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UNIVERSITY OF TARTU
Press

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

Dissertation was accepted for the commencement of the degree of Doctor of Philosophy in biomedical technology on August 13th, 2019 by the Council of the Institute of Technology, Faculty of Science and Technology, University of Tartu, Estonia.

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Commencement: Auditorium 121, Nooruse 1, Tartu, Estonia, at 12.15 on October 3rd, 2019

Publication of this thesis is granted by the Institute of Technology, University of Tartu.

ISSN 2228-0855
ISBN 978-9949-03-153-5 (print)
ISBN 978-9949-03-154-2 (pdf)

University of Tartu Press
www.tyk.ee

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LIST OF ORIGINAL PUBLICATIONS

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

II. Liisi Henno, **Eva-Maria Tombak**, Marit Orav, Jelizaveta Geimanen, Ene Ustav, Mart Ustav (2017) Analysis of human papillomavirus genome replication using two- and three-dimensional agarose gel electrophoresis. *Current Protocols in Microbiology*, 45, 14B.10.1–14B.10.37

III. **Eva-Maria Tombak**, Andres Männik, Robert D. Burk, Roger Le Grand, Ene Ustav, Mart Ustav (2019) The molecular biology and HPV drug responsiveness of cynomolgus macaque papillomaviruses support their use in the development of a relevant in vivo model for antiviral drug testing. *PLoS ONE*, 14(1), e0211235.

My contributions to the listed publications are as follows:

I. Conducted the 3D N/N/A and 2D N/N experiments related to the analysis of the linearized HPV18 genomes and the HPV18 subgenomic fragments; interpreted the results; and participated in writing of the manuscript.

II. Participated in the optimization of the 2D N/N and 3D N/N/A techniques; interpreted the results; and participated in writing of the manuscript.

III. Designed and performed all the replication experiments of the MfPV genomes; conducted the transcriptome analysis together with Andres Männik; interpreted the results; and wrote the manuscript.

LIST OF ABBREVIATIONS

9-1-1	–	Rad9-Rad1-Hus1 complex
1D	–	one-dimensional
2D	–	two-dimensional
AGE	–	agarose gel electrophoresis
ATM	–	Ataxia telangiectasia mutated
ATR	–	ataxia telangiectasia and Rad3-related
ATRIP	–	ATR interacting protein
BLM	–	Bloom syndrome protein
CFSs	–	common fragile sites
Chk2	–	checkpoint kinase 2
CIN	–	cervical intraepithelial neoplasia
COPV	–	canine oral papillomavirus
CRPV	–	cottontail rabbit papillomavirus
DDR	–	DNA damage response
D-loop	–	displacement loop
DSB	–	DNA double-stranded break
dsDNA	–	double-stranded DNA
EV	–	epidermodysplasia verruciformis
FA	–	Fanconi anemia
HPV	–	human papillomavirus
HPV + OPC	–	HPV-positive oropharyngeal carcinoma
HR	–	high-risk
ICLs	–	interstrand crosslinks
kbp	–	kilo-base pair
LR	–	low-risk
MfPV	–	Macaca fascicularis papillomavirus
MRN	–	Mre11-Rad50-Nbs1 complex
N/N	–	neutral/neutral
N/A	–	neutral/alkaline
N/N/A	–	neutral/neutral/alkaline
ND10	–	nuclear domain 10
NHEJ	–	nonhomologous end joining
ORF	–	open reading frame
PV	–	Papillomavirus

RACE – rapid amplification of cDNA ends
RDR – recombination-dependent replication
RI – replication intermediates
ROPV – rabbit oral papillomavirus
RPA – replication protein A
RT-PCR – reverse transcriptase polymerase chain reaction
SA – splicing acceptor
SD – splicing donor
SSC – squamous cell carcinomas
ssDNA – single-stranded DNA
Tdp1 – tyrosyl-DNA-phosphodiesterase type 1
TopBP1 – topoisomerase II-binding protein 1
TSS – transcription start site
URR – upstream regulatory region

1. INTRODUCTION

Papillomaviruses (PVs) are highly species-specific viruses that have coevolved with their hosts for millions of years and have become well adapted to their distinct epithelial niches; in addition, a group of specific subtypes have developed oncogenic potential. To date, over 200 human papillomavirus (HPV) types have been identified, and infections caused by these types can result in a wide variety of outcomes that are mostly asymptomatic or induce the development of benign warts and papillomas determined by the HPV type and anatomical region. However, the persistent, long-lasting infections of oncogenic HPV types might induce genetic instability and cancer development in host cells over many years. For instance, mucosal oncogenic HPVs are almost always causative agents of cervical cancer and an increasing number of several anogenital and oropharyngeal cancers, posing a considerable global health and economic burden.

To establish persistent infection, the HPV genome must first undergo several rounds of DNA replication (called the initial DNA amplification phase) upon entry into the host cell and successful partitioning into daughter cells during cell division. HPV has a small circular DNA genome that is very compact with minimal coding capacity. The virus encodes only two replication proteins, and thus, to facilitate its reproduction, it necessarily evolved strategies to hijack the host DNA synthesis machinery. Over the past several years, mounting evidence has indicated that the HPV replication centers localize close to DNA regions that are prone to replication stress and that HPV might hijack and take advantage of these cellular DNA damage and repair pathways to replicate its DNA. However, the exact viral functions and mechanisms activating these pathways remain unknown and need to be elucidated for successful anti-HPV drug development that has failed so far.

The literature overview provided within the current thesis mainly summarizes the current knowledge of the HPV DNA replication cycle, its clinical importance, cellular DNA damage response pathways together with their involvement in the HPV life cycle, and a brief overview of current in vivo animal models for studying HPV infection. The original studies on which the current thesis relies are focused on the initial replication of the HPV genomes, specifically describe the replication intermediates generated during the viral DNA replication phase and suggest the DNA replication modes behind these structures. Additionally, this research characterizes the gene expression and replication properties of the HPV-related cynomolgus macaque papillomavirus genomes as potentially valuable in the development of an in vivo animal model for preclinical testing of anti-HPV therapeutics.

2. LITERATURE OVERVIEW

2.1 General introduction to human papillomaviruses

Papillomaviruses are extremely species-specific small DNA viruses that infect the mucosal or cutaneous keratinocytes of vertebrates. To date, over three hundred PV types have been identified, of which approximately two hundred types infect humans [1]. PV research has mostly focused on human papillomaviruses because of the clinical importance of these viruses since the discovery of the association between cervical cancer and infection with specific HPV types [2].

2.1.1 Medical burden of HPVs

HPVs are widely spread among the human population, and general cutaneous HPV types are considered a part of healthy skin microflora; such infections are usually asymptomatic (e.g., HPV12, HPV14, HPV19) or cause warts or other clinically insignificant lesions (e.g., HPV1, HPV2, HPV4), which tend to be cleared by the host immune system [3]. However, some cutaneous HPV types, such as HPV5 and HPV8, have been associated with nonmelanoma skin cancer lesions in immunosuppressed [4–6] or epidermodysplasia verruciformis (EV) patients having a rare hereditary skin disorder [7,8].

Clinically, more important mucosal HPV types are the most common sexually transmitted viruses and can be divided broadly into two distinct groups based on their potential to induce malignancy progression in the host or not. The nononcogenic, called low-risk (LR), types, are mainly associated with benign lesions, anogenital warts and recurrent respiratory papillomatosis (RRP) [9]. RRP is a rare disorder in which recurrent benign papillomas arise in the respiratory tract; frequent surgical removals are required depending on the severity of the disease, and sometimes the number of surgeries may exceed 100 [10]. The most prevalent LR types are HPV6 and HPV11, which are responsible for approx. 90% of the cases of anogenital warts [11,12]. Although anogenital warts rarely have the potential to undergo malignant conversion, the recurrence rate after treatment is very high, posing substantial healthcare costs [9].

Although the productive infection of high-risk (HR) types often causes no or minimal cytological abnormalities and these lesions are usually transient and clear without intervention within 1–2 years [13], there is still a fraction of oncogenic HPV infections that persist and cause genetic instability of the host cells, promoting cancer development with a considerable worldwide burden. For instance, HR HPV types are considered to be the causative agents of virtually all cases of cervical cancer, being diagnosed in approx. half a million women every year, and nearly half of these women die from the disease [14]. The most prevalent types are HPV16 and HPV18, both accounting for approx. 70% of

cervical cancer cases worldwide [12]. Additionally, oncogenic HPVs are responsible for promoting the development of several other anogenital malignancies (e.g., anal, penile, vaginal and vulvar cancers) [15] as well as head and neck cancers (particularly oropharyngeal cancers) [16]. In recent decades, mounting evidence has shown that HR HPVs are quite frequently related to oropharyngeal carcinoma (HPV + OPC), which can account for approx. 60% of such cases; HPV16 is the predominant HPV type identified in oropharyngeal carcinoma specimens [17]. The significantly increasing incidence rate of HPV + OPC and the unavailability of highly effective screening tests (such as Pap smears) for detecting precancerous or cancerous lesions constitute serious ongoing health concerns. The current prophylactic vaccines are highly effective in preventing the infection of certain HPV types (HPV6, 11, 16, 18, 31, 33, 45, 52 and 58) [18], and a decline in the incidence of HPV infections and cervical dysplasias was observed after the introduction of the vaccines [19,20]; however, these vaccines do not cure ongoing HPV infections. The development of HPV drugs has not been successful. Considering the clinical burden of HPVs, the need for anti-HPV therapeutics to treat ongoing viral infections is strong.

2.1.2 Genomic organization of HPVs and an introduction to the viral proteins

The HPV genome is an approx. 8 kb long double-stranded circular DNA molecule that is divided into three main regions: an early and a late coding region separated by the upstream regulatory region (URR) (Fig. 1). In principle, the viral proteins expressed from the open reading frames (ORFs) of the early coding region are synthesized from the beginning of the viral life cycle, while viral late proteins (capsid proteins) are expressed only in the upper layer of an infected epithelium before virion assembly. The URR region contains the viral DNA replication origin, promoter regions, and a number of binding sites of cellular and viral transcriptional regulators. Early proteins are involved in several processes during the viral life cycle.

The E1 and E2 proteins are the only HPV-encoded factors directly involved in viral DNA replication. The E1 protein is a multifunctional protein consisting of three functional domains, and its ATP-dependent replicative helicase activity is facilitated by the C-terminal domain. The E1 protein also interacts with several cellular DNA replication proteins necessary for recruiting host DNA replication machinery to the viral origin. The N-terminal domain contains several regulatory sequences, including the nuclear localization signal and nuclear export sequences. The central DNA-binding domain facilitates E1 binding to the viral origin [21]. The initiation process of DNA replication mediated by the viral E1 and E2 proteins is described in more detail in chapter 2.2.1. The viral E2 protein is also a multifunctional protein and has a crucial role in the maintenance of viral DNA in infected keratinocytes, tethering viral genomes to mitotic chromosomes during host cell division. Moreover, the E2 protein acts as a main viral transcriptional

regulator through its binding sites within the URR region. In addition to the viral E2 full-length protein, HPVs also encode repressor forms of E2 proteins that have a C-terminal DNA-binding domain but lack the N-terminal trans-activation domain. The repression of these truncated E2 proteins is mediated by competition for binding to E2 binding sites with the URR (reviewed in [22]). For instance, the E8^E2 protein (the E8 peptide is fused in-frame with the C-terminal DNA-binding domain of E2) is a major repressor, strongly restricting viral DNA replication and transcription [23–26].

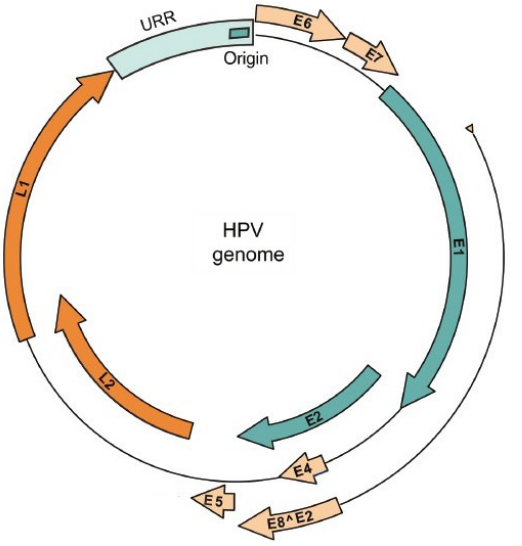

Figure 1. Schematic representation of HPV genome. The viral early open reading frames are marked with light orange, the open reading frames expressing viral replication proteins are emphasized with dark cyan. The late open reading frames are marked with dark orange. URR denotes upstream regulatory region; origin denotes approximate position of the origin sequence of viral DNA replication (adapted from [41]).

E5, E6 and E7 are viral oncogenes that reshape the host cell environment to ensure conduciveness to DNA replication and resistance to apoptotic signals, two important factors for the successful completion of the viral life cycle. E6 and E7 are the main viral proteins modulating the activity of several cellular proteins. The most important and extensively studied target of E6 proteins is the cellular tumor suppressor p53. E6 promotes its proteasome-mediated degradation and leads to the abolition of p53-mediated growth arrest and apoptosis-promoting activities [27,28]. E7 is responsible for inducing the dysregulation of cellular proliferation by most prominently disrupting the function of the retinoblastoma family proteins [29] and several other cellular proteins [30–32]. The oncogenic potential of HR HPV types mainly lies in the properties of the viral E6 and E7 proteins, in particular the higher binding affinity for their cellular targets (p53, pRb) [28,33,34]. The E5 oncoprotein is a transmembrane

protein, and its function is poorly defined. It is likely that E5 contributes to immune evasion [35] and promotes infectious cell growth by amplifying mitogenic signals from the epidermal growth factor receptor [36].

As a result of mRNA splicing, the viral E4 protein is expressed as an E1^E4 fusion protein [37]. Although the E1^E4 protein is defined as a viral early protein, its expression level is highest in the upper epithelial layers during productive infection. Furthermore, the E1^E4 protein has been shown to have the ability to bind and collapse the cytokeratin network, which might be necessary for facilitating virion release into the environment [38].

L1 and L2 are capsid proteins that are synthesized in terminally differentiated keratinocytes during the productive life cycle. L1 is the major capsid protein responsible for forming the capsid structure [39], while L2 proteins are crucial for the effective encapsulation of viral DNA and ensuring the infectivity of HPV virions [40].

2.1.3 HPV life cycle in stratified epithelium

HPV infects the basal keratinocytes of stratified cutaneous and mucosal epithelia and has evolved life cycle strategies tightly linked to the renewal program of multilayered epithelial tissue (Fig. 2). The epithelial stem cells attached to the basement membrane reside in the basal layer, the innermost layer of the epidermis, which is responsible for replenishing the stratified epithelia. When a basal keratinocyte divides, the daughter cells have two alternatives: maintain stem-cell status or undergo differentiation, leaving the basement membrane and start migrating upward to the epithelial surface. Fully differentiated keratinocytes (dead, keratinized cells) are constantly sloughed off from the tissue surface as part of the constant epithelial regeneration process [42,43]. PVs exploit this renewal process and have evolved successful strategies to manipulate many cellular signaling pathways to support the effective production of progeny virions in keratinocytes undergoing differentiation. Namely, HPV enters the epithelium through microabrasions, and the productive viral infection is expected to infect the basal keratinocytes where the virus establishes a quiescent, persistent infection (i.e., low viral DNA copy number and very limited gene expression). When infected keratinocytes undergo the epithelial differentiation program, viral oncoproteins promote a cellular environment conducive to high levels of viral DNA and protein synthesis during the productive phase of the HPV life cycle. Such viral DNA replication strategies with high viral activity mostly occur in the upper epithelium, outside of the basement membrane and away from the epithelial immune effector cells, allowing HPVs to evade the host immune system. The amplified viral DNA is packaged into virions and released inside terminally differentiated superficial keratinocytes that are sloughed off the epithelial surface. These cells are destined for death and desquamation, and no virus-mediated cell death and inflammation occur that would activate the host immune response (reviewed in [44,45]).

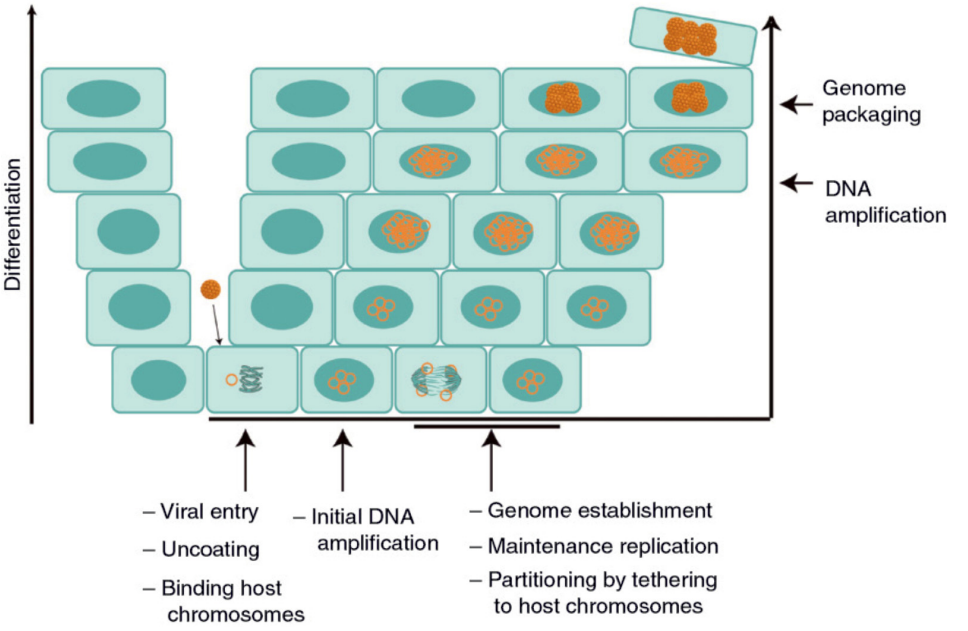

Figure 2. Schematic representation of productive HPV life cycle in a stratified epithelium. The schematic shows the differentiated layers of a stratified epithelium and the viral life cycle events corresponding to the state of infected keratinocytes. See text for details (adapted from [41]).

2.2 HPV DNA replication cycles

The complex differentiation-dependent viral life cycle involves three separate phases of viral DNA replication. The first HPV DNA replication phase occurs soon after entry into the host cell to establish persistent viral infection. It is followed by the stable maintenance phase, during which the viral genomes are maintained at a low, constant copy number. The final stage, vegetative amplification, is a differentiation-dependent DNA replication phase wherein the HPV genomes are amplified to a high copy number prior to virion assembly [46]. Mimicking this viral life cycle that is tightly linked to the host differentiation program under laboratory conditions is quite time consuming and complicated.

2.2.1 The early stages of the HPV life cycle. The initial DNA amplification

Most PVs use the clathrin-mediated endocytosis pathway to enter the host cell [47–49]. During the host cell mitosis, where the nuclear membrane is disassembled and the cellular mitotic chromosomes are available, the L2-mediated binding of the viral genome to the mitotic chromosomes assures the efficient

delivery of the L2-viral genome complex along with a subset of L1 to the host nucleus after cell division [50]. In the nucleus, the viral DNA is localized adjacent to distinct nuclear regions known as nuclear domain 10 (ND10) [51]. These organized nuclear structures consist of proteins that have IFN-inducible antiviral activity and are associated with several cellular processes, including DNA replication, transcription, and DNA repair [52]. However, HPVs, like numerous other viruses, have evolved means to circumvent this antiviral defense mechanism and likely take advantage of these subnuclear sites for effective viral genome replication [53,54]. For instance, the viral L2 reorganizes the Sp100 and Daxx proteins of the ND10 bodies to promote early viral transcription and replication of HPV genomes [55–57]. The L2 protein has been observed to mediate the recruitment of the E2 protein to the ND10 bodies, which would be necessary to initiate the early viral DNA replication and transcription [58].

The initiation of PV DNA replication is determined by the levels of the E1 and E2 proteins; both factors are sufficient and required for the initiation process at the viral origin of replication [59]. DNA replication initiation at the viral origin is an extensively described process. First, the dimeric E1 protein is recruited to the viral origin by viral E2, forming the E1-E2 complex [60] (Fig. 3). E2 is responsible for the specific binding of E1 to the viral origin, interacting with the E1 helicase domain [61,62]. Then, the E2 proteins are displaced from the complex, followed by the loading of additional E1 molecules that results in the formation of a double-trimer complex at the viral origin. This functional complex has activity that induces the local melting of the double-stranded viral origin using ATP hydrolysis energy [62,63]. The generated single-stranded DNA (ssDNA) serves as the subsequent assembly of additional E1 molecules, after which the active double-hexameric helicase complex is formed and loaded onto the single DNA strands to unwind the viral dsDNA (ATP hydrolysis-dependent process) [64,65], making DNA accessible to DNA replication factors. PVs exploit the cellular replicative machinery for viral DNA synthesis, and these factors are recruited to the PV origin by viral proteins. E1 has been demonstrated to interact with several host replication factors, such as DNA polymerase α-primase [66,67], topoisomerase I [68] and replication protein A (RPA) [69] (reviewed in [21]).

Upon formation of the replication initiation complex, the HPV genome undergoes a number of rounds of DNA synthesis to amplify viral DNA to a low copy number (approx. 50–200 copies) [70] for establishing effective viral infection. During the initial DNA replication phase, HPV genomic oligomers (containing several copies of the viral genome) appear, becoming predominant viral DNA forms over time [71–74]. Furthermore, the replication of these oligomeric genomes may initiate at only one active replication origin, ensuring the efficient replication of a large number of the HPV genomes despite the limited availability of viral replication factors [72,75]. This phenomenon would be a very beneficial strategy for the virus to maintain a high genomic copy number with minimal viral biological activity in the lower layer of the infected

epithelium where host immune effector cells reside. The oligomerization process is thought to be an important step for switching to the stable maintenance phase [71]. Hence, the first DNA amplification phase is more like a short-term burst that must be tempered to enter the quiescent, stable maintenance phase and remain undetectable to the host immune surveillance. The E8^E2 repressor protein could have an important role in preventing this runaway replication [26,76,77].

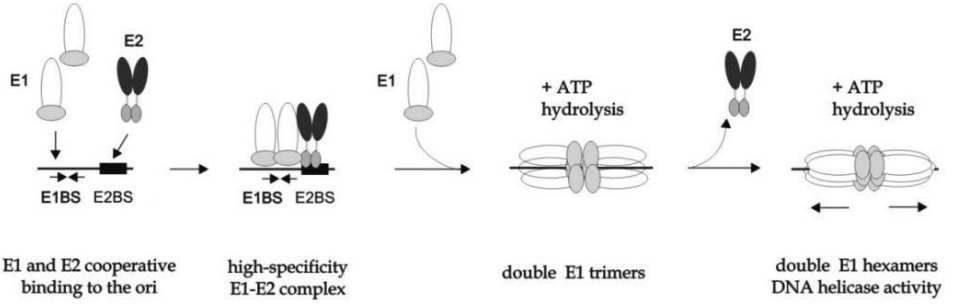

Figure 3. Assembly of the DNA replication initiation complex. The initiation of DNA replication form viral origin is an E1/E2-dependent process. See text for details. E1BS denotes E1 binding site; E2BS denotes E2 binding site (adapted from [79]).

It is generally assumed that viruses replicate via bidirectional theta structures during the initial DNA amplification. The initiation of the theta replication mode involves the formation of two replication forks at the replication origin of the circular genome that progress in opposite directions until they converge approximately 180 degrees from the origin (Fig. 4A). DNA replication is completed by the separation of the replicated DNA molecules. However, this DNA replication mode has not been confirmed experimentally, and currently, accumulating evidence has also indicated that DNA damage response and repair pathways are engaged in the initial replication of the HPV genomes [71,78]. To determine the DNA replication mechanism involved in the initial amplification, the replication intermediates (RIs) that arise during viral genome replication should be analyzed. It is technically challenging to study the HPV DNA replication occurring soon after viral entry into the host cell since the copy number of replicating viral genomes is low in the basal keratinocytes, and therefore, the viral RIs derived from patient tissue samples cannot be detected using standard molecular analysis techniques. Under laboratory conditions, the first amplification phase of PV replication is mimicked by transferring naked viral genomes into eukaryotic cells. The lack of a suitable eukaryotic cell line that readily supports the efficient replication of a full-length HPV genome has hampered the progress in determining the replication mechanism behind the initial viral amplification. Alternatively, short-term HPV replication can be reconstituted transiently in several cell lines by cotransfecting PV origin-containing plasmids and heterologous expression vectors encoding the viral replication proteins E1

and E2, which has extensively enabled the description of the viral DNA replication initiation steps and the viral/host replication factors required for the E1/E2-dependent replication of PV genomes.

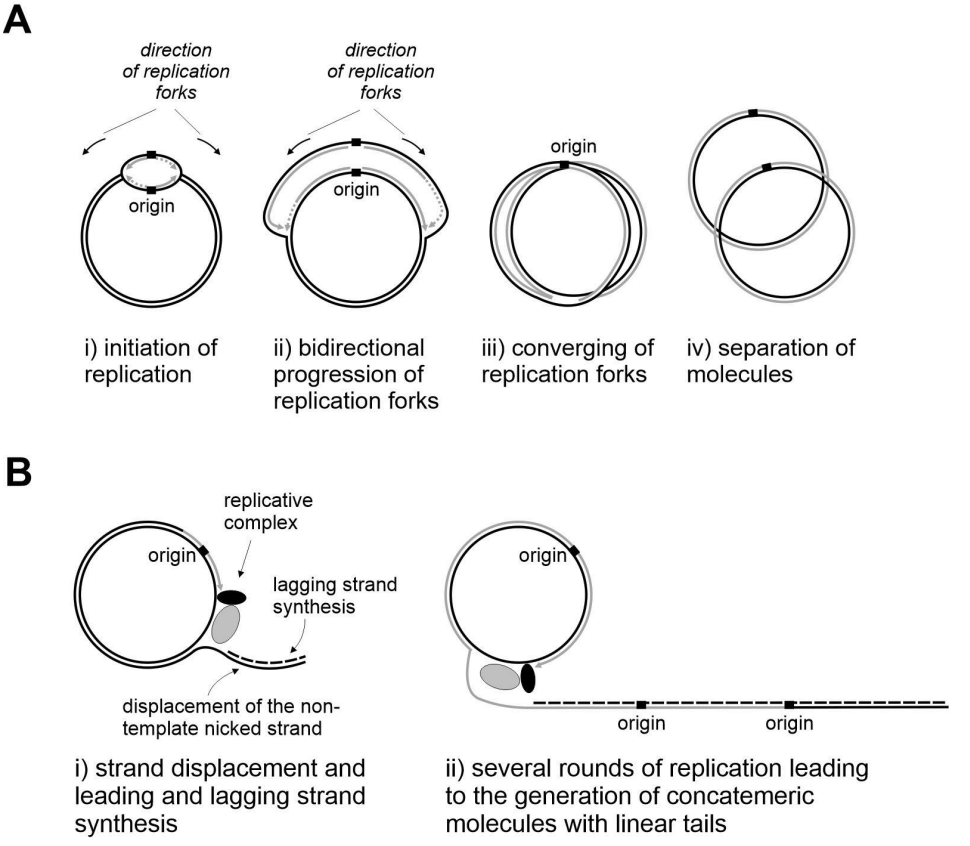

Figure 4. Schematic representation of the replication intermediates of bidirectional theta replication (A) and sigma type rolling cycle replication mode (B). Bidirectional theta replication and sigma type rolling cycle replication are the suggested replication modes of HPV genomes. See text for details. Origin denotes the approximate position of the origin sequence of viral DNA replication (adapted from [93])

2.2.2 Stable maintenance replication

After the initial amplification when a small number of viral genomes have been synthesized, the virus enters a quiescent, stable maintenance phase during which the viral DNA is stably maintained at a near constant level in subsequent divisions of the basal keratinocytes, and the viral infection can last for decades. To sustain a persistent infection, localization of the viral genomes adjacent to the active regions of host chromatin is crucial for continuous viral genome replication and for the effective partitioning of viral episomal genomes to

daughter cells during mitosis. The faithful partitioning of viral genomes to daughter cells is facilitated by the E2 activity of tethering the viral episomal genomes (through E2BSs in the viral genome) to the host mitotic chromosomes, which guarantees that viral episomes are retained in the nucleus of daughter cells after cell division [80,81].

The level of viral DNA replication must be tightly regulated during the stable viral maintenance phase to prevent the runaway DNA replication characteristic of the initial and vegetative amplification phases. Study of the HPV DNA replication of the stable maintenance stage has mainly taken advantage of the W12 (HPV16-positive) [82] and the CIN612 cell lines (HPV-31b-positive) [83], which are derived from low-grade cervical lesions harboring replicating HPV episomes or keratinocytes transfected with the HPV genomes [84–86]. Notably, HPV encodes repressors, including the E8^E2 protein necessary for regulating the level of stable maintenance DNA replication [24,76]. Additionally, the viral replication protein E1 has been demonstrated to be retained in the cytoplasm if the host cell is not in the S-phase of the cell cycle [87]. Furthermore, in certain circumstances, viral DNA replication might even proceed in the absence of the E1 protein [88,89]. However, this result contradicts the findings obtained when analyzing replication intermediates generated during the stable maintenance phase. Two-dimensional agarose gel electrophoresis (2D AGE) analysis revealed that viral DNA replication during stable maintenance phase occurs bidirectionally via theta structures and that the viral DNA replication origin is located within the URR where also the initial E1/E2 dependent viral DNA replication is initiated, thus reducing the likelihood that the E1 protein is dispensable for the initiation of stable maintenance DNA replication [75,90,91].

The maintenance replication of viral genomes occurs in S-phase along with that of cellular DNA. Two different modes have been reported for how HPV maintains the viral copy number at a near constant level in proliferating basal keratinocytes. Namely, in the W12 cell line (HPV16-positive), HPV DNA replication occurred via a strictly controlled once-per-cell cycle mechanism proposed to rely entirely on cellular DNA replication factors, while the random-choice mode was characteristic of the CIN612 cell line (HPV31b-positive), in which some DNA molecules underwent a few rounds of replication during S-phase, some only one, and some did not undergo any. Both modes result in a statistically constant viral DNA copy number per cell throughout the proliferative phase of the basal cell [92]. However, when HPV31b or HPV16 DNA was transferred into the immortalized keratinocyte cell line NIKS, a random-choice mechanism was observed for both HPV types. Moreover, overexpression of the viral E1 protein promoted the random-choice replication mode in W12 cells, indicating that HPV genomes are inherently capable of both replication modes [92]. Collectively, these results reflect that two different DNA replication mechanisms might be deployed for viral DNA replication during the stable maintenance phase, with the choice determined by the availability of viral replication factors in the host cell nucleus.

2.2.3 Vegetative viral DNA replication

When the HPV-infected basal keratinocyte starts moving upwards through the stratified epithelium and undergoes a differentiation program, the viral productive life cycle stage is initiated. The virus must transition out of the stable maintenance phase to promote extensive viral DNA synthesis and late gene expression, ensuring the effective production of infectious viral particles. The considerably increased levels of the viral replication factors, especially E1 protein, in the nucleus of the differentiating keratinocyte promotes the replication mode switch from strictly regulated stable maintenance DNA replication to unscheduled extensive vegetative DNA replication, resulting in thousands of copies of the viral genome per cell [94,95]. The E1 transcripts are transcribed from both early and late promoters during the vegetative phase; however, only the expression from the late promoter is not suppressed by the E2 protein, enabling the high expression levels of the E1 protein. Therefore, the expression of E1 at high levels would be regulated by the differentiation processes of the host cell (the activation of the viral late promoter) and guarantees that expression of the E1 proteins from the viral genomes stably maintained in basal keratinocytes is limited [46]. Additionally, for the effective vegetative amplification of the HPV genomes, the processed E1 protein is required. Namely, upon differentiation, HPV infection activates caspases, which mediate the cleavage of the N-terminus of the E1 protein at a conserved caspase cleavage motif. The caspase-mediated cleavage of E1 appeared to be nonessential for viral genome maintenance in basal cells, implying that E1 cleavage would also be crucial for regulating differentiation-dependent viral DNA replication [96,97].

There has long been evidence that upon differentiation, there is a shift in the viral DNA replication mode. To mimic viral vegetative DNA replication under laboratory conditions, HPV-positive keratinocyte lines that are induced to differentiate in high-calcium medium suspended in methylcellulose are mainly utilized [96,98]. 2D AGE analysis of RIs arising in the W12 and CIN612 cell lines revealed that the bidirectional theta replication structures were preset in undifferentiated keratinocytes, and upon differentiation, only the alternative replication structures were detectable [90]. When analyzing the in vivo DNA replication structures extracted from HPV11-positive vocal cord papilloma tissue (expected to represent vegetative amplification phase due to the high viral DNA copy number), there were also indications of an alternative DNA replication mode, although the bidirectional theta structures were predominant [99]. These additional RIs are consistent with the unidirectional DNA replication mode with the absence of specific initiation and termination sites, and therefore, a rolling circle mode of DNA replication has been proposed to be involved in the vegetative viral DNA replication [90] in which nicking of one strand generates the 3’ end, enabling leading strand synthesis, which together with lagging strand synthesis on the displaced strand template generates linear tails of concatemeric genomes [100] (Fig. 4B). However, these alternative DNA replication intermediates identified during the vegetative amplification phase are also compatible

with recombination-dependent replication (RDR) mode, which is in line with the accumulating evidence of the localization of the cellular DNA damage response and repair pathway proteins in the HPV replication foci.

2.3 DNA damage response

The integrity of the cellular genome is constantly challenged by environmental agents or lesions arising spontaneously during DNA metabolism. To counteract genomic insults to retain genome functionality, cells have evolved DNA repair and genome surveillance mechanisms, collectively forming the DNA damage response (DDR) network. In principle, when DNA lesions are discovered by DDR sensor proteins, transducer kinases are recruited to the site of damage. Then, together with mediator proteins, the signal is amplified and mediated, and through the effector kinases, the signaling pathways are activated. This process results in arrested cell cycle progression induced by activated cell cycle checkpoints to prevent cells with damaged or incompletely replicated DNA undergoing mitosis and ultimately, the influx of DNA repair proteins to the DNA lesions. For counteracting genomic insults, two major signaling pathways are utilized, depending on the nature of the DNA lesion [101].

2.3.1 DNA double-stranded breaks

dsDNA breaks (DSBs), mainly induced by exogenous chemicals, reactive metabolic intermediates, ionizing radiation, or replication stress, are considered one of the most deleterious types of DNA damage with the potential to induce chromosomal rearrangements. DSBs can be repaired through two major distinct repair mechanisms: the error-prone nonhomologous end joining (NHEJ) or the error-free homologous recombination pathway. The occurrence of homologous recombination is limited to late S to G2 phases, while NHEJ acts primarily in G1 phase, although it can function throughout the cell cycle [102]. Thus, depending on the cellular context, DSBs could be recognized by two main sensors, the Mre11-Rad50-Nbs1 (MRN) or the Ku70/Ku80 complexes, which determines the choice of repair pathway. The binding of the Ku70/Ku80 heterodimer leads to the engagement of DNA-PKcs to promote the repair of DNA damage via the NHEJ pathway (i.e., the ligation of two broken DNA ends together) [103], whereas the MRN complex (in particular the activity of Nbs1) has an important role in recruiting the ATM (Ataxia telangiectasia mutated) kinase that, through autophosphorylation, triggers the repair mechanism via homologous recombination. The checkpoint kinase 2 (Chk2), a crucial down-stream effector in the ATM signaling cascade, amplifies the DNA damage signal throughout the nucleus and promotes cell cycle arrest in response to DNA damage [103]. Homologous recombination-mediated repair proceeds in several distinct steps: upon detection of DSBs, the MRN complex, in particular

the nuclease activity of Mre11, is responsible for processing the DNA to generate the 3′ ssDNA free end. BRCA2 mediates the recruitment of the recombinase RAD51 to DSBs, and subsequently, a RAD51-ssDNA nucleoprotein filament is formed that invades the undamaged homologous DNA region, forming the displacement loop (D-loop) structure. Subsequent to D-loop formation, DNA synthesis is initiated, and the homologous DNA region serves as a template to faithfully repair the damaged DNA sequence (reviewed in [104]).

2.3.2 DNA replication stress

The DDR response is also stimulated in response to DNA replication stress manifested as stalled DNA replication forks. It may arise from different sources, including secondary DNA structures, collision with transcription complexes, common fragile sites, interstrand crosslinks (ICL), or a shortage of nucleotides [105]. Replication stress is recognized by ATR (ataxia telangiectasia and Rad3 related) kinase in the presence of DNA structures that contain ssDNA bound by RPA. The DDR response is advanced upon the localization of ATR, and its obligate partner, the ATRIP (ATR interacting protein), to the site of DNA damage, which is followed by the loading of the ring-shaped 9-1-1 complex (Rad9-Rad1-Hus1) onto the collapsed DNA replication forks. Finally, the 9-1-1 complex brings TopBP1 (topoisomerase II-binding protein 1) in close proximity to the ATR-ATRIP complex, unleashing the ATR kinase activity [106]. The activated ATR kinase, the key protein in the pathway, transduces the signal through phosphorylation of the checkpoint kinase Chk1 that induces cell cycle arrest at the G2/M checkpoint. Additionally, the ATR signaling cascade plays an important role in regulating the stability of stalled replication forks and recruiting DNA repair proteins, such as BRCA1 and Bloom syndrome protein (BLM), to the damaged sites [107,108]. Moreover, Chk1 has been shown to induce homologous recombination through the phosphorylation of Rad51 [109], and the role of ATR in promoting homologous recombination has been proposed to be restricted to replication-born DSBs [106].

The ATR and ATM signaling pathways have been thought to operate in parallel as two distinct kinase signaling cascades activated in response to DSBs and replication stress, respectively; however, more recently, accumulating evidence has shown that these two pathways might be interdependent. Namely, the MRN complex is necessary for promoting strand resection and the activation of ATR-Chk1 in response to DSBs [110]. For instance, although the MRN complex is a key component in the ATM-mediated repair of DSBs through the homologous recombination, it has been suggested to be necessary for the activation of ATR in response to stalled DNA replication forks and is also involved in activation of ATR for repairing the replication-associated DSBs [111–113]. Moreover, the MRN complex protein of Nbs1 likely has an essential role in the resolution of stalled replication forks through homologous recombination [114]. Additionally, the ATR and ATM signaling cascades intersect

with the Fanconi anemia (FA) pathway to respond to DNA interstrand crosslink (ICL) lesions. FA patients are hypersensitive to all DNA cross-linking agents due to the dysfunctional FA repair pathway. This pathway comprises at least twenty-two FA proteins and many associated factors [115] classified into groups according to their function. Stalled replication forks at the ICLs are recognized by the FA targeting components (the FANCM-FAAP24-MHF complex), which in turn recruit the FA core complex at sites of DNA damage [116,117]. The FANCM protein is responsible for the activation of ATR kinase in response to replication stress [118], resulting in the ATR-mediated phosphorylation of numerous FA proteins, including the key complex FANCI-FANCD2 (FA ID), which is necessary for its ubiquitination by the FA core complex [119]. This ubiquitination leads to the recruitment of the FA effector and other repair proteins (e.g., BRCA2, RAD51) to the lesion site to remove the ICLs and restore stalled replication forks [120–122]. The ATM kinase also mediates the phosphorylation of FANCD2, which is important for activating the intra-S-phase checkpoint [123]. Recently, FANCM has been suggested to interact with the BLM complex in stalled forks to promote both repair and replication traverse of ICLs [124]. Several studies have shown that BLM plays a role in resolving replication stress by stimulating broken DNA end resection and homologous recombination processes [125–130].

2.3.3 HPV manipulation of the DDR

HPVs encode only two replication proteins, and therefore, their DNA-reproduction mechanism is highly dependent on host factors. HPVs must have evolved strategies that enable viral DNA replication in differentiated keratinocytes that have completed S-phase. Over the past several years, an accumulation of evidence has indicated that HPV infection activates DNA damage and repair pathways. However, the DDR cascade is not activated as an indirect response to viral infection, but instead, it is a critical part of effective viral genome replication. In principle, the virus recruits DDR proteins to the specific viral nuclear foci [78,97,131,132] and likely takes advantage of the activated DNA repair factors for its genome replication; however, the exact mechanism remains to be elucidated. The knowledge of the involvement of DDR in HPV DNA replication has been mainly elucidated from studying vegetative viral DNA replication. Although the activated ATM pathway components localize in the viral replication foci throughout the viral life cycle, the ATM is necessary for HPV genome replication only in differentiated keratinocytes [78,97]. Additionally, the cellular repair factors Rad51 and BRCA1 associated with homologous recombination are localized to viral DNA foci and are required for HPV genome replication. This fact raises the possibility that recombination might be involved in HPV DNA amplification and that homologous recombination might be induced by ATM-Chk2 signaling [132,133]. However, the function of the ATM-Chk2 pathway in HPV vegetative DNA replication is somewhat

controversial. Namely, the absence of Nbs1 (a direct upstream activator of ATM kinase) blocks vegetative viral DNA replication and limits the localization of Rad51 to viral foci; however, the ATM kinase and its substrate Chk2 maintain activation upon depletion of Nbs1 in differentiating keratinocytes, indicating that Nbs1 might not contribute to vegetative viral DNA replication as an upstream regulator of ATM kinase [134]. In addition to ATM signaling, the ATR pathway components are also localized and activated at viral replication centers during the HPV life cycle [74,78], and these factors are required for HPV genome replication in both undifferentiated and differentiated keratinocytes [74]. Recently, there have been suggestions that the ATM and ATR pathways are likely interlinked [110–114]. For instance, the MRN complex (a key component in the ATM-mediated repair of DSBs through the homologous recombination) is also crucial in ATR activation in response to stalled DNA replication forks and to the replication-associated DSBs (e.g., a consequence of replication fork collapse) [111–113]. Additionally, the MRN complex protein Nbs1 is involved in the ATR-mediated resolution of stalled replication forks via homologous recombination [114]. Thus, ATR signaling may be a vital component in HPV DNA replication. For instance, ATR signaling would be activated in response to replication stress to restart stalled replication forks through homologous recombination; ATM might regulate HPV genome replication upon differentiation via different pathways than those associated with homologous recombination. For instance, ATM-Chk2-mediated E1 protein processing by caspases upon differentiation has been shown to be crucial for viral productive DNA replication but is not essential for viral genome maintenance in the basal cells [96,97]. However, these are merely speculations, and the exact mechanisms of the involvement of these interdependent DDR pathways in HPV DNA replication need further investigation, similar to the recently reported FA pathway factors.

HPV infection was identified in over 80% of squamous cell carcinomas (SSCs) from Fanconi anemia patients compared to 36% from the control group [135], implying a possibility that there could be a link between the FA repair pathway and HPV DNA replication. A number of other studies have provided additional evidence that the FA repair pathway might be implicated in HPV DNA replication strategies. The FA pathway key regulator protein FANCD2, which activates ATR-mediated phosphorylation [119], appears to play an important role in viral DNA replication. For instance, activated FANCD2 accumulates in replication foci, binds to several sites along the HPV31 episome, and is necessary for episomal maintenance in undifferentiated keratinocytes [136–138]. On the other hand, the loss of FANCD2 in differentiated keratinocytes leads to enhanced vegetative DNA replication, suggesting that the FA pathway may limit HPV genome replication upon differentiation [139]. Hence, further investigations are needed to clarify the role of the FA repair pathway in HPV DNA replication mechanisms.

Although cellular DDR pathways are clearly necessary for HPV DNA replication, the exact viral functions and mechanisms that activate DDR remain

unknown. The viral oncoprotein E7 has been implicated in the induction of DDR signaling during viral infection. E7 expression from heterologous vectors leads to the upregulation and accumulation of homologous recombination and MRN complex proteins and enhances ATM-Chk2 signaling activity through the activation of transcriptional regulator STAT5 [74,133,134]. In addition, the E1 protein has been shown to induce DDR by directly generating DSBs in host DNA, and when expressed together with E2, both viral factors form distinct nuclear foci where DDR markers are also recruited [78,87,140]. However, it appears that the E1-mediated large-scale cellular DNA damage occurs only under overexpression conditions because it was not observed during transient viral DNA replication assays (i.e., the E1 protein is expressed from the HPV genome) [78]. The E1/E2-dependent viral initial DNA amplification has also been speculated to be initiated repeatedly from the viral origin. This rapid DNA replication potentially generates onion skin-like aberrant DNA structures that activate DNA damage and repair mechanisms to resolve these DNA structures [141]. Collectively, the triggering factors and processes of DDR are only partially understood, and future investigations are needed to define the exact mechanisms.

2.4 DDR and HPV genome integration

In normal, healthy cells, DDR activation also results in the accumulation of downstream signaling factors that ordinarily trigger cell cycle arrest to enable the repair of damaged DNA, and restart occurs only if DNA lesions are eliminated. However, during HPV infection, the DDR is turned on, but the cell cycle progresses along with viral DNA replication [142]. This viral DNA replication-conductive environment and resistance to apoptotic signals are mainly mediated by the activity of the viral oncoproteins E6 and E7. However, this thoroughly reshaped cellular environment makes the host extremely susceptible to mutations and genetic instability. Additionally, the minimal coding capacity makes HPVs reliant on cellular factors with the result that viral DNA replication centers are frequently formed adjacent to common fragile sites (CFSs) [143]. Likewise, the HPV16 E1/E2/Brd4 complex has been shown to bind to host chromatin at CFSs [143]. CFSs are hypersensitive to replication stress that can be caused by a paucity of replication origins, leading to late or incomplete replication, or contain difficult-to-replicate sequences, resulting in stalled DNA replication forks [144]. Notably, CFSs are still undergoing DNA replication in the G2 phase, and DNA damage and repair factors in CFSs are engaged to maintain the stability of these regions [145,146]. ATR signaling has been demonstrated to be critical for sustaining CFS stability [147], while ATM signaling plays a role only in the absence of ATR [148]. Thus, localization close to these regions is potentially favorable for HPV genome replication. However, the close vicinity of viral and cellular DNA replication at the regions undergoing replication stress could promote accidental integration into the host genome.

This idea is in line with the notion that the integration loci of high-risk HPVs are frequently associated with CFSs in HPV-induced cancer cells [143,149,150], and in most cases, the expression of the viral oncogenes E6 and E7 is deregulated, leading to abrogative cell cycle checkpoints and continuous cellular proliferation. This reshaped cellular environment ensures a growth advantage that potentially promotes genetic instability and oncogenesis over time, which is a dead-end for virus production [151].

HPV genomes are commonly integrated into host DNA as either a single genome or tandemly in a head-to-tail orientation [152]. The dysregulation of E6 and E7 can be a result of the disruption of the E2 ORF during the integration event [153] or by methylation of the E2BSs within the URR [154]. Tandemly integrated HPV16 genomes have also been reported to generate a Brd4-dependent superenhancer-like element composed of interspersed tandem repeats of the viral URR and a cellular enhancer, facilitating abundant expression of viral oncogenes [155,156].

Although the integrated state of HPV DNA is predominant in cervical cancer cells (approx. 80%) [157,158], tumors with merely episomal viral DNA or with a mixture of both are also noted [157,159]. When biopsy specimens of precancerous lesions (CIN 1–3) were analyzed, the HPV DNA was almost always extrachromosomal [157]. In contrast to cervical cancer, in most cases (up to three-quarters) of HPV-associated oropharyngeal carcinoma, the viral genomes retained the extrachromosomal state and replicated in an E1/E2-dependent manner [160]. The substantially increasing incidence rate of HPV + OPC in developed countries over the past two decades constitutes an ongoing health concern, and unlike cervical cancer, there is currently no highly effective screening test (such as a Pap smear) for identifying potentially precancerous or cancerous lesions in this case [161]. Hence, there is a clear unmet medical need for efficient treatment solutions regarding HPV-related diseases. Since virtually all precancerous HPV-positive lesions and a significant number of malignancies contain exclusively episomal DNA, targeting E1/E2-dependent viral DNA replication might be a potential therapeutic strategy [141].

2.5 Animal models of papillomavirus infection

Model systems that precisely mimic PV infections in humans are required to evaluate the preclinical safety and efficacy of potential prophylactic or therapeutic antiviral agents. Although the availability of in vitro model systems has increased our understanding of HPV differentiation-dependent reproductive mechanisms and the interactions between viral and host proteins involved in these processes, it is evident that in vitro systems are generally unable to mimic significant aspects of natural PV infection, including interactions with the immune system that control infection, the lesion formation process, the study of viral latency, and the synergy between virus and host genetic and environmental factors. To date, several animal papillomaviruses and their hosts have been used

as models for studying the basic aspects of PV infection in humans as well as for the development of prophylactic vaccines. However, the current animal models of HPV-associated diseases have several drawbacks related to the key aspect that HPVs propagate themselves only in humans and that HPV-induced precancerous lesions arise at specific epithelial sites, which are not straightforward to model using current animal models [162].

Cottontail rabbit papillomavirus (CRPV) model has been extensively used for the development of prophylactic vaccines and has given more insights into the function of viral gene products during productive and latent infection and malignancy progression [163–169]. CRPV causes cutaneous papillomas in its natural host, whereas in domestic rabbits (New Zealand White rabbits), CRPV fails to properly finish the productive viral life cycle, producing only a small number of infectious virions or none at all [165,168,170,171]. These lesions have the potential risk for malignant transformation, and therefore, CRPV infection in domestic rabbits has been studied as a model for HR HPV-induced carcinogenesis. Although the life cycle deregulation by the E6 and E7 proteins is necessary for the development of the cancer phenotype [171–174], the malignancy still progresses to CRPV-induced skin warts, whereas the HR alpha-HPV-induced cancers most frequently arise at discrete mucosal sites [175]. HPV vaccination studies have also been conducted using canine oral papillomavirus (COPV), which is considered a mucosal model of HPV infection, although unlike HPVs, it is unable to infect genital mucosal tissue [176–179]. At the sequence level, COPV exhibits the greatest sequence homology with the HPV types causing plantar and palmar warts (e.g., HPV types 1, 63, and 65) rather than mucosal HPV types [180]. Likewise, the differences between COPV and mucosal HPV types are also mirrored in the timing of life cycle events. Namely, the late events of COPV life cycle are already triggered (i.e., the abundant expression of E4 protein) in the lowest epithelial layers, which is also a characteristic feature of related cutaneous virus types (e.g., HPV types 1, 63, and 65) [181]. In contrast, mucosal alpha-HPVs (e.g., HPV11 and 16) generally express their late proteins at high levels only in the upper half of the epithelium, which is part of complex immune evasion strategies [182–184]. In recent years, the COPV model has been superseded by rabbit oral papillomavirus (ROPV), which has played an important role in assessing natural host immunity to infections [185]. The ROPV tissue tropism and infection in New Zealand White rabbits likely resembles LR mucosal HPV11 infection in humans [181,186–188]. The life cycle organization of ROPV is closer to those of the human mucosal types, and productive viral genome amplification is induced in keratinocytes undergoing differentiation, not in the basal layer [181].

Despite the current range of animal models, a need for a preclinical model that properly mirrors medically important HR mucosal HPV infection still exists since previously described animal models mimic the infection of cutaneous or LR mucosal HPV types. The HR mucosal HPVs have different life cycle characteristics and disease associations compared to the LR types, and the current animal models mimic these aspects of the life cycle imprecisely. The

HR mucosal HPVs have different life cycle characteristics and disease associations compared to the LR types, and the current animal models mimic these aspects of the life cycle imprecisely. For instance, they have different life cycle strategies, which is reflected in the poor ability of LR to promote the development of malignancy. This capacity mainly lies in the oncogenic properties of the viral E6 and E7 proteins facilitated by differences in transcriptional regulation and viral protein function (in particular, binding affinity for tumor suppressor proteins p53 and Rb). The oncoproteins of LR types do not have the ability to stimulate extensive cell proliferation in the basal cell layers, as it is characteristic of HR HPV infections [189]. Although viral oncoproteins are required for completion of a productive life cycle, the deregulated expression of these proteins would cause the accumulation of DNA mutations and chromosome rearrangements, leading to neoplasia and over time, malignancy [190,191]. The capability of HR HPVs to undergo a productive life cycle is believed to be most influenced by the site of infection. The most common sites where HR HPV-induced cancer most frequently arise are the anal and cervical squamocolumnar junction (in case of the cervix, the region where the ectocervix transitions into the columnar cells of the endocervix), and the crypts of the oropharynx [175,192,193]. Therefore, HR HPVs appear to have different gene expression patterns at the transformation zone and the adjacent ectocervix, and thus, the frequency of cancer occurrence [162]. The current disease models mimic these specific mucosal sites inaccurately, and due to the considerable evolutionary distance between several animal PVs and the medically relevant HPV types, general conclusions can often be made only about PV-host interactions, rather than observations about distinct HPV types [192]. Therefore, there is a considerable need for a relevant in vivo model for preclinical testing designed to evaluate therapeutic strategies against clinically relevant HPV-associated lesions.

3. OBJECTIVES OF STUDY

Persistent HR HPV infection is considered to be a strong risk factor in the development of several anogenital as well as head and neck cancers. Although HPV-induced cancers are among the leading causes of cancer mortality worldwide, no effective therapeutics for ongoing HPV infection currently exist. The progress in drug development has been mainly hampered by the incomplete understanding of viral DNA replication mechanisms and the lack of relevant animal models accurately mimicking HR HPV infection in humans, which are crucial prerequisites for developing effective therapeutic strategies. Furthermore, the complex and differentiation-dependent viral life cycle has hindered studying HPV DNA replication mechanisms under laboratory conditions. For that reason, our research group has developed a human U2OS cell line-based assay system that has proven to be a useful and efficient tool for studying HPV genome replication [72].

The general objectives of the research summarized in the present thesis were to investigate the initial amplification of HPV genomes and characterize the molecular mechanisms of HPV-related cynomolgus macaque papillomaviruses (MfPVs) as attractive targets for the development of novel animal model systems for HPV drug testing. We specifically focused on the following aims:

- To analyze the DNA replication intermediates arising during the initial amplification of the HPV18 genomes using 2D and 3D agarose gel electrophoresis techniques and determine the mechanism(s) behind them.
- To establish the U2OS cell line-based model system for studying the molecular biology of the cynomolgus macaque cutaneous papillomavirus MfPV1 as well as the mucosal types MfPV5 and MfPV8.
- To study the viral DNA replication and gene expression properties of MfPV1, MfPV5 and MfPV8 and juxtapose the obtained results with their closest HPV types.
- To characterize the sensitivity of the MfPV genome replication to HR HPV-specific inhibitors.

4. MATERIALS AND METHODS

In the present study, the U2OS cell line-based in vitro model system was implemented to analyze the HPV episomal genome replication structures as well as the replication and transcription properties of the MfPV genomes. The U2OS cell line, derived from the bone tissue of a 15-year-old human female suffering from osteosarcoma, has an adherent epithelial cell-like morphology and carries wild-type p53 and pRb genes [194]. Although U2OS cells are not the natural host cells of PVs, they provide an adequate cellular environment for studying the replication of HPV episomal genomes. It has been demonstrated that the HPV18, HPV11 and HPV5 gene expression profile and oligomeric replication end products generated during the initial amplification truly reflect the situation previously described in keratinocytes [71,195–197]. The development of U2OS-based cellular assay system supporting all three viral DNA replication phases of cutaneous as well as LR and HR mucosal HPV types has been extensively described in the publication by Geimanen and colleagues [72].

All the conducted experiments of the present thesis describe processes occurring during the initial amplification phase of viral episomal genomes and detailed experimental procedures have been described in the Materials and Methods sections of publications I, II and III. In principle the viral DNA replication assay consisted of the following steps: bacterial backbone-free covalently closed circular viral DNA molecules (Ref. III, Basic Protocol 1) were transferred to U2OS cells using efficient DNA electroporation method, allowed to replicate, and at the indicated time points episomal DNA was extracted (Ref. III, Basic Protocol 2). The replicated viral DNA was analyzed using agarose gel electrophoresis (AGE) method followed by Southern blotting (Ref. III, Basic Protocol 3–5 or 6, respectively). The viral DNA replication end products were analyzed using one-dimensional (1D) AGE method. For the analysis of viral DNA replication intermediates, different electrophoresis conditions were implemented, such as 2D neutral/neutral (N/N), neutral/alkaline (N/A) and three-dimensional (3D) neutral/neutral/alkaline (N/N/A). These 2D and 3D AGE methods and related experimental procedures have been described extensively in the publication II (Basic Protocol 3–5). For the construction of the MfPV early region transcription maps, the RNA was extracted from the U2OS cells replicating MfPV genomes and analyzed using 5′ and 3′ RACE methods and RT-PCR (for detailed description, see the Materials and Methods section of publication III).

In all described studies, the viral genomes that were introduced into the U2OS cells were prepared as bacterial backbone-free covalently closed circular DNA molecules. This was conducted by adaption of the recombination-based minicircle DNA production technology developed by Kay and colleagues [198]. Relevant experimental procedures have been also described in detail in the paper II (Basic Protocol 1).

5. RESULTS AND DISCUSSION

5.1 Initial amplification of the HPV genomes proceeds through two different replication mechanisms (Ref I, II)

For determining replication modes of HPV genomes during the initial amplification phase, multidimensional AGE techniques were implemented to analyze the viral DNA replication intermediates. To perform and interpret such highly specialized analyses are challenging. Both publications Ref I and Ref II address the characterization of the replication structures of HPV genomes. The Ref II primary focuses on the technical aspects and the interpretation of the analyses while the aim of the Ref I is to determine the replication mechanism(s) behind viral initial DNA replication.

The initial amplification of the HPV genomes initiated by the viral E1 and E2 proteins from the viral replication origin within the URR [59,85,152,199–202] is assumed to proceed bidirectionally through theta-mode replication. However, this hypothesis is not confirmed by experimental data because it is very complicated to study HPV DNA replication occurring soon after viral entry into the host cell. At the same time, there are indications that DDR pathways are involved in the initial viral amplification phase. For instance, the HPV replication-dependent oligomeric genomes have been shown to be formed through homologous recombination [71], and the ATR pathway proteins are engaged in HPV replication centers during this replication phase [78]. The U2OS cell line-based model system has enabled us to study the initial replicative amplification of the HR HPV18 genome. We used two- and three-dimensional gel electrophoresis techniques to characterize the viral DNA replication mode(s) by separating branched DNA replicative intermediates from replication products. A replicating DNA molecule progressively doubles in mass, acquiring various complex topologies during the synthesis process. The 2D AGE technique contains subsequent gel electrophoresis steps: first, separating DNA molecules mainly in proportion to their extent of replication (molecular mass), and the conditions of the following second dimension favor separation on the basis of topology (Ref. II, Fig. 14B.10.2) [203]. Furthermore, a subsequent third dimension conducted under alkaline conditions enables the characterization of newly synthesized DNA by resolving nascent DNA strands from the parental strands (Ref. II, Fig. 14B.10.4) [204,205].

Circular DNA molecules typically replicate bidirectionally via theta structures, the replication mode proposed for the HPV initial DNA replication. The initiation of the theta replication mode involves the formation of two replication forks at the replication origin that progress in opposite directions until they converge. Such replicating DNA molecules in general give rise to three different types of replication intermediates: converging fork (dY), replication bubble and simple Y intermediates whose structures are determined by the position of the

endonuclease restriction site relative to the DNA replication origin (Ref. II, Fig. 14B.10.7 E–I) [203,206].

When analyzing replication intermediates generated during the initial amplification of the HPV18 episomal genomes in U2OS cells, we detected replication intermediates characteristic of bidirectional theta replication initiating from the viral replication origin within the URR and replication forks converging over the quite broad area of the viral circular genome (the end of E1 ORF to the middle of the L2 ORF) opposite of the viral DNA replication initiation site (Ref. I, Fig. 5). This observation was demonstrated by noticing the bubble-shaped RIs if the viral DNA restriction fragments containing the origin of replication were analyzed (Ref. I, Fig. 3, Fig. 5; Ref. II, Fig. 14B.10.7) or the presence of dY intermediates within DNA restriction fragments opposite the initiation site of viral DNA replication, indicating that the viral DNA replication termination occurred within this fragment (Ref. I, Fig. 3, Fig. 5; Ref. II, Fig. 14B.10.7). During the HPV18 initial DNA amplification, the accumulation of extremely branched molecules with large mass was observed, exhibiting a typical migration pattern of almost fully replicated bidirectional theta replication intermediates (Ref. I, Fig. 2–5, marked by black arrowhead; Ref. II, Fig. 14B.10.7, marked by gray bold arrow). The accumulation of these late theta intermediates proposes that the completion of bidirectional theta replication is quite slow and complex step in the initial amplification phase. Also, there might be additional populations of RIs from different origin (e.g., complex D-loop and/or X structures indicative of hemi-catenates or Holliday junctions) moving together with the late theta intermediates because of the large mass and highly complex nature of these molecules that limit their identification by agarose gel electrophoresis technique.

We also noted that the initial replication of the HPV18 genomes results in RIs from different origins that are not characteristic of bidirectional theta-mode replication (Ref. I, Fig. 2–5, marked by white arrows; Ref. II, Fig. 14B.10.7, Fig. 14B.10.8, Fig. 14B.10.9, marked by white bold arrows). These additional replication intermediates were also present in the immortalized human keratinocyte HaCat cell line [207] (Ref. I, Fig. 7) and HPV-31b-positive human cervical cell line CIN-612 derived from a CIN1 lesion (data not shown), further confirming the authenticity of these RIs and excluding the possibility that these are specific to the U2OS cell line alone. Further characterization of the additional group of RIs revealed that they are most likely consistent with a unidirectional mode of DNA replication that lacks a specific initiation site. The Y-shaped nature of the nascent strands of these additional molecules detected via N/A and N/N/A AGE techniques provides evidence that the additional replication mechanism proceeds unidirectionally (Ref. I, Fig. 3B, Fig. 4; Ref. II, 14B.10.8, 14B.10.9). This conclusion is also supported by the analysis of the HPV18 subgenomic fragments, suggesting that the migration pattern of secondary RIs shared characteristics with Y-shaped structures (Ref. I, Fig. 5; Ref. II, Fig. 14B.10.7 A). Furthermore, these additional RIs maintained the same migration pattern during 2D N/N and N/A AGE analyses regardless of the position of the

endonuclease restriction site in the HPV18 genome (Ref. I, Fig. 3–5; Ref. II, Fig. 14B.10.7 B, Fig. 14B.10.8), indicating the absence of a distinct DNA replication origin sequence, unlike that for the bidirectional theta mode of replication. Determining the direction of the viral DNA replication fork movement demonstrated that in addition to theta replication forks, DNA replication forks moving in opposite directions could also be detected, suggesting that the unidirectional mode of DNA replication does not have determined polarity and can occur in both directions (Ref. I, Fig. 6). Thus, all these considerably different characteristics of the second group replication intermediates, compared to the bidirectional theta replication intermediates, provide evidence that two replication mechanisms are involved in the initial DNA replication of the HPV18 genomes. There have been observations about different DNA replication modes within different viral DNA replication phases. Namely, the unidirectional DNA replication mode without a specific origin sequence has been previously proposed as the replication mode during the vegetative phase, while bidirectional theta replication is characteristic of a stable maintenance phase [90]. However, when in vivo viral DNA replication structures isolated from HPV11-positive vocal cord papilloma tissue were analyzed (determined as the vegetative amplification phase due to the high viral DNA copy number), there were indications of both an alternative DNA replication mode and the bidirectional theta structures, which were predominant [99].

The initial replication of HPV genomes has been reported to be initiated during S-phase and extended to the G2-phase of the host cell cycle [208]. The same indication has been described for viral vegetative DNA amplification that occurs in the G2-like phase of the cell cycle [209,210]. Although PVs encode two replication factors, E1 and E2, the synthesis and processing of viral DNA relies entirely on the cellular replication machinery [66–68]. Therefore, HPV must have evolved strategies for rapid DNA amplification during G2 phase when the cellular DNA is already replicated. Accumulating evidence shows that HPV induces the cellular DDR in the viral replication foci, and the resulting repair pathways are usurped for viral vegetative DNA amplification. DDR induction in HPV replication foci might be facilitated by viral proteins. Furthermore, the E1 protein has been demonstrated to have DNA-damaging activity, generating DNA DSBs in the host DNA [78,140]. However, it appears that the E1-mediated large-scale cellular DNA damage occurs only under overexpression conditions because it was not observed during transient viral DNA replication assays (i.e., the E1 protein is expressed from the HPV genome) [78,211]. Additionally, the viral oncoproteins E6 and E7 modulate many cellular pathways associated with checkpoint control and DNA repair [97,212,213], including E7-mediated upregulation and activation of several DNA damage and repair factors. However, DDR factors are recruited to the replication foci of the HPV18 E6 or E7 mutant genomes during the initial DNA amplification, indicating that the viral oncoproteins are not absolutely necessary for engagement of the DDR pathways to the HPV DNA replication centers [78] and are

more likely involved in the upregulation of DNA damage and repair factors to amplify the DDR signal [141].

There are indications that replication stress occurs during viral initial DNA replication, for instance, the engagement of the ATR pathway (e.g., ATRIP, TopBP1) in the HPV replication foci [78], implying the possibility that the viral DNA replication process itself is directly related to DDR activation. The notable accumulation of late theta structures indicates difficulties with the processing and separation of converged late theta RIs (Ref. I, Fig. 2–5, marked by black arrowhead; Ref. II, Fig. 14B.10.7, marked by gray bold arrow); these stalled theta DNA replication forks are a possible trigger of ATR-mediated rescue. This phenomenon is also an intrinsic part of the replication of the SV40 small circular dsDNA genomes. The converging theta replication forks also accumulate, and the ATR pathway is crucial for completing SV40 (simian virus 40) genome replication; without ATR activity, the converging replication forks stall and eventually collapse, leading to aberrant viral replication products [214]. Strikingly, a temporal difference occurs when two different populations of replicating viral DNA molecules arise during the initial amplification of the HPV18 genomes. Bidirectional theta DNA replication seems to be responsible for the early amplification of viral DNA, since bidirectional theta structures were predominant at first. However, the additional DNA replication intermediates arose over time, becoming as prevalent as theta structures (Ref. I, Fig. 2). Therefore, we propose that two different replication mechanisms are involved in the initial amplification of the HPV genomes: soon after E1/E2-mediated viral DNA replication is initiated, supposedly in the S-phase of the cell cycle [208], the second replication mechanism is initiated. The exact mechanism by which HPVs manipulate and employ cellular repair pathways needs to be clarified. However, the stalled theta replication forks most likely induce a DDR signaling cascade that triggers an influx of repair factors to the viral replication foci, and viral replication is likely restarted by recombination-dependent replication (RDR) mode, which couples the homologous recombination event to DNA synthesis. This replication mode is initiated when the free 3’ ssDNA end (which may arise when the stalled replication forks collapse) invades a homologous DNA molecule, and a processive replication fork is formed that proceeds in a unidirectional manner, enabling the synthesis of extensive DNA sequences well beyond the DNA lesion [215]. Such a replication mode would be quite beneficial for a virus since the initiation site of DNA replication is dictated by the invading DNA sequence, the origin licensing factors are not required for restarting DNA replication [216], and enabling of a rapid and high-fidelity genome amplification in the G2 phase when there is no competition for the resources is also required for cellular DNA synthesis. Several characteristics of the additional DNA replication intermediates revealed using 2D and 3D AGE techniques are compatible with RDR (i.e., a unidirectional mode without fixed polarity, the absence of a specific origin sequence), and the homologous recombination involvement in the generation of oligomeric HPV genomes during the initial amplification has been reported [71].

Remarkably, the initiation structures of the additional replication mechanism never originate from 8 kb (1n) nonreplicating molecules, but instead, RIs with a molecular mass of approximately 1.2–1.4n are first detectable (Ref. I, Fig. 2–4; Ref. II, Fig. 14B.10.7 B, Fig. 14B.10.9). This finding would be due to the extremely labile nature of these structures (as strand invasion structures could be), and therefore, we are not able to detect the initiation structures of the additional replication mechanism. Alternatively, the initiation intermediates are more complex structures with larger molecular masses compared to 1n molecules, indicating that in addition to a unidirectional DNA replication fork, unidentified complex structures may be present whose nature remains to be elucidated.

Recently, Orav et al. shed more light on the HPV DNA replication mechanisms during the initial amplification phase. They provided evidence that the FA DNA repair pathway, mainly modulated by ATR kinase activity [116,217], is involved in the processing of late bidirectional theta RIs and is required for the initiation of the unidirectional replication mechanism [218]. Namely, the viral E1 protein has been demonstrated to form a complex with the FA DNA repair pathway proteins UAF1 and USP1 [219–221], and the activity of this protein complex is necessary for the processing of late bidirectional theta RIs, which are structurally similar to ICLs. After the separation of almost fully replicated viral genomes, unidirectional DNA replication starts from the reinitiated theta replication forks, wherein Bloom helicase likely plays an important role [218].

Collectively, we propose that the initial amplification of the HPV genomes is initiated from viral DNA replication origin within the URR where two replication forks are formed and subsequently progress in opposite directions until they converge. The accumulation of late theta intermediates suggests that the completion of theta replication is complicated and rate-limiting process, thereby the replication forks stall and leading to the subsequent induction of DDR pathways. It is probably that without DDR activation, HPV could not achieve the resolution of its almost fully replicated circular genomes and efficient high-fidelity viral DNA synthesis in the G2 phase that proceeds via a unidirectional replication mode without specific origin sequence, most likely via a recombination-dependent replication mechanism.

5.2 Cynomolgus macaque papillomaviruses may serve as a highly relevant model for preclinical anti-HPV drug testing (Ref III)

Mucosal HR HPV-related cancers pose a considerable global health and economic burden; for instance, cervical cancer is among the leading causes of cancer mortality in women worldwide, and in terms of oropharyngeal cancers, the substantially increasing incidence rate (especially among men) is alarming.

Nevertheless, no relevant animal models exist that closely mimic the specific characteristics of HR HPV infection and have anatomic, genetic, and immunological similarities with humans. The current animal models have provided an essential basic knowledge of PV biology; however, principal differences among existing animal models and mucosal HR HPV infection (e.g., viral transcription regulation, life cycle organization, mucosal tropism, specific biologic context) restrict their use in developing antiviral therapeutic strategies.

The genus Alphapapillomavirus contains HPV types with oncogenic potential that are responsible for nearly all cases of human cervical cancer and a number of penile, anal, and head and neck carcinomas [16,222]. Notably, this clinically important genus also includes alpha-PV types identified in nonhuman primates [223–228]. For instance, several cynomolgus macaque (*Macaca fascicularis*) PVs (MfPVs) isolated from the cervicovaginal area (including MfPV3 and MfPV5 characterized in this study) exhibit a close phylogenetic relationship with HR HPVs (e.g., HPV16) and have the potential to induce obvious clinical symptoms similar to PV-related lesions noticed in humans [223,226,228]. The cutaneous type MfPV1 associated with aggressive papillomas on the hands and feet has also been characterized and clustered with HPV5 into the genus β-PVs, where EV-related types belong [224]. Additionally, a notable homology exists at the sequence level and in genome organization between specific MfPV and HPV types. Accordingly, these arguments strongly support the idea that MfPV genome replication in nonhuman primates could be utilized for the development of reliable animal models for HPV-related therapies. However, all of the above-mentioned conclusions have been made based on sequence analysis [224,226] because of the absence of a suitable model system. Thus, according to the previously described close phylogenetic relationship, we wondered whether the human-derived U2OS cell line could support replication of not only HPV [71,72,78,195–197,208,229–231] but also the closely related viruses isolated from nonhuman primates. Indeed, the cutaneous (MfPV1) and mucosal (MfPV5 and MfPV8) cynomolgus macaque PV genomes are clearly able to replicate in U2OS cells (Ref. III, Fig. 1). The MfPV DNA replication during the initial amplification phase is a strictly viral E1/E2-dependent process (Ref. III, Fig. 2), as is described for the HPV genomes in human keratinocytes [230,231], since the E1 or E2 mutant genomes of all three studied MfPV types alone failed to replicate, while cotransfection of these defective genomes complemented each other's defects and the replication competence of both genomes was restored (Ref. III, Fig. 2 A–C, lanes 4–12). Thus, the U2OS cell line-based assay system offers a chance to characterize these HPV-related viruses experimentally to shed more light on the biology of these viruses at the molecular level to determine their adequacy in more physiologically relevant studies for developing in vivo animal models.

5.2.1 Characterization of MfPV early gene expression during the initial DNA amplification

The fact that MfPV genomes are capable of replicating in U2OS cells provides a unique tool for experimental descriptions of MfPV gene expression during initial replication. When 5′ and 3′ RACE analyses and RT-PCR were used for the construction of the early region transcription maps of MfPV1, MfPV5 and MfPV8, it became apparent that similar to other PVs, MfPVs regulate their gene expression in a coordinated way that includes the usage of different promoters and extensive alternative splicing, enabling the expression of multiple genes from a compact viral genome. In terms of the viral transcription pattern during the initial amplification phase, the mucosal MfPV types 5 and 8 appear to be similar to mucosal oncogenic HPVs, while MfPV1 resembles most HR cutaneous beta HPVs, in particular HPV5. This result is consistent with several findings. Namely, for early gene expression, both mucosal MfPV5 and MfPV8 predominantly used the promoter region within the URR, and the transcription start site (TSS) mapped immediately upstream of the E6 gene (at nts 7982 and 7996, respectively) (Ref. III, S1 and S2 Figs.), which are compatible with those reported for several HR HPVs, including P97 for HPV16 [232,233] and P102 for HPV18 [153,196,234–236]. Additionally, less prominent TSS positioning within E6, E7, E1 and E2 ORFs was identified (Ref. III, S1 and S2 Figs.) that was also defined for HPV18 [153,196]. In contrast, cutaneous MfPV1 has a more characteristic heterogeneous TSS usage since a strong promoter region with several TSSs in the E7 ORF (Ref. III, S3 Fig) was identified. This kind of TSS usage is also typical of the closely related human Betapapillomavirus HPV5 [195].

Additionally, the viral transcripts can undergo extensive alternative splicing, for which MfPV5 and MfPV8 utilize several splicing donor (SD) and acceptor (SA) sites (Ref. III, S1 and S2 Figs.) that share clear sequence homology with the corresponding SD and SA sites of human HR types 16 and 18 [196,232,234,237]. The same conclusions can also be made for the SD and SA regions of MfPV1 (Ref. III, S3 Fig) and closely related HPV5 [195]. The polyadenylation cleavage site for early mRNAs of all three characterized MfPVs shows clear sequence homology with closely related HPVs [195,196,234] and positions near the beginning of the L2 ORF (Ref. III, S1–S3 Figs.) with a conserved upstream polyadenylation AAUAAA motif.

Expressing a shorter form of the E6 protein (termed E6*) is a distinctive feature of oncogenic mucosal HPVs (e.g., HPV types 16, 18, 31, 45). This expression is the result of an internal splicing event due to the presence of an SD and SA pair within the E6 ORF and leads to more effective translation of the E7 oncoprotein [238–240]. The transcriptome mapping experiments revealed that these spliced short E6* transcripts are also characteristic of MfPV5 (Ref. III, S1 Fig, RNAs B–D, G). Notably, although MfPV8 is isolated from precancerous lesions [226], no internally spliced E6 transcripts were detected (Ref. III, S2 Fig, RNAs A–D), most likely due to a mutation in the core motif of the

SD sequence (AG^GT → AT^GT) within the E6 ORF (GenBank ID EF558842). However, when analyzing the DNA sequence of MfPV8, oncoproteins appear to have a number of distinct features characteristic of HR HPVs. For instance, the E6 protein has a putative PDZ-binding motif in the carboxy terminus, which is unique to oncogenic HPVs (e.g., HPV16, HPV18) but is not found in low-risk HPV E6s (e.g., HPV6, HPV11). The PDZ-binding motif has been shown to be important for the interaction of several host proteins involved in cell signaling and adhesion that leads their degradation (reviewed in [241]).

Collectively, these findings demonstrate that a remarkable homology exists not only at the sequence level but also in the gene expression pattern between specific MfPV and HPV types during initial viral DNA amplification.

5.2.2 Characterization of MfPV replication properties and sensitivity to HR HPV-specific inhibitors

As mentioned above, the U2OS cell line is evidently capable of supporting E1/E2-dependent MfPV DNA replication (Ref. III, Fig. 1 and 2). While the MfPV8 genome exhibited a clear DpnI-resistant signal accumulation in U2OS cells, indicating that the viral genome copy number increased over time (Ref. III, Fig. 1, lines 12–14), the replication signal of MfPV5 slightly decreased at later time points (Ref. III, Fig. 1, lines 7–9). Although the replication of beta-PV MfPV1 genomes was obvious in U2OS cells, the DNA replication intensity was still rather modest (Ref. III, Fig. 1, lines 1–3) compared to that of the alpha types (Ref. III, Fig. 1, lines 7–9, 12–14). However, highly efficient time-dependent viral DNA replication was notable for the E8 mutant genomes of all three characterized MfPV genomes (Ref. III, Fig. 2A–C; compare lines 1–3 and 13–15), and most strikingly for cutaneous MfPV1, indicating that viral DNA replication is strongly restricted by the E8^E2 repressor protein. The E8^E2 mutant genomes of mu and beta HPVs have also been demonstrated to replicate readily in human keratinocytes, while wild-type genomes fail [26]. Moreover, the viral E8^E2 protein interacts with the cellular NCoR/SMRT-corepressor complex and thereby mediates the viral transcriptional repression and inhibition of E1/E2-dependent DNA replication [26]. This phenomenon appears to be highly conserved and has been previously described in several human and animal papillomaviruses [23,25,173,242–245]. Thus, using the E8^E2 defective genomes would facilitate studying the HPV types of which DNA replication has not been detected in human keratinocytes in vitro so far.

When the molecular state of MfPV genomes was analyzed in U2OS cells, in addition to the episomal circular monomeric viral genomes, dimeric and oligomeric forms also appeared over time (Ref. III, Fig. 3). This phenomenon is also observed during the initial amplification phase of HPV genomes. HPV genome oligomerization has been proven to be a viral DNA replication-dependent process, and it also occurs when HPV origin-containing plasmids are cotransfected with heterologous expression vectors for the viral replication proteins E1

and E2, indicating that no other viral proteins are absolutely necessary; however, homologous recombination proved to be involved in this process [71]. HPV multimeric genomes are also detectable in clinical samples obtained from HPV-associated precancerous lesions or malignancies [71,152,157,159,246,247]. This finding implies that viral genome multimerization is a general process during HPV infections in vivo, and the results indicate that this is also a distinct characteristic of MfPV DNA replication.

Based on the remarkable similarities between mucosal MfPVs and HR HPVs observed in the molecular analyses, we decided to analyze whether the previously described HR mucosal HPV-specific drug candidates can also inhibit MfPV genome replication. In fact, the MfPV8 genome replication is sensitive to compounds that inhibit only the HR HPV but not the LR (HPV11) or cutaneous HPV (HPV5) genome replication, suggesting that the molecular mechanisms underlying HR mucosal HPV and MfPV genome replication are very similar and that the replication processes likely proceed through the same pathways in the host cells. All these five HR-specific small molecule inhibitors were discovered from high-throughput screening of the NCI Diversity Set IV chemical library [230], and two compounds (NSC 305831 and NSC 88915) are previously known to inhibit tyrosyl-DNA-phosphodiesterase type 1 (Tdp1) activity [248,249]. Tdp1 is involved in the DDR signaling being crucial in releasing stalled topoisomerases from DNA lesions [250]. Additionally, the HPV-specific compounds that target viral DNA replication by antagonizing E1-E2 interactions, have been discovered [251,252]. Although these compounds are efficient in inhibiting viral DNA replication, the effect is visible only for LR HPVs (HPV6b, HPV11), supposedly because of the differences in the E1-E2 complex formation between LR and HR HPVs. Given that, an in vivo model that adequately mirrors the molecular processes characteristic of specific HPV types is a crucial prerequisite for the anti-HPV drug development.

MfPVs are quite common pathogens in cynomolgus macaques associated with naturally occurring cervical neoplasia. It has also been proved that the experimental transmission of MfPV3 from a naturally infected female (exfoliated cervicovaginal cells were collected) to naïve female macaques induced CIN progression [253]. It is noteworthy that CIN lesions in cynomolgus macaques exhibit distinctive histopathologic similarities to those observed in women [253,254], and the MfPV-induced higher-grade lesions predominantly arise at cervical transformation zone as it is also characteristic of cervical cancer in women [254]. Therefore, even if the human U2OS cell line-based model system has limitations, especially in the terms of viral immortalization or transformation potential, but still, the fact that MfPVs and HPVs share similar characteristics in gene expression and DNA replication that is also sensitive to HR HPV-specific inhibitors, would justify more physiologically relevant studies in cynomolgus macaques in purpose to confirm their HR phenotype. Additionally, the ability of HPV to establish persistent infection in cynomolgus macaque merits also investigations. Alternatively, instead of virus particles, naked viral genomic DNA transfer, such as epidermal DNA electroporation [255], could be

applied to deliver viral genomes into the basal layer keratinocytes. This delivery approach could enable the presence of viral genomes and expectantly their replication in the target tissue. Since a nonhuman primate model serves as an essential resource for developing therapeutic antiviral strategies, it deserves further research.

6. CONCLUSIONS

- When the DNA replication structures generated during the initial viral DNA amplification phase were analyzed using 2D and 3D AGE techniques, two distinct DNA replication mechanisms were revealed to be involved in HPV18 genome replication. The first is bidirectional theta-mode replication initiated from the viral DNA replication origin within URR, and the RIs of the second replication mechanism are consistent with a unidirectional DNA replication mode, most likely with RDR.
- The assembled beta and alpha MfPV genomes evidently replicated in human U2OS cells, providing a model system for studying these viruses.
- The transcription maps of the MfPV1, MfPV5 and MfPV8 genomes during the initial amplification phase were constructed. The gene expression patterns of the MfPV5 and MfPV8 genomes are considerably similar to clinically relevant HR mucosal HPVs (e.g., HPV16 and HPV18) in U2OS cells or in human keratinocytes. The transcription pattern of the cutaneous MfPV1 genomes is consistent with the gene expression of beta HPV type 5 genomes in human U2OS cells.
- The replication of all three MfPV genomes is strictly E1/E2-dependent and is strongly restricted by the E8^E2 repressor protein. In addition to monomeric genomes, oligomeric forms also appeared during E1/E2-dependent initial MfPV DNA amplification. All these observations are also characteristic of HPV genome replication.
- The mucosal MfPV8 genome replication is sensitive to HR HPV-specific small molecule inhibitors, suggesting that the molecular mechanisms underlying HR mucosal HPV and MfPV genome replication are very similar and that the replication processes likely proceed through the same pathways in the host cell.

SUMMARY IN ESTONIAN

Uurimistöö inimese papilloomiviiruse ja evolutsiooniliselt lähedase *Macaca fascicularis* papilloomiviiruse genoomi paljundamisest viirusnakkuse varajastes etappides

Inimese papilloomiviirused (HPV-d) on laialt levinud viirused ning tihti arvestatakse neid normaalse mikrofloora osana ning üldjuhul viirusnakkus tervetel inimestel märkimisväärseid kaebusi ei põhjusta. Siiski on mitmetel HPV tüüpidel väga oluline kliiniline tähtsus. Nakkuse kliiniliste ilmingute järgi, vastavalt viirusnakkuse potentsiaalile põhjustada pahaloomuliste kasvajate arengut või mitte, jagatakse viirustüübid kaheks rühmaks: kõrge ja madala riskiga tüüpideks. Madala riskiga HPV tüüpidega nakatumisele on iseloomulikud peamiselt healoomulised vohandid ehk papilloomid (nt soolatüükad, genitaalpiirkonna kondüloomid), mis aja möödudes organismi immuunvastuse mõjul taanduvad. Seetõttu on madala riskiga HPV-d suhteliselt ohutud ning nende mõju piirdub pigem kosmeetiliste probleemidega. Seevastu kõrge riskiga viirustüüpide elutsükli käigus võib leida aset nakatunud raku muutumine kontrollimatu paljunemisvõimega kasvajarakuks, mis omakorda võib aastate jooksul viia vähkkasvaja tekkeni. Kõige enam on HPV-nakkus seostatav emakakaelavähiga (99% kartsinoomi juhtudest on HPV-seoselised), mis naistel on sageduselt teine vähisurma põhjustaja maailmas. Meeste seas on kõige levinum HPV-seoseline (85% juhtudest) pahaloomuline kasvaja anaalvähk. Lisaks on kõrge riskiga HPV-de nakkus oluliseks riskiteguriks ka teiste anogenitaal- ning pea- ja kaelapiirkonna kartsinoomide puhul. Seega on tegemist kliiniliselt väga oluliste viirustega ning HPV-seoseliste haiguste puhul on selgelt määratletav täitmata ravivajadus, kuna tänaseni ei ole õnnestunud välja arendada spetsiifilisi ravimeid väljakujunenud HPV nakkuse kõrvaldamiseks. Senini on kasutusel ainult ennetava toimega vaktsiinid, mis kaitsevad teatud HPV tüüpide nakkuse eest, kuid ei oma mõju juba eelnevalt tekkinud viirusinfektsiooni tõkestamiseks.

HPV-d nakatavad limaskestade või naha epiteelkoe keratinotsüüte. Epiteelkoe kõige alumises kihis (basaalkihis) paiknevad jagunemisvõimlised keratinotsüüdid, mis tagavad koe pideva uuenemise. Raku jagunemisel tekib kaks tütarrakku, millest üks säilitab jagunemisvõime ning jääb basaalkihi koosseisu, teine tütarrakk aga eraldub basaalkihist ning migreerudes epiteelkoe ülemistesse kihtidesse toimub keratinotsüüdi diferentseerumine. Selle protsessi käigus rakud kaotavad jagunemisvõime ning lõpuks koe pindmistes kihtides rakud surevad, moodustades peamiselt sarvainest ehk keratiinist koosneva lameda pindmise kihi, mis eraldub keskkonda surnud epiteelina. Et tekiks produktiivne viirusnakkus, peab HPV nakatama basaalkihis paiknevaid jagunemisvõimelisi keratinotsüüte, milleni viirus pääseb epiteelkoe mikrovigastuste kaudu. Pärast viirusosakese rakku sisenemist ning viiruse genoomi sattumist raku tuuma toimub esmane lühiajaline viiruse genoomse DNA paljundamine (DNA replikatsioon) suhteliselt madala koopiaarvuni (50–200 viiruse DNA molekuli raku kohta).

Sellele järgneb viiruse DNA stabiilne säilimine epiteelkoe basaalkihi jagunemisvõimelistes rakkudes, mille jooksul viiruse DNA molekulide arv püsib nakatunud rakus üsna konstantsena. Selline stabiilne faas, millele on iseloomulik viiruse genoomi madal koopiaarv ning viiruse elutegevuse minimaalne avaldumine, võib kesta aastaid. See on viiruse strateegia paljuneda ja levida aktiivselt jagunevas rakupopulatsioonis, jäädes samas võimalikult märkamatuks peremeesorganismi immuunsüsteemile. Kui nakatunud rakk alustab diferentseerumist ning migreerumist epiteelkoe ülemistesse kihtidesse, korraldab viirus raku elutegevuse ümber, luues nakatunud rakus keskkonna, kus säilivad viiruse DNA paljundamiseks vajalikud tingimused. Selline rakukeskkond tagab HPV DNA kiire ja jõulise paljundamise (nn vegetatiivne DNA replikatsioon) epiteelkoe ülemistes kihtides, kus immuunsüsteemi rakke ei ole. Vegetatiivse DNA replikatsiooni käigus viiruse DNA molekulide koopiaarv raku kohta mitmekordistub, mis on oluline eeltingimus viiruse DNA pakkimiseks rakus moodustuvatesse nakatamisvõimelistesse valgulistesse struktuuridesse – viirusosakestesse ehk virionidesse, mis vabanevad väliskeskkonda koos koest eralduva pindmise epiteeliga. Seega võib HPV elutsüklis eristada kolme viiruse genoomse DNA replikatsioonifaasi: esmane lühiajaline DNA paljundamine viirusnakkuse tekkimiseks rakus, stabiilne säilimine jagunevates keratinotsüütides ning vegetatiivne DNA replikatsioon uute viirusosakeste moodustamiseks. Sellise epiteelkoe diferentseerumisest sõltuva HPV elutsükli jäljendamine laboritingimustes on keeruline ning see on oluliselt pärssinud HPV DNA replikatsiooni uurimist, mille mehhanismide mõistmine molekulaarsel tasandil on üliloluline HPV-vastaste spetsiifiliste ravimite arendamisel.

Sellistele probleemidele lahendusi otsides on meie uurimisrühm välja töötanud lihtsasti käsitletava robustse mudelsüsteemi HPV DNA replikatsiooni uurimiseks. Antud mudelsüsteem põhineb avastusel, et erinevate kõrge ja madala riskiga HPV DNA molekulide sisestamisel inimese luukasvajast pärinevatesse U2OS rakkudesse toimub viiruse DNA paljundamine, mis võimaldab uurida kõiki kolme viiruse DNA replikatsioonifaasi. Kogutud andmestik lubab väita, et antud mudelsüsteem peegeldab täpselt viirusega asetleidvaid protsesse epiteelkoes ning seega võimaldab paremini mõista, kuidas viirus paljundab oma DNA-d peremeesrakus, millised viiruse ja raku komponendid on selleks vajalikud ning millised on DNA replikatsiooni limiteerivad etapid molekulaarsel tasandil. Sellise teabe alusel on võimalik suunata ravimiarendust teadlikult, näiteks viiruse DNA paljundamise pärssimiseks.

Antud uurimistöö üks osa keskendubki viiruse esmase lühiajalise DNA replikatsioonifaasi molekulaarsel tasemel kirjeldamisele. Täpsemalt analüüsiti viiruse genoomi paljundamisel tekkivaid DNA vaheprodukte, mille struktuurne kirjeldamine võimaldab saada rohkem teavet viiruse DNA replikatsiooni molekulaarsetest mehhanismidest. Kasutades erinevaid analüüsimeetodeid tuvastati, et HPV esmasesse lühiajalisse DNA paljundamise faasi on kaasatud kaks erinevat replikatsiooni viisi. Esmalt toimub HPV DNA paljundamine nn teeta-replikatsiooni mehhanismi kaudu, mis on oma nime saanud kreeka tähestiku tähe teeta (θ) järgi, kuna antud DNA paljundamisviisi käigus tekivad sellele sarnase

struktuuriga vaheproduktid. Lisaks esineb esmases DNA paljundamisfaasis replikatsiooni vaheprodukte, mille struktuur ei ole omane teeta-mehhanismile, viidates et viiruse paljundamisel kasutatakse ka teist, erinevat DNA replikatsioonimehhanismi. Käesolevas uurimistöös saadud tulemused ei anna küll lõplikke vastuseid tuvastatud lisastruktuuride taga oleva replikatsioonimehhanismi üksikasjade kohta, kuid meie ning teiste HPV uurimisrühmade avaldatud tulemused viitavad sellele, et suure tõenäosusega on tegu rekombinatsioonist sõltuva replikatsiooniga, mis on seotud peremeesraku DNA-s tekkivate kahjustuste parandamiseks vajalike protsessidega.

Uurimistöö teises osas analüüsiti detailsemalt HPVga evolutsiooniliselt lähedase jaava makaagi (*Macaca fascicularis*) papilloomiviiruse (MfPV) DNA paljundamise ja geenide avaldumisega seotud molekulaarseid mehhanisme, kuna tõenäoliselt on tegemist sobivaimate viirustega HPV-vastaste ravimite testimiseks kasutatava loommudeli välja arendamiseks. Seni kasutatavad loommudelid, milleks on koera ja küüliku papilloomiviirused, on küll toonud märgatavat kasu ennetavate vaktsiinide väljaarendamisel ja alusteadmiste kogumisel HPV elutsükli kohta, kuid siiski on tegemist evolutsiooniliselt küllaltki kaugete organismidega. Seetõttu ei jäljenda nende papilloomiviiruste nakkus piisavalt täpselt kõrge riskiga HPV-de nakkust inimeses, mille abil saaks loomkatsetes testida HPV-vastaste ravimikandidaatide tõhusust ja ohutust enne kliinilisi katseid inimestel.

DNA järjestuse andmete põhjal on MfPV-d ja HPV-d väga sarnased, kuid seni puudus sobiv eksperimentaalne süsteem MfPV DNA replikatsiooni ja geenide avaldumise uurimiseks. Antud uurimistöö raames selgus, et HPV-de uurimiseks kasutatav U2OS-põhine mudelsüsteem on sobiv ka erinevat tüüpi MfPV-de molekulaarbioloogiliseks uurimiseks. Tulemuste põhjal võib järeldada, et kõrge riskiga MfPV ja HPV DNA paljundamine ja geenide avaldumine on molekulaarsete protsesside poolest väga sarnane. Lisaks näidati, et spetsiifiliselt kõrge riskiga HPV replikatsiooni takistavad ravimikandidaadid pärsivad sarnaselt ka jaava makaagi papilloomiviiruste DNA paljundamist. Lisaks on teada, et jaava makaakidel esineb looduses MfPV-seoselisi emakakaela düsplaasiaid (vähieelseid seisundeid) samas anatoomilises piirkonnas, kus on ka kõrge HPV tekitatud emakakaelavähi esinemissagedus naistel. Samuti esineb makaakidel nahka nakatavaid papilloomiviirusi ning antud uurimistöös näidati, et ka sellist tüüpi viiruse molekulaarsel tasemel toimuvad protsessid on väga sarnased inimesel esineva vastava evolutsiooniliselt lähedase nahka nakatava HPV-ga. Seega on antud uurimistöö raames saadud tulemused esimene samm arendustegevuses, mille eesmärgiks on välja töötada HPV-vastaste ravimite eelkliinilisteks testimisteks kasutatav uudne loommudel, mis jäljendaks erinevate kliiniliselt oluliste HPV tüüpide nakkust inimesel.

ACKNOWLEDGEMENTS

First and foremost, I am very grateful to my supervisors Ene and Mart for making it possible for me to complete what I started. Without their guidance and encouragement it would not have been achievable. My special gratitude also belongs to my first supervisor Marit – you taught me a lot over the years! An extra special thank you is reserved for Andres, whose selfless support and advice were immensely important. Thank you for everything that you are!

I would like to thank all of the former and current members of our research group and colleagues from other research groups of the Institute of Technology with whom I have had the pleasure of working with over the years. Thank you Liisi, Age, Sirle, Kadri, Marit, Matu, Tormi, Ervin, Liza and Regina – we have unforgettable memories of the times we had and many more to come. I will always cherish this period of my life. My gratitude also belongs to Ivar for his useful suggestions and comments on the thesis. I highly appreciated all of the administrative help Inge and Merike provided me.

I would also like to thank my dear cheerleaders Anett, Edi, Helena for keeping my spirits up and with whom we have had amazing times.

I owe a lot to my parents and sister for their lifelong support and constant belief in me during my endless studies. Thank you for being a safe place for me to turn to! Last but not least, the encouragement and love from my dearest Ivo and laughing boy Buula have been crucial.

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PUBLICATIONS

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List of publications:

1. Marit Orav, Jelizaveta Geimanen, **Eva-Maria Sepp**, Liisi Henno, Ene Ustav, Mart Ustav (2015) Initial amplification of the HPV18 genome proceeds via two distinct replication mechanisms. *Scientific Reports*, 5, 15952
2. Liisi Henno, **Eva-Maria Tombak**, Marit Orav, Jelizaveta Geimanen, Ene Ustav, Mart Ustav (2017) Analysis of human papillomavirus genome replication using two- and three-dimensional agarose gel electrophoresis. *Current Protocols in Microbiology*, 45, 14B.10.1–14B.10.37
3. **Eva-Maria Tombak**, Andres Männik, Robert D. Burk, Roger Le Grand, Ene Ustav, Mart Ustav (2019) The molecular biology and HPV drug responsiveness of cynomolgus macaque papillomaviruses support their use in the development of a relevant in vivo model for antiviral drug testing. *PLoS ONE*, 14(1), e0211235.

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