Mart Ustav (2015). Initial amplification of the HPV18 genome proceeds via two distinct replication mechanisms. Scientific reports 5: 15952. doi:10.1038/srep15952.

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