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Viral-host interactions in the life cycle of human papillomaviruses

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original papers, which will be referred by their Roman numerals:

I Kadaja M, **Isok-Paas H**, Laos T, Ustav E, Ustav M (2009) Mechanism of Genomic Instability in Cells Infected with the High-Risk Human Papillomaviruses. *PLoS Pathog. 2009 Apr; 5(4): e1000397.*

II Geimanen J, **Isok-Paas H**, Pipitch R, Salk K, Laos T, Orav M, et al. (2011) Development of a cellular assay system to study the genome replication of high- and low-risk mucosal and cutaneous human papillomaviruses. *J Virol. 2011 Apr; 85(7):3315–29.*

III Orav M, Henno L, **Isok-Paas H**, Geimanen J, Ustav M, Ustav E. Recombination-dependent oligomerization of human papillomavirus genomes upon transient DNA replication. *J Virol. 2013 Nov; 87(22):12051–68.*

IV **Isok-Paas H**, Männik A, Ustav E, Ustav M (2015) The Transcription Map of HPV11 in U2OS Cells Adequately Reflects the Initial and Stable Replication Phases of the Viral Genome. Virology Journal 12:59

The author's contributions to the publications are as follows:

Ref. I I performed HPV16 and HPV18 genome rearrangement analysis via restriction analysis of single-cell subclones of SiHa cells transfected with HPV16 or HPV18 genomes and participated in IF-FISH analysis; performed the immunofluorescence (IF) part of the IF-FISH methodology;

Ref. II I designed experiments and performed the subcloning of cells transfected with HPV6b and HPV11 genomes for the isolation of HPV6b- and HPV11-positive stable cell lines and performed HPV DNA analysis of the stable cell lines of low-risk HPV types;

Ref. III I performed analysis of clinical samples obtained from HPV-infected patients and used different restriction enzyme digestions to analyze the physical state of HPV DNA;

Ref. IV I designed and performed experiments, analyzed data and wrote most of the manuscript.

Invention: Method and a kit for identifying compounds capable of inhibiting human papilloma virus replication. Authors: 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. PCT/EE2010/ 000010

LIST OF ABBREVIATIONS

2D – two-dimensional
AGE – agarose gel electrophoresis
ATM – ataxia-telangiectasia mutated kinase
ATR – ATM- and Rad3-related kinase
ATRIP – ATR-interacting protein
BIR – break-induced replication mode
bp – base pair
BPV1 – bovine papillomavirus type 1
Brca1 – breast cancer susceptibility protein
Brd4 – bromodomain-containing protein 4
BS – binding site
CBM – cyclin E/A-binding motif
Cdk – cyclin-dependent protein kinase
Chk1 – checkpoint kinase-1
Chk2 – checkpoint kinase-2
CIN – cervical intraepithelial neoplasia
CS – polyadenylation cleavage site
C-terminal – carboxyl-terminal
DDR – DNA damage response
D-loop – displacement loop
DNA-PK – DNA-dependent protein kinase
ds – double-stranded
DSBs – double-stranded breaks
dsDNA – double-stranded DNA
E. coli – Escherichia coli
E1BS – E1 Binding Site
E2BS – E2 Binding Site
FDA – Food and Drug Administration
FISH – fluorescent *in situ* hybridization
H2AX – H2A histone family, member X
HFK – primary human foreskin keratinocytes
HPV – human papillomavirus
HR – homologous recombination
HR-HPV – high-risk human papillomavirus
HSIL – high-grade squamous intraepithelial lesion
IF – immunofluorescence analysis
kbp – kilobase pair
LR-HPV – low-risk human papillomavirus
LSIL – low-grade squamous intraepithelial lesion
MCM2 – minichromosome maintenance protein 2
MO – minimal origin of DNA replication
MRN – Mre11-Rad50-Nbs1 complex
Myc – myelocytomatosis oncogene

Nbs1 – nibrin 1 protein
NES – nuclear export signal
NHEJ – non-homologous end joining
NIKS – normal immortalized keratinocytes from skin
NLS – nuclear localization signal
N-terminal – amino terminal
Oligomeric (HPV) genome – viral DNA concatemers containing several copies of the HPV genome
ORF – open reading frame
ori – origin of DNA replication
Pap – Papanicolaou test for cervical screening
PaVE – papillomaviruse episteme
pRB – retinoblastoma protein
PV – papillomavirus
qPCR – quantitative real-time PCR
RACE – rapid amplification of cDNA ends
Rad – radiation-sensitive protein
RCR – rolling circle replication
RDR – recombination-dependent DNA replication
RF – replication fork
RI – replication intermediate
RPA – replication protein A
SCC – invasive squamous cell carcinoma
Sp1 – specificity protein 1
ss – single-stranded
SSB – single-stranded break
ssDNA – single-stranded DNA
TopBP1 – topoisomerase II β-binding protein 1
TSS – transcription start site
URR – upstream regulatory region
VLP – virus-like particle
wt – wild type

1. INTRODUCTION

Human papillomaviruses are small DNA viruses that have been identified as etiological agents for the induction of cancer in humans. Papillomavirus extrachromosomal genome or integrated subgenomic fragments have been found in cervical, vaginal, vulvar and anal carcinoma biopsies and also in head, neck and throat cancer tissues. HPV types that infect mucosal tissue are classified into high- and low-risk groups depending on the ability to progress from benign lesions to malignant neoplasias. Infection by low-risk HPV types is frequently asymptomatic or causes the formation of benign tumors, such as warts or condylomas. In most cases, the virus infection is eventually taken care of by the immune system. In rare cases, a persistent HPV infection is established, in which case immune responses are altered by the virus oncoproteins, and the immune system is not sufficient to remove either the virus or HPV-infected cells. This type of infection is established by HPV types that belong to the group of high-risk HPVs (HPV16, 18, 31, 33, 45, 52, etc.).

Papillomaviruses are masters at hijacking host cell replication machinery as well as cellular DNA damage response pathways to amplify viral genomes. The co-localization of homologous recombination as well as non-homologous-end-joining effector molecules to HPV DNA repair/recombination centers indicates the important role of cellular DNA damage response pathways in repairing the double-stranded DNA breaks that occur during viral DNA replication. The unlicensed replication mode characteristic of HPVs often causes the collision of replication forks. Unfinished DNA replication products are then recognized by cellular DNA damage response pathways and subjected to DNA repair. While most double-stranded breaks are repaired properly, the generation of larger than unit-sized HPV molecules (oligomers) as well as integration into host cell genomic material may be the result of failed attempts to repair DNA damage.

Integration of HR-HPV sequences into host cell genomic material is believed to be one of the critical events in the formation of cancer cell. Usually, HPVs integrate such that the regulatory role of the viral E2 protein to the viral oncogenes E6 and E7 is disrupted, allowing the E6 and E7 proteins to take control of regulating cellular growth by inhibiting cellular p53 and pRB family proteins. In addition, integration of HPV sequences into cellular DNA may disrupt genes that are responsible for the control of cell growth, thereby releasing the inhibitory role of the gene products, which can subsequently lead to the uncontrolled growth of a cell. The co-occurrence of episomal and integrated forms of HPV genomes has also been detected in cancer tissues. In these cases, the expression of replication proteins from episomal molecules may initiate DNA replication from an integrated HPV origin of replication, thereby amplifying both the integrated HPV sequences as well as the flanking cellular sequences. Depending on the location of the integration site, the amplification of cellular sequences may contribute to genomic instability. If the integration occurred in close proximity to a cellular oncogene, amplification of this

sequence may lead to the overexpression of the oncoprotein, which can again result in the uncontrolled growth of the cell.

Papillomaviruses have been studied for decades after Harald zur Hausen proposed that HPV infection may be the cause of cervical cancer. PVs are quite species- and tissue-specific, and the first objective of PV research was to find a suitable *in vitro* system through which different functions of the viral life cycle could be analyzed. A breakthrough was achieved when it was discovered that bovine papillomavirus type 1 (BPV1) genes can be expressed and genomes can be replicated in the mouse fibroblast cell line C127. Our research group has also been involved in studying the mechanisms of DNA replication of the BPV1 genome, and the results have been widely recognized and considered a major contribution to the field (Männik et al., 2002; Piirsoo et al., 1996; Ustav and Stenlund, 1991; Ustav et al., 1993). Since the organization of papillomavirus genomes is quite conserved, the information gained from BPV-1 studies, such as insights into the basic mechanisms of viral gene expression, DNA replication and the regulation thereof, can be extrapolated with caution to the majority of papillomaviruses. The study of human papillomaviruses took off when cell lines that stably maintain the PV genome as an extrachromosomal molecule were derived from patients infected with high-risk HPV types (W12-E, CIN612-9E). Furthermore, various immortalized epithelial cell lines have been isolated that support the replication of transfected HPV genomes. Despite these advances, the need for a more robust system to study papillomaviruses and to screen potential anti-HPV drugs encouraged us to continue searching for a cell line that is easy to culture and supports HPV genome replication. After HPV genomes were introduced into the human osteosarcoma cell line U2OS, the results of gene expression and DNA replication were compared to analogous studies made in keratinocytes. Surprisingly, we found that gene expression, episomal DNA replication and maintenance in U2Os cells are essentially the same as in keratinocytes. This encouraged us to further explore the use of this cell line for HPV studies. We discovered that U2OS cells are suitable to monitor HPV transient DNA replication, active mRNA transcription and translation into functional proteins. Since no therapeutic drugs against HPV infection have been marketed, we believe that U2OS cells may have a promising future in screening for chemical compounds that would reduce HPV replication or viability.

2. LITERATURE REVIEW

2.1 Papillomaviruses

Papillomaviruses are small, non-enveloped, species-specific, epitheliotropic, double-stranded DNA (dsDNA) viruses that belong to the family *Papillomaviridae*. In addition to humans, they infect other mammals (such as rabbits, rodents, bovines, horses, etc.), birds and reptiles. According to the diversity of the nucleotide sequence of the major capsid protein L1, more than 200 types of human papillomaviruses (HPV) have been classified into 5 genera: Alpha-, Beta-, Gamma-, Mu- and Nupapillomaviruses (α, β, γ, μ, and ν), which infect different regions of epithelial tissue (Bernard et al., 2010; Bzhalava et al., 2015; de Villiers et al., 2004).

HPVs infect the basal layer of epithelial tissue through microabrasion, and the infection is usually asymptomatic, but in some cases causes epithelial lesions (papillomas) such as warts on skin (β-HPVs) and condylomas on mucosa (α-HPVs). A variety of the HPVs that infect cutaneous tissue, such as HPV1 and HPV2, are often found in healthy skin samples and are therefore considered as a part of the normal microbiota of human skin. Under appropriate conditions, they may develop into a variety of clinically insignificant benign epithelial growths, such as warts or papillomas. However, certain other β-HPVs, such as HPV5 and HPV8, have been linked to non-melanoma skin cancer in immunocompromised and immunosuppressed patients with the rare hereditary disease *Epidermodysplasia Verruciformis* (EV) (Accardi and Gheit, 2014). HPV types infecting mucosal tissue (α-HPVs) are divided into high- and low-risk groups according to their ability to cause lesions that can progress into malignant neoplasias. Low-risk HPV infections cause discomfort, but overall the disease is not life threatening, and the infection will be eliminated by the immune system. By contrast, the 19 types that are classified as high-risk (HPV16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73 and 82) (Gheit and Tommasino, 2011) can establish persistent infections and have been closely linked to the development of cervical cancer, with HPV16 being the predominant high-risk type worldwide. In addition to cervical cancer, HPV16 and HPV18 have also been found in 70% of analyzed vaginal, 40% of vulvar, and 84% of anal carcinoma biopsies, as well as in head, neck, and throat cancer cells (De Vuyst et al., 2009).

Progression from a premalignant lesion to invasive cancer usually takes years to occur, indicating that cancer development is a multistep process. Although preventative cervical screening methods like Pap smear have been developed to detect potential pre-cancerous or cancerous cells in the cervix, HPV infection is not usually detected early enough to intervene at the right time. Therefore, cervical cancer is a serious health problem, especially in developing countries. It is also the second most common cause of cancer-related mortality in women worldwide. Currently, no therapeutic drugs have been marketed to cure HPV infection, and the vaccines that have been developed against nine

different HPV types have various limitations. Gardasil was the first preventative vaccine developed; it offers protection against four HPV types (HPV6, HPV11, HPV16, and HPV18) with partial cross-protection against HPV31 and HPV45 and was approved by the Food and Drug Administration (FDA) in 2006 (De Vincenzo et al., 2013; Markowitz et al., 2007). Cervarix, licensed in 2007, was the second vaccine developed and is a bivalent vaccine that provides protection against high-risk HPV16 and HPV18 (Harper et al., 2004). The third vaccine, Gardasil 9, was approved by the FDA in 2014 and protects against 9 HPV types: HPV6, HPV11, HPV16, and HPV18 which were also the targets to the first generation of Gardasil, plus HPV31, HPV33, HPV45, HPV52, and HPV58, which are responsible for the remaining 20% of cervical cancers. To date, HPV vaccines have been marketed in more than 100 countries. However, not all of the countries have included the vaccines in national vaccination programs. As vaccination is quite expensive, this often prevents widespread use of the vaccines. Vaccination is recommended for girls at a young age, before the onset of sexual activity. Women up to 45 years of age may benefit from vaccination, but the overall effectiveness is quite low, as the vaccines only help women who have not already been infected with HPV prior to vaccination. For this reason, the FDA has not approved administration of the vaccine to all women up to age 45.

2.2 Papillomavirus genome organization and viral proteins

Papillomaviruses have circular double-stranded DNA genomes approximately 8 kbp in length that typically contain 9 to 10 open reading frames (ORFs). The 8 ORFs located in the early region of the viral genome encode non-structural proteins, and the two ORFs located in the late region encode structural proteins (Fig. 1). The viral genome also contains a non-coding upstream regulatory region (URR) that is about 1 kbp in size and contains *cis* elements for DNA replication initiation and gene transcription.

The E1 protein is an ATP-dependent DNA helicase that binds to the E1 binding site (E1BS) in the URR region and is responsible for melting DNA (at viral origin) and unwinding the double helix during replication fork progression (Sedman and Stenlund, 1998; Seo et al., 1993; Yang et al., 1993). The E1 and E2 proteins are two viral factors needed for DNA replication. They are also responsible for recruiting cellular replication factors to replicate viral DNA (Chiang et al., 1992; Ustav and Stenlund, 1991; Wilson et al., 2002; Yang et al., 1993). The E1 protein can be divided into three functional regions. The C-terminal half of the protein carries an ATPase/helicase domain that can self-assemble into hexamers and interact with the E2 protein and with the cellular polymerase α-primase and topoisomerase I (Clower et al., 2006; Masterson et al., 1998; Sun et al., 1998; Titolo et al., 1999). The origin-binding domain is located in the hinge region of E1 and is required for dimerization and binding to the viral origin (Titolo et al., 2003). The N-terminal half of E1 has a regulatory role and is required for efficient viral DNA replication *in vivo* but can be dis-

pensable *in vitro*, as the C-terminal half of the protein together with the origin binding domain is sufficient to catalyze viral DNA replication (Amin et al., 2000). The N-terminal part of E1 also includes *cis* elements that regulate the nucleocytoplasmic shuttling of E1 *in vivo*; such as a bipartite nuclear localization signal (NLS), a Crm1-dependent nuclear export signal (NES), a cyclin E/A-binding motif (CBM), and specific Cdk2 phosphorylation sites (S92 and S106 in HPV31 and S89, S93, and S107 in HPV11) (Deng et al., 2004; Fradet-Turcotte et al., 2010; Ma et al., 1999; Yu et al., 2007).

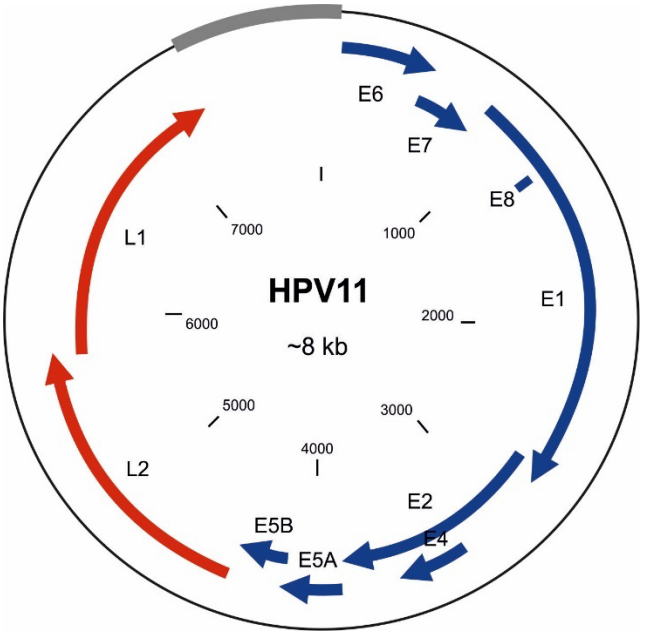

Figure 1. Schematic presentation of HPV11 genome. Genes from the early coding region are marked with blue arrows, genes from the late coding region are marked with red arrows, and the non-coding upstream regulatory region is designated with a grey box.

E2 is a multi-functional protein that has important roles in transcription regulation, in the initiation of viral DNA replication, in genome segregation during mitosis, and in nuclear retention. Depending on its concentration and configuration, E2 can act as either a transcription activator or a repressor. Full-length E2 protein at moderate concentrations acts as a transcription activator, but at higher concentrations, it acts as a transcription repressor (Abroi et al., 1996). Truncated E2, called E2C (also E8^E2), lacks a transactivation domain and is a replication and transcription repressor (Chiang et al., 1991; Lim et al., 1998; McBride et al., 1991). However, the heterodimer composed of both the full-length and truncated forms of E2 is a replication activator, meaning that a single

transactivation domain is enough to activate replication and transcription (Kurg et al., 2006). The E2 protein is also a specificity factor for E1 during the initiation of DNA replication, as it helps E1 specifically recognize the E1BS in the URR region by simultaneous binding of E2 protein with E1 and the E2 binding site (E2BS) in the URR region (Bonne-Andréa et al., 1997; Sedman and Stenlund, 1995; Ustav and Stenlund, 1991; Yang et al., 1991). In bovine papillomavirus type 1 (BPV1) and HPV18, it has been shown that E2 is also responsible for segregation of the viral genome between daughter cells through simultaneous binding with E2BSs in the PV genome and with host cell mitotic chromatin during cell division (Piirsoo et al., 1996; Ustav et al., 2015).

The functions of the E4 protein are associated with the vegetative phase of infection, and this protein is highly expressed in the parabasal layer of infected tissue. E4 contributes to genome amplification and regulation of the viral life cycle, as it suppresses cell growth and arrests cell cycle progression at the G2/M phase (Doorbar, 2013; Nakahara et al., 2002). Also, in the presence of the E1^E4 fusion protein, the cellular cytokeratin matrix collapses, whereas nuclear lamins, tubulin and actin networks remain unaffected (Doorbar et al., 1991).

The E5 protein is a transmembrane protein that interacts with growth factor receptors in the cell surface and stimulates cell growth (DiMaio and Mattoon, 2001). E5 also contributes to host cell transformation and immune evasion by down-regulating the expression of cellular proteins involved in antigen presentation at the cell surface (Ashrafi et al., 2002, 2005, 2006; Campo et al., 2010). According to sequence analysis, not all HPVs encode the E5 protein and some HPVs encode two E5 proteins (e.g., HPV11, Fig. 1).

E6 is viral oncoprotein that prevents cell cycle blockage and apoptosis by inhibiting the activity of the tumor suppressor protein p53 (Scheffner et al., 1990).

E7 is also an oncoprotein and is responsible for driving the host cell into S-phase through the degradation of pRB family proteins (Boyer et al., 1996).

The L1 protein is the major capsid protein expressed during the vegetative phase of infection in terminally differentiated epithelial cells. The coding sequence of the L1 gene is considered as the most conserved region within papillomavirus genome, and therefore, PV classification relies on differences of this gene (de Villiers et al., 2004).

L2 is minor capsid protein that is also expressed during the vegetative phase of infection in terminally differentiated epithelial cells.

2.3 The HPV gene transcription

Identification and accurate mapping of each mRNA produced by HPV is crucial for understanding the roles of viral gene products during DNA replication and RNA transcription, as well as how the expression of these gene products are regulated. Full transcription maps of various HPV types have been collected in the PaVE database (http://pave.niaid.nih.gov/), and transcription maps for

HPV5 and HPV18 in the U2OS cell-line are also available (Sankovski et al., 2014; Toots et al., 2014).

Depending on the type of HPV, the HPV genome consists of either 9 to 10 ORFs. For initiation of mRNA synthesis, there are three conserved differentiation-specific promoter regions at the end of the URR region and within the E6 and E7 ORFs. For the addition of poly-A tails to the end of mature messenger RNAs, HPV genomes utilize two (early and late) polyadenylation cleavage sites. The early promoter (P90 for HPV11, P102 for HPV18, P97 for HPV16, and P99 for HPV31) is active in undifferentiated keratinocytes and serves as the initiation region for all early transcripts (Hummel et al., 1992; Smotkin et al., 1989; Wang et al., 2011). The early genes are translated from a polycistronic mRNA that is polyadenylated from the early polyadenylation site at the end of the early gene region (Hummel et al., 1992; Remm et al., 1999). The expression of different genes from polycistronic mRNA is regulated by splicing. In the early region of the HPV11 genome, there are 3 splicing donor sites and 3 splicing acceptor sites that in combination regulate the expression of all viral early proteins (Chiang et al., 1991; Chow et al., 1987a; Nasseri et al., 1987a; Rotenberg et al., 1989a, 1989b).

The late promoter (P674-714 for HPV11, P811 for HPV18, P670 for HPV16, and P742 for HPV31) is activated after the differentiation of the host cell and is situated just before the E1 start codon in the E7 ORF (Fig. 2, P674-714) (Chow et al., 1987a; Hummel et al., 1992; Ozbun, 2002; Wang et al., 2011). While the early promoter, P90, of HPV11 contains binding sites for the E2 protein within its sequence and is therefore regulated through the binding of the viral E2 protein, the differentiation-specific late promoter does not contain E2BSs and is E2-independent. Deregulation of the late promoter provides an environment to increase replication protein levels as well as amplify viral genome load by switching from theta replication mode to aggressive rolling circle replication mode. The late region genes encode viral structural proteins L1 and L2, which are expressed at the very end of the viral life cycle, just before capsid formation. Upon differentiation, use of the early poly-A site is reduced, and late mRNAs are polyadenylated from the late poly-A site and spliced from the late splicing sites.

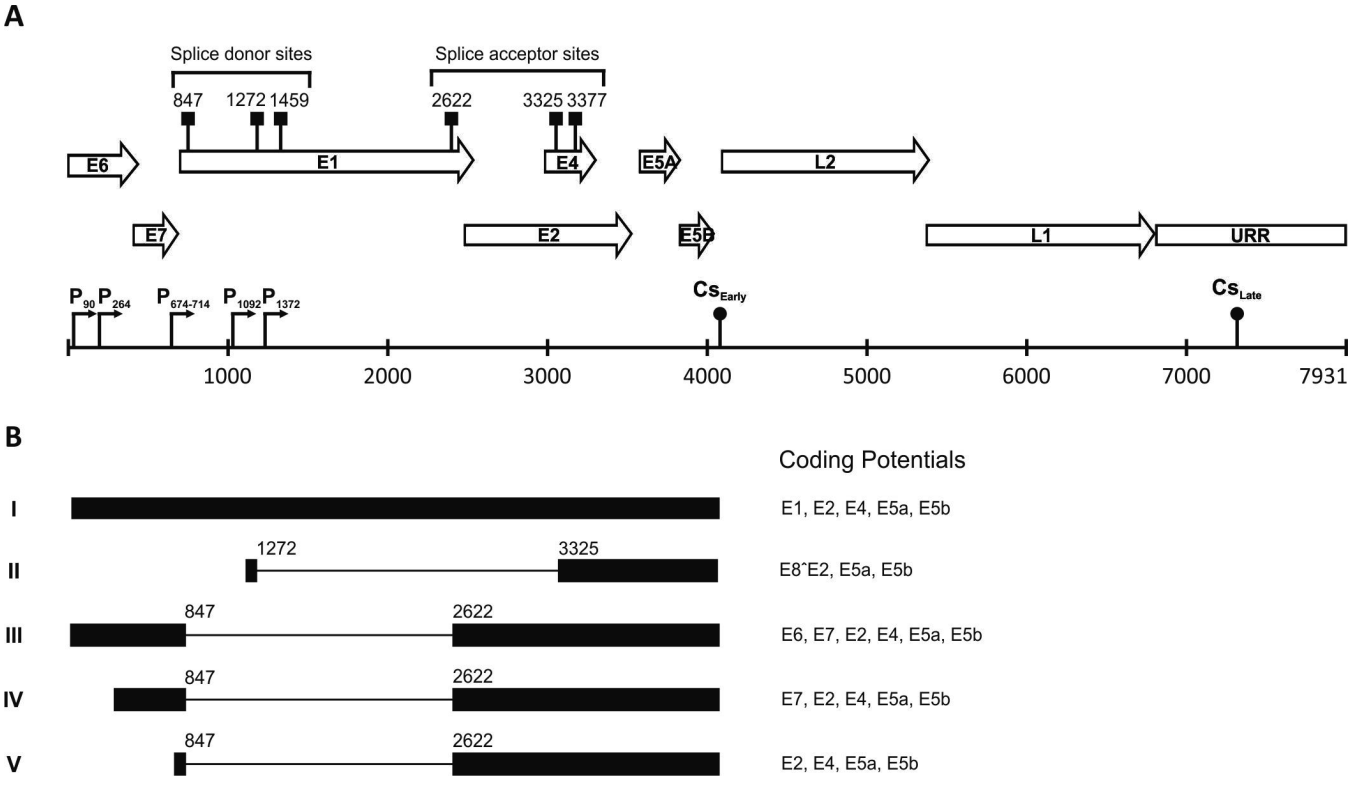

Figure 2. (**A**) A linear depiction of the HPV11 genome with ORFs, and URR, together with five promoter regions P90, P264, P674-714, P1092, P1372, two polyadenylation cleavage sites CS_{Early} and CS_{Late}, three splice donor sites at nt 847, nt 1272, and nt 1459, and three splice acceptor sites at nt 2622, nt 3325, and nt 3377. (**B**) A schematic presentation of HPV11 transcripts that potentially encode viral proteins E1, E2 and E8^E2 essential for viral DNA replication and regulation.

Chow *et al*. provided the first descriptions of HPV11 mRNA structures by characterizing HPV-specific mRNAs obtained from a human anogenital condyloma via electron microscopic R-loop mapping (Chow et al., 1987a). The results were further confirmed and amended by other groups using a variety of methods, including PCR (Chiang et al., 1991; Rotenberg et al., 1989a), cDNA cloning (Nasseri et al., 1987a), nuclease mapping (DiLorenzo and Steinberg, 1995; Smotkin et al., 1989), retrovirus-mediated gene transfer (Chiang et al., 1991; Rotenberg et al., 1989b), and RACE analysis (Renaud and Cowsert, 1996). Analyzing the mRNAs collected from an anogenital condyloma, Chow *et al*. found that the majority of the transcripts have their 5´ends located at the end of the E7 ORF, and only a few species have their 5´ends just upstream of the E6 ORF or within E1 ORF. Interestingly, 99.9% of the transcripts analyzed were polyadenylated in the early polyadenylation site, although the high copy number of the HPV11 genome and the use of the late promoter indicates that the infection might have reached the vegetative stage of the viral life cycle (Chow et al., 1987a). However, the late polyadenylation site in the end of the L2 ORF was clearly used. This agrees with the general observation that LR-HPVs produce very few virus particles and that large amount of the viral capsid proteins L1 and L2 are not needed. The most abundant transcript detected (98% of the cases) potentially encodes the viral fusion protein E1^E4, the functions of which are largely unknown. PCR analysis of HPV11 transcripts revealed that the E1^E4 protein could be encoded from two separate mRNAs: one ending at the early poly-A site and encoding only the E1^E4 protein and the other being a double-spliced late message for the major capsid protein L1, which also contains the entire coding region for the E1^E4 protein (Rotenberg et al., 1989a). The possibility of internal reinitiation during translation and the potential of this late mRNA to encode the E1^E4 protein and the L1 protein could explain the high frequency of the E1^E4 transcript in the lesion analyzed.

2.4 The HPV protein translation

In this chapter, the translation of HPV18 E1 mRNA is described. Because of the high similarity among HPV genome organization, it is assumed that other HPV types use similar translation strategies. Translation of the largest HPV gene product, the replication protein E1, is a complicated process, as it is translated from polycistronic mRNA that includes coding sequences for E6 and E7 in front of the E1 ORF (Fig. 2, species I) (Plumpton et al., 1995; Remm et al., 1999). According to the general paradigm, eukaryotic ribosomes translate the first coding sequence in mRNA most efficiently and continue at later cistrons with lower efficiency (Kozak, 1978, 1980). Although HPVs are quite conserved in terms of genome organization, there are some differences regarding the intron within the E6 ORF: HR-HPVs such as HPV16, HPV18, and HPV31 have an intron within the E6 ORF, whereas LR-HPVs such as HPV11 and HPV6 do not. However, splicing within this intron in the case of HR-HPV does not pro-

duce monocistronic mRNA because the E7 ORF remains unaffected, leaving the E1 mRNA in an at least bicistronic state (Remm et al., 1999; Smotkin et al., 1989). Interestingly, attempts to produce E1 protein from monocistronic mRNA in heterologous eukaryotic gene expression vectors have largely failed (Remm et al., 1999).

Classical eukaryotic mRNA is monocistronic, and scanning of mRNA starts from the ultimate 5′ end and does not continue after the stop codon. However, translation of polycistronic mRNA is also possible, although this occurs at a lower efficiency (Kaufman et al., 1987). A variety of mechanisms have been proposed for the translation of polycistronic mRNAs in other small viruses. Ribosomal frameshift is a mechanism for the translation of overlapping retrovirus proteins (Jacks et al., 1988). In cauliflower mosaic virus (Fütterer et al., 1993) and adenovirus (Yueh and Schneider, 1996), a ribosome jumping mechanism has been proposed in which the ribosome moves among different mRNA regions without scanning. Reinitiation or internal initiation has been proposed for the translation of non-overlapping picornavirus genes (Meerovitch and Sonenberg, 1993) and hepatitis B virus (Chang et al., 1989), and leaky scanning has been suggested to ignore the weak initiation codons for the translation of the hepatitis B virus P gene (Fouillot et al., 1993).

Results of experiments with HPV18 reported by Remm *et al*. indicated that a discontinuous scanning mechanism drives the production of the E1 protein (Remm et al., 1999). In addition to other experiments that excluded some of the unusual scanning mechanisms of E1 polycistronic mRNA (e.g. read-through, and reinitiation), Remm *et al*. generated a series of mutant E1 plasmids with false ATG codons at increasing distances from the E1 start codon to determine how far in front of the E1 gene ribosomes scan mRNA. The nearest mutation of the real E1 ATG codon (45 nt away) had only a slight effect on levels of the E1 protein, and other false ATG codons further away from the E1 gene had no effect on the translation of the E1 gene, indicating that mRNA scanning is discontinuous and happens only in front of the E1 gene (Remm et al., 1999).

The E2 protein of papillomaviruses is translated from messages that lack the entire E1 ORF, but depending on the promoter that is used during mRNA transcription, it may contain intact E6 and E7 ORFs in front of the E2 coding sequence (Fig. 2, species III-V). If the early promoter in front of the HPV11 E6 ORF is used, the corresponding E2 mRNA will contain two exons: the first exon is composed of coding sequences for the E6 and E7 proteins, and the second exon is composed of the full-length E2 ORF (Fig. 2, species III) (Chow et al., 1987a; Rotenberg et al., 1989b). The splicing from nt 847 to nt 2622 completely disrupts the E1 ORF. Mutating the splice acceptor site at nt 2622 disrupts coding of the E2 protein, demonstrating that splicing is essential for the expression of E2 (Rotenberg et al., 1989b). Furthermore, deletion of the E2 AUG start codon at nt 2723 demonstrated that this codon is indeed required for the functional expression of E2 (Hirochika et al., 1987). The full-length E2 protein can also be translated from mRNA in which transcription is started from the differentiation-dependent promoter in front of the E1 ORF (Fig. 2, species V).

In that case, the splicing pattern is the same as in the previously described E2 mRNA, but the E6 and E7 ORFs are absent (Chow et al., 1987a; Rotenberg et al., 1989b). The lack of the N-terminal transactivation domain in the truncated form of E2 (E8^E2, also known as E2C) makes it an important inhibitor of HPV transcription and DNA replication (Chiang et al., 1991; Lim et al., 1998; McBride et al., 1991). The transcription of E8^E2 starts from a poorly described promoter region within the E1 ORF, which is then spliced from nt 1272 to nt 3325 such that two exons are generated: the first exon includes the small E8 ORF, and second exon includes the E2 C-terminal DNA binding/dimerization domain (Fig. 2, species II) (Chiang et al., 1991; Rotenberg et al., 1989a). The truncated form of the E2 protein competes with full-length E2 for DNA binding, which effectively downregulates DNA replication (Zobel et al., 2003).

2.5 Papillomavirus life cycle

2.5.1 Epithelial tissue complexity and its close relationship with the HPV life cycle

The stratified epithelium is a very complex tissue composed of several cell types, including small numbers of melanocytes, Langerhans cells and Merkel cells, as well as differentiating keratinocytes (85% of the cells) that form layered sheets depending on the status of differentiation. The life cycle of human papillomavirus is tightly coupled to the differentiation process that the hosts, i.e., keratinocytes, undergo within the stratified epithelium (Fig. 3). There are two types of undifferentiated proliferating keratinocytes in the basal cell compartment, a few slow-cycling stem cells and transiently amplifying dividing cells that are the initial targets for PV infection and establishment of latent infection. To interact with basal proliferating keratinocytes, HPVs must first reach the basement membrane of the epithelium. An interaction between the carboxy-terminal part of the HPV L1 protein and host cell heparan sulfate and laminin 5 receptors is required for the effective infection of HPV (Giroglou et al., 2001; Joyce et al., 1999). Virus entry into the host cell is mediated via clathrin-dependent endocytosis (Day et al., 2003). Release of the HPV genome from the endosome as well as genome transport to the nucleus seem to be important additional roles of the viral L2 protein (Richards et al., 2006). After invading the cell nucleus, the HPV genome must undergo a few rounds of unlicensed replication to quickly increase the viral genome copy number. As soon as persistent infection is established, papillomaviruses switch to a stable maintenance phase in which viral genomes replicate in concert with host cell DNA and are partitioned to daughter cells. In these cells, viral gene expression is kept at very low levels that are sufficient to replicate and maintain viral genomes, enhance cellular proliferation, and evade host immune defenses. When the basal cells divide, the daughter cells either remain in the basal layer and continue dividing or move upwards and begin the process of differentiation, which eventually

leads to complete stratification and to cell death. In the cells that differentiate, the third phase in viral infection is switched on; in this phase, viral late gene expression is activated and vegetative viral DNA amplification is initiated. Herein, large numbers of viral genomes are synthesized for packaging into viral capsids. One obstacle with this strategy is that HPVs need to synthesize viral DNA in differentiated cells, which normally exit the cell cycle upon differentiation. HPV-positive suprabasal cells are able to override normal checkpoint controls and remain active in the cell cycle by expressing the E6 and E7 viral oncoproteins, which interact with host cell regulatory proteins, allowing for the synthesis of cellular replication factors that are necessary for viral DNA replication. It is believed that an additional important function of the E7 oncoprotein is the re-activation of host cell DNA replication machinery to support viral genome replication in terminally differentiated keratinocytes (Cheng et al., 1995).

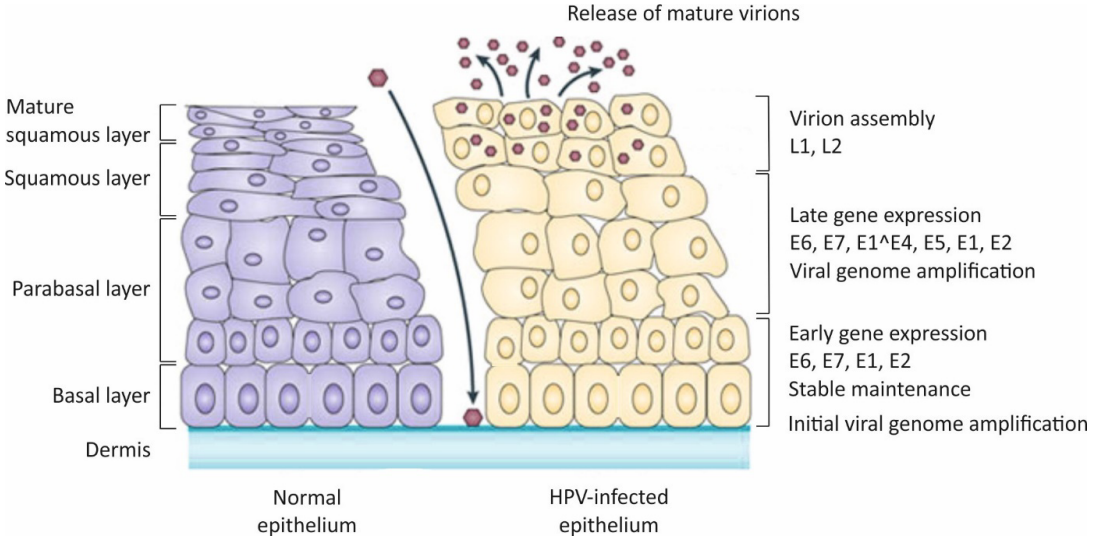

Figure 3. Simplified overview of the course of HPV infection in stratified epithelium. Healthy epithelium is shown on the left with keratinocyte differentiation stages. HPV-infected epithelium is shown on the right, and the viral life cycle phases that are dependent on host cell differentiation are indicated. HPVs infect keratinocytes in the basal layer of the epithelium through microabrasions. After entering the host cell nucleus, the viral early genes are expressed, and the viral genome is amplified to establish a persistent infection. During the stable maintenance phase, viral protein expression is downregulated, and viral genomes are replicated in synchrony with host cell genetic material and divided almost equally between two daughter cells. After the differentiation of an HPV-positive cell, the vegetative phase of infection is switched on. The expression of HPV oncoproteins alter cell cycle control and push a cell that normally would exit the cell cycle into S phase, allowing viral genome amplification. In the uppermost layers of the epithelium, viral structural proteins are expressed, viral genomes are encapsidated, and virions are released to the environment. The figure is adapted from (Moody and Laimins, 2010).

2.5.2 Triphasic replication model for papillomavirus genome replication

Depending on the differentiation status of infected cells, replication of the papillomavirus genome can be characterized by three fundamentally different mechanisms: first, transient viral genome amplification replication; second, a stable viral genome maintenance replication and third, vegetative amplification replication.

2.5.2.1 Transient amplification replication

After invading to host cell viral genetic material is released into the host cell nucleus. The viral early promoter in the upstream regulatory region is activated, and the viral early proteins needed for cell transformation (E6 and E7) and for viral genome replication (E1 and E2) are expressed. To establish successful papillomavirus infection, the viral DNA copy number is quickly increased to approximately 50 to 100 copies per cell (Hebner and Laimins, 2006).

Studies of different papillomaviruses have demonstrated that the E1 and E2 proteins are the only viral proteins required for the initiation of DNA replication from the viral origin, which is located in the non-coding upstream regulatory region (URR) (Ustav and Stenlund, 1991; Ustav et al., 1991). The HPV viral replication origin contains four E2-specific palindromic motifs (5′-ACCGNNNNCGGT-3′, which corresponds to the E2 binding site, E2BS) and an adjoining A/T-rich sequence containing a binding site for the E1 protein (Piirsoo et al., 1996; Ustav et al., 1993, 1991). The minimal origin (MO) for replication initiation requires an A/T-rich sequence and one adjoining E2 binding site or two E2BSs alone (Lu et al., 1993; Sverdrup and Khan, 1995). Initiation of viral DNA replication relies on the capacity of the E2 protein to specifically bind to the E2BS at the origin of replication and to simultaneously interact with the viral E1 helicase (Abbate et al., 2004; Androphy et al., 1987). The E1 protein is recruited to the viral origin as a dimer, which is facilitated by E1-E2 complex formation (Fig. 4) (Frattini and Laimins, 1994; Mohr et al., 1990; Sedman and Stenlund, 1995). When E1 binds to the E1BS at the A/T-rich segment in the URR, the E2 protein is displaced, and E1 forms a double-trimer complex (Sanders and Stenlund, 1998). The E1 double-trimer melts the dsDNA at the origin of replication, after which a double-hexamer is formed around each DNA strand (Fig. 4) (Schuck and Stenlund, 2005). The resultant E1 double hexamers are needed throughout the replication process as ATP-dependent replication helicases to unwind the DNA double-helix ahead of replication fork progression (Schuck and Stenlund, 2005; Wilson et al., 2002).

During the nonproductive phase of infection, viral DNA replication is initiated bidirectionally via theta structures (replication intermediates that resemble the Greek letter “theta” – θ) at the replication origin. In this process, two replicating forks move away from the origin to drive DNA synthesis in the 5′ to 3′ direction, with continuous synthesis of the leading strand and discontinuous synthesis of the lagging strand (Okazaki fragments) (Fig. 5) (Auborn

et al., 1994; Flores and Lambert, 1997). On a circular DNA molecule, the synthesis is terminated 180 degrees opposite of the initiation site, resulting in two daughter molecules, each of which has one strand from the parental DNA molecule and a second newly synthesized strand (Auborn et al., 1994; Flores and Lambert, 1997; Gilbert and Cohen, 1987; Yang and Botchan, 1990).

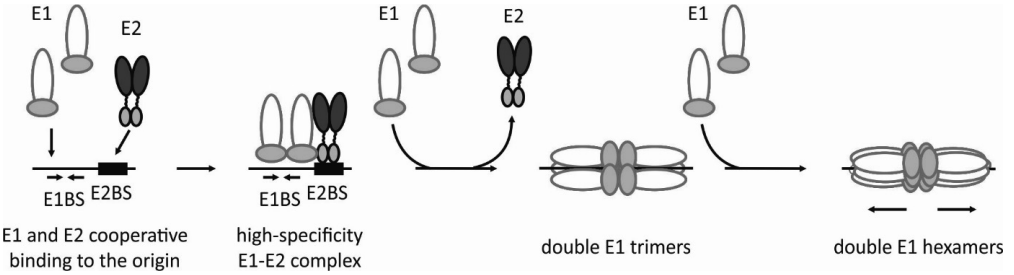

Figure 4. A schematic overview of viral initiation complex assembly. Cooperative binding of the E1 and E2 proteins to the E1 and E2 binding sites (E1BS and E2BS) within the URR region results in the formation of a high-specificity E1:E2 complex. Adjacent E1 proteins are recruited to the complex such that double E1 trimers are formed and E2 proteins are released. Additional recruitment of E1 proteins is required for the conversion of the E1 trimers into double E1 hexamers, which have DNA helicase activity. The figure is adapted from (Kurg, 2011).

Since papillomaviruses are highly dependent on cellular replication machinery for doubling their genetic material, the E1 hexamers also serve as platforms for the assembly of host DNA replication factors such as DNA polymerase α/primase, replication protein A (RPA), and topoisomerases I and II (Chow and Broker, 1994; Clower et al., 2006; Masterson et al., 1998; Park et al., 1994; Titolo et al., 1999; Wilson et al., 2002).

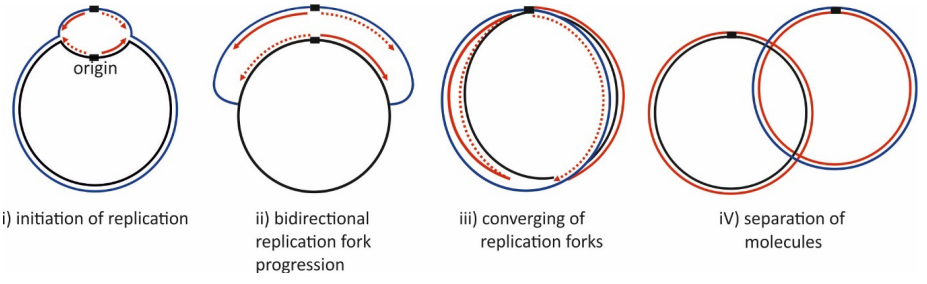

Figure 5. Simplified overview of bidirectional theta replication mode. Replication is initiated with the assembly of two replication forks at the origin of replication. The bidirectional progression of the replication forks converges 180 degrees opposite the initiation site, at which point the daughter molecules are separated and the replication process is finalized.

2.5.2.2 Stable viral genome maintenance replication

The initial amplification replication stage that occurs during the establishment of infection is believed to be rapid, and as soon as infection is ensured, papillomaviruses switch into the latent phase of infection. During latent infection in the basal and parabasal layers of the epithelium, the expression of viral replication proteins is downregulated, and the viral genome is replicated simultaneously with host cell genetic material (once per cell cycle). During mitosis, HPV genomes are attached to mitotic chromatids to ensure the effective segregation of synthesized HPV genomes into daughter cells. The partitioning of HPV genomes is mediated by the viral E2 protein, which simultaneously binds with E2BSs at the HPV URR region and with mitotic chromosomes to link viral genomes to host cell genetic material during cell division (Ustav et al., 2015).

The latent phase of infection has been extensively studied in the HPV-positive cell lines W12-E and CIN612-9E, which were both isolated from naturally infected human tissues (Bedell et al., 1991; Stanley et al., 1989). Comparative studies of HPV replication during the latent phase of infection in W12-E and CIN612-9E cells revealed contradictory results: in the HPV16-positive W12-E cell line, a once-per-S-phase replication mode was observed, whereas viral DNA replication occurred via a random-choice mode in the HPV31-positive CIN612-9E cell line (Hoffmann 2006). In some cases, the replication of HPV31 genomes in CIN612-9E cells was initiated more than once per S-phase, but some genomes replicated only once, and there was a fraction of viral genomes that failed to replicate. The random-choice replication mode in W12-E cells occurred when E1 protein levels increased, indicating that over-expression of the E1 protein is enough to overcome the restriction of once-per-S-phase replication during the latent phase of HPV16 infection (Hoffmann et al., 2006). When HPV16 and HPV31 genomes were transfected into the HPV-negative keratinocyte cell line NIKS, the random-choice replication mode was observed (Hoffmann 2006). The random-choice replication mode of HPV16 indicates that HPVs are capable of replicating by both replication mechanisms and that the mode of replication depends on E1 protein level and on the cells that harbor HPV episomes.

2.5.2.3 Vegetative amplification replication

When an infected basal cell divides and one of the daughter cells detach from the basal membrane, differentiation begins. Normally, these cells exit the cell cycle and stop dividing. However, cells that are infected with HPV continue the cell cycle, even those in the uppermost layers of stratified epithelia. This continued cycling is mediated by the viral oncoproteins E6 and E7, which alter cell cycle regulation and push cells into active cycling. These proteins therefore ensure that the host cell replication factors that are needed for viral DNA replication are continuously synthesized. During the vegetative phase of infection, viral episomes are amplified to high copy numbers, presumably through multi-

ple rounds of replication in S-phase-arrested cells (Hoffmann et al., 2006). The high load of HPV episomes initiates the expression of the late capsid proteins L1 and L2, and single HPV genomes become encapsidated in viral particles (Frattini et al., 1996). The newly formed virions are then shed into the environment when the top layer of epithelium degrades.

The mechanisms underlying the switch from the latent phase of infection to the vegetative phase are largely unknown. However, the processes that take place during the vegetative phase can be monitored *in vitro* under certain culture conditions that mimic the three-dimensional architecture of the stratified epithelium. The productive phase of HPV16 and HPV31b infection can be studied in the naturally infected keratinocyte cell lines W12-E and CIN612-9E, respectively. Suspending the cells in methylcellulose, using high-calcium medium or culturing the cells as organotypic rafts are three methods that induce host cell differentiation *in vitro*, allowing the extensive study of the final processes that occur during the vegetative phase of infection. It has been suggested that a switch in the mode of DNA replication occurs during the productive phase of infection: in differentiated HPV16-positive W12-E cells, a switch to unidirectional rolling circle replication (RCR) has been suggested (Flores and Lambert, 1997). A study of replication intermediates (RIs) of HPV16 genome replication produced during the productive phase of infection indicated that DNA replication in this phase does not follow the bidirectional replication mode that is present during the initial genome amplification and latent phase of infection. Indeed, DNA structures detected via 2D AGE analysis correspond to the rolling circle replication mode (Flores and Lambert, 1997). Bubble and double Y-shaped DNA RIs, which are characteristic products that result when two replication forks move away from each other in bidirectional replication mode, were absent, and only Y-shaped replication intermediates, which are characteristic of a unidirectional replication fork, a hallmark for rolling circle replication mode, were present (Flores and Lambert, 1997). The same replication mode has been presumed to occur in the differentiated HPV31-positive CIN612-9E cell line (Flores and Lambert, 1997). The switch to unidirectional replication mode may be a solution for escaping the unfavorable conditions that occur after host cell differentiation. While the theta replication mode requires initiation in every round of DNA replication, in rolling circle replication, one initiation event leads to the generation of multiple daughter DNA molecules. This method optimizes the use of already limiting levels of cellular factors for the generation of large amounts of viral genomes.

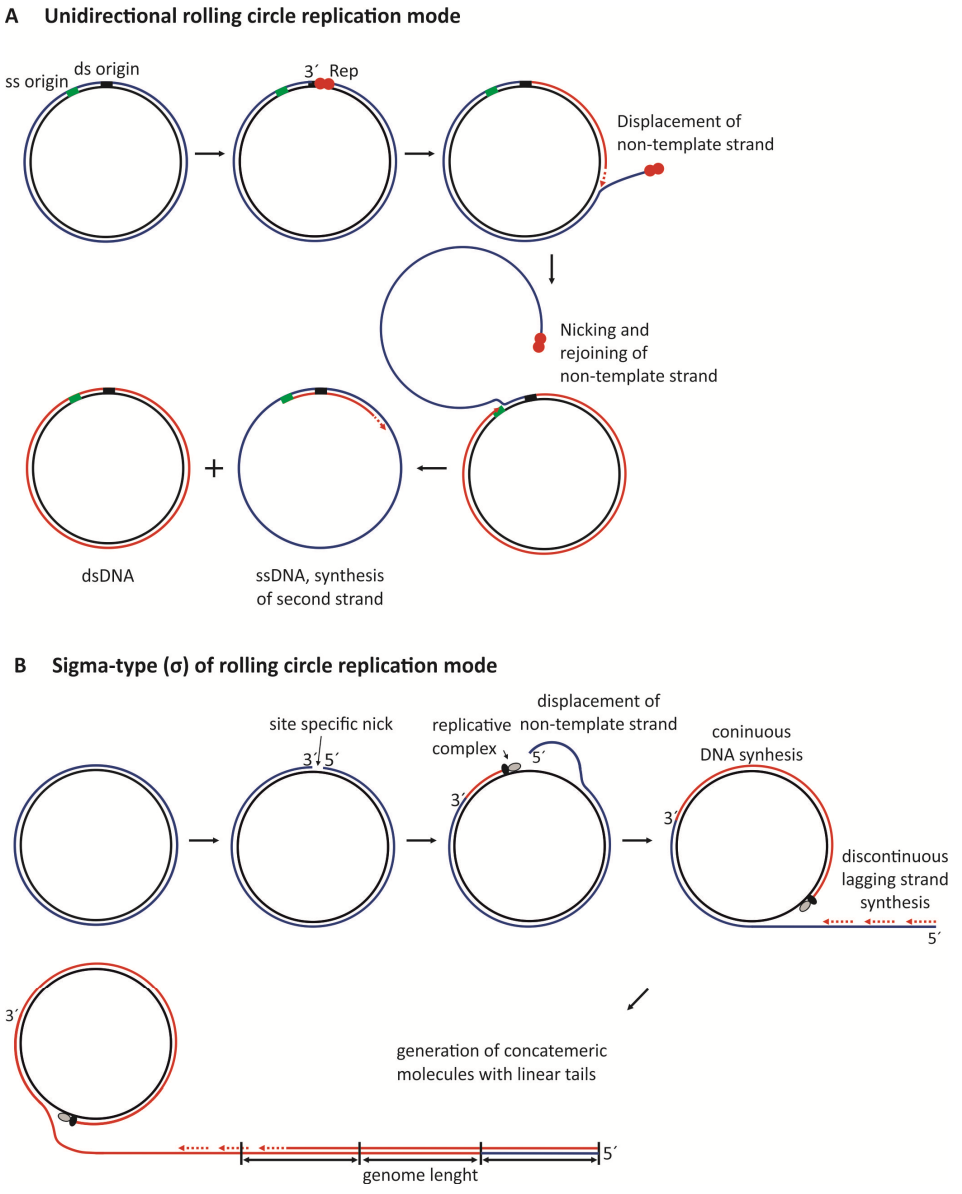

Figure 6. (**A**) Simplified overview of unidirectional rolling circle replication mode. Replication is initiated at the double-strand (ds) origin with a site-specific nick. The free 3′ end serves as a primer for DNA synthesis, while the unnicked strand serves as a template. Unidirectional replication proceeds with the simultaneous displacement of the non-template strand around the plasmid. Once the replication machinery reaches the initiation site, the displaced single strand is nicked at the 5′ end and recircularized. Leading-strand synthesis is completed when the replication complex returns to the ds origin. Replication is then initiated at the single-strand (ss) origin of the displaced strand. (**B**) Simplified overview of sigma-type rolling circle replication. The free 3′ end that serves as a primer for the replicative complex is generated by a site-specific nick.

The leading strand is synthesized with the simultaneous displacement of the non-template strand. Upon reaching the initiation site, leading-strand synthesis is not terminated but proceeds for several rounds, generating long concatemeric tails. Discontinuous synthesis of the lagging strand in concert with leading-strand synthesis converts the newly synthesized ssDNA strand into dsDNA.

Rolling circle replication mode is common for circular bacterial plasmids and ssDNA viruses. Unidirectional RCR requires one replication complex, which is formed on ssDNA through a site-specific nick (by the specific initiator protein Rep) of the dsDNA at the replication origin (Fig. 6A) (Ruiz-Masó et al., 2015). The free 3′-OH DNA end generated through the nick serves as a template for the replication complex, which extends the strand by moving from the 5′ to 3′ direction, with the simultaneous displacement of the non-template strand. The replication fork proceeds until the initiation/nicking site is reached, after which the leading-strand synthesis is terminated and the non-template strand is released from the newly synthesized molecule. For lagging-strand synthesis, the released ssDNA strand is used as a template, and dsDNA synthesis is initiated from a specific ssDNA replication origin (Ruiz-Masó et al., 2015).

Sigma-type (σ-type) RCR generates multimeric linear dsDNA tails and can quickly amplify viral genomes. This replication mode is used during bacteriophage λ late replication (Bastia and Sueoka, 1975). In this mode, leading-strand synthesis is not terminated upon reaching the nick site but proceeds for several rounds, generating long concatemeric dsDNA tails (Fig. 6B). The lagging strand is synthesized simultaneously with the leading strand; otherwise, replication intermediates with ssDNA tails would be generated.

2.6 Overview of cellular DNA damage response pathways

Eukaryotic cells harbor two major DNA damage response (DDR) pathways to maintain intact genomic material during the duplication of their genetic material. Activation of the DDR can originate from two types of DNA breakage: double-stranded and single-stranded breaks (DSBs and SSBs, respectively). DSBs activate the ataxia-telangiectasia mutated (ATM)-dependent pathway, which is characterized by the phosphorylation of ATM and its downstream effector kinase Chk2. SSBs, however, lead to activation of the Rad3-related (ATR) pathway, which results in activation of the ATR kinase and phosphorylation of its cognate downstream effector kinase Chk1. Upon their activation, the signal transduction of both checkpoint pathways triggers the activation of hundreds of proteins, primarily by phosphorylation at Ser/Thr-Glu motifs, that block cell cycle progression and facilitate DNA repair (Abraham, 2001; Maréchal and Zou, 2013; Shiloh, 2001).

2.6.1 The ATM signaling pathway

The ATM signaling pathway stalls the cell cycle to repair DSBs though two basic mechanisms: homologous recombination (HR) and non-homologous end joining (NHEJ). Which of these repair mechanisms is used depends on the phase of the cell cycle and on the nature of the DSB. HR is an "error-free" repair mechanism that is primarily used when a DSB is caused by a replication blockage in late S or G2 phase. NHEJ is an "error-prone" repair mechanism that is used when DSBs occur in packed chromosomes (Shrivastav et al., 2008). Since NHEJ is active during the entire cell cycle, the preference for HR during S and G2 phase is assured by different regulatory mechanisms. For example, one regulatory element between the HR and NHEJ pathways is the Mre11 endonuclease, which is responsible for resectioning the DNA 3´ end at a DSB to activate HR and repress NHEJ (Dimitrova and de Lange, 2009; Shibata et al., 2014). It is believed that when the homologous recombination pathway becomes compromised and cannot complete DNA repair, the repair process is switched to NHEJ.

One reason why DSBs occur in S phase is replication fork collision during DNA replication. These breaks can be repaired via HR when sister chromatids, which sequences can be used as a template are available (Shrivastav et al., 2008). The homologous recombination pathway is initiated when the MRN complex (Mre11-Rad50-Nbs1), which functions as a sensor for DSBs, registers a DNA break and resections the 3´ DNA end of the DSB through Mre11 endo-nuclease activity (a crucial step in HR) (Dimitrova and de Lange, 2009; Shibata et al., 2014). Rad50 provides a protein scaffold that maintains the proximity of the two resectioned DNA ends (Hopfner et al., 2000; Williams and Tainer, 2005). Subsequently, Nbs1 activates ATM kinase (Difilippantonio et al., 2005), which leads to the phosphorylation of a number of proteins, such as Brca1, Chk2, and p53, which mediate the effects of ATM on DNA repair, cell-cycle arrest, apoptosis, and other downstream processes (Maréchal and Zou, 2013). When DNA repair via HR is chosen, the resectioned ssDNA end invades the homologous sequence and through the mediation of a number of proteins (e.g., Rad51) forms a displacement loop (D-loop) structure (Fig. 7) (Krejci et al., 2012). Finally, the replication complex is recruited onto the D-loop structure, DNA replication is initiated, and the break is repaired. The HR process takes time but results in the reconstitution of the original sequence (Thompson and Schild, 2001).

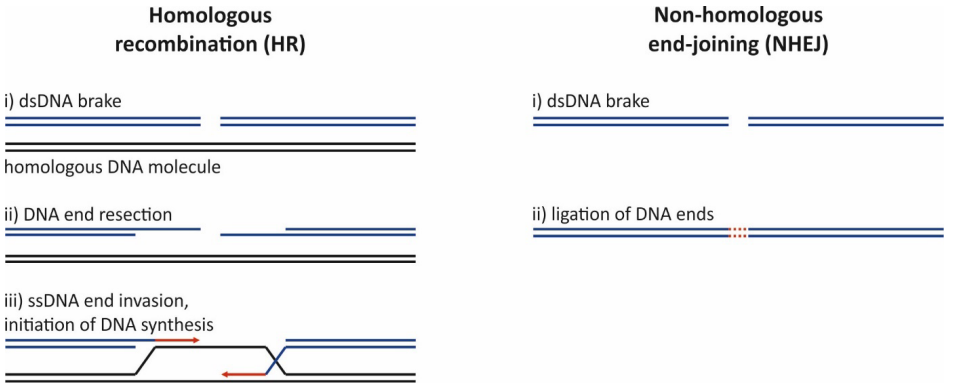

Figure 7. Simplified overview of the two fundamentally different ATM pathways used to repair double-stranded DNA breaks. In homologous recombination, the resectioning of broken DNA ends and the alignment of the broken DNA with homologous sequences results in the reconstitution of the original sequence. In non-homologous end joining, the broken DNA ends are aligned and ligated together with no need of homologous sequences.

Non-homologous end joining is used when DSBs result from the activity of endogenous factors (e.g., nucleases) or exogenous factors such as oxidative stress and ionizing radiation. In such cases, "clean" DSBs with complementary overhangs are available, and NHEJ can simply align and ligate two DNA ends together. NHEJ is a rapid process and functions throughout the cell cycle, as it does not require a homologous chromosome (Moore and Haber, 1996; Sonoda et al., 2006). However, when complementary overhangs are not available and DNA ends cannot be precisely rejoined, NHEJ typically generates small deletions or sometimes small insertions by modifying the broken DNA ends (Shrivastav et al., 2008). Therefore, NHEJ may be the cause of genomic mutations and can also cause genomic instability over time (Lieber, 2008).

The NHEJ pathway is activated when a Ku70/80 heterodimer binds with DSBs that occur during G0, G1 or in early S phase (Lieber, 2008). Once the Ku-DNA complex is formed, the conformation of the Ku70/80 heterodimer changes to promote interactions with other key components (nuclease, polymerases and ligase) (West et al., 1998). After the formation of Ku:DNA end complex Ku interacts with endonuclease complex Artemis-DNA-PKcs. The autophosphosylation of DNA-PKcs triggers the conformational change in DNA-PKcs that regulates the access and also the endonuclease activity of Artemis to the DNA overhangs (Goodarzi et al., 2006; Meek et al., 2004; Uematsu et al., 2007). To complete repair process of the broken DNA end via NHEJ, the polymerases μ and λ, and the ligase complex of XLF-XRCC4-DNA ligase IV are recruited to the DNA end repair complex (Ahnesorg et al., 2006; Lieber, 2008). Since, NHEJ repairs DSBs through various scenarios, the enzymes that drive the NHEJ pathway have to be flexible in function.

2.6.2 The ATR signaling pathway

To ensure that each DNA replication origin is used only once per cell cycle, eukaryotic cells have acquired several origin licensing mechanisms. If the DNA re-replication is initiated during S phase and the accumulation of ssDNA or ssDNA-dsDNA junctions occurs, the ATR signaling pathway is activated (Liu et al., 2007). The quick response of the ATR checkpoint implies that ATR detects abnormal chromosomal structures at the initial steps of DNA re-replication and suppresses re-replication immediately after it is activated. The ATM checkpoint pathway, however, is activated at a much later stage, presumably after the ATR-mediated pathway has become compromised. The inhibition of re-replication is very important to avoid re-replication forks from running into leading forks, as it generates unfinished DNA fragments that are highly recombinogenic and may cause chromosomal rearrangements (Liu et al., 2007).

RPA-coated ssDNA is the key structure that recruits the ATR-ATRIP (ATR interacting protein) complex to damaged DNA. In response to these events, ATR phosphorylates not only its effector kinase Chk1 but also many other downstream proteins, such as p53, Brca1, and Rad17, which collectively inhibit DNA replication or mitosis and promote DNA repair and recombination (Zou and Elledge, 2003). ATR also directly inhibits DNA re-replication by phosphorylating the RPA2 and MCM2 proteins, which are essential for DNA unwinding and during DNA replication initiation and elongation (Liu et al., 2007). The phosphorylation of the RPA2 subunit prevents RPA from localizing to replication centers, and the replication initiation and elongation by RPA is subsequently compromised (Olson et al., 2006; Vassin et al., 2004). MCM2 helicase activity is inhibited when ATR site-specifically phosphorylates MCM2 at S108 in response to multiple forms of DNA damage and stalling of replication forks (Cortez et al., 2004).

2.6.3 Recombination-dependent DNA replication mode

Recombination-dependent DNA replication mode (RDR, also known as break-induced replication [BIR] in yeast) can be associated with sigma-type rolling circle replication (Weller and Sawitzke, 2014a). RDR couples a unidirectional replication mode and the homologous recombination pathway, providing a mechanism for the maintenance of chromosome ends in budding yeast and in other eukaryotes that lack telomerase activity (Llorente et al., 2008). Prokaryotes with linear DNA also use RDR to finish replicating linear DNA ends. RDR replication has been characterized in *E. coli* (Kuzminov, 1995), in bacteriophage SPP1 (infects *Bacillus subtilis*) (Lo Piano et al., 2011), during the bacteriophage T4 late replication step (Kreuzer and Brister, 2010; Liu and Morrical, 2010), and in herpes simplex virus (Weller and Sawitzke, 2014b).

RDR also contributes to the rescue of collapsed replication forks (RFs) that generate dsDNA breaks with ssDNA overhangs. RDR is initiated by the invasion of a single strand into a homologous DNA molecule, followed by the

assembly of anew replication fork, after which DNA replication is initiated. Normally, the RF is disassembled and the elongation of DNA replication is finished when the break is repaired, but in RDR, the end of the invading single strand can be extended for hundreds of kilobases and DNA synthesis can proceed until the end of the chromosome is reached (Malkova and Ira, 2013).

The initial steps in the induction of RDR overlap with the induction of the HR repair pathway in many aspects. Recombination-dependent replication of dsDNA breaks requires a free 3′ end to trigger the initiation of leading-strand synthesis. Similarly to HR, the resectioning of free 3′ DNA ends is mediated by Mre11 nuclease (in eukaryotic cells) (Shibata et al., 2014). The resultant 3′-ssDNA overhangs act as substrates for strand invasion mediated by RecA in bacteria or by Rad51 in eukaryotes and ultimately form a D-loop structure (Asai et al., 1993; Davis and Symington, 2004; Malkova et al., 2005). The mechanisms driving replication fork assembly and DNA synthesis have been studied in great detail in bacteria. In bacteria, the D-loop structure is recognized by the mediator protein PriA, and with the help of other proteins, the replicative helicase dnaB and the primase dnaG are loaded onto the D-loop structure (Marians, 2000). Once the helicase and primase are loaded, the polymerase Pol III is recruited, and DNA synthesis of the leading strand is initiated. The lagging strand is synthesized synchronously with the leading strand (Kreuzer, 2000). In eukaryotes and yeast, the process of replication fork assembly is not well understood. Initial studies of BIR in yeast have shown that almost all of the factors that are essential for regular replication initiation in S-phase as well as for unwinding of the DNA double helix (Mcm2-7) are also needed in BIR (Lydeard et al., 2010). Only the components that are exclusively needed for formation of the pre-replication complex (cdc6 and Orc) are dispensable in BIR (Lydeard et al., 2010).

2.7 The connection between HPV replication and the cellular DNA damage response

Cellular DNA damage response pathways are often activated by active viral DNA replication as replication intermediates are sensed as aberrant DNA molecules (Lilley et al., 2005; Stracker et al., 2002). Additionally, various viral proteins induce the DNA damage response and produce replication stress by interfering with cell cycle regulation (Bester et al., 2011). Viruses have adapted mechanisms to address such host responses and intervene or inactivate cellular pathways that might hinder viral DNA replication. Likewise, viruses are also able to take advantage of host defenses, such as the ATM and ATR pathways, in various aspects of viral DNA replication (Chaurushiya and Weitzman, 2009).

In human papillomaviruses, the activity of the E1 protein is sufficient to induce cell cycle arrest and activate cellular DDR pathways (Fradet-Turcotte et al., 2011; Sakakibara et al., 2011). The ATPase/helicase and origin-binding functions of E1 and its nuclear accumulation cause early S-phase arrest and

activate the DNA damage response, specifically the ATM pathway (Fradet-Turcotte et al., 2011). To prevent cell cycle arrest during the maintenance phase of the viral genome in undifferentiated cells, E1 has to be maintained in the cytoplasm, being transported to the cell nucleus only when it is required for DNA replication (Fradet-Turcotte et al., 2010, 2011; Yu et al., 2007). The formation of the E1-E2 protein complex balances the induction of the DDR by E1, as E2 reduces the nonspecific binding of E1 but does not prevent E1-induced cell cycle arrest. In ATM-deficient cells, in which activation of the DDR pathway is compromised, E1 still inhibits the cell cycle (Fradet-Turcotte et al., 2011). It seems that the capacity of E1 to trigger cell cycle arrest and activate DDR pathways are two mechanistically distinct events. Interestingly, inhibition of the ATM pathway has no effect on viral DNA replication during the latent phase of HPV infection, although activation of the ATM pathway can be clearly detected (Moody and Laimins, 2009). More importantly, although the ATM pathway is dispensable during the latent phase, it is essential for amplification of the viral genome during the vegetative phase of infection; inhibition of the ATM pathway completely blocks HPV genome amplification in differentiated cells (Moody and Laimins, 2009). While some viruses, such as adenovirus, delocalize or degrade components of the MRN complex (Stracker et al., 2002), HPV maintains them at high levels throughout the vegetative phase (Moody and Laimins, 2009). The active phosphorylation of Chk2 (an important transducer of the ATM signaling pathway), Nbs1 and Brca1 (both of which play important roles in DNA repair and in regulating S and G2 checkpoints) as well as the co-localization of these effectors to HPV replication foci indicates the important role of the MRN complex during the vegetative phase of HPV infection. In differentiated cells, where the levels of cellular components needed for DNA replication are limited, the activation of DNA repair mechanisms is very beneficial, as all the components required for DNA synthesis are then delivered to viral recombination/replication foci. Additionally, activation of the ATM pathway induces S or G2/M cell cycle arrest, which provides an environment that allows multiple rounds of viral genome re-replication.

The importance of homologous recombination proteins in active HPV DNA replication centers has raised the question what mode of replication occurs during the vegetative phase of replication. Although clear evidence shows that there is a switch from bidirectional theta replication to unidirectional replication after host cells are induced to undergo differentiation (Flores and Lambert, 1997), mechanisms other than rolling circle replication may be involved in the duplication of HPV genomes during the vegetative phase of infection. Several HPV replication intermediates that are characteristic to recombination-dependent replication give rise to the possibility that homologous recombination-related DNA replication may play a role in HPV DNA duplication during the vegetative phase of infection (Sakakibara et al., 2013). RDR is a combination of unidirectional replication and homologous recombination, which govern the conditions that occur during the vegetative phase of infection. Additionally, the possibility to extend DNA replication for hundreds of kilobases makes RDR an

efficient and attractive way for viruses to quickly amplify their genetic material. For circular DNA molecules, RDR can give rise to concatemeric DNA multimers that must later (in the case of HPV) be processed into unit-sized circular genomes. Oligomeric forms of the HPV genome have been shown to be present in various transiently transfected cell lines (Orav et al., 2013), and HPV oligomers have also been detected in clinical samples obtained from patients with permanent HPV infection (Boshart et al., 1984; Cullen et al., 1991; Dürst et al., 1985; Orav et al., 2013). The involvement of recombination-dependent replication in the production of HPV oligomers is suggested to occur as the combination of heterogenic HPV molecules within the formed oligomers was observed (Orav et al., 2013).

The possible connection between the non-homologous end joining repair pathway and HPV DNA replication is still controversial. While the ATR factor DNA-PK does not exhibit significant co-localization to HPV genome replication centers (Gillespie et al., 2012), clear co-localization of the Ku70/80 heterodimer to HPV replication foci has been detected (Kadaja et al., 2009). Because the homologous recombination pathway seems to be the most prevalent pathway during S and G2 phase, NHEJ may serve as an alternative for the repair of DSBs when HR is somehow compromised. Additionally, because NHEJ is an "error-prone" mechanism that may generate rearranged genomes, it is probably beneficial for HPV to not use this pathway for the repair of DNA breaks.

In addition to the ATM pathway, the ATR pathway also responds to DNA replication stress and is likely to be involved in HPV genome replication. The key components of the ATR pathway, namely, ATR-interacting protein (ATRIP) and topoisomerase IIβ-binding protein 1 (TopBP1), have been shown to co-localize at HPV replication centers during initial amplification of the viral genome (Kadaja et al., 2009; Reinson et al., 2013). Additionally, localization of the DNA damage response marker γH2AX to viral replication centers indicates the possibility that the ATR pathway is activated.

2.8 Physical state of HPV DNA during infection and the progression from normal virus infection to cervical cancer

Permanent HPV infection is the most significant event in the malignant transformation of human cervical epithelium. The progression from benign cellular changes to invasive cancer is graded from the formation of a low-grade squamous intraepithelial lesion (LSIL, cervical intraepithelial neoplasia CINI) to a high-grade squamous intraepithelial lesion (HSIL, CINII and CINIII). The progression from stage CINIII to squamous cell carcinoma (SCC) exclusively occurs as the result of an untreated infection. However, not all HSILs progress to invasive cancer: approximately 35% of HSILs undergo complete regression in a time frame of 4-6 months (Melnikow et al., 1998; Trimble et al., 2005). The physical state of the HPV genome in these lesions is highly dependent on the type of HPV and on the differentiation stage of the infected tissue (Hudelist et

al., 2004). Generally, normal infection proceeds through the episomal HPV molecules, and the virus does not need to integrate into the host genome to be fully functional. In normal epithelium and in CINI/CINII stage tissues, the HPV genome exclusively exists as an extrachromosomal molecule. However, in stage CINIII tissues, subgenomic parts of HR-HPV genomes are often integrated into the host chromosome. In HPV18 and several other HR-HPVs (-31, -33, -52b, and -58), the progression to cervical cancer is highly dependent on the integration step (Hudelist et al., 2004), as cells carrying integrated HR-HPV gain a growth advantage due to cell immortalization (Jeon et al., 1995; Romanczuk and Howley, 1992). At the same time, HPV16 genomes are generally found both in an episomal form and in a mixed episomal/integrated form and are rarely found only in an integrated form in cervical biopsy probes (Arias-Pulido et al., 2006).

In cancer cells, the HPV genome is found integrated into the host chromosome in a way that approximately half of the HPV genome is lost. Extensive mapping of the integration locus of HPV16 has revealed that the viral genome is primarily disrupted within the HPV16 E2 hinge region (Arias-Pulido et al., 2006). Disruption of the E2 ORF releases the repressor activities of this gene product on the E6 and E7 oncogenes. The increased expression of the E6 and E7 proteins, which bind and inactivate p53 and pRB-family proteins, leads to a growth advantage of the affected cells (Jeon and Lambert, 1995; Jeon et al., 1995; Romanczuk and Howley, 1992). In HPV16, however, where strict integration occurs in a low percentage of tumors and the viral genome typically exists as both an episomal and integrated form, the E2 protein may regulate E6 and E7 expression in *trans*, and a growth advantage of cells carrying only integrated forms of the HPV16 genome may not be detectable (Arias-Pulido et al., 2006). Using a W12-E culture model, Pett et al. demonstrated the coexistence of episomal and integrated HPV16 DNA in the same cell during early passages and the subsequent loss of episomal plasmids in later passages (Pett et al., 2006). The loss of E2-expressing HPV plasmids resulted in a growth advantage and in the spontaneous selection of cells containing only integrated HPV16 (Pett et al., 2006). Similar results were obtained with cells carrying a single copy of integrated HPV33 (Peitsaro et al., 2002).

LR-HPVs, like HPV6 and HPV11, do not tend to integrate into cellular DNA and have only been detected in an episomal form regardless of lesion grade.

The integration locus on the host chromosome has also been the focus of several studies. Analysis of cervical tumor probes revealed that HPV integration sites are randomly distributed over the whole genome, with a clear preference for genomic fragile sites (Thorland et al., 2000; Wentzensen et al., 2004). Fragile sites are transcriptionally active sites, intragenic regions or gene-dense regions that, under appropriate culture conditions or exposure to certain chemical agents (e.g., aphidicolin, a DNA polymerase inhibitor), exhibit a tendency to form chromosome or chromatid gaps and breaks or other chromosomal abnormalities. The lack of replication origins in very long gene areas frequently cause

collisions of replication machinery, and cells often proceed to mitosis with incompletely replicated regions of DNA. In G2 phase, there needs to be a mechanism to keep chromatin open to DDR pathways to finish DNA replication/repair. The localization of the cellular chromosome-associated protein Brd4 (bromodomain-containing protein 4) to fragile sites is believed to mediate the accessible chromatin environment (Jang et al., 2014). Brd4 is also an important cellular partner for papillomavirus E2 protein. The interaction of the E2 protein with Brd4 regulates the transcriptional activity of E2 and is also required to ensure the tethering of PV genomes to host mitotic chromosomes to ensure the persistence of viral episomes in infected cells (Baxter et al., 2005; Kurg et al., 2006; You et al., 2004). In addition to the genome partitioning function of the E2-Brd4 interaction, it is believed that this complex also tethers viral replication foci adjacent to fragile sites (Jang et al., 2014). The association with fragile sites gives another opportunity for the virus to recruit the cellular DDR pathway, which is already activated by the replication stress occurring in fragile sites, to amplify viral genomes. However, by replicating its genome in the proximity of unstable chromosome regions, the virus greatly increases the chances of accidental integration of viral DNA into these sites.

The human genome contains a number of hotspots for HPV integration. For example, the region of the c-Myc gene at chromosomal position 8q24 is one site where HPV frequently integrates (Couturier et al., 1991). Structural alterations and/or overexpression of the c-Myc gene occur with high frequency in cervical carcinomas (Ocadiz et al., 1987; Riou et al., 1984), and amplification of the c-Myc gene in the HPV16-immortalized keratinocyte cell line resulted in an increased growth rate compared with the parental W12-E cell line (Crook et al., 1990). MYC overexpression has been related to co-amplification of the Myc gene and integrated HPV sequences (Peter et al., 2006). A low level of MYC overexpression has been characterized in HeLa cells harboring integrated HPV18 sequences in chromosome band 8q24 (Dürst et al., 1987; Lazo et al., 1989). A decrease in the levels of the myc gene product was observed in HeLa cells when an integrated HPV18 fragment located ~500 kbp downstream of the myc gene was knocked out, supporting the hypothesis that integration of HPV influences MYC expression via a long distance chromatin interaction (Zhang et al., 2016).

2.9 Multiple HPV infections in cervical neoplasias

Multiple human papillomavirus genotypes often coexist within infected epithelia and are frequently detected together in samples of various grades of cervical neoplasia. For women, age is one of the most important factor associated with multiple HPV infection (Antonsson et al., 2014). There is a U-shaped curve for HPV prevalence versus age, with the highest multiple infection rate being found among women 55 or older, followed by women younger than 25 (Wang et al., 2016; Yip et al., 2010). In contrast, the single HPV infection rate is highest

among women who are 25-34 years old (79%) (Wang et al., 2016). Multiple infection among young women might result from high levels of sexual activity and has also been associated with the ongoing development of HPV-type-specific acquired immunity (Kjaer et al., 2000; Schiffman and Castle, 2003). Among older women, fluctuations of menopausal hormones along with decreased immunity and diminished ability to eliminate and inhibit the virus may together play an important role in multiple HPV infection (Althoff et al., 2009).

A study of the prevalence of multiple infections with HR-HPV genotypes found that in 65% of the cases in Northern China, the most common arrangement of multiple infection was HPV16 in combination with other HR-HPVs (Wang et al., 2016). Single HPV infection was found in 25% of the samples. The frequency of multiple HPV infections in Northern China were highest in inflammatory cervical samples (41.0%), followed by cervical cancer (15.7%), CINI (7.2%), and CINII–III (1.2%) (Wang et al., 2016).

The clinical importance of multiple infections in the progression of cervical neoplasia remains a controversial area of investigation. In some studies, these infections have been associated with a high risk for the development of cervical cancer (Cuschieri et al., 2004; Herrero et al., 2005; Pista et al., 2011; Spinillo et al., 2009; Trottier et al., 2006), whereas other studies have reported no increased risk for CIN compared with single-type infections (Kay et al., 2003; Levi et al., 2002; Muñoz et al., 2003; Sandri et al., 2009; Wang et al., 2016).

Due to the limited technical capabilities for identifying multiple infections in the same cell, double infection cases have thus far only been identified in the same tissue probe. However, under laboratory conditions, the expression of HPV replication proteins in cell lines harboring integrated HPV sequences induces overamplification of the genomic locus where the HPV origin has been integrated (Kadaja et al., 2007). It is possible to show that transfected HPV circular genomes are not only replicating in HeLa and SiHa cells but also mobilize the integrated HPV origin for DNA replication, which leads to the induction of genomic rearrangements within the integrated locus. Overamplification can cause excisions, rearrangements and *de novo* integration of the replication products of integrated HPV and flanking cellular sequences.

Additionally, irrespective of the type of integrated HR-HPV, other types of HPV replication proteins, including LR-HPV types (HPV6b and HPV11), can also induce DNA replication from an integrated HPV replication origin (Kadaja et al., 2007). Therefore, integration of HR-HPV and episome loss may silence an HPV infection, allowing a cell to be re-infected by a new virus. The possibility of double infection of cells harboring integrated HPV sequences causing genomic instability may contribute to the formation of HPV-related cervical carcinogenesis and needs further investigation.

2.10 In vitro cell culture systems to study HPV gene expression and viral DNA replication

Papillomaviruses are extremely host- and tissue-specific and infect cells in the basal layer of stratified epithelia. The three-step life cycle of PV can be mimicked in organotypic cell culture models that undergo differentiation. These cultures are prone to spontaneous differentiation and are therefore quite variable in culture conditions; furthermore, maintaining the cultures is time consuming and very expensive. The lack of suitable cell culture systems has hindered the study of different aspects of human papillomavirus infection. Preliminary knowledge about papillomavirus gene expression, individual viral protein functions and viral DNA replication has been collected from studies of bovine papillomavirus type 1 in the mouse fibroblast cell-line C127, transformed by the BPV1 oncoprotein E5, which turned out to be a suitable model system (Law et al., 1981; Neary and DiMaio, 1989; Yang et al., 1985). Although there are differences in genomic organization and host preference, the overall structure of the papillomavirus genome is very conserved. Therefore, information gained from BPV1 studies, including the basic mechanisms of viral gene expression, the interactions of viral *cis* and *trans* elements (Abroi et al., 1996; Kurg et al., 2006; Piirsoo et al., 1996; Sedman et al., 1997), the modes of DNA replication and the regulation thereof (Männik et al., 2002; Ustav and Stenlund, 1991; Ustav et al., 1993), and the segregation to daughter cells (Piirsoo et al., 1996), can be taken as a general principle when studying HPV.

The development of virus-like particles (VLPs) for various viruses has offered the opportunity to study the initial steps of viral infection as well as interactions between virus and host cell for basic and clinical research. However, the development of infectious HPV pseudovirions in native organotypic cultures has had very little success. The methods used are technically demanding, time-consuming, and variable, and they produce relatively low virus yields (Dollard et al., 1992; McLaughlin-Drubin et al., 2003, 2004; Meyers et al., 1992). In recent years, methods to produce papillomavirus pseudovirions independent of epithelial cell differentiation have been developed (Buck et al., 2004; Pyeon et al., 2005). As a first approach, BPV1 capsid proteins were expressed from expression vectors in transiently transfected 293TT human embryonic kidney cells, and the reporter plasmids were encapsidated into bovine papillomavirus VLPs (Buck et al., 2004). However, there is a strong limitation on the size of a target plasmid, as only DNA ranging from 6 to 7 kbp in size can be packed into pseudovirions, which is much less than the natural viral genome size of approximately 8 kbp. A second approach enables the encapsidation of full-length papillomavirus DNA into HPV16 VLPs using the same 293T(T) cell line as the previous study (Pyeon et al., 2005). Pseudovirions resulting from this approach were highly infectious toward their natural host, epithelial cells: they were >1000 times more infectious than pseudoviruses isolated from the labor-intensive and time-consuming organotypic raft culture method. Why one of these approaches leads to the encapsidation of the full-length HPV genome or a

same-size reporter plasmid into virus particles and the other results in the packaging of a shortened genome is unknown. Presumably, either HPV16-based VLPs have different packaging constraints than BPV1-based VLPs or differences in the DNA being packaged contribute to differences in packaging efficiency (Pyeon et al., 2005). The development of HPV VLPs is crucial for understanding how papillomaviruses interact with receptors at the cell surface, enter the cell, and infect the cell, but applying this approach for other HPV research purposes has not been successful. Therefore, other methods (e.g., electroporation, lipofection) have been used to effectively transport viral DNA into cells in culture.

For HPV, a number of immortalized epithelial cell lines have been developed that enable short-term DNA replication studies and gene expression analysis. Monolayer epithelial cell lines containing integrated HR-HPVs, such as HeLa (HPV18-positive), SiHa (HPV16-positive) and CaSki (HPV16-positive) cells, were obtained from samples of naturally infected human cervix. HPV-negative cell lines, such as the C33A and 293 lines, that support the expression of HPV proteins and DNA replication from heterologous plasmids are all convenient and cost-effective model systems to identify the *cis* elements necessary for initiation of viral DNA replication and to study transient viral DNA replication and viral protein expression and function. Co-transfection of E1 and E2 expression plasmids and an URR-containing plasmid allowed monitoring of the effects of E1 and E2 to episomal URR plasmid replication as well as to integrated HPV URR (in HPV-positive cell lines) (Kadaja et al., 2007). However, expressing these viral proteins in near-native concentrations and monitoring of viral genome replication are still quite challenging in these cells.

Primary human foreskin keratinocytes (HFKs) are considered the most relevant native host cells for HPV infection and have therefore been widely used. Transfection of HFKs with different HR-HPV types results in a high frequency of cells that maintain viral genomes as extrachromosomal elements (Frattini, Lim et al. 1996; Frattini, Lim et al. 1997). The growth of these cells in organotypic cultures enabled the monitoring of differentiation-dependent expression of viral late genes, viral genome amplification, and virion biosynthesis. Ironically, however, these cells are extremely difficult to infect, as they have high levels of basal autophagy, which serves as a host defense pathway to inhibit HPV infection (Griffin et al., 2013). Additionally, HFKs should not be cultured for longer than 4 weeks, as significant morphological changes and growth arrest occur after 6 weeks (Griffin et al., 2014). Moreover, the isolation of primary human keratinocytes from various patients with different genetic backgrounds may produce different results with regard to HPV research.

Normal immortalized keratinocytes from skin (NIKS) is another physiologically relevant cell culture model that has been used to investigate HPV infection. NIKS are a spontaneously immortalized near-diploid cell line that has a normal growth and differentiation program (Allen-Hoffmann et al., 2000). As immortalized cells, establishment of genetically modified stable cell lines is possible. However, NIKS are quite difficult to culture compared to HFK or

HaCaT cells, as they require feeder cells and various growth factors to support healthy growth without spontaneous differentiation.

The spontaneously immortalized HaCaT cell line has been widely employed as a keratinocyte cell model due to its ease of use and near-normal phenotype. Under typical culture conditions, HaCaT cells have a partially to fully differentiated phenotype due to high calcium concentrations in standard media and in fetal bovine serum (Deyrieux and Wilson, 2007). While HaCaT cells might be useful for initial experiments, data obtained from these cells should be further confirmed with normal keratinocytes, such as NIKS or HFKs.

Two organotypic cell culture systems established from naturally infected human cervical tissues have become powerful tools for studying the stable and vegetative life cycle phases of the two HR-HPV types (HPV16, HPV31) that originally infected these cells. The first, HPV16-positive W12-E cell line was isolated from a low-grade cervical lesion (CINI) that in early passages maintains HPV16 genomes as extrachromosomal molecules at approximately 100 to 200 copies per cell and exhibits a typical keratinocyte morphology. But when grown in the flank of a nude mouse, an epithelial lesion with CINI/II histological features is formed (Stanley et al., 1989). Ultrastructural analysis of the lesion indicated that the tissue had stratified morphology and exhibited characteristics of differentiation. The lower layer of the lesion contained undifferentiated cells with normal nuclei, the upper layer contained condensed nuclei and keratin bundles, and the uppermost layer had lost cell nuclei (Flores and Lambert, 1997). Therefore, the W12-E cell line is a valuable culture system that represents an accurate keratinocyte differentiation model and thus allows monitoring of the carcinogenic progression of HPV16-associated infection. However, long-term cultivation of W12-E cells generally leads integration of the HPV16 genome, which ultimately results in the complete loss of episomal HPV16 genomes (Stanley et al., 1989). Therefore, to obtain reproducible results, the physical state of HPV16 episomes needs to be specified prior to any analysis.

The second organotypic cell line derived from a CINI lesion contains episomal HPV31b genomes and is called CIN612-9E. These cells differ from W12-E cells in that they do not stratify when maintained in high-calcium and high-serum medium (Flores and Lambert, 1997). However, when suspended in semisolid medium and grown at the air-liquid interfaces of *in vitro* collagen raft cultures, the cells differentiate and histologically resemble a low-grade cervical lesion (Bedell et al., 1991; Flores and Lambert, 1997). In the CIN612-9E cell line, the progression of HPV31b-associated infection can be followed, with a switch in the mode of replication being observed upon differentiation (Flores and Lambert, 1997). Amplification of the HPV31b genome is observed in distinct foci in the upper layer of the stratified epithelium, but no capsid protein synthesis can be detected, despite the presence of transcripts from the late region of HPV-31b (Bedell et al., 1991).

Due to the low oncogenic activity of LR-HPV E6 and E7 proteins, no immortalized LR-HPV-positive cell lines are available. Most knowledge of

low-risk HPV types has been gained by transfecting already immortalized human keratinocytes or other cell lines capable of supporting viral DNA replication and gene expression (Del Vecchio et al., 1992; Fang et al., 2006; Mungal et al., 1992; Thomas et al., 2001). The development of organotypic cultures carrying LR-HPV genomes has not been effective, as LR-HPVs do not immortalize cells *in vitro*, although they have been shown to increase the life span of infected cells (Thomas et al., 2001).

3. OBJECTIVES OF STUDY

The transformation of HPV-infected cell into a genetically unstable cancer cell is believed to be triggered by the viral integration to a host chromosome. The mechanisms underlying the integration of HPV DNA are not well understood, however, the generation of abberant DNA molecules during HPV DNA replication and the co-localization of DDR pathway key components to HPV DNA repair/recombination centers may play a role in this process. Moreover, viral DNA integration encompasses only a few viral genomes, while the majority remain extrachromosomal molecules. The co-occurrence of two forms of viral DNA in one cell is a real threat to genomic stability, as episome-derived replication proteins can initiate DNA replication also from the integrated HPV origin of replication.

Although HPV vaccines that successfully prevent infection of 9 HPV types have been developed, there remains an unmet need for anti-viral drugs that can cure women and men who are already infected with HR-HPV. Whilst, infection with LR-HPV seems less important due to its low oncogenic activity, it still causes women to suffer under discomfort, and therefore the development of drugs to treat the infection is also relevant. Currently, approximately 8 billion dollars are spent annually to cure HPV infection in the US alone. The development of anti-viral drugs has been hindered for years because of the lack of suitable and cost-effective cell culture systems that would enable the screening of thousands of chemical compounds that might target the vital functions of the virus. Currently available native organotypic keratinocyte cell culture models for the study of papillomaviruses are quite unstable, as they are very sensitive regarding the availability of feeder cells and various growth factors that can support healthy growth without causing spontaneous differentiation. It has been our goal for several years to develop a simpler system for the study of different papillomaviruses and for the future screening of chemical compounds. The general aim of our group has been to search for an HPV-negative monolayer cell line that is cost-effective, robust in culture, has high transfection efficiency and viability, and effectively replicates the HPV genome at near-native protein levels. Transfection of HPV genomes into previously used immortalized epithelial cell lines (e.g., HeLa, SiHa, CaSki, and HaCaT) did not meet this goal as very large quantities of transfected DNA are needed to monitor HPV genome replication at detectable levels.

The general objectives of this thesis were to further characterize the usefulness of the human osteosarcoma cell line U2OS for molecular studies of HPVs and to elucidate the factors surrounding genomic instability in HPV-positive cell lines in the presence of HPV replication proteins.

The specific goals of my studies were as follows:

1) To explore the consequences of host cell genomic DNA re-replication after the initiation of HPV E1 and E2 protein-dependent DNA replication from an integrated HR-HPV origin

2) To investigate the physical state of HPV16 and HPV18 genomes in patient clinical samples
3) To study the transient, stable and amplificational replication of HPV6b and HPV11 in U2OS cells and to produce and characterize stable cell lines harboring episomal HPV6b and HPV11 genomes
4) To compile a complete transcriptome map of HPV11 in U2OS cells, thereby providing additional reliability for the use of U2OS cell-based assays for HPV research
5) To elucidate the importance of the E6/E7 leader sequence in front of the HPV11 E1 ORF for the synthesis of stable and functional E1 protein

4. MATERIALS AND METHODS

The materials and methods used in this thesis are described in detail or referred to in the primary source within the associated research articles. As most of the experiments presented in this thesis were performed using U2OS cells, the cell line and methods used for handling the cells are briefly described herein.

The U2OS (originally 2T) line is derived from a moderately differentiated sarcoma of the tibia from a 15-year-old girl in 1964 and exhibits an epithelial cell-like morphology with functional tumor-suppressor genes p53 and retinoblastoma protein pRb, which are often mutated in cancer cell lines (Isfort et al., 1995; Ponten and Saksela, 1967; Wesierska-Gadek and Schmid, 2005). Characterization of oncogenes in U2OS cells revealed increased expression of the c-myc oncogene positioned in the 8q24 chromosome band (Isfort et al., 1995). U2OS cells are deficient in inhibition of cell division–even in crowded culture–possibly due to overexpression of the c-myc gene that causes uncontrollable expression of various genes, including genes involved in cell proliferation.

In Ref. I and II, U2OS cells were transfected with HPV genomes that were excised from bacterial vectors, religated, and concentrated. In Ref. III and IV, the method used to produce HPV genomes was changed, and minicircles of HPV plasmids were yielded using a previously established protocol (Kay et al., 2010). In brief, HPV genomes were inserted into a pMC.BESPX vector and transformed into *E. coli* strain ZYCY10P3S2T. The culture was grown until optimal density, after which an equal volume of induction mix was added. Subsequent inoculation for 5 h at 32°C allowed the bacteria to excise the bacterial backbone and religate the HPV genome. Plasmid DNA was extracted and gel purified to separate circular viral genomes from bacterial DNA.

Although U2OS cells are not the native host of HPV, transfecting the cells with HPV genomes allowed us to monitor transient viral DNA amplificational replication, and subsequent single-cell subcloning allowed us to monitor the stable maintenance of viral genomes. The third phase (vegetative phase) of the HPV life cycle can be monitored in cells that can be induced towards differentiation. Usually, in moderately differentiated cultures (such as U2OS cells), the vegetative phase of HPV infection cannot be induced. Interestingly, by culturing U2OS cells under dense culture conditions without splitting but with regular feeding, the unfavorable conditions do not trigger apoptosis. Instead, cell proliferation is slowed down, and the cell cycle is presumably stalled in G2 phase. These conditions turned out to be ideal for HPV to re-initiate genome replication multiple times, as the second amplification replication phase, which is characteristic of the vegetative phase of infection, was observed. It should be noted that viral structural proteins are not expressed in U2OS cells, and viral particles are not produced. However, the lack of infective viral particles provides additional laboratory safety while handling the cells.

5. RESULTS AND DISCUSSION

5.1 HPV replication proteins initiate replication from integrated HR-HPV replication origin and activate DNA damage response pathways (Ref. I)

The coexistence of a replicating viral episome and integrated HPV sequences containing the potential replication origin is a quite usual phenomenon in HPV16-positive cancer cells. Additionally, other HPV types like HPV18, HPV31, HPV33, HPV52b, and HPV58, which are often found integrated into host chromosomes, also have a short period of time when episomal and integrated HPV genomes coexist in the same cell. Integration of the functional HPV origin within the host cell chromosome is a serious threat to genomic stability. Under laboratory conditions, it has been demonstrated that episome-derived HPV replication proteins initiate replication from the integrated HPV origin, and the replication complex moves into the flanking cellular sequences up to 12.6 kbp in both directions from the HPV origin (Kadaja et al., 2007). Because papillomaviruses do not follow a once-per-cell cycle replication mode (Hoffmann et al., 2006; Ravnan et al., 1992), multiple unscheduled replication initiations from the integrated HPV origin may occur, resulting in rearrangements like deletions, duplications or even cross-chromosomal translocations of cellular DNA sequences by host cell repair/recombination machinery.

To investigate the possibility of cross-chromosomal translocation of an integrated HPV locus *in vitro*, we transfected SiHa cells with HPV16 or HPV18 genomes and performed single-cell subcloning. Herein, HPV genomes (instead of E1 and E2 expression vectors) were used to demonstrate that more physiological concentrations of replication proteins trigger DNA replication from the integrated HPV locus. Restriction analysis of total DNA from the subclones demonstrated *de novo* integration of newly synthesized replication products (Fig. 8E; Ref. I). Two HPV16-transfected subclones with new restriction patterns were identified: they represented the integration of either transfected HPV16 plasmid into the host chromosome or the excision and re-integration of the integrated HPV16 sequences (Fig. 8E, lanes 7-12; Ref. I). More importantly, a new HPV16-specific restriction pattern was observed in one subclone transfected with the HPV18 genome, indicating the re-location of integrated HPV16 sequences (Fig. 8E, lanes 16-18; Ref. I). Parallel Southern blot analysis with an HPV18-specific probe revealed that, similarly to HPV16, a newly synthesized replication product from episomal HPV18 genome is also probably integrated into the host chromosome (8F, lanes 4-6; Ref. I). FISH analysis of subcloned SiHa cells transfected with HPV18 E1 and E2 expression plasmids revealed a new integration site of integrated HPV16 with the entire q-arm of chromosome 13 (Fig. 7B; Ref. I), giving more evidence that episome-derived replication proteins induce genomic instability via replication initiation from the integrated HPV origin.

The process of how HPV integration is carried out is not well understood, but host cell DNA damage response pathways clearly play an important role in this event. Upregulation of the histone γH2AX is a hallmark of DNA double-strand breaks in eukaryotic cells. Co-transfection of HPV18 E1 and E2 proteins into HeLa cells considerably elevates the formation of the phosphorylated form of γH2AX, indicating to the activation of a cellular response to the DNA DSBs generated by replication from the integrated HPV origin (Figure 6A, panel c, lane 1; Ref. I). Co-localization of ATRIP to the HPV replication foci indicates the presence of RPA-coated ssDNA (Fig. 5; Ref. I). However, the weak co-localization of phosphorylated Chk1 (S317) to HPV replication centers (Fig. 5; Ref. I) might refer to the poor activation of the ATR pathway, which is not sufficient to prevent the re-replication of integrated HPV sequences. This might be due to the limited targets available in HPV repair/recombination centers compared to the complex cellular DNA replication-initiation mechanisms. However, the co-localization of ATM and Chk2 to integrated HPV DNA replication repair/recombination centers (Fig. 5; Ref. I) and the phosphorylation of Chk2 kinase (Fig. 6B, panel b, lane 1; Ref. I) in HeLa cells transfected with HPV18 E1 and E2 expression plasmids highlight the important role of the ATM pathway in resolving DNA damage. We believe that since the ATR signaling pathway is not fully functional, the ATM pathway is probably the pathway of choice for HPV DNA repair. Activation of the ATM pathway induces S or G2/M cell cycle arrest, which provides an environment to re-replicate viral genomes multiple times. This is most beneficial in the vegetative stage of the viral life cycle, where large numbers of viral genomes are needed.

Depending on the phase of the cell cycle, the ATM pathway is divided between two principal repair mechanisms: homologous recombination (HR), which is activated in late S-phase or in G2-phase when sister chromatids are available, and non-homologous-end-joining (NHEJ), which does not need any homology and can repair dsDNA breaks at any phase of the cell cycle. The primary sensors to detect DSB in the HR pathway are in the MRN (Mre11-Nbs1-Rad50) complex, and in case of NHEJ, the primary sensor is the Ku70/80 heterodimer. Co-immunostaining of HR or NHEJ complex proteins with the E1 protein, which represents the integrated HPV DNA replication centers (Fig. 2; Ref. I), revealed that all components of the MRN complex as well as the Ku70/80 heterodimer co-localize within foci of the E1 protein (in HeLa cells transfected with HPV18 E1 and E2 expression plasmids) (Fig. 4; Ref. I). These data demonstrate that both repair mechanisms are recruited to the repair/recombination centers of the integrated HPV locus to resolve HPV-derived DNA breaks. Although, HR is the primary DNA repair mechanism in late S and G2 phase, we propose that the slow “error-free” HR pathway for repair of HPV-derived DSBs is easily saturated and that DSBs are then repaired by the “error-prone“ NHEJ pathway. Although NHEJ plays important role in maintaining genomic stability and preventing tumorigenesis, it is also responsible for the majority of cross-chromosomal translocations. *De novo* synthesized sequences can therefore be randomly integrated into the host genome and, depending on

the location, can cause genomic instability, which then can lead to uncontrollable cell growth and to the formation of cancer tissue.

Mucosal HPVs classified into the low-risk group due to their lower oncogenic activity have been considered harmless regarding progression to malignancy. However, it has been demonstrated that the E1 and E2 replication proteins originating from episomal low-risk HPV6b or HPV11 are capable of starting replication from an integrated high-risk HPV URR in HeLa and SiHa cells (Kadaja et al., 2007). The possibility that cells containing integrated HPV sequences could be the targets of *de novo* infection of another type of papillomavirus (including LR-HPV) should not be underestimated. In such cases, episomal and integrated HPV DNA co-exists in the same cell. Whether there is a benefit for the HPV type that re-infected the cell needs further clarification, as episomal forms of HPV have a tendency to be lost soon after integration. A "hit-and-run" mechanism can be used to describe the situation: although "the newcomer" will not complete its life cycle, the expression of replication proteins and the damage done by amplifying the integrated HPV locus and its flanking cellular sequences remain. The possibility that low-risk HPVs indirectly cause genomic rearrangements needs to be further analyzed. Therefore, it is necessary to study LR-HPVs in parallel with HR-HPVs to better understand differences between the groups, including in the viral life cycle, gene expression and DNA replication.

5.2 Transient, stable and amplificational replication of the low-risk HPV11 genome can be monitored in U2OS cells (Ref. II, Ref. IV)

Knowledge gained from studies with organotypic raft cultures has had a very large impact on understanding of how HPV infection runs its course. The downside of organotypic culture is largely technical, as the cultivation method is time-consuming and strict growth conditions make the results prone to variability. Furthermore, organotypic cultures are not suitable for screening thousands of chemical compounds to find anti-viral drugs that target important vital HPV functions. It has been our goal for several years to find an alternative simplified cell culture system that stably replicates HPV genomes. Previous studies with monolayer epithelial cells (C33A, 293, HaCaT, HeLa, and SiHa) have been suitable for monitoring DNA replication of plasmid containing HPV replication origin in the presence of the E1 and E2 replication proteins expressed from expression plasmids but in general have lacked the power to monitor the replication of HPV genomes in the presence of native expression levels of replication proteins. After transfection of U2OS cells with various types of HPV E1 and E2 expression vectors together with origin-containing plasmids, we realized that this system is highly effective in initiating HPV DNA replication and made it possible to monitor viral DNA replication at much lower concentrations of replication proteins than in previously used cell lines (HeLa, SiHa, etc.). Most

importantly, the replication of HPV genomes can be easily followed in this cell system.

Regardless of the fact that bone cells are not the native hosts of HPV, the effective replication of various alpha (HPV6b, 11, 16, and 18) and beta (HPV5 and 8) HPV genomes can be easily monitored in U2OS cells without the addition of expression plasmids for the E1 and E2 proteins. The levels of viral replication proteins expressed from viral promoters are sufficient to initiate DNA replication from the viral origin of replication. Transient DNA replication of the above-mentioned HPV types was followed 4 days with continuous growth of the replication signal (Fig. 1A, B, C, and D; Ref. II). There were differences regarding replication signal intensity, but in general, all HPVs tested herein efficiently replicated their genomes in U2OS cells. The robustness of U2OS cells is the greatest advantage over other cell culture systems, as previously used cell lines could not support the genome replication of various HPV types, especially low-risk α-HPVs and β-HPVs. Additionally, compared to organotypic cell culture models, the U2OS transfection system is simple, safe and easy to use, and it can be adapted for automated industrial use to screen HPV replication inhibitors.

Stable maintenance of extrachromosomal LR-HPV molecules has always been a limitation of *in vitro* cultures. Stable cell lines carrying extrachromosomal molecules of high-risk HPV16 or HPV33 have long been used under laboratory conditions; however, no cell lines carrying LR-HPVs have been described. Single-cell subcloning of HPV18-positive (and HPV16-positive) U2OS cells has been very successful in creating stable clones that have been used to further describe the transcripts of HPV18, to characterize the oligomeric forms of the HPV18 genome and also to perform initial viral DNA replication inhibitor studies. The stability of HR-HPV-positive clones likely arises from the switch from initial amplificational replication of the HPV genome to a stable maintenance phase. To analyze the stability of HPV11 genome copy number over time, the possible growth advantage of untransfected cells was excluded by using selective growth conditions. For that, plasmids containing a selection marker and the HPV11wt genome were co-transfected into U2OS cells. Subsequent cultivation in Geneticin-containing media eliminated untransfected cells and enriched the culture with Geneticin-resistant cells. Post selection, the cells were regularly passaged and the density of the culture was kept at 70–80%. Total DNA was extracted every 2 days, and the samples were analyzed by Southern blotting. In this process, the downside of the adapted U2OS transfection system was revealed: the replication signal of the HPV11wt genome gradually decreased in a time-dependent manner from days 10 to 21 post transfection (Fig. 1B, lanes 1-6; Ref. IV), indicating to the instability of genome copy number maintenance. LR-HPV genome stability was also an issue when developing single-cell subclones. Although selection of HPV6b and HPV11-positive clones with different genome copy number was generated (Fig 2C; Ref. II), the overall effectiveness of isolating HPV11- and HPV6b-positive clones from cells carrying the selection marker alone was 29% and 15%, respectively, compared to the

45% of isolation efficiency obtained for HR-HPV-positive subclones. Additionally, in some of the LR-HPV-positive clones, the loss of extrachromosomal HPV molecules was observed after multiple passaging (Fig 2D, clone 6b #41; Ref. II). Few of the clones stably maintained LR-HPV genomes at low concentrations (Fig 2D, clone 6b #11; Ref. II), and in one case, a possible integration event of the HPV11 genome and the loss of episomal molecules was observed (Fig 2D, clone 11 #3.13; Ref. II). The integration of LR-HPV is very uncommon and has not described before. The loss of episomal LR-HPV molecules may reflect to the lower capability of the E2 protein to effectively bind to HPV molecules and to mitotic chromosomes during cell division compared to HR-HPVs. As a consequence, we believe that the instability of LR-HPV clones is a natural feature of this HPV type, while HR-HPVs are considered to have more stability in both copy number maintenance and in generating persistent infection in humans.

In terminally differentiated keratinocytes, papillomaviruses switch to the vegetative phase of the viral life cycle, in which DNA copy number is quickly amplified. Depending on the culture conditions, different approaches can be used to mimic the differentiation program of keratinocytes *in vitro*: (i) by growing keratinocytes in organotypic raft cultures where cells are grown at an air-liquid surface and where daughter cells can migrate only vertically or (ii) by suspending cells in semi-solid media (Flores et al., 1999, 2000, Frattini et al., 1996, 1997). However, by cultivating moderately differentiated U2OS cells in dense culture conditions, the second rapid DNA amplification of HPV genomes can be observed (Fig. 3; Ref. II; Fig. 1B; Ref. IV). For closer investigation, equal numbers of cells per plate from a selection of HPV18 (18 #1.13), HPV16 (16 #2.5), HPV11 (11 #74), HPV6b (6b#82) and HPV5 (5#23) cell clones were seeded and maintained without passaging but with regular feeding for up to 12 days (Fig. 3; Ref. II). A similar study was also carried out with Geneticin-resistant U2OS cell pools initially co-transfected with HPV11 genomes and a selection marker plasmid (Fig. 1B, lanes 7-11; Ref. IV). In both studies, total DNA was extracted at 2-day intervals during the growth period. The samples were analyzed by Southern blotting, and replication signals were measured using a PhosphorImager and ImageQuant software (Ref. II) or with qPCR analysis (Ref. IV). With these experiments, the different behaviors of the HPV types were again revealed: DNA replication intensity increased most with the HPV18 #1.13 cell clone (approximately 10-fold), a general increase in genome copy number was observed for the HPV16 and HPV11 cell clones, and minimal increase in copy numbers were detected for the HPV6b and HPV5 clones (Fig 3C and G; Ref. II). In general, similar results were obtained with HPV11 cell pools that, in addition to HPV11-positive cells, also contained cells harboring only the selection marker. However, only the stable maintenance of the HPV11 genome could be monitored in dense culture conditions (Fig. 1B, lanes 7-11; Ref. IV), and the overall effect of changing the culture conditions on HPV11 genome replication was not observed (Fig. 1B, compare lanes 1-6 with 7-11; Ref. IV). The reason that dense culture conditions trigger HPV genome ampli-

fication may be the result of cell cycle arrest in G2 phase. Since HPVs do not follow once-per-cell-cycle replication mode (Hoffmann et al., 2006) and HPV replication activity has also been described in G2 phase, it is logical to assume that cell cycle arrest creates the perfect environment for HPV to amplify the viral genome. However, subsequent analysis of HPV5 and HPV8 clones did not exhibit an amplificational replication mode during a 12-day cultivation period in confluent culture conditions (Sankovski et al., 2014). The incapability of β-HPVs to amplify their genomes in confluent conditions may reflect to the lack of certain critical factors for the amplification of HPV5 and HPV8 genomes in U2OS cells.

5.3 Papillomavirus DNA replication is regulated through the activity of the E8^E2 fusion protein (Ref. IV)

Papillomavirus DNA replication is regulated through transcription, translation, post translational modifications, and nuclear localization of the E1 and E2 replication proteins, as well as through the expression of the E8^E2 fusion protein, which is a negative regulator of viral DNA replication. In order to test the effect of the HPV11 E8 ORF-containing fusion protein on HPV11 DNA replication, a mutant HPV11E8- genome was generated via a single nucleotide substitution of ATG to ACG at the E8 start codon at nt 1242 in the HPV11 genome. The mutation of the E8 ATG start codon in the HPV11 genome disrupts the translation of the E8^E2 fusion protein. Transfection of the mutated HPV11E8- genome into U2OS cells considerably increased transient DNA replication compared to the HPV11wt genome (Fig. 1A, compare lanes 4-6 with 1-3; Ref. IV). The same effect was found with other HPVs tested in our lab, although the intensity of the replication signal was quite variable and depended on the HPV type. For the HPV18E8- genome, a 10-fold increase in replication signal was detected (Toots et al., 2014), and for the HPV5E8- genome, even higher levels of replication signal (up to 100-fold) have been described (Sankovski et al., 2014). The ability to increase the replication signal through a simple single nucleotide substitution has been beneficial in studies with HPV18 in which the replication signal was enhanced in order to detect the mode of replication through a transient DNA replication assay by describing replication intermediates using 2D electrophoresis (Orav et al., 2015) and to detect the oligomerization of HPV genomes (Orav et al., 2013).

A slight decrease in HPV11E8- genome replication signal was detected in a stability assay in which Geneticin-resistant U2OS cell pools, initially transfected with the HPV11E8- genome and a selection marker plasmid, were cultivated in actively dividing culture for 21 days (Fig. 1B, lanes 12-17; Ref. IV). A similar loss in genome copy number was also observed for the HPV11wt genome (Fig. 1B, lanes 1-6; Ref. IV). Parallel analysis of HPV11wt and E8- cell pools in dense culture conditions where equal number of cells were seeded and maintained without passaging but with regular feeding for 11 days showed that,

while HPV11wt genome copy number was stably maintained under these conditions (Fig. 1B, lanes 7-11; Ref. IV), HPV11E8- genome DNA replication was amplified, with an increase in replication signal of 5- to 6-fold (Fig. 1B, lanes 18-22; Ref. IV).

In HPV11 transcript analysis using an E8-specific forward primer, we detected two differently spliced E8 ORF-containing transcripts. Sequencing analysis revealed that (i) E8^E1 is composed of the E8 ORF, the E1 3´ end and the intact E2 ORF, and (ii) E8^E2 contains the E8 ORF and the 3´ end of the E2 ORF. To evaluate the role of both E8 ORF-containing proteins in the regulation of HPV11 DNA replication, we constructed expression plasmids for E8^E1 and E8^E2 (for details, see the Mat/Meth in Ref. IV). Co-transfection of increasing amounts of these expression vectors with the HPV11E8- genome indicated that the expression of the E8^E1 protein did not affect the replication of the HPV11 genome (Fig. 1C, lanes 2-5; Ref. IV). It seems like E8E1 is not needed for the regulation of DNA replication and may be important in other parts of the viral life cycle. However, a strong reduction of HPV11E8- genome replication was observed after the E8^E2 expression vector was introduced into U2OS cells (Fig. 1C, lanes 7-9; Ref. IV).

5.4 Recombination-dependent oligomerization of HPV genomes *in vitro* and *in vivo* (Ref. III)

Episomal HPV molecules containing several copies of covalently closed circular HPV genomes (further referred to as oligomers) have been described as early as the 1980s (Boshart et al., 1984). The factors driving the formation of oligomeric HPV molecules and why these molecules are formed are not well understood, as the virus functions through its monomeric form, and ultimately only unit-size genomes are packed into viral particles. We can only speculate that the oligomeric forms provide an advantage when viral DNA is segregated between daughter cells or when the viral load is quickly amplified during the vegetative phase of infection. Oligomeric forms can result from active sigma-type rolling circle replication that generates long, linear head-to-tail dsDNA forms that are then recognized and circularized by host cell DNA damage repair (DDR) pathways. For example, a σ-type replication mode is used by phage λ to produce the tandem repeats required for λ DNA packaging (Weller and Sawitzke, 2014b). Oligomeric forms can also be the product of homologous recombination-dependent replication. Unlike rolling circle replication, which needs one template to produce linear multimeric DNA forms, homologous recombination combines two separate molecules into one.

In studies of HPV DNA replication in U2OS cells, we noticed that in addition to monomeric HPV molecules, higher forms of HPV molecules were also present (Ref. II). After closer investigation, it became clear that those forms were head-to-tail concatemers of HPV genomes (Fig. 2; Ref. III). To investigate whether HR-dependent or rolling circle replication is used to produce oligo-

meric HPV molecules, two distinguishable HPV18 molecules (HPV18wt and HPV18E) or HPV11 mutant genomes (HPV11E1- and HPV11E2-) were transfected into U2OS cells. Co-transfection of HPV18wt and the truncated form of HPV18E (plasmid without the late ORFs) produced oligomers containing both forms of HPV18 genomes (Fig. 5b, lanes 5-8; Ref. III). Co-transfection of replication-incompetent HPV11E1- and HPV11E2- also produced oligomers that contained both genome variants (Fig. 6, lanes 13-16; Ref. III). The production of heterogenic molecules is characteristic only to homologous recombination-dependent replication. Oligomeric forms of HPV DNA are also produced in other eukaryotic cell lines, such as HeLa, SiHa and C33 A cells (Fig. 7; Ref. III). It seems that the oligomerization of HPV molecules is formed in all cell lines that also support HPV genome replication.

In collaboration with the Women's Clinic of the University of Tartu Hospital, we analyzed cervical brush or colposcopy probes collected from women with persistent infection of HPV16 or HPV18. Via restriction analysis of total DNA with subsequent 1D or 2D electrophoresis and the detection of HPV-specific signal by Southern blotting, we identified 3 samples containing both oligomeric and monomeric forms of HPV molecules (Fig. 8; Ref. III). Taken together, the fact that oligomeric forms of HPV molecules can be produced *in vitro* and the presence of such forms *in vivo* indicates that oligomerization of HPV genomes seems to be a naturally occurring event. Large viruses such as vaccinia virus encode resolvase (Garcia et al., 2000) and herpes simplex virus encodes alkaline nuclease (Martinez et al., 1996) to cleave long concatemeric replication intermediates back to unit-sized viral genomes. For small viruses such as HPV, the factors that assist in this process are not known. Therefore, the cleavage of HPV oligomers is probably mediated by the host cell. The processes through which HPV manipulates or adapts host factors to resolve oligomeric molecules remain to be understood.

5.5 Mapping of HPV11 transcripts in U2OS cells (Ref. IV)

mRNA splicing is an important modification mechanism among viruses with small compact genomes, as one sequence often encodes various proteins with different functionality. HPV11 has 3 splicing donor sites and 3 acceptor sites within its early region that in combination form a set of proteins essential for successful progression through the viral life cycle. Knowledge about HPV11 transcripts has been gained from studies with human clinical samples (Chow et al., 1987a; Nasseri et al., 1987a; Rotenberg et al., 1989a; Smotkin et al., 1989) and from studies with the transiently transfected human squamous carcinoma cell line SCC-4 (Renaud and Cowsert, 1996). Although the transient replication of various HPV types as well as the expression of functional E1 and E2 proteins from native promoters within HPV genomes has been described in U2OS cells (Fig. 1; Ref. II), little was known about the overall gene expression of HPV in these cells. Recently, transcription maps of HPV5 and HPV18 were created

(Sankovski et al., 2014; Toots et al., 2014), and a part of this thesis focused on describing the transcripts of HPV11 expressed from the HPV11 genome in transfected U2OS cells. A thorough characterization of HPV11 transcripts expressed in U2OS cells and subsequent comparison with transcripts characterized by others would add more reliability to the results obtained with the U2OS cells and would further show the suitability of these cells for studies with human papillomavirus.

After introducing HPV11wt and E8- genomes into U2OS cells by transfection, polyA+ mRNA was extracted, and 3′ RACE, 5′ RACE or RT-PCR analysis with HPV11-specific primers was performed (Ref. IV). As we have demonstrated that different culture conditions trigger stable or amplificational replication of HPV genomes, the same two culture conditions were introduced during HPV11 transcript analysis to gain insight into the changes that occur at the transcriptional level. For that, U2OS cells were transfected with the HPV11wt genome together with a Geneticin-resistant plasmid, and the cells were cultivated under selective media and maintained under subconfluent and confluent conditions.

For 3′ RACE analysis, polyadenylation cleavage sites (CS) were mapped to nt 4384, where early-region transcripts are cleaved, and to nt 7458, which is necessary for the cleavage of late-region mRNAs (Fig. 3; Ref. IV). Both CSs have also been described in previous studies with HPV11 transcripts (Chow et al., 1987b; Nasseri et al., 1987b). Interestingly, use of the early CS does not change significantly over time in U2OS cells (Fig. 3A; Ref. IV), indicating that viral early proteins are needed throughout the entire period that was under analysis. At the same time, increased use of the late CS was found at later time points and under dense culture conditions (Fig. 3B; Ref. IV), which may indicate that viral structural proteins are needed for the production of viral particles characteristic of the vegetative phase of the viral life cycle. However, 5′ RACE analysis with specific late-region HPV11 primers was used for the detection of mRNA products encoding viral structural proteins composed of mixed mRNA products that exhibited irregular splicing patterns (data not shown). We believe that late mRNAs are unstable and are easily degraded in U2OS cells, which is why late structural proteins are not synthesized. The same phenomenon was also found following transcript analysis of HPV18 and HPV5 in U2OS cells: although the use of late CS was clearly upregulated, no intact mRNAs encoding late viral proteins were detected (Sankovski et al., 2014; Toots et al., 2014).

The HPV11 transcriptional start sites (TSS) were mapped through 5′ RACE analysis with different HPV11-specific reverse primers located in exon regions previously characterized by others. With this method, the TSSs were clustered into 5 promoter regions: P90, P264, P674-714, P1092, and P1372. The P1092 and P1372 TSSs are quite rarely used and have not been extensively defined as promoter regions in studies with HPV11. However, a transcriptional start site at nt 1374 has been suggested in studies with SCC-4 cells with autonomously replicating HPV11 genomes, and depending on its 3′ end, the E2, E4, E5a, and E5b proteins are encoded (Renaud and Cowsert, 1996). A potential transcriptional

start site of P1092 identified herein could be used for the expression of the E8^E2 fusion protein. A short exon within the E1 ORF that fuses with the C-terminal end of the E2 ORF has been previously described in HPV11 by Chow et al. in 1987, and transcriptional start sites other than the ones in front of the E1 gene have also been suggested (Chow et al., 1987a). Similar use of a promoter has previously been reported in studies with HPV1 (Palermo-Dilts et al., 1990) and HPV16 (Milligan et al., 2007), but a lack of detection mechanisms or additional structural information has hindered the subsequent characterization of this region. For BPV1, thorough analysis to describe the promoter region within the E1 ORF was carried out, and in combination with multiple experiments such as primer extension, S1 nuclease mapping, and RNase protection analysis, the promoter region (P3) was mapped to the first half of the E1 gene at the N-terminal end (Choe et al., 1989). It has also been reported that the P3 promoter region is used for the transcription of mRNAs that express the strong transcriptional and replicational repressor E8^E2, described earlier by Lambert et al. (Lambert et al., 1987).

The promoter regions located in the E6 and E7 ORFs, P90, P264, P674-714 are divided as early and late promoters that are utilized in a differentiation-specific manner. In undifferentiated basal keratinocytes, the E2-dependent promoter P90 is used to transcribe the early transcripts needed for host cell transformation and viral DNA replication. In the progression of the viral life cycle and after host cell differentiation, the E2-independent late promoter P674-714 is activated. The switch from early to late promoter is not strict, as the differentiation of host cells increased the abundance of both the P90 and P674-714 transcripts, and only isolated suprabasal cells almost exclusively contained transcripts starting from the late promoter (DiLorenzo and Steinberg, 1995). In transfected U2OS cells, all of the previously described promoters can be detected with a clear prevalence of the P90 promoter over the P264 and P674-714 promoter regions (Fig. 4A; Ref. IV), indicating to the early stages of the viral life cycle. The activity of the P90 promoter region varies at different time points: the signal is stronger in samples cultured for 10 days compared to those cultured for 4 days (Fig. 4A and C, compare lane 2 with 1; Ref. IV). Under dense culture conditions where amplificational DNA replication has been described and presumably higher levels of replication proteins are needed, use of the P90 promoter region is enhanced (Fig. 4A and C, lane 3; Ref. IV). Use of the late promoter region also becomes more evident at later time points and in dense culture conditions (Fig. 4A; Ref. IV). However, increased P90 promoter signal at the same probes indicate that although the viral life cycle may have progressed further and late promoter activity was upregulated, it has not reached the phase where early promoter activity is minimized, as has been described in suprabasal cells by DiLorenzo and Steinberg (DiLorenzo and Steinberg, 1995). In transcript analysis of HPV11 from condylomata acuminata, mRNAs were predominantly initiated from the late promoter region P674-714, which may indicate that the infection reached the end of the viral life cycle, at which point use of the P90 promoter region is downregulated (Chow et al., 1987a).

Regulation of the P264 promoter has been a controversial topic. Some studies have shown that transcripts started from this promoter are more abundant in suprabasal cells (Stoler et al., 1989), whereas others have detected higher abundance in the two to three lowest epidermal layers (Iftner et al., 1992). *In vitro* studies with cells cultured in low-calcium media (conditions that minimize differentiation and stratification) showed that the abundance of P264 transcripts was lower or equal to the transcripts started from the early P90 promoter (DiLorenzo and Steinberg, 1995). In raft cultures where cells were induced towards differentiation with a high calcium concentration, the abundance of P264 transcripts remained unchanged, while P90 and P674-714 transcript levels both increased. Treatment of laryngeal papilloma cell culture with increasing concentrations of retinoic acid, which also induce cell differentiation, led to a concentration-dependent decrease in the levels of P264 transcripts, but the abundance of P90 transcripts was less affected (DiLorenzo and Steinberg, 1995). These results show that LR-HPV promoters are differentially regulated, suggesting that independent regulation of the levels of E6, E7 and other viral proteins is important for efficient progression through the viral life cycle. In transfected U2OS cells, the P264 promoter region is rarely used compared to the early promoter P90. However, a slight increase in the use of the P264 promoter was observed at later time points and under dense culture conditions (Fig. 4A; Ref. IV). At the same time, the P90 and P674-714 promoters were also upregulated.

Splicing of papillomavirus mRNAs is the key event in producing proteins with various purposes from the same sequence. The splicing donor and acceptor sites were characterized in HPV11-transfected U2OS cells by sequencing cDNAs obtained by 5´ RACE or by RT-PCR methods using polyA+ mRNA extracts as a template. With these methods and with various HPV11-specific primers, we were able to detect three splice donor sites at nt 847, nt 1272, and nt 1459, and three acceptor sites at nt 2622, nt 3325, and nt 3377, which are identical to those previously found in human condylomas, in laryngeal papillomas and in transfected SCC-4 culture. The data demonstrate the presence of transcripts containing splice junctions representing all possible combinations of detected splice donor and acceptor sites: 847/2622, 847/3325, 1272/2622, 1272/3325, 1272/3377, 1459/2622, 1459/3325, and 1459/3377 (Fig. 4D, E, F, and Fig. 5). Additionally, using 5´ RACE with HPV11-specific primers from the latter exon enabled the identification of TSSs together with the splice sites (Fig 4D, and E; Ref. IV). Data regarding the transcripts detected during transient HPV11 replication are summarized in Figure 8, with the transcripts that were previously detected by others being designated by the numbers on the right. With this set of mRNAs in transfected U2OS cells, there is the potential to encode all the major early-region proteins described to date. Therefore, the transcript map created for HPV11 supports the use of this cell system as a model for investigating various aspects of the HPV life cycle.

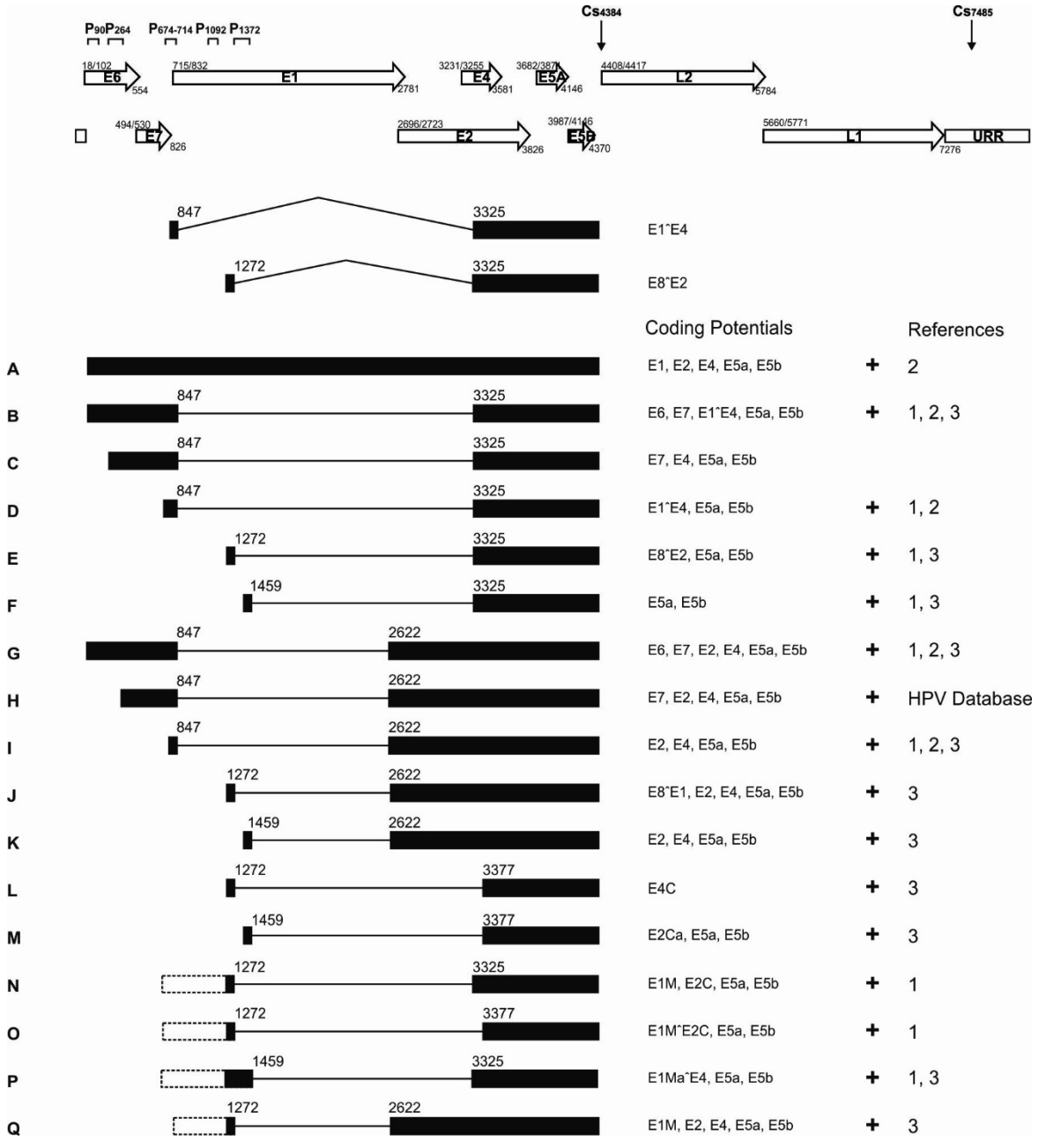

Figure 8. HPV11 transcription map obtained from transiently transfected U2OS cells. The HPV11 ORFs, LCR, promoters (P90, P264, P674-714, P1092, and P1372) and polyadenylation CSs (Cs 4384 and Cs 7485) are shown at the top of the figure. All HPV11 transcripts from transiently transfected U2OS cells (indicated with letters from A-Q, shown at left) are presented, showing the exons (solid boxes), introns (lines) and splicing donor and acceptor sites (nt numbers in HPV11 genome). The inferred 5′ ends of the RT-PCR products (N-Q) are indicated with dashed boxes. The coding potential is

described to the right of each transcript, and references are provided when similar transcripts were found by others, namely, Chiang et al., 1991 (1), Chow et al., 1987 (2), and Renaud et al., 1996 (3).

5.6 Role of the E6/E7 ORF in the production of functionally active E1 protein (Ref. IV)

Preliminary R-loop studies with HPV11 transcripts derived from condyloma acuminata suggested that the E1 protein is translated from monocistronic mRNA that starts from the late promoter region P674-714 (Chow et al., 1987a). The early promoter region P90, which produces polycistronic mRNA, was believed to be used for the production of oncoproteins E6 and E7 (Chow et al., 1987a). Although knowledge about the viral factors (E1 and E2) needed for BPV1 genome replication has been available since the beginning of the 1990s (Ustav and Stenlund, 1991), *in vitro* HPV replication studies using E1 expression vectors took quite a long time to get going due to poor expression levels of E1, until Remm *et al*. described using an HPV18 model that biologically active E1 protein levels can be produced by introducing the E6/E7 ORFs in front of the E1 gene in E1 expression plasmids (Remm et al., 1999). This structural organization mimics transcription from the early promoter region in front of the E6 ORF, which produces polycistronic mRNA, with the E1 ORF being the third coding region. The effective expression of the E1 protein from the designed E1 plasmid shows the crucial regulatory role of the E6/E7 region. However, little is known about the *cis* elements responsible for E1 protein expression. Since HPVs are very conserved in genomic structure, the E6/E7 ORFs were also introduced into HPV6b, HPV11, and HPV16 E1 plasmids, and the effective expression of the E1 protein in transfected HeLa cells was observed (Kadaja et al., 2007). Efficient replication from a homologous URR-containing plasmid as well as replication initiation from the integrated HPV18 sequence could be detected in all of the HPV types under investigation (Kadaja et al., 2007).

To further evaluate the role of the E6/E7 region in HPV11 E1 protein expression, we designed an E1 plasmid that lacks the E6/E7 region (Leader-) and compared the results with an HPV11 E1 plasmid that contains the E6/E7 region (Leader+), as designed by Kadaja *et al*. (Kadaja et al., 2007). Similarly to Remm *et al*., the HPV11 E1 protein expression plasmids containing the E6 and E7 ORF showed higher E1 protein levels (Fig. 6A, Leader+; Ref. IV) and supported URR plasmid replication more effectively (Fig. 6C, lanes 1-6; Ref. IV) than the E1 expression plasmid lacking the E6/E7 region (Fig. 6A and C, Leader-; Ref. IV) (Remm et al., 1999). Interestingly, while Kadaja *et al*. introduced high concentrations (2.5 and 10 μg) of Leader+ plasmids into HeLa cells to obtain detectable E1 protein levels, U2OS cells require far less plasmid (up to 1 μg) to obtain even higher levels of E1 protein expression than those observed in HeLa cells. This again shows the suitability of the U2OS system, as was previously described in studies with various HPV genomes (Fig. 1; Ref. II).

In high-risk HPV subtypes such as HPV16, HPV18, and HPV31, the E1 protein is translated from spliced polycistronic mRNA, which carries partial sequences of the E6 ORF (generating E6* protein) (Milligan et al., 2007; Ozbun and Meyers, 1998; Wang et al., 2011). We believe that splicing from the E6 ORF may contribute to the effective expression of the E1 protein. In LR-HPVs, such an intron has not been described. However, cloning of an intron in front of the E1 coding region in an HPV11 E1 expression plasmid and the effective splicing of translated mRNA (Fig. 7, Int1; Ref. IV) increased E1 protein expression and the URR plasmid replication signal to levels comparable to those produced with the Leader+ plasmid (Fig. 6A, compare lanes 7-9 with 1-3, and Fig. 6C, compare lanes 13-18 with 1-6; Ref. IV). Expression of E1 protein was even more intense from a plasmid containing two introns in front of the E1 coding sequence than from the Leader+ or Intron1 plasmids (Fig. 6A, Intron2; Ref. IV). However, the toxicity of high E1 protein levels also seemed to be a burden for host cells, as the replication signal of the URR plasmid dropped in the presence of higher levels of E1 protein (Fig. 6C, lanes 25-26; Ref. IV). Interestingly, 75% of mRNAs translated from the plasmid with the double intron were spliced from the second intron, leaving the first intron intact, whereas 25% were spliced from both introns, and no mRNAs spliced only from the first intron were detected (Fig 7B, Int2, signal e; Ref. IV). In addition to the detected splicing event of the E1 intron plasmids, the Leader+ plasmid that contains the E6/E7 region and the viral native promoters P264 and P674-714 showed a weak signal at approximately 750 bp, and sequence analysis of this HPV11-specific mRNA revealed that native promoters were used to transcribe E1 mRNAs, although at low levels (Fig. 7, L+, signal b; Ref. IV).

6. CONCLUSION

1) The observation of new integration sites of HPV16 sequences with flanking cellular sequences in SiHa cells transfected with the E1 and E2 expression vectors indicate that replication initiation from integrated HPV origins may lead to host cell genomic rearrangements. As a result of the re-replication of integrated HPV16 sequences, the co-localization of homologous recombination and non-homologous end joining effector molecules (MRE complex, Ku70/80, ATM, ATRIP, and Chk2) to HPV repair/recombination loci indicate to the formation of double-stranded breaks. The produced replication intermediates were detected as aberrant DNA molecules and were subjected to cellular repair/recombination pathways. Most of the ds breaks were repaired properly, but in some cases, DNA rearrangements were generated. Our work suggests that the *de novo* integration of HPV16 loci in SiHa cells is mediated by cellular DNA damage response pathways. This provides additional evidence in support of the hypothesis that amplification of integrated HPV loci together with flanking cellular sequences in pre-cancerous lesions (LSIL and HSIL) could occur if episomal-derived replication proteins are present.
2) The oligomerization of HPV genomes observed in U2OS cells is consistent with findings from clinical samples. Analysis of HPV16- or HPV18-positive cervical brush or colposcopy probes confirmed that oligomerization of HPV genomes is a naturally occurring event. Oligomerization of HPV genomes could be generated through a recombination-dependent replication mode or through homologous recombination. In the first case, DNA replication is not terminated when the replication complex reaches the replication initiation site but continues for several rounds of replication on circular molecules. This type of replication can produce only homologous oligomeric DNAs. In the second case, homologous recombination combines two molecules together and can produce heterogeneous oligomers. In U2OS cells, both recombination variants were detected.
3) The transient replication of mucosal low-risk (HPV6b, HPV11) and high-risk (HPV16, HPV18) α-HPVs as well as the replication of cutaneous β-HPVs (HPV5, HPV8) can be easily monitored in U2OS cells. Stable maintenance of HPV6 and HPV11 subclones as well as HPV11 cell pools is complicated, as genome copy number has a tendency to continuously decrease over time. The amplificational replication of HPV6 and HPV11 subclones in confluent U2OS culture can be detected. In the case of the HPV11 cell pool, confluent culture conditions rather helped stably maintain HPV11 genome copy number than triggered DNA amplification. At the same time, clones of HR-HPV types were stably maintained under normal culture conditions (70–80% confluency), whereas dense culture conditions triggered active amplification replication.

4) The identification of HPV11 transcripts with transcriptional start sites, polyadenylation cleavage sites, and splice sites in U2OS cells and the similarity of the transcription pattern to previous findings in clinical samples and in keratinocyte cell lines provides additional support for the use of U2OS cell-based assays in HPV research.
5) Expression of the HPV11 E1 protein from polycistronic mRNA produces more stable and functionally active E1 protein than E1 protein translated from monocistronic mRNA. Although HPV11 does not contain an intron within the E6 region such as the HR-HPVs, E1 expression levels and the replication of URR-containing plasmid were positively affected when an intron was introduced into the monocistronic E1 expression vector. The viral promoters within the E6/E7 region are also active as mRNAs with TSSs at the P264 or P674-714 promoter regions are transcribed from polycistronic E1 expression vector. However, the contribution of this activity to the expression of functionally active E1 protein is unknown because the origin of the functional E1 protein cannot be distinguished.

SUMMARY IN ESTONIAN

Viiruse ja peremeesraku interaktsioonid inimese papilloomiviiruse elutsüklis

Papilloomiviirused on väga laialdase levikuga ning pea kõik inimesed nakatuvad mingil eluetapil selle viirusega. Üldiselt kulgeb HPV infektsioon üsna kergelt, põhjustades nahal või limaskestadel healoomulisi vohandeid, soolatüükaid (β-HPV) või kondüloome (α-HPV). Kliiniliselt oluliseks peetakse aga papilloomiviirusi seetõttu, et selle viiruse infektsiooniga on seotud enamik emakakaela vähi juhtumitest. Arvatakse, et emakakaela vähi tekke üks olulisemaid etappe on raku nakatumine kõrgesse riskigruppi kuuluva papilloomiviiruse subtüübiga. Kui soolatüükaid ja kondüloome põhjustavad madala riskiga inimese papilloomiviiruste subtüüpide infektsioon aja möödudes kaob, siis emakakaela vähki põhjustavate viiruste infektsioon muutub kergesti pikaajaliseks. Sellisesse faasi jõudnud nakkuse ravivõimalused on aga väga piiratud, mistõttu on HPV infektsiooni varajane avastamine eduka ravi seisukohalt ülioluline.

Papilloomiviiruse infektsioon kulgeb tavaliselt läbi ekstrakromosomaalse rõngasmolekuli ning viiruse elutsükli edukaks lõpetamiseks ei ole integreerumine peremeesraku pärilikku materjali (DNA) tingimata vajalik. Vähirakkude geneetilisel uurimisel on aga muuhulgas HPV nakkuse tuvastamisele avastatud ka asjaolu, et väga paljudel juhtudel on papilloomiviiruse genoomi osad integreerunud peremeesraku kromosoomidesse, muutes HPV DNA üheks osaks peremeesraku pärilikust materjalist. HPV genoomi osaline integratsioon rikub ära aga mitmete viiruse jaoks oluliste valkude ekspressiooni (s.h. DNA replikatsiooniks ja virioni moodustamiseks vajalike valkude sünteesi). Seega peetakse integreerumist viiruse seisukohalt tupikteeks. Peremeesraku seisukohalt aga peetakse pahaloomuliseks kasvajaks arenenud rakkude DNAs tuvastatud suuri ja tagasipöördumatuid muutusi just viiruse integratsiooni tagajärjeks. Viiruse integratsioon võib toimuda mistahes kromosoomi piirkonda, ka nendesse piirkondadesse, mis on peremeesraku kasvu ja jagunemise kontrolli seisukohalt väga olulised.

Kuna nakatunud rakk sisaldab keskmiselt 100 ekstrakromosomaalset viiruse genoomi koopiat ning massilist genoomide integratsiooni tavaliselt ei toimu, tekib olukord, kus ühes rakus eksisteerib kaks erinevat viiruse DNA vormi: üks, puudulike geenidega integreerunud vorm ja teine, täiesti funktsionaalne ekstrakromosomaalne vorm. Kuigi integreerunud viiruse DNAlt ei saa enam ekspresseerida DNA paljundamiseks vajalikke replikatsioonivalke, siis ekstrakromosomaalselt molekulilt replikatsioonivalkude sünteesiks mingeid takistusi ei ole. Selline olukord on aga peremeesraku genoomse stabiilsuse seisukohalt väga ohtlik, kuna integreerunud HPV järjestused sisaldavad viiruse DNA paljundamiseks vajalikku alguskohta (replikatsiooni *origin*-i), mis ei ole rakuliste regulatsiooni faktoritega kontrollitav. Sellelt järjestuselt alustatud DNA replikatsioon, põhjustab nii integreerunud HPV DNA kui ka sellega külgnevate peremeesraku genoomi osade kontrollimatut paljundamist (amplifikatsiooni).

Olenevalt integratsiooni kohast, võivad integreerunud HPV järjestusega külgnevad alad sisaldada onkogeene või muid raku kasvu ja jagunemisega seotud regulatoorseid geene, mille amplifikatsioon võib põhjustada raku genoomset ebastabiilsust ning kontrollimatut kasvu ja jagunemist. Lisaks võib põhjustada HPV-dele omane litsentseerimata DNA re-replikatsioon replikatsiooni komplekside takerdumisi, mis viib ebatüüpiliste DNA replikatsiooni produktide kuhjumiseni raku tuumas. Rakulise DNA reparatsiooni radade võtmemolekulide lokalisatsioon HPV DNA replikatsiooni tsentritesse viitab rakuliste mehhanismide olulisele rollile tekkinud kaheahelaliste DNA katkete parandamisel. Kui enamik kaheahelalisi DNA katkeid parandatakse õigesti, siis antud väitekirjas kirjeldatud viiruse integratsiooni koha translokatsioon uude kromosoomi piirkonda viitab ebaõnnestunud katsele parandada DNA amplifikatsiooni tagajärjel tekkinud DNA produkte. Seega, integreerunud HPV järjestuste amplifikatsioon ja peremeesraku vastusena aktiveeritud DNA reparatsiooni mehhanismid võivad viia peremeesraku genoomse ebastabiilsuseni ja vähkkasvaja tekkeni.

Inimese papilloomiviiruste uurimine nende looduslikes peremeesrakkudes on üsna keerukas just keratinotsüütide keeruka elutsükli tõttu, mis on aluseks nahkkoe kihistunud struktuuri moodustumisel. Kui diferentseerumata basaalsed keratinotsüüdid on pidevas jagunemises ja vastutavad basaalrakkude populatsiooni uuenemise eest, siis diferentsieerumisradadele suunatud rakud läbivad kontrollitud rakusurmaga lõppeva elutsükli. Mitmes eri diferentseerumisastmes olevate keratinotsüütide populatsioon tekitab nahkkoe kihistunud struktuuri, mille peamiseks ülesandeks on tagada tugev (ja läbimatu) barjäär sise- ja väliskeskkonna vahel. Kuna viirus nakatab diferentseerumata basaalseid keratinotsüüte ja pakib oma viiruspartiklid alles terminaalselt diferentseerunud rakkudes, on papilloomiviirused kohanenud kõikide peremeesraku eluetappidega. Lühidalt, papilloomiviirus nakatab basaalseid keratinotsüüte mikrovigastuste kaudu ning peale viiruse geneetilise materjali jõudmist raku tuuma, toimub esmalt aktiivne viirusgenoomi paljundamine. Peale optimaalse genoomi koopiaarvu saavutamist, lülitub viirus ümber stabiilsele genoomi säilimisele ning jääb ootama peremeesraku diferentseerumist. Sel ajal toimub HPV- ja peremeesraku geneetilise materjali sünkroonne paljundamine ning viiruse genoomide peaaegu ühtlane jaotumine tütarrakkude vahel. Terminaalselt diferentseerunud rakkudes toimub taaskord aktiivne genoomi paljundamine (amplifikatsioon) ning viimase etapina viiruspartiklite moodustumine. Laboritingimustes on keratinotsüütide kasvatamine ja elutsükli mimikeerimine tehniliselt keerukas, ajakulukas ja küllaltki kallis. Lisaks ei ole otstarbekas niigi kapriisset rakukultuuri kasutada laiamahuliseks kemikaalide skriinimiseks, et leida HPV elutsüklit pärssivaid komponente. Meie laboris välja töötatud sääreluu kasvaja rakuliiinil U2OS põhinev HPV uurimismudel osutus edukaks mitmete HPV subtüüpide (β-HPV5, β-HPV8, α-HPV6b, α-HPV11, α-HPV16, α-HPV18) transientse, stabiilse kui ka amplifikatsioonilise DNA replikatsiooni jälgimisel. Kuna U2OS rakud ei ole looduslikud papilloomiviiruse peremeesrakud, pidasime vajalikuks kaardistada viiruse geenide avaldumist, et tõsta U2OS rakusüsteemi usaldusväärsust HPV uuringutes. Antud töös kirjeldati HPV11 geenide ekspressiooni, kuid sarnane

analüüs on läbi viidud ka HPV5 ning HPV18 subtüübiga. Kõiki saadud tulemusi on võrreldud viiruse loomulikest peremeesrakkudest, keratinotsüütidest, saadud tulemustega ning nende sarnasuse tõttu oleme arvamusel, et U2OS süsteem on sobiv keskkond papilloomiviiruste uurimiseks ning oma lihtsuse ja odavuse poolest ideaalne ka esialgsete ravimi kandidaatide skriinimiseks.

Lisaks tuvastati U2OS süsteemis 1n HPV molekulide sünteesi kõrval ka suuremaid, 2n ja rohkem, järjestikku paigutunud HPV genoomi koopiatega rõngasmolekule (oligomeerid). Sarnaseid vaheprodukte on täheldatud nii varasemates kui ka antud töö raames kogutud HPV-positiivsetest kliinilistest koeproovidest. HPV-spetsiifiliste oligomeeride tekke täpsemal uurimisel tekkis hüpotees, et nende moodustumisel võib olla oma roll rakulise rekombinatsioonilise DNA replikatsiooni laadil. Oligomeersed HPV molekulid võivad osutuda kasulikeks, et tagada kiire HPV genoomi koopia arvu tõus vegetatiivses viiruse elutsükli faasis kui ka viiruse genoomi jagunemisel kahe tütarraku vahel. Kuna viiruspartiklisse pakitakse vaid üks HPV genoom, pole täpselt teada, milliseid mehhanisme kasutatakse oligomeersete DNA vormide konverteerimisel tagasi 1n HPV genoomideks.

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Method and a kit for identifying compounds capable of inhibiting human papilloma virus replication; Owners: Icosagen Cell Factory OÜ; Authors: 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

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Leiutis

Method and a kit for identifying compounds capable of inhibiting human papilloma virus replication; Omanikud: Icosagen Cell Factory OÜ; Autorid: 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; Prioriteedi number: PCT/EE2010/000010; Prioriteedi kuupäev: 19.05.2010.

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