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The role of the Protein Kinase CKII in the life cycle of Human Papillomaviruses

Master´s Thesis (30 EAP)

Gene Technology

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Abstract

The role of the Protein Kinase CKII in the life cycle of Human Papillomaviruses

Cervical cancer is the fourth most common cancer in women and virtually all cases can be traced back to a preceding infection with a high-risk HPV (human papillomavirus) type. Worldwide 10% of all women are infected, with the hotspots being South America and Africa. The best way to fight cervical cancer is to prohibit its formation. Currently, only vaccines are available to achieve this, but these are not the ultimate solution, as they are not able to clear an existing HPV infection. If we want to reduce the cancer risk of the millions of women infected with HPV today, we need a supplement, which is able to clear a persisting HPV infection reliably. In this thesis it is shown that the catalytic subunit of the protein kinase CK2 plays an important role in the initial amplification phase of HPV and an interference leads to lesser replication rates of viral genomes. Additionally, a CK2 inhibitor (CX-4945) is tested for its ability to inhibit CK2 and through this to hinder HPV replication. It is shown that this compound could be the basis for the long sought anti-HPV drug.

key words: HPV, HPV replication, protein kinase CK2, CK2α, CX-4945

CERCS code: B230 (microbiology, bacteriology, virology, mycology)

Kokkuvõte

Proteiinkinaas CK2-e roll inimese papilloomiviiruse elutsüklis

Emakakaelavähk on maailmas levimuselt neljas vähiliik ning peaaegu kõiki juhtumeid põhjustab varasem infektsioon inimese papilloomiviirusega. Üle maailma on sellega nakatanud ligikaudu 10% kõikidest naistest. Parim viis emakakaelavähi vastu võitlemiseks, on selle arenemise takistamine, kuid tänapäevani on vaktsineerimine *HPV* (ingl. k. *human papillomavirus*) vastu ainuke ennetusmeetod. Vaktsineerimine ei tõkesta aga olemasolevat nattust ning vaktsiinide kättesaadavus arengumaades on piiratud. See tähendab, et kui tahame vähendada miljonite naiste emakakaelavähki haigestumine, siis on vaja ravimit, mis suudaks käimasoleva *HPV* infektsiooni kõrvaldada. Käesolevas töös näidati, et proteiinkinaas CK2-e katalüütiline subühik omab tähtsat rolli HPV algses amplifikatsiooni faasis. Selle ensüümi tööd häirides, saab viiruse genoomi replikatsiooni taset efektiivselt alandada. Lisaks katsetati CK2-e inhibiitori (CX-4945) võimet läbi CK2-e inhibeerimise HPV replikatsiooni takistada. Leiti, et just CX-4945 võiks olla potentsiaalse *HPV* vastase ravimi baasiks.

märksõnad: HPV, HPV replikatsioon, proteiinkinaas CK2, CK2α, CX-4945

CERCS-i kood: B230 (mikrobioloogia, bakterioloogia, viroloogia, mükoloogia)

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<u>Used abbreviations</u>

AMP – ampicillin

ATM – ataxia telangiectasia mutated

BPV – bovine papillomavirus

CDC – Centers for Disease Control and Prevention (USA)

CK2 – casein kinase 2

CK2α – casein kinase 2 subunit alpha

CK2α′ – casein kinase 2 subunit alpha prime

CK2β – casein kinase 2 subunit beta

CMV – cytomegalovirus

CRISPR/Cas – Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated nucleases

CX-4945 – 5-[(3-Chlorophenyl)amino]benzo[c][2,6]naphthyridine-8-carboxylic acid

DMEM – Dulbecco/Vogt modified Eagle's minimal essential medium

ECL – enhanced chemiluminescence

EBV – Epstein–Barr virus

FCS – foetal calf serum

HPV – human papillomavirus

IC_{50} – half maximal inhibitory concentration

IMDM – Iscove’s modified Dulbecco’s medium

KM – kanamycin

LB – lysogenic broth

p53 – cellular tumour antigen p53

PDB – RCSB Protein Data Bank

PDZ – acronym combining the first letters of three proteins – PSD95, Dlg1, zo-1

pRb – retinoblastoma protein

PV – papillomavirus

siRNA – small interfering RNA

TB – terrific broth

TBP – TATA-Box binding protein

URR – upstream regulatory region

WHO – World Health Organisation

Introduction

Human Papillomaviruses (HPV) are small DNA viruses that infect various epithelial tissues. HPV infections are normally cleared within a few months, without any symptoms appearing, however, in some rare cases, genital warts, benign tumours, or even cervical cancer develop. These HPV types, which are known to cause cancer, are called high-risk types. HPV infections are a pandemic issue and worldwide 10% of women carry the virus. This is especially a problem in the less-developed regions of the world, where the infection rate is high and systems for cancer screenings are not implemented. Almost all cervical cancer cases can be traced back to a previous infection with different risk types of human papillomaviruses. Cervical cancer is worldwide the fourth most common cancer in women and every year roughly 500 000 women are diagnosed, while 270 000 die, which accounts for about 7.5% of all cancer deaths. To this day there is no drug available that would heal an HPV infection, although the demand for one is huge.

In this thesis the importance of the protein kinase CK2 in early HPV replication is investigated. In addition, the effect of an CK2 inhibitor (CX-4945) is tested, as this compound could be used in the first anti-HPV drug. The fact that CX-4945 reached the second phase in clinical trials already as an anti-cancer drug makes this even more promising.

Literature overview

1. Human papillomaviruses (HPV)

The human papillomaviruses are DNA viruses that belong to the *Papillomaviridae* family and are subdivided into five genera: α-papillomaviruses (Alpha-PV), β-papillomaviruses (Beta-PV), γ-papillomaviruses (Gamma-PV), μ-papillomaviruses (Mu-PV) and ν-papillomaviruses (Nu-PV). The HPV classification into first genera and then species and types is based on sequence similarities in the L1 region of the HPV genome (see chapter 1.2 HPV genome), which is highly conserved. Up-to-date there are over 200 different HPV types described (Kocjan *et al.*, 2015).

Viruses of different genera infect different parts of the human body, for example, viruses of the α-genera infect mucosal while viruses of the β-, γ-, μ-, or ν-genera infect cutaneous tissues. From this it follows, that different HPV types cause warts and papilloma in very different anatomical sites, like the genital region or in the oral cavity. The risk of these benign tumours to turn into a malign form depends highly on the HPV type, so that different HPV types are in addition divided into a high-risk and a low-risk group (Bernard *et al.*, 2010), (de Villiers *et al.*, 2004).

1.1. Clinical data

The primary way of HPV transmission is during sexual practices, so that most people are infected soon after their onset of sexuality. An HPV infection is the most common viral infection of the reproductive tract and the fact that simple skin-to-skin genital contact is enough for transmission, explains the high infection rates in human populations all over the world. (Kjaer *et al.*, 2001).

Especially women, who are in danger of developing cervical cancer due to an HPV infection, are the object of studies on HPV infections and it got clear that HPV infections prevail most notably in countries with a lower average-income. This brings Africa and Central America to the top of the list with more than 20% of women carrying an HPV infection (Figure 1). In Europe, on the other hand only 6.6% of women are infected. The WHO explains this situation, with better education about sexual practices and more frequent use of condoms in developed countries (Crow, 2012).

More often than not, HPV infections are asymptomatic. The infection will clear itself spontaneously without any treatment usually within a few months, so that about 90% of the patients are clear of the virus within 2 years. In some few cases, genital warts are formed and although hardly resulting in the death of the patient, should these be treated with care, as they are highly infectious. On the other hand, certain infections with high-risk HPV types (most commonly 16 and 18) might persist and lead to precancerous lesions and, if left untreated, progress to cancer (Moscicki *et al.*, 2006).

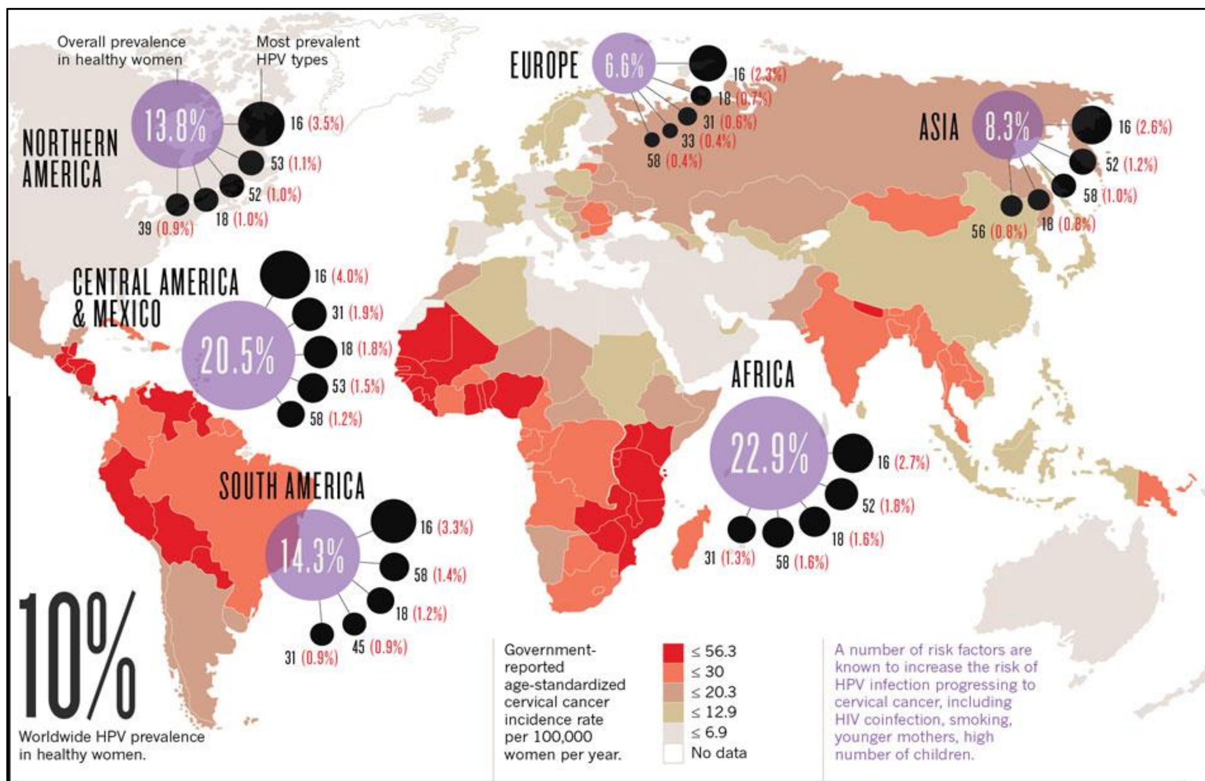


Figure 1: Percentage of women carrying an HPV infection broken down for every continent

This figure shows the percentage of women being infected with HPV broken down for every continent. It gets clear that HPV infections prevail most notably in countries with a lower average-income, making Africa and Central America top of the list with more than 20% of women carrying an HPV infection. In Europe, on the other hand, with its higher level of development the amount of women with an HPV infection is only 6.6%, that is almost a fourth less, than in Africa. The WHO explains this situation, with better education about sexual practices and more frequent use of condoms in developed countries.

The figure also provides information on the most prevalent HPV types found in infected women. With HPV 16 being the most common HPV type on every continent. It also states additional risk factors for a HPV infection to process to cervical cancer.

(Crow, 2012)

The most common cancer type resulting of an HPV infection is cervical cancer (Figure 2) and almost all cases can be traced back to an initial HPV infection (Walboomers *et al.*, 1999). Typical symptoms appear only in an advanced stage, when the tumour grows bigger due to ulceration and these might include irregular and abnormal vaginal bleeding, back, leg or pelvic pain and vaginal discomfort (Crow, 2012).

According to the WHO, of all the types of HPV at least 13 are cancer-causing (high-risk types) and two of these types alone, HPV 16 and 18, are responsible for 70% of precancerous cervical lesions and cervical cancers. Cervical cancer is the fourth most common cancer in women worldwide and in the year 2012 alone, 530 000 new cases were estimated, while in the same year, roughly 270 000 patients died. This accounts for 7.5% of all worldwide cancer deaths (GLOBOCAN, 2012), (Crow 2012).

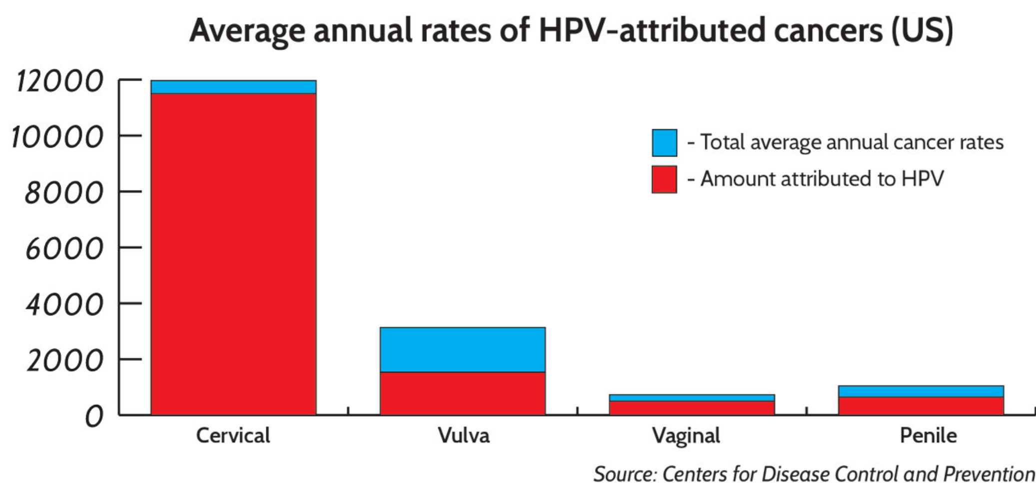


Figure 2: Average annual rates of HPV-attributed cancers in the USA

From this figure, it becomes clear how high the percentage of HPV-attributed cases in different cancers is. Especially cervical and vaginal cancers, but also penile cancers are virtually always connected to an HPV-infection. In vulva cancers, about 50% of the cases can be connected to HPV. This numbers show how important a prevention of HPV infection is in order to decrease the cancer cases in the genital area.

This figure also shows the correlations of occurrence of different cancer types of the genital area, with cervical cancers occurring about three times more often than the second numerous cancer type, vulva cancer.

(Centers for Disease Control and Prevention, USA)

In high-developed countries, where women are screened for precancerous lesions, 80% of cervical cancers are prevented, as cervical cancer develops slowly (15 – 20 years) and precancerous lesions can be treated. However, in developing countries, where programs to screen women are missing, the disease frequently advances until symptoms develop, with the

prospects for these late-stage cases often being poor. Globally 52% of cervical cancer patients die, with the majority of these being residents of developing countries (Shieh *et al.*, 2016).
There is growing evidence that HPV infections are also linked to other cancer forms, for example to cancer of the anus, vulva, vagina and penis (Figure 2), although those cases occur very scarcely compared to cervical cancer (Bansal *et al.*, 2016).
Today there are different vaccines available, with the newest (nine-valent vaccine) having the potential to prevent almost 90% of cervical cancers as it grants a protection against nine high-risk HPV types (6, 11, 16, 18, 33, 45, 52 and 58) (Joura *et al.*, 2015). Clinical trials showed that the vaccines are very effective and should be used before the first sexual intercourse, as they cannot treat HPV infections or HPV associated diseases. A vaccination of girls in the age between 9 and 13 years is seen as the most cost-effective way to deal with cervical cancer (WHO), as this reliably prevents the girls from developing cervical cancer. Some countries started to vaccinate boys as well, as this helps to prevent genital warts and by that, the fast spread of the virus (Harper *et al.*, 2006), (Villa *et al.*, 2006), (Villa *et al.*, 2005).
There is, as illustrated above, a huge problem with HPV-attributed cancers worldwide and some cancer types are even on the rise, according to the Annual Report to the Nation on the Status of Cancer (Jemal *et al.*, 2013). The fact that people tend to be suspicious with vaccines, as they fear them to cause severe sicknesses, is counterproductive and does not help to lower the occurrence of HPV-attributed cancers (Bratic, 2016).
This means that there is an unmet medical need for a supplement that is able to stop a prevailing HPV infection and eliminates the virus from the patient's body. This would not only help to curtail the worldwide HPV infection cases, but also to prevent the thousands of cases of cancer in the genital area every year.

1.2. HPV genome

Human papillomaviruses are 45 – 55 nm in size and lack a cap. Their DNA is double-stranded, circular and almost 8 000 base pairs long. Even though a family with many representatives, show the genomes of the different HPV types a high grade of similarity and they can be separated into three distinct segments (Figure 3). The early region (E), which covers roughly 50% of the viral genome, is the biggest of these and contains the open reading frames for proteins, expressed early in the lifecycle of HPV. E1 is a DNA helicase necessary for HPV replication and E2 is a regulatory protein, which controls viral gene expression, replication and

segregation. E5, E6 and E7 are oncoproteins, whose function lies in the transformation process and, last, E4, which controls the release of the virions.

The second largest segment is the late region (L), which covers about 40% of the genome and contains the reading frames for the L1 and L2 proteins. These are structural proteins and encode the proteins of the virion's cap.

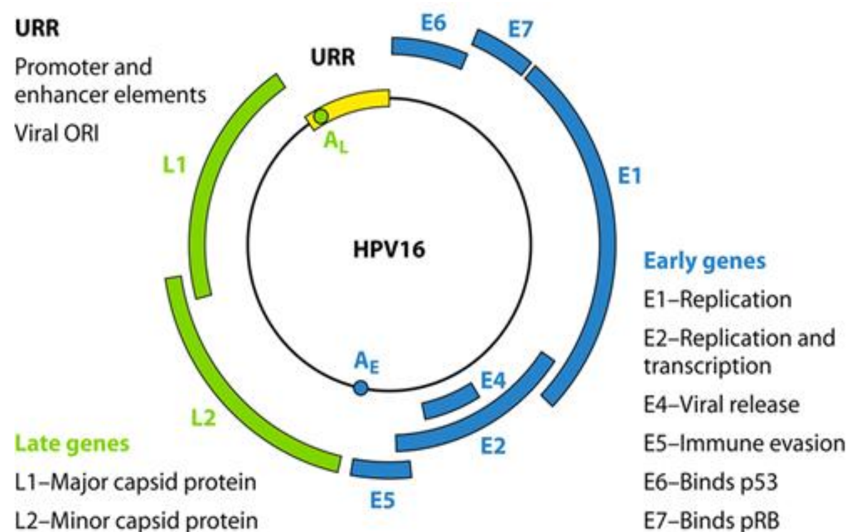


Figure 3: Genome organisation of HPV type 16

All HPV types share a similar genome organization. It is separated into three distinct regions: The early (E; blue) region, the late (L; green) region and the upstream regulatory region (URR; yellow).

The early region contains the open reading frames of proteins expressed early in the life cycle of the virus. Among these are E5, E6 and E7, the oncoproteins, which operate the transformation process, E1 and E2, which are regulatory proteins of the transcription and E4, which controls the release of the virions.

The late region contains the open reading frames of the two capsid proteins: L1, the major and L2, the minor capsid protein.

The upstream regulatory region controls the early gene transcription and replication and contains promotor and enhancer elements, as well as the viral origin of replication.

As all HPV transcripts are polycistronic, are A_E and A_L the common polyadenylation sites of the early or respectively the late region.

(Stanley, 2012)

The smallest part is the upstream regulatory region (URR), which spans around 10% of the viral genome and includes promotor and enhancer elements, as well as the viral origin of replication. It controls the early gene transcription and replication (Lee and Laimins, 2007), (de Villiers *et al.*, 2004), (Stanley, 2012).

1.3. Life cycle of HPV

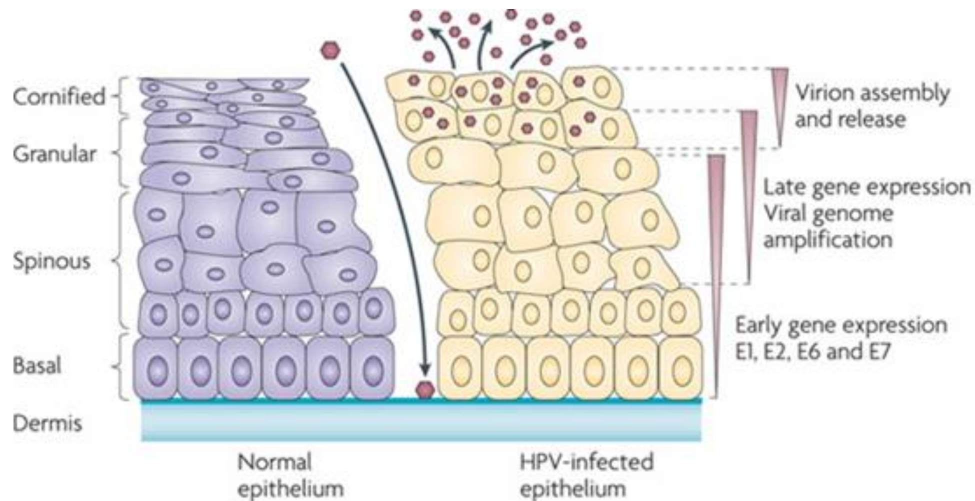


Figure 4: HPV life cycle

This figure shows the complete life cycle of HPV from infection until virion release.

To establish a perpetual infection, HPV needs to infect a cell in the basal layer of a stratified epithelium. This is possible via micro lesions. After reaching the host cell's nucleus, early viral genes are expressed. These include E1 and E2, the viral replication factors, and E6 and E7, the viral oncogenic proteins. In this basal layer cells the replication rate of viral genomes is kept low and can persist over a long period. Only upon cell differentiation, late HPV genes are expressed and then in the outer parts of the epithelium virions assembled and released.

(Moody and Laimins, 2010)

As the genome of HPV is of very limited size, it is clear that HPV highly depends on proteins and factors of the host cell to complete its life cycle (Figure 4). Without them HPV is neither able to maintain viral genome copy numbers in undifferentiated cells nor to amplify viral genomes in differentiated cells (Moody and Laimins, 2010).

Human papillomaviruses infect stratified squamous epithelia, where the basal layer became exposed after an injury (Culp *et al.*, 2006). The viral genomes remain in basal cells as extrachromosomal plasmids and replicate together with the host's DNA during S-phase and this proceeds into the G2-phase as well (Wang *et al.*, 2009), (Reinson *et al.*, 2013), (Reinson *et al.*, 2015). After entering the nucleus of basal cells, the viral genome copy number increases swiftly until about 50 – 100 copies per cell are reached (initial amplification) (Peh *et al.*, 2002). Now this number is maintained continuously through the infection (maintenance replication) (Penrose and McBride, 2000), until the host cell starts to differentiate (Bedell *et al.*, 1991).

Then the vegetative amplification takes place, which means a high-level replication of viral genomes (Figure 5). At the same time capsid protein production is initiated (Schwartz, 2013), which leads to virion assembly and then, virion release. It is crucial for the process of amplification that the differentiating cell remains in an active cell cycle, as HPV genomes only are amplified during host cell replication (Galloway and Laimins, 2015).

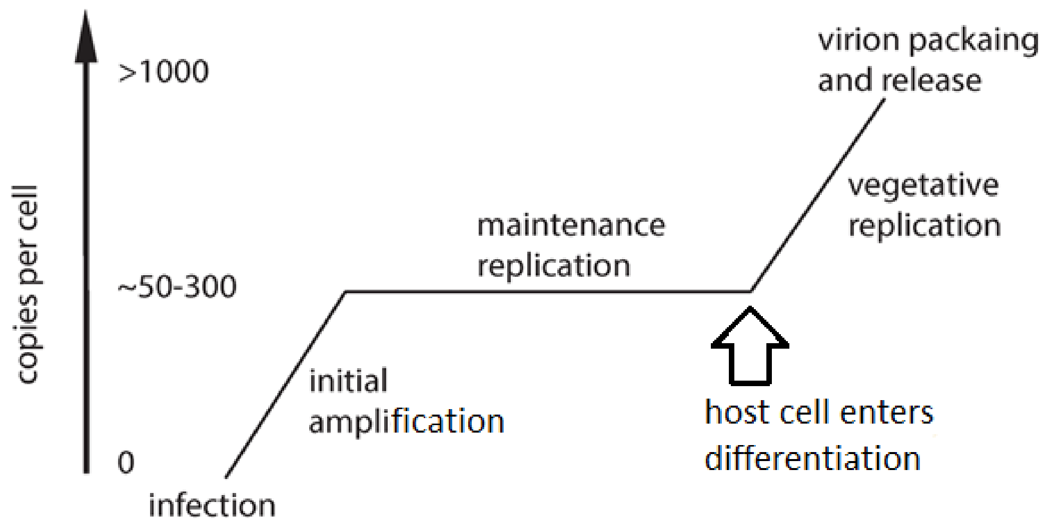


Figure 5: HPV genome copy number variation throughout the infection

The number of HPV genomes varies highly throughout the different stages of an HPV infection. Right after infection, it rises swiftly to 50 – 300 copies per cell. This phase is called the initial amplification. During maintenance replication the replication rate is kept low and the copy number kept stable. Only upon host cell differentiation a steep increase in the replication rate and thus copy number can be seen. The produced copies of the HPV genome are then packed into virions and released.

(Fisher, 2015; modified)

One part of the host's cell cycle that is required for the genome amplification of high-risk HPV types is the ataxia telangiectasia mutated (ATM) pathway (Moody and Laimins, 2009). Normally, this pathway works as a tool to repair double strand breaks, but in with HPV infected cells, factors of this pathway are constitutively activated and recruited to the HPV genomes (Gillespie *et al.*, 2013).

1.3.1. E1 and E2, viral replication proteins.

Two replication proteins, E1 and E2, are required in all life stages of HPV to execute viral replication (Egawa *et al.*, 2012). E1 is a replicative helicase and E2, besides other functions, recruits E1 to the replication origin of the viral genome (Mohr *et al.*, 1990), (Ustav and Stenlund, 1991), (Ustav *et al.*, 1991). The origin is situated in the upstream regulatory region (URR) of the viral genome and contains binding sites for E1 as well as for E2 (Ustav *et al.*, 1991). With the help of E2, E1 binds to the replication origin and, after E2 left the site, unwinds the DNA. Now the replication machinery of the host is used to replicate the viral DNA (Sanders and Stenlund, 1998).

1.3.2. E6 and E7, viral oncogenic proteins

These proteins make sure that the environment in the host cell stays suitable for viral replication. They promote cellular proliferation, delay cellular differentiation and help HPV to escape the immune system of the host (McLaughlin-Drubin *et al.*, 2012). Without them a productive replication is impossible (Flores *et al.*, 2000), (Chow *et al.*, 2009).
E6 causes, amongst other things, a degradation of p53 by recruiting the cellular ubiquitin ligase E6AP, which leads to a ubiquitination of p53. Normally, p53 arrests cells after DNA damage in order to make a reparation possible (Chen *et al.*, 1995). In addition, the E6 proteins from high risk HPV types contain PDZ (acronym combining the first letters of three proteins – PSD95, Dlg1, zo-1) binding domains, which allow them to interact with other PDZ domain containing factors, which control differentiation and cell polarity (Ganti *et al.*, 2015).
HPV host cells, keratinocytes, exit the cell cycle at the onset of differentiation. But an active cell cycle is needed in order to replicate the HPV genome. That is why the viral oncogene E7 induces the degradation of pRB family proteins, which in turn allows the cell, through the release of E2F, to enter the S-phase of the cell anew. E2F factors are involved in cell cycle regulation and DNA synthesis and are with the help of E7 constitutively active (Münger *et al.*, 1991), (Huh, *et al.*, 2007).
In HPV-induced cancers, both oncogenic proteins are usually expressed, where they synergistically promote oncogenesis. Apart from being essential for providing a suitable environment for HPV, they are also essential for an HPV infection to turn into cancer (Mesri *et al.*, 2014).

1.3.3. Initial amplification

HPV enters a dividing cell of the basal layer of the epithelium through micro-lesions and its genome is transported is transported to the nucleus (Culp *et al.*, 2006), (Day, *et al.*, 2004). After entry, the HPV genome is amplified and the viral genome copy number reaches up to a few hundred copies per cell (Figure 5) (Peh *et al.*, 2002).

During this stage, HPV replication is controlled by the E8^E2 protein. This protein is a repressor of viral transcription and replication and might help to prohibit a too high level of viral genome replication. This is useful in order to keep cellular viability stable and to avoid detection by the immune system (Stubenrauch *et al.*, 2000).

1.3.4. Maintenance replication

During this phase, the viral genomes replicate during S-phase by average once per cell cycle with the host's genome in order to keep a constant copy number (Hoffmann et al., 2006). HPV replication could be controlled, during this stage, by different mechanisms. The E1 protein is, for example, outside of the S-phase, kept in the cytoplasm (Fradet-Turcotte et al., 2010) and might even be obsolete during maintenance replication (Egawa et al., 2012). E8^E2 is another key protein in the replication rate control mechanism and regulates both transcription and replication, by strongly repressing the activity of the viral early promoter. This repression is totally independent of E2 (Stubenrauch et al., 2000).

It was shown that in BPV1 also the phosphorylation of E2, which recruits E1 to the replication site, is a limiting factor during the maintenance replication (Penrose and McBride, 2000). This could also be true for different HPV types and is for this thesis especially of interest, as a protein kinase CK2 inhibitor is used to inhibit HPV replication.

Following to replication, HPV genomes are equally divided between the daughter cells. It is stated that E2 binds to several E2 binding motifs in the viral URR while binding to the host chromatin as well. This would ensure a distribution of the HPV genomes between the daughter cells (Ilves et al., 1999). While BPV1 is mostly used as a model for viral gene distribution in papillomaviruses, most likely, this process applies to all PVs, with only a few small differences (McBride et al., 2006).

1.3.5. Vegetative replication

The third HPV genome replication stage – vegetative amplification takes place in differentiated keratinocytes. During this stage, HPV genomes undergo rapid amplification and genome copy number reached up to a few thousand copies per cell (Bedell *et al.*, 1991). This rapid amplification is possible due to the activation of late promoter which results in high levels of E1 and E2 protein (Burnett *et al.*, 1990).

Additionally, the expression of the capsid proteins is initiated. This is regulated through differentiation-dependent changes in polyadenylation site usage and through alternate splicing (Schwartz, 2013), but also microRNAs from the host cell are used to regulate processes in this phase (Cai *et al.*, 2006). These processes include a variety of pathways involving proliferation, differentiation and innate immune surveillance. As a large number of these cellular microRNAs are modified by viral proteins, one can expect the regulation to be highly complex (Gunasekharan and Laimins, 2013).

2. U2OS cell line

U2OS cells are derived from moderately differentiated osteosarcoma, they have an adherent epithelial morphology and express the genes for pRb and p53 (Pontén and Saksela, 1967), (Niforou *et al.*, 2008).

Although HPV genomes were already isolated for a quite long period of time, the investigation of HPV was hindered by a missing adequate model system, due to HPV's complex life cycle. HPV highly depends on the differentiation of basal epithelial cells to keratinocytes and uses mainly the host's replication machinery, as itself expresses only two replication factors (see chapter 1.3 Life cycle).

For studies on basic mechanisms of animal papillomaviruses the mouse fibroblast cell line C127 was used, as it is able to maintain PV genomes as free plasmids at a constant number. This method, however, is not suitable for researching differentiation specific functions of HPV (Law *et al.*, 1981). This is possible in human cell lines only, but most established and widely used human cell lines do not support HPV genome replication. As one possible reason for this, a lack of regulated expression of viral proteins from the HPV genome, was stated (Geimanen *et al.*, 2011).

After analysing different human cell lines for their ability to support E1 and E2 dependent replication and to maintain HPV genomes as extrachromosomal plasmids, it got clear that the U2OS cell line is especially suitable for this. This is true, as it also allows the investigation of events leading to amplification of the viral genome, which occurs in the later stages of the life cycle of HPV (Geimanen *et al.*, 2011).

Another way to study the life cycle of human papilloma viruses it through infecting primary keratinocytes. However, these cells have to be transformed by the virus, thus only oncogenic HPV types could be studied. As U2OS cells do not have to be transformed by HPV, they allow to study the life cycle of high-risk, low-risk and cutaneous HPV types (Geimanen *et al.*, 2011).

3. Protein kinase CK2

The protein kinase CK2 (casein kinase 2) is a serine/threonine kinase, which is constitutively expressed in mammalian cells and phosphorylates a number of substrates. Through this, CK2 takes part in many cellular processes, such as cell cycle progression, apoptosis, transcription and viral infection (St-Denis and Litchfield, 2009).

This being said, it becomes clear, that the name ‘casein kinase’ is misleading, as this enzyme is a protein kinase, which is not linked to casein in the kinome. This situation facilitates the confusion with the genuine casein kinase G-CK, which really is responsible for the phosphorylation of casein (Tagliabracci *et al.*, 2012).

When extracted from living tissues, CK2 is a heterotetrameric holoenzyme (Figure 6), consisting of two catalytic (α) and two regulatory (β) subunits (Hathaway and Traugh, 1979). The catalytic subunit exists in two isoforms: α (CK2α) and α’ (CK2α’), with the latter isoform being less common. Although highly homologous, two separate genes encode the two forms (Ackermann *et al.*, 2005) and usually both genes are active, with their relative expression depending on the tissue type (Xu *et al.*, 1999).

As CK2 is a constitutively active enzyme, the regulatory β (CK2β) subunit rather plays a role in determining substrate specificity, with different substrates having a different effect on the enzyme’s phosphorylation efficiency (Pinna, 2002), but due to a lack of homology between the CK2β and other known proteins (Jakobi *et al.*, 1989), it is rather difficult to ascertain it’s role.

After individual CK2 subunits were analyzed in living cells, dynamic events were documented, which means that different multi-molecular assemblies of this enzyme are possible. The

majority of the subunits is not bound to a holoenzyme but moving freely and also entering the nucleus individually, not in the form of a pre-assembled holoenzyme (Filhol *et al.*, 2003), (Filhol *et al.*, 2005).

The minimal consensus sequence of CK2 is S/T-X-X-Acidic, so that it phosphorylates serine and threonine residues proximal to acidic amino acids (Pinna, 1990), wherefore it can use both, ATP and GTP as a phosphate donor (Allende and Allende, 1995).

One important function of CK2 is that it works as a global anapoptotic agent, which means that it is strongly involved in the regulation of the cell death ratio (Ruzzene and Pinna, 2010). From this is follows that CK2 is more abundant in cancers, as an unbalanced cell death and survival ratio is characteristic for cancerous cells (Guerra and Issinger, 1999). This fact highlights the importance of CK2 in anti-cancer drug research and after developing several CK2 inhibitors, one of them, CX-4945 (systematic name: 5-[(3-Chlorophenyl)amino]benzo[c][2,6] naphthyridine-8-carboxylic acid; also known as Silmitasertib), reached the clinical trial phase (Pierre *et al.*, 2011).

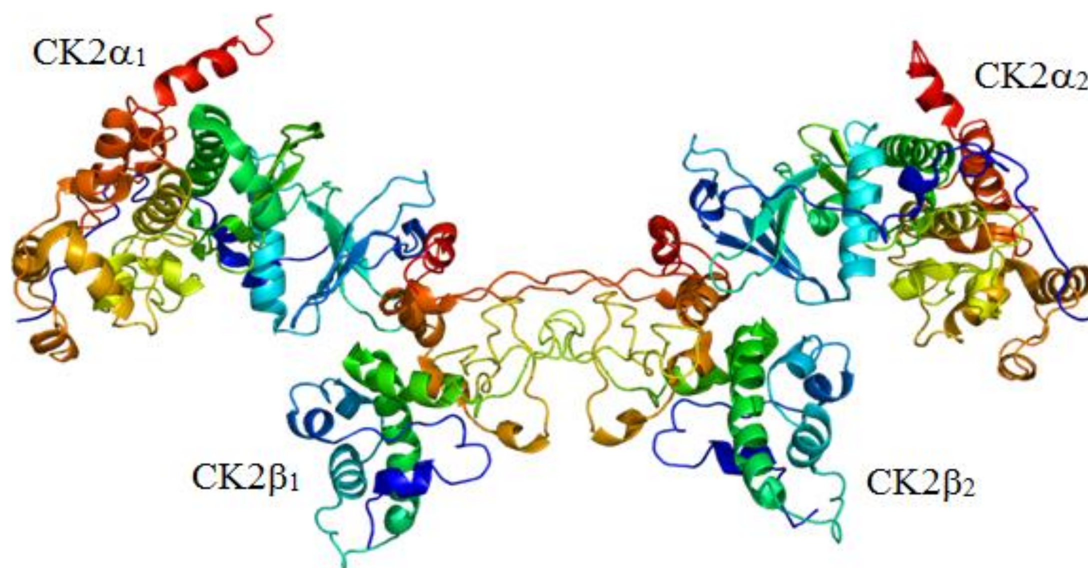


Figure 6: 3D Structure of the protein kinase CK2

The 3D structure of the protein kinase CK 2 holoenzyme as provided from the RCSB Protein Data Bank.

The holoenzyme consists of 4 subunits, with two being regulatory (β) and two being catalytic (α). 2 isoforms of the catalytic subunit has been documented (α and α'), which are highly homologous, yet still encoded in 2 separate genes.

(PDB code 4MD8)

3.1. CK2 and HPV

It was stated that several viruses, amongst other also HPV, use phosphorylation of different proteins by CK2 in different stages of their life cycle (Firzlaff *et al.*, 1989). This phosphorylation can have various affects, with one of them being regulation of viral enzyme activity. The inhibiting activity of HPV's oncogenic protein E7 on the DNA binding activity of TATA-Box binding proteins (TBP) is, for example, increased after phosphorylation by CK2. This results in a drop of TBP DNA binding activity and explains the inhibitory effects of E7 on transcription (Maldonado *et al.*, 2002).
Furthermore, it is shown that the E1 and E2 proteins are regulated by a phosphorylation of the protein kinase CK2. While these findings were mainly done in bovine papilloma viruses (BPV), they seem to be valid for several HPV types as well, as multiple possible phosphorylation sites are also found in the amino acid sequences of the E1 and E2 proteins of several HPV types (Schuck *et al.*, 2013). This is of great significance, as it implies that phosphorylation by CK2 could have an effect on the life cycle of HPV. Different phosphorylation patterns of the replication proteins E1 and E2 might influence the activity of the protein through the rate of viral genome replication. If this consideration would be proven to be correct then, through a change in CK2 activity, it would be possible to lower the HPV replication rate and maybe even to hinder it totally.

4. Enzyme inhibitors

Enzyme inhibitors are molecules that bind to enzymes and by this decrease the enzyme's activity. There are different types of inhibition, which can be reversible or irreversible. If the inhibitor binds non-covalently to the enzyme, then the inhibition is reversible. The inhibition is irreversible, if it reacts with and alters the enzyme. The mechanisms of inhibition are manifold, with typically the inhibitor stopping the substrate from entering the reaction site or hindering the enzyme to catalyse the reaction (Berg *et al.*, 2002).

4.1. CX-4945 (Silmitasertib)

A downregulation of the protein kinase CK2, which is overexpressed in many cancers, leads to a reduction of cancer cell viability and an increase in the apoptosis rate (Faust *et al.*, 2000), (Wang *et al.*, 2001). Thus, CK2 inhibitors could be used as anti-cancer drugs. The first CK2 inhibitor was developed by Cylene Pharmaceuticals Inc. and is known under the abbreviation CX-4945 (Figure 7), or under its trivial name Silmitasertib (systematic name: 5-(3-chloroanilino)benzo[c][2,6]naphthyridine-8-carboxylic acid). Today an orally administered drug based on this compound is in the second phase clinical trials for cancer treatment (Siddiqui-Jain *et al.*, 2010), (Pierre *et al.*, 2011).

Silmitasertib is thought to establish polar bonds with hydrophobic residues in the small and flat binding site of the active configuration of the α as well as the α’ subunit of the protein kinase CK2. If measured, Silmitasertib and CK2α show a strong interaction, with the dissociation constant of the inhibitor being K_i=0.38. This strong binding results in a reduction of enzyme activity and through that in altered phosphorylation patterns downstream of CK2 (Sarno *et al.*, 2005), (Ferguson *et al.*, 2011).

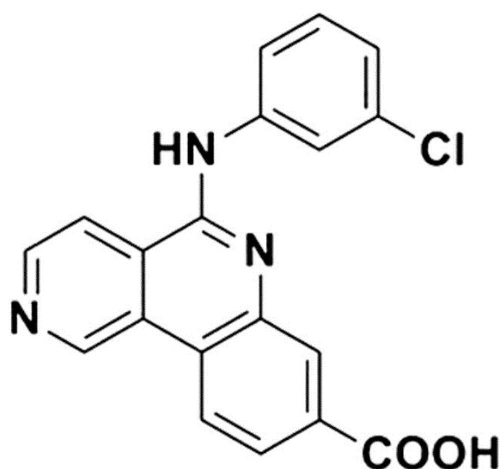


Figure 7: structural formula of CX-4945 (Silmitasertib)

This ATP-competitive inhibitor of the α and of the α’ subunit of the protein kinase CK2 has as an anti-cancer drug already reached the second clinical trial phase. Due to its strong binding with the binding site of CK2α and CK2α’ it leads to a drop in CK2 enzyme activity and through this to altered phosphorylation patterns.

(Siddiqui-Jain *et al.*, 2012)

Experimental section

1. Objectives

The principal objective of this thesis is to understand the role of the protein kinase CK2 in the life cycle of human papillomaviruses:

- First it is shown that the protein kinase CK2 has a general effect on the replication of HPV genomes, by using U2OS cell lines defective for the regulatory (α) subunit of CK2.
- After the confirmation, that CK2 indeed has an impact on HPV genome replication, the question arose, if E2, the transcriptional regulator of HPV, is still able to downregulate HPV gene expression if the regulative (α) subunit of CK2 is defective.
- Due to the effect of a defective regulatory (α) subunit of CK2 on the HPV replication, a CK2 inhibitor (CX-4945) was tested, as it is a possible drug candidate against HPV. Its IC_{50} was determined.
- As a drop in CK2 activity resulted in a drop in HPV genome replication, the reverse experiment was to be conducted, in order to show the general significance of the results. This was planned by doing an overexpression of the regulatory (α) subunit of CK2, as well as by a rescue experiment with expressing the regulatory (α) subunit of CK2 from plasmids in the defective U2OS lines.

2. Material and methods

2.1. Cell culture

Former works of the Ustav group showed that U2OS cells are a good system to study different stages of an HPV infection (Geimanen *et al.*, 2011). Thus are all cell lines used for this thesis based on the U2OS cell line which is derived from the osteosarcoma of a young girl. U2OS cells were obtained from the American Type Culture Collection (ATCC no: HTB-96). Apart from the U2OS wild type, three other cell lines were used, two of them, named U2OS alpha and U2OS prime, were developed in the laboratory of Professor Mauro Salvi from the University of Padova. These lines are defective for the α subunit of the protein kinase CK2 by CRISPR/Cas. The α subunit exists, as mentioned above, in two highly homologous forms (α and α´) and the two cell lines, respectively, are not able to produce functional proteins of either the α (U2OS alpha) or the α´ (U2OS prime) subunit. The forth cell line that is used is the U2OS EBNA cell line, which contains stably the gene for the EBNA1 protein. This protein of the Epstein-Barr virus (EBV) enables to replicate a plasmid (oriP), which contains an EBV latent origin of replication. This replication imitates the replication of the genome of the cell and can be used to compare the replication of the genome of the cell with the replication of extrachromosomal HPV genomes.
Different U2OS cell lines were cultured either in IMDM (Iscove's modified Dulbecco's medium; Naxo OÜ; U2OS wild type and EBNA) or DMEM (Dulbecco/Vogt modified Eagle's minimal essential medium; Naxo OÜ; U2OS α and α´) Both media were supplemented with FCS (fetal calf serum; final amount 10% of medium), penicillin (100 IU/ml) and streptomycin (100 ng/ml). The cells, maintained in sub-confluent conditions, were grown at 37°C and 5% CO_2, on 100 mm, 60 mm and 6-well (30 mm) dishes, filled with 8 ml, 3 ml or 2 ml of medium.

2.2. Transformation using heat shock

After thawing competent *E. coli* DH5α cells on ice, 0.5 µg of plasmid DNA were added and the mixture kept on ice for another 15 minutes. The heat-shock was executed by incubating the cells at 37°C for 3 minutes and then on ice for 1 minute. 750 µl of LB medium were added and the cells incubated at 37°C for 30 minutes, before plating them on LB (lysogenic broth) plates containing the corresponding antibiotic for selection and then grown overnight at 37°C.

The following day, *E. coli* colonies were picked and grown overnight (37°C and 220 rpm) in 3 ml of liquid LB medium with a 50 µg/ml concentration of the corresponding antibiotic (ampicillin 100, kanamycin 25).
The next day the samples were centrifuged at 5000 g for 5 minutes and the plasmids extracted with the "FavorPrep^{TM} Plasmid Extraction Kit" (Favorgen Biotech Corp.). To check if the extracted plasmids were correct, the plasmids were cut with a linearizing enzyme (depending on the plasmid) and after that a gel electrophoresis (0.8% TAE gels) was run.

2.3. HPV mini-circle preparation

This procedure was proposed by Kay *et al.* (2010).
In order to obtain functional HPV genomes (HPV mini-circles) for transfection, first plasmids had to be duplicated in an *E. coli* cell line (DH5α). Transformation was performed as described above. The following day the colonies were picked but then grown in 3 ml of liquid LB (as described above) for only about 7 hours. 200 µl of this pre-grown culture were transferred to 100 ml of TB (terrific broth) medium containing 50 µg/ml kanamycin and The culture was grown for 12 – 16 hours at 37°C and 220 rpm.
The next morning the induction mixture was added, which triggers the outrecombination between the attB and attP sites so that the plasmid backbone is removed, which results in a mini-circle, resembling the functional viral genome of HPV. This mixture contained 100 ml LB medium, 4 ml 1M NaOH and 200 µl filtered 20% L-arabinose. The samples were grown for 8 hours at 32°C and 220 rpm.
After this period, the cultures were centrifuged for 15 minutes at 4°C and 5000 g and the mini-circles extracted using the Nucleobond® Xtra Maxi EF kit (Macherey-Nagel).

2.4. Transfection by electroporation

Per transfection one 100 mm 70-90% confluent plate of U2OS cells was used. The cells were washed with 2 ml 1xPBS, then detached through dissolving their extracellular matrix with 2 ml trypsin and gathered into a 50 ml tube containing as much medium as trypsin was used to detach the cells. After centrifuging for 5 minutes at 20°C and 300 g, the medium was removed and the

cell pellet resuspended in as much medium as it was necessary to execute all the transfections (250 µl per transfection).
The transfection mixture consisted of the plasmid or mini-circle, which was to be transfected, and of 50 µg carrier DNA. This mixture was added to the cuvette, as well as 250 µl of the cells. A voltage of 220 V and a capacity of 975 µF were applied (Gene Pulse II, Bio-Rad). Right after electroporation, the cells were transferred into 2 ml medium, suspended, centrifuged (5 minutes, 20°C, 300 g) and the pellet was resuspended in 1 ml medium and transferred to a cell culture dish of suitable size.

2.5. Total DNA extraction

The medium was removed and the cells were washed twice with 1xPBS. The cells were lysed with 0.5 ml Sol IV (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 10 mM EDTA, 0.2% SDS; supplemented with proteinase K (0.2 µg/ml)) solution and incubated for at least 18 hours at 37°C.
An equal amount of phenol-chloroform (1:1) was added and the samples were vortexed twice, before centrifuging them for 2 minutes at 16 000 g. The upper phase was transferred into a new tube and the DNA was precipitated with 2 volumes of 96% EtOH. Another incubation step at -20°C for 15 – 30 minutes was conducted.
The samples were centrifuged at 4°C for 20 minutes at 16 000 g, afterwards the supernatant was removed and the DNA pellet dried. The pellet was resuspended in 300 µl TE (10mM Tris ph8.0, 1 mM EDTA) buffer, containing 20µg/ml RNase, vortexed and incubated at 37°C for 45 – 60 minutes.
Then the DNA was precipitated by adding NaCl up to a concentration of 200 mM, by adding two volumes of 96% EtOH and by incubating at -20°C for 30 minutes. After incubation the samples were centrifuged at 4°C for 15 minutes at 16 000 g, the supernatant was removed and the pellet washed with 0.5 ml 70% EtOH. Another centrifugation for 3 minutes at 16 000 g was executed.
The DNA was reconstituted in TE (10mM Tris pH 8.0, 1 mM EDTA) buffer.

2.6. Episomal DNA extraction – the HIRT method

The medium was removed and the cells were washed twice with 1xPBS. Then the cells were lysed by adding 800 µl HIRT solution (0.5% SDS, 50 mM Tris pH 8.0, 10 mM EDTA) and incubating them for 15 minutes at room temperature. Then 200 µl 5M NaCl were added dropwise and the dishes incubated at 4°C overnight.

After collecting the lysate into 1.5 ml tubes, the lysate was centrifuged at 4°C for 25 minutes at 16 000 g. The supernatant was transferred into new tubes, 600 µl isopropanol were added and the probes were incubated at -20°C for 30 minutes. After incubation, the samples were centrifuged once more at 4°C for 10 minutes at 16 000 g and the supernatant removed. The pellet was suspended in 200 µl Sol IV (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 10 mM EDTA, 0.2% SDS), containing 50 µg/ml proteinase K.

After incubating at 56°C for 30 minutes an equal amount of phenol-chloroform (1:1) was added, the samples were vortexed and centrifuged for 2 minutes at 16 000 g. The upper phase was transferred into a new tube and the DNA precipitated by adding twice as much 96% EtOH and incubating at -20°C for 30 minutes.

The samples were centrifuged at 4°C for 10 minutes at 16 000 g, the supernatant was removed and the pellet washed by adding 700 µl 70% EtOH and centrifuging at 4°C for 3 minutes at 16 000 g. After that the EtOH was removed and the pellet dried for 2 – 5 minutes at room temperature and then suspended in TE buffer, containing 20 µg/ml RNase A.

The samples were incubated at 37°C for 60 minutes.

2.7. Southern blot

If the genetic material for the Southern blot was a total DNA extraction, the DNA amount was normalized by the total DNA content.

In order to eliminate the input DNA, the samples were incubated for at least 12 hours with DpnI and with linearizing enzymes (HPV 11: HindIII, HPV 16: ScaI, HPV 18: BglI). Then the DNA was separated by gel electrophoresis on a 1% TAE gel in 1xTAE buffer (4-5 h, 70 V or 16 h, 30 V). The gel was incubated for 30 minutes in Sol A (0.5 M NaOH, 1.5 M NaCl) solution on a shaker, in order to denaturize the DNA, and afterwards neutralized for 30 minutes in Sol B (1 M Tris pG 7.4, 1.5 M NaCl) solution. The transfer of the DNA onto a Hybond-N$^+$ filter (Amersham Pharmacia Biotech) was executed for 6–18 hours, using the capillary transfer

method in 10xSSC buffer (1.5 M NaCl, 150 mM $Na_3C_6H_5O_7$). The DNA was cross-linked to the filter in an UV Stratalinker 1800 (Stratagene). To avoid unspecific binding, the filter was pre-hybridized for at least 45 minutes at 65°C with a pre-hybridization solution (30% 20xSSC, 10% 50xDen, 5% 10%SDS, 200 µg/ml denatured carrier DNA).
As the hybridisation probe a linearized HPV genome was used, which was radiolabelled using the DecaLabel™ DNA Labeling Kit (Fermentas) and α32P-dCTP ("Du Pont NEN"). The filter was rinsed in the hybridisation solution for at least 18 hours at 65°C. Afterwards several washing steps were conducted. First, was the filter washed twice for 5 minutes in washing buffer 1 (2xSCC, 0.1% SDS), followed by 15 minutes in washing buffer 2 (1xSSC, 0.1% SDS) and finally twice for 10 minutes in washing buffer 3 (0.1xSSC, 0.1% SDS).
The filters were kept for about 2 hours in a x-ray cassette (Kisker Biotech) at -80°C and then the outcome revealed in a phosphoimager (Typhoon Trio, GE Healthcare) and the resulting pictures quantified (Image Quant).

2.8. Western blot

After removing the medium and washing the dishes twice with 1xPBS, lysing solution (2xLaemmli (62.5 mM Tris, pH 6.8; 2% SDS; 10% glycerol; 5% 2-mercaptoethanol; 0,001 % bromphenolblue), 200mM DDT) was added (300 µl to 60 mm petri dishes; 150 µl to 30 mm dishes). The lysate was collected, transferred into 1.5 ml tubes and denatured at 100°C for 10 minutes.
After incubation, the tubes were vortexed thoroughly and hold at -20°C for a short time, to let them cool down. The samples were run on a 10% acrylamide gel in 1xSDS buffer at 80 V and, after the samples passed through the stacking gel (10 – 15 minutes), at 120 V for about an hour (resolving gel). A Prestained Protein MW Marker (Thermo Fisher Scientific) was loaded as well.
The transfer of the proteins onto the membrane (Immobilon®-P polyvinylidene difluoride membrane (Millipore Coorp.)) took place in a blotter (Trans-blot® SD Semi-Dry Transfer Cell; Bio-Rad), where 15 V were applied for 20 minutes. Before the transfer, the gel was incubated in Semi-Dry Met (60 mM Tris, 40 mM CAPS, pH 9.6, 15% methanol) for 20 minutes while the membrane was activated with methanol and then incubated in Semi Dry Met for 10 minutes.
Now the filter was washed for 5 minutes in a Western blot washing solution (0.05M Tris pH 7.5, 0.15M NaCl, 0.1% Tween20), before being incubated in a 5% skimmed milk blocking

solution (5% skimmed milk powder, PBS/0.1% Tween20) for one hour. Two 5-minute washing steps were conducted, before adding a 2% skimmed milk solution containing the antibody. In this solution, the filter was incubated for one hour and afterwards washed three times for 5 minutes in a Western blot washing solution. If necessary, a 2% skimmed milk solution containing the secondary antibody was prepared, the filter incubated in it for an additional hour and then, again, washed 3 times.
The signal was made visible with an ECL (enhanced chemiluminescence) cassette on radiographic films (Fuji), by mixing the ECL reagents 1:1 (luminol/enhancer solution, peroxide solution; Naxo OÜ) and immediately drizzling the solution all over the membrane. The radiographic films were developed after 1, 3 and 10 minutes of exposition.

2.9. Dual-Luciferase® Reporter (DLR™) Assay (Promega)

First, the medium was removed and the cells washed twice with 1xPBS. Now 250 µl of five times diluted Passive Lysis Buffer were added into each of the 30 mm petri dishes. After incubating for 15 minutes at room temperature and tilting the dishes from time to time, the probes were ready to be measured. The system of this assay uses two different luciferases (firefly (*Photinus pyralis*) and Renilla (*Renilla reniformis*)) in order to measure the amount of the protein of interest and normalise the signal of the different samples.
The cells were transfected with URR-Luc and a non-specific pRL-Tk reporter plasmid, which expresses Renilla luciferase, for normalisation. As according to the manufacturer’s protocol, first 50 µl of Assay Reagent II (LAR II) was added to 20 µl of the lysate and then the firefly luciferase reporter´s luminescence measured with a Glomax 20/20 luminometer (Promega). This value correlates directly with the amount of the protein of interest in the lysate. Immediately after this first measurement, this reaction was stopped and at the same time, the Renilla luciferase reaction was started, by adding 50 µl of the Stop & Glo® Reagent (prepared from Stop & Glo® Buffer and 50x Stop & Glo® Substrate (Renilla luciferase)) to the tube. A second measurement was conducted. This second luminescence signal was then used to normalise the results of the first luminescence measurement.

3. Results and discussion

3.1. Effect of the protein kinase CK2 on the replication of HPV

Schuck *et al.* showed 2013, that the life cycle of BPV is partly regulated through a phosphorylation of the viral proteins E1 and E2 by the protein kinase CK2. As several HPV proteins also show possible phosphorylation sites for CK2 (Firzlaff *et al.*, 1989), the theory arose that CK2 could also play a role in the life cycle of HPV.

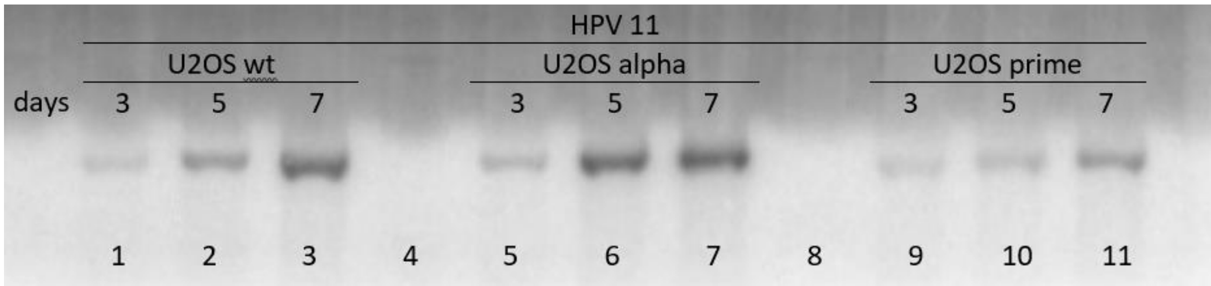
In order to examine this, wild type HPV genomes (mini-circles) of two high-risk (16 and 18) and one low-risk (11) type were transfected into U2OS cell lines (HPV 11: 2µg; HPV 16: 5µg; HPV 18: 3µg). The cell lines used were wild type U2OS, as a control, where the expression of CK2 is not impaired, as well as two mutant U2OS cell lines, named ‘U2OS alpha’ and ‘U2OS prime’ (developed in the laboratory of Professor Mauro Salvi from the University of Padova). In these, the gene for the catalytic α (CK2α) or respectively for the catalytic α’ (CK2α’) subunit of the protein kinase CK2 is mutated.

The cells were grown in IMDM (wild type) or DMEM (U2OS alpha and prime) medium and the total DNA was extracted 3, 5 and 7 days after transfection. The extracted DNA was incubated for at least 12 hours with linearizing enzymes (HPV 11: HindIII, HPV 16: ScaI, HPV 18: BglI) and with DpnI, to eliminate the input DNA. After separation of the DNA fragments by length on a 1% TAE gel and transfer of the DNA to a filter, a linearized and radioactively labelled HPV probe was used to mark the viral DNA fragments. The filters were then revealed in a phosphoimager and the resulting pictures quantified All the experiments were conducted twice.

The results show that while for HPV 11 (Figure 8) and 18 (Figure 10) the amount of viral DNA is drastically higher in the U2OS wild type (blue) as well as in the U2OS alpha line (orange), compared to the U2OS prime line (grey). This effect is clearly visible in panel A and was also affirmed by quantification (Panel B).

For HPV 16 (Figure 9), on the other hand, no difference can be spotted in the increase of viral DNA between the three used cell lines.

A



B

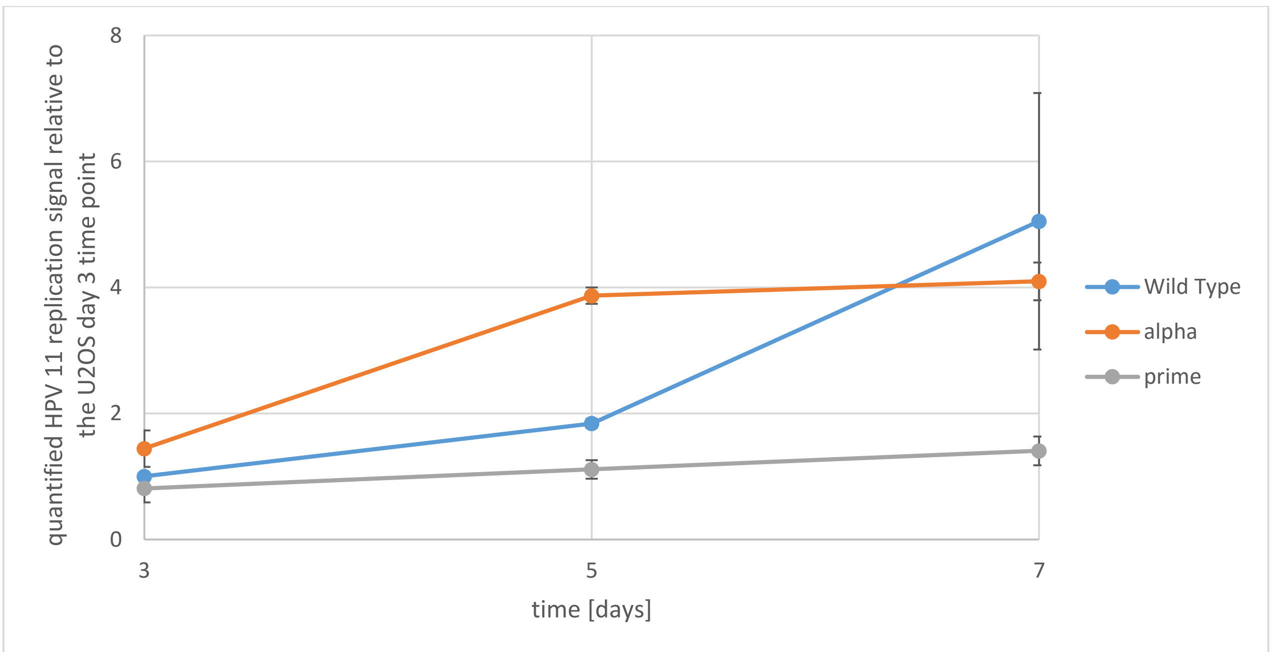


Figure 8: Replication signal of the HPV 11 in the U2OS wild type and the U2OS alpha and prime cell lines (A: Replication signal; Panel B: Quantified replication signal)

Panel A shows the replication signal of the viral HPV 11 DNA. Lines 1, 2 and 3 show the time points (3, 5, 7 days) for the wild type cell line, lines 5, 6 and 7 for the alpha line and 9, 10 and 11 for the prime cell line. It is apparent that the amount of viral DNA increases in the wild type as well as in the alpha cell line over the period of the experiment, while in the prime line this increase is significantly lower.

This results were confirmed via quantification, with the results being shown in Panel B. The increase in the amount of viral DNA for the wild type (blue) and the alpha line (orange) is significantly higher than the increase in the prime cell line (grey). The error bars show the standard deviation of the two independent experiments, which were conducted.

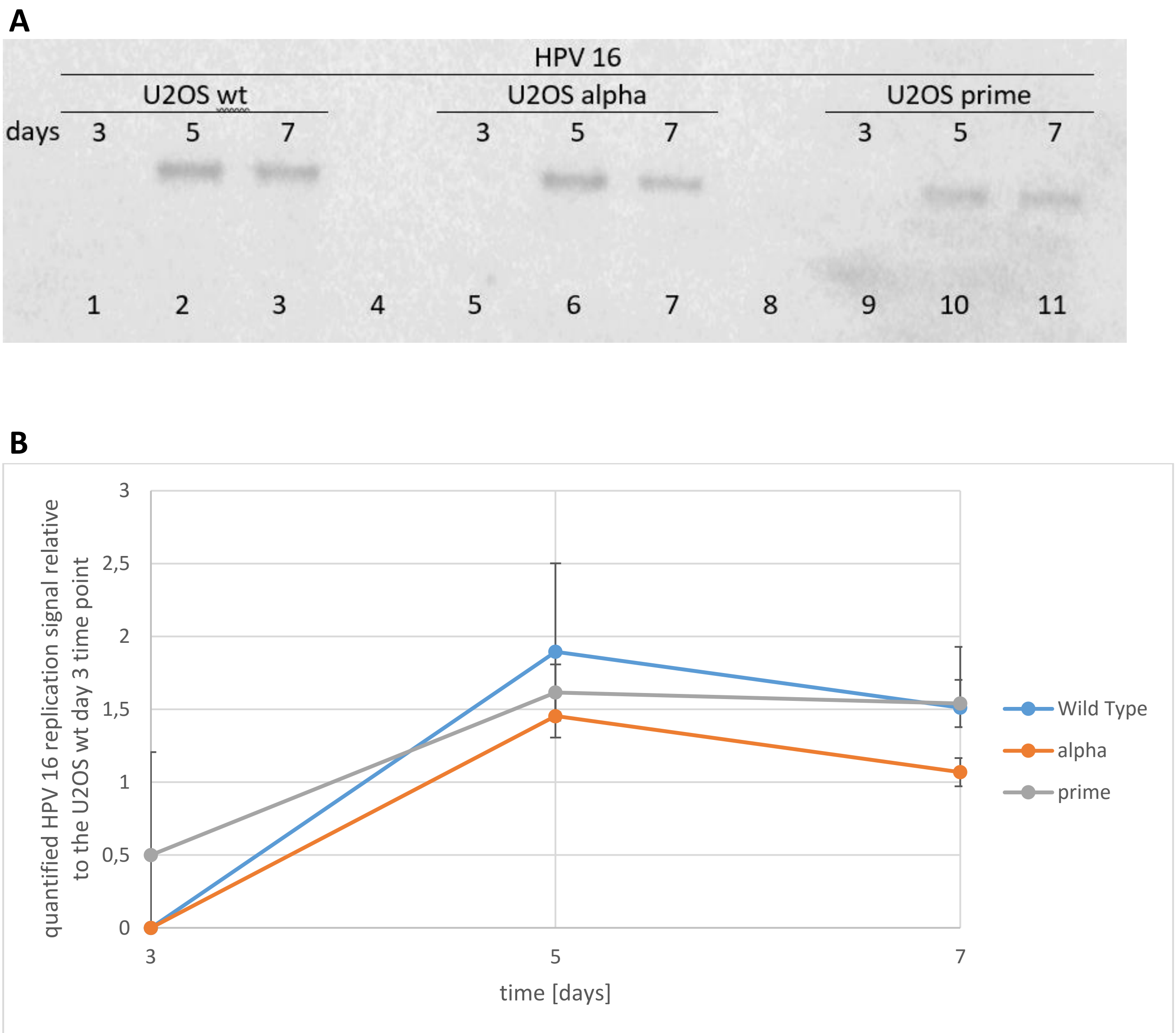


Figure 9: Replication signal of the HPV 16 genome in the U2OS wild type and the U2OS alpha and prime cell lines (A: Replication signal; Panel B: Quantified replication signal)

Panel A shows the signal of the viral HPV 16 DNA. Lines 1, 2 and 3 show the time points (3, 5, 7 days) for the wild type cell line, lines 5, 6 and 7 for the alpha line and 9, 10 and 11 for the prime cell line. The results show that there is no large difference in the increase of the amount of viral DNA between the three different cell lines.

This results were confirmed via quantification, with the results being shown in Panel B. The increase in the amount of viral DNA for the wild type (blue), the alpha (orange) and the prime (grey) cell line do not considerably differ from each other. The error bars show the standard deviation of the two independent experiments, which were conducted.

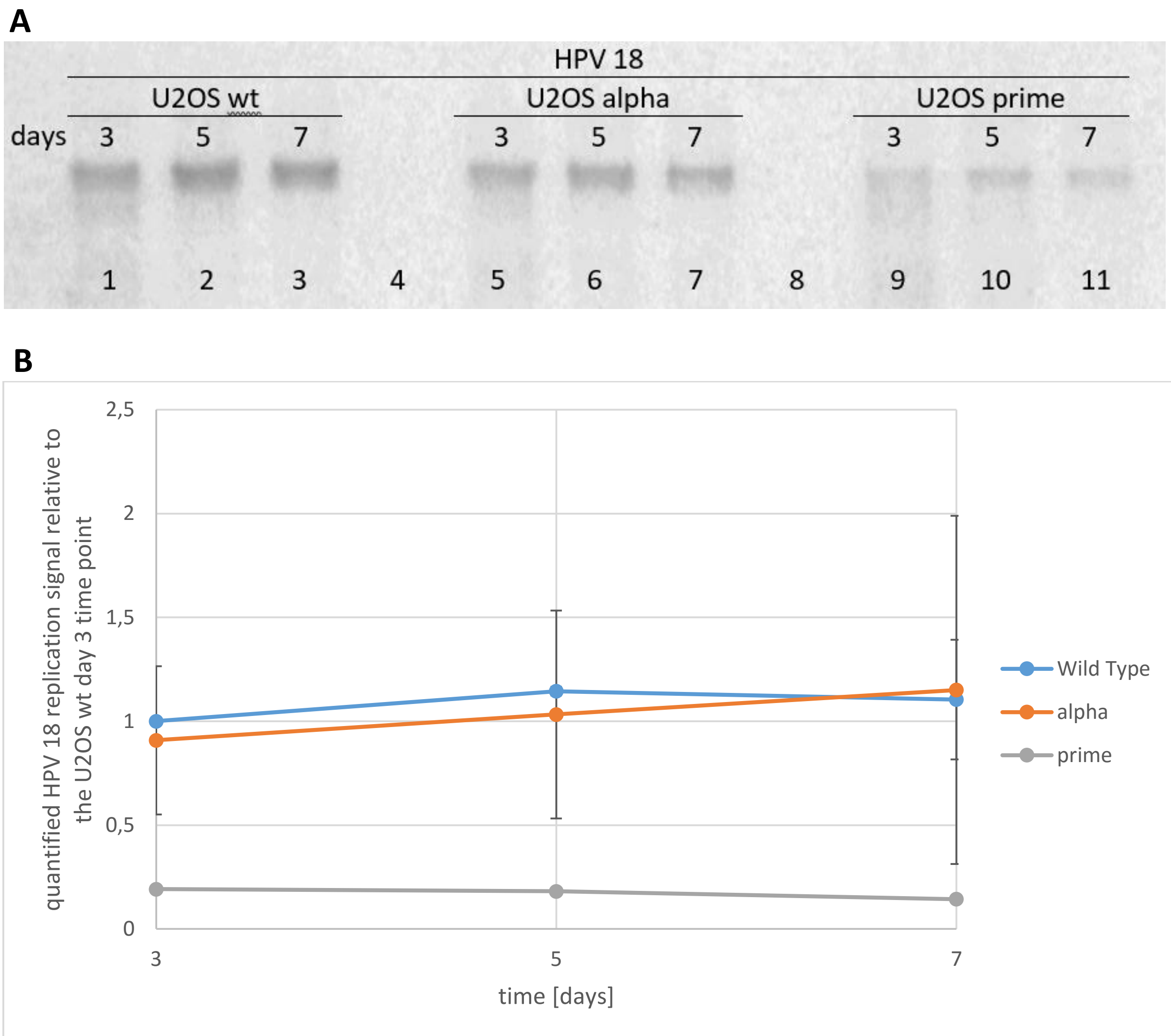


Figure 10: Replication signal of the HPV 18 genome in the U2OS wild type and the U2OS alpha and prime cell lines (A: Replication signal; Panel B: Quantified replication signal)

Panel A shows the signal of the viral HPV 18 DNA. Lines 1, 2 and 3 show the time points (3, 5, 7 days) for the wild type cell line, lines 5, 6 and 7 for the alpha line and 9, 10 and 11 for the prime cell line. It is apparent that the amount of viral DNA in the wild type as well as in the alpha cell line is over the period of the experiment on a significantly higher level, than in the prime cell line.

These results were confirmed via quantification, with the results being shown in Panel B. The level of the amount of viral DNA in the wild type (blue) and the alpha line (orange) is significantly higher than the level in the prime cell line (grey). The error bars show the standard deviation of the two independent experiments, which were conducted.

These results imply that, as only in the U2OS prime cell line an effect on viral genome replication during the initial amplification is visible, the α' subunit of the protein kinase CK2 (CK2α') plays an important role in the genomic replication of HPV 11 and 18. If this subunit of CK2 is mutated and not fully functional, a far less effective genomic replication is to be expected.
Furthermore, it can be stated, that contrarily to HPV 11 and 18, in HPV 16 the regulatory subunit of the protein kinase CK2 does not have an integral role in the genomic replication. A mutation of this subunit does not impair viral gene replication whatsoever. A possible reason for this is that in HPV 16 another system is used to regulate the viral genome replication and a phosphorylation by CK2 is not necessary for a successful replication.
Another possible explanation is that due to the, in comparison with the other two HPV types, considerably lower level of HPV 16 replication in U2OS cells, it was not possible to show the influence of the α' subunit of the protein kinase CK2 (CK2α') on the initial amplification of HPV 16.
The error bars for the HPV 18 type in the U2OS alpha line are exceptionally big, so that an influence of the α subunit of the protein kinase CK2 (CK2α) on the initial amplification of HPV 18 can only be reliably ruled out, after conducting more identical experiments.

3.2. Early protein E2 transcription regulation in CK2α deficient cell lines

Kurg *et al.* showed in 2010, that E2 is a negative regulator of HPV 18 gene expression and strongly reduces the gene expression controlled by the viral full length URR. This fact together with the results of the previous experiment (i.e. CK2 is needed for successful viral genome replication) might indicate that a phosphorylation of E2 by CK2 could be necessary for E2 to work as a regulator of HPV genome expression.

That is why, a two-luciferase reporter assay was used to investigate if E2 is able to regulate transcription in the two CK2α deficient cell lines as well. Three plasmids were transfected together with 50 µg of carrier DNA into wild type U2OS, as well as into alpha and prime lines. The first plasmid (pRL-Tk; 25 ng) encodes for a Renilla-luciferase product and is used for normalization, while the second plasmid (pQMN-H18-E2) was transfected in different concentrations (0 ng, 10 ng, 50 ng, 100 ng, 500 ng) and encodes for E2, which is the transcriptional regulator of HPV 18. The last one (URR-Fluc; 100 ng) encodes a firefly-

luciferase product, controlled by the full-length HPV18 URR region. The cells were grown for 24 hours and then lysed, the substrates of the luciferases added and the luminescence measured in a luminometer. The experiment was conducted twice.

From figure 11 it is easily spotted, that while in the wild type (blue) the luminescence falls with a growing amount of transfected E2 plasmid, in both mutated U2OS lines (U2OS alpha: orange and U2OS prime: grey), the luminescence stays at a high level. This means that when CK2 is not functional, the ability of the E2 protein of HPV 18 to regulate viral gene expression is hampered.

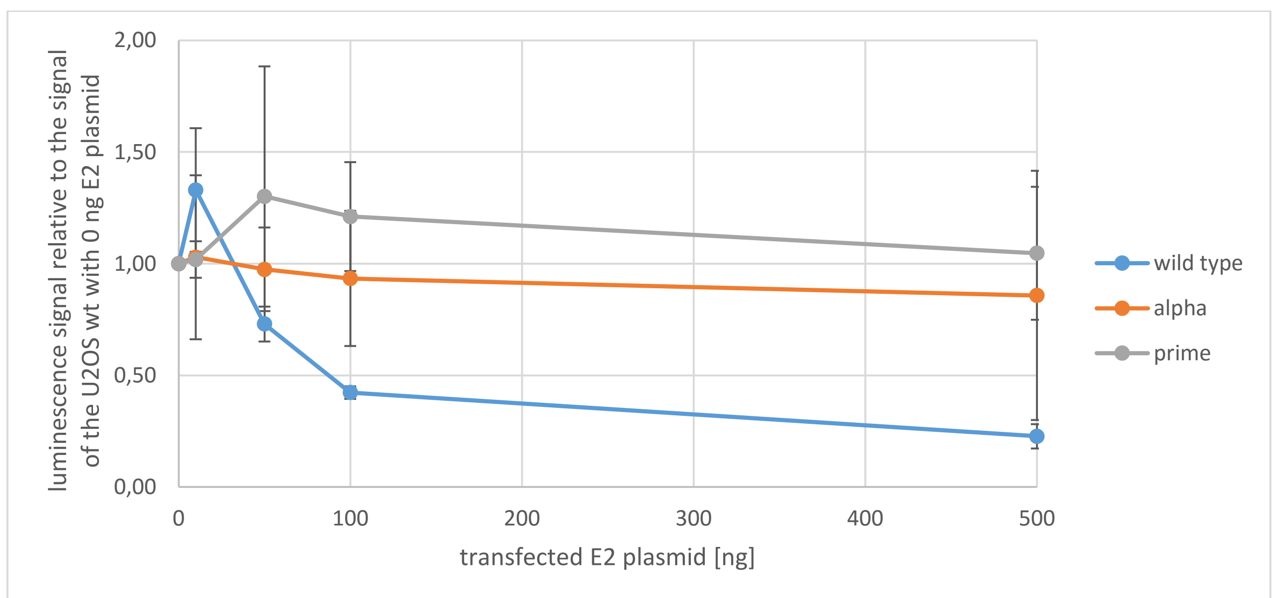


Figure 11: E2 transcription regulation in CK2α deficient cell lines

In graph for the U2OS wild type (blue graph) a strong inhibition of viral gene replication is visible with a growing amount of transfected E2 plasmid. This is not true for the other two cell lines (orange: U2OS alpha; grey: U2OS prime), where the level of replication only fluctuates slightly, no matter how much E2 plasmid was transfected. The error bars show the standard deviation of the two independent experiments, which were conducted.

The results of this experiment indicate, that, as expected, CK2 mediated phosphorylation of E2 is required for E2 to perform as a regulator of viral genome expression, as only with an intact version of the regulatory subunit of CK2 (U2OS wild type: blue) a decrease in viral replication was detected. No matter how much E2 plasmid was transfected, in those cell lines with a not

functioning version of the regulatory subunit of CK2 (U2OS alpha: orange; U2OS prime: grey) no inhibition of viral replication is seen.

Another conclusion to make is that for E2 to perform as a regulator of viral genome expression both subunits are relevant. It does not matter, which of the two isoforms of the regulatory subunit of CK2 is mutated, in both cell lines the same effect becomes visible and so it can be concluded that both are of same importance.

3.3. CX-4945 effect on HPV 18 replication

In order to validate the previous findings, that CK2 plays an important role in the initial amplification of HPV, an inhibitor for CK2 (CX-4945) was used in different concentrations (0 µM, 0.1 µM, 0.19 µM, 0.38 µM, 0.75 µM, 1.5 µM, 3 µM, 6 µM). The appropriate amount of inhibitor was added to the medium in the cell culture dishes after transfection.

2 µg of HPV 18 wild type genomes (mini-circles) were transfected into the U2OS EBNA cell line, together with 1 µg oriP and 50 µg of carrier DNA. U2OS EBNA cells stably express the EBNA1 protein of the Epstein-Barr virus which can be used to facilitate the replication of oriP, as this plasmid contains the EBV latent origin of replication. EBNA1-dependent replication is initiated once per cell cycle (Kirchmaier and Sugden, 1995) and thus its replication mechanism is different from the amplification of HPV replication and mimics the cellular genomic DNA replication. The EBNA1-dependent replication could be thus used as an internal control to determine the specificity of the compound.

The cells were grown for 5 days, with a medium change at the third day (the inhibitor was added anew) and then the total DNA extracted. In order to linearize the viral genome and oriP, the extracted DNA was incubated for at least 12 hours with ScaI and BstXI, as well as with DpnI, to eliminate the input DNA. After separation of the DNA fragments by length on a 1% TAE gel and transfer of the DNA to a filter, a radioactive HPV18-specific and a ori-P specific probes were used to mark the DNA fragments. The filters were then revealed in a phosphoimager and the resulting pictures quantified. The experiment was conducted twice.

The results of the southern blot (Figure 12A) show that the higher the concentration of the CK2 inhibitor CX-4945 gets, the more the replication rate of HPV 18 declines. Especially the three highest concentrations (1.5 µM, 3 µM, 6 µM) cause a noteworthy decline. The EBNA1-dependent replication rate, on the other hand, is not affected at all and continues at the same

pace in all the samples in spite of a growing CX-4946 concentration. This replication mimics, as described above, the genomic replication.

These findings are affirmed after quantification, shown in Figure 12B, where the trend line for HPV (blue) drops with growing inhibitor concentration, while the trend line for EBNA (orange) even rises.

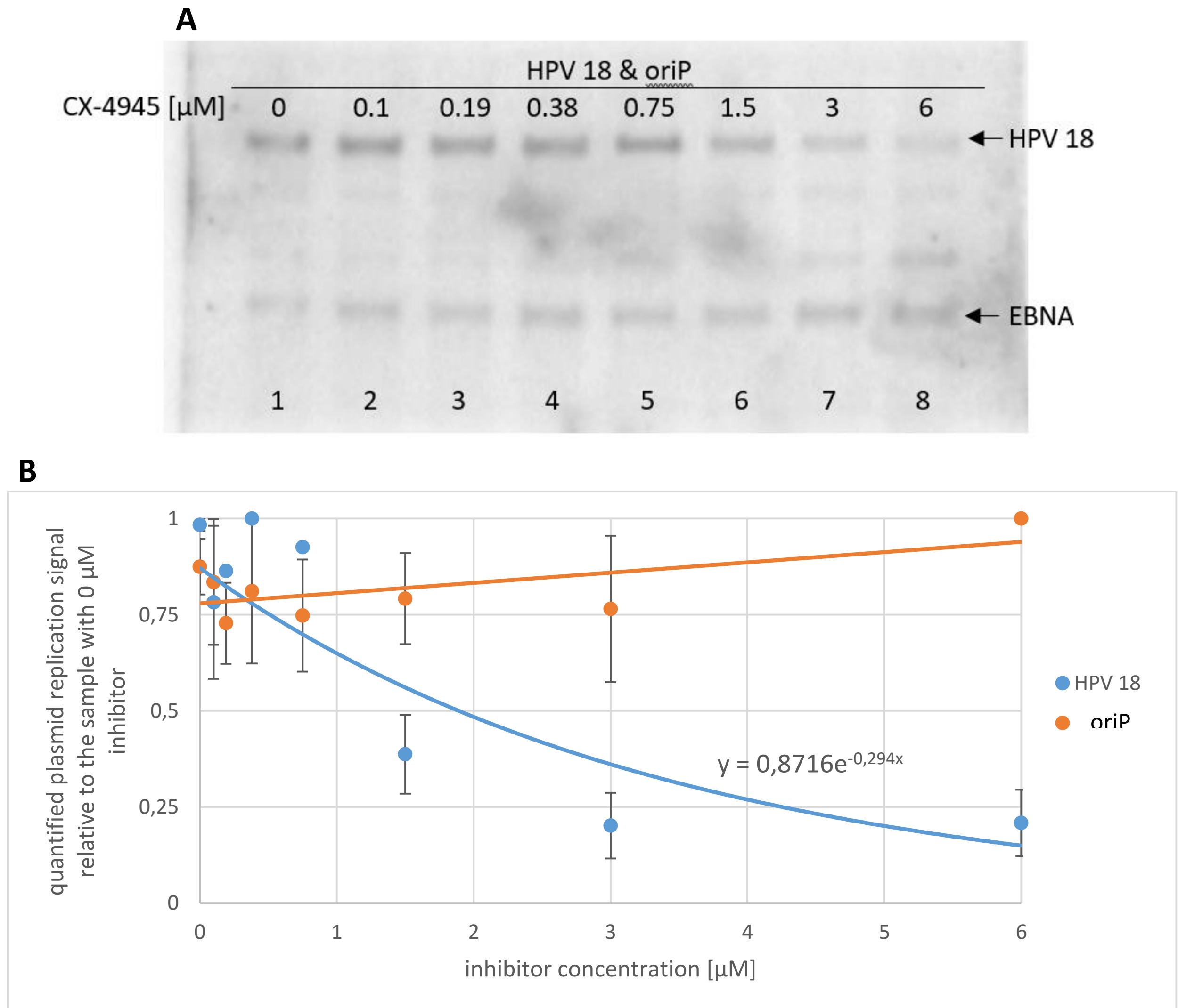


Figure 12: Effect of CX-4945 on HPV 18 replication (A: Replication signal; Panel B: Quantified replication signal)

Panel A shows the signal of the viral HPV 18 DNA, as well as the signal for oriP, which contains an EBV latent origin of replication and thus imitates the replication of the genomic DNA of the cell in the U2OS EBNA cell line. It is apparent that the signal for HPV 18 weakens with a higher CX-4945 concentration, while the signal for oriP (EBNA) remains unaltered.

These results were affirmed by quantification (Panel B), with the signal of the viral HPV DNA (blue graph) declining at higher inhibitor concentrations, while the signal for oriP (orange graph) shows no signs of decrement. The error bars show the standard deviation of the two independent experiments, which were conducted.

The formula for the HPV trend line ($y = 0.8716e^{-0.294x}$) was used to calculate the IC_{50}:

$$IC_{50}: 0.5 = 0.8716e^{-0.294x}$$

$$IC_{50}: 1.89\ \mu M$$

These results validate the outcome of the experiments illustrated above (3.1 Effect of the protein kinase CK2 on the replication of HPV) and strengthen the theory that the protein kinase CK2 is important in the amplification of the viral genome of HPV, as an inhibitor of this kinase is able to inhibit viral genome amplification as well.

These results are even more of interest, as up-to-date, there is no specific treatment of an HPV infection available. The protein kinase CK2 inhibitor CX-4945, however, is a potent candidate for an anti-HPV drug, as it inhibits selectively the viral genome replication and, as it was seen from the results, quite effectively. The host cell genomic replication, on the other hand, is not inhibited, as shown by the unaltered replication of oriP even at high inhibitor concentrations. This inhibitor is, as mentioned above, already in the second phase of clinical trials as an anti-cancer drug, which makes the prospects even more promising.

3.4. CX-4945 effect on E1 and E2 activity

To investigate if the protein kinase CK2 has an effect on the ability of the viral proteins E1 and E2 to initiate replication from the viral origin of replication, 250 ng of the p18URR plasmid were transfected into U2OS wild type cells, together with 25 ng of an E1 (p18 E1) and 25 ng of an E2 (p18 E2) coding plasmid. p18URR contains only the upstream regulatory region (URR) of HPV 18 and its replicational behaviour is thought to be similar to the behaviour of an HPV wild type genome, while p18E1 and p18E2 express E1 and E2 (Dale, 1954) under the control of a strong CMV promotor. This experimental set-up allows to examine the replication properties of E1 and E2 and to determine if the phosphorylation of these proteins by CK2 is necessary for the initiation of HPV replication.

Different concentrations (0 µM, 1.5 µM, 3 µM, 6 µM) of the CK2 inhibitor CX-4945 were added after transfection and episomal DNA was purified after 24 and 48 hours using the HIRT extraction method. In order to linearize HPV18URR plasmid, the extracted DNA was incubated for at least 12 hours with BglI as well as with DpnI, to eliminate the bacterially produced input

DNA. After separation of the DNA fragments by length on a 1% TAE gel and transfer of the DNA to a filter, a radioactive HPV18URR-specific probe was used to mark the DNA fragments. The filters were then revealed in a phosphoimager and the resulting pictures quantified. The experiment was conducted twice.

A 100 pg control and size-marker (linearized URR H18 plasmid) was loaded on the gel as well.

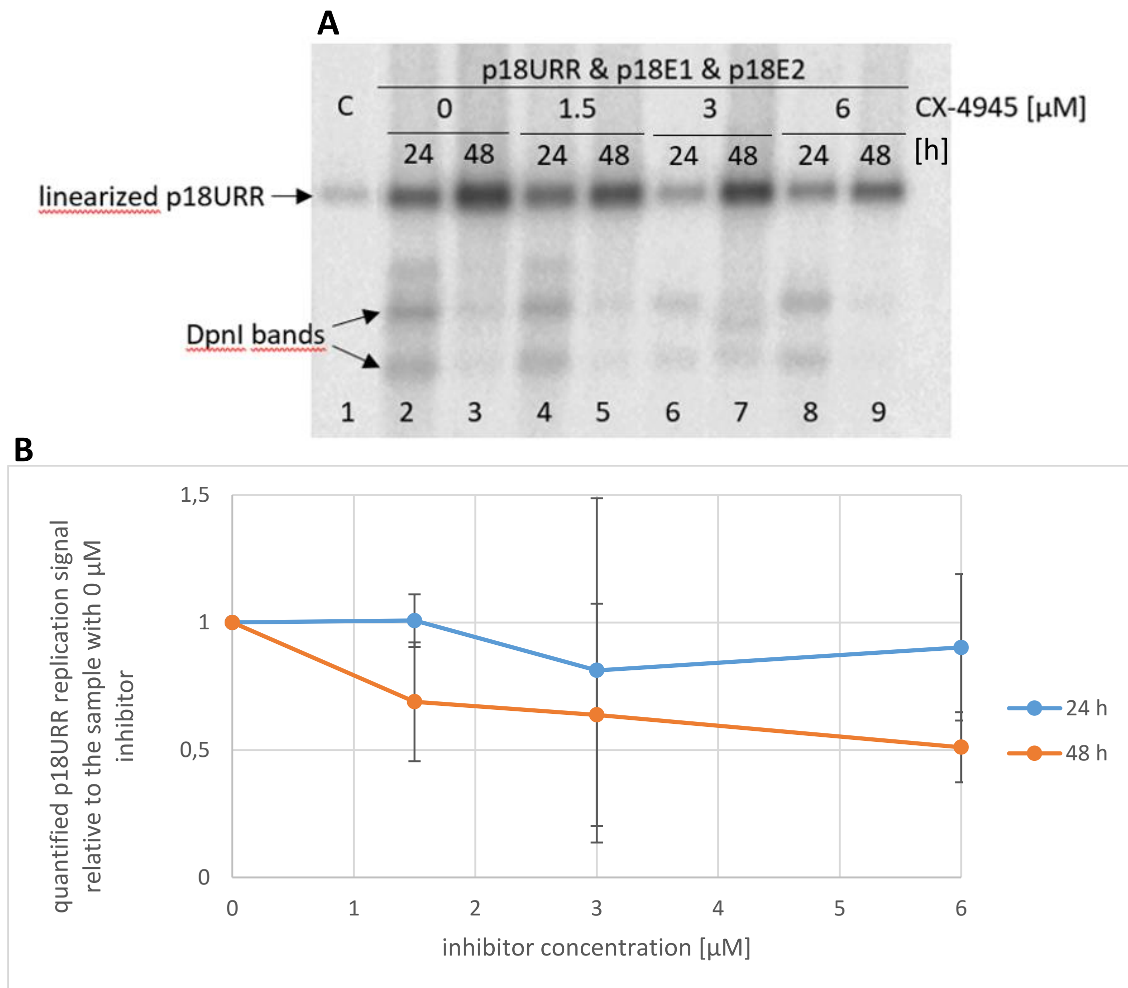


Figure 13: Effect of CX-4945 on E1 and E2 activity (A: Replication signal; Panel B: Quantified replication signal))

Panel A shows the signal for the linearized pURR18 plasmid, from which it gets clear that there is no significant change in the degree of replication even if high concentrations of the casein kinase inhibitor CX-4945 are added. The visible variations are to be explained with the technique used (HIRT extraction), where it is not possible to normalize the extracted DNA. The bands visible below the pURR18 signal are remains of the input DNA, which got digested by DpnI.

This results were affirmed by quantification (Panel B), where the blue graph shows the p18URR replication at different inhibitor concentrations (0 µM, 1.5 µM, 3 µM, 6 µM) after 24 hours of growth and the orange graph shows the p18URR replication at different inhibitor concentrations (0 µM, 1.5 µM, 3 µM, 6 µM) after 48 hours of growth. The visible variations are, again, to be explained with the technique used (HIRT extraction), where it is not possible to normalize the extracted DNA. The error bars show the standard deviation of the two independent experiments, which were conducted.

Considering the possible unequal amount of DNA loaded (due to HIRT extraction), it can be said that there is no significant effect of the inhibitor on the replication visible (Figure 13A). The DpnI bands are a result of the input DNA being digested by DpnI. This outcome is also supported by the quantification (Figure 13B).

These results show that the protein kinase CK2 has no direct effect on the E1 and E2 dependent replication in HPV or, in other words, CK2 does not regulate the transcriptional regulator E1 and E2´s ability to bind to their binding sites. It rather implicates that CK2 has an effect on the ability of E2 to influence HPV 18 gene expression and through that on HPV replication.

3.5. Preliminary test: Overexpression of the CK2α and CK2α' subunits

The next logical step was to try and overexpress both isoforms of the regulatory subunit of CK2 (CK2α and CK2α'), in order to investigate if the effect seen in the previous experiments (lack of CK2 activity leads to less HPV replication) works also the other way (additional CK2 activity leads to more HPV replication?). Also a rescue experiment was planned, where the missing activity of the regulatory subunit in the U2OS alpha respectively U2OS prime cell line was to be balanced with an expression of those subunits from extrachromosomal vectors.
In order to conduct these experiments, CK2α and CK2α' expressing plasmids were needed and these were sent by Professor Mauro Salvi from the University of Padova. First it had to be shown that those plasmids effectively express CK2α and CK2α' in U2OS wild type cells. Different amounts of CK2α respectively CK2α' expressing plasmids (0 ng, 100 ng, 500 ng, 1000 ng) were transfected into U2OS wild type cells. The cells were grown for 24 hours and then a western blot performed.

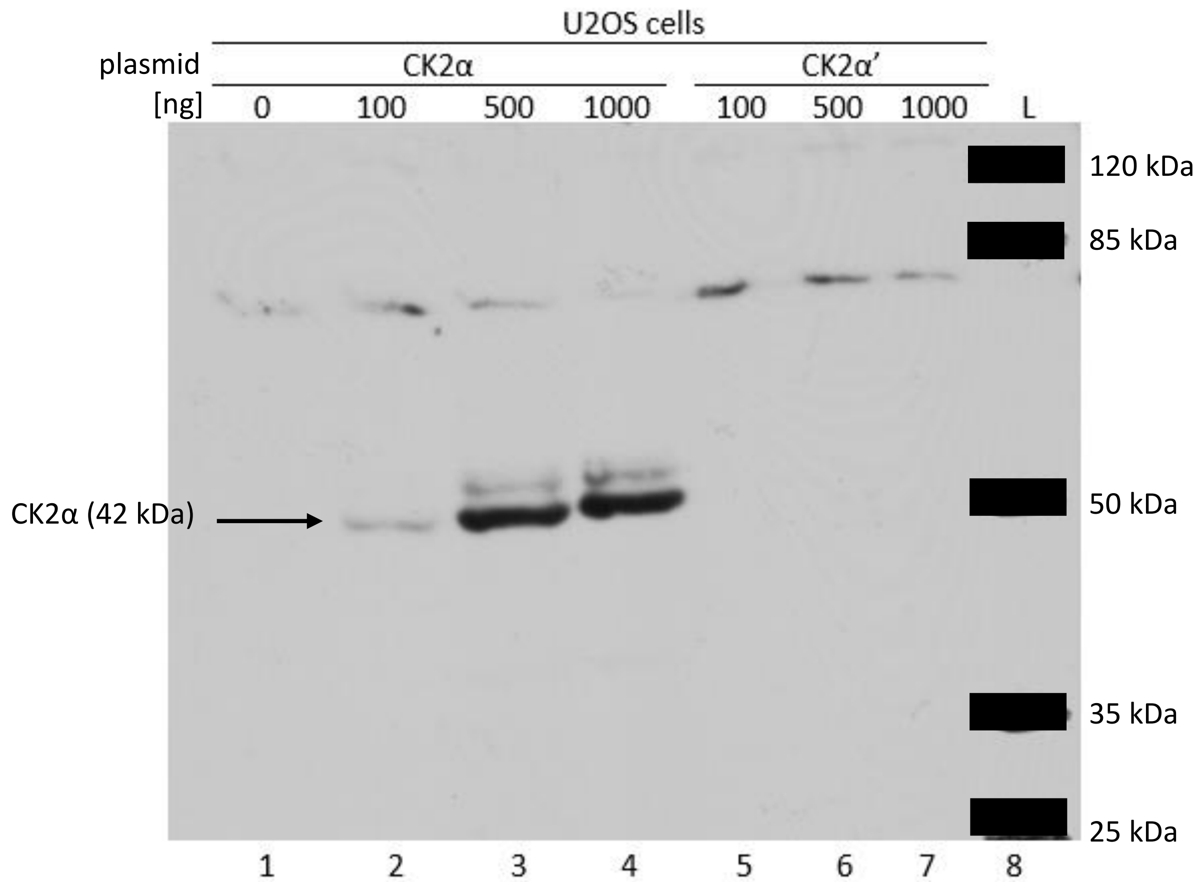


Figure 14: Overexpression of CK2α and CK2α' in U2OS cells

As it is seen from the figure, CK2α is detectable for all three plasmid amounts used (lines 2, 3 and 4). While the signal for 100 ng is very low, with 500 ng as well as 1000 ng a strong signal is seen. No signal was detectable for CK2α' (lines 6, 7 and 8), no matter how much plasmid was transfected (L – ladder (Prestained Protein MW Marker) (line 8); both proteins ~42 kDa; exposition time: ~15 minutes).

As seen from Figure 14, only the CK2α subunit is solidly expressed, while for CK2α' no signal could be detected. This means that in order to perform further experiments, first, a functional CK2α' expressing plasmid needs to be created.

4. Outlook

The results of this thesis offer a lot of suggestions for further experiments. More experiments need to be conducted, to assure the importance of the protein kinase CK2 for HPV replication. These experiments could be, for example, an overexpression of the regulatory subunit of CK2, or a rescue experiment with CK2α and CK2α' expressing plasmids in the CK2α defective cell lines. Other possible experiments are a CK2 knock-out with siRNA or a mutation of the active sites of the protein kinase CK2.

After assuring the observed effect of CK2 on HPV replication, it could be investigated how exactly this mechanism works and where exactly the protein kinase CK2 is needed in HPV replication. This could be achieved by mutating the casein kinase CK2 sites in E2 or in other HPV proteins.

Moreover, all the experiments were performed during HPV's initial amplification only. It would be of interest to examine if CX-4945 also is able to inhibit HPV replication during maintenance and vegetative replication in the later stages of HPV's life cycle.

Most certainly, the long-term goal of experiments on the effect of CX-4945 on HPV replication is the development of an anti-HPV drug, because currently there is none available.

Another question this thesis might offer an answer to is that, thus far, the HaCat cell line, which are immortal keratinocytes, did not prove to be a good model system for studies of HPV, although this cell line should provide a more natural environment for the virus. One hypothesis could be that, as CK2 activity has proven to be of high importance for both replication and translation, this could be the limiting factor for HPV replication and CK2 activity might be too low in HaCat cells. One way to prove this hypothesis is to co-transfect HPV genomes and CK2 expression plasmids into HaCat cells. If the hypothesis is correct then this should enable HPV to persist also in HaCat cells at a higher level.

Summary

To summarise this thesis, it can be said that the protein kinase CK2 indeed has a noteworthy effect on the replication of HPV. This is true for the HPV types 11 and 18 and propably for other types as well, but further experiments need to be performed to investigate that.

In addition, it was shown that the regulatory subunit of CK2 (CK2α) is the part that plays the important role in the dependence of HPV on CK2, with especially the α' subunit being of significance. The level of viral genome replication is at a significantly lower level, if this subunit is defective.

Another outcome of this thesis is that the viral replication protein E2 needs CK2 phosphorylation in order to work as a negative regulator of viral genome replication. Although CK2 phosphorylation has no direct effect on E1 and E2 dependent replication in HPV, it seems to have an influence on E2's ability to influence HPV gene expression and through that on HPV replication.

Furthermore, CX-4945, a protein kinase CK2 inhibitor, was successfully used to inhibit the replication of HPV 18 during the initial replication phase. This inhibition seems to be selective for viral genome replication as the replication of another plasmid (oriP), which mimicked genomic replication, was not affected. With a determined IC_{50} of 1.89 µM, this compound could be a possible solution to meet the need of an anti-HPV drug. The fact that CX-4945 reached the second phase in clinical trials as an anti-cancer drug makes this finding even more promising.

As an outlook it can be stated that the results of this thesis offer a lot of suggestions for further experiments. Not only are more experiments needed to assure the findings of this thesis, but also to investigate the mechanism behind these findings.

Additionally, the results presented in this thesis might offer an explanation for the weak replication levels of HPV in HaCat cells which should offer this virus a more natural environment. More experiments are needed, to examine if the CK2 levels in HaCat cells are in fact lower than in U2OS cells.

All these suggested experiments are very interesting from the point of understanding the mechanisms of the life cycle of HPV, but the long-term goal should be the development of the first anti-HPV drug. This would help to prevent many cervical cancer cases, which are the reason for many cancer deaths worldwide. The results of this thesis show that CX-4945 is a promising candidate for this undertaking and more experiments are needed to bring this to a successful end.

Proteiinkinaas CK2-e roll inimese papilloomiviiruse elutsüklis

Matthias Mauch

Kokkuvõte

Peaaegu kõiki emakakaelavähi juhtumid saab taandada varasemale infektsioonile erinevate inimese papilloomiviiruse (ingl. k.: *human papillomavirus, HPV*) tüüpidega. Tavaliselt paranetakse sellisest infektsioonist iseeneslikult paari kuu jooksul, ilma et sümptomeid üldse esineks. Siiski võib harva esineda tüükaid ja teisi healoomulisi vohamisi ning osadel juhtudel võib ka emakakaelavähk välja areneda. Emakakaelavähki põhjustavaid *HPV* tüüpe kutsutakse kõrge riskiga *HPV* tüüpideks. *HPV* infektsioon on pandeemiline probleem ning sellega infitseeritud on ligikaudu 10% kõikidest naistest. Enim on nakatumisi ja surmajuhtumeid maailma vähem arenenud regioonides, kus infektsioonimäär on kõrge ja puuduvad igasugused emakakaelavähi sõeluuringuid. Emakakaelavähk on maailmas levimuselt neljas vähiliik ning ühes aastas nakatub sellesse umbes 500 000 naist. Samal ajal sureb sellesse 270 000 naist, mis moodustab 7,5% kõikidest vähisurmadest maailmas. Tänase päevani on vaktsineerimine ainus võimalus *HPV* vastu võitlemiseks ning hoolimata sellest, et maailmas on suur nõudlus *HPV* ravimi järgi, ei ole seda senini luua suudetud.

Käesolevas magistritöös viidi läbi erinevad eksperimendid *HPV* ning proteiinkinaasi CK2-ga. Näidati, et proteiinkinaas CK2 on *HPV* varases replikatsioonis hädavajalik ning ilma selleta ei saa *HPV* oma elutsüklit lõpuni läbida. Samuti suudeti näidata, et CK2-e funktsiooni eest vastutab eelkõige proteiinkinaasi CK2 regulatoorne subühik α’. Proteiinkinaasi CK2 inhibiitori CX-4945 lisamisega suudeti samuti *HPV* varast replikatsiooni takistada. CX-4945, triviaalnimetusega „Silmitasertib“, reageerib kovalentselt CK2 regulatoorse subühiku seondumissaidiga ja omab kõrget dissotsiatsioonikonstanti, mis tähendab, et see inhibeerib usaldusväärselt proteiinkinaasi CK2 fosforüleerimist.

Selle magistritöö tulemused pakuvad ideid paljudele järgmistele katsetele, milles oleks võimalik näiteks proteiinkinaasi CK2 täpset seost *HPV* varase replikatisooniga uurida, ehk kus kohas ja millal CK2 oma tööd teeb. Kindlasti on CX-4945 huvipakkuv ka sellepärast, et tegemist on potentsiaalse ravimi kandidaadiga, sest see näib olevat tugev inhibiitor *HPV* replikatsioonile ja jõudis vähiravimina juba kliiniliste uuringute teise etappi.

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