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MART TOOTS

Novel Means to Target Human Papillomavirus Infection

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Novel Means to Target Human Papillomavirus Infection

UNIVERSITY OF TARTU
Press

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

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LIST OF ORIGINAL PUBLICATIONS

I. **Mart Toots**, Andres Männik, Gaily Kivi, Mart Ustav Jr., Ene Ustav, Mart Ustav 2014 The Transcription Map of Human Papillomavirus Type 18 During Genome Replication in U2OS Cells. PLoS One, 9 (12), e116151.

II. Tormi Reinson, **Mart Toots**, Meelis Kadaja, Regina Pipitch, Mihkel Allik, Ene Ustav, Mart Ustav 2013 Engagement of the ATR-dependent DNA Damage Response at the Human Papillomavirus 18 Replication Centers During the Initial Amplification. Journal of Virology, 87 (2), 951–964.

III. Tormi Reinson, Liisi Henno, **Mart Toots**, Mart Ustav Jr., Mart Ustav 2015 The Cell Cycle Timing of Human Papillomavirus DNA Replication. PLoS One, 10 (7), e0131675.

IV. **Mart Toots**, Andres Männik, Karl Mumm, Tarmo Tamm, Kaido Tämm, Andres Tover, Mart Ustav Jr., Mart Ustav 2016 Identification of Several High-Risk HPV Inhibitors and Drug Targets Through High-throughput Screening Using a Novel Assay System. Manuscript

My contributions to the publications are as follows:

I I designed and performed the experiments regarding HPV regulator proteins E2C-1 and E2C-2; participated in data analyses and writing of the manuscript.

II I performed, together with Tormi Reinson most of the experiments; I helped to analyze the data and participated in writing of the manuscript.

III I performed the analyses showing that HPV18 replication foci are present in the cells that are in G2 phase of the cell cycle because they exhibit cytoplasmatic expression of Cyclin B1.

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

Inventions:

ASSAY SYSTEM TO IDENTIFY HPV REPLICATION INHIBITORS IN HT-SCREEN;
Authors: Mart Ustav, Ene Ustav, Andres Männik, **Mart Toots**, Mart Ustav Jr., Andres Tover
Applicant – Icosagen Cell Factory OÜ. Submitted 04/2015.

HUMAN PAPILLOMAVIRUS REPLICATION INHIBITORS
Authors: Mart Ustav, **Mart Toots**, Andres Männik, Andres Tover, Ene Ustav
Applicant – Icosagen Cell Factory OÜ. Submitted 05/2015.

LIST OF ABBREVATIONS

HPV	–	Human Papillomavirus
PV	–	Papillomavirus
HR-HPV	–	High-Risk Human Papillomavirus
LR-HPV	–	Low-Risk Human Papillomavirus
EBV	–	Ebstein-Barr Virus
ORF	–	open reading frame
URR	–	upstream regulatory region
E2BS	–	E2-binding site
HIV	–	Human Immunodeficiency Virus
FDA	–	Food and Drug Administration
UV	–	ultraviolet light
DDR	–	DNA damage response
HR	–	Homologous recombination
NHEJ	–	Non-homologous end joining
MMEJ	–	Microhomology-mediated end joining
PI3K/PIKK	–	Phosphoinositide 3-kinase
ATM	–	Ataxia telangiectasia mutated
ATR	–	Ataxia telangiectasia and Rad3 related protein
DNA-PK	–	DNA-dependent protein kinase
DSB	–	double-stranded DNA break
Ad	–	Adenovirus
LT	–	large T antigen
BPV-1	–	Bovine Papillomavirus type 1
PHK	–	primary human keratinocyte
pRb	–	retinoblastoma
HTS	–	High-throughput screening
TSS	–	transcription start site
CS	–	polyadenylation cleavage site
PAS	–	polyadenylation site
MRN	–	Mre11-Rad50-Nbs1 complec
AAV	–	Adeno-associated Virus
IF	–	immunofluorescence analyses
Fluc	–	Firefly luciferase
Rluc	–	Renilla luciferase
RSV	–	Rous Sarcoma Virus
FMDV	–	Foot-and-mouth Disease Virus
qPCR	–	quantitative Real-Time-PCR
Top1(2)cc	–	Topoisomerase 1(2) cleaving complex
PAR	–	ADP-ribose polymers

CPT	–	Camptothecin
RACE	–	rapid amplification of cDNA ends
VCIP	–	Vascular endothelial growth factor and type 1 collagen inducible protein
IRES	–	internal ribosome entry site
Tdp1	–	Tyrosyl-DNA Phosphodiesterase 1

INTRODUCTION

Papillomaviruses infect the epithelial tissues of various species (humans, other mammals, birds and reptiles). In many cases, papillomavirus infection is asymptomatic or results in various benign lesions, such as warts that can be cleared by the immune system. The same is true in the case of humans; at some point during their lives, they acquire infections from Human Papillomaviruses (HPVs) that may or may not lead to signs of infection in the form of benign tumors, which will be cleared by the immune system. However, in the case of infections in which high-risk oncogenic HPVs are not cleared, persistent infections are established, which can, in rare cases, lead to the formation of cancerous lesions. This course of infection has been described for cervical cancer most frequently, as well as for other organs. Because HPV infections are very common, the overall number of cervical cancer cases caused by these viruses is very high. With at least half a million new cases every year, cervical cancer is the fourth-most common cancer among women.

There are preventive vaccines available against different HPV subtypes, but to date, no effective treatment against ongoing infections has been developed. Several social and economic aspects limit the successful use of HPV vaccines, and there is a clear, unmet medical need for new, specific antivirals.

HPV infection and gene expression are tightly linked to the keratinocyte differentiation program, which significantly narrows the number of suitable model systems for studying the viral life cycle. Recent studies that were performed in our lab identified the human osteosarcoma cell line U2OS as a model system for analyzing the complete replication cycle of many different HPVs. Part of this work characterizes the transcription map of HPV type 18 in U2OS cells and concludes that the HPV18 gene expression profile at the transcriptional level is, in fact, identical to the one described in its natural host. Given that U2OS cells can be grown easily without feeders in a cost-effective manner and that they have been shown to be useful for HPV studies, they may be a perfect cellular platform for identifying new anti-HPV drugs.

Early-stage drug development is usually performed by screening chemical libraries containing thousands of compounds. This type of high-throughput screening requires specific systems that measure the compound's effects on the target of interest rapidly. As part of this study, we have established this type of cellular system to monitor the genome replication of different HPVs. Using this system allowed us to identify several new compounds that specifically inhibit the replication of oncogenic high-risk HPVs.

LITERATURE REVIEW

About Papillomaviruses

Papillomaviruses are host and tissue-specific DNA viruses that infect most mammals; in addition, these viruses also infect other amniotes, such as birds and reptiles. Papillomaviruses infect cells called keratinocytes at the basal layer of the epithelial tissue, where the viruses often establish very prolonged infections. Human Papillomaviruses (HPVs) have been widely studied, and to date, more than 200 different types have been described (Bzhalava et al. 2015).

Burden of HPV infection

Most people become infected with HPVs at some point during their life. These viruses may even be considered part of the normal skin microbiota because these infections can be asymptomatic, and many different HPVs can be identified from seemingly normal epidermis (Antonsson et al. 2003). HPVs can cause benign lesions, such as warts or condylomas, on various epithelial tissues. These lesions, although uncomfortable and not cosmetically pleasing, are rarely life-threatening. They can persist for up to a year and are usually resolved by the host's immune system (Doorbar et al. 2012). Warts are very common in the population, and there are specific beliefs about them in Estonian folklore and likely in other cultures, as well. According to certain beliefs, to rid oneself of warts, one must rub the warts against a surface lit by light that originated from a full moon. During the 19th century, Estonian farmers believed that warts may even be a good thing. It was said that you must not remove the warts from children's faces because children with warts will be smart and successful (http://herba.folklore.ee/).

Although most HPV infections are not lethal, these viruses are still widely studied and considered to pose a serious health risk to and burden on society. The reason is because of HPV's association with various cancers, most commonly with cervical cancer. In the 1950s and 60s, the research that uncovered the cause of cervical cancer was initiated. Cervical cancer was found to be more prevalent among young, sexually active women. Cancer itself is not contagious, so there had to be a distinctive causative agent. This agent was found in the early 1980s when DNA originating from various types of HPVs was detected in cervical cancer lesions (Durst et al. 1983, Boshart et al. 1984). However, HPV was not detected, likely because of a lack of suitable methods, in a significant number of cervical cancer lesions. The most prominent association between cervical cancer and HPV infection was found in 1999, when HPV DNA was detected in 99.7% of analyzed cervical carcinoma samples worldwide (Walboomers et al. 1999). In addition to cervical cancer, HPVs are also associated with other cancers, such as those of the head and neck or penile or anal cancers (Chaturvedi 2010). Most HPV types are not oncogenic

and are classified as low-risk HPVs (LR-HPV). The vast majority of cancer cases are related to mucosal high-risk HPVs (HR-HPV) from genus alpha (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68), with type 16 being the most prevalent around the world (Figure 1). Cervical cancer is the seventh-most common cancer globally, with approximately half a million new diagnoses every year and 250,000 deaths (Munoz et al. 2003, Stanley 2006, Ferlay et al. 2010). In addition to HR-HPVs from genus alpha, a growing number of studies suggest that cutaneous beta HPVs may be related to the development and progression of skin cancers (Akgul et al. 2006, Feltkamp et al. 2008).

In addition to causing serious health problems, HPVs are an economical burden; an estimated 3.4 billion dollars are spent on the diagnoses and treatments of HPV-related cancers in the United States alone, and additional funds are spent on treating benign papillomas (Insinga et al. 2004). The significant medical and socioeconomic burden created by HPV makes it an important subject for both basic and applied scientific research.

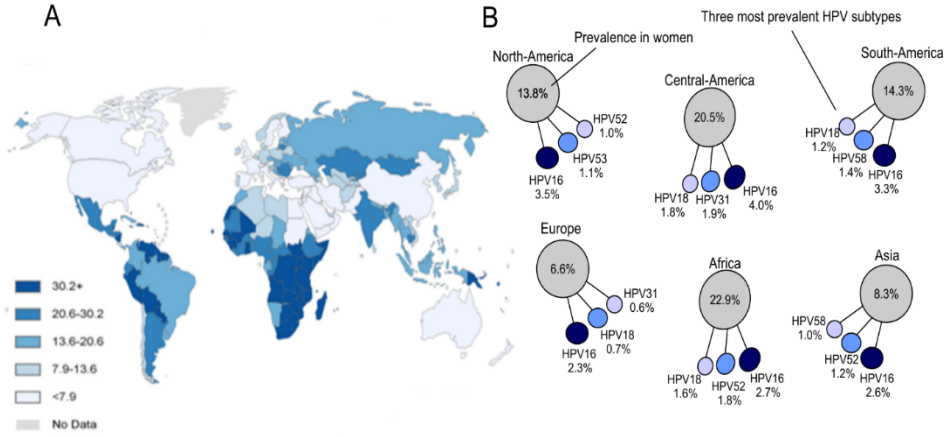

Figure 1. A: Global cervical cancer incidence as the age-standardized rate (ASR). The ASR is expressed per 100 000 thousand females in this figure, which shows how many cervical cancer cases there would be if a given population has a standard age structure. B: The percentage in the gray circle indicates the cervical HPV prevalence in women in different regions. The three most prevalent types are shown for each region (small circles), with HPV16 being the most prevalent worldwide. Adapted from (Ferlay et al. 2010, Crow 2012, Jung et al. 2015).

Current status of HPV therapy

There is no magic cure for ongoing HPV infections, but there are several preventive vaccines that have been developed against various HPV subtypes: Gardasil, Cevarix and Cevarix 9. These vaccines prevent infection with up to 9 different HPV types, including the most prevalent HPVs, 18 and 16 (which make up 60–70% of overall cervical cancer cases) (Pahud and Ault 2015).

These vaccines have been shown to prevent HPV infection successfully; however, various social and economic factors have held back the success of these vaccines. Because HR-HPVs are sexually transmitted, the vaccination must be done at an early age (9–13 years) to be effective, before the start of sexual activity. A number of debates, misbeliefs, preconceptions and religious beliefs prevent the vaccination of a sufficient number of individuals to reduce the spread of the virus. Because the development of the vaccines is expensive, the price per patient is very high, as well; thus, HPV vaccination programs should be subsidized by governments. Most of the cancer cases around the world are in developing countries, where there is not sufficient funding available. Efficient protection against HPV requires several vaccination doses, which may be difficult to achieve in reality because of the potential shortage of vaccines and the simple loss of interest in vaccinating patients (Stubbs et al. 2014, White 2014, Nicol et al. 2015, Pahud et al. 2015, Wigle et al. 2016).

These issues with the vaccination suggest that there is a need for therapeutics that would be effective and specific against ongoing HPV infection; they should be widely available, possibly targeting a wide range of different HPV subtypes. The currently approved invasive therapies (cryotherapy, electrosurgery, laser therapy or larger excision procedures) against HPV lesions are based on the elimination of the infected tissue together with viral DNA. These therapies do not eliminate HPV-infected tissue completely, which leads to an approximately 40% chance of recurring infection (von Krogh et al. 2001, Lacey et al. 2013, Rosales and Rosales 2014).

Because of its tissue specificity, non-lytic life cycle and low level of viral protein expression, HPV effectively evades recognition by the host immune system. Thus, immune system stimulants (such as imiquimod) have been used to suppress HPV infections, but there is still a 50% chance of recurring infection (Maw 2004, Maw 2004).

Because the current therapeutic or prophylactic approaches are not sufficient for treating HPV-related infections, large-scale research for identifying better drugs or ways to eliminate HPV infections is needed.

Overview of the HPV life cycle

The HPV life cycle is tightly linked to keratinocyte differentiation programs (a simplified overview of the life cycle is shown in Figure 2). HPVs infect proliferating cells known as keratinocytes in the basal layer of the epithelium through micro-wounds. For entry into keratinocytes, HPV virions bind to heparan sulfate on the cell membrane. After this binding, conformational changes occur in the HPV virion, which lead to the exposure of the N-terminal part of capsid protein L2. The exposure of the N-terminus of L2 is a necessary step for infection because it reveals the binding motif for furin, which cleaves the L2 at the furin consensus site (Giroglou et al. 2001, Richards et al. 2006, Raff et al. 2013). Interestingly, different HPV types use different mechanisms

for entering the cells. For example, HPV16 primarily uses clathrin-mediated endocytosis, and HPV 31 prefers caveolar endocytosis. However, HPVs may use alternative pathways for entry in some cases. However, furin cleavage subsequently results in capsid uncoating in the late endosomes, which in turn allows the genomes to be released from the endosomes into the cytoplasm (Letian and Tianyu 2010). The next steps in the entry process are not completely understood. It appears that the microtubule-dependent transport system is involved in delivering the viral genome to the nucleus. Some studies suggest that HPV16 and BPV-1 L2 proteins contain the nuclear localization signal. The L2 proteins, in conjugation with the viral genome, interact with the dynein motor complex used for transporting various cellular cargos, and they deliver the genome to the nucleus. In some cases, the nuclear envelope has to be destroyed for viral genome entry (Darshan et al. 2004, Fay et al. 2004, Florin et al. 2006, Pyeon et al. 2009). Regardless of the mechanism, the HPV genome will be transported to the cell nucleus, where the infection will be established.

During the first replication stage, which is known as initial amplification, the HPV genome copy number reaches a few hundred copies per cell (Del Vecchio et al. 1992, Sverdrup and Khan 1994). Next, stable maintenance or latent replication is triggered, during which the HPV genome copy number remains largely constant, and the viral DNA is replicated on average once per cell cycle, perhaps solely by cellular replication proteins (Egawa et al. 2012). During this stage, viral gene expression levels are low, which helps to prevent the activation of the host immune response.

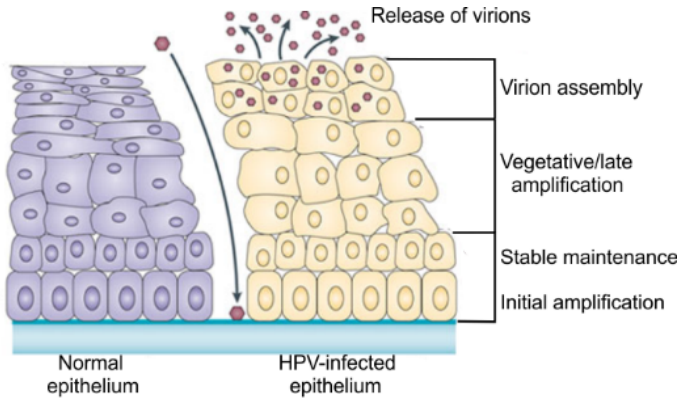

Figure 2. Simplified schema of the HPV life cycle. HPV virions enter proliferative basal epithelial cells through microabrasions, and the viral genome is transported to the nucleus, where it is replicated up to 300 copies per cell. An almost-stable HPV copy number and very low level viral gene expression are maintained until the cells undergo a differentiation program. Differentiation triggers the vegetative/ late amplification of the HPV genome in the upper parts of the epithelium, during which a sufficient amount of viral genomes is produced. In the uppermost epithelium layer, viral genomes are packed into capsids, and virions will be released (marked in red). Adapted from (Moody and Laimins 2010).

This type of replication is reminiscent of the latent replication of many DNA viruses, such as the Epstein-Barr Virus (EBV) (Adams 1987, Kirchmaier and Sugden 1995). To distribute HPV genome molecules evenly between daughter cells upon cell division, viral DNA is tethered to mitotic chromosomes for efficient partitioning. During epithelium differentiation, some cells remain proliferative in the basal layer, whereas others start to differentiate and move upwards in the epithelial layers. The HPV genome remains in both of these cells. When the differentiating cell reaches the upper layers of the epithelium, the vegetative or late amplification phase of HPV replication is triggered. Differentiation results in significant upregulation of viral gene expression, and HPV DNA is rapidly replicated multiple times per cell cycle to generate new genera of genomes (Bedell et al. 1991, Grassmann et al. 1996). In the terminally differentiated epithelium, the viral capsid proteins are synthesized, and a new generation of viruses is released from the cells (Stubenrauch and Laimins 1999, Kadaja et al. 2009).

HPV genome organization

The HPV genome organization and structure are conserved among all known HPV virus types. The HPV genome is a circular double-stranded DNA that is approximately 8 kbp in size and consists of eight or nine early open-reading frames (ORF) and two late ORFs (Figure 3). Early ORFs encode for proteins that are involved in replication, regulation of gene expression and modulation of cellular environment to make it more suitable for the virus. The late region encodes for structural proteins L1 and L2, which form a capsid. In addition to ORFs, the HPV genome contains a non-coding upstream regulatory region (URR) that contains the replication origin and several binding-sites for cellular regulator proteins, as well as E2 (reviewed in (Doorbar et al. 2012)).

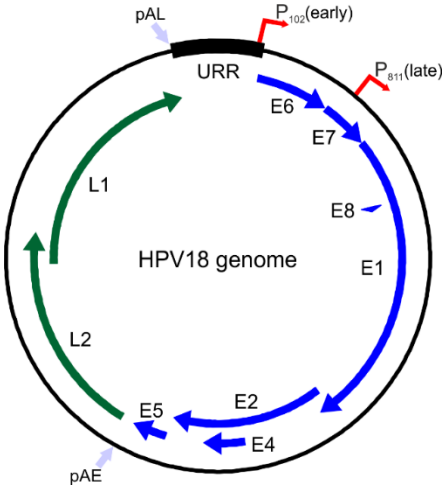

Figure 3. HPV18 genome, circular ~8000 bp dsDNA. Early ORFs are shown in blue, and late ORFs, in green. The non-coding upstream regulatory region (URR), which contains the replication origin, is shown in black. The primary promoter regions are in red, and the positions of the early (pAE) and late (pAL) polyadenylation elements are indicated in light purple arrows.

Possible ways to target HPV replication

"Classical" anti-viral compounds usually inhibit some key process during infection that is performed by proteins encoded by the virus itself. This approach results in the higher expected specificity of the compound and thus minimizes potentially lethal/toxic side effects. During Human Immunodeficiency Virus (HIV) infection, viral DNA is integrated into the host genome by specific virus-encoded integrase. This enzyme has been a good target for HIV inhibition because a compound called Raltegravir was approved by the United States Food and Drug Administration (FDA) in 2007 (Grinsztejn et al. 2007, Steigbigel et al. 2008). The inhibition of viral DNA polymerases has been successful, as well; Aciclovir (the first successful anti-viral compound) specifically inhibits the Herpes virus DNA polymerase but not the cellular one (Elion et al. 1977, James and Prichard 2014).

To replicate their genomes, HPVs largely depend on cellular proteins. Because HPVs do not encode their own polymerase, the use of compounds such as Aciclovir is not possible. HPVs encode only two essential proteins for viral DNA replication, that is, E1 and E2 (Ustav and Stenlund 1991, Seo et al. 1993). The E1 protein is a DNA helicase (Yang et al. 1993), and E2 is a multi-functional protein involved in replication, gene expression regulation and the partitioning of the viral genome (Androphy et al. 1987, Seo et al. 1993, Stenlund 2003, McBride et al. 2012, Ustav et al. 2015). To initiate HPV genome replication from the specific viral origin, the E1 protein has DNA melting, ATP hydrolysis and specific DNA binding functions, although the last function is not crucial for replication initiation (Blitz and Laimins 1991, Reinson et al. 2013). For HPV genome replication initiation, the E1 and E2 proteins form a complex. E2 then directs E1 to the site of viral origin by binding to specific E2-binding sites (E2BS). After directing E1 to the origin, E2 dissociates from the DNA-bound complex. Binding and ATP hydrolysis result in the formation of the double-hexameric E1 structure (Stenlund 2003). For efficient replication, cellular proteins are incorporated into HPV replication complexes. The E1 protein is shown to be bound to Topoisomerase I, DNA polymerase alpha and ssDNA binding protein RPA which are all incorporated into the HPV replication complex (Park et al. 1994, Han et al. 1999, Clower et al. 2006). Because of the crucial roles of E1 and E2 proteins in HPV DNA replication, a large amount of research has been performed to identify potential compounds that may somehow interfere with the functions of these proteins. The compounds that inhibit the ATP hydrolysis function of the E1 protein have been characterized, but they have not been approved for use as HPV antivirals (Faucher et al. 2004, White et al. 2005). Another possibility would be to target the interaction between the HPV E1 and E2 proteins. The crystal structure of the E1/E2 complex has been characterized, which allows the design of these compounds (Abbate et al. 2004). This approach led to the discovery of the first HPV-specific inhibitors. Unfortunately, those inhibitors were "too specific", as they were capable of inhibiting LR-HPV 11 or 6 but not HR-HPVs such as 18

and 16 (White et al. 2003, Yoakim et al. 2003). In conclusion, targeting E1 and E2 proteins has thus far not resulted in the development of suitable anti-viral compounds against HPV infection.

Both E1 and E2 interact with numerous cellular proteins for an effective HPV life cycle. Several studies analyzing these interactions have been performed (Ma et al. 1999, Muller et al. 2012, Archambault and Melendy 2013). The E2 protein interacts with cellular transcription factor Brd4, which is crucial for the regulation of viral transcription, segregation and replication (Chang et al. 2014, Jang et al. 2014). Targeting specific interactions of HPV and cellular proteins results in the specific inhibition of HPV infection and, at the same time, does not inhibit key cellular processes. However, this approach may have unwanted "side-effects". For example, the inhibition of E2-dependent transcriptional regulation may upregulate the promoter that drives the expression of HPV oncoproteins and actually increase the risk of cancer development (Desaintes et al. 1997, Francis et al. 2000).

Oncogenic HR-HPVs primarily differ from LR-HPVs in terms of the oncogenic potential of HPV oncoproteins E5, E6 and E7 (Munger et al. 1991). The E5 protein has weak cell transformation abilities, and it has been suggested that E5 induces keratinocyte immortalization and modulates apoptotic pathways (Stoppler et al. 1996, Kabsch and Alonso 2002). During normal HPV replication, E2 represses the expression of E6 and E7. In HPV-induced cancers, the viral genome is usually integrated into host chromosomes quite often so that E2 expression is lost (Baker et al. 1987, Durst et al. 1987). The elevated expression of E6 and E7 stimulates cell cycle progression and cell proliferation and prevents apoptosis (for review, see (McLaughlin-Drubin and Munger 2009, Roman and Munger 2013, Mighty and Laimins 2014)). E6 protein is best known for its ability to cause the degradation of tumor suppressor gene p53 (Scheffner et al. 1990). E7 protein's most "famous" function is likely its destabilization of pRb, to induce cell cycle progression (Berezutskaya et al. 1997, Jones and Munger 1997). E7 also causes a delay in mitosis and helps to create a suitable environment for DNA synthesis by downregulating anaphase-promoting complex activities (Yu and Munger 2013). Compounds that target the functions of HPV oncoproteins have caused growth suppression and cell cycle arrest in HPV-related cancer cells (Tan et al. 2012). Although E6 or E7 inhibitors may be useful in HPV-related cancer therapy, they will not eliminate the viral infection entirely. The HPV genome can replicate without E6 or E7 expression in proliferating basal cells, resulting in the long-term presence of viral DNA and increasing the risk of cancer development.

DNA Damage Response – guardian of the genome

Cellular DNA is constantly attacked by various endogenous and exogenous agents, such as ultraviolet light (UV), ionizing radiation or errors in DNA replication and transcription. Enzymes such as topoisomerases also make breaks in DNA to decrease the torsional stress needed to gain access to DNA molecules for replication or transcription. As many as tens of thousands of DNA lesions can occur every day in each cell (Hoeijmakers 2009). If unrepaired, these lesions may lead to senescence, apoptosis, or, in the worst cases, to cancer progression because of possible mutations in cellular oncogenes. When a protein is, for example, non-functional, cells can usually synthesize a new protein and degrade the old one. However, DNA has to be replicated very precisely once per cell cycle, and mutated DNA cannot not be replaced merely by synthesizing a new molecule. Therefore, cells have sophisticated pathways to ensure the integrity of DNA; they are known as the DNA damage response (DDR) (Greenberg 2011). When DNA damage occurs, the first step in the DDR is recognition of the lesion, which is a very fast process that occurs up to a minute after the damage occurs. After recognition of the lesion, a number of DNA repair proteins will be localized to the damage site, which will be repaired by different mechanisms depending on the nature of the lesion. DDR activation results in cell cycle stoppage, and if there is large-scale DNA damage that cannot be repaired, apoptosis pathways are activated (Siddiqui et al. 2015). The accumulation of DNA damage may eventually lead to cancer progression when apoptosis is inhibited (either by the mutations of the genes in apoptotic pathways or by some pathogens) and the cells will continue to divide. The most dangerous lesions are DNA double-stranded breaks (DSBs), which can be repaired through the following three main mechanisms: homologous recombination (HR), non-homologous end joining (NHEJ) or microhomology-mediated end joining (MMEJ). The choice between these pathways is connected to the cell cycle phase, and HR is usually more active during the S and G2 phases of the cell cycle (Symington and Gautier 2011).

The key proteins in the DDR network are phosphoinositide 3-kinases (PI3Ks or PIKKs) consisting of ataxia telangiectasia-mutated (ATM), ataxia telangiectasia and Rad3-related protein (ATR) and DNA-dependent protein kinase (DNA-PK). ATM is mostly activated during DSBs, whereas ATR is related to both DSBs and replication stress (stalled or collided replication forks (Ward and Chen 2001, Lee and Paull 2005, Cimprich and Cortez 2008)). The activation of ATM or ATR results in lesion repair primarily by HR. DNA-PK is mostly associated with NHEJ (Smith and Jackson 1999).

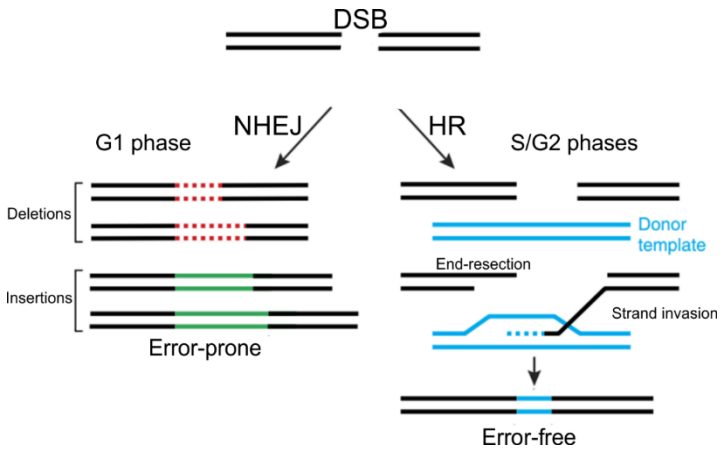

Figure 4. Comparison of two major DNA repair pathways: non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ is an error-prone repair pathway in which double-stranded DNA break (DSB) ends are ligated. NHEJ is primarily active in the G1 phase of the cell cycle. Homologous recombination (HR) is an error-free repair pathway in which single-stranded 3' DNA ends are generated by end-resection, after which strand invasion occurs in the intact homologous donor template. The donor molecule is used as a template for DSB repair. This pathway is primarily active during the S and G2 phases of the cell cycle. Adapted from (Sander and Joung 2014).

DDR and viral infection

Many very different viruses cause activation of the DDR during their life cycles (extensive reviews can be found in (Turnell and Grand 2012, Hollingworth and Grand 2015)). Whether activation is the "side-product" during viral infection, is carefully controlled by the viruses or is somehow beneficial for their life cycle is often unclear. Different viral proteins are known to degrade, relocate or inhibit cellular DDR proteins to avoid the activation of antiviral responses or to inhibit viral replication. Adenoviruses (Ad) degrade a number of proteins related to DDR to avoid the concatenation of its linear genome (Stracker et al. 2002, Blackford et al. 2010). However, some Ad types include DDR proteins in their replication centers to help facilitate viral replication (Forrester et al. 2011). Polyomaviruses need the activation of ATM kinase for effective replication, and their primary replication protein, the large T antigen (LT), is shown to activate it (Dahl et al. 2005, Shi et al. 2005). HPVs also activate ATM kinase during vegetative amplification, and this activation seems to be necessary for viral replication (Moody and Laimins 2009). Several cellular proteins involved in HR-dependent DNA repair are recruited to HPV replication centers, and it has been suggested that HPVs employ HR to replicate their own genomes (Gillespie et al. 2012, Orav et al. 2015). Several viruses also modulate the cellular environment to induce proliferation and prevent apoptosis by regulating the

DDR network. Both SV40 LT and HPV E6 are known to induce p53 degradation to inhibit apoptosis (Mietz et al. 1992, Scheffner et al. 1993).

DDR in cancer cells

Some components of DDR are mutated in almost all cancer cases; thus, DNA repair is less effective than in normal cells (O'Connor 2015). In addition, cancer cells divide very rapidly; therefore, more DNA damage may occur because of the faster overall DNA replication rate. Potential anti-cancer compounds targeting DDR proteins are, in essence, causing significant DNA damage throughout the cell cycle, which eventually leads to replication or mitosis catastrophes. Cancer cells are more prone to DNA damage, and some specific lesions occur only in tumor cells and occur either very rarely or not at all in normal cells. However, cells have specific DDR pathways available for repairing these lesions. One example of such a pathway involves topo-isomerase-induced DNA damage (reviewed in (Pommier 2013, Pommier et al. 2014)). Some of those pathways may also be necessary during replication of oncogenic viruses, such as HPV, polyomaviruses or adenoviruses. Targeting these pathways would then effectively kill the virus and cancer simultaneously.

Regulation of HPV gene expression

Given that the HPV genome is conserved among different types of viruses, HPV gene expression may be broadly similar, as well. Complete transcription maps for HPV16, HPV18 and HPV31 have been constructed (they are available in the PaVE database (http://pave.niaid.nih.gov/) and in (Wang et al. 2011)). The early HPV promoter (p97 for HPV16, p99 for HPV31 and p102 for HPV18) drives the expression of all early transcripts except E4 (Smotkin et al. 1989, Hummel et al. 1992, Wang et al. 2011). The early promoter is active in undifferentiated keratinocytes during the early stages of HPV infection. Early transcripts are generated as polycistronic mRNAs that are all polyadenylated at the early polyadenylation site that is situated upstream of the late region, and they are spliced using different early splice donor and acceptor sites (Johansson and Schwartz 2013). The late promoter (p670 for HPV16; p742 for HPV31 and p811 for HPV18) is situated in the ORF of E7 and is activated only in differentiated keratinocytes (Hummel et al. 1992, Grassmann et al. 1996, Ozbun and Meyers 1998, Wang et al. 2011). In differentiated cells, early polya-denylation signal efficiency is reduced, and late mRNAs (coding for the structural proteins L1 and L2) are generated and are polyadenylated at the late polyadenylation site and spliced using late splice donor and acceptor sites (Johansson et al. 2013). Because the L1 and L2 proteins are immunogenic, their expression must be controlled; thus, they're synthesis is "allowed" only immediately before capsid formation. The E2 protein controls the timing of

HPV late mRNA production by inhibiting early polyadenylation (Johansson et al. 2012).

In addition to the full-length E2 protein, papillomaviruses encode for other transcriptional regulators that control PV replication levels by modulating viral gene expression. One of the most studied regulators is the E8^E2 protein, which is conserved among many PVs. It's expression is shown for BPV-1 and HPV types 1, 5, 11, 16, 18, and 31 (Choe et al. 1989, Rotenberg et al. 1989, Doorbar et al. 1990, Palermo-Dilts et al. 1990, Stubenrauch et al. 2000, Kurg et al. 2010, Sankovski et al. 2014). The E8^E2 protein lacks the N-terminal transactivation domain of E2 that is substituted with the short (~11 amino acids) E8 peptide. The E8^E2 regulator forms heterodimers and competes with the full-length E2 to bind to the E2BS, thereby inhibiting replication (Zobel et al. 2003). Mutant HPV genomes lacking in E8^E2 protein expression can replicate up to 10 times more efficiently than their respective wt. viral genomes. In addition to directly competing with E2 for DNA binding, the E8 domain of the E8^E2 protein interacts with cellular transcription regulators. These interactions result in the downregulation of viral gene expression from promoters that are distal to the E2BSs (Stubenrauch et al. 2001, Fertey et al. 2010). Most of the studies describing the functions of HPV negative regulators have been performed by analyzing the initial amplification phase. It is still unclear whether E8^E2 is needed for the stable maintenance of various HPVs. E8^E2 expression is necessary for stable infection of HPV31 and HPV18, whereas it is dispensable for HPV16 (Stubenrauch et al. 2000, Lace et al. 2008, Kurg et al. 2010). A recent study indicates that E8^E2 controls the vegetative amplification of HPV16, as well (Straub et al. 2014). In addition to E8^E2, BPV-1 encodes another truncated version of E2 protein, namely E2C or E2-TR (Lambert et al. 1987). The E2C represses viral gene expression and replication similar to the E8^E2 protein. These types of proteins have not yet been characterized for many HPVs, but one study suggests that E2C proteins are able to inhibit the replication of HPV11 (Liu et al. 1995).

The negative regulators of PV gene expression and replication show how elegantly the virus has "learned" to exist in its host cell. The virus knows exactly how efficiently it should replicate or express viral proteins to avoid significantly damaging the host cell, as well as getting caught by the host immune system.

Model systems used for studying the HPV life cycle

HPVs are tissue and cell type-specific viruses that infect only proliferating basal cells (basal keratinocytes) in the epithelium. Therefore, most commonly used eukaryotic cell lines do not allow researchers to address most aspects of the HPV life cycle. Early studies about papillomavirus replication were performed in mouse fibroblast cell line C127, which was transformed by the E5 oncoprotein of Bovine Papillomavirus type 1 (BPV-1) (Law et al. 1981, Yang et

al. 1985, Neary and DiMaio 1989). With regard to HPV replication, many studies have been performed in primary human keratinocytes (PHK) that were transformed with HR-HPV genomes. PHK cells can be isolated from various epithelia, and the most readily available cells are from neonatal foreskins (Chow and Broker 1997). The culturing of PHK cells is relatively time-consuming, and because these cells are not transformed, they can be grown for a limited amount of passages. The isolation of PHK cells from different patients results in great genetic variance between clones, which may eventually result in mixed results in terms of HPV research. However, the transfection of these cells with HR-HPV genomes results in the formation of transformed cell clones, which have been widely used to study the stable maintenance and vegetative amplification of HPV (Frattini et al. 1996, Flores et al. 1999). The HR-HPV life cycle can also be studied in cell lines that were established from various HPV-induced lesions; for example the HPV31 life cycle has been studied in cells that were isolated from human cervical intraepithelial neoplasia (CIN). These cell lines contain HPV31 DNA in its episomal state, although various clones differ significantly in their cell growth properties and HPV genome copy number. However, the differentiation-dependent replication properties of HPV can still be studied in these cells (Bedell et al. 1991, De Geest et al. 1993). Another widely used cell line for studying the stable maintenance of HPV is W12, which is derived from low-grade cervical lesions. This cell line contains episomal HPV16 genomes, with 100 – 200 copies per cell at early passages, but the HPV genome tends to integrate readily in these cells at later passages (Stanley et al. 1989, Pett et al. 2004, Pett et al. 2006). Because HR-HPVs can effectively transform the cell, obtaining cell clones from various models is somewhat effective. By contrast, LR-HPVs have low transforming abilities, and their induced differentiation patterns differ from those of HR-HPVs (Thomas et al. 2001). Therefore, studying the replication stages of LR-HPVs is more challenging.

Most of the problems with the cellular systems described in this work are largely technical; their cultivation is relatively time-consuming and expensive. Moreover, the generation of HPV-positive cells is an inefficient and slow process. Part of the work that was performed in our lab was to screen various eukaryotic cell lines to find alternatives to the already available model systems for studying HPV genome replication. The aim was to identify the cell line(s) that can be grown quickly and transfected with high efficiency and would be suitable for studying the replication stages of HR, LR and cutaneous HPV genomes. The most suitable cell line was U2OS, an adherent cell line with an epithelial cell-like morphology. The U2OS cells were derived from a moderately differentiated sarcoma of the tibia of a 15-year-old girl in 1964. Different analyses show significant chromosomal rearrangements, mostly on chromosomes 8, 17 and 20. However, the tumor-suppressor genes p53 and retinoblastoma (pRb), which are often mutated in various cancer cell lines, are completely functional (Isfort et al. 1995, Bayani et al. 2003, Wesierska-Gadek and Schmid 2005, Niforou et al. 2008). After the U2OS cells were transfected

with either HR, LR or cutaneous HPV genomes, the initial amplification was monitored in these cells for approximately a week. By maintaining HPV-positive U2OS cells in subconfluent conditions for longer periods, the stable maintenance phase was studied. By growing these cells at 100% confluency for at least five days, differentiation-like changes, such as the induced expression of involucrin, occur, and late amplification of the HPV genome replication is turned on. Viral copy numbers increase rapidly, and gene expression is up-regulated, as well (Geimanen et al. 2011). This work suggests that the U2OS-based system is somewhat unique, allowing researchers to study different aspects of HPV replication (Figure 5). Since description of HPV genome replication in U2OS, several other studies have been performed in these cells to characterize different parts of the HPV life cycle, such as segregation or gene expression regulation (Ben et al. 2015, Lo Cigno et al. 2015, Ustav et al. 2015). U2OS cells are easy and cost-effective to cultivate, and they have high transfection efficiency as well as HPV replication levels and are therefore a good alternative to the "classical" model systems used for HPV research to date. In addition to analyzing HPV genome replication, a number of studies regarding HPV replication have been performed by measuring the replication properties of URR-containing plasmids facilitated by the expression of E1 and E2 proteins from heterologous expression vectors (Chiang et al. 1992). URR plasmid replication assays can be performed in various cell lines due to the abnormally high expression levels of E1 and E2.

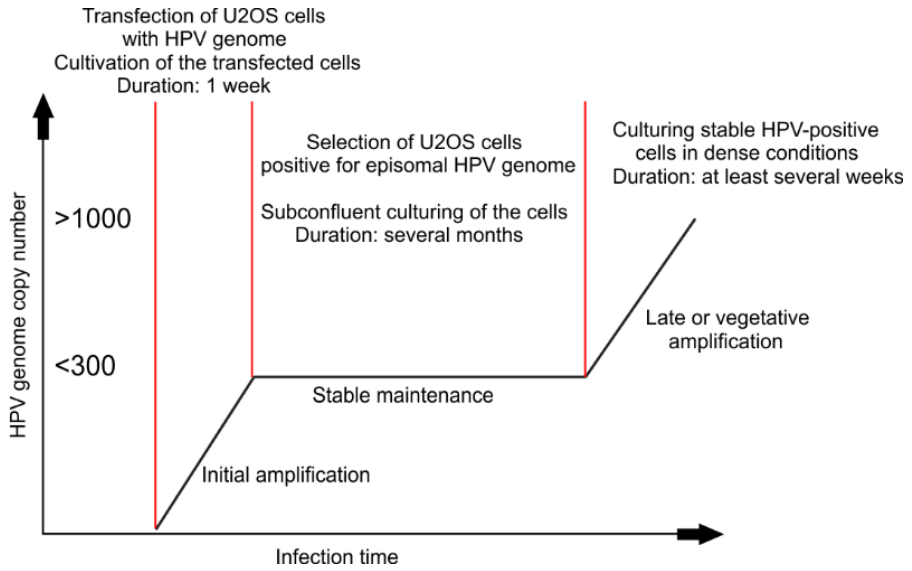

Figure 5. Different stages of HPV replication and ways to study them in U2OS cells.

HPVs are important human pathogens, and there is no cure for an ongoing HPV viral infection. Thus, there is a need for active compounds that target HPV replication. A widely used technique to identify novel compounds is High-Throughput Screening (HTS). HTS involves analyses of thousands of compounds that originate from various available chemical libraries (Mayr and Bojanic 2009). Analyzing thousands of compounds for the identification of potential HPV inhibitors by using classical techniques for DNA replication measurement would be impossible because of the need for a significant amount of biological material and extensive hands-on time. Different HTS-compatible model systems have been developed that use very sensitive and easily quantifiable marker genes for measuring the effect of chemical compounds on target of interest. There are a number of these types of systems available for HPV studies, as well. In one of those models, the luciferase reporter gene is added to the URR plasmid, which can then be used to measure the E1 and E2-dependent initiation of replication more easily (Fradet-Turcotte et al. 2010). The primary issue with the so-called URR replication assay is that the expression of E1 and E2 proteins is usually significantly higher than it is during HPV genome replication, and their expression is not controlled by viral promoters. Because this system allows only the study of the E1/E2-dependent initiation of HPV replication, many potential compounds that inhibit other parts of the viral life cycle will not be found. A HaCat cell line based system has been developed, in which the stable maintenance of HPV11 can be monitored for limited passages (Wang et al. 2014), but this system does not use any easily quantifiable reporter genes, which makes conducting HTS somewhat difficult. Another HTS-compatible model uses HPV pseudoviruses that express reporter genes (Huang et al. 2012), which should be very powerful for finding compounds that inhibit the very early stages of HPV infection. Although there are a number of model systems available for conducting HTS screens to identify HPV inhibitors, none of them are capable of mimicking the complete replication cycle and other aspects of the HPV life cycle, such as gene expression, segregation, cellular transformation and differentiation.

AIMS OF THIS STUDY

HPVs are the primary causative agents of cervical cancer as well as other cancers, such as those of the head and neck or anal or penile cancer. Despite the existence of several preventive vaccines, HPVs are still widespread and cause thousands of cancer cases each year. Thus, there is a clear need for effective antivirals that target HPV infection.

The general objective of this study was to develop a means for identifying novel HPV inhibitors and characterizing their mechanisms of action.

Specifically, we performed the following actions:

- Described the HPV18 transcription map and regulation of gene expression in U2OS cells, thereby providing validation for this cellular system for HPV genome molecular studies;
- Described the mechanism and potential outcome E1 expression leading to DNA Damage Response (DDR) activation during HPV genome replication;
- Generated the HPV genome-based model system, which can be used in high-throughput screens to identify novel HPV inhibitors in U2OS as well as in keratinocytes; and
- Used the developed model system to screen and identify new, specific HPV inhibitors and identify the targets for these compounds in the viral replication cycle.

MATERIALS AND METHODS

This study was performed in U2OS cells, which have been useful for studying the replication and other aspects of different parts of the HPV life cycle (Geimanen et al. 2011, Lo Cigno et al. 2015, Ustav et al. 2015). To study the initial amplification of HPV, U2OS cells were transfected with HPV18 minicircle genomes, and replication was monitored for up to one week. For later stages of HPV replication, stable cell lines bearing episomal viral genomes must be generated. To study the role of individual viral proteins during HPV replication, genomes containing frameshift or start codon mutations in their respective ORFs were generated.

HPV replication levels were measured by Southern blotting and quantitative PCR analyses. IF and FISH analyses were used for visualization and localization studies of HPV replication foci and of viral or cellular proteins.

RACE (rapid amplification of cDNA ends) analyses were used to map HPV18 promoter regions, polyadenylation and splicing patterns.

A novel method for conducting a high-throughput screening of chemical compounds for their ability to inhibit HPV replication was developed. A monoclonal U2OS cell line #10.15 expressing Firefly luciferase and GFP was generated. This cell line allows researchers to measure cell growth and viability. HPV genomes expressing Renilla luciferase (Rluc) were engineered so that the levels of Rluc expression would correlate with changes in viral copy number, which is termed here as a marker genome (a full description of the modified genome is presented in the results and discussion section below).

The screening of chemical library Diversity Set IV from the National Cancer Institute of the United States was conducted using the model system developed during this study. The basic outline of the screening process is shown in Figure 6.

The compounds were divided into four categories based on their effect, as shown in Figure 1, panel C as follows:

1. DMSO samples and non-active compounds showed high expression for both Renilla and Firefly luciferases.
2. Toxic compounds (and Actinomycin D samples) killed all of the cells; thus, both the Firefly and the Renilla luciferase readouts are at background levels.
3. Mildly toxic compounds decreased the cellular viability, and with it the HPV replication levels to a different extent. The inhibition of HPV was thus non-specific.
4. Compounds specifically inhibiting Renilla, but not Firefly luciferase, were chosen for further analyses (as described below in the results and discussion section)

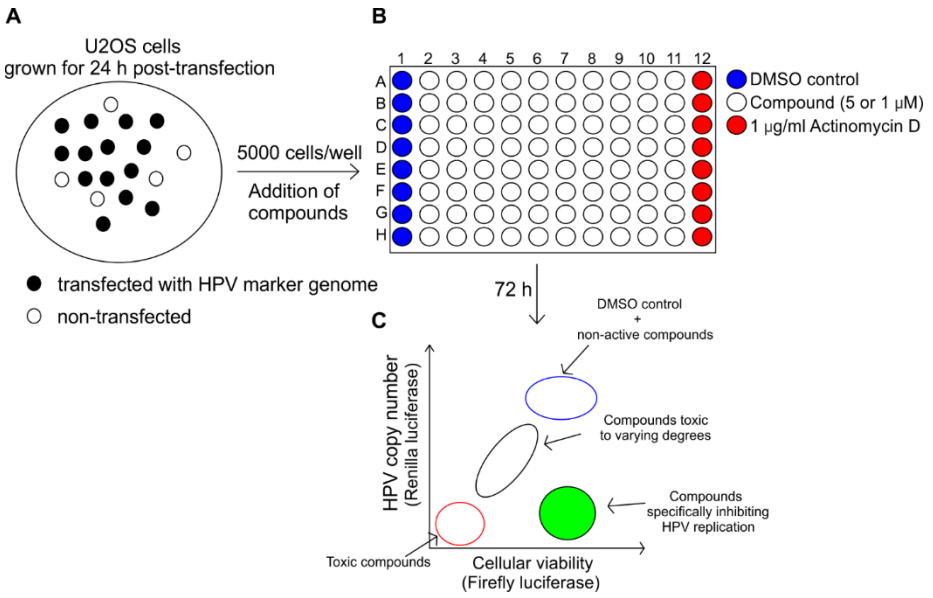

Figure 6. The basic principle underlying the drug screen by using the model system developed during this study. A: U2OS #10.15 cells were transfected (by electroporation, with a transfection efficiency of up to 80%) with the HPV18-Rluc-E2 marker genome and plated on 100 mm tissue culture plates for 24 hours, for recovery. B: The cells were detached from the 100 mm tissue culture plates and seeded at a density of 5000 cells/well onto 96-well plates. Column 1 is for vehicle control DMSO, and column 12, for Actinomycin D (the 1 µg/ml concentration was extremely toxic to the cells and was used for the pipetting control and for background reads of the luciferase signal). Compounds from the chemical library were added to columns 2–11 at the following two concentrations: 5 µM and 1 µM. The cells were grown in the presence of these compounds for 72 hours C: Both the Firefly (Fluc; cellular viability) and the Renilla (Rluc; HPV copy number) luciferases were measured in the plates using a Dual-Glo Luciferase Assay System kit and a GloMax Microplate luminometer (Promega) according to the manufacturer's instructions. The Fluc and Rluc values from each well were blotted on an XY-scatter diagram. A PerkinElmer JANUS automated workstation was used for the steps shown in panels B and C.

In addition to the wt and #10.15 cells, U2OS cells stably expressing EBV EBNA1 protein (U2OS-EBNA1) were also used in this study. This cell line was used to study the EBNA1-dependent replication of the plasmid containing the EBV replication origin. The EBNA1-dependent replication of the oriP plasmid (described in (Sugden and Warren 1988)) differs significantly from that of HPV genome replication, as it occurs only once during the cell cycle and is controlled by the cellular replication proteins. Therefore, EBNA1-dependent replication can be used as an internal control when studying a compound's effect on HPV replication because the replication mechanisms differ (principle on Figure 7).

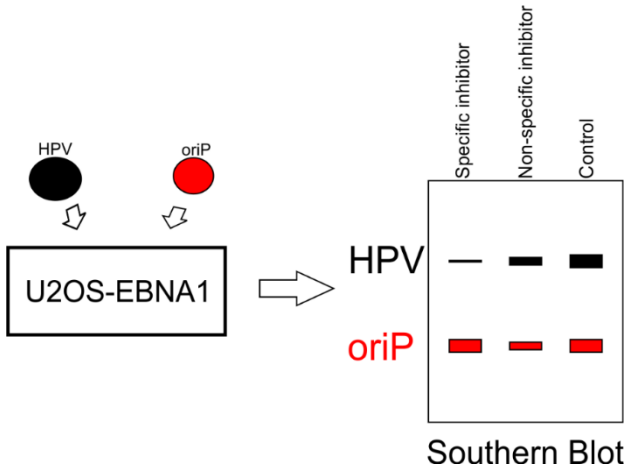

Figure 7. HPV and oriP replication in U2OS-EBNA1 cells. The cells are co-transfected with the HPV genome (black) and oriP plasmid (red), and the replication of both plasmids is analyzed by Southern blotting. With the specific inhibitor, the HPV replication level is lower than the control when the oriP replication level is unchanged. Non-specific inhibitors lower the replication levels of both plasmids compared with the control samples.

RESULTS AND DISCUSSION

HPV18 gene expression and regulation in U2OS cells

The previous studies performed in our lab showed that U2OS could be used to study the different replication stages of HR, LR and cutaneous HPV genomes. Genetic studies indicate that HPV replication proteins E1 and E2 are both necessary and sufficient for HPV genome replication (Geimanen et al. 2011). However, not much was known about the gene expression and transcription map of HPV18 in U2OS cells. Recently, a full transcription map for a productive HPV18 life cycle in keratinocytes was published (Wang et al. 2011), and we used this map as a reference when characterizing the transcription map for HPV18 in U2OS cells. After introducing the HPV genomes to U2OS by transfection, the initial amplification could be monitored for up to seven days. The subsequent selection of HPV-positive U2OS cells and their maintenance under subconfluent conditions allows researchers to monitor the stable maintenance phase. The culture of HPV-positive cells under confluent conditions triggers viral genome amplification that is reminiscent of the vegetative amplification that occurs in differentiated keratinocytes.

The transcription map for the initial amplification of HPV18 in U2OS was analyzed using U2OS cells that were transiently transfected with HPV18 minicircle genomes. Analyses of transcripts that were present during stable maintenance and late amplification were performed in HPV-positive U2OS cell line #1.13, which contained multiple stably maintained copies of the HPV18 genome, as described in (Geimanen et al. 2011).

To map the HPV18 transcription start sites (TSS) in U2OS cells, 5'RACE analyses were performed with polyA+ RNA obtained from U2OS cells that were transfected with the HPV18 genome (for analyses of the initial amplification), as well as from the #1.13 cell line. Taken together, the TSSs were clustered into five promoter regions, indicating that the early region of HPV18 consists of five promoter regions, termed P_{102}, P_{520}, P_{811}, P_{1193} and P_{3000} (I, Fig. 3). The activity of promoter P_{102} was previously described in various cell lines (Schneider-Gadicke and Schwarz 1986, Thierry et al. 1987, Romanczuk et al. 1990). Promoter region P_{811} was described in HPV-18-infected raft cultures as a late promoter (Wang et al. 2011). P_{520} and P_{1193} were not extensively defined as promoter regions previously; however, the transcripts initiated from these regions have been detected before, in HPV18-positive keratinocytes (Wang et al. 2011). The promoter region P_{3000} spans a large area in the ORF of E2 from nt. 2918 to 3426, which, as described in detail later in the text, encodes regulatory proteins for HPV18 gene expression. This promoter has not been extensively studied, but it was described in one study that was conducted in vitro and in HeLa cells (Karlen and Beard 1993).

The polyadenylation cleavage sites (CSs) for HPV18 as transfected to keratinocytes were mapped at nt. 4270 (for early transcripts) and nt. 7300 (for late transcripts), as described in (Wang et al. 2011). The 3'RACE analyses of

initially amplified HPV18 in U2OS cells showed that there is a CS region between nt. 4253 and nt. 4272, most frequently at nt. 4270 (I, Fig. 4A). Particularly interesting analyses were performed in the #1.13 cell line to determine whether culturing this cell line under confluent conditions would reflect the late amplification and the usage of late polyadenylation sites (PASs). Analyses of PASs during stable maintenance but primarily during late amplification support the extensive usage of late PASs with a CS at approximately nt. 7300, which were not used during the initial amplification of HPV18 in U2OS cells (I, Fig. 4B).

A summary of the detected transcripts during HPV 18 replication is shown in Figure 8. The transcripts that were also detected during the productive life cycle of HPV18 in keratinocytes (Wang et al. 2011) are marked by asterisks.

Papillomaviruses encode different regulator proteins that control the levels of viral gene expression and thus play an important role in regulating the virus genome copy number. The most common and thoroughly studied regulator is the E8^E2 protein. E8^E2 consists of a short E8 product (typically 11 amino acids) fused into the C-terminus of the E2 protein, which contains DNA binding and dimerization domains but lacks the transactivation domain. The E8^E2 protein has been shown to repress PV early promoters and inhibits E1 and E2-dependent viral replication (Stubenrauch et al. 2000, Zobel et al. 2003, Lace et al. 2008). This type of HPV genome copy number regulation seems to be conserved, given that the E8^E2 protein has been characterized for BPV-1 as well as for various HPVs (types 1, 5, 11, 16, 18 and 31) (Choe et al. 1989, Rotenberg et al. 1989, Doorbar et al. 1990, Palermo-Dilts et al. 1990, Stubenrauch et al. 2000, Kurg et al. 2010). Our analyses of the P_{3000} revealed the existence of two similar transcripts that were generated from this promoter, which are known here as E2C-1 (RNA I in Figure 1) and E2C-2 (RNA U in Figure 1; 80% of mRNA from P_{3000}). Both E2C variants contain only the C-terminal part of the E2 protein. E2C-1 is a spliced variant that consists of a short peptide starting from nt. 3253 in the E2 ORF (11 amino acids) that is spliced into the C-terminus of E2, whereas E2C-2 starts from position 3426 and is an unspliced C-terminal E2 protein. Similar truncated variants of E2 are described for BPV-1 and HPV11, and it has been suggested that these proteins also act as repressors for Papillomavirus gene expression by competing to bind to the E2BS in the URR region or to form heterodimers with the full-length E2 (Lambert et al. 1987, Choe et al. 1989, Lambert et al. 1989, Vaillancourt et al. 1990, Liu et al. 1995).

We analyzed a potential role for the HPV18 genome's replication of the two E2C proteins. First, we mutated the ATG start-codons for E2C-1 or E2C-2 alone or in combination, and we compared the replication levels of those mutant genomes with the wt. or HPV18 E8^E2 mutant genome (E8-). As expected, the E8-genome replication was approximately 10 times higher than that of the wt. (I, Fig. 7, panels A and B). The E2C-1 mutation did not have any significant effect on HPV18 genome replication, but suprisingly the E2C-2 mutant resulted in approximately 40% lower replication.

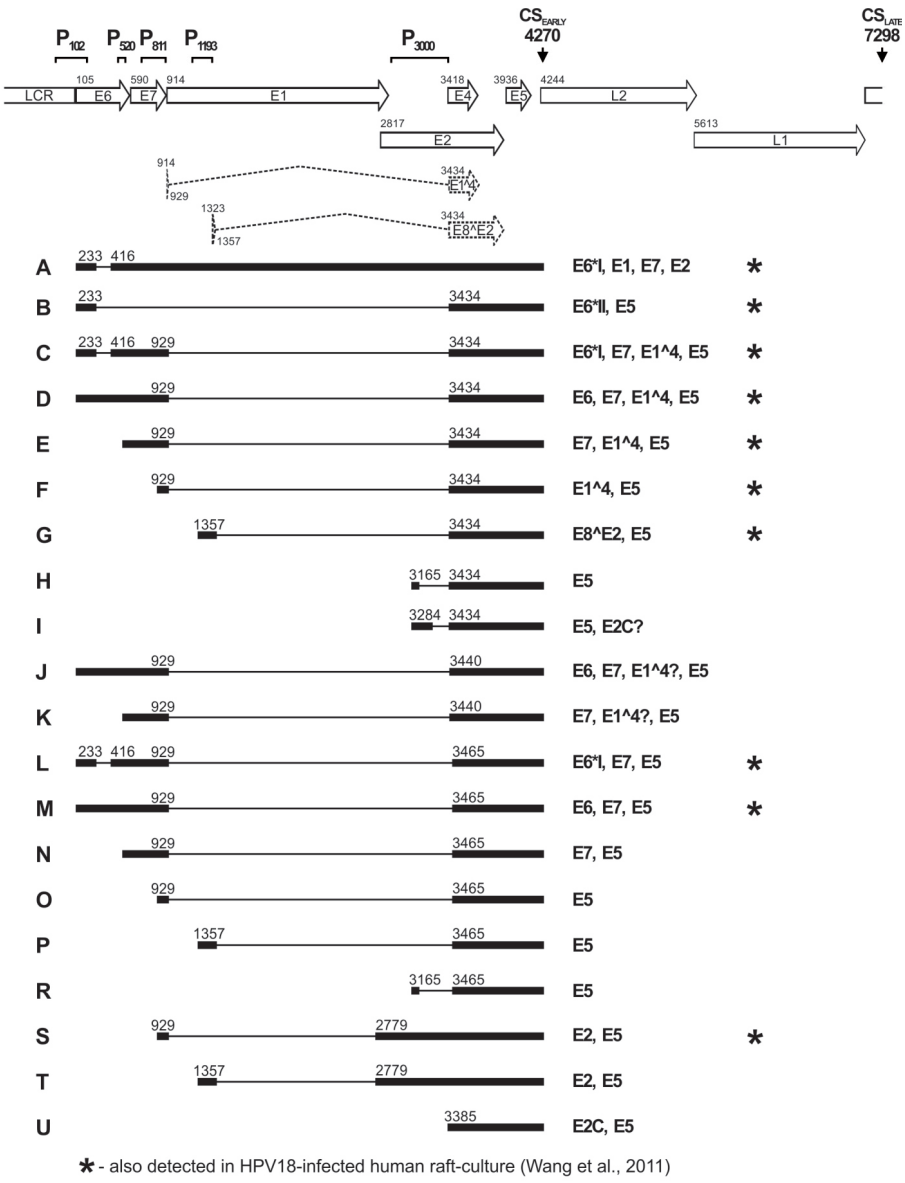

Figure 8. Summary of the HPV18 transcripts that were mapped in transiently transfected U2OS cells and in subclone #1.13 cells by 5′ RACE analyses. Top, a linear depiction of the HPV18 genome with the ORFs, LCR, E1^E4 and E2^E8 coding sequences spanning over two exons (dashed line), along with the promoter regions P_{102}, P_{520}, P_{811}, P_{1193} and P_{3000} and the major polyadenylation cleavage sites CS 4270 and CS 7298. All transcripts depicted herein (which are designated with letters A-U, as shown at left) are shown with exons (solid boxes) and introns (lines) and with the splicing donor and acceptor sites (nt. numbers). The coding potential is described to the right of each transcript. The RNA species previously described in HPV18-infected raft cultures are indicated by asterisks (Wang et al. 2011).

The HPV18 with both E2C proteins mutated replicated similarly to the wt. HPV genome. To characterize the roles of the E2C proteins, we analyzed the replication of HPV18 genomes where E8^E2, as well as E2C-1 or E2C-2, was mutated. These double mutant genomes exhibited approximately 3-fold higher replication levels than the HPV18 genome containing only the E8^E2 mutation. These results suggest that E8^E2 is the primary negative regulator for HPV18 and that the E2C variants are "back-up" regulators (I, Fig. 7, panels A and B).

An extremely high viral replication intensity may have a negative effect on the host's viability and eventually become a barrier to productive HPV infection. Moreover, high replication and gene expression levels may activate the host's immune response and eventually lead to elimination of HPV infection. Therefore, it is vital for HPV to control its replication levels, and HPV18 seems to encode three regulator proteins for that reason. However, not all HPVs have this sophisticated regulation pathway because the E2C proteins are not very conserved among the different HPV types. Analyses of 302 PV types revealed that only the α7 family HPVs (types 18, 39, 45, 59, 68, 70, 85 and 97) and HPV67 (α9 family) have the potential to encode E2C-2. The start-codon for E2C-2 was also present in MfPV4, MfPV5, MfPV10 and PhPV1 Papillomaviruses. As for E2C-1, only HPV18 seems to encode for this protein. Perhaps there is actually no need for other regulator proteins besides E8^E2 for most HPV types, which is the reason why E2C proteins are not conserved among all HPVs.

The HPV18 mutant that does not express E2C2 exhibited an approximately 40% lower replication level relative to that of the wt. genome, which was an unexpected phenotype because truncated E2 proteins are supposed to be negative regulators. This reduction in replication may indicate that in addition to being a repressor, E2C-2 may also upregulate HPV gene expression by modulating specific promoters. An activation potential has been described for BPV-1 E2C protein (Lace et al. 2012). We analyzed the transcriptional regulation of HPV18's full-length E2, E8^E2, E2C-1 and E2C-2 on different promoters. All of the E2 proteins efficiently downregulate transcription from full-length HPV18 URR to the same extent (I, Fig. 8, panel B). In addition to the effect on full-length URR, we also analyzed the effects of various E2 proteins on the transcription of a promoter region containing E2-binding sites 3 and 4, a minimal-TK and a TATA box. This configuration should be more suitable because of its lower baseline activity, which allows researchers to distinguish among the effects of E2 proteins and cellular factors on transcription. The results indicate that HPV18 E2C-2 did activate the transcription up to 4-fold, whereas other E2 proteins did not activate it at all or even repressed it (I, Fig 8, panels C and D). Depending on the stage of infection or some other aspect, HPV18 E2C-2 can either up or downregulate viral gene expression and thus control the viral genome copy number.

In conclusion, this work shows that the HPV18 gene expression in U2OS cells is almost identical to that of keratinocytes. Similar results were obtained from analyses of transcription maps for HPV11 and HPV5 in U2OS cells

(Sankovski et al. 2014, Isok-Paas et al. 2015). U2OS cells provide an adequate cellular environment for HPV replication and are therefore suitable for studying various aspects of the HPV life cycle. Because U2OS cells can be grown easily and cost-efficiently, they provide an excellent platform for identifying novel HPV inhibitors.

DNA damage response is activated during HPV replication

As with the replication of many other viruses, HPV replication takes place in distinct foci of the host cell nucleus. During HPV stable maintenance and vegetative amplification, the DNA damage response (DDR) was shown to be activated in these foci and several cellular DDR regulator proteins, such as ATM kinase, BRCA1, Rad51 and members of the MRN complex are present there (Moody et al. 2009, Gillespie et al. 2012). We were curious as to whether there is an HPV protein that is responsible for activating the DDR. One of the early markers of DDR activation and the presence of DNA double-stranded breaks (DSBs) is the phosphorylated form of histone H2AX, which is called gamma-H2AX (reviewed in (Kuo and Yang 2008). Studies performed in our lab have suggested that the HPV E1 protein activates the DDR in HPV-positive HeLa cells (Kadaja et al. 2009). Expression analyses of HPV18 replication proteins E1 and E2 in U2OS cells indicated that gamma-H2AX levels are significantly up-regulated primarily because of the expression of E1. Furthermore, E1's ability to induce DDR activation is conserved among HR and LR HPVs (II, Fig. 1). Although gamma-H2AX activation is usually connected to DSBs, this factor can be activated in the absence of DNA lesions, as well (Soutoglou and Misteli 2008). To further characterize the nature of E1-dependent DDR activation, we performed comet assay (neutral single-cell gel electrophoresis). If DSBs are present, then the DNA is fragmented and migrates from the nucleus during gel electrophoresis, forming a comet tail. The measurement of the length and brightness of the tail allows researchers to estimate the extent of the DSBs (Singh and Stephens 1998). Analyses of the extent of DNA damage by comet assay showed that the expression of HPV18 E1 protein causes DSBs in cellular DNA and that the level of damage is increased when E1 and E2 are expressed together (Figure 9), likely because E2 stabilizes E1 expression (II, Fig. 1). As a result of E1-dependent DNA damage, cell cycle progression is blocked primarily in S-phase and, to a lesser extent in, G2, as well (II, Fig. 5).

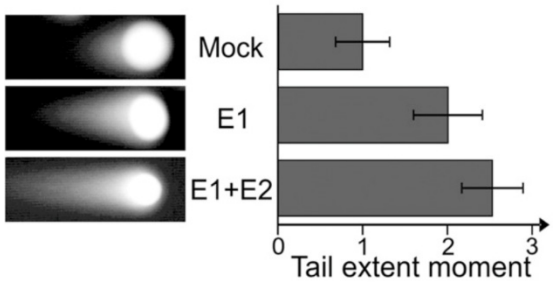

Figure 9. HPV18 E1 induces DNA double-stranded breaks in cellular DNA. A comet assay was performed using cells that were transfected with the expression vectors for HPV18 E1 alone or supplemented with E2; the extent of DNA damage was calculated and is given relative to the signal obtained from empty transfection. The error-bars represent standard deviations from three independent experiments.

The exact mechanism of how E1 causes DSBs is unclear. Our experiments show that the DNA melting and ATP hydrolysis, but not the origin-specific DNA binding activities, of the E1 protein are necessary for either replicating HPV DNA or activating DDR (II, Fig. 2). U2OS cells are HPV-negative; thus, the presence of HPV E1 and E2 proteins may lead to the uncontrolled (and non-specific?) replication of cellular DNA. The expression of E1 and E2 proteins results in the formation of a DNA replication complex similar to the one used for HPV genome replication, and this complex then replicates cellular DNA. The replication fork movement may eventually be stalled, or the forks may collapse, which leads to the formation of DSBs. The formation of DSBs lead to the recruitment of cellular DNA repair proteins. DSBs are likely repaired through homologous recombination (HR) because this pathway is shown to be more active in S-phase in response to replication-dependent DNA damage (Petermann and Helleday 2010, Kakarougkas and Jeggo 2014).

The previous experiments were performed by using heterologous expression vectors for HPV18 E1 and E2 in the absence of HPV DNA. We next analyzed the activation of the DDR during the replication of the HPV18 genome in U2OS. For those analyses, the HPV18 E8-genome, which replication level is approximately 10 fold higher than that of the wild type to increase the sensitivity of the analyses, was used. During HPV genome replication, E1 and E2 accumulate in distinct nuclear foci where viral DNA is replicated (Swindle et al. 1999). We analyzed the HPV18 genome replication foci by immuno-fluorescence (IF) analyses and fluorescent in situ hybridization (FISH) assays, and we showed that the viral genome in U2OS cells is also replicated in distinct foci. We observed that most of the DDR activation measured by gamma-H2AX is localized in these foci, indicating that there is no wide-spread DNA damage present in the cells. When HPV DNA is present in the cells, E2 preferably binds to the viral genome, and it directs E1 to the viral origin to facilitate HPV, but not cellular DNA, replication (II, Fig. 7A and Figure 10). The absence of a

large-scale DDR during HPV replication may be beneficial to the virus for several reasons. A low-level DDR does not cause significant changes in cellular viability or hamper the progression of the cell cycle, which would allow the HPV genome to replicate more efficiently. Damaged DNA sends out signals to "invite" various DNA repair and replication proteins to remove the lesions. DDR activation may be the way how HPV recruits various cellular proteins for its own genome replication. It is unclear whether the DNA damage as such is the signal for DNA repair proteins or if some HPV proteins interact with cellular proteins and thus involve them in the viral replication complex. The HPV oncoproteins E6 and E7 have been shown to interact with various DDR-related proteins, such as pRb and p53 (Werness et al. 1990, Heck et al. 1992, Jones et al. 1997). However, the role of E6 and E7 is mostly associated with modulating the cellular environment by making it more suitable for HPV replication. E7 drives cells to cycle more actively, and E6 makes cells more tolerant to DNA damage and prevents the activation of apoptotic pathways (Lilley et al. 2007, Wallace and Galloway 2014). In the case of initial amplification, E6 and E7 are not needed for DDR activation in HPV18 replication foci (II, Fig. 7B and Figure 10).

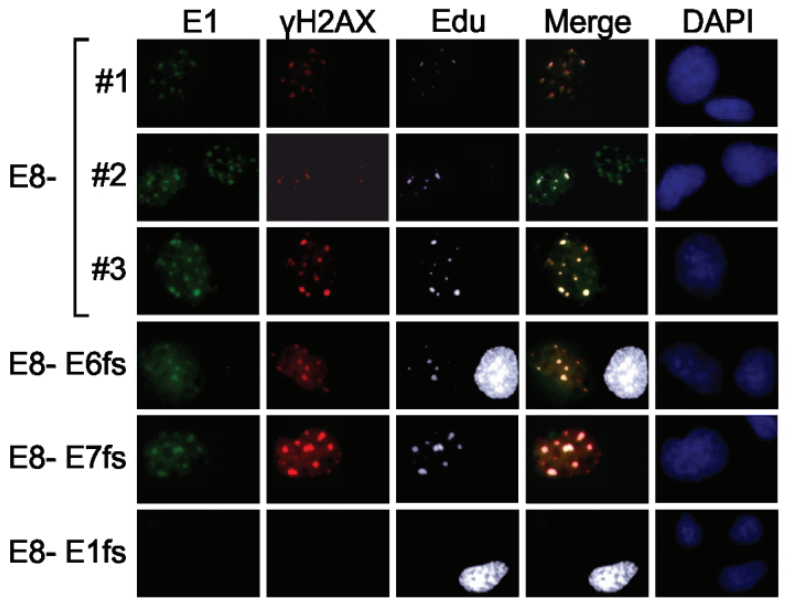

Figure 10. HPV18 genome replication takes place in distinct foci. An IF analyses of HPV18 replication centers detected by E1 protein. DNA synthesis measured through incorporation of EdU (white) show that HPV18 positive cells, only viral but not cellular DNA is synthesized. DDR is activated in the HPV18 replication foci measured by the localization of gamma-H2AX (red).

During initial amplification, oligomeric forms of HPV genomes are generated by homologous recombination-dependent DNA repair. Analyses of the replication intermediates during the initial amplification suggested that at some point during DNA synthesis, there is a switch from bidirectional replication to HR-dependent unidirectional replication (Orav et al. 2013, Orav et al. 2015). Perhaps there is thus some type of DNA replication stress occurring during HPV replication, such as replication fork collapse, and the virus has "learned" to use it for effective genome amplification. In addition, this process does not even require any of the viral proteins to facilitate secondary HR-dependent replication. It is known that during the stable maintenance and vegetative amplification of HPVs, ATM-dependent DDR is activated and necessary for viral replication (Moody et al. 2009). However, during HPV's initial amplification, ATM seems to be dispensable because the inhibition of ATM by its specific inhibitor KU55933 did not alter HPV replication levels (II, Fig. 6, panel E). There is a strong possibility for replication stress to occur during the initial amplification of the HPV genome. It has been suggested that histone H2AX is phosphorylated by ATR kinase during DNA replication stress (Ward et al. 2001). We decided to analyze whether this may be the case during HPV initial amplification, as well. We performed IF analyses on the HPV replication centers and showed that two essential proteins in the ATR-pathway called TopBP1 and ATRIP are both localized in the HPV replication foci (II, Fig. 8). It is possible that during bidirectional E1/E2-dependent replication, there is a fork collision or other type of DNA replication stress, ATR is activated, and HR-dependent repair pathways are activated.

HPV genome replication is extended beyond the S-phase

In eukaryotic cells, DNA is replicated only during the S-phase of the cell cycle. However, during the life cycle of many different viruses, cell cycle progression is halted in the G2 phase. For example, it is known that the Adeno-associated Virus (AAV) single-stranded DNA genome causes cell cycle arrest in G2 (Cotmore and Tattersall 1994). G2 arrest may be beneficial for HIV-1, as well because provirus integration, a necessary stage in the HIV life cycle, happens more efficiently in G2 (Groschel and Bushman 2005). During the infection with murine and SV40 polyomaviruses, arrest in G2 may be used to generate a pseudo-S-phase-like environment in which cellular DNA synthesis has been completed, but all the necessary replication proteins are still readily available (Lehman et al. 1994, Lehman et al. 2000). Different HPV proteins also cause cell cycle arrest in G2 phase (II, Fig. 5, panel A; (Davy et al. 2002, Nakahara et al. 2002). There is evidence that during HPV vegetative amplification, at least some viral DNA synthesis takes place during the G2 phase of the cell cycle (Wang et al. 2009). We measured *de novo* DNA synthesis by detecting EdU, a modified nucleotide analog that is incorporated into newly synthesized DNA. Analyses of HPV replication foci by tracking the presence of E1 protein and

EdU revealed that no cellular DNA was synthesized in the cells where HPV DNA was replicated. EdU incorporation took place only in HPV DNA foci, and the overall EdU signal was much lower than that of HPV-negative cells (II, Fig. 7, panel B and Figure 10). This finding may indicate that either HPV inhibits cellular DNA synthesis or its replication continues after the S-phase.

During our study, we developed a novel assay to measure the rate and timing of DNA synthesis. The basic principle of this assay is described in III, Fig. 2, panel A. The idea is to measure only the DNA that is synthesized during a specific short period, such as one hour. The nucleotide analog EdU is added to the cells and incubated for the desired time, and DNA is purified. Next, from the total purified DNA, only *de novo* synthesized DNA will be extracted using biotinylated EdU and streptavidin beads, which can be quantified by qPCR analyses. This assay allows researchers to measure DNA synthesis during different cell cycle stages. The cells will be blocked in G1 or G2 phases, for example, and then released to continue the cell cycle synchronously. EdU is added and the rate of *de novo* DNA synthesis can be measured at the desired time points. Using this assay, we have shown that the HPV initial amplification is initiated in S-phase and is continued in G2 phase, where cellular DNA synthesis is finished (III, Fig. 3). To confirm that the initial amplification of the HPV18 wt. genome takes place in G2 phase, we performed an IF analysis and labeled the HPV18 replication foci with anti-E1 antibodies. Most of the cells that were positive for HPV18 replication foci showed strong cytoplasmatic staining of Cyclin B1 (Figure 11), indicating that these cells are in G2 phase (Pines and Hunter 1991).

Although the initial amplification of HPV takes place during the S and G2 phases of the cell cycle, our analyses on stable maintenance indicate that during this stage, viral DNA is replicated simultaneously with cellular DNA (III, Fig. 3, panel F) and is not continued during G2-phase.

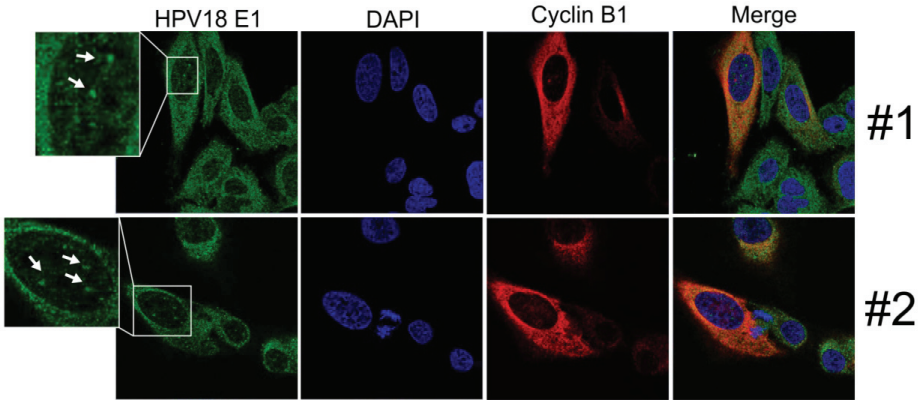

Figure 11. HPV18 initial amplification takes place in G2 phase of the cell cycle. Sample images from two different IF analyses showing HPV18 wt. genome replication foci detected by E1 protein (green) present in cells that are in G2 phase of the cell cycle, which is detected by tracking the cytoplasmatic expression of Cyclin B1 (red).

Differences in cell cycle timing between the amplification and stable phases of HPV replication may indicate that HPV uses different replication mechanisms during different replication phases. During initial and vegetative amplification, a rapid increase in the viral copy number is needed, and multiple E1/E2-dependent initiation events from the same viral genome occur during one cell cycle. It may be possible that the replication of all viral genomes could not be finished during S-phase and that G2 provides additional time for HPV amplification. During stable maintenance, however, the HPV genomes are likely replicated solely by cellular proteins (E1 may be dispensable for this phase, as shown in (Egawa et al. 2012)), which is done only during S-phase.

Novel model system for high-throughput screening of HPV replication inhibitors

Although there are various model systems available for HTS against various stages of HPV infection (as described in the literature review), none of those allow researchers to analyze the replication of full-length wt. HPV genomes. We propose a dual-component universal model system that would allow the monitoring of cellular viability and HPV replication simultaneously in the same assay. Given that U2OS cells are excellent for studying HPV replication, the first component of such a model system is the modified U2OS cell line. To evaluate the effects that the analyzed compounds may have on cellular viability, we have inserted the coding sequences for the reporter genes Firefly luciferase (Fluc) and GFP into the U2OS genome, which resulted in a clone #10.15. Analyses of this cell line indicate that both Fluc and GFP are easily detectable and allow researchers to evaluate cell growth and viability. Moreover, these modifications in U2OS cells have not hampered their ability to support HPV genome replication relative to unmodified cells (IV, S1).

The second component of this model system would be an engineered HPV genome, which would express some easily detectable and quantifiable marker genome. Our initial attempts to generate this type of marker genome were concentrated on modifying the late region of the HPV genome. HPV genomes lacking the late region encoding ORFs of L1 and L2 replicate at a similar efficiency to that of the wt. genomes (Geimanen et al. 2011). We generated the 1st generation of marker genomes in which most of the late region was substituted with various reporter genes under the control of Rous Sarcoma Virus (RSV) LTR promoter (IV, S2, panel A). Analyses of this marker genome showed that additions made to replace the late region had almost completely abolished the viral replication (IV, S2, panel C). The transcriptional analyses of HPV18 in U2OS cells (see text above) indicated the presence of L2-containing transcripts during all stages of HPV replication. Completely substituting the late region may therefore interfere with the transcription. The design of the 2nd generation marker genomes left the elements in the ORF of L2 necessary for the correct polyadenylation of early transcripts intact. Full-length Vascular

endothelial growth factor (VEGF) and type 1 collagen inducible protein (VCIP) internal ribosome entry site (IRES) that was shown to be active in U2OS cells was added in front of the marker gene to promote its translation (Blais et al. 2006). Two variants were generated, and they differed in the presence of the heterologous polyadenylation signal (IV, S2, panel B) and were analyzed similarly to that of 1st generation marker genomes. Because the 2nd-generation marker genomes did not replicate much more efficiently than the 1st generation genomes (IV, S2, panel C), the addition of non-HPV sequences to the late region seemed to greatly interfere with viral gene expression, and functional HPV marker genomes could not be generated.

We then turned to the early region of the HPV genome and attempted to insert marker genes there. In this case, we added the coding sequence of Renilla (Rluc) luciferase to the ORF of E2, immediately after the overlapping region of E1 and E2. The Renilla sequence was followed by the Foot-and-mouth Disease Virus (FMDV) 2A peptide coding sequence, after which a full-length E2 coding sequence started again. FMDV 2A peptide causes "cleavage", which, in the case of this type of HPV marker genome, leads to the translation of the following two proteins: Renilla luciferase, which contains ~20 amino acids from the N-terminus of E2 and full-length E2 containing an additional amino acid, proline, in the beginning. The FMDV 2A causes "ribosome skipping", in which a peptidyltransferase is inhibited, resulting in the release of nascent protein, and translation is reinitiated from the amino acid proline (Ryan and Drew 1994, Donnelly et al. 2001). A schematic design and the working principles of this marker genome (HPV-Rluc-E2) are shown in Figure 12.

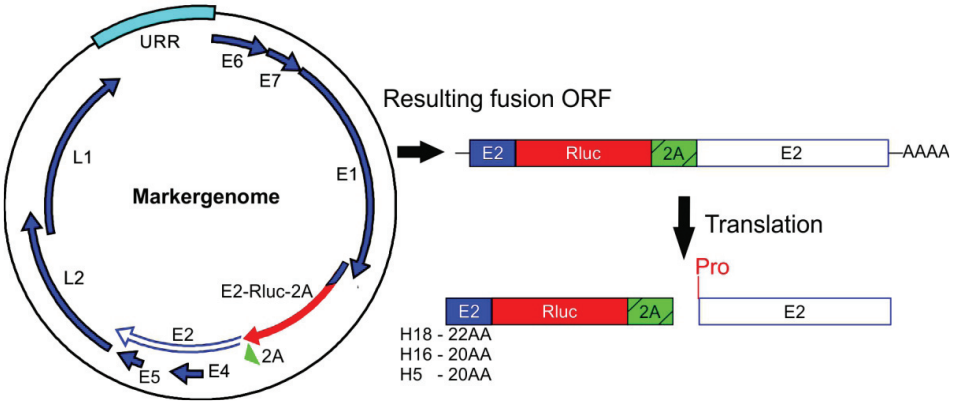

Figure 12. Schematic design and working principles of the HPV-Rluc-E2 marker genome. The coding sequence of Renilla luciferase is inserted into the ORF of E2 immediately after the overlapping region of E1 and E2. Coding sequences of FMDV 2A and full-length E2 follow. This cassette is transcribed as a single mRNA. During translation two functional proteins (Renilla luciferase, which contains 20–22 amino acids of N-terminal E2 and 2A, and functional E2, which contains an additional proline in the N-terminus) are synthesized by "cleavage" of FMDV 2A

Based on the principle described in Figure 4, the marker genomes for HPV18, HPV16 and HPV5 were generated. A comparison of these marker genomes with the respective wt. genomes showed that in the case of HPV18 and HPV16, the replication properties were largely the same, whereas for HPV5, the marker genome showed approximately three-fold higher replication (IV, Fig. 2). The enhanced replication of the HPV marker genome may result from the enhanced splicing/overall gene expression or stability of E2 protein as additional proline residues are known to enhance it (Matthews et al. 1987). We then analyzed the expression of Renilla luciferase from these marker genomes and showed that the expression of the Renilla luciferase correlates with the changes in the viral copy number that occur during HPV replication (IV, Fig. 2, panels D and E). Comparative transcription analyses of HPV18 wt. and Rluc-E2 marker genome (IV, S4) showed that the promoter activity and splicing patterns in the early viral region are very similar.

The previous experiments with Rluc-E2 marker genomes were conducted during the initial amplification phase of HPV replication. The selection of U2OS cell clones containing episomal HPV genomes allows researchers to study the stable maintenance and late amplification stages of viral genome replication, as well (Geimanen et al. 2011). Thus, we generated two cell lines based on U2OS #10.15 that contain episomal HPV18-Rluc-E2 marker genomes. Analyses of those cell lines indicate that both stable maintenance and late amplification can be studied by measuring the Renilla luciferase expression originating from the HPV marker genome and the Firefly luciferase originating from the U2OS genome for measuring cell growth (IV, Fig. 3).

The identification of novel compounds and drug targets usually starts with HTS. The analyses of thousands or even millions of compounds for their potential inhibitory effect on HPV replication using “classical” DNA replication measuring methods, such as Southern Blot or qPCR, would be impossible. Therefore, there is a need for special model systems that would allow researchers to describe the compound’s effect on HPV infection as well as on cellular viability. To date, there has been no such system (to our knowledge) that would allow the analysis of the entire replication cycle of the HPV genome in a way that is suitable for HTS and to identify drugs that would inhibit an ongoing infection. The system described here allows researchers to evaluate the HPV copy number changes during all three replication stages by measuring Renilla luciferase expression. The key aspect of this system is the way in which Renilla luciferase is produced: the expression of the HPV genes is completely under the control of viral transcription. The addition of the Renilla luciferase sequence to the HPV genome has not altered any of the important aspects of that virus replication cycle that we can detect. Conducting HTS using this system would allow researchers to identify the novel HPV replication inhibitors, as well as the drug targets, that are most effective against the virus.

Novel ways to target high-risk HPV replication

The second major part of the work performed in manuscript IV was to conduct an HTS using the novel HPV genome-based model system described in the previous section. We chose the HPV18-Rluc-E2 marker genome as the model and chemical library Diversity Set IV from the National Cancer Institute of the United States, consisting of ~1500 biologically active compounds belonging to different chemical classes. Screening was performed to identify compounds that are capable of specifically inhibiting the initial amplification phase of HPV18. Compounds at 5 μM and 1 μM concentrations were analyzed, and approximately 80 compounds (5% of the overall compounds) that specifically reduced the levels of Renilla (originating from the HPV18-Rluc-E2 marker genome, an indication of HPV replication), but not Firefly luciferase (originating from the U2OS genome, an indication of cellular viability), were chosen. The compounds were tested during the initial amplification of the HPV18 wt. genome by Southern blotting and qPCR, and the five most potent compounds were subsequently chosen, with IC50s (the concentration needed for 50% inhibition) ranging from 2.5 – 80 μM (IV, Fig. 4). The effect of those compounds on the stable maintenance and late amplification of HPV18 was also analyzed using a monoclonal U2OS cell line containing episomal HPV18 #1.13, as described in (Geimanen et al. 2011). Four of those compounds showed a clear concentration-dependent inhibition of stable maintenance, and three also inhibited the late amplification of HPV18 (IV, S7).

These results indicate that the luciferase-based HPV model system described in the previous section can be used for conducting HTS to identify HPV inhibitors. The screening resulted in the identification of five novel HPV inhibitors, and three of those could be used to target an already established infection.

Two of the compounds identified during the screen are known inhibitors of the protein Tyrosyl-DNA Phosphodiesterase 1 (Tdp1), which could be used to inhibit the growth of various cancer cells (Antony et al. 2007, Dexheimer et al. 2009). Tdp1 protein is an important protein in the Topoisomerase 1 pathway. Topoisomerases are enzymes that relax tightly packed nucleic acids to release torsional stress. Relaxed nucleic acids are accessible for replication and transcription complexes. Topoisomerases are part of the Top1 or Top2 cleaving complexes (Top1(2)cc). Several endogenous or exogenous DNA-damaging agents cause the entrapment of Top1cc-s, which in turn interferes with replication (or transcription) fork progression (Snapka 1987, Tsao et al. 1993, Desai et al. 2003, Pommier 2013). Tdp1 is then needed for the release of entrapped Top1cc as it hydrolyzes the phosphodiester bond between Top1 and DNA, thereby releasing the cleaving complex (Interthal et al. 2001, Das et al. 2009, Murai et al. 2012). For its activation and localization to the correct sites on DNA, Tdp1 has to be activated by PARP1 protein (Das et al. 2014). PARP1 catalyzes the addition of ADP-ribose polymers (PAR) to its target proteins (including Tdp1), thereby regulating their cellular localization and biological

activities (Malanga and Althaus 2004, Park and Cheng 2005, Schreiber et al. 2006). The downregulation of Tdp1 by shRNA and PARP1 by shRNA or its specific inhibitor ABT-888 (Penning et al. 2009) resulted in a significant decrease in HPV18's initial amplification (IV, Fig. 5). Based on these analyses, we can conclude that both Tdp1 and PARP1 are valid drug targets for inhibiting HPV replication. The inhibition of these proteins would specifically inhibit HPV replication because Tdp1 is not absolutely necessary during normal cellular DNA replication.

To characterize the specificity of the compound targeting HPV replication, additional analyses were performed in U2OS cells expressing Epstein-Barr Virus (EBV) EBNA1 protein. The expression of EBNA1 will allow researchers to monitor the replication of oriP plasmid (described in (Yates and Guan 1991, Kirchmaier et al. 1995)). EBNA1-dependent replication is initiated once per cell cycle, whereas HPV genome amplification occurs throughout the S and G2 phases of the cell cycle. Differences in replication mechanisms between HPV and EBV make oriP plasmid replication an ideal internal control for the compounds. Our analyses clearly show that HPV replication is inhibited in the presence of these compounds, whereas EBNA1-dependent oriP plasmid replication levels are similar to those of the control sample (IV, Fig. 6).

Camptothecin (CPT) is an inhibitor of Topoisomerase I, which stabilizes the Top1cc-DNA complex, and its analogs could be used as anti-cancer therapeutics (Hsiang et al. 1985, Redinbo et al. 1998, Keil et al. 2015). The stabilization of Top1cc with CPT and the deactivation of Tdp1 results in a more enhanced inhibition of the related targets, as has been suggested previously (Keil et al. 2015). We also tested the five compounds that were identified during the HTS of NCI Diversity Set IV together with CPT. Four compounds showed more effective inhibition of initial HPV18 amplification, but not EBNA1-dependent oriP, when supplemented with CPT (IV, Fig. 6). These results suggest that four out of five compounds either inhibit Tdp1 directly, or some related protein in the pathway responsible for releasing Top1cc from DNA. It seems that Top1cc gets trapped at some point during HPV DNA replication or transcription and fails to release this complex, resulting in replication fork collapse and the likely formation of aberrant DNA replication intermediates (a schematic model is shown in Figure 13).

It is not clear how and why Top1cc will be entrapped on the HPV genome or how Tdp1 is recruited to release it. However, this entrapment seems to occur only during the replication of HR-HPV genomes because none of the five compounds inhibited the initial amplification of LR-HPV11 or cutaneous HPV5 but were effective against HR-HPV16 (IV, Fig. 7). There may be different cellular proteins involved in HR-HPV genome replication or slight differences in the replication mechanism from the LR or cutaneous HPV types. The HR-HPV genome sequences may contain some specific elements (sites) where Top1cc will become entrapped. Another possibility may be that some HR-HPV protein interacts with Tdp1/PARP1 and thus involves these proteins in HPV replication. The major differences between LR and HR HPVs result in different

functions/transforming abilities of oncoproteins E6 and E7. It is known that E6 and E7 are able to activate the DDR and interact with DNA repair proteins (Duensing and Munger 2002, Iftner et al. 2002, Rogoff et al. 2004, Banerjee et al. 2011). We analyzed the effect of the five compounds on the initial amplification of HPV18 mutant genomes that do not express E6, E7 or both, and the replication of mutant genomes was inhibited to the same extent as for wt. HPV genome replication (data not shown). It seems that E6 and E7 are not responsible for the involvement of Tdp1 in HR-HPV genome replication.

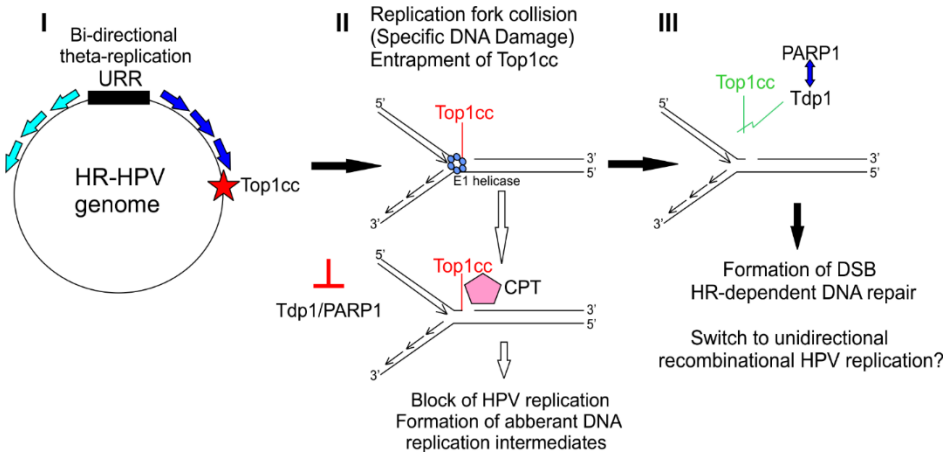

Figure 13. Proposed model for the role of Tdp1 and PARP1 proteins in High-risk (HR) HPV genome replication. I: E1 and E2 dependent bi-directional replication is initiated from the HPV replication origin in the URR region. II: The Topoisomerase I cleaving complex (Top1cc) will be entrapped in at least one site in the HR-HPV genome because of specific DNA damage or replication fork collapse. III: Tdp1, which is activated through PARylation by the PARP1 protein, cleaves the phosphodiester bond between Top1cc and DNA, thereby releasing the complex. The replication fork collision will be repaired by homologous recombination-dependent DNA repair, and replication mode is switched to unidirectional recombination-dependent replication (described in (Orav et al. 2015)).
When Tdp1 and/or PARP1 are inhibited and Top1cc is stabilized by camptothecin (CPT), the replication forks will collide, HPV genome replication will be blocked, and eventually, aberrant DNA replication intermediates form.

It has been shown that both HPV replication proteins E1 and E2 bind to Topoisomerase I and stimulate its activity. The C-terminal helicase domain of HPV E1 protein, specifically the last ~60 amino acids in HPV11 E1, was necessary for Topoisomerase binding (Clower et al. 2006, Clower et al. 2006). A comparison of the C-terminus E1 mutants of HPV11 and BPV1 showed that although deletions were deleterious for BPV-1 replication, only 45% lower

replication was seen in the case of HPV11 (Bergvall et al. 2016). Perhaps the interaction between the E1 protein from HR-HPVs and Topoisomerase I is crucial for replication, similar to BPV-1, whereas the interaction is (partially) dispensable in LR and cutaneous HPVs. Specific mapping and mutational analyses of E1-Topoisomerase I interactions would describe why the compounds analyzed during this work are HR-HPV-specific inhibitors. Regardless, differences in the replication of LR- and HR-HPVs may be the reason why oncogenic HPVs integrate more readily into the host genome.

The problem with the HPV replication inhibitors identified thus far is that they are not effective against HPV-related cancers. Targeting only HPV-related cancerous lesions would help to reduce or eliminate the tumor but not necessarily the replicating HPV genomes in the basal epithelium. Tdp1, PARP1 and Topoisomerase I inhibitors have a negative effect on cancer cell viability. It may be possible to target HPV genome replication and HPV-related cancers using the same drugs, making the treatment more effective and reducing the risk of potential side effects.

CONCLUSIONS

1. HPV18 gene expression in U2OS cells is almost identical to gene expression in HPV's natural host keratinocytes. Therefore, U2OS cells provide an adequate environment for the HPV life cycle and may be used as an alternative model system for studying various stages of the HPV life cycle. Because U2OS cells can be cultured rapidly and cost-effectively, they provide an excellent platform for the development of anti-HPV drugs.
2. The overexpression of HPV replication protein E1 induces double-stranded DNA breaks which leads to the fragmentation of genomic DNA measured by the comet assay. Due to the large-scale DDR activation, cell cycle progression is halted in the S and G2 phases of the cell cycle, measured by flow cytometry analyses.
3. HPV18 genome is localized into distinct foci detected through FISH analyses of HPV genomic DNA in U2OS cells. These foci are HPV replication centers as their formation is E1-dependent and there is an active DNA synthesis measured through incorporation of EdU. There is no large-scale E1-dependent DDR activation, damage of cellular DNA and arrest of the cell cycle as E1 protein is localized in the HPV replication foci.
4. HPV18's initial amplification is extended beyond the S phase to the G2 phase of the cell cycle. This extension may be beneficial for the virus because it leaves more time to amplify viral DNA.
5. The insertion of the Renilla luciferase coding sequence into the ORF of HPV E2 protein will allow researchers to monitor viral genome replication by measuring the activity of Renilla luciferase. This type of HPV replication analyses is rapid and sensitive; thus, these types of viral genomes can be used in high-throughput screens to identify novel anti-HPV compounds.
6. The high-throughput screening of the NCI Diversity Set IV chemical library with the HPV18 marker genome led to the identification of five novel HR-HPV-specific inhibitors. Analyses of these inhibitors identified Tdp1 and PARP1 as essential cellular proteins and valid drug targets for HR-HPV replication. Because inhibition of Tdp1 and PARP1 is also effective against cancer cells, compounds targeting this pathway may be effective against HPV replication as well as cervical cancer.

SUMMARY IN ESTONIAN

Uued võimalused Inimese papilloomiviiruse replikatsiooni tõkestavate ühendite kirjeldamiseks

Praktiliselt kõik inimesed nakatuvad teatud eluetapis Inimese papilloomiviirustega (HPV). HPVd on väga laialdase levikuga ning nende evolutsioon on toimunud koos inimeste evolutsiooniga. Mõningatel juhtudel loetakse neid isegi osaks normaalsest naha mikrofloorast. Lisaks inimestele on Papilloomiviiruseid leitud veel teistelt imetajatelt aga ka lindudelt ja roomajatelt.

Infektsioon Papilloomiviirusega võib kulgeda asümptomaatiliselt, sagedaselt põhjustavad need viirused ka healoomulisi kasvajaid, kõige tuntumad nendest on soolatüükad. Enamikel juhtudel möödub HPV infektsioon immuunsüsteemi toimel aasta jooksul. Siiski on soolatüükad ebamugavad ning koledad, seega on nende ravimiseks läbi aegade kasutatud vägagi erinevaid, tänapäeval naljakatena tunduvaid, viise. Eestlaste kogemuste põhjal pidi soolatüükaid hästi ravima nende hõõrumine vastu pinda, mida valgustab täiskuu valgus. Tänapäeval kasutatakse siiski pigem papilloomiviiruste poolt põhjustatud infektsioonikollete eemaldamist kas laserteraapia, krüoteraapia või immuunsüsteemi stimulaatoritega. Selliste teraapiatega ei eemaldata üldjuhul kogu infektsioonikollet ning ~50% tõenäosusega sümptomid korduvad.

Lisaks healoomulistele kasvajatele võib infektsioon HPV-dega tekitada ka mitmesuguseid halvaloomulisi kasvajaid, millest kõige levinumaks on emakakaelavähk. Emakakaela vähi tekkeks peab HPV infektsioon muutuma pikaajaliseks. Pikaajaliseks muutub küll ainult väga väike osa nakkusi, kuid HPVd on üle maailma väga levinud ning põhjustavad ligikaudu pool miljonit uut vähijuhtumit aastas. Nüüdseks on välja töötatud kolm preventatiivset vaktsiini, mis ennetavad kuni üheksa erineva HPV tüübiga nakatumist. Vaktsineermine HPV vaktsiiniga ei ole siiani piisavalt levinud, see on kallis ning ei ole efektiivne juba käimasoleva infektsiooni tõkestamiseks. Siiani puuduvad spetsiifiliselt HPV paljunemist tõkestavad ravimid.

Papilloomiviirused naktavad mitmesuguseid epiteelkudesid ning viiruse elutsükkel on tihedalt seotud tema peremeesraku – keratinotsüüdi differentseerumisega. Keratinotsüütide kasvatamine laboritingimustes on tehniliselt keerukas, ajamahukas ning küllaltki kallis. Meie laboris töötati hiljuti välja inimese osteosarkoomi (sääreluu kasvaja) rakuliinil U2OS põhinev mudelsüsteem, mis võimaldab uurida mitmesuguste HPV tüüpide kogu replikatsioonitsüklit. U2OS rakke on võimalik väga lihtsalt, kiiresti ja odavalt kasvatada ning seeläbi on sellisel mudelsüsteemil klassikaliste HPV uurimiseks kohandatud mudelite ees mitmeid eelisi. Kuivõrd U2OS rakud ei ole HPV loomulikud peremeesrakud, ei pruugi uuringud nendes peegeldada looduses viirusega toimuvaid protsesse. Selle töö raames analüüsiti HPV tüüp 18 geenikespressioonimustrit U2OS rakkudes võrrelduna keratinotsüütidega. Tulemustest selgus, et viiruse geenikespressioon U2OS rakkudes on autentne ning U2OS rakud on sobiv keskkond HPV erinevate elutsükli etappide uurimiseks.

Uute ravimikandidaatide ning märklaudade otsinguid alustatakse tänapäeval keemiliste raamatukogude uurimisega. Keemilised raamatukogud sisaldavad tuhandeid erinevaid keemilisi ühendeid, mille analüüsimisega loodetakse leida mõni ühend, mis uuritavat protsessi tõkestab. Klassikalised DNA replikatsiooni mõõtmise meetodid aga ei ole piisavalt lihtsad, kiired ja odavad, et analüüsida tuhandete keemiliste ühendite mõju HPV genoomi replikatsioonile. Antud töö raames loodi selliseks analüüsiks sobiv mudelsüsteem. See põhineb viiruse DNA koopianumbri kaudsel hindamisel läbi ensüümi Renilla lutsiferaas, mis on sisestatud HPV genoomi, aktiivsuse mõõtmise. Renilla lutsiferaasi aktiivsuse mõõtmine on väga lihtne, kiire, tundlik, suhteliselt odav ning võimaldab oluliselt väiksema töömahuga hinnata tuhandete keemiliste ühendite sobivust HPV replikatsiooni tõkestamiseks. Töös näidati, et Renilla lutsiferaasi aktiivsus korreleerub viiruse genoomi koopianumbri muutustega ning ei muuda viiruse geeniekspressiooni ega genoomi replikatsiooni mehhanisme.

Töös loodud mudelsüsteemi kasutati ~1500 keemilist ühendit sisaldava raamatukogu analüüsiks, mille tulemusena identifitseeriti viis seni kirjeldamata spetsiifilist HPV replikatsiooni inhibiitorit. Viie ühendi märkaludade analüüsides leiti, et neli ühendit inhibeerivad valku türosüül DNA difosfoesteraas (Tdp1) või teisi selle valgu aktiivsusega seotud protsesse. DNA on rakkudes tihedalt kokku pakitud ning edukaks replikatsiooniks või transkriptsiooniks on vaja see lahti harutada ning molekuli kokkukeerdumisel tekkinud pingeid maandada. Selleks on rakud ensüümid nimega Topoisomeraasid, mis tekitavad DNA molekuli üheahelalisi katkeid muutes seeläbi DNA kättesaadavaks replikatsiooni/transkriptsiooni kompleksidele. Teatud juhtudel (näiteks DNA replikatsioonikahvli "kokku kukkumisel" või spetsiifiliste DNA kahjustuste korral) jäävad Topoisomeraasi kompleksid kovalentselt DNA-ga seotuks nind blokeerivad edasise replikatsiooni või transkriptsiooni. Normaalselt jagunevates keharakkudes see eriti tihti ei juhtu, küll aga kõrge DNA replikatsiooni tasemega vähirakkudes. DNA-le kovalentselt seotud Topoisomeraasi komplekside vabastamiseks ongi vajalik Tdp1 valgu aktiivsus. Töös näidati, et ka HPV genoomi replikatsiooni käigus jääb Topoisomeraasi kompleks viiruse DNA külge kovalentselt seotuks ning Tdp1 on vajalik selle vabastamiseks ning seeläbi replikatsiooni jätkamiseks. Tdp1 valgu aktiivsuse inhibeerimine või valgu taseme mahareguleerimine blokeerib spetsiifiliselt vähki tekitavate HPVde replikatsiooni.

Selle töö tulemusena selgus, et U2OS rakud on sobilik mudelsüsteem Inimese papilloomiviiruse replikatsiooni ja geeniekspressiooni uurimiseks. Täna lihtsusele ja odavusele sobivad need rakud ka uudsete HPV nakkus tõkestavate keemiliste ühendite leidmiseks, kasutades selle töö käigus välja arendatud mudelsüsteemi.

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PUBLICATIONS

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Mart Toots, Andres Männik, Gaily Kivi, Mart Ustav Jr., Ene Ustav, Mart Ustav 2014 The Transcription Map of Human Papillomavirus Type 18 During Genome Replication in U2OS Cells. PLoS One, 9 (12), e116151.

Tormi Reinson, **Mart Toots**, Meelis Kadaja, Regina Pipitch, Mihkel Allik, Ene Ustav, Mart Ustav 2013 Engagement of the ATR-dependent DNA Damage Response at the Human Papillomavirus 18 Replication Centers During the Initial Amplification. Journal of Virology, 87 (2), 951–964.

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Mart Toots, Andres Männik, Karl Mumm, Tarmo Tamm, Kaido Tämm, Andres Tover, Mart Ustav Jr., Mart Ustav 2016 Identification of Several High-Risk HPV Inhibitors and Drug Targets Through High-throughput Screening Using a Novel Assay System. Manuscript

Inventions:

ASSAY SYSTEM TO IDENTIFY HPV REPLICATION INHIBITORS IN HT-SCREEN;
Authors: Mart Ustav, Ene Ustav, Andres Männik, **Mart Toots**, Mart Ustav Jr., Andres Tover
Applicant – Icosagen Cell Factory OÜ. Submitted 04/2015.

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Publikatsioonid:

Mart Toots, Andres Männik, Gaily Kivi, Mart Ustav Jr., Ene Ustav, Mart Ustav 2014 The Transcription Map of Human Papillomavirus Type 18 During Genome Replication in U2OS Cells. PLoS One, 9 (12), e116151.

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