UNIVERSITY OF TARTU

Faculty of Science and Technology

Institute of Technology

Lidiia Zaikina

Exploring the impact of small-molecule compounds on human papillomavirus type 5 replication and host cell differentiation

Bachelor's Thesis (12 ECTS)

Curriculum Science & Technology

Supervisor:

M.Sc. Elina Lototskaja-Perepelenko

Tartu 2024

Exploring the impact of small-molecule compounds on human papillomavirus type 5 replication and host cell differentiation

Abstract:

Human papillomavirus (HPV) is the most widely spread sexually transmitted virus worldwide. Different types of HPVs can cause skin lesions and cancers. HPV5 belongs to beta papillomavirus and is commonly found in healthy skin, while also associated with skin cancer in patients with immune deficiency or a genetic disorder, *Epidermodysplasia verruciformis*. Vaccines preventing infection with the most carcinogenic mucosal HPV types exist, but they are inefficient against cutaneous HPVs. Also, there is no effective treatment for existing infections, which emphasizes the need to develop antiviral drugs.

The present thesis focuses on two small-molecule compounds (#1 and #2) identified through a high-throughput screening performed prior to this study. The aim was to confirm their inhibitory effect on HPV5 replication and to determine the effect of the identified inhibitors on the differentiation of human osteosarcoma (U2OS) cells and human primary epithelial keratinocytes (HPEKs), which are used as HPV model host cells. Both compounds showed a strong inhibitory effect on the HPV5 replication in U2OS cells, and the inhibitory activity of the compound #2 was confirmed also in HPEKs. However, in contrast to the compound #2, the compound #1 upregulated the HPV5 replication in HPEKs, which was explained by the differentiation-inducing effect. Thus, the compound #2 was chosen as a potential HPV5 replication inhibitor for future investigation.

Keywords: human papillomavirus (HPV), HPV 5, inhibition, replication, differentiation

CERCS: B230 Microbiology, bacteriology, virology, mycology.

Väikeste molekulaarsete ühendite mõju uurimine inimese papilloomiviirus tüüp 5 replikatsioonile ja peremeesrakkude diferentseerumisele

Lühikokkuvõte:

Inimese papilloomiviirus (HPV) on kõige laialdasemalt sugulisel teel leviv viirus maailmas. Erinevad HPV tüübid võivad põhjustada nii nahakahjustusi kui ka vähki. HPV5 kuulub beeta-papilloomiviiruste hulka ja seda leidub tavaliselt tervel nahal, kuid see on seotud ka nahavähiga patsientidel, kellel on immuunpuudulikkus või geneetiline häire, Epidermodysplasia verruciformis. On olemas vaktsiinid, mis ennetavad nakatumist kõige kantserogeensemate limaskestade HPV tüüpidega, kuid need ei ole tõhusad nahka nakatavate HPV-de vastu. Samuti puudub tõhus ravi olemasolevate nakkuste vastu, mis rõhutab vajadust arendada viirusevastaseid ravimeid.

Käesolev töö keskendub kahele väikesele molekulaarsele ühendile (#1 ja #2), mis tuvastati enne käesoleva uuringu läbiviidud kõrge läbilaskevõimega sõeluuringu käigus. Eesmärgiks oli kinnitada nende pärssivat mõju HPV5 replikatsioonile ja määrata kindlaks tuvastatud inhibiitorite mõju inimese osteosarkoomi (U2OS) rakkude ja inimese primaarsete epiteelkeratinotsüütide (HPEK) diferentseerumisele, mida kasutatakse HPV mudelrakkudena. Mõlemad ühendid näitasid tugevat pärssivat mõju HPV5 replikatsioonile U2OS rakkudes. Ühend #1 avaldas HPEK-des väiksemat mõju HPV5 replikatsioonile, mida selgitati diferentseerumist soodustava toimega. Saadud tulemuste põhjal valiti ühend #2 tulevaseks uurimiseks potentsiaalseks HPV5 replikatsiooni inhibiitoriks.

Võtmesõnad: inimese papilloomiviirus (HPV), HPV5, pärssimine, replikatsioon, differentseerumine

CERCS: B230 Mikrobioloogia, bakterioloogia, viroloogia, mükoloogia

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ACTB – beta-actin

AP – alkaline phosphatase

cDNA – complementary DNA

CSPD – chemiluminescent alkaline phosphatase

DMSO – dimethyl sulfoxide

DNase – deoxyribonuclease

dNTP – deoxynucleotide triphosphates

dsDNA – double-stranded DNA

E – early (genomic region or protein)

EDTA – ethylenediaminetetraacetic acid

EV – *Epidermodysplasia verruciformis*, a genetic disorder

FBS – fetal bovine serum

FMDV – foot-and-mouth disease virus

GAPDH – glyceraldehyde 3-phosphate dehydrogenase

HPEK – human primary keratinocytes

HPV – human papillomavirus

HPV5 – plasmid vector containing the sequence of HPV type 5 genome

HTS – high-throughput screening

IMDM – Iscove's Modified Dulbecco's Medium

L – late (genomic region or protein)

MAPK – mitogen-activated protein kinase

MHC-II – a class of major histocompatibility complex molecules

Nluc – Nano luciferase

ORF – open reading frame

ori – origin of replication

p53 – tumor suppressor protein 53

PBS – phosphate-buffered saline

pRb – retinoblastoma tumor suppressor protein

PVs – Papillomaviruses

RNase – ribonuclease

SD – standard deviation

qPCR – quantitative real-time PCR

U2OS – human osteosarcoma epithelial cells

URR – noncoding upstream regulatory region

VLP – virus-like particle

wt – wild type

INTRODUCTION

Human papillomaviruses (HPVs) are double-stranded DNA viruses infecting mucosal or cutaneous keratinocytes. HPV stands as the most widespread sexually transmitted infection globally. HPV types can be divided into two main groups: high-risk which are carcinogenic, and low-risk types associated with warts and papillomas. The majority of the sexually active population are likely to contract HPV at some time in their lives. Infection can exhibit no symptoms or lead to oncogenesis – with HPV being found in more than 90% of cervical malignancies, which ranks fourth in cancer-related deaths in women.

While currently vaccination against several high-risk HPV types is available, there is no credible, safe, and well-tested treatment for existing infection. Existing vaccines are designed to prevent future infections and are not effective in treating an individual who is already infected with HPV. This makes the development of medication targeting HPV activity highly necessary. One potential solution would be HPV replication inhibition as this would mean decreased production of viral proteins affecting the formation of warts, papillomas, and oncologies. Compounds inhibiting the HPV replication cycle do exist. However, we have to thoroughly study their effect not only on the replication of HPV but also on the host cells in order to avoid unpredictable consequences for the living cells.

Previous to the current thesis, two small-molecule compounds inhibiting HPV replication were selected based on high-throughput screening results. The aim of the thesis is to confirm the ability of these compounds to inhibit HPV5 replication. HPV5 infects cutaneous epithelium and was chosen for this study due to its capability to cause skin malignancies in patients with immune deficiency. An essential part of this study is the determination and comparison of the effects of the compounds on two model cell lines. U2OS (human osteosarcoma) is widely used because it effectively supports HPV5 replication. The HPEK (human primary epithelial keratinocytes) cell line is HPV host cells – keratinocytes.

1 LITERATURE REVIEW

1.1 Papillomaviruses

Papillomaviruses are a large and diverse group of viruses in the *Papillomaviridae* family. They are characterized by non-enveloped capsids, protecting them. Their genetic material is double-stranded circular DNA (de Villiers *et al*., 2004). Papillomaviruses infect various animals: mammalians, birds, reptiles, and fish (Moreno-Lopez *et al*., 1984; López-Bueno *et al*., 2016). Around 450 PV types exist in the phylogenetic tree, but this number has dramatically changed and may grow further. According to the PV genome's highly conserved L1 ORF gene, PVs can be classified into different types (de Villiers *et al*., 2004). The L1 gene encodes a protein, which is a building block for the PV capsid. It is used for papillomaviruses classification because it is highly conserved, but, at the same time, it varies enough to differentiate between types of the virus (Bernard *et al*., 2010). Also, as L1 is one of the main parts of PV capsid, it can be targeted or utilized in various treatments, such as HPV vaccines.

1.2 Human papillomaviruses

Human papillomaviruses (HPVs) are a large group of Papillomaviruses with more than 200 types. HPV can be divided into five genera: alpha, beta, gamma, mu, and nu (Figure 1) (de Villiers *et al*., 2004). HPV infects keratinocytes of cutaneous or mucosal epithelium. Oncogenic HPVs are high-risk, and low-risk types are not oncogenic (Muñoz *et al*., 2003).

Many high-risk mucosal HPVs belong to alpha papillomaviruses, such as 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 types. Most mucosal HPV types are found in this genus (de Villiers *et al*., 2004). Mucosal alpha HPV types 16 and 18 significantly contribute to cervical cancer (Walboomers *et al*., 1999). The high-risk alpha HPV types are known to be the main reason for invasive cervical cancer; but they also cause penile, vulvar, anal, and vaginal cancers (Muñoz *et al*., 1992). Alpha HPVs are associated with genital and oral infections, while other types with nongenital infections (Rosa *et al*., 2013). HPV types 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, and 81 are considered low-risk (Muñoz *et al*., 1992). These types cause skin lesions: warts or papillomas. Although in most cases, low-risk HPV infections are not a dangerous health concern, rarely HPV types 6 and 11 can lead to recurrent respiratory papillomatosis, potentially causing breathing difficulties (Gissmann *et al*., 1982).

Other HPV genera, primarily beta HPVs, are mostly cutaneous but have also been found at mucosal sites (Forslund *et al*., 2013). Most cutaneous HPVs are considered low-risk, meaning they are not typically associated with cancer development. Cutaneous HPV types are associated with different warts: common, plantar, and flat. However, it was discovered that HPV5, which is a cutaneous beta-papillomavirus, contributed to skin cancer development in patients with immune deficiency (Lutzner *et al*., 1980). Beta papillomaviruses are part of a genus that consists of five cutaneous species. Beta HPVs do not integrate into the host genome but rather exist within the cell as episomes (Feltkamp *et al*., 2008). Such characteristic allows them to persist in the host without causing immediate harm, therefore, they are commonly associated with normal skin. However, certain types of beta HPVs, such as 5 and 8, were detected in skin cancers and lesions, which suggests they play a certain role in the development of the disease (Orth, 2008). These discoveries were made in patients with compromised immunity or a rare genetic disorder - *Epidermodysplasia verruciformis* (EV).

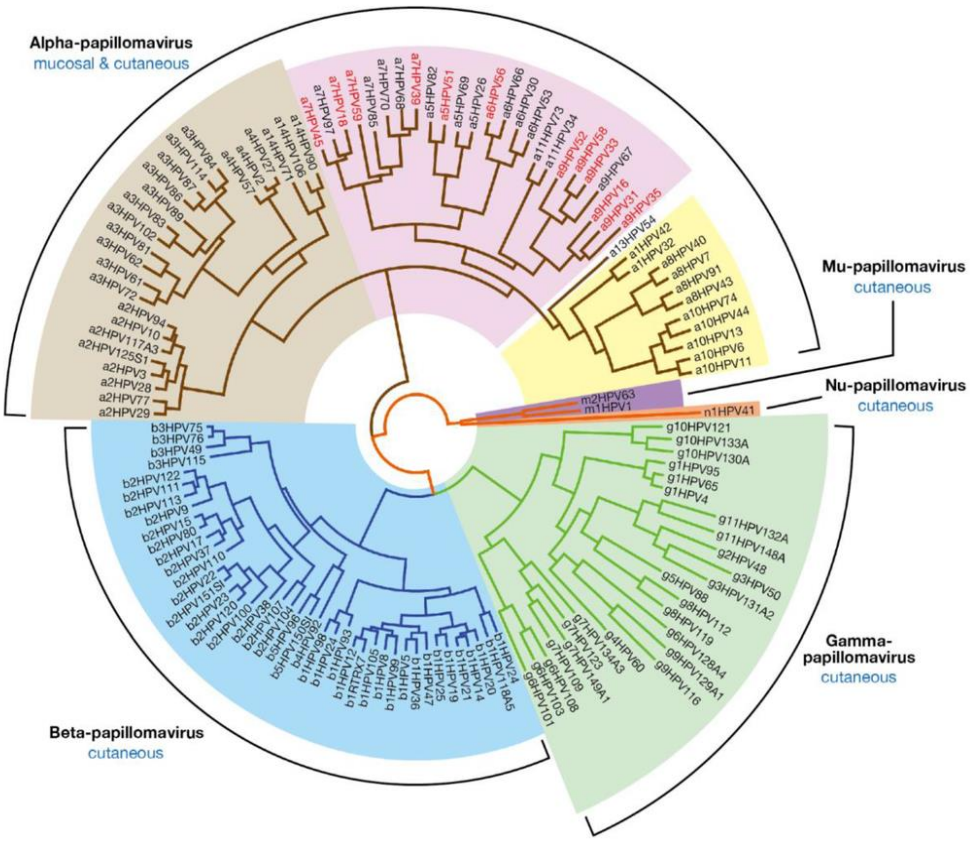


Figure 1. HPVs belong to alpha, beta, gamma, mu, and nu papillomaviruses. Low-risk cutaneous (beige), low-risk mucosal (yellow), and high-risk (pink) groups of alpha papillomaviruses are present in the picture. The HPV types marked with red text are high-risk, and they are proven to be carcinogenic. Beta papillomavirus types are shown in blue (Egawa *et al*., 2015).

1.3 HPV genome

HPV genome size is usually around 8000 base pairs and comprises eight open reading frames (ORFs) in almost all HPVs (Graham, 2010). The viral genome consists of three main parts: early proteins, late proteins, and the noncoding upstream regulatory region (URR). The URR contains the origin of replication, E1 and E2 binding sites, and several transcription factors. The early region is coding six proteins that play a regulatory role (Figure 2).

E1 and **E2** proteins are necessary for genome replication. They form a complex that binds to the origin of replication at specific sites, and E2 plays a crucial role in ensuring E1 reaches the origin of replication (ori) (Ustav & Stenlund, 1991; King *et al*., 2011; Sedman & Stenlund, 1995). E1 protein has a helicase activity necessary for replication initiation (Yang *et al*., 1993). E2 interacts with other proteins and is vital in transcription control and replication initiation. It is a factor that can activate or repress transcription (Thierry & Yaniv, 1987).

The **E4** protein is encoded by E1^E4 transcript – spliced from E1 and E4 gene products. It is vital for virus release and transmission. This is why, despite being an early protein, the highest level of E4 is detected in the late stages of the viral cycle and outer layers of the epithelium (Doorbar, 2013). There is evidence that E4 also inhibits cell proliferation in the G2 stage of the cell cycle (Davy *et al*., 2002).

E5 protein differs between high-risk and low-risk HPV types (DiMaio & Petti, 2013). In general, its' transforming ability is limited. However, it contributes to and amplifies the oncogenic activity of E6 and E7 proteins in high-risk virus types (Bravo & Alonso, 2004). E5 is expressed in the early stages of the viral lifecycle (Chang *et al*., 2001). The specific feature of beta HPV is the absence of E5 ORF (de Villiers *et al*., 2004).

E6 viral protein is one of two main oncoproteins of HPV. It degrades the p53 – tumor suppressor that initiates cell apoptosis in case of DNA damage and activates DNA repair mechanisms. This way, cells with damaged DNA keep proliferating, and the cell cycle does not stop, causing uncontrolled cell growth. The degradation happens because of the complex with the E6-AP ubiquitination enzyme, p53, and E6 protein, marking p53 for destruction by the ubiquitin-proteasome pathway (Crook *et al*., 1991).

The **E7** protein is another HPV oncoprotein. It has similarities with E6 but targets and degrades another tumor suppressor - pRb (Dyson *et al*., 1989). pRb inhibits cell cycle

progression from the G1 to the S phase by forming a complex with E2F and stopping it from inducing DNA replication. However, in the case of HPV infection, E7 binds to pRb, disassembling its complex with transcription factor E2F, which leads to uncontrolled DNA replication and premature transfer of cells into the S phase (Chellappan *et al*., 1992). Essentially, E6 and E7 activity disrupts the normal cell cycle, and the cells cannot exit the cycle or stop replication even if DNA damage accumulates, contributing to oncogenesis (Sund *et al*., 2022).

The **E8^E2** is a protein encoded by the E8^E2 transcript, which is a spliced gene product of E8 and E2 ORFs. It acts as a repressor, inhibiting transcription of early viral genes. The repression is caused by E8^E2 binding to E2 sites or by forming inactive heterodimers with E2. It limits the amplification of initial HPV plasmids, which may be necessary to balance HPV replication and differentiation of cells (Lace *et al*., 2008).

The late region has two proteins, which participate in viral capsid assembly, providing protection and transmitting the virus. **L1** and **L2** are major and minor capsid proteins, correspondingly. They self-assemble to form a capsid structure, and the viral DNA can be encapsidated within this structure. (Graham, 2010).

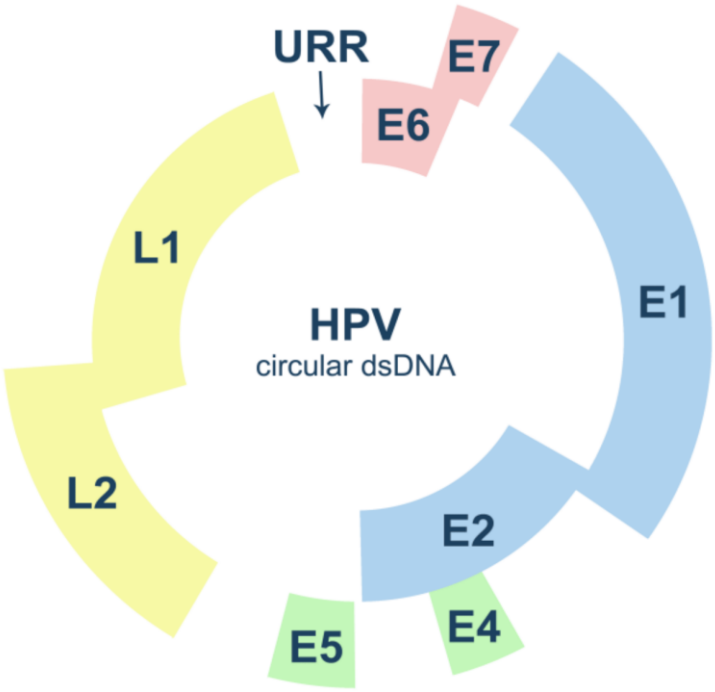


Figure 2. Organization of HPV genome. The HPV genome consists of three main parts: early proteins, late proteins, and URR (noncoding upstream regulatory region). Early ORFs are E1, E2, E4, E5, E6, and E7. They are necessary for the regulation of the HPV life cycle. Late ORFs are L1 and L2. Their products are necessary for virus capsid formation. URR contains the origin of replication, enhancers, and promoters, and E1 and E2 binding sites (Ferreira *et al*., 2020).

1.4 HPV infection cycle

HPV targets the stratified epithelia's basal layer, including the skin, anogenital tract, and oral cavity. The HPV lifecycle starts with the infection. The virus uses microscopic wounds or hair follicles to access the basal layer. To attach to the surface, the help of heparan sulfate proteoglycans (HSPGs) is necessary (Kines *et al*., 2009).

The first stage is the initial amplification, also known as transient amplification (Figure 3). Once inside the basal cell, the viral DNA replicates to a low copy number without disrupting the normal cell cycle (Fisher, 2015). The E1 protein initiates replication due to its' helicase activity and ability to influence replication factors of the host cell (Yang *et al*., 1993). With E2 guiding E1 to the ori, E1 assembles into a double-hexamer and can unwind DNA (Bergvall *et al*., 2013). Here, in the nucleus of infected cells, the HPV genome amplifies until a 50-300 copy number is reached.

Then, the viral DNA goes into the maintenance replication state (Figure 3). HPV maintains a low-copy number as the episomal form, which can happen for a long time in the latent state (Penrose & McBride, 2000). This latent state can persist for years, even decades before the virus becomes reactivated (Maglennon *et al*., 2011). E2 connects cell chromatin with viral DNA, which is necessary for proper maintenance replication, ensuring proper distribution of viral genomes during cell division and preventing loss of viral DNA from the nucleus (Ilves *et al*., 1999). Here, in some cases with alpha HPVs, it can also integrate into the host cell's genome.

In normal circumstances, basal cells migrate upwards together with differentiating, but in the case of HPV infection, while dividing, E6 and E7 target tumor suppressor proteins, allowing the cell cycle to continue (Sund *et al*., 2022).

In the vegetative replication stage, with the process of migration and differentiation, viral DNA amplifies to a high copy number together with capsid protein gene expression, which is necessary for new virions (Figure 3). When infected cells reach the top of the epithelium layer, viral DNA is packed into virions, which can be released by shedding dead differentiated cells (Peh *et al*., 2002).

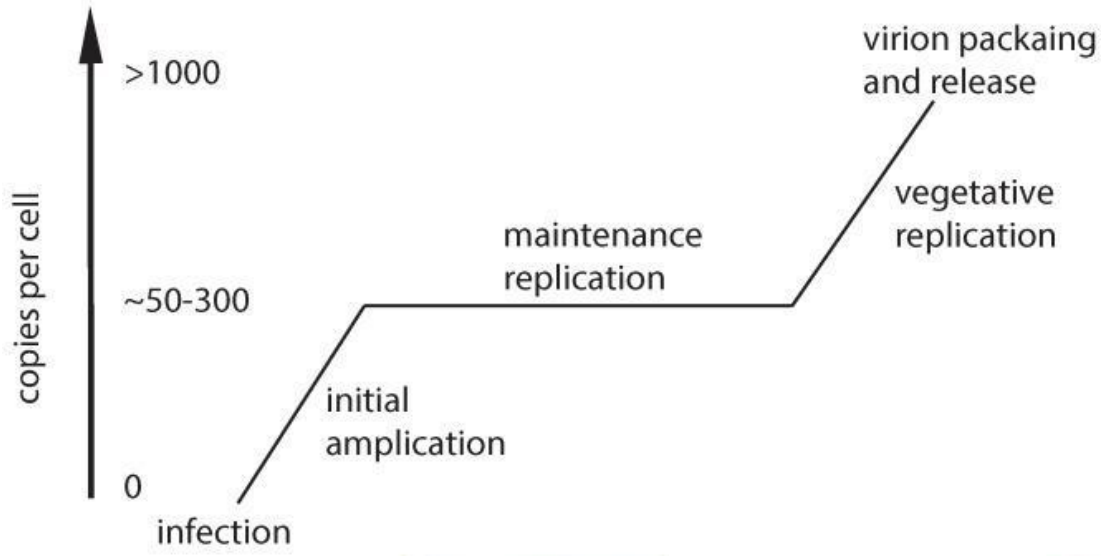


Figure 3. HPV life cycle. After infection, viral DNA is amplified to the level of 50-300 copies per cell, and then this number is maintained throughout the maintenance stage. During the maintenance replication stage, HPV can stay in the latent state for years. After that, vegetative replication elevates the copy number to more than a thousand copies per cell. The last event is the creation and release of virion (Fisher, 2015).

1.5 Existing vaccines targeting HPV

1.5.1 Vaccines preventing infection

The most widely used vaccines are L1 VLP (virus-like particle) ones. They stimulate the immune response against the L1 capsid protein of HPV (Zhou *et al*., 1991). VLPs do not cause infection but provoke a strong humoral immune response by mimicking the structure of the actual virus. Currently, three vaccines are used: bivalent Cervarix (16 and 18 HPV types), quadrivalent Gardasil (HPV16, 18, 11, and 6), and nine-valent Gardasil 9 (HPV6, 11, 16, 18, 31, 33, 45, 53, and 58). The effect of these vaccines may fade over time. Therefore, booster vaccination is necessary. Although some vaccines can protect from HPVs causing 90% of cervical cancers, they cannot treat existing infections (Yang & Bracken, 2016). As all of these vaccines target the capsid part, the antibodies they generate only target the intact virus and cannot act against the viral DNA in the cell after HPV infection.

1.5.2 Vaccines treating existing HPV infection

Although studies targeting the achievement of safe vaccines treating the existing HPV infection are ongoing, we currently do not have a reliable vaccine for this goal.

The peptide vaccines are often not very immunogenic, and it is hard to predict the immunogenicity of specific MHC-II-restricted peptides (Filipazzi *et al*., 2012). Protein-based vaccines are not very efficient (Einstein *et al*., 2007). DNA-based vaccines also cause weak immune reactions, and there is a risk of chromosomal integration (Kim *et al*., 2004). RNA replicon-based vaccines are difficult to prepare, cause transfected cells to undergo apoptosis, and are not immunogenic enough. Tumor cell-based vaccines show weak presentation of antigens, are laborious to prepare, and pose safety concerns for tumor injections (Chang *et al*., 2000). More immunogenic vaccines exist, such as bacterial and viral vector ones. However, they could harm the patient, and neutralizing antibodies can be generated, leading to decreased efficiency (Condit *et al*., 2016). Dendritic cell-based vaccines are personalized and expensive, and their quality cannot be controlled well (Abakushina *et al*., 2021).

1.6 Infected keratinocytes differentiation

Cellular differentiation is a process by which a stem cell becomes specialized. Because of differentiation, a cell can eventually become a multicellular organism with several systems with different functions. As a cell differentiates, it goes to a path determined by the environment, chemical signals, and mechanical forces (Kurpinski *et al*., 2006). In the differentiation process, genes responsible for a particular path must be activated, and others must be silenced (Maines *et al*., 2007).

In the case of keratinocytes, stem cells reside in the basal layer of epithelium. Stem cells start to divide in the basal layer, and their daughter cells can go into differentiation (Régnier, 1986). Here, the keratinocyte usually exits the cell cycle and starts migrating upwards. Then mitogen-activated protein kinase (MAPK) signaling pathway sends signals that trigger specific gene expression patterns (Efimova *et al*., 2003). If the cell is infected with HPV, it does not exit the cell cycle and keeps dividing while moving upwards because of the action of E6 and E7 viral proteins. The pRb and p53 are destructed by viral proteins, and they cannot

start apoptosis of cells with damaged DNA or control DNA replication. Tumor cells can start developing under these circumstances (Sund *et al*., 2022).

During differentiation, keratinocytes start activating genes encoding for essential structural proteins like keratins, moving upwards, and can no longer divide in the normal state (Candi *et al*., 2005). Produced structural proteins become a part of the outer shell of mature keratinocytes and form a protective layer later on (Zingkou *et al*., 2022). At the same point, when the cell enters the differentiation stage, HPV enters a vegetative replication state and reaches a high-copy number of more than a thousand copies per cell (Kadaja *et al*., 2009). HPV capsid proteins for later virion building are also expressed at this stage (Zhao *et al*., 2005).

After HPV DNA and capsid parts are ready, virions can be assembled in the keratinocyte nucleus (Doorbar *et al*., 2015). Enzymes break compounds and parts of the cell to remodel the keratinocyte. Keratinocytes become fully differentiated, full of keratin, losing or partially degrading their nucleus. Eventually, cell death happens. Then, the dead cells detach from the skin in the continuous cycle of epithelial renewal (McCall & Cohen, 1991). Furthermore, with these dead keratinocytes full of HPV virions, HPV can continue spreading (Bryan & Brown, 2001).

1.7 Differentiation markers

The differentiation process has its' own markers. This way, it can be determined if the markers are present in the cell – the differentiation has started. HPV replication and its effect on cells in our laboratory is usually studied within U2OS (osteosarcoma) cells, which are a convenient model for such studies due to their availability, support of HPV replication, and suitable growth rate (Geimanen *et al*., 2011). Keratinocytes are HPV host cells, thus studying HPEK cell line could be an approximation for real world events. Therefore, while studying the differentiation of infected cell lines, it is crucial to investigate the presence of both osteoblast and keratinocyte differentiation markers.

1.7.1 Osteoblast differentiation markers

RUNX2 is a transcription factor that is essential for osteoblast differentiation. Mice with RUNX2 deficiency had a decrease in the number of osteoblasts and a reduction in osteoblast

surface. Additionally, these mice had a decrease in the expression of osteoblast marker genes. RUNX2 plays a role in the expression of osteopontin, bone sialoprotein, osteocalcin (BGLAP), osteoprotegerin, Rankl, and other genes (Rashid *et al*., 2024; Li *et al*., 2012). RUNX2 plays a crucial role in determining the fate of multipotent mesenchymal cells by simultaneously inhibiting adipogenesis, chondrocyte differentiation and inducing osteoblast differentiation (Enomoto *et al*., 2004; Maeno *et al*., 2011).

SP7 (osterix) is a transcription factor containing zinc finger. It is necessary at later stages of differentiation for bone matrix production, acting downstream of RUNX2, as it is not expressed in cells lacking RUNX2. Mice lacking SP7 could not form bones (Nakashima *et al*., 2002). SP7 also regulates the enhancer activity of RUNX2 and induces other genes needed for bone formation, such as osteocalcin, for example (Kawane *et al*., 2014; Nakashima *et al*., 2002)

BGLAP (osteocalcin) is a bone gamma-carboxyglutamic acid-containing calcium-binding protein. It constitutes up to 15% of mature bone proteins, except collagen (Price *et al*., 1976). It is primarily expressed in osteoblasts and is strongly associated with osteoblast differentiation (Ducy & Karsenty, 1995). Osteocalcin is necessary to correctly align hydroxyapatite crystals, ensuring proper formation of bone matrix and strong bones (Moriishi *et al*., 2020).

1.7.2 Keratinocyte differentiation markers

Specific markers for keratinocyte differentiation are, for example, K10, K14, involucrin and loricrin. Keratinocytes are HPV host cells, so by studying the presence of these markers, we can assess whether the differentiation process is happening inside the infected cells.

K14 (keratin 14) is a protein found primarily in the basal layer of the epidermis. This layer places stem cells that continuously divide, generating new cells constantly for differentiation. K14 acts as a marker for early differentiation stages. Its presence indicates that a cell is either a stem cell or has just begun its journey toward becoming a mature keratinocyte (Alam *et al*., 2011). As well, K14 interacts with other differentiation markers, which seems necessary for normal differentiation (Sümer *et al*., 2019).

K10 (keratin 10) is another keratin protein that appears as differentiating keratinocytes move further away from the basal layer. It forms the intermediate layer of the epidermis, providing

additional structural support and contributing to the overall barrier function of the skin (Poumay & Pittelkow, 1995). The presence of K10 indicates a more mature stage of differentiation compared to involucrin.

Involucrin is a protein that is expressed when keratinocytes begin to differentiate and migrate upwards from the basal layer (Banks-Schlegel & Green, 1981). It plays a critical role in forming a waterproof barrier on the outermost layer of the skin. Involucrin helps connect other proteins, creating a sturdy protective layer (Steinert & Marekov, 1997). Involucrin indicates a more advanced differentiation stage than K14, signifying the cell's transition toward its final protective function.

Loricrin is a structural protein that appears in the outermost layers of the epidermis. It is also, just as involucrin, forms the barrier protecting the body from external threats. Loricrin expression signifies the terminal stage of differentiation (Yoneda *et al*., 1992).

2 EXPERIMENTAL PART

2.1 AIMS OF THE STUDY

The general purpose of this thesis was to study the impact of two small-molecule compounds identified during high-throughput screening of an NCI Diversity set VI library of small molecular weight compounds on HPV5 replication in HPEK and U2OS cells.

The step-by-step aims of the study are:

- analysis of HPV5 replication in U2OS cells and HPEK treated with two chosen compounds;
- analysis of the established differentiation markers expression levels in the compound-treated U2OS cells and HPEK to assess potential differentiation induction.

2.2 MATERIALS AND METHODS

2.2.1 Plasmids used in the present study

HPV5-Nluc – the plasmid was generated on the basis of the HPV5 genome (Appendix 2). The sequences inserted into the plasmid were the codon-optimized Nluc and the self-processing 2A peptide of the foot-and-mouth disease virus (FMDV) after the 72nd nucleotide of the E2 ORF, corresponding to the E1 stop codon. The full-length wt E2 ORF begins after the 2A sequence (Piirsoo *et al*., 2019).

HPV5-NlucE8^{-} – the HPV5-Nluc plasmid, containing a point mutation in the E8 first AUG and, therefore, deficient in the E8^E2 gene expression (Appendix 3).

2.2.2 Cell cultures and used medium

U2OS (human osteosarcoma) cells were cultivated in Iscove's Modified Dulbecco's Medium (IMDM, *Corning Inc.*), containing 10% of FBS (fetal bovine serum) and 1% of penicillin-streptomycin (*Sigma-Aldrich*).

Human primary keratinocytes (HPEKs) were propagated in a Defined Keratinocyte-SFM Medium (DKSM, *Gibco*). HPEKs were detached using 0.025% Trypsin-EDTA solution. The cells were incubated at 37 ˚C temperature and 5% CO2 on 10 cm plates (*Corning Inc*.).

2.2.3 Cells transfection

The day prior to transfection, U2OS cells grown on 10 cm cell culture dishes were split and seeded on two plates. The next day, the medium was discarded, and cells were washed with 1 ml of PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4). Then, the cells were detached using 800 µl 0.25% Trypsin-EDTA solution. Cells were transferred into the fresh medium, and the cell suspension was centrifuged for 5 minutes at 1000 rpm using Eppendorf Centrifuge 5810R (*Thermo Fisher Scientific*). The supernatant was discarded. Cells were resuspended in 500 µl of fresh medium and subjected to two transfections with either HPV5-Nluc or HPV5-NlucE8^{-} genomes using 30 ng of the plasmid per one well of a 96-well plate. DNA was mixed with 250 µl of cell suspension, and 30 µg of salmon sperm carrier DNA was added to each mixture. Both mixes were then transferred to the electroporation cuvettes and electroporated at 975 µF and 220 V using the Gene Pulser XCell machine (*Bio-Rad Instruments*). The cuvettes stayed for approximately 30 seconds, and 200

μl of fresh growth medium was added. The contents of each cuvette were transferred to 15 ml falcons pre-filled with 6.5 ml of fresh medium. Then, 70 μl per well of the transfected cell suspension was transferred to 96-well plates (*Corning Inc.*) for luciferase assay, and the cells transfected with the HPV5-Nluc genome were also transferred to 6-well plates for further treatment with the investigated chemical compounds.

For HPEK cell transfection, cells were plated to the 96-well plates two days prior to transfection. On the day of transfection, the medium for HPEK plates was renewed. For HPEK transfection, two mixes were prepared. The first mix contained 380 μl of the supplements-free medium, 9 μl of Plus reagent (*Invitrogen*), and 6 μg of HPV5-Nluc plasmid. The second one consisted of 380 μl of the supplements-free medium and 11.4 μl of lipofectamine (Lipofectamine LTX, *Invitrogen*). Both mixes were incubated for 2-3 minutes at room temperature, mixed and incubated for an additional 5 min at room temperature. Finally, 10 μl of the transfection mix was added to each well.

2.2.4 Cell treatment

The U2OS and HPEK cells were treated with reagents 24 hours post-transfection and further. The compounds were applied to cells at 96-well and 6-well plates – for luciferase assay and the assessment of differentiation markers by qPCR, respectively.

U2OS cells were treated with 20 μM and 40 μM #1 and #2 compounds diluted in the normal growth medium. DMSO was used as a control, as the compounds were dissolved in DMSO. 3.6 μl of DMSO was dissolved in 6 ml of medium for the control treatment. The medium containing compounds was renewed every two days. For the luciferase assay, three timepoints were taken: 48-, 72-, and 96 hours. The cells were incubated for 48- and 72 hours to analyze the differentiation markers.

HPEKs were treated with 12 μM compound #1 and 15 μM compound #2 diluted in the normal growth medium, and DMSO was used as a control. $CaCl_2$ diluted in a keratinocyte supplements-free medium to a final concentration of 1.5 μM was used to induce differentiation of HPEKs. Three timepoints were taken for luciferase assay: 72-, 96-, and 120 hours and two timepoints, 48- and 72 hours, for the analysis of the differentiation markers.

2.2.5 Luciferase assay

The cells transfected with the HPV5-Nluc or HPV5-NlucE8$^-$ genome and treated with the compounds were washed with PBS and lysed in 40 µl of Cell culture Lysis Buffer (*Promega*) per well for 10-15 minutes at room temperature. 12 µl of the cell lysate from each well was transferred to the new 96-well plate to analyze Nluc, and alkaline phosphatase (AP) activities, and total protein concentrations. Nano-Glo Luciferase Assay System Kit (*Promega*) was used to measure the luciferase activity. The Nluc substrate furimazine was diluted in Luciferase Assay Buffer in a 1:600 ratio, and 24 µl per well of the diluted substrate was added to the lysates. Then, the luciferase activity was measured using the GloMax 96 Microplate Luminometer (*Promega*).

For the AP activity measurement, 24 µl of CSPD substrate (*Applied Biosystems, Tropix*) per well were added to 12 µl of the lysates, and the mixtures were incubated for 10 minutes at room temperature. The chemiluminescence was further measured with a GloMax 96 Microplate Luminometer (*Promega*). The AP activity was used to normalize the Nluc activity.

A BCA protein assay kit (*Pierce*) was utilized to measure the total protein concentrations. Reagents A and B were mixed in a 50:1 ratio to compose the working reagent (WR). Next, 4 µl per well of BSA standards and the lysates were pipetted on a 96-well plate. BSA standards were used in the following concentrations: 2 mg/ml, 1.5 mg/ml, 1 mg/ml, 0.75 mg/ml, 0.5 mg/ml, 0.25 mg/ml, and 0.125 mg/ml. 40 µl WR was added to each well, followed by 30 minutes of incubation at 37 ˚C. An absorbance was measured at a wavelength of 562 nm using a microplate reader and Magellan data analysis software (*Sunrise, Tecan*).

2.2.6 Extraction of RNA

Total RNA was extracted using a quick-RNA Miniprep kit (*Zymo Research*) The cells were washed with PBS and lysed in 300 µl of RNA Lysis Buffer for 5 minutes. The lysate was then transferred to a Spin-Away filter in a collection tube and centrifuged at 10000 x g for 30 seconds. 1 volume of 96% ethanol (300 µl) was mixed with the flow-through. The mixture was transferred to a Zymo-Spin IIICG column and centrifuged for 30 seconds at 10000 x g, and the flow-through was discarded. 400 µl of RNA Prep Buffer was added to the column, centrifuged with the same speed and duration as previously, and the flow-through was discarded. Then, 700 µl of Wash Buffer with ethanol was added, and the flow-through was

discarded after centrifugation. The second wash with 400 µl of the Wash buffer finalized with the 2-minute centrifugation at the maximum speed. Then, the column was transferred to a new microtube, and total RNA was eluted in 30 µl of DNase/RNase-Free water added to the center of the column matrix, followed by centrifugation for 1 minute at maximum speed. RNA concentrations were measured using a NanoDrop 1000 Spectrophotometer (*Thermo Fisher Scientific*).

Next, total RNA was treated with DNase. For this, approximately 5000 ng of RNA were mixed with 4 µl of 10x Turbo DNase buffer (*Thermo Fisher Scientific*), 1.5 µl of Turbo DNase (*Thermo Fisher Scientific*), and DNase/RNase free water in a volume required to fill up the reaction to 40 µl. Then, the mix was incubated at 37 ˚C for one hour. For DNase inactivation, 15 mM EDTA was added, and the mixture was incubated at 75 ˚C for 10 minutes. After incubation, half of the current reaction volume of 7.5 mM LiCl was added. The tubes were vortexed thoroughly and incubated overnight at -20 ˚C. Next day, the samples were centrifuged for 25 minutes at 4 ˚C at the maximum speed using MicroCL 21R Microcentrifuge (*Thermo Fisher Scientific*). The supernatant was discarded, and 30 µl of 75% ice-cold ethanol was added. Centrifugation at 4 ˚C for 10 minutes was performed. The ethanol was then carefully removed, and the tubes stayed open for a couple of minutes to fully evaporate the ethanol. Then, the RNA was dissolved in 13 µl of DNase/RNase-free water, and concentrations were measured.

2.2.7 Complementary DNA synthesis

RevertAid First Strand cDNA Synthesis Kit (*Thermo Fisher Scientific*) was used to synthesize complementary DNA (cDNA). 1500 ng of the DNase-treated and precipitated RNA was taken, mixed with 1 µl oligo(dT) primer, and DNase/RNase-free water up to total volume of 12 µl. The mixture was incubated at 65 ˚C for 5 minutes, chilled on ice, spun down, and placed back on ice. 5x Reaction Buffer in a volume of 4 µl, 1 µl of RiboLock RNase Inhibitor (20 U/µl), 2 µl of 10 mM dNTP Mix, and 1 µl of RevertAid M-MuLV RT (200 U/µl) were added with the total volume of the reaction of 20 µl. The mixture was gently mixed, centrifuged briefly, and then incubated at 44 ˚C for 60 minutes. The reaction mix was incubated at 70 ˚C for 5 minutes to terminate it.

2.2.8 qRT-PCR

Gene expression levels were measured using qRT-PCR (qPCR). The reaction volume of 10 µl contained 2 µl of 5x EvaGreen (Solis Biodyne), 0.5 µl of the synthesized cDNA, 6.5 µl of DNase/RNase free water, and 1 µl of the 10 pmol/µl primer mixes diluted in water (Appendix 1). Each sample was analyzed in 3 replicates using the 384-well qPCR plates (*Roche Diagnostics*). The following program for qPCR was performed using the QuantStudio Real-Time PCR System (*Thermo Fisher Scientific*): 12 min of denaturation and DNA polymerase activation at 95 ˚C, 40 cycles of amplification (denaturation 95 ˚C 15 s, annealing, 60 ˚C 15 s, synthesis 72 ˚C 15 s). Expression levels of the housekeeping genes GAPDH or ACTB were measured for normalization of the target gene mRNA expression levels.

2.3 RESULTS

2.3.1 Replication efficiency of the HPV5-Nluc genome in U2OS cells and HPEKs treated with the compounds

Before this study, two compounds inhibiting HPV5 replication were identified through a high-throughput screening (HTS) of an NCI Diversity set VI library consisting of 1584 compounds. To confirm the ability of these two compounds to inhibit HPV5 replication, U2OS cells and HPEKs were transfected with the HPV5-Nluc genome. 24 hours post-transfection, the cells were treated with two different concentrations of the compounds. We used luciferase assay to determine the level of genome replication, because the Nluc region was inserted in used genomes. It was shown that for the genomes constructed this way, the luciferase activity correlates with the viral genome copies number (Piirsoo *et al.*, 2019).

For U2OS, #1 and #2 compounds were both used in 20 µM and 40 µM concentrations, and timepoints were taken at 48-, 72-, and 96 hours post-transfection. Luciferase activity was measured as described in the methodology part 2.2.5. The data were normalized to AP activity that represents a number of viable cells in a sample (Figure 4).

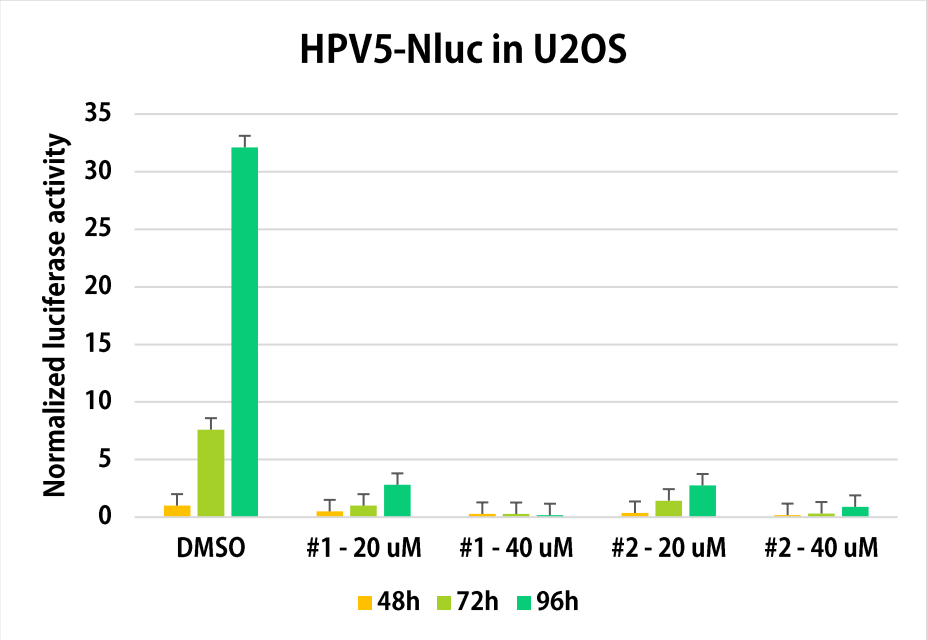


Figure 4. Luciferase assay of HPV5-Nluc genome in U2OS cells treated with DMSO as control, #1 and #2 compounds in 20 µM and 40 µM concentrations for 48-, 72-, and 96 hours post-transfection. The data were normalized using AP activity. The first timepoint data for DMSO were set as 1, and the data from other samples were calculated relative to this. The data is presented as the average mean + standard deviation (SD) of four replicates.

For HPEKs, lower concentrations of the chemicals were used: 7.5 µM and 15 µM for the #1 compound and 4 µM and 8 µM – for the second in order to avoid any potentially toxic effects to highly sensitive and delicate primary cells. As some treated HPEKs displayed unusual morphology, 1.5 mM $CaCl_2$ was used as a control to induce differentiation in HPEK cells. Cells were treated for 72-, 96-, and 120 hours. The data were obtained through a luciferase assay and normalized to total protein concentrations measured using BCA assay as described in the methodology part 2.2.5 (Figure 5).

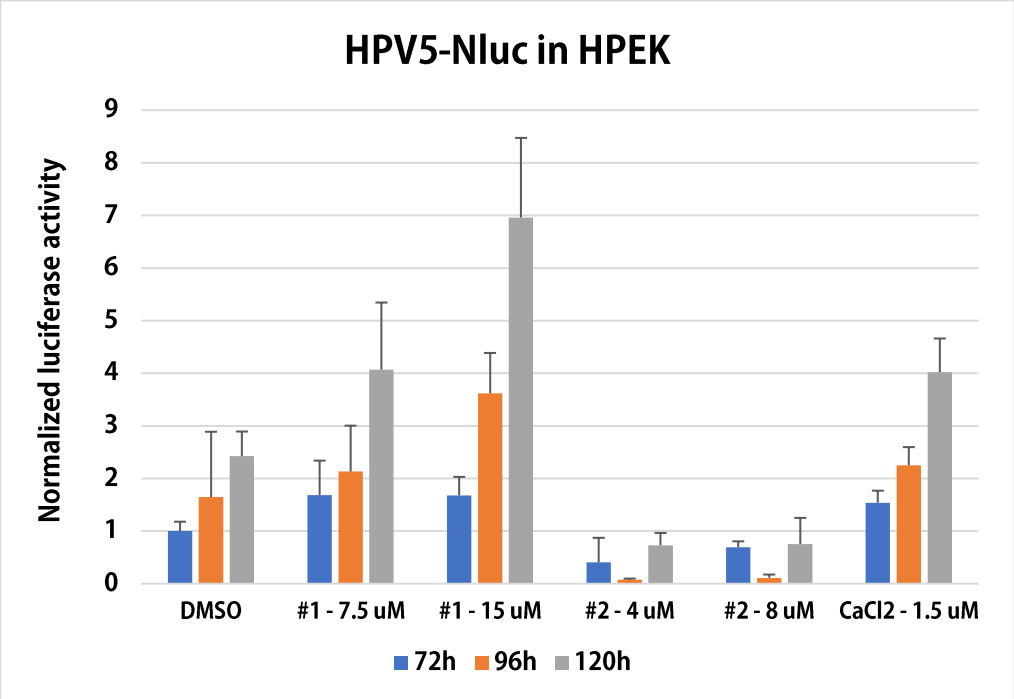


Figure 5. Luciferase assay of HPEKs transfected with the HPV5-Nluc genome and treated with DMSO as control, #1 compound in 7.5 µM and 15 µM concentrations, #2 compound in 4 µM and 8 µM concentrations for 72-, 96-, and 120 hours post-transfection, and 1.5 mM $CaCl_2$ treatment. The data were normalized to total protein concentrations. The first timepoint data for DMSO were set as 1, and the data from other samples were calculated relative to this. The data is presented as the average mean + SD of four replicates.

In both cell types, HPV5-Nluc genome replicated efficiently, and normalized Nluc activity increased in time 30-fold in U2OS cells and 2-fold in HPEKs treated with DMSO. In U2OS cells, both compounds were able to inhibit HPV5 replication in a concentration-dependent manner (Figure 4). However, in HPEKs, compound #1 failed to inhibit the HPV5-Nluc replication (Figure 5). Moreover, treatment with compound #1 resulted in elevated HPV5-Nluc replication efficiency compared to the samples treated with DMSO. The effects of

compound #1 were even higher than that of $CaCl_2$, which is known to induce differentiation of HPEKs accompanied by an increase in HPV copy numbers. As it has been expected, treatment with $CaCl_2$ resulted in an increase in HPV5 copy numbers compared to the control samples (Figure 5). The compound #2 inhibited replication of HPV5 also in HPEKs, and its efficiency was similar to that observed in U2OS cells (Figure 5).

2.3.2 Replication of HPV5 genome deficient in the E8^E2 repressor protein in U2OS cells

To evaluate whether the compounds influence the E8^E2 inhibitor protein, two genomes, HPV5-Nluc and HPV5-NlucE8$^-$, were transfected simultaneously. An experiment performed only with one genome may not be comparable with another because many factors influence the results, such as different transfection efficiency, status of cells, and dilution of Nluc substrate.

The Nluc activity was measured in U2OS cells transfected with the HPV5-NlucE8$^-$ (Figure 6) and compared to such activity in cells transfected with the HPV5-Nluc (Figure 4). The average inhibition percentage in both cases was calculated and compared (Table 1). When comparing the effect of the compounds on two genomes, we do not see drastic differences, except for the #2 compound 20 μM concentration. This can be explained by HPV5-NlucE8$^-$ extremely high copy numbers due to deficiency in inhibitor protein E8^E2., In this case, higher concentrations of an inhibitor are needed to achieve a similar inhibitory effect. The conclusion can be made that the compounds do not influence the E8^E2 protein.

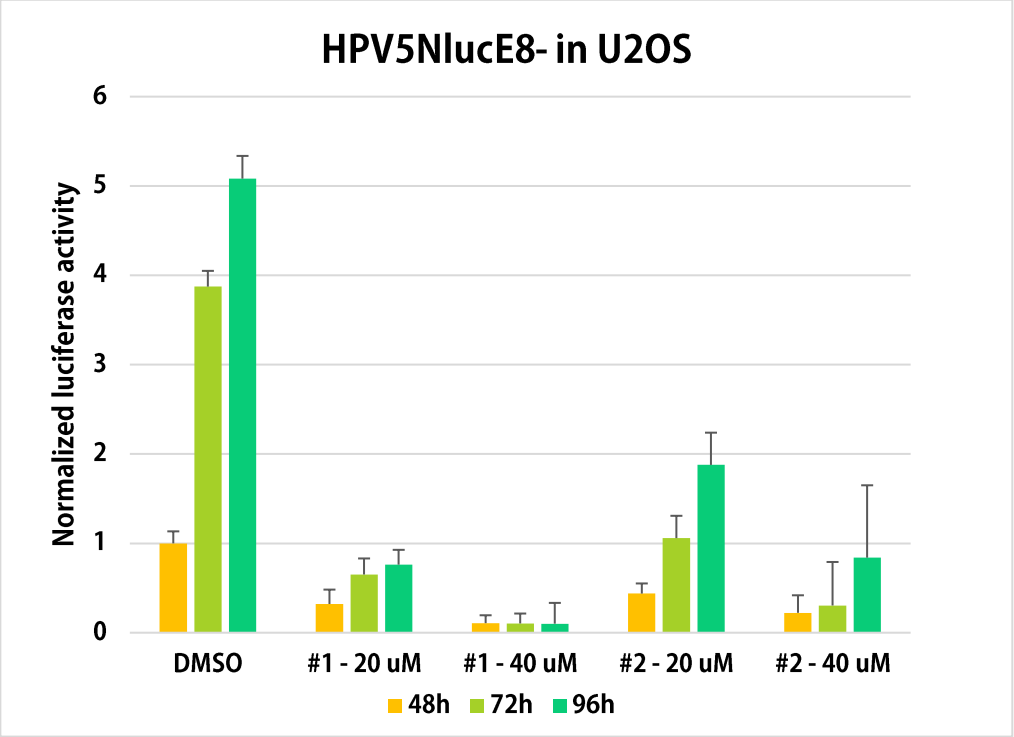


Figure 6. Luciferase assay of HPV5NlucE8$^-$ genome in U2OS cells treated with DMSO as control, and #1 and #2 compounds in 20 µM and 40 µM concentrations. Nluc activity was measured 48-, 72-, and 96 hours post-transfection. The data were normalized to AP activity. The first timepoint data for DMSO were set as 1, and the data from other samples were calculated relative to this. The data are presented as the average mean + SD of four replicates.

Table 1. Comparison of inhibitory effects of #1 and #2 compounds on two genomes. The numbers represent the average percentage of inhibition from three timepoints: 48-, 72-, and 96 hours. The first timepoint data for DMSO were set as 100%, and the data from other samples were calculated relative to this.

	HPV5-Nluc	HPV5-NlucE8$^-$
#1 - 20uM	32	38
#1 - 40uM	15	8
#2 - 20uM	21	51
#2 - 40uM	8	10

2.3.3 Levels of transcripts of the osteoblast and keratinocyte differentiation factors in U2OS cells

As the HPV5 replication levels in the case of $CaCl_2$ and #1 compound treatments were similar, qPCR was performed to determine levels of differentiation markers for osteoblasts and keratinocytes in U2OS cells treated with the studied compounds. Total RNA was obtained from U2OS treated with DMSO and both compounds in 20 µM and 40 µM concentrations. Then, cDNA was synthesized, and mRNA expression levels of the differentiation markers were determined using qPCR as described in methodology parts 2.2.7 and 2.2.8, respectively.

First, expression levels of the RUNX2, SP7, and BGLAP differentiation markers that control osteoblast differentiation were measured in the osteosarcoma U2OS cells (Figure 7). The results showed that compound #1 inhibited the expression of all studied genes involved in osteoblast differentiation at least 5-fold, and compound #2 demonstrated a slight inhibitory effect only on BGLAP expression and had no significant effect on the expression of RUNX2 and SP7 transcription factors.

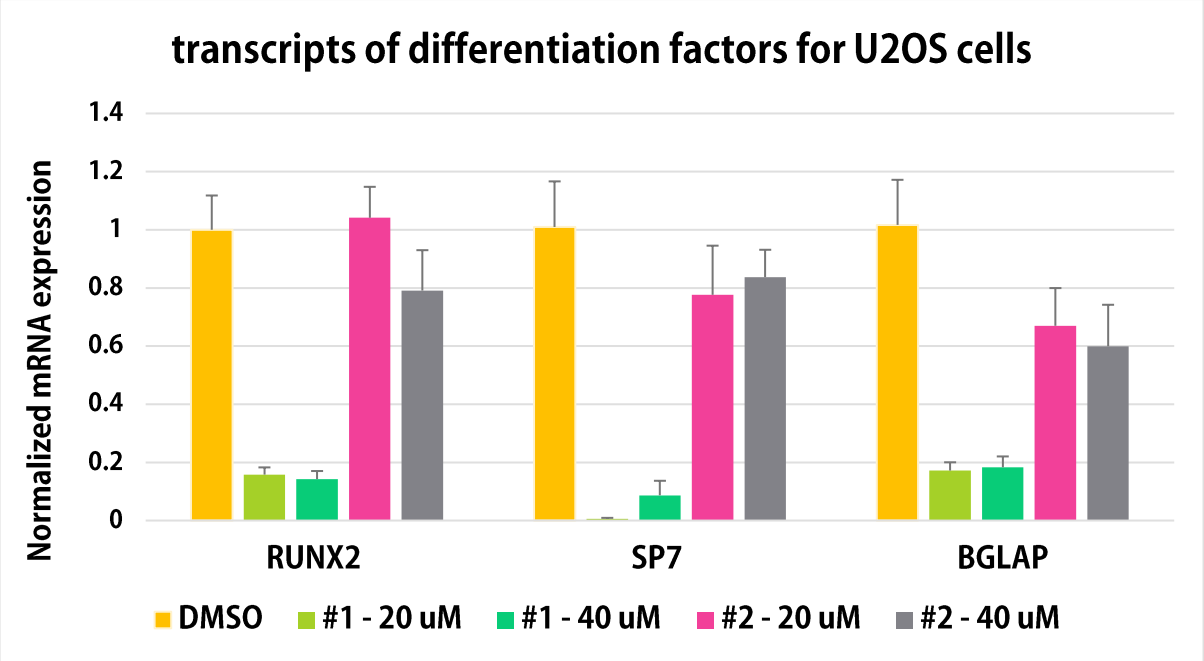


Figure 7. mRNA expression levels of the osteoblast differentiation factors RUNX2, SP7, and BGLAP in U2OS cells treated with DMSO as control, #1 and #2 compounds were measured using qPCR for 48 hours post-transfection. The data were normalized to the housekeeping gene GAPDH. The data for DMSO were set as 1, and the data from other samples were calculated relative to this. The data are presented as the average mean + SD of three replicates.

It has been previously described that U2OS cells express keratinocyte differentiation markers in dense culture (Geimanen *et al.*, 2011). Therefore, we analyzed expression levels of the keratinocyte differentiation factors in U2OS cells using qPCR and oligonucleotides specific for keratin-14, involucrin, keratin-10, and loricrin (Figure 8). Treatment of U2OS cells with the #1 compound resulted in a 3-5-fold increase in keratin-14 transcripts compared to the DMSO data, and the level of involucrin transcripts was 2-3-fold higher compared to the control. In contrast, expression of the loricrin decreased in response to the compound #1, and expression level of the keratin-10 varied remaining similar to the control. For the compound #2, transcript levels, compared with DMSO treatment, are either decreased or similar for all differentiation factors, except for keratin-10 in case of higher concentration #2 compound, which exhibits small growth.

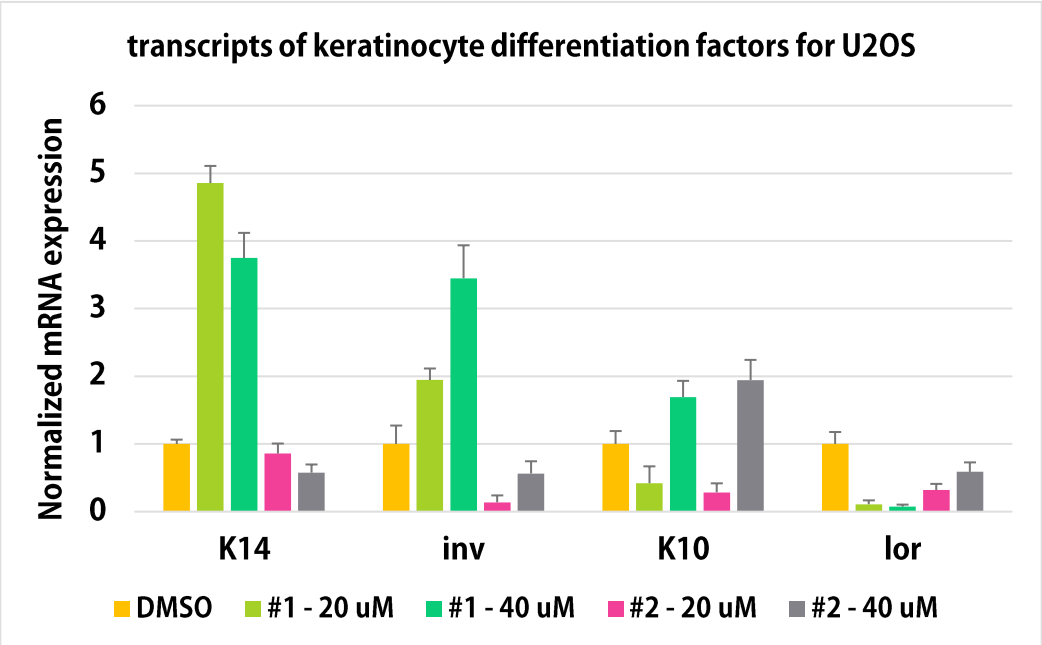


Figure 8. mRNA expression levels of the keratinocyte differentiation factors K14 (keratin-14), inv (involucrin), K10 (keratin-10), and lor (loricrin) in U2OS cells treated with DMSO as control, #1 and #2 compounds were measured using qPCR for 48 hours post-transfection. The data were normalized to the housekeeping genes GAPDH and ACTB. The data for DMSO was set as 1, and the data from other samples were calculated relative to this. The data are presented as the average mean + SD of three replicates.

2.3.4 Levels of keratinocyte differentiation factors transcripts in HPEK cells

Next, I investigated the impact of the studied compounds on the differentiation of HPEKs, given their role as host cells for HPV, and since the #1 compound up-regulated HPV5-Nluc replication (Figure 5). HPEK cells were treated with DMSO, 12 μM of the #1 compound, 15 μM of the #2, and 1.5 μM $CaCl_2$ as a differentiation inducer. A comparative analysis of the effects of these compounds on cellular differentiation was performed. The gene expression levels were measured at two timepoints – 24- and 48 hours post-treatment. After 24-hour treatment, it is evident that $CaCl_2$ induced expression of all studied genes except K10 (Figure 9A), and the levels of the differentiation markers increased further 48 h post-treatment (Figure 9B). Compound #1 induced expression level of loricrin and involucrin. Moreover, at the 48-hour timepoint, loricrin levels continued to increase, reaching a level more than 40-fold higher than that of the DMSO control, while $CaCl_2$ exhibited only a 10-fold increase relative to DMSO-treated samples (Figure 9B). The transcription of involucrin increased to a level 2-fold higher compared to the DMSO-treated control cells, which is less than the approximately 7-fold increase obtained in response to $CaCl_2$. For compound #2, for both timepoints and all factors the levels were close to the DMSO control, except for the loricrin level at 48 hours. There, loricrin exhibited an almost 6-fold increase relative to DMSO-treated samples.

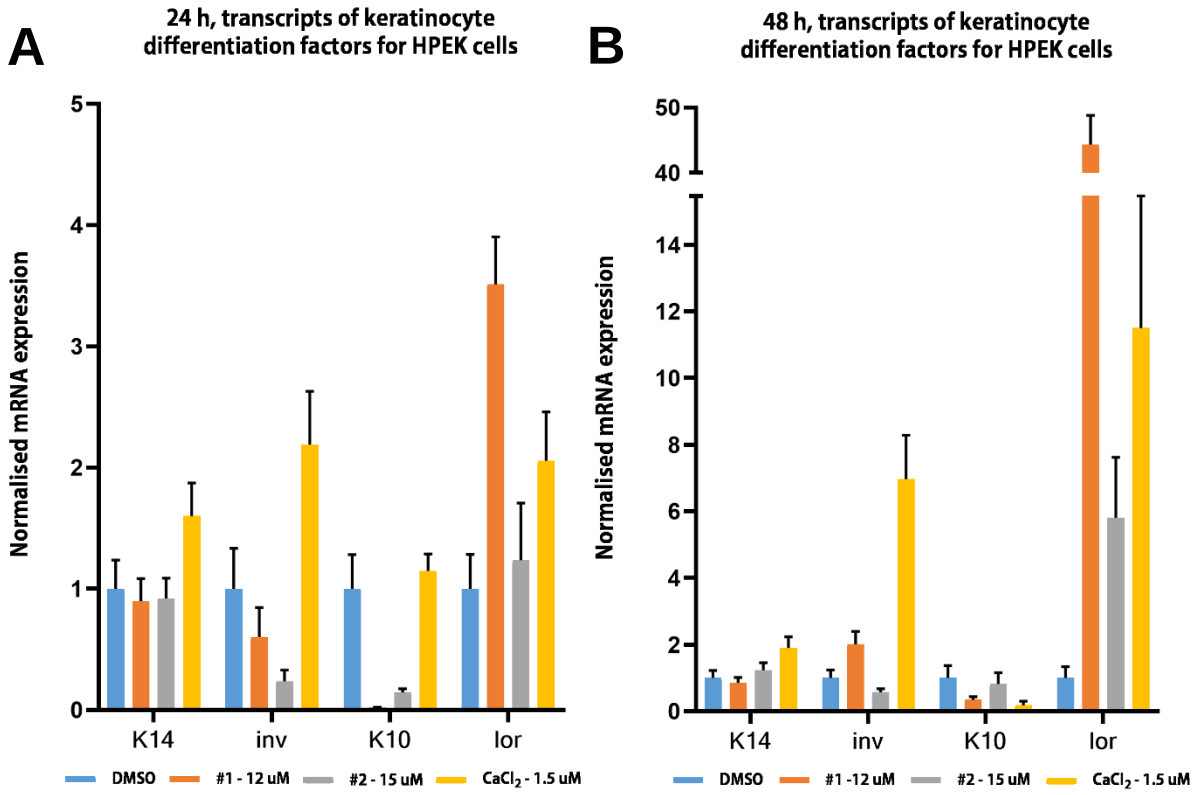


Figure 9. mRNA expression levels of the keratinocyte differentiation factors K14 (keratin-14), inv (involucrin), K10 (keratin-10), and lor (loricrin) in HPEK cells treated with DMSO as control and $CaCl_2$ as differentiation control, #1 and #2 compounds were measured using qPCR for 24-hour treatment (A) and 48-hour treatment (B). The data were normalized to the housekeeping genes GAPDH and ACTB. The data for DMSO was set as 1, and the data from other samples were calculated relative to this. The data are presented as the average mean + SD of three replicates.

2.4 DISCUSSION

HPV infection is widely spread among sexually active people, with more than 80% of them either infected or will be in the future (Chesson *et al*., 2014). HPV can also be transmitted through household contacts or from mother to child during pregnancy (Hong *et al*., 2013; Antonsson *et al.,* 2003). While usually present asymptomatically on the skin of healthy people, the HPV5 type studied in this work can induce skin carcinomas in people with weakened immunity, for instance, after organ transplantation or more susceptible to the virus due to the genetic *Epidermodysplasia verruciformis* disease (Orth, 2008). Although vaccines against some types of oncogenic mucosal HPVs have been developed and widely used, they are ineffective against cutaneous types and existing infections. At present, there are no reliable antivirals to cure HPV-associated diseases. This indicates the importance of developing treatments that could inhibit HPV activity.

In this thesis, the impact of two small-molecule compounds on HPV5 replication in U2OS and HPEK cells was studied and confirmed. These compounds were previously identified in high-throughput screening of the NCI Diversity Set VI library. In addition, it has been found that the inhibitors have no impact on activity of the E8^E2 repressor protein, since similar effects were obtained analyzing replication efficiency of the HPV5-Nluc and HPV5-Nluc-E8$^-$ genomes in U2OS cells. While investigating the effect of compounds on HPV5 genome replication, it was discovered that the #1 compound does not inhibit HPV5 in HPEKs as it does in U2OS cells. The inability of the compound #1 to inhibit HPV5 replication in HPEKs led us to the hypothesis that this effect could be caused by the cell differentiation, since HPV genome copy numbers increase in differentiated cells when the virus enters the vegetative amplification phase of its infection cycle.

The compounds' ability to induce differentiation of U2OS cells and HPEKs was analyzed. It was revealed that U2OS did not differentiate in the osteoblast direction, but osteosarcoma cells have exhibited transcription of keratin-14 and involucrin – keratinocyte differentiation markers. This implies additional advantages of utilizing U2OS cells as a model system for HPV research. For the HPEK – HPV host cells, observations confirmed their differentiation in the case of the #1 compound, as mRNA levels of differentiation markers increased – loricrin expression level was even 40 times higher than in the control, and involucrin expression level increased two times in response to the compound #1. Also, expression of K14 and involucrin was upregulated in response to the compound #1 in U2OS cells. In contrast, effects of the

compound #2 on HPEK and U2OS differentiation were negligible. However, further analysis is needed to confirm these observations.

Taken together, the results indicate that both compounds inhibit HPV5 replication in U2OS cells. However, the #1 compound does not inhibit the replication of HPV5 in HPEKs, probably due to the induction of their differentiation. The #2 compound inhibits viral genome replication in both cell lines without significant adverse effects on the cells themselves; therefore, it was chosen for further studies. The #1 compound has been excluded from further studies due to its inefficiency in HPEKs and its ability to induce HPEK differentiation.

CONCLUSION

This thesis aimed to confirm the inhibitory effect of two small-molecule compounds identified through a high-throughput screening on HPV5 replication. Also, to determine the effect of the identified inhibitors on the differentiation of U2OS cells and HPEKs.

The study resulted in the following findings:

- The studied reagents inhibit replication of HPV5 in U2OS cells
- The studied reagents do not cause differentiation in osteoblast direction of U2OS cells
- The #1 reagent leads to increased transcription of keratin-14 and involucrin in U2OS cells
- The #1 reagent does not inhibit HPV5 replication in HPEK, likely due to its induction of HPEK differentiation

ACKNOWLEDGMENTS

I am genuinely grateful to Elina Lototskaja-Perepelenko for her exceptional guidance, patience, and responsiveness; having such a great supervisor is not possible to be overrated. I also express my sincere gratitude to Alla Piirsoo for the opportunity to work on an exciting topic in the Piirsoo group and for valuable advice and reviews. I appreciate the experiences I gained and the new techniques I learned. I am thankful for all the dearest support from my family and the people closest to me. I could not wish for better outcomes, topic, or people that helped me throughout this challenging path.

REFERENCES

Abakushina, E. V., Popova, L. I., Zamyatnin, A. A., Jr, Werner, J., Mikhailovsky, N. V., & Bazhin, A. V. (2021). The Advantages and Challenges of Anticancer Dendritic Cell Vaccines and NK Cells in Adoptive Cell Immunotherapy. *Vaccines*, *9*(11), 1363. https://doi.org/10.3390/vaccines9111363

Alam, H., Sehgal, L., Kundu, S. T., Dalal, S. N., & Vaidya, M. M. (2011). Novel function of keratins 5 and 14 in proliferation and differentiation of stratified epithelial cells. *Molecular biology of the cell*, *22*(21), 4068–4078. https://doi.org/10.1091/mbc.E10-08-0703

Antonsson, A., Karanfilovska, S., Lindqvist, P. G., & Hansson, B. G. (2003). General acquisition of human papillomavirus infections of skin occurs in early infancy. Journal of clinical microbiology, 41(6), 2509–2514. https://doi.org/10.1128/JCM.41.6.2509-2514.2003

Banks-Schlegel, S., & Green, H. (1981). Involucrin synthesis and tissue assembly by keratinocytes in natural and cultured human epithelia. *The Journal of cell biology*, *90*(3), 732–737. https://doi.org/10.1083/jcb.90.3.732

Bergvall, M., Melendy, T., & Archambault, J. (2013). The E1 proteins. *Virology*, *445*(1-2), 35–56. https://doi.org/10.1016/j.virol.2013.07.020

Bernard, H. U., Burk, R. D., Chen, Z., van Doorslaer, K., zur Hausen, H., & de Villiers, E. M. (2010). Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. *Virology*, *401*(1), 70–79. https://doi.org/10.1016/j.virol.2010.02.002

Bravo, I. G., & Alonso, A. (2004). Mucosal human papillomaviruses encode four different E5 proteins whose chemistry and phylogeny correlate with malignant or benign growth. *Journal of virology*, *78*(24), 13613–13626. https://doi.org/10.1128/JVI.78.24.13613-13626.2004

Bryan, J. T., & Brown, D. R. (2001). Transmission of human papillomavirus type 11 infection by desquamated cornified cells. *Virology*, *281*(1), 35–42. https://doi.org/10.1006/viro.2000.0777

Candi, E., Schmidt, R., & Melino, G. (2005). The cornified envelope: a model of cell death in the skin. *Nature reviews. Molecular cell biology*, *6*(4), 328–340. https://doi.org/10.1038/nrm1619

Chang, E. Y., Chen, C. H., Ji, H., Wang, T. L., Hung, K., Lee, B. P., Huang, A. Y., Kurman, R. J., Pardoll, D. M., & Wu, T. (2000). Antigen-specific cancer immunotherapy using a GM-CSF secreting allogeneic tumor cell-based vaccine. International journal of cancer, 86(5), 725–730. https://doi.org/10.1002/(sici)1097-0215(20000601)86:5<725::aid-ijc19>3.0.co;2-k

Chang, J. L., Tsao, Y. P., Liu, D. W., Huang, S. J., Lee, W. H., & Chen, S. L. (2001). The expression of HPV-16 E5 protein in squamous neoplastic changes in the uterine cervix. *Journal of biomedical science*, *8*(2), 206–213. https://doi.org/10.1007/BF02256414

Chellappan, S., Kraus, V. B., Kroger, B., Munger, K., Howley, P. M., Phelps, W. C., & Nevins, J. R. (1992). Adenovirus E1A, simian virus 40 tumor antigen, and human papillomavirus E7 protein share the capacity to disrupt the interaction between transcription factor E2F and the retinoblastoma gene product. *Proceedings of the National Academy of Sciences of the United States of America*, *89*(10), 4549–4553. https://doi.org/10.1073/pnas.89.10.4549

Chesson, H. W., Dunne, E. F., Hariri, S., & Markowitz, L. E. (2014). The estimated lifetime probability of acquiring human papillomavirus in the United States. Sexually transmitted diseases, 41(11), 660–664. https://doi.org/10.1097/OLQ.0000000000000193

Condit, R. C., Williamson, A. L., Sheets, R., Seligman, S. J., Monath, T. P., Excler, J. L., Gurwith, M., Bok, K., Robertson, J. S., Kim, D., Michael Hendry, R., Singh, V., Mac, L. M., Chen, R. T., & Brighton Collaboration Viral Vector Vaccines Safety Working Group (V3SWG) (2016). Unique safety issues associated with virus-vectored vaccines: Potential for and theoretical consequences of recombination with wild type virus strains. Vaccine, 34(51), 6610–6616. https://doi.org/10.1016/j.vaccine.2016.04.060

Crook, T., Tidy, J. A., & Vousden, K. H. (1991). Degradation of p53 can be targeted by HPV E6 sequences distinct from those required for p53 binding and trans-activation. *Cell*, *67*(3), 547–556. https://doi.org/10.1016/0092-8674(91)90529-8

Davy, C. E., Jackson, D. J., Wang, Q., Raj, K., Masterson, P. J., Fenner, N. F., Southern, S., Cuthill, S., Millar, J. B., & Doorbar, J. (2002). Identification of a G(2) arrest domain in the E1 wedge E4 protein of human papillomavirus type 16. Journal of virology, 76(19), 9806–9818. https://doi.org/10.1128/jvi.76.19.9806-9818.2002

DiMaio, D., & Petti, L. M. (2013). The E5 proteins. *Virology*, *445*(1-2), 99–114. https://doi.org/10.1016/j.virol.2013.05.006

Doorbar J. (2013). The E4 protein; structure, function and patterns of expression. *Virology*, *445*(1-2), 80–98. https://doi.org/10.1016/j.virol.2013.07.008

Ducy, P., & Karsenty, G. (1995). Two distinct osteoblast-specific cis-acting elements control expression of a mouse osteocalcin gene. *Molecular and cellular biology*, *15*(4), 1858–1869. https://doi.org/10.1128/MCB.15.4.1858

Dyson, N., Howley, P. M., Münger, K., & Harlow, E. (1989). The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science (New York, N.Y.)*, *243*(4893), 934–937. https://doi.org/10.1126/science.2537532

Efimova, T., Broome, A. M., & Eckert, R. L. (2003). A regulatory role for p38 delta MAPK in keratinocyte differentiation. Evidence for p38 delta-ERK1/2 complex formation. *The Journal of biological chemistry*, *278*(36), 34277–34285. https://doi.org/10.1074/jbc.M302759200

Egawa, N., Egawa, K., Griffin, H., & Doorbar, J. (2015). Human Papillomaviruses; Epithelial Tropisms, and the Development of Neoplasia. *Viruses*, *7*(7), 3863–3890. https://doi.org/10.3390/v7072802

Einstein, M. H., Kadish, A. S., Burk, R. D., Kim, M. Y., Wadler, S., Streicher, H., Goldberg, G. L., & Runowicz, C. D. (2007). Heat shock fusion protein-based immunotherapy for treatment of cervical intraepithelial neoplasia III. Gynecologic oncology, 106(3), 453–460. https://doi.org/10.1016/j.ygyno.2007.04.038

Enomoto, H., Furuichi, T., Zanma, A., Yamana, K., Yoshida, C., Sumitani, S., Yamamoto, H., Enomoto-Iwamoto, M., Iwamoto, M., & Komori, T. (2004). Runx2 deficiency in chondrocytes causes adipogenic changes in vitro. *Journal of cell science*, *117*(Pt 3), 417–425. https://doi.org/10.1242/jcs.00866

Feltkamp, M. C., de Koning, M. N., Bavinck, J. N., & Ter Schegget, J. (2008). Betapapillomaviruses: innocent bystanders or causes of skin cancer. *Journal of clinical virology: the official publication of the Pan American Society for Clinical Virology*, *43*(4), 353–360. https://doi.org/10.1016/j.jcv.2008.09.009

Ferreira, A. R., Ramalho, A. C., Marques, M., & Ribeiro, D. (2020). The Interplay between Antiviral Signalling and Carcinogenesis in Human Papillomavirus Infections. Cancers, 12(3), 646. https://doi.org/10.3390/cancers12030646

Filipazzi, P., Pilla, L., Mariani, L., Patuzzo, R., Castelli, C., Camisaschi, C., Maurichi, A., Cova, A., Rigamonti, G., Giardino, F., Di Florio, A., Asioli, M., Frati, P., Sovena, G., Squarcina, P., Maio, M., Danielli, R., Chiarion-Sileni, V., Villa, A., Lombardo, C., … Rivoltini, L. (2012). Limited induction of tumor cross-reactive T cells without a measurable clinical benefit in early melanoma patients vaccinated with human leukocyte antigen class I-modified peptides. Clinical cancer research : an official journal of the American Association for Cancer Research, 18(23), 6485–6496. https://doi.org/10.1158/1078-0432.CCR-12-1516

Fisher C. (2015). Recent Insights into the Control of Human Papillomavirus (HPV) Genome Stability, Loss, and Degradation. Journal of clinical medicine, 4(2), 204–230. https://doi.org/10.3390/jcm4020204

Forslund, O., Johansson, H., Madsen, K. G., & Kofoed, K. (2013). The nasal mucosa contains a large spectrum of human papillomavirus types from the Betapapillomavirus and Gammapapillomavirus genera. *The Journal of infectious diseases*, *208*(8), 1335–1341. https://doi.org/10.1093/infdis/jit326

Geimanen, J., Isok-Paas, H., Pipitch, R., Salk, K., Laos, T., Orav, M., Reinson, T., Ustav, M., Jr, Ustav, M., & Ustav, E. (2011). Development of a cellular assay system to study the genome replication of high- and low-risk mucosal and cutaneous human papillomaviruses. *Journal of virology*, *85*(7), 3315–3329. https://doi.org/10.1128/JVI.01985-10

Gissmann, L., Diehl, V., Schultz-Coulon, H. J., & zur Hausen, H. (1982). Molecular cloning and characterization of human papilloma virus DNA derived from laryngeal papilloma. *Journal of virology*, *44*(1), 393–400. https://doi.org/10.1128/JVI.44.1.393-400.1982

Graham S. V. (2010). Human papillomavirus: gene expression, regulation and prospects for novel diagnostic methods and antiviral therapies. *Future microbiology*, *5*(10), 1493–1506. https://doi.org/10.2217/fmb.10.107

Hong, Y., Li, S. Q., Hu, Y. L., & Wang, Z. Q. (2013). Survey of human papillomavirus types and their vertical transmission in pregnant women. BMC infectious diseases, 13, 109. https://doi.org/10.1186/1471-2334-13-109

Ilves, I., Kivi, S., & Ustav, M. (1999). Long-term episomal maintenance of bovine papillomavirus type 1 plasmids is determined by attachment to host chromosomes, which Is mediated by the viral E2 protein and its binding sites. Journal of virology, 73(5), 4404–4412. https://doi.org/10.1128/JVI.73.5.4404-4412.1999

Kadaja, M., Silla, T., Ustav, E., & Ustav, M. (2009). Papillomavirus DNA replication - from initiation to genomic instability. *Virology*, *384*(2), 360–368. https://doi.org/10.1016/j.virol.2008.11.032

Kawane, T., Komori, H., Liu, W., Moriishi, T., Miyazaki, T., Mori, M., Matsuo, Y., Takada, Y., Izumi, S., Jiang, Q., Nishimura, R., Kawai, Y., & Komori, T. (2014). Dlx5 and mef2 regulate a novel runx2 enhancer for osteoblast-specific expression. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research*, *29*(9), 1960–1969. https://doi.org/10.1002/jbmr.2240

Kim, J. W., Hung, C. F., Juang, J., He, L., Kim, T. W., Armstrong, D. K., Pai, S. I., Chen, P. J., Lin, C. T., Boyd, D. A., & Wu, T. C. (2004). Comparison of HPV DNA vaccines employing intracellular targeting strategies. *Gene therapy*, *11*(12), 1011–1018. https://doi.org/10.1038/sj.gt.3302252

Kines, R. C., Thompson, C. D., Lowy, D. R., Schiller, J. T., & Day, P. M. (2009). The initial steps leading to papillomavirus infection occur on the basement membrane prior to cell surface binding. Proceedings of the National Academy of Sciences of the United States of America, 106(48), 20458–20463. https://doi.org/10.1073/pnas.0908502106

King, L. E., Dornan, E. S., Donaldson, M. M., & Morgan, I. M. (2011). Human papillomavirus 16 E2 stability and transcriptional activation is enhanced by E1 via a direct protein-protein interaction. *Virology*, *414*(1), 26–33. https://doi.org/10.1016/j.virol.2011.03.002

Kurpinski, K., Chu, J., Hashi, C., & Li, S. (2006). Anisotropic mechanosensing by mesenchymal stem cells. Proceedings of the National Academy of Sciences of the United States of America, 103(44), 16095–16100. https://doi.org/10.1073/pnas.0604182103

Lace, M. J., Anson, J. R., Thomas, G. S., Turek, L. P., & Haugen, T. H. (2008). The E8--E2 gene product of human papillomavirus type 16 represses early transcription and replication

but is dispensable for viral plasmid persistence in keratinocytes. *Journal of virology*, *82*(21), 10841–10853. https://doi.org/10.1128/JVI.01481-08

Li, Y., Ge, C., Long, J. P., Begun, D. L., Rodriguez, J. A., Goldstein, S. A., & Franceschi, R. T. (2012). Biomechanical stimulation of osteoblast gene expression requires phosphorylation of the RUNX2 transcription factor. *Journal of bone and mineral research: the official journal of the American Society for Bone and Mineral Research*, *27*(6), 1263–1274. https://doi.org/10.1002/jbmr.1574

López-Bueno, A., Mavian, C., Labella, A. M., Castro, D., Borrego, J. J., Alcami, A., & Alejo, A. (2016). Concurrence of Iridovirus, Polyomavirus, and a Unique Member of a New Group of Fish Papillomaviruses in Lymphocystis Disease-Affected Gilthead Sea Bream. *Journal of virology*, *90*(19), 8768–8779. https://doi.org/10.1128/JVI.01369-16

Lutzner, M., Croissant, O., Ducasse, M. F., Kreis, H., Crosnier, J., & Orth, G. (1980). A potentially oncogenic human papillomavirus (HPV-5) found in two renal allograft recipients. *The Journal of investigative dermatology*, *75*(4), 353–356. https://doi.org/10.1111/1523-1747.ep12531131

Maeno, T., Moriishi, T., Yoshida, C. A., Komori, H., Kanatani, N., Izumi, S., Takaoka, K., & Komori, T. (2011). Early onset of Runx2 expression caused craniosynostosis, ectopic bone formation, and limb defects. *Bone*, *49*(4), 673–682. https://doi.org/10.1016/j.bone.2011.07.023

Maglennon, G. A., McIntosh, P., & Doorbar, J. (2011). Persistence of viral DNA in the epithelial basal layer suggests a model for papillomavirus latency following immune regression. Virology, 414(2), 153–163. https://doi.org/10.1016/j.virol.2011.03.019

Maines, J. Z., Park, J. K., Williams, M., & McKearin, D. M. (2007). Stonewalling Drosophila stem cell differentiation by epigenetic controls. *Development (Cambridge, England)*, *134*(8), 1471–1479. https://doi.org/10.1242/dev.02810

McCall, C. A., & Cohen, J. J. (1991). Programmed cell death in terminally differentiating keratinocytes: role of endogenous endonuclease. *The Journal of investigative dermatology*, *97*(1), 111–114. https://doi.org/10.1111/1523-1747.ep12478519

Moreno-Lopez, J., Ahola, H., Stenlund, A., Osterhaus, A., & Pettersson, U. (1984). Genome of an avian papillomavirus. *Journal of virology*, *51*(3), 872–875. https://doi.org/10.1128/JVI.51.3.872-875.1984

Moriishi, T., Ozasa, R., Ishimoto, T., Nakano, T., Hasegawa, T., Miyazaki, T., Liu, W., Fukuyama, R., Wang, Y., Komori, H., Qin, X., Amizuka, N., & Komori, T. (2020). Osteocalcin is necessary for the alignment of apatite crystallites, but not glucose metabolism, testosterone synthesis, or muscle mass. *PLoS genetics*, *16*(5), e1008586. https://doi.org/10.1371/journal.pgen.1008586

Muñoz, N., Bosch, F. X., de Sanjosé, S., Herrero, R., Castellsagué, X., Shah, K. V., Snijders, P. J., Meijer, C. J., & International Agency for Research on Cancer Multicenter Cervical Cancer Study Group (2003). Epidemiologic classification of human papillomavirus types associated with cervical cancer. *The New England journal of medicine*, *348*(6), 518–527. https://doi.org/10.1056/NEJMoa021641

Muñoz, N., Bosch, F. X., de Sanjosé, S., Tafur, L., Izarzugaza, I., Gili, M., Viladiu, P., Navarro, C., Martos, C., & Ascunce, N. (1992). The causal link between human papillomavirus and invasive cervical cancer: a population-based case-control study in Colombia and Spain. *International journal of cancer*, *52*(5), 743–749. https://doi.org/10.1002/ijc.2910520513

Nakashima, K., Zhou, X., Kunkel, G., Zhang, Z., Deng, J. M., Behringer, R. R., & de Crombrugghe, B. (2002). The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell, 108(1), 17–29. https://doi.org/10.1016/s0092-8674(01)00622-5

Orth G. (2008). Host defenses against human papillomaviruses: lessons from epidermodysplasia verruciformis. Current topics in microbiology and immunology, 321, 59–83. https://doi.org/10.1007/978-3-540-75203-5_3

Penrose, K. J., & McBride, A. A. (2000). Proteasome-mediated degradation of the papillomavirus E2-TA protein is regulated by phosphorylation and can modulate viral genome copy number. *Journal of virology*, *74*(13), 6031–6038. https://doi.org/10.1128/jvi.74.13.6031-6038.2000

Peh, W. L., Middleton, K., Christensen, N., Nicholls, P., Egawa, K., Sotlar, K., Brandsma, J., Percival, A., Lewis, J., Liu, W. J., & Doorbar, J. (2002). Life cycle heterogeneity in animal models of human papillomavirus-associated disease. *Journal of virology*, *76*(20), 10401–10416. https://doi.org/10.1128/jvi.76.20.10401-10416.2002

Piirsoo, A., Piirsoo, M., Kala, M., Sankovski, E., Lototskaja, E., Levin, V., Salvi, M., & Ustav, M. (2019). Activity of CK2α protein kinase is required for efficient replication of some HPV types. PLoS pathogens, 15(5), e1007788. https://doi.org/10.1371/journal.ppat.1007788

Poumay, Y., & Pittelkow, M. R. (1995). Cell density and culture factors regulate keratinocyte commitment to differentiation and expression of suprabasal K1/K10 keratins. *The Journal of investigative dermatology*, *104*(2), 271–276. https://doi.org/10.1111/1523-1747.ep12612810

Price, P. A., Otsuka, A. A., Poser, J. W., Kristaponis, J., & Raman, N. (1976). Characterization of a gamma-carboxyglutamic acid-containing protein from bone. *Proceedings of the National Academy of Sciences of the United States of America*, *73*(5), 1447–1451. https://doi.org/10.1073/pnas.73.5.1447

Rashid, H., Smith, C. M., Convers, V., Clark, K., & Javed, A. (2024). Runx2 deletion in hypertrophic chondrocytes impairs osteoclast mediated bone resorption. *Bone*, *181*, 117014. https://doi.org/10.1016/j.bone.2024.117014

Régnier, M., Vaigot, P., Darmon, M., & Pruniéras, M. (1986). Onset of epidermal differentiation in rapidly proliferating basal keratinocytes. *The Journal of investigative dermatology*, *87*(4), 472–476. https://doi.org/10.1111/1523-1747.ep12455517

La Rosa, G., Fratini, M., Accardi, L., D'Oro, G., Della Libera, S., Muscillo, M., & Di Bonito, P. (2013). Mucosal and cutaneous human papillomaviruses detected in raw sewages. *PloS one*, *8*(1), e52391. https://doi.org/10.1371/journal.pone.0052391

Sedman, J., & Stenlund, A. (1995). Co-operative interaction between the initiator E1 and the transcriptional activator E2 is required for replicator specific DNA replication of bovine papillomavirus in vivo and in vitro. The EMBO journal, 14(24), 6218–6228. https://doi.org/10.1002/j.1460-2075.1995.tb00312.x

Steinert, P. M., & Marekov, L. N. (1997). Direct evidence that involucrin is a major early isopeptide cross-linked component of the keratinocyte cornified cell envelope. *The Journal of biological chemistry*, *272*(3), 2021–2030. https://doi.org/10.1074/jbc.272.3.2021

Sümer, C., Boz Er, A. B., & Dinçer, T. (2019). Keratin 14 is a novel interaction partner of keratinocyte differentiation regulator: receptor-interacting protein kinase 4. *Turkish journal of biology = Turk biyoloji dergisi*, *43*(4), 225–234. https://doi.org/10.3906/biy-1904-37

Sund, D. T., Brouwer, A. F., Walline, H. M., Carey, T. E., Meza, R., Jackson, T., & Eisenberg, M. C. (2022). Understanding the mechanisms of HPV-related carcinogenesis: Implications for cell cycle dynamics. *Journal of theoretical biology*, *551-552*, 111235. https://doi.org/10.1016/j.jtbi.2022.111235

Thierry, F., & Yaniv, M. (1987). The BPV1-E2 trans-acting protein can be either an activator or a repressor of the HPV18 regulatory region. *The EMBO journal*, *6*(11), 3391–3397. https://doi.org/10.1002/j.1460-2075.1987.tb02662.x

Ustav, M., & Stenlund, A. (1991). Transient replication of BPV-1 requires two viral polypeptides encoded by the E1 and E2 open reading frames. *The EMBO journal*, *10*(2), 449–457. https://doi.org/10.1002/j.1460-2075.1991.tb07967.x

de Villiers, E. M., Fauquet, C., Broker, T. R., Bernard, H. U., & zur Hausen, H. (2004). Classification of papillomaviruses. *Virology*, *324*(1), 17–27. https://doi.org/10.1016/j.virol.2004.03.033

Walboomers, J. M., Jacobs, M. V., Manos, M. M., Bosch, F. X., Kummer, J. A., Shah, K. V., Snijders, P. J., Peto, J., Meijer, C. J., & Muñoz, N. (1999). Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *The Journal of pathology*, *189*(1), 12–19. https://doi.org/10.1002/(SICI)1096-9896(199909)189:1<12::AID-PATH431>3.0.CO;2-F

Yang, D. Y., & Bracken, K. (2016). Update on the new 9-valent vaccine for human papillomavirus prevention. Canadian family physician Medecin de famille canadien, 62(5), 399–402.

Yang, L., Mohr, I., Fouts, E., Lim, D. A., Nohaile, M., & Botchan, M. (1993). The E1 protein of bovine papilloma virus 1 is an ATP-dependent DNA helicase. *Proceedings of the National*

Academy of Sciences of the United States of America, *90*(11), 5086–5090. https://doi.org/10.1073/pnas.90.11.5086

Yoneda, K., Hohl, D., McBride, O. W., Wang, M., Cehrs, K. U., Idler, W. W., & Steinert, P. M. (1992). The human loricrin gene. *The Journal of biological chemistry*, *267*(25), 18060–18066. https://doi.org/10.1016/S0021-9258(19)37152-2

Zhao, K. N., Gu, W., Fang, N. X., Saunders, N. A., & Frazer, I. H. (2005). Gene codon composition determines differentiation-dependent expression of a viral capsid gene in keratinocytes in vitro and in vivo. *Molecular and cellular biology*, *25*(19), 8643–8655. https://doi.org/10.1128/MCB.25.19.8643-8655.2005

Zhou, J., Sun, X. Y., Stenzel, D. J., & Frazer, I. H. (1991). Expression of vaccinia recombinant HPV 16 L1 and L2 ORF proteins in epithelial cells is sufficient for assembly of HPV virion-like particles. Virology, 185(1), 251–257. https://doi.org/10.1016/0042-6822(91)90772-4

Zingkou, E., Pampalakis, G., & Sotiropoulou, G. (2022). Keratinocyte differentiation and proteolytic pathways in skin (patho) physiology. *The International journal of developmental biology*, *66*(1-2-3), 269–275. https://doi.org/10.1387/ijdb.210161gs

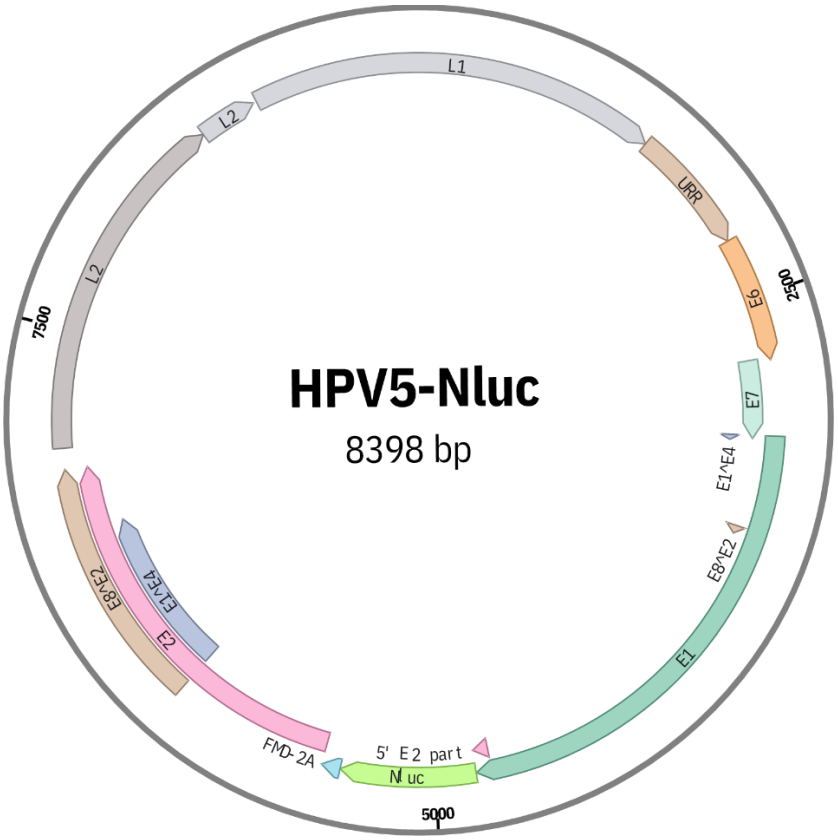
SUPPLEMENTARY DATA

Appendix 1. Primers used in the study

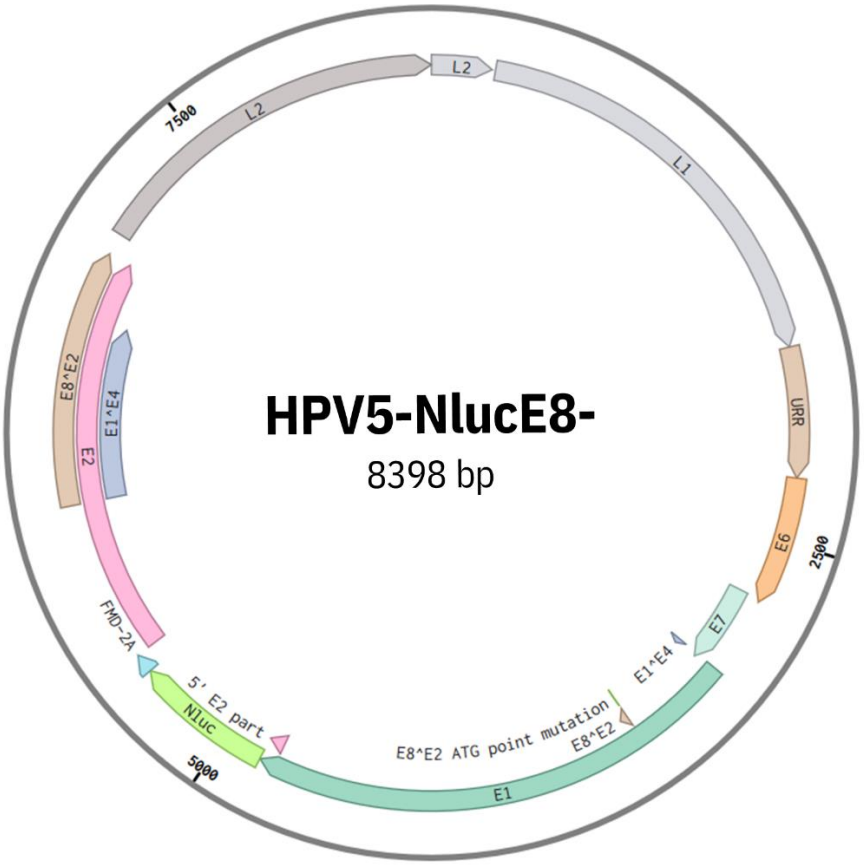
№	Primer	Sequence 5’-3’
1	ACTB F	CAGAGCCTCGCCTTTGCCGA
2	ACTB R	ATCCATGGTGAGCTGGCGGC
3	BGLAP F	GTCCAAGCAGGAGGGCAGCGAG
4	BGLAP R	GTGAGCTCAATCCGGACTGTGAC
5	GAPDH F	CTCTCTGCTCCTCCTGTTCGAC
6	GAPDH R	TGAGCGATGTGGCTCGGCT
7	IVL F	TTCCTCCTCCAGTCAATACC
8	IVL R	CTGTAAAGGGACTGCCTGAG
9	KRT10 F	GGGCTCTGGAAGAATCAAAC
10	KRT10 R	CTTCAGATCGACAATGCCAG
11	KRT14 F	GAAGTGAAGATCCGTGACTG
12	KRT14 R	CAGTGGACAATGCCAATGTC
13	LOR F	CACCCTTCCTGGTGCTTTG
14	LOR R	GGACTGCGTGAAGACCTCT
15	RUNX2 F	CCAAGTAGCAAGGTTCAACGATC

16	RUNX2 R	CCAAGTAGCTACCTATCACAGAG
17	SP7 F	CATCTGCCTGGCTCCTTGGGAC
18	SP7 R	CCATGCTGACGGCAGCGTGCAG

Appendix 2. HPV5-Nluc genome



Appendix 3. HPV5-NlucE8^{-} genome



NON-EXCLUSIVE LICENCE TO REPRODUCE THESIS AND MAKE THESIS PUBLIC

I, Lidiia Zaikina,

1. grant the University of Tartu a free permit (non-exclusive licence) to: reproduce, for the purpose of preservation, including for adding to the DSpace digital archives until the expiry of the term of copyright, my thesis:

“Exploring the impact of small-molecule compounds on human papillomavirus type 5 replication and host cell differentiation”,

supervised by Elina Lototskaja-Perepelenko,

2. I grant the University of Tartu the permit to make the thesis specified in point 1 available to the public via the web environment of the University of Tartu, including via the DSpace digital archives, under the Creative Commons licence CC BY NC ND 4.0, which allows, by giving appropriate credit to the author, to reproduce, distribute the work and communicate it to the public, and prohibits the creation of derivative works and any commercial use of the work from ***22/05/2026*** until the expiry of the term of copyright,

3. I am aware that the author retains the rights specified in points 1 and 2.

4. I confirm that granting the non-exclusive licence does not infringe other persons’ intellectual property rights or rights arising from the personal data protection legislation.

Lidiia Zaikina

22/05/2024