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Adeno-associated virus-based heterologous replicon technology for detection and quantification of adeno- and herpesvirus infections

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Zusammenfassung

Die Entwicklung von Resistenzen gegen bestimmte Medikamente steigt bei Herpes- und Adenovirus Infektionen, vor allem bei Kindern und immungeschwächten Personen, stark an. Gegen beide Viren gibt es trotz klinischem Bedarf nur eine unzureichende Auswahl an antiviral wirksamen Substanzen, welche oft mit schädlichen Nebenwirkungen einhergehen. Zusätzlich sind phänotypische Tests zur Ermittlung von Resistenzen zeitaufwendig, schwer quantifizierbar und mit langen Wartezeiten bis zur Auswertung verbunden. In dieser Arbeit haben wir ein neues, konditionelles Replikonsystem konstruiert, welches auf dem Genom des Adeno-assozierten Virus (AAV) beruht. Unser Ziel war es, ein Reportersystem zu etablieren, um virale Resistenzen gegen zugelassene Medikamente und neue antivirale Wirkstoffe effektiv zu detektieren.

Dabei nutzen wir die natürliche Eigenschaft des AAV, eine lytische Replikation nur in Abhängigkeit einer Überinfektion mit anderen Viren wie dem Herpes-simplex-Virus (HSV) oder dem Adenovirus (Ad) durchführen zu können. Um ein induzierbares Expressionssystem zu entwickeln, wurden die strukturell kodierenden Sequenzen des AAVs mit einem Reportergen ausgetauscht, ohne die entsprechende Promotersequenz des Virus-Genoms zu verändern. Die Expression dieses Reportergens wird bei diesem sogenannten AAV Replikon Vektor nur durch eine Infektion mit den entsprechenden Wildtyp-Viren induziert. Infektionen aller getesteten Ad-Serotypen, wie auch Infektionen durch HSV-1, HSV-2 und das humane Cytomegalievirus (HCMV) konnten das AAV Replikon Reportersignal signifikant induzieren, während bei fehlender Infektion ein Signal nur knapp über dem Hintergrundsignal gemessen wurde. Für unterschiedliche Anwendungen haben wir verschiedene Einführungs-möglichkeiten des AAV Replikon Vektors in entsprechende Zelllinien getestet: transiente und stabile Transfektion und Transduktion durch Verpackung des AAV Replikon Vektors in AAV Vektoren. Unter allen Versuchsbedingungen führte die Induktion des Replikon Vektors durch Infektion zu unterschiedlichen Reporterexpressionsniveaus.

Um eine Virusinfektion zu therapieren, ist das schnelle und präzise Testen eines effektiven antiviralen Wirkstoffes an Patienten-Virusisolaten essentiell. Deshalb haben wir mit dem AAV Replikon System einen phänotypischen Wirkstoff-Resistenztest für HSV etabliert und mit diesem bereits 21 HSV Patientenisolate auf ihre Resistenz gegen das Referenzmedikament Acyclovir getestet. Dabei waren wir in der Lage, die Ergebnisse des als Standard geltenden 5 -7 Tage dauernden Plaque-Reduktions-Tests für alle HSV-1 Isolate bereits nach 24 Stunden, und für HSV-2 nach 48 Stunden quantitativ zu bestätigen.

Zusätzlich haben wir mit dem AAV Replikonsystem ein Wirkstofftestsystem etabliert, welches auch im Hochdurchsatz-Screening-Format genutzt werden kann. In einem ersten Vorversuch wurden 22 unbekannte und 2 bekannte Wirkstoffe auf deren Wirksamkeit gegen Ad und HSV-1 getestet. Das Replikonsystem konnte dabei reproduzierbar die bekannten Wirkstoffe detektieren und zusätzlich neue interessante Wirkstoffgruppen als direkte Inhibitoren der Virusreplikation in vitro identifizieren.

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Summary

Due to long-term chemoprevention, the risk of immune compromised patients to suffer from an infection of therapy-resistant herpes simplex virus (HSV) or adenovirus (Ad) is increasing. Therefore, fast and reliable tests for drug resistance of these viruses become more and more important in clinical praxis. Additionally, because of the lack of approved antivirals in case of human Ad and the limitation of the therapeutic target range for antiviral therapy of HSV infections, there is an unmet need for new antivirals improving the infectious mortality in this patient group.

Here, we constructed a new conditional expression system that is based on the adeno-associated virus (AAV) genome replication. This system provided an appropriate reporter system for replication of large DNA viruses, for testing their drug resistance and for screening of new antiviral substances. The multiplication of AAV is dependent on super-infection with other viruses such as herpes- or adenoviruses. The helper functions provided by these viruses induce the replication of the previously silent AAV turning its lytic gene expression on. We employed this natural switch to regulate the expression of a reporter gene, replacing the AAV Cap gene, depending on the infection with herpes or adenoviruses. By replacing the coding sequences for the structural AAV proteins with a Gaussia luciferase (GLuc) or a green fluorescence protein (GFP) open reading frame, we constructed a genetic element. This genetic element retained the extremely low basal activity in absence, and inducibility in the presence of helper virus infection. Instead of AAV production, reporter gene expression is turned on dependent on this infection. We coined this genetic element as AAV replicon in analogy to similar virus replication-based reporter systems, which were established for assessing RNA virus replication.

After successful introduction of the AAV replicon into permissive cells the reporter gene expression was specifically activated upon infection with different human Ad serotypes, HSV-1, HSV-2, and human cytomegalovirus (HCMV). Almost no induction of the signal was measured without infection. In this study, we characterized the responsiveness of the AAV replicon system using different delivery methods and target cell lines and tested feasibility of different applications such as resistance testing, trans-complementation and drug screening.

A fast diagnosis of drug resistance of patient isolates towards certain antiviral drugs is absolutely essential for the decision on effective therapy. Therefore, a phenotypical drug resistance test using the AAV replicon vector system was established for HSV. By testing 21 clinical isolates of HSV we showed that the AAV replicon-based test can differentiate between acyclovir sensitive and resistant strains already after 24 hours (for HSV-1) and 48 hours (for HSV-2) after virus isolation, compared to the gold standard plaque reduction assay, which takes 5-7 days.

Furthermore, the AAV replicon vector system was used to generate a drug susceptibility test system, which can be applied in a high-throughput screening format. In a first approach, 24 kinase inhibitors were tested for their ability to inhibit Ad and HSV. The system was reproducibly detecting known inhibitors and could even identify new interesting compound groups.

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1. Introduction

1.1. Microbial drug resistance

Antimicrobial resistance is a natural mechanism of pathogens to overcome treatments with antimicrobial drugs. It occurs due to adaptive genetic changes of the pathogen microorganisms under selection pressure. Especially prolonged treatment, overuse and misuse of antimicrobials are accelerating this process in all kind of microorganisms, such as in bacteria, viruses, fungi, and parasites. Emergence and spreading of antimicrobial resistance mechanisms occur globally and influence the ability of treating common infectious diseases [1] [2].

Antibiotic resistance of bacteria can be preserved by intrinsic or acquired mechanisms. Intrinsic mechanisms are obtained by naturally occurring genes like multidrug-resistant active efflux systems and modification of antibiotic target sites [3]. Acquired mechanisms in contrast include the transfer of resistance genes on plasmids, bacteriophages, transposons, and similar mobile genetic material [4]. Consequences for humans infected with emerged pathogens resistant to multiple antibiotics, are prolonged illness, disability, and death [5] [6]. Some nosocomial pathogens are even untreatable if they become resistant to all antimicrobials available [7].

Antimicrobial susceptibility testing is important to meet the challenge of fast treatment. Bacterial infection, for example, currently have to be treated with broad-spectrum antibiotics before the antimicrobial susceptibility test result of the isolated pathogen is available. Preferred testing methods at the moment are broth microdilution test, agar dilution, disk diffusion, and gradient diffusion methods. These methods take 16–24 hours. More rapid results (3.5–16 hours) can be obtained by automated instrument methods, like ultra-high-throughput bacterial growth assays, for which materials and devices are commercially available [8] [9]. In clinical practice, every hour can be crucial for the mortality rate of patients if the effective antimicrobial treatment is not started as soon as possible [10] [11]. Globally, the world is facing an increasing emergence of multi-drug resistances, while the average time span of developing a new antimicrobial treatment takes 10–12 years [7]. Future prospects concentrate on alternatives to antibiotics, like modulating the host immune response by

altering inflammation and autophagy, or on combinations of antibiotics and so-called antibiotic resistant breakers [12].

1.1.1. Viral resistance development

Viruses have the evolutionary advantage to generate a remarkable genetic diversity by a fast adoption to new host and environmental changes. The necessary viral mutations in their genomes are dependent on multiple viral- and host-specific processes [13]. Emergence of resistant strains is therefore a common result of therapeutic selection pressure [14]. In this perspective, an antiviral drug must fulfill several requirements. The two most important requirements are safety and potency. Since the viral life cycle is dependent on cellular functions, it is important to specifically inhibit either a viral function or a cellular pathway that is essential for virus growth, without causing deleterious effects on the host cell functionality itself. Most antivirals therefore target viral enzymes such as proteases, nucleic acid synthesizing proteins or other viral targets essential for viral reproduction. For a potent antiviral drug, it is important to avoid even modest replication of the virus. Due to their fast adopting capacities, viruses can easily acquire resistance in presence of an inhibitor that does not fully block virus replication. If the drug concentration is insufficient to completely inhibit virus replication, the remaining virus population can expand and gain fitness due to genetic variation. In patients that are not treated with an alternative antiviral drug in time, the uncontrolled expansion of the resistant mutant virus can be fatal [15].

In the last two decades, potent antiviral drugs were approved for treatment of viral infections. Some antivirals can clear infections from the patient or persistent viruses can at least be controlled effectively by antivirals. Unfortunately, development of resistance has been documented for nearly all clinically used antivirals. The mechanisms of how viruses circumvent drug therapy involve either a gene mutation of the target site of the antiviral drug or a gene mutation of antiviral drug activators [14]. The randomly appearing frequent mutation rate and the fast replication of viral genomes lead to a large pool of variants called quasispecies. The respective fitness of the quasispecies determines whether a mutation leads to a replication-competent and additionally resistant virus mutant. Therefore, once the replication of the virus is not fully repressed, the possibility of resistance development is very high [16]. This is the reason for resistance occurring with an increased probability especially in immunocompromised patients under long term low dose antiviral prophylaxis. Extensive

immunosuppression for example, has to be initiated in transplant patients due to the management of rejection in solid organ transplant recipients or hematopoietic stem cell transplant recipients. This is similar for the treatment of leukemia and autoimmune diseases [17] [18]. In the following chapter, the mechanisms behind resistance development of viruses as well as strategies for dealing with resistance are discussed for selected important viruses.

1.1.2. Antiviral drug-resistant tests

The risk for patients to suffer from drug-resistant viruses should be detected as fast and as accurate as possible. There are two means of determining drug resistance: either by genotypic or by phenotypic assays. Genotypic assays detect mutations in the viral genome pools that are known to cause resistances. Phenotypic assays measure the actual drug resistance of patient derived infectious virus. The choice which assay to use for drug resistance detection is dependent on the genome size of the respective virus and the ability to culture the virus in vitro.

Genotypic testing has several technical advantages. However, novel or so far uncharacterized mutations that lead to resistance as well as the overall replication fitness of mutated viruses, cannot be detected with this method. Especially under long term therapy of patients, the choice of antiviral drugs based on genotyping can be challenging because of the increasing occurrence of cross resistance and multiresistance. Furthermore, the implementation of new antiviral drugs on the market will lead to new and complex patterns of yet unknown mutations [19]. Notably, genotyping of resistance is only applicable for drugs that are in longtime use and therefore sufficient data are available to connect resistance with specific mutations. This connection needs to be generated or proven by a phenotypic test. Hence, a standardized phenotypic testing would be necessary for each virus of interest.

Phenotypic assays have the advantage of detecting decreased susceptibility of a viral mutant to a certain antiviral agent compared to a wild-type strain. The drug concentration necessary to inhibit wild-type viral growth by 50% is called IC_{50} value. For evaluation of the phenotypic drug resistance, the increase of the IC_{50} of the viral isolate strain compared to the wild-type strain is important. The general IC_{50} threshold (so called cut-off), which defines therapeutic resistance, is dependent on the respective assay and determined by careful assessment of standard strains and isolates [17].

The standard clinical practice test for hepatitis B virus (HBV) antiviral susceptibility is the genotypic resistance test, because it is fast and convenient. Since most of HBV drugs on the market are nucleosid- or nucleotid analogs, the detection concentrates mainly on HBV polymerase gene mutations [17]. Genotypic resistance mutation tests include standard sequencing of polymerase chain reaction (PCR) products, real-time PCR, reverse hybridization, restriction fragment length polymorphism, single genome sequencing, and ultra-deep pyrosequencing. These assays differ up to 20% concerning the sensitivity of the minor subpopulation of mutant HBV viruses [20] [21]. Since the genome size of HBV is small, the number of mutations leading to resistance is better investigated compared to viruses with a larger genome size. The database for genetic resistance is based on and controlled by phenotypic tests.

For HBV, the phenotypic testing method is limited in clinical use because of the absence of a fully permissive HBV infectious cycle in a cell culture system. Nevertheless, there are different methods to determine the phenotypic resistance of patient derived HBV isolates to learn more about drug-resistant HBV phenotypes. This includes the transient transfection method, where clinical isolated HBV genomes are either amplified or cloned and transfected to hepatocyte-derived cell lines in the presence of antiviral drugs [22]. Another method is the transduction of recombinant baculoviruses encoding drug-resistant HBV clones. Like the transient transfection method, this method is very work-intense but good for cross-resistance testing [19]. To define and characterize the fitness of a certain HBV mutant, a stable human hepatoma cell line, which promotes differentiation and phenotypic stability, can be constructed for every isolated mutant [23] [24].

In the case of human immunodeficiency virus (HIV) the high rate of replication in vivo leads to an accumulation of innumerable genetically distinct quasispecies with a high genetic variability in individuals [25]. HIV drug resistance is mainly tested via sequencing-based genotyping methods, as they offer reduced costs and faster turnaround times compared to phenotypic cell-based methods. Despite the disadvantages of phenotypic assays, they are used for HIV-testing and are able to define antiviral resistance to any drug without prior knowledge of the corresponding mutations. The most advanced phenotypic tests are based on construction of pseudotyped viruses by homologous recombination of patient derived coding sequences for integrase, protease, and reverse transcriptase into a standardized virus

backbone. The spread of these recombinant HIV infection is measured in the presence of different drugs and compared to wild-type standards. Significant increase of spread reveals presence of coding sequences with resistance phenotype. Unfortunately, the sensitivity of this methodology allows a detection of resistant viruses only if they constitute more than 10% to 20% in one patient sample [26-28]. Overall, the data of phenotypic tests provide experimental proof for the correlation between genome mutations and drug resistance. These characterizations are very important for the interpretation of genotypic data and improvement of performing HIV genotyping based predictions [29]. In case of assays for HIV susceptibility testing, the greatest sensitivity for drug resistance is reached by next-generation sequencing based methods. The increased sensitivity thereby is achieved by massively increased coverage of sequencing data, which generates three to four orders of magnitude more identification of mutations compared to the Sanger-sequencing based method [30] [31] [32]. This allows the description of the drug resistance potential for a given HIV pool more exactly, including the genotypes with extremely low frequency. However, one of the major limitations of next-generation sequencing, especially for lower volume laboratories, is the necessary start-up and running costs. Nevertheless, the utility of next-generation sequencing for human viral pathogens in general is expected to increase in the future [33].

In contrast to HIV and HBV, the resistance determination of larger DNA viruses like herpes simplex viruses (HSV) or adenoviruses (Ad) is more challenging due to their large genome size and therefore aggravated conditions to detect mutations leading to resistance. However, for HSV, a phenotypic assay is standard because patient derived viral isolates can be grown easily in vitro. In case of other herpesviruses, like cytomegalovirus (CMV) or varicella zoster virus (VZV), in vitro growth abilities are limited and therefore neither genetic nor phenotypic methods can be easily applied for testing drug resistance. For Ad, genotypic and phenotypic drug resistance testing is limited to specialized laboratories. Further information on resistance tests for HSV and Ad can be found in chapter 1.2.2.7 and chapter 1.2.3.5.

1.2. Biology of large DNA viruses and their inhibitors

Herpesviruses and adenoviruses belong to the large class of double-stranded viruses. Both groups of viruses are able to induce AAV replication and therefore their growth potential can be tested by an AAV based replicon system.

1.2.1. Herpesviruses

In order to structure the relationship of herpesviruses into a taxonomical framework, the International Committee on Taxonomy of Viruses (ICTV) classified the order of *Herpesvirales* into three families called *Alloherpesviridae*, *Herpesviridae* and *Malacoherpesviridae*. This classification was made according to their biological characteristics, like having a linear double-stranded DNA genome, a capsid of about 100 nm with icosadeltahedral shape, a proteinaceous matrix called tegument, and an glycoprotein-containing lipid envelope [34]. Amongst those, the family of Herpesviridae consists of three subfamilies: *Alphaherpesvirinae* (with the genera of *Iltovirus*, *Mardivirus*, *Scutavirus*, *Simplexvirus*, and *Varicellovirus*), *Betaherpesvirinae* (with the genera of *Cytomegalovirus*, *Muromegalovirus*, *Proboscivirus*, *Roseolovirus*) and *Gammaherpesvirinae* (with the genera of *Lymphocryptovirus*, *Macavirus*, *Percavirus*, *Rhadinovirus*) [35].

The transmission route of Herpesviridae ranges from physical contact to aerosol spread. Severe symptoms after infection are often limited to immunocompromised hosts [36]. A characteristic observed among all herpesviruses is their ability to establish a life-long latent state after primary infection. Interestingly, herpesviruses show a high species specificity due to a long-lasting coevolution between the viruses and their hosts. This may be the reason why the pathology of primary herpesvirus infection is normally relatively mild. For example, in humans, although the average adult population is infected with 3-4 out of the 9 human herpesviruses, symptoms of primary herpesvirus infections are rarely observed [37].

The nine known human herpesviruses are Human alphaherpesvirus 1 (also called herpes simplex virus type 1 (HSV-1)), Human alphaherpesvirus 2 (also called herpes simplex virus type 2 (HSV-2)) and Human alphaherpesvirus 3 (also called varicella zoster virus (VZV)), which belong to *Alphaherpesvirinae*, Human betaherpesvirus 5 (also called cytomegalovirus (CMV)) and Human betaherpesvirus 6A, 6B, and 7, belonging to *Betaherpesvirinae*- and Human gammaherpesvirus 4 (also called Epstein-Barr virus (EBV)) and Human gammaherpesvirus 8 (also called Kaposi's sarcoma-associated herpesvirus (KSHV)), which belong to the *Gammaherpesvirinae* subfamily [37, 38]. The clinical outcome of infections and the tissue tropism in vivo and cell tropism in vitro differs amongst these herpesviruses.

Human *Alphaherpesvirinae* have a restricted host specificity and are characterized by their short reproduction cycle and rapid spread in cell culture. During latent state, these viruses are

maintained in the nervous system in vivo. They may cause neurological diseases upon both primary infection and reactivation [38] [39] [40]. In contrast, human *Betaherpesvirinae* possess a very broad cell tropism compared to *Alphaherpesvirinae* subfamily. They are able to replicate in vitro in epithelial cells, fibroblasts, endothelial cells, and smooth muscle cells, whereas their replication cycle takes longer (reviewed in [41]). Viruses remain latent in bone marrow-derived hematopoietic or endothelial cells [42] [43]. Human *Gammaherpesvirinae* subfamily has the highest restriction of host cell tropism both in cell culture and in vivo amongst the human *herpesviruses*. EBV infects either B cells or epithelial cells by tropism switching after propagation in one or the other cell types [44] and latently infected cells are resting memory B cells used by the virus in a non-pathogenic persistence state [45].

1.2.2. Herpes simplex viruses

There are two known human members of the Simplexvirus genus: HSV-1 and HSV-2, common and endemic worldwide. In Germany, the seroprevalence of HSV-1 in the population older than 15 years is relatively high, being 76.3% in females and 75.2% in males, in contrast to the seroprevalence of HSV-2 being only 18% in women and 13.8% in men [46]. In some regions in developing countries, the seroprevalence of HSV-1 and HSV-2 is even up to 100%. In principle the prevalence of infection is strongly dependent on age, geographic regions, population subgroups, and pre-infections with other endemic human viruses like HIV [47] [48].

Usually HSV-1 is already transmitted during childhood mainly due to oral contact with body fluids of infected persons around the mouth (orolabial) causing oral herpes. However, a proportion of HSV-1 infections can as well be transmitted via the genital or anal area and cause genital herpes. In contrast, HSV-2 is mainly sexually transmitted also leads to genital herpes infections. Infections with both HSV species persists lifelong and, in most cases, asymptomatic. Reactivation from latency is associated with mild symptoms in about 30% of latently infected patients and characterized by recurrent eruptions of painful blisters or ulcers at the respective site. Immunocompromised patients, such as HIV infected patients, and solid or bone marrow transplant recipients are at high risk to develop herpes simplex keratoconjunctivitis and herpes simplex encephalitis, where the latter goes along with a frequency of 5 in 1 million people. Perinatal HSV infections of neonates lead to a life threating systemic disease. Herpes infections of neonates, immunocompromised patient and herpes encephalitis is associated with high mortality [49] [50] [51].

1.2.2.1. Genome organization of HSV

The genome of HSV consists of a linear, double stranded DNA, which contains two unique regions named unique segment long (U_L) and unique segment short (U_S). Both are flanked by an internal repeat sequence (IR) and a terminal repeat sequence (TR) (see Figure 1). HSV produces 4 roughly equimolar genomic isomers, which differ by inversion of the long and short components. Furthermore, the HSV genome has three origins of replication depicted as ORI_L and ORI_S. The latter one is present twice [52] [53].

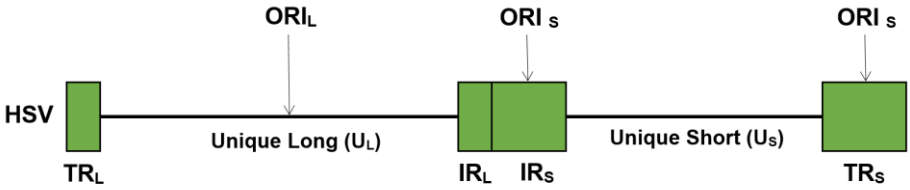


Figure 1: The structure of HSV-1 genome.
Genome configuration depicting IR_S and TR_S. (modified from [54]).

1.2.2.2. Lytic and latent infection cycle

Like all *Herpesviridae,* HSV has a biphasic life cycle consisting of a lytic and a latent state. During latency in neurons of sensory ganglia, gene expression is limited, and viral production is completely inhibited. Only microRNAs and latency-associated transcripts (LATs) are abundantly accumulated whereby LATs are suggested to suppress viral lytic genes as primary function [55-57]. Especially stress or neuronal damage can lead to periodic viral reactivation throughout the lifetime of a host. During a productive lytic phase, virions are retrogradely transported along neuronal axons resulting in a release of an infectious virus in the axonal termini, where it has access to permissive peripheral tissue to cause recurrent lesions [58]. Here, viral genes are expressed in a certain cascade manner of at least three coordinated kinetic classes: immediate-early (IE) genes, early (E) genes and late (L) genes [59] [60].

HSV virions consist of an outer envelope containing 13 distinct viral envelope glycoproteins for viral attachment and entry, and host-cell derived lipids and membrane proteins. A proteinaceous layer termed tegument links the envelope to the inner viral capsid. This inner icosahedral capsid of 100 nm size contains 150 hexons and 12 pentons in an icosahedral symmetry (T=16). The linear double-stranded DNA is comprised in the inner nucleoprotein core [61] [62]. The attachment of HSV-1 is upon binding of the viral glycoprotein gC to cell surface heparin sulfate (HS). Viral glycoprotein gB mediates fusion of the viral envelope with

the cell membrane [63]. In case of HSV-2, the glycoprotein gB is involved in both mechanisms of binding and penetration of HSV [64]. Then the viral envelope is fused to the cellular membrane and the tegument with the capsid is released into the cytoplasm [65] [66]. The microtubule-mediated transport mechanism transfers the viral nucleocapsid to the nucleus and naked DNA is then released at a nuclear pore [67]. The linear DNA is converted into a covalently closed circular form [68].

Transcription of HSV genes is solely dependent on the host RNA polymerase II (pol II) since the virus does not encode its own RNA polymerase. Gene expression is conducted by the formation of an activator complex by the viral protein VP16 with the host cell factor (HCF) and the octamer-binding transcription factor-1 (Oct-1). This complex binds to IE gene promoters, recruits other transcriptional factors and stimulates directly the transcription of IE genes ICP0, ICP4, ICP22, ICP27 and ICP47 [69] [70]. Then the early phase of transcription is stimulated mainly by ICP4 [70] [71] and ICP22 [72]. The E genes are involved in viral genome replication during the viral lytic cycle. HSV encodes for its own polymerase helicase and single stranded DNA binding protein ICP8, which are mandatory for the viral DNA replication [73]. The final late phase is characterized by expression of viral structural genes after onset of the DNA genome replication [74].

1.2.2.3. Viral DNA replication

The onset of DNA replication in HSV leads to significant reduction of E gene expression whereas L genes start to be expressed in very high amounts. Seven viral proteins are essential for viral DNA synthesis: the viral DNA polymerase complex composed of a catalytic subunit Pol U_L30 and a processivity subunit U_L42, the origin-binding protein U_L9, the DNA binding proteins ICP8 (U_L29) and the helicase/primase complex is based on $U_L5/U_L8/U_L52$ (reviewed in [53]). Detection of nucleoprotein complexes into the nucleus leads to the recruitment and formation of cellular nuclear substructures called ND10 protein foci, which are disrupted by IE protein ICP0 [75] [76]. The initiation step at the beginning of DNA synthesis is started by U_L9 and ICP8 with distortion or destabilization at one of the three Oris. During the elongation phase, H/P complex is recruited to unwind duplex DNA and for synthetizing short RNA primers for initiation of the DNA replication step [77]. Then the two-subunit polymerase is recruited to the fork to catalyze leading- and lagging-strand synthesis [78]. The lagging strand is looped back to the fork. Leading and the lagging strands, which consists of so-called Okazaki

fragments, are synthesized in cycles [79] [80]. Whether DNA replication initially proceeds by a rolling circle amplification or by a theta type mechanism remains as one of the challenges in the field [81].

1.2.2.4. Viral assembly and egress

After DNA replication, the viral DNA is incorporated into the accumulated and pre-assembled viral capsids [82, 83]. Maturated nucleocapsids then egress to the cytoplasm. First an envelopment takes place at the inner nuclear membrane. Then there follows a de-envelopment of the nucleocapsid at the outer side of the nuclear membrane [84]. In this process the capsids acquire a transient (also called “primary”) envelope. Primary enveloped virions can be found only in the peri-nuclear space. After the fusion of the primary envelop to the outer nuclear membrane, the nucleocapsids are released to the cytosol where they acquire most of their tegument. Their morphogenesis is continued in the trans-Golgi derived membrane compartment, which is enriched in viral glycoproteins. There they acquire their permanent (secondary) envelop, which can be found in infectious particles. During the secondary envelopment the virus particles are budding to secretory vesicles and the resulting virions are entering the extracellular space ready to initiate a new infection cycle [85, 86].

1.2.2.5. Immunity against herpes simplex viruses

Since HSV are ancient and very well adapted human pathogens, their immune evasion mechanisms are complex. Both, the innate and the adaptive immune response are activated by the virus. As a first line of defense against HSV the innate immune response is induced in all cells that are infected and mainly rely on the type I interferon response. This innate immune response against HSV also involves activity of multiple immune cell types [87]. Most importantly, natural killer (NK) cells respond by cytokine production and cell killing to the recognition of HSV infected cells. Additionally, plasmacytoid dendritic cells (pDCs) produce type I IFN and thereby support the cell intrinsic antiviral immune responses [88, 89]. The adaptive immune response shows an important role in controlling disease progression, latency, and limiting of viral spread. Key players in the cellular response are CD8+ T cells [90] [91]. In the absence of other immune effectors, CD4+ T cells as well as the humoral immunity have been shown to play only a minor role in protection [92].

1.2.2.6. Antiviral drugs against herpes simplex virus infections

The most commonly used drugs against HSV are nucleoside and nucleotide analogues, sharing a common molecular mechanism of inhibiting the viral DNA polymerase.

Acyclovir and valacyclovir:

Among the 25 by the US Food and Drug Administration (FDA) approved antiviral nucleoside analogues, Acyclovir (ACV) was the first efficient and selective antiviral agent against herpesvirus infections back in 1982. Still, it is a commonly used drug and the primary choice in treatment of HSV infections. ACV consists of a guanosine derivate with an acyclic side chain declared as 9-[(2-hydroxyethoxy)methyl]guanine. The specificity relies on the fact that mainly the viral encoded thymidine kinase (TK) performs phosphorylation of the acycloguanosine to a monophosphate (ACV-MP) (Figure 2). This reaction is not efficiently catalyzed by any nucleotide kinases belonging to the host nucleoside salvage pathway. After this initial step, however, the host cellular GMP kinase and the host nucleoside diphosphate kinase (NDP), respectively, can further phosphorylate the ACV-MP resulting in the biologically active intermediate of acyclovir, which is the ACV-triphosphate (ACV-TP) [93] [94]. The ACV-TP then serves as an alternative substrate of the natural nucleoside dGTP for the interaction with the viral DNA polymerase. If ACV-TP is incorporated into the DNA at its 3' terminus, a further chain elongation is impossible because the nucleoside analogue lacks the hydroxyl group needed for this step [95] [96].

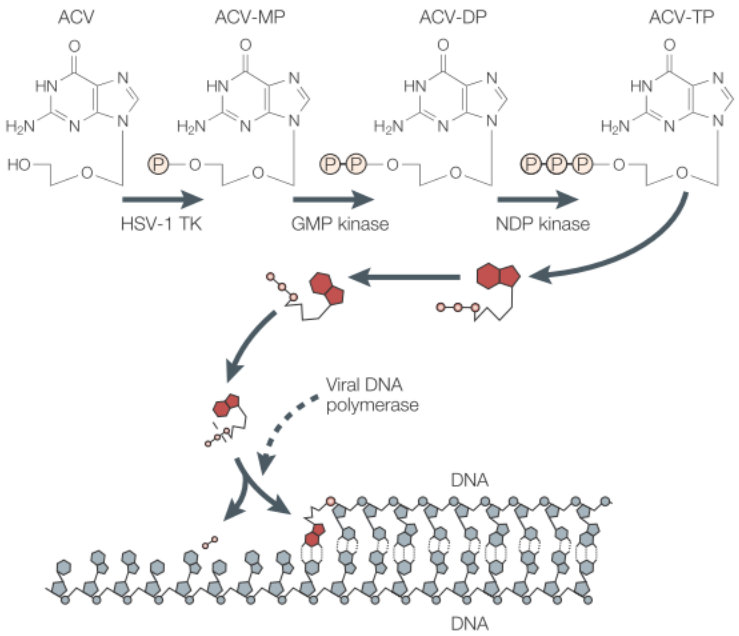


Figure 2: Chain termination mechanism of Acyclovir. Different viral and cellular kinase phosphorylation steps are needed before the ACV-triphosphate is recognized by the viral DNA polymerase. Figure reprinted from [97].

Furthermore, the HSV DNA polymerase-associated 3' -> 5'-exonuclease activity is not able to excise the incorporated ACV-monophosphate residues [98]. In principle, ACV is very potent against HSV-1 but only half as potent against HSV-2. In vitro, the half inhibitory dose (IC_{50}) against HSV-1 is 0.09 - 4 µM and in case of HSV-2 it is 0.1 – 9.8 µM [99]. Due to its low oral absorption, this drug is applied topically against labial herpes and intravenously to fight systemically genital and labial herpes and herpes encephalitis [100] [101].

The low oral uptake is the reason why the L-valyl-ester prodrug of acyclovir called valacyclovir was developed, which shows a better oral bioavailability (54% versus 12 – 20%) [99] [100]. After oral intake, valacyclovir is first carried out by the human intestinal transporter. In the intestine it is then converted to ACV by ester hydrolysis. ACV and valacyclovir are both very well tolerated drugs [102].

Penciclovir and famciclovir:

Another drug used in the clinic is penciclovir and its prodrug famciclovir, which shows improved oral bioavailability. Penciclovir is an acyclic guanine derivative and famciclovir is the inactive prodrug of penciclovir with an additional diacetyl ester. Similar to ACV, penciclovir leads to a limited chain elongation during viral DNA replication by virtue of the triphosphate hydroxyl group. The advantage of penciclovir over acyclovir is the higher stability of the active triphosphate as well as the longer time frame it persists inside the target cell [103] [104].

Because the antiviral mechanisms of ACV and penciclovir are similar, resistance to one of these agents has an impact on the other drug as well [105].

Foscarnet:

The similarity of ACV and penciclovir is the reason why foscarnet, with the chemical name phosphonoformic acid (PFA), is used as a second in line drug. It inhibits the viral DNA polymerase by mimicking the structure of pyrophosphate and therefore blocking the pyrophosphate-binding site during DNA chain elongation without being incorporated. It does not belong to the nucleoside or nucleotide analogues. During its mode of action, it is independent from viral and cellular kinase activation in contrast to ACV. In addition, compared to host cellular enzymes foscarnet shows a 100-fold increase against viral derived enzymes [106]. The drug is supplied intravenously and it is associated with nephrotoxicity and hemoglobin disturbances [107].

Cidofovir:

Another second line therapy is cidofovir, which has a very broad antiviral mechanism of action against several DNA viruses. It consists of an acyclic phosphonate nucleotide analogue. Here an initial phosphorylation of the viral kinase is not required because it has already a single phosphate group attached. It is sequentially phosphorylated to its active triphosphate form by the cellular kinases. As a consequence, cidofovir selectively inhibits the viral DNA polymerase due to the 25- to 50-fold greater affinity compared to the cellular one [108] [109] [110].

New drugs:

New drugs target different sides of the virus life cycle. 1-docosanol for example is a 22-carbon-long saturated fatty alcohol, which prevents the fusion of the viral capsid with the host cell membrane and is used topically against recurring labial herpes infections [111]. Another current approach of special novel small molecules, which are currently in development, belongs to the class of helicase-primase inhibitors. This class of inhibitors are based on the viral helicase-primase enzyme complex, which is essential and has no eukaryotic homologue. Thus it is a very interesting target for novel drugs against HSV [101]. The two most promising helicase-primase inhibitors are amenamevir and pritelivir [112] [113].

Since current treatments of HSV infections generally show limited efficacy, new and more competent antiviral drugs are needed. Especially for immunocompromised patients with a

higher probability of developing an acyclovir resistant HSV infection, therapies with an increased oral bioavailability, less toxic effects, and higher efficacy are urgently needed.

1.2.2.7. Antiviral drug resistance of HSV

Although drugs against HSV are widely used, the prevalence of resistance in immunocompetent hosts is less than 1%. In contrast, 3.5% to 14% of immunocompromised hosts are affected by drug-resistant HSV infections (reviewed in [17]).

Two genes of HSV are currently targeted by approved antiviral therapy: either the U_L23 gene encoding the 376-amino-acid (aa) HSV TK protein or the U_L30 gene encoding the HSV DNA-polymerase enzyme. Since the TK protein is dispensable, the probability of gene mutations in this gene is 95% in contrast to the essential U_L30 gene [114] [115]. TK mutations can lead to different outcomes like TK-negative or -low-producer mutants, which show no or reduced TK activity. In the minor cases, the mutation leads to TK-altered mutants, which are able to circumvent phosphorylation of ACV or PCV [116] [117]. The essential sites for enzyme activity are the nucleoside-binding site, the ATP-binding site, one cysteine at codon 336, which is responsible for the structure of the active site, and six highly conserved domains [118]. The DNA polymerase consists of 1235 aa with eight conserved regions, whereas the regions associated with resistance are most likely region II and III [119] [114]. Since in general the U_L23 gene and the U_L30 gene reveal a polymorphism pattern, it is not easy to differentiate between mutations having no influence and mutations leading to resistance [99]. Most of the resistant viruses show cross resistance to other nucleoside analogues and even resistance to both ACV and foscarnet can been found [120] [121] [122].

1.2.2.8. Assays for HSV drug resistance

As described in the last chapter, in case of detecting HSV resistance in patient isolates, genotypic tests reveal only already characterized mutations. If treatment failure points toward resistance development, a phenotypic test should be performed to define a possible responsive drug for counselling and management of the patient.

The “gold standard” for phenotypic determination of antiviral susceptibility of HSV-1 and HSV-2 isolates is definitively the plaque reduction assay (PRA). For testing the antiviral drug effect on HSV, the appropriate patient-derived clinical specimen has to be propagated in a

cultured cell line. Afterwards, the viral inoculum is grown in the presence of serial dilutions of the applicable drug. The time needed to make a defined statement due to viral plaque numbers is usually 2-3 days. For manual counting of the single plaques, the cells have to be fixed and stained. All in all, a final conclusion about the susceptibility of an isolated virus takes 3-7 days [123]. The disadvantage of this method is that it is time-consuming, labor intensive, and due to its manual read out, the results are very subjective.

Alternative methods are evaluating the antigen expression after virus multiplication in vitro, like the sandwich enzyme-linked immunosorbend assay (ELISA) or the microplate *in situ* ELISA called MISE [124] [125] [126]. Other established methods are based on DNA hybridization [127] or fluorescence activated cell sorting (FACS) analysis of late gene expression [128]. A similar method like PRA is a genetically marked Vero cell line that responds to HSV infection with β-Galactosidase expression [129]. The DNA reduction assay (DRA) combines pre-cultured clinical samples treated with antivirals and nucleic acid detection with HSV-1 specific quantitative real-time PCR by measuring the viral DNA concentration in the cell lysates. The cell count and lysis are corrected via beta-globin PCR [130].

1.2.3. Human adenoviruses and their replication

Adenoviruses (Ad) were detected in human tonsils and adenoids in tissue culture in 1953 by Wallace Rowe and Robert Huebner when they were searching for the causative agent of ‘common cold’ [131]. In general adenoviruses are important pathogens of human and animals. Furthermore, they are very interesting as vectors for gene therapy.

According to the ICTV, the family of Adenoviridae consists of 5 genera: Atadenovirus (infecting a broad range of hosts like ovine and bovine), Aviadenovirus (infecting birds), Ichtadenovirus (the only species infecting sturgeons), Mastadenovirus (infecting mammals) and Siadenovirus (infecting birds and frogs). The genus of Mastadenovirus consists of 36 species infecting different mammals, including the 7 species of human adenovirus species, currently coined Human mastadenovirus A-G. The human adenovirus species consist of different serotypes. All in all up-to-date 71 different human adenovirus serotypes were described [132] [35]. The classification is based on classical serotyping via neutralization testing and hemagglutination patterns among other biological attributes [133] [134].

The transmission route of human Ad ranges from physical contact to aerosol spread. In general, the virus shows a high stability outside the host and can still be infectious after several weeks at room temperature or after one week at 36°C [135]. Human Ad are the causative agent of a multitude of diseases including respiratory infections, gastroenteritis and epidemic keratoconjunctivitis [136] [137] [138]. The different Ad serotypes and their specific tropism are summarized in Table 1.2.3.1. Dependent on serotype of the infecting human Ad and the immune status of infected individuals, the disease manifestation varies from mild localized lesions to life-threatening disseminated diseases. Like in the case of HSV infections, the frequency of diseases caused by human Ad increases in immunocompromised patients, like allogeneic hematopoietic stem cell recipients, solid organ transplantation recipients, or bone marrow recipients [139] [140]. 5–47% of immunocompromised patients were found to be infected with Ad and out of these, 6–70% died [141]. In immunocompromised children, the mortality rate is even greater than 50% [142].

Table 1.2.3.1: Differentiation of human adenovirus types by species and tropisms.

Species	Type	Tropism
A	12, 18, 31	Enteric, respiratory
B1	3, 7, 16, 21, 50	Respiratory, keratoconjunctivitis
B2	14, 11, 34, 35, 55	Renal, respiratory, keratoconjunctivitis
C	1, 2, 5, 6, 57	Respiratory, keratoconjunctivitis, hepatic, lymphoid
D	8-10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-49, 51, 53, 54, 56, 70	Keratoconjunctivitis, enteric
E	4	Respiratory, keratoconjunctivitis
F	40, 41	Enteric
G	52	Enteric

Since for human Ad it was shown that the virus is able to be excreted after acute infection for several months or years, it is suggested that Ad is able to establish a persistent infection [143]. This point is supported by the ability of adenoviruses to infect the urinary tract, lymphatic tissues, like tonsils and adenoids, and the gastrointestinal tract permanently [144] [145] [146].

1.2.3.1. The organization of the adenovirus genome

The genome of *Mastadenovirus* genus consists of a linear double-stranded DNA of 34-36 kbp, which has two inverted terminal repeats (ITRs) on both ends of the genome as well as a terminal protein (TP) covalently linked to the 5’ end of the ITR at each strand [147].

The genomes of Ad are organized in 8 units transcribed by the RNA polymerase II, which are located at both DNA strands (Figure 3). The transcription units are temporally regulated and can be divided into: five early genes expressed before onset of DNA replication (E1A, E1B, E2, E3, E4); the delayed early genes expressed independent of DNA synthesis (IX and Iva2); and one late transcription unit expressed after replication of Ad DNA (L1-L5) [148]. Additionally, there are two RNA polymerase III dependent transcription units encoding virus-associated RNAs I and II (VAI and VAII RNAs).

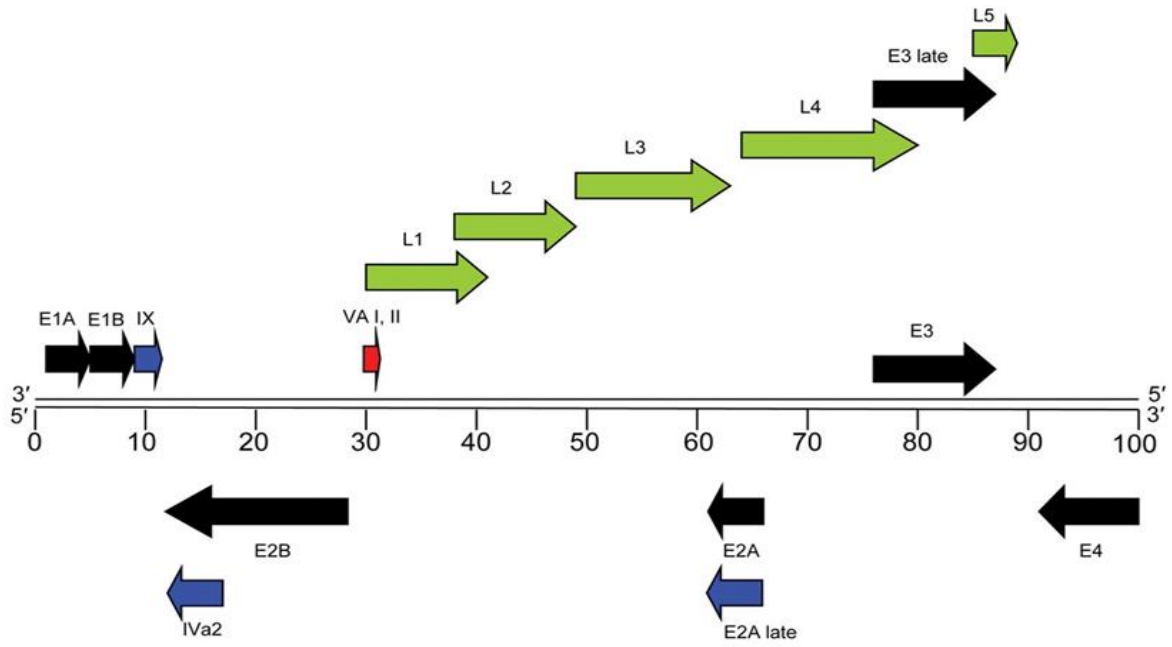


Figure 3: Genome organization of human adenovirus.
Black arrows highlight early protein gene regions, blue arrows indicate delayed early genes, green arrows show late transcription units and red arrows denote the VA-RNA I and II. Adopted from [149].

In principle, the human Ad life cycle can be divided in an early and late phase. In the early phase, regulatory proteins are expressed. Regulatory proteins are responsible for activating transcription of other viral genes, for avoiding antiviral host mechanisms, and for altering host protein expression. The first transcription unit expressed shortly after cell entry is the E1A gene. The two major proteins encoded by E1A are transcription modulators and force the host cell to enter the S-phase [150] [151]. The gene products of E1B are the proteins called 19K and 55K, which both prevent apoptosis of the host cell by inhibiting the tumor suppressor protein p53 as well as using p53-independent apoptosis [152] [153]. From the two early regions E2A, three proteins are encoded by alternative splicing: TP, which is responsible for a unique protein-priming mechanism, the DNA polymerase, and the ssDNA binding protein (DBP) [154] [155] [156]. Proteins encoded by E3 have immunomodulatory functions [157]. The gene

products of the E4 gene, which encodes 18 distinct mRNAs due to alternative splicing mechanisms, are associated with DNA replication, transcription and in the regulation of cell cycle signaling [158] [159]. The VA RNAs are responsible for inhibition of the interferon-induced PKR and for the block of cellular micro-RNA machinery [160] [161]. As soon as all proteins responsible for viral DNA replication are synthetized, the late phase starts with DNA replication on the one side and transcription of late virus genes on the other side. The late viral genes encode the structural proteins and the proteins that are necessary for the maturation of viral particles [162].

1.2.3.2. Adenoviral DNA replication

The DNA replication of human Ad depends on a unique mechanism among viruses. It starts between 5 to 8 hours after infection of the cell. The DNA replication is catalyzed by the viral DNA polymerase (POL) and involves other essential viral components such as the pre-terminal protein (pTP), which forms a heterodimer with the POL. The DBP is the viral single strand binding protein and essential in stabilization of replication intermediates [163] (Figure 4). The initiation of the process starts when a deoxycytidine monophosphate (dCMP) is covalently linked to the pTP serine hydroxyl group by the adenovirus polymerase [164]. The pTP has a high affinity for ssDNA. In presence of the two cellular factors octamer-binding transcription factor-1 (Oct-1) and nuclear factor-1 (NF1), it binds to the origin of DNA replication and functions as a primer for replication [165] [166]. The DBP most likely plays a key role for the unwinding of the DNA, although the full mechanism has not yet been understood [167]. Then a 5’ to 3’ elongation process starts and the new growing strand displaces the old one [168]. Subsequently, the new dsDNA serves as template for the following replication rounds. In a second step, the displaced single strand, which is protected by DBPs, forms a panhandle structure by hybridization of the complementary sequence of the two ITRs. The strand synthesis is then primed by new pTP binding and takes place in the same way as the 5’ to 3’ strand synthesis [169] [170]. DNA replication is finalized by a viral protease cleaving pTP to TP. The resulting progeny DNA is finally packaged into the virion [171]. The protein priming and the missing lagging strands (and Okazaki fragments) clearly differentiate the human Ad replication machinery from the mammalian DNA replication as well as for the replication strategy of most DNA viruses.

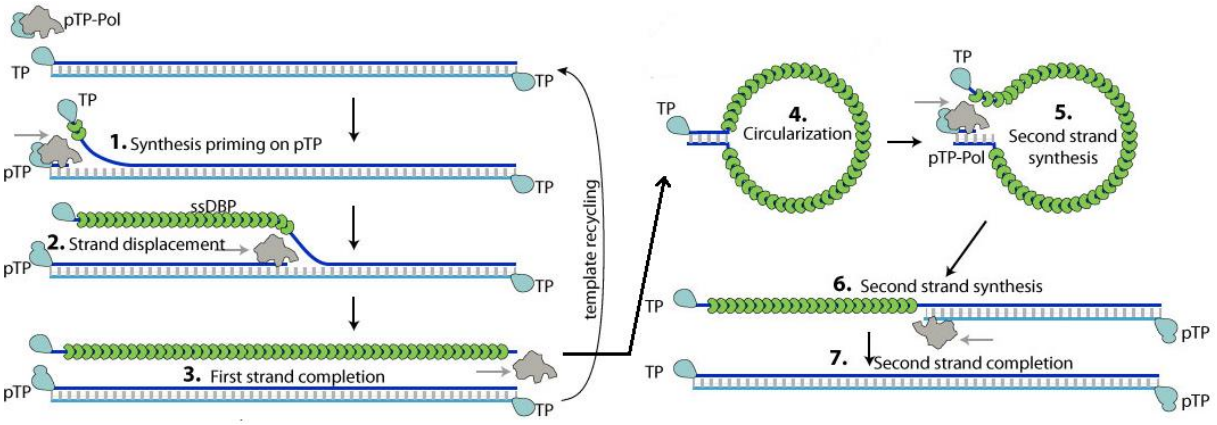


Figure 4: Adenovirus genome replication.
Terminal protein precursor (pTP) and viral DNA polymerase form a heterodimer. This binds to viral DNA and induces DNA replication by priming. During elongation process, one strand is displaced by new synthesized DNA and protected from degradation by binding of DNA binding Protein (DBP). Panhandle structure is formed by hybridization and second strand is synthesized. Figure reprinted from ViralZone with permission [172].

1.2.3.3. The adenovirus virion

Ads consist of icosahedral particles of about 90 to 100 nm in size, which are composed of 13 structural proteins denoted by roman numerals (II-X). The 252 capsomeres comprise the major structural component: the trimeric hexon proteins and 12 pentons, consisting of a pentamer and a penton base. There is a trimeric fibre protein at each capsid vertices projecting from the penton base that mediates the initial attachment to the host cells. Species C Ad enter the host cell via the primary coxsackie B virus and adenovirus receptor (CAR) on the cell membrane [173]. Although most Ad species are able to bind CAR, they primarily use several additional receptors: CD46 as well as the cadherin protein Desmoglein-2 (DSG-2) and the sialic acid-containing proteins were identified as entry receptors for different Ad species [174]. Subsequently, the penton base promotes interaction with cellular αv integrins, causing the entry of the virus via clathrin-mediated endocytosis. Other minor components of the capsids are IIIa, VI, VIII and IX. After internalization within the endosomes, the acidification leads to the release of the dismantled virus particle due to disruption of the endosomal membrane by protein pVI [175]. Inside the capsid, the viral double-stranded DNA genome is associated with five basic polypeptides: V, VII and X (also called Mu), which form the virus core, Iva2, responsible for genome packaging and the TP [149]. After transportation along the microtubules, the viral DNA is imported into the nucleus where DNA replication of the virus takes place [162] [176]. Since translation to proteins occurs in the cytoplasm, but the assembly

of progeny occurs in the nucleus, protein VI serves to shuttle the proteins over the nuclear pore complex back into the nucleus [177]. There, assembled virions with the packaged DNA must go through a maturation process by processing several viral proteins with the viral cysteine protease [178]. As the nucleus finally is fully packed with virions, the cytoskeleton is disrupted leading to rounding up of cells and in the end to cell lysis [179] [180].

1.2.3.4. Induction immune cascade

Human Ad infection induces release of type I interferons (IFN) in infected cells: IFNα and IFNβ, which cause an antiviral state in neighboring cells [181] [182]. At the level of the host, however, the immune response upon symptomatic Ad infection is initiated by the release of proinflammatory cytokines IL-6, IL-1β and additionally the tumor necrosis factor alpha (TNF-α) into the bloodstream. Other important innate immune mechanisms include the activation of cytotoxic immune cells [183]. Macrophages are attracted at first to different infected organs. They are trapping the invading viruses and play an important role in preventing viremia and systemic spread of Ad infection [184] [185] [186]. Further cytotoxic innate immune cells are chemo-attracted by different macrophage-derived cytokines and chemokines, whereby IL-1α/IL-1R1 pathway is the most important [187]. The adaptive immune response plays a crucial role in controlling the dissemination of adenovirus infection. Both humoral and cytotoxic adaptive immune mechanisms are involved in humans to contain adenovirus infection. It is believed that the serotype-specific antibody response is protective against reinfection with the same serotype.

1.2.3.5. Antiviral drugs against adenovirus infections

Until now, no approved drug against Ad is available and therefore only broadly active antivirals such as cidofovir or ribavirin are used in therapy of adenoviral diseases. Cidofovir acts as an Ad DNA replication inhibitor by mimicking a triphosphate nucleotide substrate for Ad DNA polymerases after activation by cellular kinases (see also 1.2.2.6). It is active against all species of human adenoviruses in vitro. Several clinical case studies have already indicated an efficient treatment of acute adenoviral keratoconjunctivitis as well as adenoviral infections in immunocompromised children and adults with cidofovir [188] [189] [190] [191] [192]. It was shown that the time point of treatment after infection plays a key role concerning the antiviral efficacy of cidofovir [193]. Additionally, combination-therapies with e.g. intravenous

immunoglobulin therapy (IVIG) demonstrate promising results [194]. The disadvantage using cidofovir for therapy of adenovirus infections is a high rate of non-responders and a limited oral bioavailability. This is the reason why an intravenous administration is necessary. Moreover, cidofovir tends to be accumulated in renal tubule cells to toxic levels and therefore this drug is associated with nephrotoxicity [195] [196].

Another broad-spectrum antiviral agent, which is used against both RNA and DNA viruses, is ribavirin. In vitro activity is shown mainly against Ad of species C, which is the clinically most relevant species in humans [197]. It is a purine nucleoside analogue, but up to now, there has been no consensus about the mechanism of action that would explain the major antiviral activity of ribavirin [198]. According to the "European guidelines" ribavirin is not an approved drug for the treatment of Ad infections since it failed to show consistent activity against various different Ad serotypes.

A new anti-Ad therapy showed a major advantage in recent years, concerning upcoming drugs against Ad: Brincidofovir (3-hexadecyloxy-1-propanol-cidofovir), developed by Chimerix, received Fast Track designation from the FDA for the treatment of Ad, CMV and smallpox virus. In addition, it obtained Orphan Medicinal Product Designation from the European Commission for the prevention of Ad- and CMV-disease. Brincidofovir (previously named CMX001) is a lipid-linked derivative of cidofovir and can be orally administered because of a lipid moiety. The drug is cleaved only within the cells to Cidofovir and cannot exit cells readily. Since it is not transported by an organic anion transporter, it does not show any nephrotoxicity because there is not re-accumulation in renal tubules [199] [200].

Patients suffering from T-cell-specific diseases, such as T-cell-depleted grafts, severe lymphopenia, and hematopoietic stem cell transplantation, especially associated in children, have a high risk for fatal Ad infections [201]. Successful treatment with Ad-specific cytotoxic T-lymphocytes from donors (CTL) have been reported [202] [203].

1.2.3.6. Assays for virus resistance

Failing to control Ad infections in risk groups with systemic Ad infection occurs frequently. A timely start of monitoring human Ad loads, especially in vulnerable patient groups, is important to prevent progression of invasive and disseminated diseases and to control the susceptibility of Ad in immune compromised patients [204]. The role of viral resistance

development in these therapy failures is unknown. Detection methods for different Ad serotypes include quantitative PCR (qPCR), Ad-specific viral antigen assays, and viral culture. Evaluation by real time PCR is a fast and easy-to-handle test. However, the current lack of broad knowledge concerning the drug resistance mutations in Ad does not allow genotyping of resistance. A reliable phenotypic resistance test is an urgent need in the field to generate the information pool needed for the genetic approaches. The basis of the current standard phenotypic tests, the culture of Ad, is work and time intense. It can take one to four weeks. Additionally, in some cases, the viral isolate does not grow well in cell culture [205].

For evaluation of new anti-Ad-targets it is important to have a cell culture model for screening the antiviral activity of a certain compound. The most commonly used methods for in vitro antiviral assays in the case of Ad are: the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based method, the plaque assay, the yield reduction assay, and the real-time PCR. These methods can be applied at any step of the viral replication cycle [206] [207]. Also, several other methods can be used like non-replicative vectors with a reporter gene, as well as other cell-based assays that concentrate on specific steps of the Ad life cycle. Furthermore, biosensor method using capacitance sensor arrays, computation method and animal model can be used for detection of Ad [208].

1.3. Replicon-based reporter systems in virology

Replicons are genetic elements that are amplified by virus infection. In general, they are comprised of a viral origin of replication (ORI) and a transgene. The ORI itself is the starting point of DNA- or RNA-replication. The dependence of the initiation of replication on certain viral elements, which are missing from the replicon itself, is the key factor for the specificity and the inducibility of replicon systems. As a consequence, the transgene is only expressed, if this deficiency is rescued by a co-infecting complementation proficient virus. These systems can provide platforms for new viral vaccines, specific detection system of infectious RNA viruses, drug susceptibility testing, studies on viral replication, and pathogenesis [209] [210] [211] [212].

Especially in the field of RNA virus research and diagnostics, replicon-based systems are already playing important roles as specific live cell-based reporter systems for detection of infectious particles. In contrast to traditional diagnostic tools, replicon-based reporter systems

have the advantage of a great sensitivity towards infectious viruses and are furthermore a robust, fast, cost effective, and specific technology [213]. They can easily be applied for testing of potential new antiviral inhibitors in a high throughput format.

Unlike RNA viruses, replicon-based reporter vectors for DNA viruses are more difficult to establish due to the challenges of viral DNA delivery into the nucleus and the dependence of the DNA virus transcription on the host RNA polymerases. Until now, only one replicon-based gene expression system for a DNA virus was published [214].

1.3.1. RNA virus replicon systems

RNA virus replicons have the advantage that replication and transcription of the genetic information are mainly confined to the cytosol. If nuclear localization was needed, the transgenes could not be transcribed by the host RNA polymerases. The replicons derive from either positive- or negative-strand RNA viruses with at least one essential gene deletion. Besides the viral ORIs and the transgene, the RNA virus replicons normally encode for the specific RNA replicase and many viral co-factors too. RNA replicons can be used for several approaches such as trans-complementation of essential viral genes, for tracking of viral infections, and for the production of recombinant viruses [211].

1.3.2. DNA virus replicon systems

The only published DNA virus-based replicon vector is specific for the murine CMV (MCMV) replication. It is based on an episomal vector and constitutes a conditional gene expression system that depends on viral DNA replication. The true-late genes of herpesviruses are only expressed after onset of lytic DNA replication and silenced in the absence of tightly regulated viral factors [214]. For the construction of the MCMV based replicon vectors, an episomal transgene was coupled to the MCMV origin of lytic replication (oriLyt). Since the oriLyt is activated upon a MCMV infection, the transgene expression relies on a co-infection of the cell with a wild-type MCMV. While the reporter gene expression is silenced in the absence of a lytic MCMV infection, an active infection leads to an induction of the transgene expression by more than 1000-fold. Mohr et al. used this mechanism as a tool for effective complementation of toxic viral late genes without the need of an additional inducer. Furthermore, the MCMV replicon-based vector could be exploited in vitro for intracellular immunization against MCMV

infection. In principle, the MCMV replicon vector shows a virus dose dependent induction of the transgene and therefore is able to provide a quantitative reporter tool for productive MCMV infection [214]. The disadvantage of the MCMV replicon system is that it is dependent on host histone-deacetylase-induced silencing process for optimal inducibility and therefore specific stable cell lines have to be generated for each application. Moreover, the published strategy could not be applied to human CMV (Zsolt Ruzsics, personal communication).

1.4. Adeno-associated virus as basis for a replicon vector

The Adeno-Associated Viruses (AAV) replicate only when the host cells are infected by another DNA virus. This natural switch built into the AAV's genetic system provided a promising platform to develop a replicon system responding to DNA virus infections.

1.4.1. Adeno-Associated Virus and their replication

In 1965, a small parvovirus was discovered as contamination of an Ad preparation and was therefore named adeno-associated virus [215]. This virus belongs to the *Parvoviridae* family, which has been isolated from many species, including humans. The genus to which the AAV is assigned to is named *Dependovirus* because it does not productively infect cells in vitro without a coinfection by a so-called helper virus. Viruses, known to serve as helper viruses for AAV are Ad, many herpesvirus strains like HSV-2 [216], HCMV [217], VZV [218] EBV, HHV6 [219] and pseudorabies virus (PrV) [220]. Other virus families, like human papillomavirus (HPV) [221] or an insect virus called baculovirus [222] were also shown as inducers of AAV productive cycle. Vaccinia virus is known as a sub-helper for AAV replication and packaging but fails to activate AAV promoters [223].

AAVs have a non-enveloped, icosahedral capsid of about 20-26 nm in diameter. Their genome consists of a linear, single-stranded DNA with approximately 4.7 – 6 kb. 13 human- and primate- specific AAV serotypes are currently known and the seroprevalence within the human population is very high (about 80%) [224]. Although no disease or pathogenic properties could be associated to AAV infections, the virus can be readily isolated from many different human samples. $CD3^+$ T lymphocytes were recently suggested as putative site of AAV persistence in humans [225].

1.4.1.1. AAV genome organization

The AAV particles carry a single stranded 4.8 kbp large linear DNA genome. At each end of the genomes, there are identical ITRs of about 150 bp, which form secondary structures by base pairing with one another [226]. The cis elements in the ITRs are necessary for AAV replication and for packaging of the AAV genome into the capsids [227]. Within the ITRs there are 3 open reading frames (ORFs), which encode the non-structural Rep gene, the structural Cap gene, and the assembly activating protein (AAP) gene [228]. Via unspliced and single-spliced forms, two out of four mRNAs encoding for the Rep proteins are transcribed by 2 different promoters and by additional alternative splicing of either transcript. All in all, the Rep gene encodes for four different proteins: Rep78 and Rep68, which are regulated by the promoter p5; Rep52 and Rep40 where transcription of both is activated by p19. Rep68 is the truncated version of Rep78 and Rep40 the one of Rep52 [229]. The Rep gene product is important for regulation of viral transcription, replication, encapsidation, integration of viral genome into the host chromosome, rescue from the latent state, and packaging of DNA.

The p40 promoter regulates the Cap gene that encodes the three structural proteins VP1, VP2 and VP3, all residing in the same reading frame [225] [230]. Responsible for the activation of p40 are the two larger Rep proteins in the presence of essential factors provided by large DNA viruses (see 1.4.1.3) [231] [232]. A nested alternative ORF within the Cap gene encodes an assembly-activating protein (AAP). A nonconventional start site initiates its translation [228].

1.4.1.2. Latent infection of AAV

In the absence of an additional helper virus, AAV establishes a latent state in its host cell. This is either characterized by chromosomal integration, mostly within the AAVS1 site located on the human chromosome 19, or by persistence in an extrachromosomal ds DNA episome [233] [234]. After entering the cell, the single stranded DNA is completed to a double stranded DNA in the host cell nucleus. Within the ITR there is a Rep-binding site (RBS) that allows binding of the two large Rep proteins of AAV [235]. Rep68 and Rep78 exhibit endonuclease activity, which is needed for unwinding the DNA and integration of a single-stranded DNA nick nearby a specific terminal resolution site (TRS) at the ITR [236]. In the AAVS1, a homologous DNA sequence to the RBS was found. This 33 bp sequence is sufficient for AAV integration and encompasses the RBS and the TRS element [237].

1.4.1.3. Productive replication cycle of AAV

Suitable conditions such as the infection of the same cell with a defined helper virus or, to a much lower extend, due to metabolic inhibitors, UV irradiation or genotoxic compounds lead to an initiation of a lytic replication cycle of AAV [238]. If the AAV genome is latently integrated into the host genome, the induced Rep gene products lead to its rescue [239]. As starting point, AAV p5 and p19 promoters are trans-activated by specific viral helper factors [240] [241]. As a consequence, the expression of the large Rep proteins lead to the replication of the AAV genome [242]. AAVs replicate their genome over unidirectional strand-displacement replication (Figure 5) [243] [244] [245]. Two motifs within the ITRs are important for replication: the TRS and the RBS. The ITRs have the ability for self-annealing and therefore build secondary structures on both ends of the genome, which provide unidirectional DNA synthesis due to a base-paired 3'OH group. Then, the replication of the AAV strand is done dependent on the infecting helper virus by either the viral or the cellular DNA polymerase with support by cellular proteins, which are involved in this process. As soon as the DNA template is copied, the terminal resolution step is initiated by binding of the Rep proteins at the RBE within the ITRs on both ends of the AAV genome. Their endonuclease activity is responsible for introducing a site-specific single stranded nick at the TRS. This leads to a free 3'-OH serving as a primer for new DNA synthesis [246]. The additional helicase activity provided by the REP proteins is another function in the replication process and is important for rendering the TRS site to single-stranded and therefore providing the access for introducing the nick into the TRS [236]. As a result, a double stranded full length AAV genome as well as a single stranded full length AAV displacement product is generated [247].

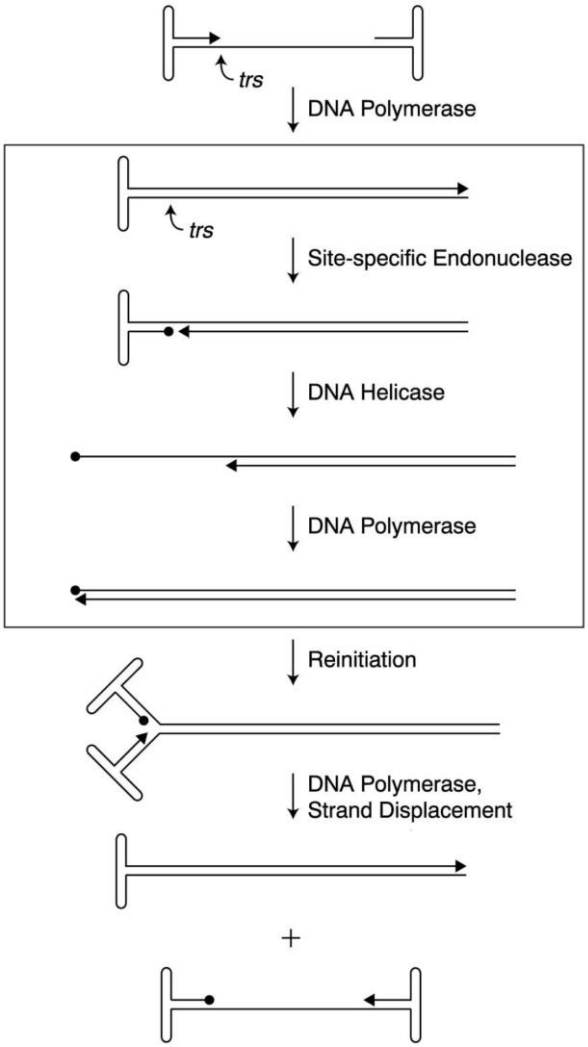


Figure 5: AAV Genome Replication.
Model of ssDNA replication of AAV genome showing the steps that lead to generation of multiple AAV genome copies. Illustration taken from [246].

The transcription of the AAV capsid proteins is controlled by p5 dependent Rep proteins in the presence of helper virus. Here, the binding of the cellular factor Sp1 to the larger Rep proteins promotes induction of the p40 promoter. In the presence of helper virus functions and wild-type AAV Rep proteins, there are three sequence elements essential for p40 promoter activity: the two Sp1 binding sites at -50 (Sp1-50) and -70 (GGT-70) bp upstream of the start of p40 and the TATA-box at -30. Another important element for maximal Rep mediated p40 activation is the CARG-like element at -140 in the p19 promoter, which cooperates with the Sp1-50 site of the p40 promoter [232] [231].

After the expression of the capsid proteins, the AAP protein directed capsid assembly takes place in the nucleus and genomes with both positive and negative polarity are packaged into the preformed AAV particles by assistant of the small Rep proteins Rep52 and Rep40 [227].

1.4.1.4. Ad helper functions for productive AAV replication

A productive AAV replication is dependent on helper functions by different helper viruses. Isolated adenoviral gene products that are able to induce AAV replication are E1A, E1B, E2A, E4ORF6, and the VA-RNA [248]. The different Ad proteins reveal different tasks: E1A is needed for the activation of the AAV promoters as transcription factor, for driving the host cell into S-phase of the cell cycle and stabilization of p53, which leads to apoptosis [249] [241]. The E2A encoded protein DBP binds the ssDNA of AAV together with Rep gene products in the nuclear replication centers and serves for viral DNA replication and furthermore for viral mRNA processing and export [250] [251] [252]. It is also important for activating p5 transcription of AAV [253]. By inhibiting the interferon-inducible eIF-2 protein kinase, the Ad derived VA-RNA circumvents this cellular anti-viral mechanism, which would block AAV protein translation [254] [255]. E1B55K together with E4ORF6 prevents apoptosis and counteracts E1A by forming a complex with p53 that leads to degradation [256] [257]. Furthermore, the two proteins form a heterodimer in order to export AAV and Ad late mRNAs from the nucleus and at the same time to inhibit the transition of Ad early genes as well as cellular mRNAs [258]. In case of Ad induction, the DNA polymerase responsible for AAV replication is of cellular origin [259].

1.4.1.5. HSV helper functions for productive AAV replication

Opposed to Ad, HSV-1 helper functions are directly involved in AAV genome replication. As helper functions for AAV, the ternary HSV-1 helicase/primase (HP) complex and the major ss DNA-binding protein ICP8 are sufficient for a productive AAV2 replication [260] [261]. For the initiation of AAV genome replication, ICP8 forms a ternary complex by co-localization with Rep78 and the AAV ssDNA within nuclear replication centers [262] [263]. In addition, a few other HSV-1 gene products enhance AAV replicative cycle. ICP0 protein was shown to activate Rep gene expression in latently AAV infected cells [264]. The proteins ICP4 and ICP22 act synergistically with ICP0 and therefore support the enhancing effect. Furthermore, the HSV-1 DNA polymerase complex (U_L30/U_L42) was proven to increase the AAV DNA replication substantially [260]. Another HSV-1 protein, the exonuclease (U_L12), has recently been shown

to intervene with AAV genome replication. U_L12 increases the resolution of AAV replicative forms and in addition it is involved in AAV particle production [265].

The helper effect of HPV is dependent on E1, E2 and E6 genes, which are able to increase AAV replication in vitro. The main contributor was shown to be the helicase/ATPase domain of the carboxy-half of E1 [266] [221].

1.5. Objectives

The aim of this work was the construction of a novel replicon-based gene expression system based on the genome of the Adeno-associated virus. This system had to fulfill several criteria:

1. Expression of the AAV replicon system transgene should be induced by wild-type virus infection without further modification of the viral genome.
2. There should be no homology between the AAV-based replicon system and the inducing virus to avoid recombination events.
3. Several delivery methods of the AAV replicon vector should be tested for different applications of the system.

The newly generated AAV replicon-based system had to be tested for several applications:

1. *Trans*-complementation of the toxic HAd5 late protein pVI by the AAV replicon vector should be tested in order to have a tool for studying essential viral genes in a chronological correct expression kinetic.
2. A fast phenotypical diagnostic assay for detecting drug-resistance in patient-derived viral samples should be generated in order to decrease the treatment start time for the patient and furthermore to get the most effective therapy according to the sensitivity of the infecting virus.
3. A drug susceptibility test system should be established in order to test new drugs against herpes- and adenoviruses in a high-throughput screening format ensuring reliable quantitative results for data analysis.

2. Material

2.1. Devices

BD FACSCanto™ II	Biosciences, San Jose, CA, USA
Biofuge Pico	Heraeus Centrifuges, Buckinghamshire, E
Bio-Photometer	Eppendorf, Hamburg, D
CASY® cell counter	Schärfe System, Reutlingen, D
Centrifuge 5417 R	Eppendorf, D
Centrifuge Avanti^{R} J-26 XPI	Beckman Coulter GmbH, Krefeld, D
Centrifuge Avanti^{TM} J-20 XP	Beckman Coulter GmbH, Krefeld, D
Fluorescence microscope DMI4000B	Leica, Wetzlar, D
Gene Pulser^{TM}	Bio-Rad, D
Incubator B5050E	Haereus instruments, D
Incubator BB16CU	Haereus instruments, D
Bakterienschüttelschrank Certomat BS1	Satorius, Göttingen, D
Light Cycler ® 2.0	Roche, Mannheim; D
Light microscope Axiovert 40L	Zeiss Carl AG, D
Magnetrührer RCT basic	IKA® Labortechnik, D
Microplate Luminometer XFluor4_V4_51	Tecan, Grödig, A
Multifuge 3 S-R	Heraeus Centrifuges, Buckinghamshire, E
Multikanalpipette, 12-Kanal (100 µL, 30-300 µL)	Eppendorf, Hamburg, D
NanoDrop™ ND-1000 Spectrophotometer	Peqlab, Erlangen, D
NanoPhotometer® P-Class P330	Implen, Westlake Village, USA
Optima LE-80K Ultracentrifuge	Beckman Coulter GmbH, Krefeld, D
PH-Meter 430	Cornig, Miami, USA
Photo documentation apparatus	Bio-rad, München, D
Shaking water bath GFL 1092	Burgwedel, D
T Gradient PCR Machine	Biometra, Göttingen, D
Thermomixer 5436	Eppendorf, Hamburg, D
Vortex-Mixer M51	IKA® Labortechnik, D

2.2. Consumables

96 well plate flat CC ST W/Lid	Thermo Electron, Langenselbold, D
Caesium Chloride Disposable PD 10 Desaltin	Sigma-Aldrich, Deisenhofen, D
CASYcups	OLS OMNI Life Science
Cell culture dish (20 cm2; 55 cm2; 145 cm2)	Corning, Durham, USA
Cell culture plate (6-, 12-, 24-, 48-, 96-well)	Corning, Durham, USA
Cell scrapers	Costar, Bodenheim, D
Combitips plus (5 mL, 10 mL)	Eppendorf, Hamburg, D
CryoTube™ Vials	nunc, Langenselbold, D
Electroporation cuvettes	Bio-Rad, D
epDualfilter tips (2-100 µL; 20-300 µL)	Eppendorf, Hamburg, D

Eppendorf tubes	Eppendorf, Hamburg, D
Falcons conical tubes (15 mL, 50 mL)	Becton Dickinson, Heidelberg, D
Inoculation Loops	Sarstedt, Nürnbrecht, D
LightCycler® Capillaries (20 µL)	Roche, Mannheim, D
Mikroschraubenröhre 0.5 mL	Sarstedt, Nürnbrecht, D
Pipettes (5 ml, 10 ml, 25 ml)	Kuhnle, Karlsruhe, D
Reaction tubes (0.5 mL, 1.5 mL, 2 mL)	Eppendorf, Hamburg, D
Ultracentrifugation tubes	Beckman Coulter GmbH, Krefeld, D

2.3. Reagents and biochemicals

Acetone	Roth, Karlsruhe, D
Acetic acid	Sigma-Aldrich, Deisenhofen, D
Acycloguanosine	Sigma-Aldrich, Deisenhofen, D
Agar	BD Falcon™, D
Agarose	Thermo Fisher Scientific, MA, USA
Alcaline Phosphatase (CIP)	NEB, Frankfurt/Main, D
Ammonium sulfate (> 99.5%, p.a.)	Fluka Chemie, Buchs, CH
Ampicillin	Sigma-Aldrich, Deisenhofen, D
Bacto™ Agar	Becton Dickinson, Heidelberg, D
Bacto™ Tryptone	Roth, Karlsruhe, D
Bacto™ Yeast Extract	Becton Dickinson, Heidelberg, D
Benzonase® Nuclease	Sigma-Aldrich, Deisenhofen, D
Boric acid	Sigma-Aldrich, Deisenhofen, D
Carboxymethyl Cellulose Sodium Medium	Sigma-Aldrich, Deisenhofen, D
Cell culture lysis reagent (5x)	Promega, Madison, USA
Cesium chloride, Optical Grade	Sigma-Aldrich, Deisenhofen, D
Chloramphenicol	Sigma-Aldrich, Deisenhofen, D
DMEM-Dulbecco's Modified Eagle	Thermo Fisher Scientific, MA, USA
DNA ladder (100 bp, 1 kbp)	NEB, Frankfurt/Main, D
dNTPs	NEB, Frankfurt/Main, D
Ethanol	Roth, Karlsruhe, D
Ethylen-Diamin-Tetra-Acetic acid (EDTA)	Fluka, Karlsruhe, D
Fetal calf serum (FCS)	PAN-Biotech, Aidenbach, D
Fomaldehyde	Roth, Karlsruhe, D
Foscarnet sodium hexahydrate	Sigma-Aldrich, Deisenhofen, D
FuGene® HD Transfection Reagent	Promega, Madison, USA
Glycerin	Roth, Karlruhe, D
Glycin	Roth, Karlsruhe, D
Hepes (1M) buffer	Thermo Fisher Scientific, MA, USA
Hygromycin B solution	Sigma-Aldrich, Deisenhofen, D
Isopropanol	Merck, Darmstadt, D
Kanamycin	Sigma-Aldrich, Deisenhofen, D
L-Arabinose	Sigma-Aldrich, Deisenhofen, D
L-Glutamin	Thermo Fisher Scientific, MA, USA
Lipofectamine2000	Thermo Fisher Scientific, MA, USA

L-Plus Arabinose Crystalline	Sigma-Aldrich, Deisenhofen, D
Luciferase Cell Lysis Buffer	NEB, Frankfurt/Main, D
Methanol	Roth, Karlsruhe, D
Newborne calf serum (NCS)	PAN-Biotech, Aidenbach, D
Opti-MEM™ I Reduced Serum Medium	Thermo Fisher Scientific, MA, USA
PEI "Max", MW 40.000	Polysciences, Eppelheim, D
Penecillin/Streptomycine	Thermo Fisher Scientific, MA, USA
Phenol-Chloroform	Roth, Karlsruhe, D
Phosphate buffered saline (PBS)	Thermo Fisher Scientific, MA, USA
Proteinase K	Quiagen, Hilden, D
Q5® High-Fidelity DNA Polymerase	NEB, Frankfurt/Main, D
Restriction enzymes and buffers	NEB, Frankfurt/Main, D
Sodium acetat	Merk, Darmstadt, D
Sodium chloride	Merk, Darmstadt, D
sodium dodecyl sulfate	SERVA, Heidelberg, D
SuperFect Transfection Reagent	Quiagen, Hilden, D
Tris-HCl (pH 8.0)	Sigma-Aldrich, Deisenhofen, D
Tris	Sigma-Aldrich, Deisenhofen, D
Trypsin/EDTA Solution (TE)	Thermo Fisher Scientific, MA, USA
T4 DNA Ligase	NEB, Frankfurt/Main, D

2.4. Commercial Kits

BioLux® Gaussia luciferase Assay Kit	NEB, Frankfurt/Main, D
DNeasy™ Blood and Tissue Kit	Quiagen, Hilden, D
Dual-Luciferase® Reporter Assay System	Promega, Madison, USA
Expand High Fidelity PCR System	Roche, Mannheim, D
illustra ™ plasmidPrep Mini Spin Kit	GE Healthcare, Freiburg, D
NucleoBond® PC100 Plasmid DNA Purification Kit	Macherey-Nagel, Düren, D
Plasmid Maxi Kit	Quiagen, Hilden, D
QIAquick PCR Purification and Gel Extraction Kit	Quiagen, Hilden, D
QuantiTect SYBR Green PCR Kit	Quiagen, Hilden, D

2.5. Culture Media for bacteriology and cell culture

Table 2.5.1: LB medium

LB medium
10 g Bacto Tryptone
5 g Bacto yeast extract
5 g NaCl
add 1 L ddH_2O

Table 2.5.2: LB agar

LB agar
7.5 g Agar
in 500 mL LB medium

Table 2.5.3: TE buffer

TE buffer
10 mM Tris, pH 8
1 mM EDTA

Table 2.5.4: TAE buffer

TAE buffer
40 mM Tris-HCl
1 mM EDTA (pH 8.0)
20 mM acetic acid
add 1 L ddH_2O

Table 2.5.5: TBE buffer

TBE buffer
90 mM Tris
2.5 mM EDTA (pH 8.0)
90 mM boric acid
add 1 L ddH_2O

Table 2.5.6: Freezing medium

Freezing medium
10% DMSO
60% FCS
30% DMEM

Table 2.5.7: 1 x Hepes buffer

1 x Hepes buffer
100 mM of HEPES
20 mM of $MgCl_2$
add 100 mL ddH_2O

2.6. Oligonucleotides

All oligonucleotides for cloning and sequencing used were either synthesized by Metabion (Martinsried, D) and additionally HPLC purified if the sequence exceeded 33 nt or purchased from GATC for sequencing directly.

List of oligonucleotides:

Name	Comments	Sequence 5' - 3'
AVfor	Sequencing primer for AAV replicon vector	CCGTGTCAGAATCTCAACCC
AVrev	Sequencing primer for AAV replicon vector	CATGCTTTGCATACTTCTGCC
REPfor1	Sequencing primer for AAV replicon vector	AGAAGCTGCAGCGCGACTTTCTGAC
REPrev1	Sequencing primer for AAV replicon vector	GGTGATCAGATCAAAAACTTCAGCCAGGTAC ATG
REPfor2	Sequencing primer for AAV replicon vector	GAGCTGGTCGGGTGGCTCGTGG
REPrev2	Sequencing primer for AAV replicon vector	GGTGGGCAAAGGATCACGTGG
pBR3	GATC sequencing primer for AAV replicon vector	TCCCCATCGGTGATGTC
5ITRfor	Sequencing primer for AAV replicon vector	GAAGTGGCGAGCCCGATCTTCCCC
HYGfor	Forward PCR primer for pAV1-Hyg	GTGTGGGCCCTGTGGTATGGCTGATTATGATC CTC
HYGrev	Reverse PCR primer for pAV1-Hyg inclusive a multiple cloning site (MCS)	GTGTCTCGAGGACACTCTCTCTGAGCTAGCTT CGTACGGATATCACCGGTAACTTAAGTTGCGG CCGCGTGTGGAAAGTCCCCAGGCTCCCC
Bgfp-rev	Sequencing primer for pAV1-GFP-Hyg	GGACGAGCTGTACAAGTAAAGCGGCCGCGAC
LUCfor	Forward PCR primer for pAV1-GLuc-Hyg	GTGTAGATCTCGGGATCCACCGGTCGCCACCT CCGGAGGCGGCGGCTCCGAAGACGCCAAAAA CATAAAGAAAGG
LUCrev	Reverse PCR primer for pAV1-GLuc-Hyg	CTCTGGCACAAAATCGTATTCATTA
pEGFP_N	Sequencing primer for pAV1-GFP-Hyg	CTGGTCGAGCTGGACGG
M13_FP	GATC sequencing primer for pL-MTT-P and pL-SV40L-P	TGTAAAACGACGGCCAGT
M13_RP	GATC sequencing primer for pL-MTT-P and pL-SV40L-P	CAGGAAACAGCTATGACC
AVfor2seq	Sequencing primer for pAV1-GLuc-P40M-MTT-TPL and pAV1-GLuc-P40M-SV40L-TPL	GCTGCCGATGGTTATCTTCC

GLucrevseq	Sequencing primer for pAV1-GLuc-P40M-MTT-TPL and pAV1-GLuc-P40M-SV40L-TPL	TTGTTCTCGGTGGGCTTGGC
AVfor3seq	Sequencing primer for pAV1-GLuc-P40M-MTT-TPL and pAV1-GLuc-P40M-SV40L-TPL	ATGTGGATTTGGATGACTGC
H3-PPVIT-zeo2	Forward PCR primer for ETPCR_DELpVI	CTGGCGGCGACATGGACGCATACATGACACA CATACGACACGTTAGCTATTGAACTGCTGATC TTCAGATCCTC
H5-PPVIT-zeo	Reverse PCR primer for ETPCR_DELpVI	TAAAAAGTCTGGACTCTCACGCTCGCTTGGTC CTGTAACTATTTTGTAGACGTTTACAATTTCGC CTGATGCG
pVI-3seq	Sequencing primer for pBA5-FRT-ΔpVI	CTCAGGTACTCCGAGGCGTCC
pVI_5seq	Sequencing primer for pBA5-FRT-ΔpVI-zeo	ATCCTGCCCCTCCTTATTCC
BAC_for	Sequencing primer for pBA5-FRT- Che-ΔpVI-zeo	GCCGTGCCGGCACGTTAACC
pVI_5seq	Sequencing primer for pBA5-FRT- Che-ΔpVI-zeo	ATCCTGCCCCTCCTTATTCC
APVIfor	Forward PCR primer for pAV1-PVI-Hyg	GTGTGCTAGCATGGAAGACATCAACTTTGCGT CTC
APVIrev	Reverse PCR primer for pAV1-pVI-Hyg	GGGTGCAATCCCTGAAGCGCCGACGATGCTT CTGAGCGGCCGCACAC

List of real time PCR primer:

Name	**Sequence 5’ - 3’**
QGLucfor	AAGTTCATCCCAGGACGCTGCC
QGLucrev	ACACTGCACGTTGGCAAGCCCT
GAPDHfor	TGGTATCGTGGAAGGACTCA
GAPDHrev	CCAGTAGAGGCAGGGATGAT

2.7. Enzymes for molecular biology

All restriction endonucleases used during this study were purchased from New England Biolabs (NEB) (Frankfurt/Main, D) and applied according to the manufacturer’s instructions.

2.8. Plasmids

pLITMUS28	**NEB, Frankfurt/Main, D**
pAV1	(REF: Laughlin et al 1983) ATCC 37215
pTRE2Hyg	Clontech, Mountain View, CA, USA
pGluc	NEB, Frankfurt/Main, D
pEGFP-N1	Clontech

pB45neo-F-tet	Derived from pBMGNeo [267]. The papillomavirus derived sequences and the MTT promoter used in this study were identical to pBMGNeo
pSVL-mo FceRI	Newly synthetized sequence by Addgene (Cambridge, MA, USA)
pMK-T-P40M(syn)	Newly synthetized by Invitrogen GeneArt Gene Synthesis; mutated human Ad-5 p40 promoter (Thermo Fisher Scientific, Waltham, MA USA)
pMK-T-TPL(syn)	Newly synthetized by Invitrogen GeneArt Gene Synthesis; 5'-untranslated tripartite leader sequences (Thermo Fisher Scientific, Waltham, MA USA)
pO6-ie-zeo	GenBank AccNr: AY700022.1 [268]
pO6-A5-CMV-mCherry	Sirion Biotech, Martinsried, Germany
pCP20	Chloramphenicol and ampicillin resistant genes, temperature sensitive replication of yeast Flp recombinase gene [269]

2.8.1. Plasmids that were constructed in this study

Name (Resistance)	Comments
pAV1-Hyg	Generated by inserting the amplicon PCRHyg –MCS (PCR template on pTRE2Hyg using primer pair HYGfor and HYGrev) replacing the Gap genes into pAAV1 with a hygromycin cassette and a multiple cloning site (MCS) with *ApaI/XhoI.*
pAV1-GLuc-Hyg	Generated by inserting the amplicon PCR-GLuc (PCR template on pGLuc using primer pair LUCfor and LUCrev) inot pAV1-Hyg after restriction with NheI/NotI using the sites in the cloned MCS.
pAV1-GFP-Hyg	The *EGFP* gene was excited from the plasmid pEGFP-N1 and inserted into pAV1-Hyg after treatment with *NheI/NotI* using the sites in the cloned MCS of pAV1-Hyg.
pL-SV40L-P	Generated by inserting the amplicon PCRSV40Lpro (PCR template on pSLV-mo using primer pair SV40LPfor (#1505) and SV40LPrev (#1506)) into pLitmus28 after restriction with *XhoI/AgeI*
pL-P40M-SV40L	The mutated p40 promoter from plasmid was excised from the plasmid pKM-T-P40M and inserted into pL-SV40L-P after treatment with *XhoI/BglII*.
pL-P40M-SV40L-TPL	The TPL sequence was excised from the plasmid pKM-T-TPL and inserted into pL-P40M-SV40L after treatment with *AvrII/AgeI.*
pAV1-GLuc-P40M-SV40L-TPL	The P40M-SV40L-TPL sequence was excised from the plasmid pL-P40M-SV40L-TPL and inserted into pAV1-GLuc-Hyg after treatment with *NheI/BsteII.*
pL-MT-P	Generated by inserting the amplicon PCR MTTpro (PCR template on pB45neo-F-tet using primer pair MTTfor (#1507) and MTTrev (#1508)) into pLitmus28 after restriction with *XhoI/AgeI*
pL-P40M-MT	The mutated p40 promoter was excised from the plasmid pKM-T-P40M and inserted into pL-MTT-P after treatment with *XhoI/BglII*.
pL-P40M-MT-TPL	The TPL sequence was excised from the plasmid pKM-T-TPL and inserted into pL-P40M-MT after treatment with *AvrII/AgeI.*

pAV1-GLuc-P40M-MT-TPL	The P40M-MT-TPL sequence was excised from the plasmid pL-P40M-MT-TPL and inserted into pAV1-GLuc-Hyg after treatment with *NheI* (fulcut) */BsteII* (partial cut).
pAV1-pVI-Hyg	Generated by inserting the amplicon PCRAd5_ppVI (PCR template on pBA5-FRT using primer pair APVIfor and APVIrev) into pAV1-Hyg after treatment with *NheI/NotI*.

2.9. Bacterial artificial chromosomes

2.9.1. Published BACs and BACs available in the group

Name	Comments
pBA5-FRT	Derived from [270] BAC carrying the Ad5 genome and an FRT site for homologous recombination; left ITR, the encapsidation signal, the E1 and E3 region are deleted;

2.9.2. BAC's cloned during this study

Name	Comments
pBA5-FRT-ΔpVI-zeo	Generated by homologous recombination to replace the *pVI* gene of pBA5-FRT by a zeocin resistance cassette using the amplicon ETPCR_DELpVI (PCR template on pO6-ie-zeo using primer pairs H5-PPVIT-zeo and H5-PPVIT-zeo2)
pBA5-FRT-Che	Ectopic insertion of pO6-A5-CMV-mCherry into pBA5-FRT via Flp/FRT system.
pBA5-FRT- Che-ΔpVI-zeo	Ectopic insertion of pO6-A5-CMV-mCherry into pBA5-FRT-ΔpVI-zeo via Flp/FRT system.

2.9.3. Viruses

Name	Comments
Human adenovirus type 5 wild-type (Ad5-WT)	German reference center, Albert Heim, Hannover, D
Ad5-Che	SIRION Biotech, Martinsried, D
Human Ad-12	Kindly provided by Prof. A. Ehrhardt, Lehrstuhl für Virologie und Mikrobiologie, Witten, D
Human Ad-3	Kindly provided by Prof. A. Ehrhardt, Lehrstuhl für Virologie und Mikrobiologie, Witten, D
Human Ad-11	Kindly provided by Prof. A. Ehrhardt, Lehrstuhl für Virologie und Mikrobiologie, Witten, D
Human Ad-9	Kindly provided by Prof. A. Ehrhardt, Lehrstuhl für Virologie und Mikrobiologie, Witten, D
Human Ad-17	Kindly provided by Prof. A. Ehrhardt, Lehrstuhl für Virologie und Mikrobiologie, Witten, D

Human Ad-4	Kindly provided by Prof. A. Ehrhardt, Lehrstuhl für Virologie und Mikrobiologie, Witten, D
HSV-1 strain F	Kindly provided by Dr. G. Jäger, Max von Pettenkofer-Institut, München, D [271]
HSV-1 res	Kindly provided by Dr. G. Jäger, Max von Pettenkofer-Institut, München, D Resistant control strain used in the viral diagnostic department of the MvP.
ACV+ HSV-1 isolate # 824670	Kindly provided by Dr. V. Kapper-Falcone, Institut für Virologie, Freiburg, D
ACV+ HSV-1 isolate # 842369	Kindly provided by Dr. V. Kapper-Falcone, Institut für Virologie, Freiburg, D
ACV+ HSV-1 isolate # 842913	Kindly provided by Dr. V. Kapper-Falcone, Institut für Virologie, Freiburg, D
ACV+ HSV-1 isolate # 854014	Kindly provided by Dr. V. Kapper-Falcone, Institut für Virologie, Freiburg, D
ACV+ HSV-1 isolate # 838500	Kindly provided by Dr. V. Kapper-Falcone, Institut für Virologie, Freiburg, D
ACV+ HSV-1 isolate # 847890	Kindly provided by Dr. V. Kapper-Falcone, Institut für Virologie, Freiburg, D
ACV+ HSV-1 isolate # 848759	Kindly provided by Dr. V. Kapper-Falcone, Institut für Virologie, Freiburg, D
ACV- HSV-1 isolate # 852044	Kindly provided by Dr. V. Kapper-Falcone, Institut für Virologie, Freiburg, D
ACV- HSV-1 isolate # 846206	Kindly provided by Dr. V. Kapper-Falcone, Institut für Virologie, Freiburg, D
ACV- HSV-1 isolate # 861747	Kindly provided by Dr. V. Kapper-Falcone, Institut für Virologie, Freiburg, D
ACV- HSV-1 isolate # 845708	Kindly provided by Dr. V. Kapper-Falcone, Institut für Virologie, Freiburg, D
ACV- HSV-1 isolate # 854437	Kindly provided by Dr. V. Kapper-Falcone, Institut für Virologie, Freiburg, D
ACV- HSV-1 isolate # 860929	Kindly provided by Dr. V. Kapper-Falcone, Institut für Virologie, Freiburg, D
ACV- HSV-1 isolate # 845531	Kindly provided by Dr. V. Kapper-Falcone, Institut für Virologie, Freiburg, D
ACV+ HSV-2 isolate # 17-168	Kindly provided by Dr. V. Kapper-Falcone, Institut für Virologie, Freiburg, D
ACV+ HSV-2 isolate # 17-604	Kindly provided by Dr. V. Kapper-Falcone, Institut für Virologie, Freiburg, D
ACV+ HSV-2 isolate # 17-742	Kindly provided by Dr. V. Kapper-Falcone, Institut für Virologie, Freiburg, D
ACV+ HSV-2 isolate # 18-650	Kindly provided by Dr. V. Kapper-Falcone, Institut für Virologie, Freiburg, D
ACV- HSV-2 isolate # 18-161	Kindly provided by Dr. V. Kapper-Falcone, Institut für Virologie, Freiburg, D
ACV- HSV-2 isolate # 18-553	Kindly provided by Dr. V. Kapper-Falcone, Institut für Virologie, Freiburg, D

HCMV (TB40-BAC4)	Wild-type BAC derived endotheliotropic HCMV strain [272]; Kindly provided by PD Dr. rer. nat. Barbara Adler, Max von Pettenkofer-Institut, München, D

2.9.4. Recombinant viral particles

Name	Comments
rAAV1-CMV-GFP	SIRION Biotech, Martinsried, D
rAAV1/2-CMV-GFP	SIRION Biotech, Martinsried, D
rAAV2-CMV-GFP	SIRION Biotech, Martinsried, D
rAAV4-CMV-GFP	SIRION Biotech, Martinsried, D
rAAV5-CMV-GFP	SIRION Biotech, Martinsried, D
rAAV6-CMV-GFP	SIRION Biotech, Martinsried, D
rAAV2-REP-GLuc	SIRION Biotech, Martinsried, D
rAAV1/2-R-GLuc	SIRION Biotech, Martinsried, D

3. Methods

3.1. Propagation of recombinant DNA in E. coli

3.1.1. Culturing recombinant E. coli

For propagation of plasmid DNA or viral BAC DNA in bacteria, the respective *Escherichia coli* strains carrying the recombinant DNA were amplified. The strains for harvesting recombinant DNA were propagated either in liquid culture using low-salt Lucia Broth (LB) medium or, if isolation of single colonies was needed, on LB-agar plates in the presence of appropriate antibiotics. Antibiotics used were: Ampicillin (amp) at concentrations of 100 µg/mL for selection of high copy plasmids, and 50 µg/mL for selection of low copy plasmids and BACs, Chloramphenicol (cm) was used at concertation of 25 µg/mL, Kanamycin (kn) at 50 µg/mL, and Zeocin (zeo) at 30 µg/mL. DH10B and PIR1 cells were cultured at 37°C and SW102 were cultured at 32°C. For optimal aeration liquid cultures were propagated under constant shaking at 160 rpm.

Glycerol stocks of the bacterial strains with the DNA of interest were long-term stored. For this purpose, 600 µL of the respective culture were added to 400 µL of 60 % (v/v) sterile glycerol and after rigorous vortexing they were stored at -80°C. To recover the strains from the glycerol stocks they were either plated or recovered in liquid pre-culture o.n.

Table 3.1.1.1: Used bacterial strains

E. coli strain	Genotype
DH10B	F − mcrA Δ(mrr−hsdRMS−mrcBC) Φ80lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu) 7697 galU galK rpsL nupG λ − (Invitrogen, Karlsruhe, D)
PIR1	F − Δlac169 rpoS(Am) robA1 creC510 hsdR514 endA recA1 widA(ΔmluI):pir-116 (Invitrogen, Karlsruhe, D)
SW102	DH10B [λc1857 (cro-bioA)<>Tet] gal490 ΔgalK [273]

3.1.2. Preparation of electro-competent bacteria

For the preparation of electro-competent DH10B and PIR1 cells, 2 x 200 mL pre-warmed LB-medium was inoculated with 2 mL of overnight pre-cultures (see chapter 3.1.1) and kept at

37°C constantly shaking at 160 rpm until reaching the middle logarithmic phase at OD_{600} of about 0.5. The bacterial cultures were then incubated on ice for 30 min and centrifuged to collect the bacteria at *6000 x g* for 10 min at 4°C. All further steps were subsequently performed on ice with pre-cooled equipment and buffers. The received pellets were combined and re-suspended in 200 mL of 10 % (v/v) glycerol. The bacteria were collected again by centrifugation as above. This washing step was repeated two times to remove as much salt residuals as possible. After the last centrifugation step, the pellet was re-suspended in 1 mL of fresh 10 % (v/v) glycerol and aliquoted to 65 µL/tube in 1.5 ml Eppendorf tubes. The bacteria were either transformed directly by electroporation or snap-frozen in liquid nitrogen and stored at -80°C.

For preparation of electro-competent SW102 *E.coli*, 100 mL of LB were inoculated with 2 mL of the pre-culture and grown at 32°C constantly shaking at 160 rpm until the OD_{600} of 0.55 to 0.6 was reached. In case of homologous recombination, the expression of the recombinases was heat induced at incubating the bacterial culture 42°C for 15 min in a shaking water bath. The bacterial cultures were then incubated on ice for 10 min and centrifuged to collect the bacteria at *6000 x g* for 10 min at 4°C. All further steps were subsequently performed on ice with pre-cooled equipment and buffers. The received pellets were combined and re-suspended in 50 mL of pre-cooled ddH_2O. The bacteria were collected again by centrifugation as above. This washing step was repeated two times to remove as much salt residuals as possible. After the last centrifugation step, the pellet was re-suspended with 250 µL of ddH_2O and aliquoted to 65 µL/tube in 1.5 ml Eppendorf tubes. The electro-transformation in case of SW102 cells was always done directly after the preparation of competent cell.

3.1.3. Transformation of electro-competent bacteria

Aliquots of electro-competent cells were either taken directly after their preparation or were thawed on ice from frozen aliquots for 15 min. Then approximately 25 – 50 ng DNA was transferred to pre-cooled 2 mm electroporation cuvettes. The prepared electro-competent cells were added directly to the DNA and mixed. Bacteria cells were electro-shocked at 2.5 kV (25 µF/200 Ω). Right after pulsing, 1 mL of pre-warmed LB w/o antibiotics was added and suspension was incubated for 1 h at the appropriate temperature (32°C or 37°C) using a thermo-mixer. Appropriate amounts of transformed cultures were then plated on LB-agar plates with selective antibiotics and cultured o.n. at 32°C or 37°C respectively.

3.1.4. Isolation of DNA from bacteria

3.1.4.1. Small scale isolation of plasmid DNA

Single clones from the o.n. plate culture were picked and inoculated in 2 mL LB completed with the appropriate antibiotics. The cultures were incubated o.n. at 32°C or 37°C, at 160 rpm. Plasmid DNA was purified from saturated o.n. cultures using the illustra ™ plasmidPrep Mini Spin Kit according to the manufacturer's instructions.

3.1.4.2. Large scale isolation of plasmid DNA

For isolation of plasmid DNA in large quantities o.n. 200 mL of LB was inoculated with either 100 µL of an o.n. culture from small scale preparations or from a scratch of a glycerol stock (see chapter 3.1.1) together with the appropriate antibiotics. Plasmids were purified according to the manufacturer's instructions using the NucleoBond® PC100 Plasmid DNA Purification Kit (Macherey-Nagel). Plasmid DNA pellet was dissolved in 200 µL TE buffer at 4°C o.n. and then stored at -20°C.

3.1.4.3. Small scale isolation of BAC-DNA

A single bacterial clone was picked and cultured in 10 mL LB in the presence of the appropriate antibiotics for selection of the BAC DNA in SW102 or DH10B strains at 32°C or 37°C, respectively, with shaking at 160 rpm o.n. Bacterial cultures were centrifuged at 3500 rpm for 15 min at RT and then pellets were re-suspended in 300 µL of resuspension buffer P1 of the Plasmid Maxi Kit (QIAGEN) and transferred to 2 mL Eppendorf tubes. 300 µL of lysis buffer P2 of the Plasmid Maxi Kit (QIAGEN) was added and mixed by gentle inversion. After an incubation time of 5 min at RT, 300 µL of neutralization buffer P3 of the Plasmid Maxi Kit (QIAGEN) was added to precipitate proteins and chromosomal DNA. Samples were incubated for 10 min on ice before pelleting the precipitate by centrifugation at *13,000 x g* for 10 min at 4°C. Supernatants were transferred into a new 2mL tube with containing 1 mL of phenol-chloroform for BAC DNA extraction. After inversion, another centrifugation with *13.000 x g* for 5 min followed. The DNA containing upper aqueous phase was transferred to a new tube and for precipitation 900 µL of isopropanol was added and mixed by inversion a few times. Then the precipitates were collected by centrifugation at *13.000 x g* for 20 min. The pellet was washed with 1 mL of 70% ethanol during centrifuged at *13.000 x g* for 10 min to remove salts

residuals. Then supernatant was discarded, the pellet was air-dried for 10 min, and finally the BAC DNA was dissolved in 60 µL TE buffer o.n. at 4°C. Purified BAC DNA was stored at 4°C.

3.1.4.4. Large scale isolation of BAC-DNA

NucleoBond® Xtra Midi/Maxi Kit of Macherey-Nagel was used for isolation of BAC DNA in large scale volume as described in the manufacturer's instructions for low copy plasmids. Then precipitated BAC DNA was eluted in 100 µL TE buffer o.n. at 4°C.

3.2. Analysis and cloning of recombinant DNA

3.2.1. Determination of DNA concentration

Isolated nucleic acid concentrations were determined by measuring the optical density (OD) at 260 nm and 280 nm by using the NanoPhotometer® P-Class P330. The purity of DNA was measured using the ratio between OD_{260}/OD_{280} that should be optimally in 1.8 for DNA preparations. For analyzation of the concentration in ng/µL, the OD_{260} value had to be multiplied with the factor 50 ng/µL and with the dilution factor.

3.2.2. Ethanol precipitation

To concentrate purified DNA or for removal of reaction components from DNA solutions, the DNA fragments were precipitated by addition of 1/10 volume of 3 M sodium acetate (pH 5.3) and three volumes of 100% ethanol. Then the mixture was incubated for 1 h on ice and centrifuged at *13.000 x g* at 4°C for 30 min. The pellet was washed with 70% ethanol and centrifuged another time *13.000 x g* at RT for 10 min. After air-drying of the pellet at RT, the DNA was dissolved in the required volume using 10 mM Tris-HCl buffer (pH 8.0).

3.2.3. Polymerase chain reaction (PCR)

To amplify DNA fragments for mutagenesis and cloning, a touch down PCR protocol was used (see Table 3.2.3.1). In this approach, the PCR cycling starts at high annealing temperatures, ensuring high specificity. During the run the annealing temperature is decreased gradually, allowing high productivity of the amplification. This approach also avoids the necessity of the PCR optimization for most of the primer pairs. In general, the PCR reaction was set up using

the manufacturer's protocol of Expand High Fidelity PCR Kit using a reaction volume of 100 µL and 3-25 ng template DNA.

Table 3.2.3.1: Program of touch down PCR:

	step	temperature	time	cycles
1.	initial-denaturation	95°C	5 min	
2.	denaturation	95°C	30 sec	
3.	annealing	67°C	30 sec	-1°C every cycle
4.	elongation	72°C	2 min	17 x back to 2.
5.	denaturation	95°C	30 sec	
6.	annealing	45°C	45 sec	
7.	elongation	72°C	2 min	20 x back to 5.
8.	final-elongation	72°C	7 min	
9.	end	4°C	∞	

3.2.4. Restriction enzyme digest

DNA restriction endonucleases from NEB were used for either restriction fragment analysis or for the preparation of linear DNA fragments. All restriction endonucleases used were purchased from NEB and performed according to the manufacturer's instructions. For restriction pattern analysis, 300-600 ng of plasmid DNA was digested with 10 U of the appropriate restriction enzyme in a total volume of 20 µL and incubated for 2 h. For preparative digestion for cloning of plasmid DNA, 1 µg of DNA was digested at the same conditions as for restriction pattern analysis for at least 3 h.

For analysis of BACs 50 µL (1-2 µg) of BAC mini preparations (see chapter 3.1.4.3) were restricted in 60 µL reaction volumes using 10-50 U restriction enzyme o.n. For the digestion of BACs for reconstitution of viruses, BAC DNA was ethanol precipitated (see chapter 3.2.2) to a concentration of 10-12 µg and diluted in 10 µL. The restriction with PacI enzyme was done using 10 x reaction buffer and 10-50 U PacI restriction enzyme in a total volume of 40 µL to linearize the DNA. Incubation was done at 4°C o.n.

3.2.5. Agarose gel electrophoresis

To analyze the fragment lengths after restriction of plasmid or BAC DNA, the restriction fragments were separated by agarose gel electrophoresis. Plasmid derived fragments were analyzed using 1 % agarose/ TAE gels containing 1 µg/mL ethidium bromide, which were run in TAE buffer. DNA fragments were separated by a fast electrophoresis protocol adjusted to

the length and amount of expected fragments using 80-120 V and a duration of 20-60 min. BAC DNA, on the other hand, was separated on a 0.8% agarose/ TBE gel with ethidium bromide staining for visualization in TBE buffer for 16 h at 80 V. Separated bands were visualized under UV-light and documented by the Eagle-eye imaging system.

3.2.6. Purification of DNA from agarose gel

After separation of fragments by preparative restriction enzyme digestion, a selected fragment was excised from the gel by usage of non-mutagenic UV-transillumination. The DNA was then purified with the Qiaquick Gel Extraction Kit as described by the manufacturer's instructions. The isolated fragments were eluted by 35 µL using 10 mM Tris-HCl buffer (pH 8.0).

3.2.7. Blunting of DNA ends

For ligation of incompatible restriction sites, 5' overhangs were filled and 3'overhangs removed using the DNA polymerase I, Large (Klenow) Fragment of NEB according to the manufacturer's protocol. After a 15 min incubation at a temperature of 25°C of, the reaction was stopped. This was achieved by adding EDTA to a final concentration of 10 mM. Then the enzyme was heat inactivated for 20 min at 75°C. Then the blunted fragment was purified via QIAquick PCR Purification Kit according to the manufacturer's instruction for further cloning steps.

3.2.8. Ligation of DNA fragments

The ligation of DNA fragments was achieved by mixed vector and insert in a molar ratio of 1:3, respectively, with 2 units of NEB T4 DNA ligase and the T4 DNA ligase reaction buffer using 100 ng of linearized vector DNA in a volume of 20 µL in total. An additional control reaction, containing water instead of insert DNA, was also prepared. Then reaction mix was incubated o.n. in +4°C. 2 µL of the ligated DNA were in a further step used for transformation into 60 µL of electro-competent bacteria.

3.2.9. DNA sequencing

For verifying the recombinant DNA constructs, 500 ng of generated DNA-plasmids were sent to GATC Biotech (Konstanz, D) for Sanger sequencing along with sequencing primers flanking the region of interest. 2000 ng of purified BAC-DNA were sent to Sequiserve (Vaterstetten, D) for Sanger sequencing with optimized BAC protocols along with sequencing primers flanking the region of interest.

3.3. Mutagenesis of BAC DNA

Bacterial artificial chromosomes (BACs) enable the preservation and mutagenesis of the genome of large DNA viruses in *E.coli*. Here the advanced methodology of bacterial genetics to the cloned viral genomes can be applied for the generation of recombinant viruses with virtually any mutation. The fertility factor (F-factor) constitutes the backbone of the BAC vector and harbors *repE* and *repF* gene products regulating the origin of replication S (oriS) to allow maintenance of only one BAC copy per cell, reducing unwanted recombination events [274].

3.3.1. Homologous recombination of BACs

For the deletion of the pVI gene in the adenovirus BAC DNA, the technique homologous recombination was used to manipulate the pBA5-FRT BAC vector coding for an E1/E3 deleted Ad5 genome, which can be propagated in 293 cells [270]. To do so, the pVI gene was replaced by the marker for zeo resistance. The zeo resistance cassette from pori6ie-zeo was amplified by PCR using primers that were flanked by 50 nt homologies to the sequences up- and downstream to the pIV coding sequences. The PCR product was column purified, DpnI digested and precipitated with ethanol.

For the homologous recombination, the pBA5-FRT BAC was introduced to the E. coli strain SW102. In this E. coli strain cis-acting λ prophage recombineering system can be induced by heat shock [273]. The SW102 harboring the pBA5-FRT BAC vector were prepared for electro-transformation as described in chapter 3.1.2 and were transformed with 0.2-0,5 µg of the prepared recombination fragments containing the zeo cassette and the homologies. Transformed cells were incubated with 1 mL LB medium w/o antibiotics for 1.5 h at 32°C. Then the cells were transferred to cm and zeo containing LB-plates and incubated o.n. at 32°C.

Single colonies were picked on the next day and incubated to perform BAC mini-preparations. Subsequently, BAC mini-preparations were tested by restriction fragment length polymorphism (RFLP) and then sequences verified.

3.3.2. Flp/FRT recombination system

In order to insert DNA sequences at a pre-determined site in the viral BAC DNA, recombination between two FRT sites was performed by the Flp recombinase to introduce a mCherry expression cassette as a marker gene into the Ad5 BACs. The Ad-BACs used in this study carried an FTR- site at the position of their deleted E1 region, the place where usually transgenes are inserted in first generation Ad vectors. The mCherry expression cassette was cloned into pO6-A5-CMV vector, which carried a conditional origin of replication (RK6) and an FRT site. For the replication of this RK6 phage based pO6 plasmid, the presence of the phage π-protein trans-complemented in the *E.coli* strain PIR1 is necessary [275]. In other bacterial strains lacking π-protein, these plasmids cannot be maintained. Therefor all cloning steps for pO6-A5-CMV-mCherry were performed in PIR1 cells, whereas FLP-mediated insertion was done using DH10B strain for propagation of the BAC DNA.

To carry out Flp recombination DH10B cells carrying the recombinase helper plasmid pCP20 were co-transformed with 100 ng of the pBA5-FRT constructs and the pO6-A5-CMV-mCherry plasmid. In order to induce Flp recombinase expression, the transformed cells plated onto LB plates at 43°C and selected with cm and kan o.n. to isolate the BACs with the integrated donor plasmids. Single colonies were picked on the next day and were analyzed by BAC mini-preparations, which were tested for single copy insertion of the donor plasmid by RFLP and then correct insertions were verified by sequencing.

3.4. Tissue culture

3.4.1. Cultivation of mammalian cell lines

All mammalian cells were maintained under sterile conditions in an incubator providing a temperature of 37°C, humidity of 95% and 5% CO_2 concentration. Cells were passaged as indicated in Table 3.4.1.1. Adherent cells were splitted by removing the old DMEM medium, washing once with PBS and detaching the cells from the plate by addition of 0.25% trypsin/ EDTA in an accurate amount according to the size of the respective well or culture dish for 5

min at RT. Reaction of detaching was stopped by addition of the respective and appropriate amount of fully supplemented DMEM medium for resuspension of the cells. After centrifugation at 1200 rpm (*311 x g*), cell pellets were re-suspended again using appropriate amount of the full medium and a defined proportion of the cells were aliquoted and seeded on new cell culture plates with fresh DMEM medium. To ascertain a defined number of cells, 100 µL of the aliquoted cells were analyzed by addition of 1 mL of CASYton and measurement using the CASYCell Counter & Analyzer.

Table 3.4.1.1: Used cell lines and culture conditions.

Cell lines	Type and source	Cultivation medium	Split ratio, interval
293A	Human embryonic kidney cells, ATCC® CRL 1573™	DMEM 10% FCS, P/S	1:8 2-3 days
LE2D8	Stable 293A derived cell line containing pAV1-GLuc-Hyg; constructed in this study	DMEM 10% FCS, P/S	1:8 2-3 days
U-2 OS	Human osteosarcoma cell line, ATCC® HTB-96™	DMEM 10% FCS, P/S	1:8 2-3 days
911	Human embryonic retinoblast cell line, RRID:CVCL_1K15	DMEM 10% FCS, P/S	1:6 2-3 days
Vero	Cercopithecus aethiops kidney epithelial cell line, ATCC® CCL-81™	DMEM 10% FCS, P/S	1:10 2-3 days
A549	Human lung carcinoma cell line, ATCC® CCL-185™	DMEM 10% FCS, P/S	1:10 2-3 days
HFF	Human foreskin fibroblasts, PromoCell NHDF-c; C-12300	DMEM 10% FBS, Q, P/S	1:3 7 days

P/S, 1.3 % (w/v) streptomycin 0.6 % (w/v), penicillin; Q, 0.3 mg/mL L-glutamine;

3.4.2. Cryopreservation of mammalian cell lines

For maintaining of stock cells in liquid nitrogen permanently, cells were grown on 3 x 145 cm^2 dishes to 80-90% confluence and harvested by trypsinization. Then the cells were pelleted by centrifugation at 311 x *g* for 5 min and resuspended in 9 mL freezing medium. For freezing, the cell suspension was aliquoted in cryo-tubes, 1 mL each. The cell aliquots in cryo-tubes were kept in isopropanol filled cryo-boxes for 48 h at -80°C to allow gentle freezing of the cells. Then the frozen cells were long-term stored in liquid nitrogen.

The frozen cell aliquots in the cryo-tubes were thawed at 37 °C using a water bath. Then the cells of each aliquot were transferred to 9 mL fresh DMEM medium and centrifuged at 311 x

g for 5 min. Washed cell pellets were subsequently re-suspended with 10 mL appropriate medium and seeded on a new 10 cm cell culture dish.

3.4.3. Transfection of cultured mammalian cells

Four different transfection reagents were used in the course of this study: polyethylenimine (PEI) (Polysciences), Lipofectamine2000 (Invitrogen), FuGene HD® (Promega) or SuperFect (Quiagen). For the usage of PEI and Lipofectamine2000, cells were seeded onto 10 cm dishes. For FuGene HD® or SuperFect the same was done onto 6-well plates. For all three transfection reagents, this was done one day before the experiment. On the day of transfection, the confluency should reach 80 %. In parallel to all transfections, an EGFP expressing control plasmid was co-transfected for evaluation of transfection efficacy.

293A cells transfection with PEI was performed using a 6-well plate format for seeding on the day before transfection. A total of 6 µg of DNA was used per well, consisting of 1.5 µg of AAV1-GLuc-Hyg plasmid DNA, 0.75 µg of EGFP control plasmid and for the rest Litmus28 as tracer DNA for increasing transfection efficacy was taken. DNA was suspended in 100 µL Opti-MEM, 15 µL of PEI were added, vortexed and transferred to the cell culture medium after an incubation time of 40 min at RT. After incubation time on the cells of 3 h medium was changed to fresh complete medium.

SuperFect was used as transfection reagent for the AAV replicon vector in 293A, 911 and U-2 OS cells seeded on a 6-well plate to 60 - 80% confluency. Here cells were transfected in duplicates with 2 µg DNA consisting of 0.5 µg pAV1-GLuc-Hyg, 0.25 µg pO6-A5-ORI-GFP plasmid and 1.25 Litmus28 as stuffer DNA according to manufacturer's instructions. After an incubation time of 3 h, the DNA-SuperFect mixture was removed and changed to 2 mL fresh complete medium.

Transfection of 293A, 911, U-2 OS and Vero cells with FuGene HD® was performed in duplicates in 6-well plate format after seeding of the cells onto a 6-well plate to 60 – 80% confluency. In total 6 µg of DNA were transfected. For *trans*-complementation experiments, 1.5 µL PacI (60 U) restricted and ethanol precipitated BAC DNA, 0.5 µg of AAV1-GLuc-Hyg DNA, 3.85 µg of stuffer DNA and 0.15 µg of pO6-A5-ORI-GFP control plasmid were mixed, whereas for transfection of AAV replicon vector, and 0.75 µg of pO6-A5-ORI-GFP control plasmid were mixed with 100 µL of Opti-MEM and vortexed. Then 15 µl of FuGene HD® reagent was carefully

added and immediately vortexed bevor incubation for 30 min at RT. Then the DNA-transfection mixture was added drop wise to the cell culture supernatant.

Duplicate transfected cells were harvested and pooled together after 72 h and 3E+04 cells per well were seeded on a 96-well plate.

3.4.4. Construction of stable cell lines

To generate stable cell lines, 293A cells were transfected either with pAV1-GLuc-Hyg or with pAV1-EGFP-Hyg. Cells of one 6-well plate were transferred to a 10 cm cell culture dish and hygromycin (200 µg/mL) was added for selection towards the AAV replicon vector containing cells. Cells that did not show positive transfection of the AAV1-GLuc-Hyg usually died within 4 - 5 days. Single cell clones were subcloned using limiting dilution by seeding the cells on day 7 (0.5, 3 and 10 cells per well) onto a 96-well plate under Hyg selection. Single cells that started to proliferate were tagged and after confluency were transferred to a 24-well plate and then to a 6-well plate. The stable cells were frozen after 4 passages and tested for their inducibility after 5 passages by Ad infection with either a Luciferase assay or via FACS analysis.

3.4.5. Cryopreservation of transfected cells

Transfection of pAV1-GLuc-Hyg into 293A cells with FuGene HD® as transfection reagent was done as described in chapter 3.4.3. Then transfected cells were pooled after 48 and 72 h post transfection (h p.t.). Cells were pelleted by centrifugation at 1200 rpm (311 x *g*), supernatants were removed, and cells were re-suspended in freezing media (70% DMEM, 20% FBS and 10% DMSO) and seeded onto 96-well plates at a density of 3E+04 cells/well. Subsequently, the plates were sealed and then rapidly frozen in -80°C.

For thawing of the plates 150 µL of pre-warmed growth medium was added directly to each well and cells were incubated for 2 hours under normal cell culture conditions. Then, the supernatants were removed and 100 µL of growth medium was added to each well. The inducibility of the introduced AAV replicon after thawing the transfected cells was tested by infection 4, 6, 20 and 26 h after thawing with different MOIs of Ad and HSV-1.

3.4.6. Antiviral drug treatment of cultured cells

For the treatment of cells with different antiviral drugs as listed in Table 3.4.6.1, stock solutions were prepared in DMEM and stored in 200 µL aliquots at -20°C and used only once after thawing in indicated concentrations.

Table 3.4.6.1: Stock solutions of antiviral drugs.

drug	abbreviation	Storage concentration
Acyclovir	ACV	166.67 µg/mL
Ganciclovir	GCV	15 mg/mL
Foscarnet	PFA	300 µg/mL
Cidofovir Hydrate	CDV	15 mg/mL

3.4.7. Extraction of genomic DNA from cultured mammalian cells

DNA extraction from cultured cell lines was performed before analyzing the genomic DNA via quantitative real-time PCR. To do so, 2 wells of a 6-well plate were transfected with the AAV replicon vector and 3 days later seeded onto a 12-well plate. Then the cells were either infected or kept mock infected by addition of the same volume of complete medium. Extraction of the genomic DNA was performed with the DNeasy Blood & Tissue Kit. This was done according to the manufacturer's instructions (purification of total DNA from animal cells).

3.4.8. Quantitative real-time PCR

Quantitative real-time PCR reaction (qPCR) was performed for analyzing GLuc expression upon virus infection after cell extraction. For the reaction 5 µL of the purified DNA were mixed in duplicates with 10 µL of QuantiTect SYBR® Green PCR master mix and with the forward and reverse primers of either GLuc or the housekeeping gene gapdh (all primers are listed in chapter 2.6). The qPCR was performed using the LightCycler® for amplification. Thermal cycling conditions comprised 15 min at 95°C, 45 amplification cycles of 15 s at 95°C (denaturation), 30 s at 58°C (annealing) and 30 s at 70°C (elongation). Melting curve analysis followed with 15 s at 65°C and at the end a cooling phase to 37°C was done. Relative quantification of GLuc amplification was performed in relation to uninfected controls and normalized to the housekeeping gene *gapdh* using the delta CT method [276].

3.4.9. Flow cytometry

For analyzation of the EGFP AAV replicon stable cell line, EGFP expression was measured via Fluorescence-based flow cytometry assay. Typically, AAVG clones were seeded 1:4 in duplicates in a new 6-well plate and infected 24 h later or mock treated. After 3 days cells were harvested by digestion with Trypsin/EDTA and washed with PBS. Cell pellets were re-suspended in 0.8 - 2 mL PBS and measurement was done using the BD FACS Canto™ II cytometer.

3.5. Virological methods

3.5.1. Virus infection of cultured cells

For infection of cells under standard conditions, the appropriate cell line was seeded to a confluency of about 90% o.n. in cell culture dishes or a total number of 3E+04 cells per well were seeded onto a 96-well plate for infections 4 - 6 h before infection. Appropriate amounts of virus stocks or inocula were diluted in complete medium to achieve the respective MOIs and applied correspondingly to the cells.

3.5.2. Reconstitution of viruses from BACs

For reconstitution of adenovirus from viral BACs permissive cells were transfected with the corresponding BAC DNA after manipulation of the viral genome. Positive control of the mutant Ad BAC lacking the *pVI* gene (pBA5-ΔpVI-Che) was the Ad5-WT BAC having Cherry expression cassette (pBA5-Che BAC). To this end, 293A or 911 cells were seeded onto a 6-well plate one day before transfection as described in chapter 3.4.1. The successful transfection was controlled by the EGFP expressing control plasmid as well as by the mCherry expression of the BAC mutants with a fluorescence microscopy. 4 days after transfection, the supernatants of all cells were removed in each well and then the cells together with the propagated viruses in the 6-well plate were immediately stored at -80°C. For both, virus trans-complementation and for virus reconstitution from BACs for production of virus stocks, viral suspension was thawed by adding 700 µL pre-warmed complete media to each well and transfer to +37°C incubator. After 15 min, cells were scraped from the plates and transferred with the media to new 2 mL Epis. Then three cycles of freezing (5 min in liquid nitrogen) and thawing (10 min at 37°C)

followed before the suspension was centrifuged again at *6.000 x g* at RT for 10 min. Sn was collected for further processing.

3.5.3. Preparation of viral inocula

For preparation of low titer virus inocula optional amount and size of cell culture dishes with the appropriate cells grown to 90% confluency were infected with either a reconstituted virus BAC or a previous virus stock. Ad were propagated in 293A cells upon infection at a MOI of 1. Harvesting of lysed cells and Sn followed 1 day after complete cytopathic effect was observed. The pooled suspension was centrifuged at *6.000 x g* at 4°C for 15 min. Except of 2 mL the Sn was collected, and an aliquot was stored in -80°C for safety. The cell pellet was re-suspended in the remaining 2 mL Sp. Then three cycles of freezing (5 min in liquid nitrogen) and thawing (10 min at 37°C) followed before the suspension was centrifuged again at *6.000 x g* at 4°C for 15 min. Viruses in the Supernatants were harvested and Aliquots of 250 µL were stored at -80°C. Virus titer was analyzed by endpoint titration (chapter 3.5.5).

For propagating HSV, Vero cells were infected at MOI 0.01 and cell-virus suspension was collected 4 d p.i., harvested and frozen at -20°C. After a thawing process to 37°C, suspension was vortexed for 15 sec and centrifuged at *6.000 x g* at 4°C for 15 min. Then the Supernatants were aliquoted using a volume of 100 µL and stored at -80°C.

3.5.4. Preparation of high-titer virus stocks

Large scale adenovirus stocks were purified using the caesium chloride (CsCl) purification method. To do so, first a seed stock of the virus had to be generated to be able to propagate a bulk stock. Therefore 150 µl of either a previous virus stock or of the 2nd lysate after BAC reconstitution (chapter 3.5.2) were used to infect 293A cells grown to a confluency of 80 – 90 % in a 15 cm culture dish. One day after cytopathic effect was reached 100% of the cells, all cells were detached by pipetting up and down. Then suspension was centrifuged at *6000 x g* for 5 min at 4°C. Supernatants were removed and replaced with 4 mL of fresh DMEM. The virus particles were realized from the infected cells by three cycles of freezing (5 min in liquid nitrogen) and thawing (10 min at 37°C). The lysates were clarified by centrifugation at *6.000 x g* at 4°C for 10 min and stored immediately at -80°C.

For the bulk preparation of Ad, 20 x 15 cm dishes of 293A cells grown to 80 - 90 % confluency and were infected with 150 µL of the thawed seed stock. When full CPE was evident, cells were detached as before and harvested together with the supernatant. After centrifugation *6.000 x g* at 4°C for 15 min, the supernatant was transferred to an extra tube and the remaining cell pellet was diluted with 3 - 4 mL of this supernatant. The rest of the supernatant was removed. Three cycles of freezing (5 min in liquid nitrogen) and thawing (10 min at 37°C) followed, then centrifugation at *6.000 x g* at 4°C for 15 min was done, supernatant was transferred to a new tube to remove cell debris and kept on ice. Then 25 U Benzonase per mL lysate was used and incubated at 37°C for 30 min. Then the lysate was kept on ice again.

The CsCl purification was done by preparing a CsCl gradient in an Ultraclear Beckman tube. To do so, first 3 mL of δ=1,2 CsCl solution (13.85 g CsCl, 50 mL PBS) were added to a fresh tube and then 3 mL of δ=1,4 CsCl solution (30.5 g CsCl, 50 mL PBS) were added underneath the δ=1,2 CsCl layer w/o disturbing the interface. Then the prepared viral Supernatant was carefully added to the top of the gradient and the tube was filled up with δ=1,2 CsCl almost to the rim. Centrifugation at *32000 x g* at 4°C for 1.5 h followed. After the centrifugation step three different layers should be visible within the CsCl gradient, containing cell debris in the top layer, empty virions in the second layer and in the lowest layer there should be properly packaged virions. The lowest layer was aspirated by puncturing the tube with a hollow needle into a 2 mL syringe.

For the step of desalination of the virus stock, a sephadex column was prepared by 5 x pre-washing steps with 1 x Hepes buffer. Then the virus was loaded onto the column and desalinated virus eluted in the void volume was aliquoted and stored in -80°C.

3.5.5. Endpoint dilution assay

An endpoint dilution assay was performed to determine the infectivity of the virus preparation [277]. 293A cells were used to estimate the infectivity Ad5 and its vectors and Vero cells for HSV-1. The cells were seeded in 96-well plates to a density of 10 % in 100 µL complete medium the day before infection. Next day 10-fold virus dilution series were prepared in twelve parallel wells (one dilution step for each row) in an extra 96-well plate starting with a 100-fold dilution in complete medium. 100 µL/well from each dilution between 10^{-2} - 10^{-9} were transferred to the cell cultures plate using the multichannel pipette. For determining virus titer of Ad and

HSV, the cytopathic effect of each virus dilution was observed for each infected well after a 1-week incubation period, whereas HCMV was analyzed after 9 days. The calculation of the $TCID_{50}$/mL comprises the dilution were 50% of the cells show a cytopathic effect using the following formula:

$$TCID_{50} = A\text{-}D\ (S\text{-}0.5)$$

A - Log of highest dilution showing CPE in over 50 % of the wells
D - Log of dilution factor
S - ratio of: number of wells per row with CPE versus number without CPE

For validation of the HSV resistance or sensitivity towards ACV, 10^4 cells of the stable replicon cell line LE2D8 were seeded onto two 96-well plates and 4 h later treated in duplicates with 96, 48, 12, 6 and 1 µg/mL of ACV in 50 µL completed medium. Next the cells were infection with either the sensitive HSV-1 WT (ACV sens) or the ACV resistant HSV-1 (ACV res) at a MOI of 0.035 or mock in a volume of 50 µL. Subsequently after an incubation time of 2 days the supernatants were collected, and virus load was analyzed in duplicates by endpoint dilution assays in Vero cell line.

3.5.6. *Gaussia* luciferase assay

Bioluminescence measurement of *Gaussia* luciferase (GLuc) reporter gene expression was performed to quantify the induction of the GLuc carrying AAV replicon vectors upon virus infection. First, the GLuc assay solution was prepared according to the manufacturer's protocol using the BioLux® *Gaussia* luciferase Flex Assay Kit. Aliquots of 10 mL were stored in -20°C and thawed to RT upon usage.

For the assay, supernatants were collected in different time points after induction and stored in -80°C. Right before the assay they were thawed to RT. 20 µL of each sample in technical triplicates was transferred to a white 96-well plate. Then 50 µl of the GLuc assay solution was added carefully into each well and incubated for 5 min covered for light protection. For the evaluation of GLuc expression, the emitted light was measured using an integration time of 1 second by the Tecan luminometer. For analyzing the fold induction of GLuc, the measured relative light unit (RLU) values were compared to the values measured in the supernatants of the AAV replicon vector treated but non-infected cells.

4. Results

4.1. Construction of the AAV based replicon

Replicon vectors represent replication proficient artificial genetic elements that are induced specifically in the presence of a viral infection. One major application of replicons utilizes their infection-dependent activation to regulate reporter genes, which can be used to trace infection with native viruses. The known replicon vectors are restricted to respond to infection with one specific virus species that can activate the homologous origin of replication embedded in the replicon constructs. To expand the replicon response to more than one virus species, we decided to choose the AAV2 genome as a basis for a replicon system reacting to infection with large DNA viruses. The replication of AAV is known to be naturally dependent on co-infection with helper viruses like adeno- or herpesviruses [215] [216]. Furthermore, the genome replication of AAV can be induced by a helper virus infection not only on the single stranded (virion) DNA, but also on a circular double stranded, or linear double stranded template [239]. For our purpose we used the plasmid pAV1 [278] containing the complete 4.7-kb genome of AAV2 (Figure 6.A first panel). This plasmid encodes all important *cis* elements, the two ITRs, and the rep gene for the helper virus infection induced AAV DNA replication on double stranded templates. Since the structural gene of AAV is kept silent in the absence of helper virus infection but is expressed at very high levels during AAV lytic replication cycle in the presence of helper virus infection, we aimed to use the properties of p40 regulation for controlling the expression of the transgene of interest. We wanted to delete the coding region of the Gap gene to prevent formation of infectious AAV particles during replicon induction but preserve the *cis*-elements, which are involved in regulating the expression of the Gap gene. Thus, to construct an AAV replicon vector, a hygromycin resistance cassette was inserted into pAV1, replacing all Cap coding sequences upstream of the p40 transcription initiation site. This hygromycin cassette (HYG) can be used for selection of stable cell lines carrying the AAV replicon. The hygromycin cassette is under the control of an SV40 early promoter, which is a constitutive promoter initiating transcription independently of activation of any AAV promoter. Next, on the 5’ end of the HYG transcription unit, we flanked a multiple cloning site (MCS) to facilitate insertion of transgenes. The MCS is now located directly downstream to the p40 promoter and upstream to the HYG. Afterwards, to test the responsiveness of our

replicon, two different ORFs were inserted between p40 and the hygromycin cassette using the MCS as insertion site. The transgene was either coding for a *Gaussia* luciferase (GLuc) or an enhanced green fluorescence protein (EGFP), resulting in pAV1-GLuc-Hyg or pAV1-GFP-Hyg, respectively (Figure 6A second and third panels).

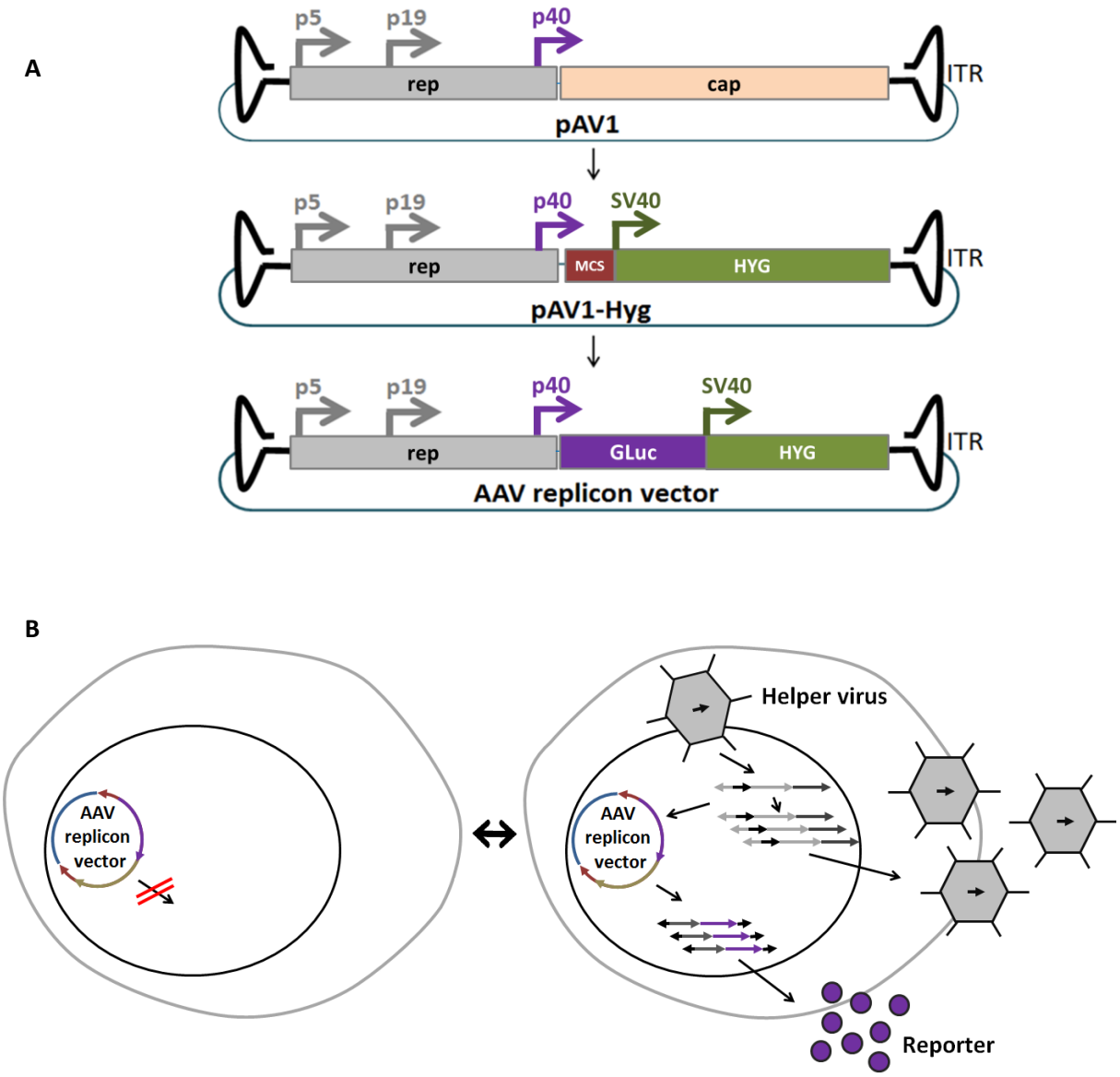


Figure 6: Cloning and Concept of the AAV replicon vector.
(A) Cloning of the pAV1-GLuc-Hyg replicon vector was done by deletion of the Cap coding locus of the plasmid pAV1 containing the whole AAV 2 genome. Instead a hygromycin resistance cassette under the control of a SV40 early promoter and either of the transgene GLuc or EGFP were introduced downstream of the inducible AAV p40 promoter. (B) Concept of the conditional gene expression by AAV replicon vectors. Delivery of the AAV replicon vector should show no or very low level of transgene expression. But, infection with a helper virus should activate DNA replication of the AAV replicon vector and consequently induce the p40 promoter to drive expression of the reporter gene.

The replicon vector without an accompanying virus infection should have a very low or no transgene expression. However, as soon as the replicon carrying cell is infected with an inducing (helper) virus infection, the lytic AAV genome replication should start. The replication should activate the p40 controlled reporter gene expression, which then can be measured by the assay specific to the actual reporter gene (Figure 6B). This allows assaying the virus infection indirectly by using simple, well-developed, automated methods for the reporter detection instead of more complicated or time-consuming methods for direct detection of infectious viruses.

To examine whether the mechanism of transgene expression of the AAV replicon vector was in fact regulated by helper virus infection, we transfected three different cell lines transiently with the newly constructed pAV1-GLuc-Hyg vector using SuperFect as transfection reagent. 293A and 911 cells carry the functional E1 gene of adenovirus type 5, which complements the deletion of the E1 region of first-generation adenovirus type 5 derived recombinant viruses. We used recombinant Ad5-Che that carry deletions of the E1 and the E3 region (the latter is not essential for Ad replication in cell culture), and expresses mCherry as transgene in this study [279]. In contrast to 293A and 911 cells, the U-2 OS cell line does not carry complementing genes for adenoviruses and therefore replication of the E1 deficient Ad5-Che vector is not supported in this cell line. Researching not only the general inducibility but also the dependence on a productive helper virus replication, we tested the inducibility of our AAV replicon in all three cell lines upon infection with Ad5-Che and Ad5 wild-type virus (Ad5-WT). Ad5-WT should be permissive in all three cell lines. Therefore, transfected cells were seeded 72 h post transfection (p.t.) onto 96-well-plates and infected 4 h later with either the E1 deficient Ad5-Che at a multiplicity of infection (MOI) of 100, the Ad5-WT virus at a MOI of 1 or kept non-infected (n.i.). The difference of the chosen MOIs between Ad5-Che and Ad5-WT were considering the expected limitation in productivity of the E1 deficient Ad5-Che. The supernatants were collected 48 h post infection (p.i.) to complete the infectious cycle of Ad5. Then, a bioluminescence assay was performed to evaluate the activity of the secreted luciferase by light emission, measured by luminometry and the results were expressed in relative light units (RLU) (Figure 7).

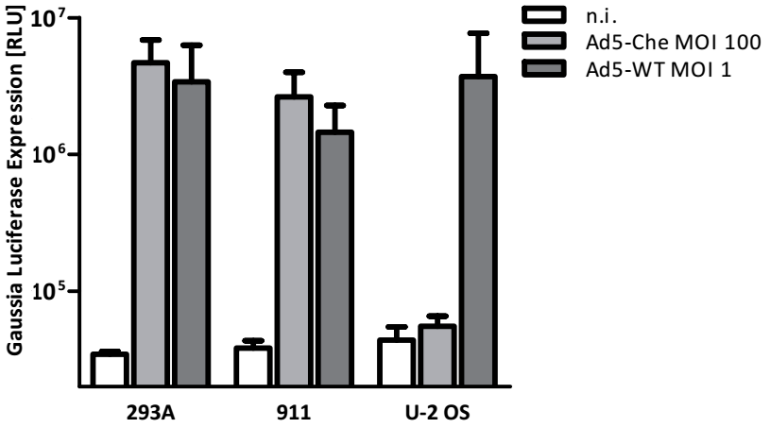


Figure 7: Productive Ad infection induced AAV replicon vector expression.
293A, 911 and U-2 OS cells were transfected with pAV1-GLuc-Hyg and infected 72 h post transfection with either Ad5-Che at MOI 100 or Ad5-WT at MOI 1 or kept non-infected (n.i.). 48 h p.i. the RLU values were measured in a bioluminescence assay. Data from three independent experiments are shown; the error-bars represent means ± SD;

Infection-dependent induction of luciferase signal was observed after infection with Ad5-WT in all cell lines and after infection with the E1 deficient Ad5-Che only in 293A and 911 cells, which complemented the essential gene E1. Here, the GLuc expression increased by approximately 60- to 135-fold. In contrast, replicon transfected but non-infected cells or U-2 OS cells infected with Ad5-Che showed extremely low levels of luciferase activity. The light signal measured in transfected but non-infected cells were comparable to background levels that can be observed in non-transfected cells (for example in 293A cells these were 39000 [+/- 6000] for transfected but non-infected and 26000 [+/- 2900] for non-transfected cells) demonstrating the tightness of the p40 promoter control on transgene expression.

Next, we wanted to test whether a productive Ad5 infection is able to induce amplification of the replicon vector, in order to determine the role of AAV genome replication in the observed response. To test the replicon vector amplification, we set up a semi-quantitative real-time PCR. We transiently transfected 293A cell line with pAV1-GLuc-Hyg 72 h before subsequent infection with Ad5-Che or keeping them uninfected. We harvested the infected and uninfected cells 24 and 48 h p.i. and extracted total cell associated DNA. Vector DNA copy numbers were analyzed by performing a semi-quantitative RT-PCR (described in chapter 3.4.8). The back calculation of the vector copy number (CN) from the observed CT values confirmed an increase of replicon vector DNA copies from 2/haploid host genome to 145/haploid host genome (24 h p.i.) and from 1/haploid host genome to 214/haploid host genome (48 h p.i) after infection with Ad5-Che (Figure 8).

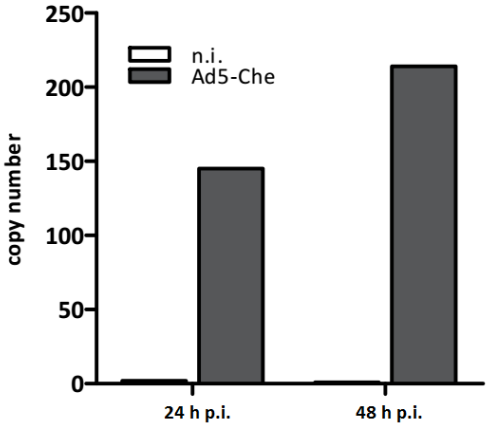


Figure 8: Amplification of the AAV replicon vector after Ad5-Che infection.
293A were transfected with pAV1-GLuc-Hyg and infected 72 h post transfection with Ad5-Che at MOI 1 or kept non-infected (n.i.). 24 and 48 h p.i. copy number of the PCR products *gapdh* (cellular housekeeping gene) and vector encoded GLuc gene (*Gaussia* luciferase gene) were determined by semi-quantitative RT-PCR. Vector copy numbers were calculated relative to the haploid host genomes by the ΔCT method. Shown is one representative experiment out of 3 replicates.

Since a robust amplification of the replicon encoded GLuc gene only appeared in virus-infected cells, the full activation of the replicon vector occurred in parallel with the infection-dependent AAV replication after infection with activating viruses.

These data strongly indicated that the transgene induction of the AAV replicon vector was dependent on a productive helper virus infection.

4.2. Characterization of the AAV replicon system

The initial reporter gene induction data were promising with regard to the responsiveness of the newly constructed AAV replicon vector. However, the data were highly variable. For further applications, such as trans-complementation of essential viral genes and in diagnostic or screening approaches as reporter for viral replication, the system had to be further optimized regarding its reproducibility. First, we aimed to test the different methodologies for the delivery of the AAV replicons in target cells.

Since the AAV replicon was significantly inducible after transient transfection, we primarily wanted to optimize this method for delivering the replicon because this system is very flexible, allows to target different cell types and requires no modification or further processing of the easy-to-produce plasmid preparations. In addition, transient transfection allows further characterization of the genetic elements of the replicon required for its performance. To get

a simpler protocol and a better reproducibility, a stable cell line could be constructed for specific applications as second step. Moreover, since recombinant AAV genomes can be packaged into recombinant AAV particles, we wanted to test a new, to our knowledge never applied before, approach to deliver replicons using transduction.

4.2.1. Delivery of the AAV replicon by transient transfection and its applications

The previous experiment showed that the induction of the AAV replicon vector was dependent on infection of Ad5 after transient transfection of pAV1-GLuc-Hyg with SuperFect as transfection reagent in 293A, 911 and U-2 OS cells. We furthermore wanted to test the AAV replicon response using different transfections reagents. After that, we wanted to explore which promoters are useful in the replicon vector context and, finally, which viruses can significantly induce the replicon system.

4.2.1.1. Comparing transfection reagents

Transient transfection generally allows flexibility concerning different cell lines and viruses. However, the various methodologies of transfection induce the innate immune response differently. To evaluate the optimal transfection reagent for further studies on the AAV replicon system, we wanted to test the major groups of different chemical transfection reagents. Therefore, SuperFect, which consists of activated dendrimers, was compared with two other reagents belonging to other chemical classes: JetPEI, based on polyethylenimine, and FuGene HD®, consisting of a proprietary blend of lipids.

At first the replicon vector pAAV-GLuc-Hyg was introduced with the respective reagent into 293A cells. For all reagents we used 5 µg total DNA, which was very close to the suggested DNA amounts by the vendors of the different reagent in 6-well plate format. To evaluate the primary transfection efficacy, 0.25 µg of pO6-GFP was co-transfected. After 3 days the transfected cells were trypsinized and either seeded on 96-well plates at a density of 30.000 cells per well or subjected to FACS analysis to control the transfection efficiency. 6 h later the seeded cells were infected with Ad5-Che. 48 h p.i. the AAV replicon derived GLuc expressions were measured in the supernatants to characterize the replicon response.

After transient transfection, FACS analysis, using the three different transfection reagents, revealed a transfection efficiency of 94.93% using PEI, 41.57% using SuperFect and 97.23% using FuGene HD® reagents with a standard deviation of 2.9, 10.2 and 1.5, respectively. Data of the GLuc measurement showed a significant induction of the AAV replicon system in 293A cells irrespective of the transfection reagent (Figure 9).

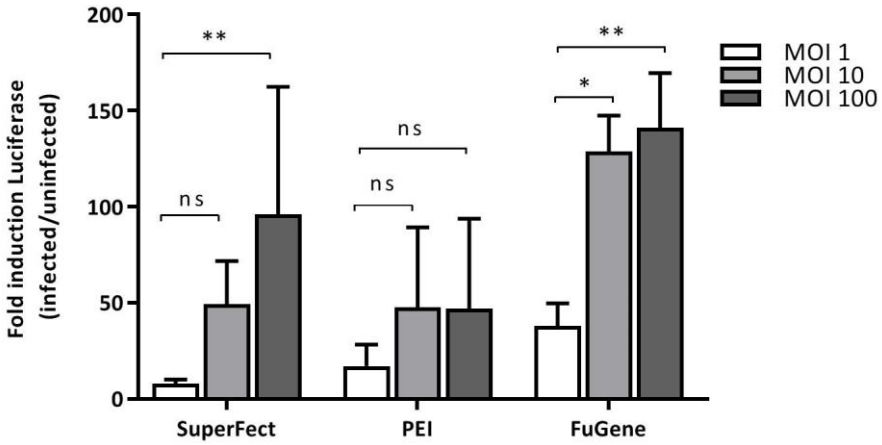


Figure 9: Comparison of three different chemical transfection reagents.
293A cells were transfected with pAAV-GLuc-Hyg, using SuperFect, jetPEI or FuGene as transfection reagent. Cells were infected 72 h p.t. with Ad5-Che at indicated MOI and 48 h p.i. a bioluminescence assay was performed. The induction upon infection was calculated by comparing the RLU values recorded after measurements of infected to non-infected supernatants Data from three independent experiments are shown; the error-bars represent means ± SD; (Two-Way-ANOVA was performed for statistical analysis; ns: $p > 0.05$, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$)

As noted above, the variation of reporter gene expression between three independent experiments was very high in the case of SuperFect and jetPEI. Using SuperFect, the GLuc expression was induced 8-fold after infection at MOI 1, 49-fold at MOI 10 and 96-fold at MOI 100 compared to non-infected but transfected cells. The calculated standard deviation between the different experiments was 2, 22 and 66 for the respective virus loads. Similarly, jetPEI as transfection reagent resulted in a 17-fold induction at MOI 1 and in a 47-fold induction at both MOI 10 and MOI 100 whereas the standard deviation was 11, 42 and 47. However, transfection with FuGene achieved the highest GLuc induction of 38-, 129- and 141-fold after infection with MOI 1, 10 and 100, respectively. Accordingly, transfection with FuGene resulted in the best induction and the best reproducibility of replicon vector induction. Based on these observations we decided to use FuGene in further experiments because it combines the highest efficacy of transfection, best inducibility, and best reproducibility among the reagents tested.

4.2.1.2. Trans-activation of different viral promoters during infection

Next, we wanted to test the importance of the p40 promoter for the inducibility of the AAV replicon. Different potential applications may need different expression levels of the regulated gene of interest, which can most efficiently be regulated by using different promoters. In the context of our basic replicon, both the genome replication and transactivation of the natural p40 promoter were responsible for the induction of the transgene expression in presence of helper functions. To evaluate the intrinsic features of the AAV p40 promoter for the replicon response, we inserted two other well-characterized constitutive promoters into the replicon construct and tested their inducibility upon Ad infection. We choose a viral promoter, the simian virus 40 major late promoter (SV40L) [280], and a cellular mouse metallothionein I promoter (MT) [281] as model promoters.

To do this, the AAV p40 promoter was inactivated by mutation of the TATA sequence of p40 in order to avoid transgene expression activation due to the AAV specific promoter (Figure 10) [232]. The mutated P40 promoter was synthetized and cloned in an extra plasmid together with either the SV40L or the MT promoter sequences, and a tripartite leader sequence for increasing the efficiency of GLuc mRNA export [282]. Then, the cassettes were transferred to the pAV1-GLuc-Hyg vector instead of the AAV p40 promoter on the 5' end of the GLuc ORF. These constructs should be regulated mainly by induction of vector amplification similarly to the published constitutive promoter based MCMV replicon.

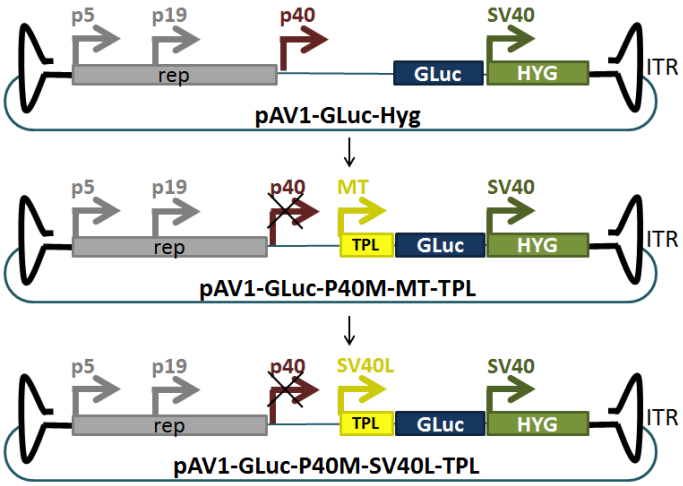


Figure 10: Cloning of two different constitutive promoters instead of the AAV p40 promoter.
For the cloning of the constructs pAV1-GLuc-P40M-SV40L-TPL and pAV1-GLuc-P40M-MT-TPL, the AAV replicon p40 promoter was eliminated in the pAV1-GLuc-Hyg construct by insertion of a mutated p40 promoter together with either the MT or the SV40L promoter fused to a tripartite leader sequence upstream to the transgene (GLuc) ORF.

The AAV replicon plasmids with the respective promoters, pAV1-GLuc-P40M-SV40L-TPL and pAV1-GLuc-P40M-MT-TPL, were co-transfected with the tracer plasmid pO6-A5-CMV-GFP into 293A cells. After controlling the transfection efficiency, the cells were split into 96-well plates and 72 h after transfection the cells were either infected with the Ad5-Che at different MOIs or kept non-infected. 48 h p.i. the GLuc expression was measured.

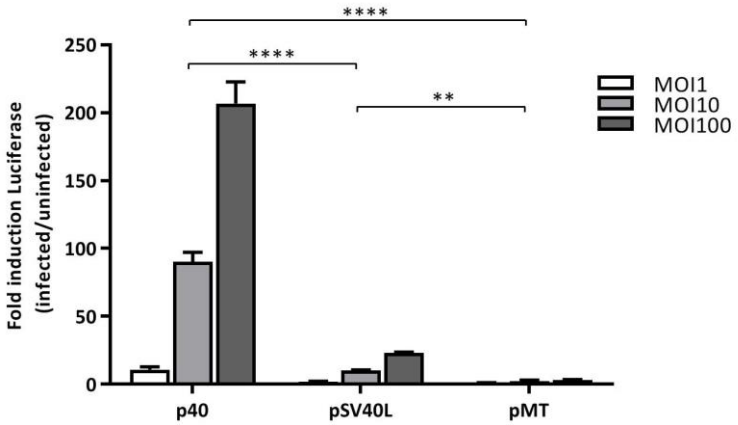


Figure 11: *Trans*-activation of different viral promoters by infection.
293A cells were transfected with pAAV-GLuc-Hyg (p40) or the respective constructs with the simian virus 40 major late promoter pSV40L or the metallothionein I promoter pMT and infected with different dose of Ad5-Che or kept non-infected. The supernatants were collected 48 h p.i. and a bioluminescence assay was performed. The induction upon infection was calculated by comparing the RLU values recorded after measurements of infected to non-infected supernatants. Data from three independent experiments are shown; the error-bars represent means ± SD; (Two-Way-ANOVA was performed for statistical analysis; **: $p < 0.01$, ****: $p < 0.0001$)

The background GLuc expression levels of non-infected AAV p40 promoter transfected cells were 1.9-fold lower compared to SV40L promoter transfected cells and 1.8-fold lower than the MT promoter transfected cells. The MT promoter driven replicon appeared to be none-inducible with Ad5-Che infection at any MOI tested (Figure 11). The simian virus promoter SV40L showed 2-, 10- and 23-fold induction of GLuc expression after infection with Ad5-Che at MOI 1, 10 and 100, respectively. Nevertheless, the highest increase of the transgene expression was significantly demonstrated by the AAV specific promoter p40, where GLuc expression was increased 11-, 90- and 207-fold. This data show that p40 promoter-specific factors play an important role in the replicon response. The p40 promoter showed the lowest background activity, it expressed the highest level of transgene product upon induction, and consequently showed the highest inducibility and also the highest expression levels among the promoters tested. Therefore, the promoter p40 of AAV was chosen for all further experiments in these studies. If lower transgene expression levels are favored upon induction (toxicity), the pSV40L based constructs may provide a useful alternative of the p40 construct.

4.2.1.3. Induction of the AAV replicon system by different human adenovirus serotypes

Several adenovirus serotypes have been shown to provide helper functions for AAV [283] [284]. To test different viruses as inducers, we first tested how efficient helper functions were provided by different Ad. To this end, selected serotypes were tested, representing 5 out of 7 human Ad species for their ability to induce the AAV replicon response.

At first, we wanted to determine the optimal virus load and time kinetics that is required to induce a significant AAV replicon response by wild-type Ad5, which belongs to species C. We used this serotype as a model inducer. With this information we could limit the test conditions to compare the helper efficiencies of different Ad serotypes. Therefore, the AAV replicon vector was transiently transfected in 293A cells and 72 h p.t. the cells were seeded into 96-well plates and, 4 hours later, infected with Ad5 at different MOIs to find out the minimal and maximal inducible infection dose. The supernatants of infected and non-infected cells were collected 1, 2, 3, and 4 d p.i. and afterwards the reporter gene expression was evaluated by bioluminescence measurement in the supernatants.

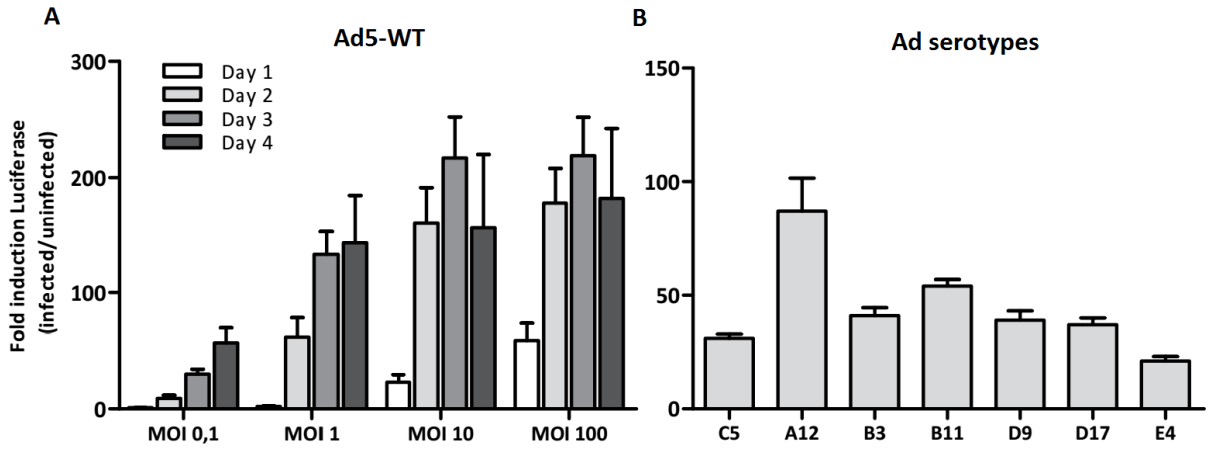


Figure 12: Induction of the AAV replicon by different Ad serotypes upon transfection.
(A) Cells were infected with Ad5 at indicated MOI 72 hours post transfection (h p.t.). The supernatants were collected at different time points and a bioluminescence assay was performed. Fold induction of infected compared to non-infected RLU values were calculated. (B) Inducibility of GLuc after infection with different adenovirus species and serotypes, species A – serotype Ad12 (A12), species B – serotype Ad3 and Ad11 (B3 and B11), species C – serotype mutant Ad5-Che (C5), species D – serotype Ad9 and Ad17 (D9 and D17) and species E – serotype Ad4 (E4), in comparison with Ad5-Che mutant virus at MOI 1 was examined 3 d p.i. by collecting the supernatants and comparison of infected versus non-infected RLU values. Data from three independent experiments are shown; the error-bars represent means ± SD;

The results revealed that the best dynamic range of induction can be achieved with a dose of MOI 1 (Figure 12A) for Ad5 infection. The induction for higher MOIs (MOI 10 and 100) reached a plateau of about 180-fold after 3 d p.i. and at the lowest dose (MOI 0.1) the levels of induction were very low. At MOI 1 in contrast, we received a clear dose response until collection at 3 d p.i. The induction of reporter gene expression was 133-fold and doubled within a 24 h period. The replication cycle of the specific Ad serotypes may differ from each other. Therefore, we decided to take 3 d p.i., which is the latest time point of significant increase observed by Ad5. Having an average replication cycle of 24h this is one of the best growing serotypes.

In order to test the responsiveness of the AAV replicon to different Ad serotypes we transfected 293A cells transiently with pAV1-GLuc-Hyg. Then, the transfected cells were seeded on 96-well plates and infected with 7 different Ad serotypes: Ad12 of species A, Ad3 and Ad11 of species B, Ad9 and Ad17 both belonging to species D, and Ad4 of species E. As control, and representing species C, we used Ad5-Che infection at MOI of 1. After analysis of the transgene expression 3 d p.i. by bioluminescence assay, the height induction by all tested Ad serotypes was compared to non-infected cells (Figure 12B). The infection with Ad12 of species A showed the highest induction of GLuc expression (87-fold), whereas the Ad5-Che infection induced the GLuc expression 31-fold, similarly as observed before (Figure 9). Interestingly, most Ads, besides Ad12, displayed the same range of induction. Ad species B viruses for example induced the bioluminescence expression 41- and 54-fold, whereas Ad species D viruses showed a 39- and 37-fold induction. Species E derived virus Ad4 was demonstrated to induce the least GLuc expression with only 21-fold. The data indicated that the AAV replicon system is generally applicable for assays of human adenovirus infections.

4.2.1.4. Induction of the AAV replicon by herpes simplex viruses

As Ad, herpesviruses are also known helper viruses for AAV. Therefore, we were primarily interested in testing if the AAV replicon vector is inducible by herpes simplex viruses. Both herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2) can productively infect Vero cells, the standard cell line culturing these viruses in clinical virology. Therefore, we decided to test these herpesviruses first for their ability to induce AAV replicons upon transient transfection of Vero cells.

We transiently transfected Vero cells with pAV1-GLuc-Hyg according to the protocol used for 293 cells and seeded those 72 h later into a 96-well plate at density of $3x10^4$/well. After 4 h, the cells were infected with either HSV-1 or HSV-2 using different doses. To examine the GLuc expression over time, supernatants were collected after 1, 2, and 3 days after infection. The induction of luciferase expression of HSV infected cells were normalized to the values obtained in supernatants of non-infected cells (Figure 13A).

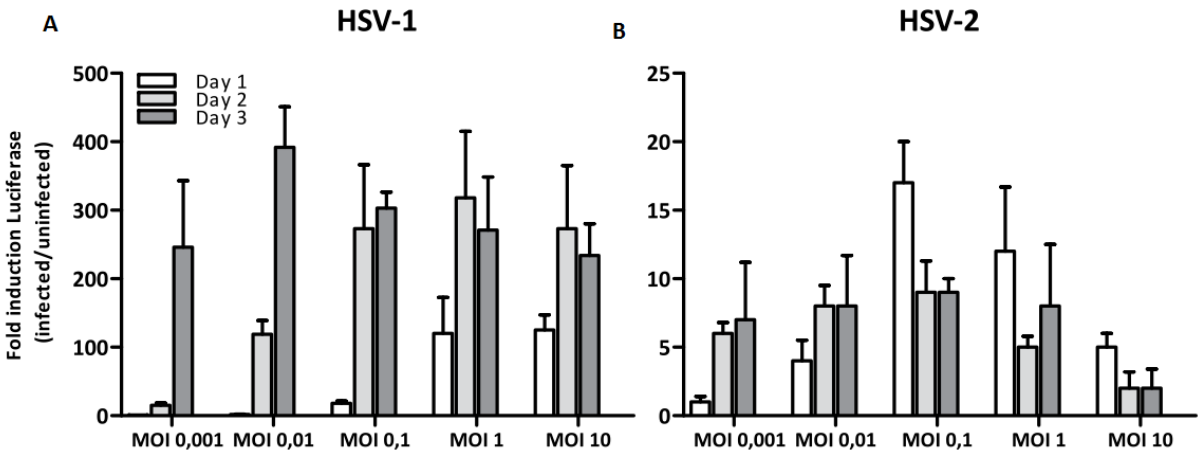


Figure 13: Infection with HSV-1 and HSV-2 activates reporter gene expression.
Vero cells were transfected with pAAV-GLuc-Hyg and infected with different particle numbers of (A) HSV-1 and (B) HSV-2. The supernatants were collected at different time points and a bioluminescence assay was performed. Fold induction of infected compared to non-infected RLU values were calculated. Data from three independent experiments are shown; the error-bars represent means ± SD;

The experiments revealed that 3 d p.i. the AAV replicon assay was highly inducible at a very low infection dose, achieving a plateau after infection at MOI 0.01. For example, infection of Vero cells at MOI of 0.001 induced the replicon encoded reporter gene expression 246-fold, compared to a 392-fold induction with infection at MOI of 0.01. Infection with the lowest dose, at MOI 0.01, showed a significant induction after 2 d p.i. (119-fold). At higher HSV-1 infection density (MOI 0.1 or more), a significant induction was already detected after 24 h p.i. With MOI 0.1 and MOI 1 the induction was 18-fold and 120-fold, respectively.

However, induction of the transiently transfected AAV replicon vector with HSV-2 was much lower than after infections with HSV-1 (Figure 13B). In this case, the induction of GLuc was at maximum 17-fold at a MOI of 0.1. Taken together, the reactivity of the transiently transfected AAV replicon system is excellent using HSV-1 infection in Vero cells. On the other hand, HSV-2 barely induced the replicon-encoded transgene under the same conditions.

4.2.2. Construction and characterization of stable cell lines carrying AAV replicons

Despite the flexibility of the transient transfection concerning the investigation of the AAV replicon in different cell lines, the reproducibility of the assay needed to be improved. Especially applications, such as phenotypic tests in virus diagnostics seek high level of standardization. Therefore, we wanted to test whether it is possible to generate cell lines that stably maintain the AAV replicon. To this end we wanted to generate cell lines carrying different reporter genes under the control of AAV replicon. For quantitative detection of virus infection using flow cytometry (FACS), the enhanced green fluorescent protein gene (EGFP) was selected and incorporated to the AAV genome as described earlier (chapter 4.1). EGFP is known to be a very stable protein, with a half-life of 24 h [285] and can easily be detected in the presence of low level induction due to its propensity to accumulate. The other transgene we wanted to test with this system, was the GLuc, which we could test using the construct we used in the transient assays. The advantage of this reporter gene is its secretion from mammalian cells into the cell culture medium, which allows testing without the need of cell lysis [286]. The sensitivity of GLuc is very high and, due to its great stability, the possibility of collecting the supernatants at different time points and measure their bioluminescence later, makes it a useful tool for high throughput applications.

4.2.2.1. Establishment of an EGFP expressing AAV replicon cell line

293A cells were chosen as a platform to construct the EGFP reporter based stable AAV replicon cell line. This cell line can be infected with both Ad and HSV. Therefore, the pAV1-EGFP-Hyg was transfected into 293A cells using FuGene, and individual cell clones were isolated by limiting dilution in the presence of hygromycin for selection. 25 cell clones named AAVG#1-25 were isolated, propagated until a stable stage of growth (usually 6 passages) and frozen for further testing. After establishing the clones based on their hygromycin resistance, they were tested for their ability to express EGFP upon Ad infection using FACS analysis.

To analyze the AAVG clones, they were recovered from their frozen stocks and either infected with Ad5-Che using a MOI of 100 or kept mock infected. FACS analysis of the non-infected and infected cell clone was done 2 d p.i. Among these cell clones, all showed an induction of green fluorescence upon Ad infection compared to non-transduced 293A cells, which had a low level

autofluorescence (1%). Most of the clones showed a higher proportion of green fluorescence positive cells after infection, ranging from 11% to 72%, and indicating Ad5 infection induced expression of EGFP. Interestingly, AAVG#7 did show a high proportion of green fluorescence that was not further induced by Ad infection. Out of these cell clones the 4 most promising clones were selected for further analysis: AAVG#9, AAVG#16, AAVG#20 and AAVG#K, together with the non-inducible but GFP fluorescence positive clone AAVG#7. In order to compare the inducibility of their GFP expression, the different AAVG clones were either infected with Ad5-Che at a MOI of 100 or with HSV at a MOI of 1. After 48 h p.i., EGFP expression in the cells was analyzed by FACS (Figure 14).

As expected, AAVG#7 showed green fluorescence independently of virus infection. The other selected clones demonstrated a similar increase of transgene expression of 4.4-fold (AAVG#9), 3.8-fold (AAVG#16), 3.3-fold (AAVG#20) and 3.5-fold (AAVG#K) after Ad5-Che infection. In general, the induction after Ad5-Che infection was slightly higher compared to the induction of EGFP expression after HSV-1 infection, which was 2.5-fold (AAVG#9), 3.5-fold (AAVG#16), 1.5-fold (AAVG#20) and 3.6-fold (AAVG#K). Considering the insignificant effect of Ad5 and HSV-1 on the reporter gene expression of the AAVG clones, these clones appeared to be minimally reactive and therefore represented no advantage over the assay based on the transient transfection of AAV replicon.

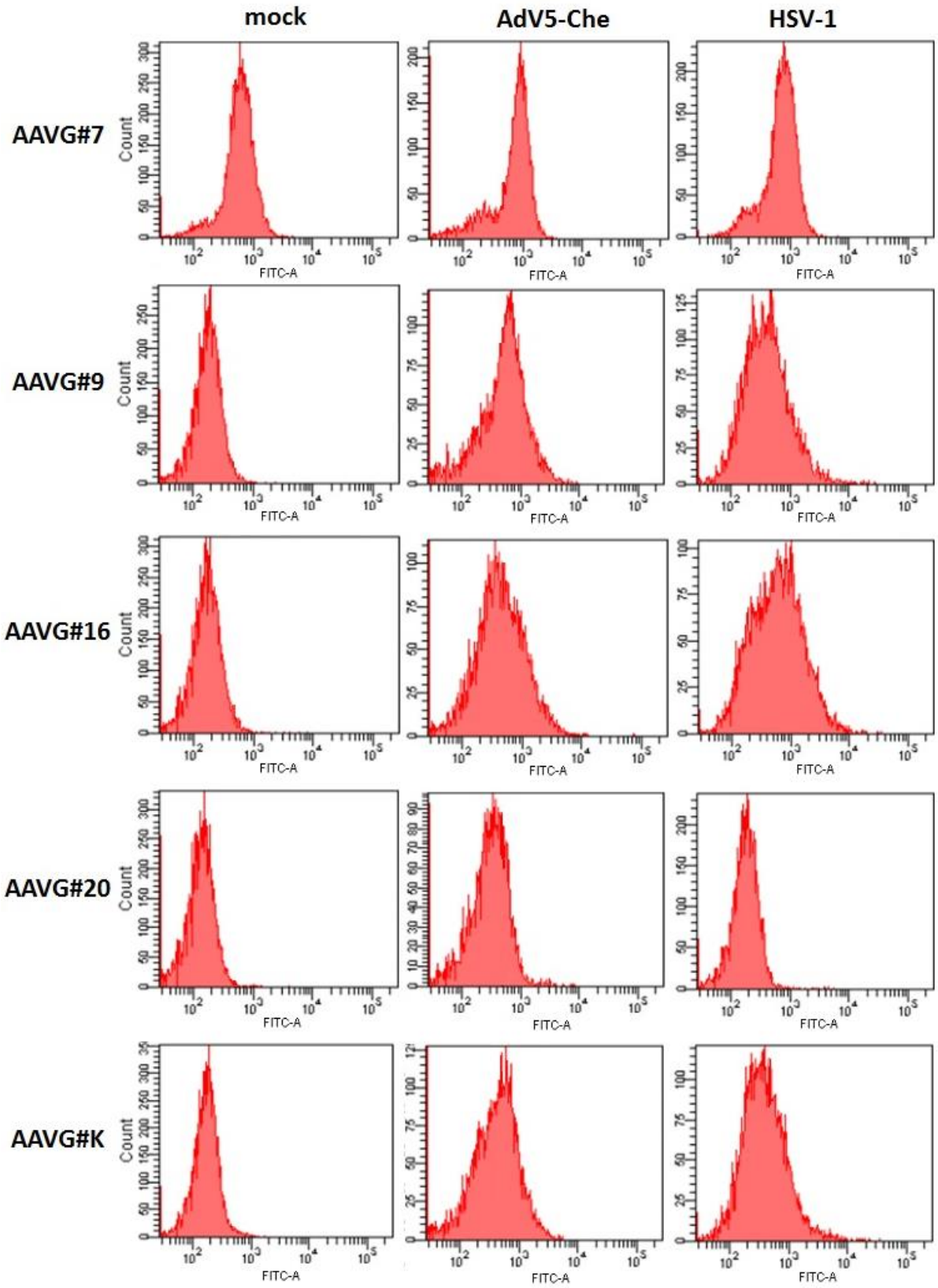


Figure 14: FACS analysis of stable AAV EGFP replicon clones.

The stable cell lines AAVG#7, AAVG#9, AAVG#16, AAVG#20 and AAVG#K were either non-infected (n.i.), infected with the mutant virus Ad5-Che at MOI 100 or with HSV-1 at MOI 1. EGFP capacities were determined 48 h p.i. using FACS analysis. Shown is one representative experiment out of two;

4.2.2.2. Construction of a GLuc expressing AAV replicon cell line

Besides EGFP, GLuc represents another possible transgene for the construction of an inducible stable cell line. To construct stable AAV replicon clones with GLuc as transgene, the vector pAV1-GLuc-Hyg was transfected into 293A cells and single clones were selected by limiting dilution under hygromycin selection. Overall, 66 clones were isolated, propagated and tested for the inducibility of the transgene expression upon Ad infection. For testing the conditional expression of GLuc, the clones were seeded into 96-well plates at $3x10^4$/well density and either kept non-infected or infected with the recombinant Ad5-Che at a MOI of 100. 48 h p.i. bioluminescence assays of the collected supernatants were performed and compared to the respective values obtained in non-infected cultures. Only 1 clone out of the 66 tested was responding to the Ad5-Che infection with an about 30-fold induction of GLuc expression.

This clone, LE2D8, was the only one we further analyzed to define the best conditions of future applications. LE2D8 cells were either infected with Ad5-Che at MOI 100 on the same day of seeding (day 0), or within the following 3 days (day 1 – day 3). The supernatants were collected at different days, ranging from 1 day p.i. to 4 days p.i., in order to test the optimal induction time (Figure 15).

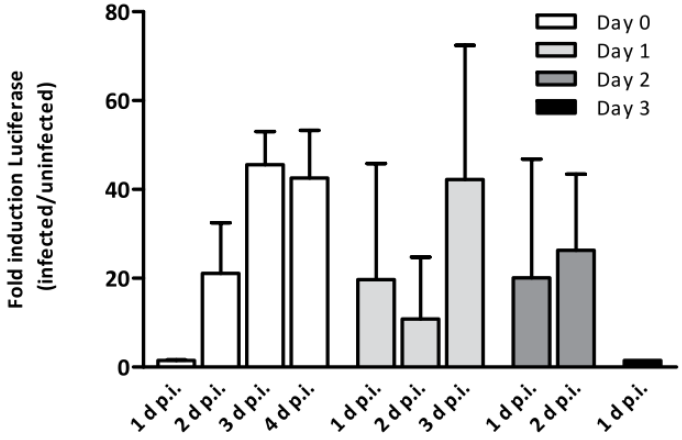


Figure 15: Characterization of LE2D8.
Stable cell line LE2D8 was infected with the Ad5-Che using a MOI of 100 at the same day (Day 0) to day 3 after seeding, following supernatants collection at different time points (1 – 4 days post infection (d p.i.)). A bioluminescence assay was performed, and the inductions of infected cells were calculated by normalizing to the values of non-infected cells. Data from three independent experiments are shown; the error-bars represent means ± SD;

The highest induction of the transgene upon Ad infection was detected when LE2D8 cell clones were infected at the same day of seeding and analysis of the bioluminescence done on day 3

after transduction. Here, the induction of luciferase expression was 40-fold. Infection on the day of seeding generally showed an increase of the signal from day 1 to day 3 post infection of 1.5-fold, 13-fold and 40-fold. At day 4 the expression of GLuc was slightly decreasing to 35-fold. If cells were infected 24 hours after seeding, the variation between the experiments started to increase considerably. Here, the induction of the transgene was 20-fold (1 day-), 11-fold (2 days-) and 42-fold (3 days after infection) with a standard deviation (SD) nearly as high as the values itself (26, 14 and 30). When the stable cell line was transduced 2 days after seeding, the induction was 20-fold with a SD of 27 after collection at day 1 - and 26-fold with a SD of 17 at day 2 post transduction. However, when the infection was performed 3 days after seeding, no induction of GLuc was detected.

In summary, the reproducibility of the experiments was better when all were infected on the day of seeding, while infection on later days after seeding gave unsatisfactory results for Ad5-Che induction.

Concerning the overall time for one assay, the possibility of immediate infection after seeding resulted in a shortened assay duration. The bioluminescence after infection of the stable cell line was lower when compared to results after infection of transiently transfected cells using the same infection conditions (see Figure 7). However, the loss of reproducible inducibility culturing the cells together with the extremely low cloning efficiency indicated the instability of the AAV replicon construct in stable cell lines.

4.2.2.3. Human Ad5 and HSV-1 infection induce the AAV replicon in the cell line LE2D8

Based on these findings, the link between the time point of analysis and signal strength was nevertheless further evaluated. Therefore, the inducibility of the LE2D8 cell line infected by Ad5-Che and HSV-1 was investigated at different MOIs right after seeding. The supernatants of Ad5-Che infected cells were collected and analyzed from 24 to 84 h p.i. and of HSV-1 infected cells from 12 – 60 h p.i. (see Figure 16).

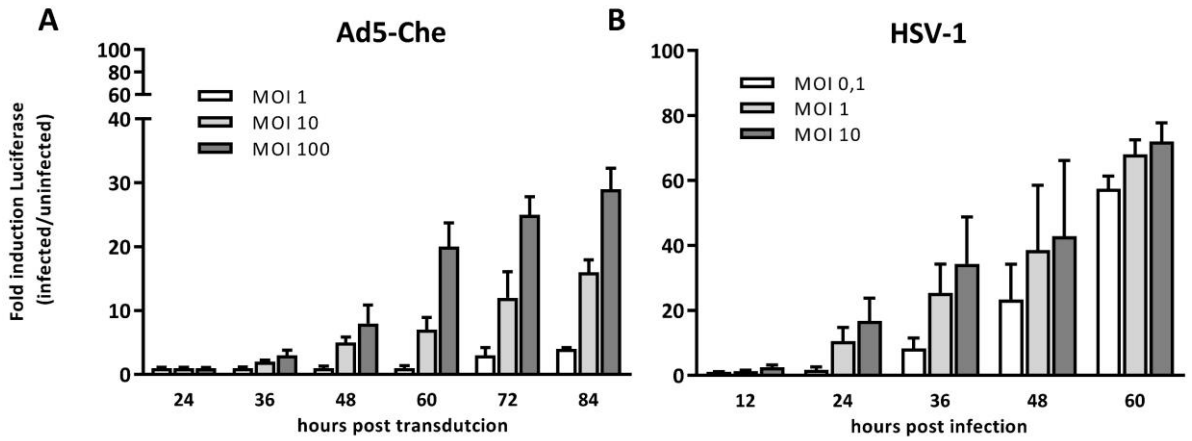


Figure 16: Infection of LE2D8 with HAd5-Che and HSV-1.
(A) The stable cell line LE2D8 was infected with the mutant virus Ad5-Che at a MOI of 1, 10 and 100 or kept mock infected and at 24, 36, 48, 60, 72 and 84 h post transduction the induction of GLuc was measured. (B) Infection of LE2D8 with HSV-1 at a MOI of 0.1, 1 and 10 or no infection was done. Then supernatants collection and measurement of GLuc induction at different time points (12, 24, 36, 48, 60 h p.i.) followed. The induction of the bioluminescence for both viruses was calculated by comparing infected and non-infected values. Data from three independent experiments are shown; the error-bars represent means ± SD;

Both viruses showed a time dependent increase that was not reaching the plateau in the tested time. Detailed analysis of Ad5-Che revealed that the expression of GLuc was highly dependent on the virus dose. The induction after infection at a MOI of 1 was not significant. There was no induction compared to the non-infected cells within the first 24 h p.i. and an increase to 4-fold followed only at 84 h p.i. At a MOI of 10, the expression of the transgene was higher and increased to 16-fold at 84 h p.i. The highest values of the bioluminescence compared to non-infected cells with an increase of 29-fold after 84 hours were reached with the highest dose of Ad5-Che. In contrast, the induction of the stable AAV replicon cell line by infection with HSV-1 was less dependent on the virus dose, compared to Ad5-Che infections. This indicates that HSV-1 infection reached the induction limit faster and at much lower virus load. The induction of GLuc could be already detected 24 h p.i. after infection at a MOI of 1 and 10. The induction compared to non-infected cells was 11-fold and 17-fold. When LE2D8 cells were infected with HSV-1 at a MOI of 0.1 the earliest time point of a significant bioluminescence signal was 36 h p.i. with an induction of 8-fold compared to non-infected cells. The highest values were reached 60 h p.i. Here, the induction of GLuc was 58-fold (MOI 0.1), 68-fold (MOI 1) and 72-fold (MOI 10).

Overall, the induction of the stable cell line LE2D8 revealed lower bioluminescence compared to the transient system. Nevertheless, the signal strength showed a clear correlation between time after infection and MOI for both viruses tested.

4.2.2.4. The replicon response in LE2D8 cells by various Ad serotypes and HSV-2 is limited

Based on the data from the transient transfection, we were interested to find out whether the response in the stable AAV replicon cell line was also inducible by different serotypes of Ads. Since the induction of GLuc after Ad5-Che infection of the stable cell line LE2D8 showed lower values compared to infection of transiently with pAV1-GLuc-Hyg transfected cells, the aim was a proof of principle and the usefulness of LE2D8 for approaches testing different adenovirus serotypes. Infection of LE2D8 cells was performed, as before, right after seeding and the bioluminescence signal was measured 3 d p.i. Afterwards the induction of GLuc was calculated compared to non-infected cells. A marginal increase of the signal could be seen for all viruses. The highest induction of GLuc, 15-fold, was reached after Ad12 infection, just like after the transient transfection. These results confirmed that the replicon response in LE2D8 recapitulate the induction seen after transient transfection of the AAV replicon but in a lower level (see Figure 17). However, a lower level of inducibility was not observed when the LE2D8 cell line was used to test HSV-2.

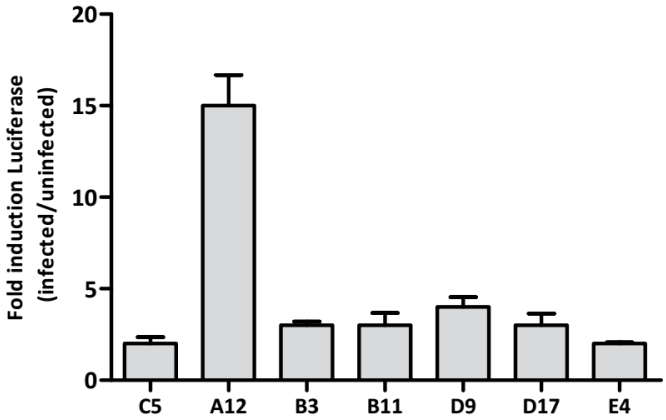


Figure 17: Infection of LE2D8 with different HAdV-serotypes.
The stable cell line LE2D8 was transduced with either species C – serotype mutant Ad5-Che (C5) as control or with species A – serotype Ad12 (A12), species B – serotype Ad3 and Ad11 (B3 and B11), species D – serotype Ad9 and Ad17 (D9 and D17) and species E – serotype Ad4 (E4) at MOI 1 or kept mock infected. 3 d p.i., the induction of GLuc was measured. The fold induction of GLuc was calculated for all viruses by comparing infected and non-infected RLU values. Data from three independent experiments are shown; the error-bars represent means ± SD;

To find out whether LE2D8 cells would be a good tool to test HSV-2, they were seeded into a 96-well plate and infected 4 h later with HSV-2. The virus was a patient isolate (used with permission), expanded in cell culture and used at a dilution of 1:10. The GLuc expression was subsequently measured after 48 and 72 h p.i. Compared to transient transfection of Vero cells, the induction of LE2D8 upon HSV-2 infection indicated no significant signal increase (Figure **18**).

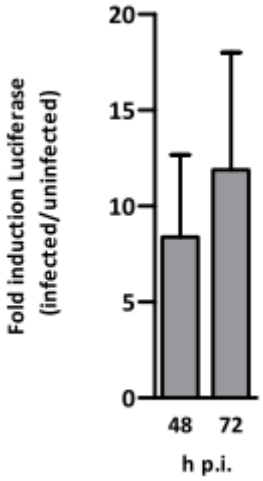


Figure 18: Infection of LE2D8 with HSV-2.
The stable cell line LE2D8 was infected with HSV-2 using 1:10 diluted viruses. 48 and 72 h p.i., the induction of GLuc was measured. The fold induction of GLuc was calculated by comparing infected and non-infected RLU values. Data from three independent experiments are shown; the error-bars represent means ± SD;

4.2.3. Induction of frozen AAV replicon vector transfected cells

Since generating cell lines that stably maintain AAV replicons did not yield a real alternative to transient transfection, we explored another way of controlling experiment-to-experiment variations.

In order to do so, we transfected a large batch of 293A cells with pAV1-GLuc-Hyg and re-suspended them after either 48 or 72 hours in standard freezing medium at a density of $3x10^4$ /100 μL and seeded them in 96 well plates. The plates were then frozen to -80°C. Right after thawing, fresh medium was added and then the cells were incubated for 2 hours under normal cell culture conditions. After that, supernatants were exchanged to fresh medium. Six h after thawing, the cells were ready to be infected with Ad5-Che at different MOIs. The GLuc signals in the supernatants were analyzed 48 h p.i. and compared to non-infected cells, which were treated the same way. The results showed that freezing and thawing of transfected cells for an induction experiment is possible (Figure 19A). The induction was higher when the

transfected cells were frozen already at 48 h post transfection. Interestingly, independent of the freezing time-point after transfection, the signal was not reflecting the number of viral particles used for infection. If the cells were frozen 48 h after transfection, infection at MOI of 1 resulted in a 126-fold induction of GLuc, whereas the signal decreased to 99-fold and 67-fold at MOI 10 and MOI 100. The same effect, but lower, could be seen if the cells were frozen 72 h after transfection. Here, the signal strength decreased from 73-fold at MOI 1, to 59-fold at MOI 10 and 46-fold at MOI 100. It seems that a combination of freezing and thawing procedure and infection with high dose of Ad has a negative impact on the replicon response.

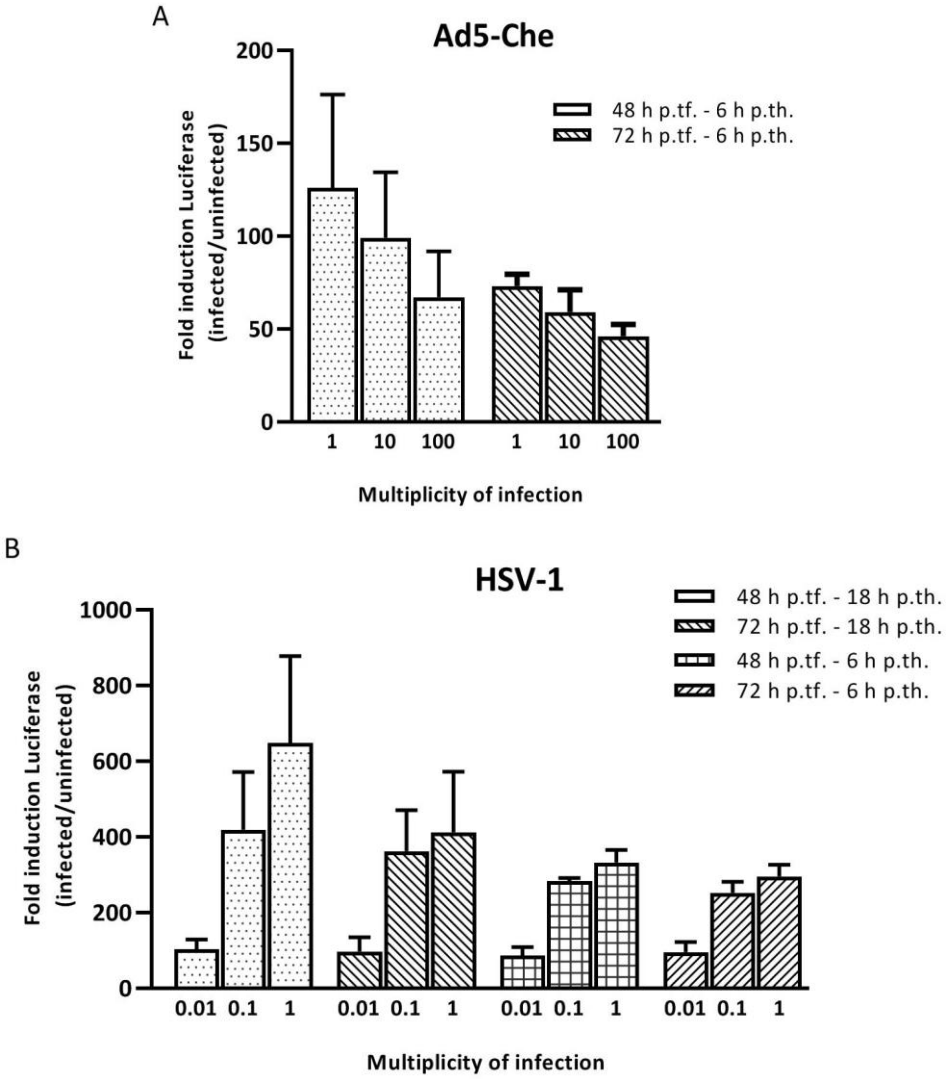


Figure 19: Frozen AAV replicon transfected cells are induced by helper virus infection.
293A cells were transfected with pAV-GLuc-Hyg, and either after 48 or 72 h post transfection (p.tf.) the cells were seeded in a 96 well plate format and frozen. (A) Cells were thawed and 6 h post thawing (p.th.) infected with Ad5-Che at indicated MOI. (B) After thawing of the cells, they were infected either 6 or 18 h p.th. with HSV-1 at indicated MOI. The induction of GLuc in the supernatants of Ad5-Che and HSV-1 infected and non-infected cells were measured. The fold induction of GLuc was calculated for all samples by comparing infected and non-infected RLU values. Data from three independent experiments are shown; the error-bars represent means ± SD;

Consequently, we infected the pre-transfected, frozen and thawed cells with HSV-1 at different MOIs to proof whether this effect can be seen using another helper virus as well. For this, AAV replicon transfected cells in the prior frozen plates were infected 6- or 18 h after thawing. The second time point for infection was chosen in order to let the cells recover for a longer period before infection to avoid possible toxic effects of infection with higher MOIs (Figure 19B). Here MOI of HSV-1 correlates with the strength of induction of GLuc expression. Cells frozen 48 h after transfection and infected 18 h after thawing at MOI 0.01, 0.1 and 1, showed the highest induction of GLuc expression (103-, 419- and 649-fold, respectively). Freezing the plate 72 h post transfection resulted in a lower induction of the signal. If the cells were infected 6 h after thawing, the bioluminescence signals were independent of the freezing time point after transfection and were about 90-, 270- and 315-fold, respectively. The correlation of the time of infection after freezing and toxicity effects seen with Ad at higher viral load could not be confirmed with HSV-1.

In conclusion, freezing of already transfected and seeded cells is functioning very well. The cells in the 96-well plate format could easily be prepared in a larger amount of plates and thawed in the respective quantity. The freezing and thawing of the AAV replicon transfected cells can be used as an alternative to save time after transfection and to have a ready to use system. Nevertheless, this new approach is restricted to cell lines that can be well transfected and are robust enough to withstand this protocol.

4.2.4. Induction of the AAV replicon vector packaged into recombinant AAV2 particles

The so-far tested transfection-based methods for delivery of the AAV replicon did not fulfill all standardization characteristics and applicability requirements for the development of a reporter assay on virus growth. This can be due to the poor reproducibility of transfection based experiments. In addition, delivery of all previously described plasmid-based replicons is limited to transient or stable transfection. This restricts the use to only well transfectable cell lines for the replicon assays. Testing the inducibility of AAV replicon by other viruses, such as human cytomegalovirus (HCMV), which are permissive to only a limited set of target cells, is inhibited by the poor transfectability of these cells.

In our AAV replicon vector, the ITRs are required as cis elements for the incorporation of its genome into recombinant AAV (rAAV) particles. This allows to test whether the AAV replicon can be packaged and delivered as recombinant AAV particle. For this purpose, we analyzed the transduction efficacy of different rAAV particle serotypes for their ability to transduce 293A cells, Vero cells and HFF (Human foreskin fibroblasts) cells. This was done to test whether packaging systems other than AAV2 are beneficial for the target cell range for the replicon assay. Next, we tested if the AAV replicon vector can be packaged into recombinant AAV particles by standard co-transfection methodology using AAV2 based packaging plasmids [287].

4.2.4.1. Transduction efficiency of different recombinant AAV serotypes on Vero and HFF cells

To identify the best rAAV serotype for transduction of Vero cells and HFF cells we used rAAV particles carrying EGFP under the control of a CMV promoter (rAAV-CMV-GFP). We tested recombinant capsids derived from AAV serotype 1, 1/2 (a hybrid between serotype 1 and 2), 2, 4, 5 and 6. First, both Vero and HFF cells were transduced with 10000 particles per cell (p/c) of the 6 different rAAV-CMV-GFP serotypes. We then analyzed the transduction efficiency of the different rAAV serotypes in the cell lines 48 h and 6 days post transduction using fluorescence microscopy (Figure 20). The particle rAAV2-CMV-GFP showed the highest number of transduction efficiency.

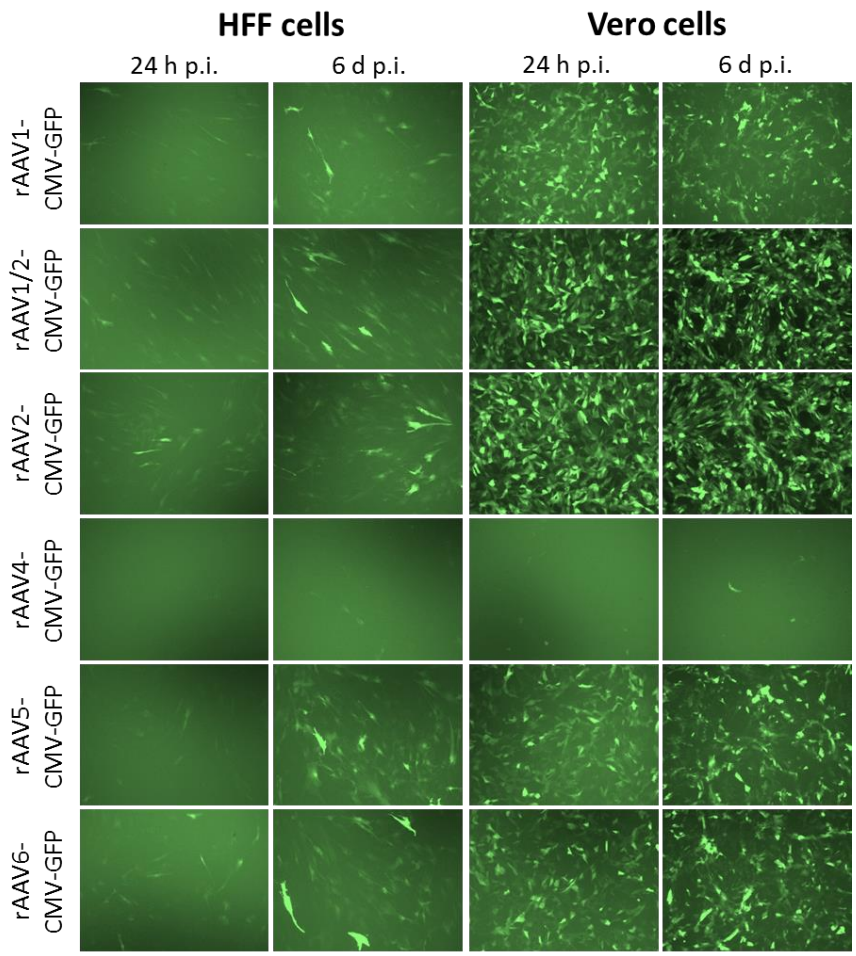


Figure 20: Analysis of transduction efficacy of different AAV serotypes.
HFF and Vero cells were transduced with 10,000 particles per cell (p/c) of different rAAV-CMV-GFP serotypes, namely 1, 1/2, 2, 4, 5 and 6. All cells were analyzed for their ability to be transduced by fluorescence microscopy 48 h and 6 days post transduction.

Consequently, the recombinant AAV2-CMV-GFP particles were further analyzed for their optimal transduction dose, applying of 100, 1000 and 10000 p/c. We also included two additional cell lines - 293A and A549 - which are both possible options to carry out replicon induction experiments after Ad infections. After transduction, the cells were analyzed by fluorescence microscopy after 24, 48 and 72 h post transduction. In all cell lines the GFP signal obtained correlated with the dose of recombinant particles (Figure 21).

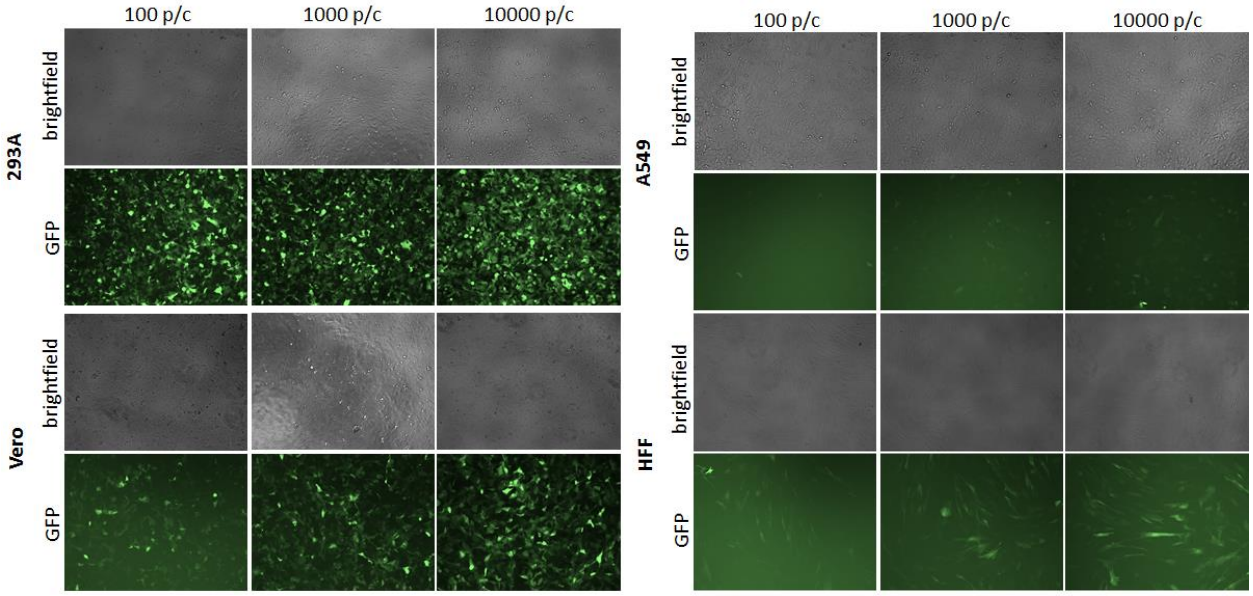


Figure 21: Analysis of transduction efficacy of rAAV2 particles.
293A, Vero, A549 and HFF cells were transduced with 100, 1000 and 10000 particles per cell (p/c) of rAAV2-CMV-GFP. 48 h post transduction, cells were analyzed for their transduction capacity by fluorescence microscopy.

The observed transduction efficiency was different for each cell line. 48 h post infection, the efficacy of GFP expression in 293A, Vero and HFF cells was high, whereas after the same timespan A549 cells revealed a low transduction rate of rAAV2-CMV-GFP, which then slightly increased after 72 h (Figure 22).

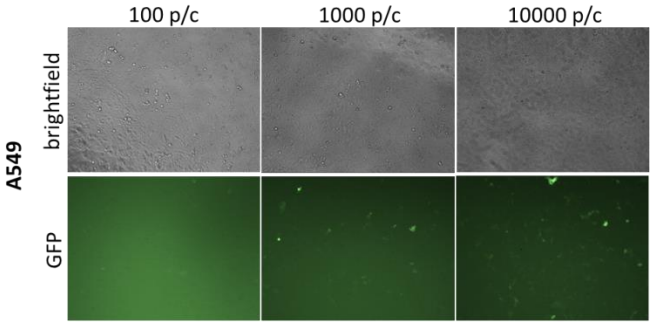


Figure 22: Analysis of transduction efficacy of A549 cells with rAAV2 particle after 72 h.
A549 cells were transduced with 100, 1000 and 10.000 particles per cell (p/c) of rAAV2-CMV-GFP. 72 h post transduction, cells were analyzed for their ability to be transduced by fluorescence microscopy.

In conclusion, the serotype 2-derived rAAV particle showed an acceptable transduction rate in at least one cell line of interest.

Subsequently, we tested the production of serotype 2 rAAV particles carrying the AAV replicon. The AAV replicon vector pAV1-GLuc-Hyg contains two ITRs serving as viral origin of

replication and packaging signal. These two ITRs flank the rep gene and the transgene under the control of the p40 promoter, which should be transferred as DNA vector genome into the rAAV particle. For the replicon containing rAAV2 particle production we used the helper plasmid pDP2rs, which provides the structural and non-structural genes of AAV2 and the Ad helper functions [287]. The AAV replicon containing serotype 2-derived recombinant AAV particles we named rAAV2-REP-GLuc.

The semi-quantitatively analyzed ITR differed from the GLuc genome copy numbers along different productions of rAAV2-REP-GLuc. These irregularities also revealed a difference between ITR and GLuc sequences of 14- and 24-fold in single productions of rAAV2-REP-GLuc batches as well as differences between batches concerning the gene copy number of GLuc (Table 4.2.4.1.1).

We did not investigate the reason for these irregularities and decided to use the GLuc-PCR based genome counts instead of the commonly used ITR-counts for the particle number calculations, since the gene copy number measured using the GLuc gene corresponded to the expression data (see later).

Table 4.2.4.1.1: Comparison of ITR and GLuc copy number by quantitative PCR.

	ITR copy number	Gluc copy number
rAAV2-REP-GLuc batch 1	N/A	$3{,}40 \times 10^{10}$
rAAV2-REP-GLuc batch 2	$2{,}38 \times 10^{11}$	$5{,}76 \times 10^{9}$
rAAV2-REP-GLuc batch 3	$3{,}98 \times 10^{11}$	$2{,}29 \times 10^{10}$
rAAV2-REP-GLuc batch 4	$4{,}25 \times 10^{11}$	$2{,}17 \times 10^{10}$

4.2.4.2. Induction of rAAV-based replicon response

We selected 293A cells for a first functional test of the transduced rAAV2-REP-GLuc, since this cell line showed appropriate induction rates for both Ad and HSV-1 infections after replicon transfection. Therefore, 293A cells were seeded onto 96-well plates at a density of 3×10^4 per well and right after seeding transduced with 1 to 10000 p/c of rAAV2-REP-GLuc. After an incubation time of 6 hours, the transduced cells were infected with either Ad5-WT at MOI 10, HSV-1 at MOI 0.1, or kept non-infected. 48 h later a bioluminescence assay was performed to measure the GLuc induction and to compare the induction of infected to non-infected but transduced cells (Figure 23).

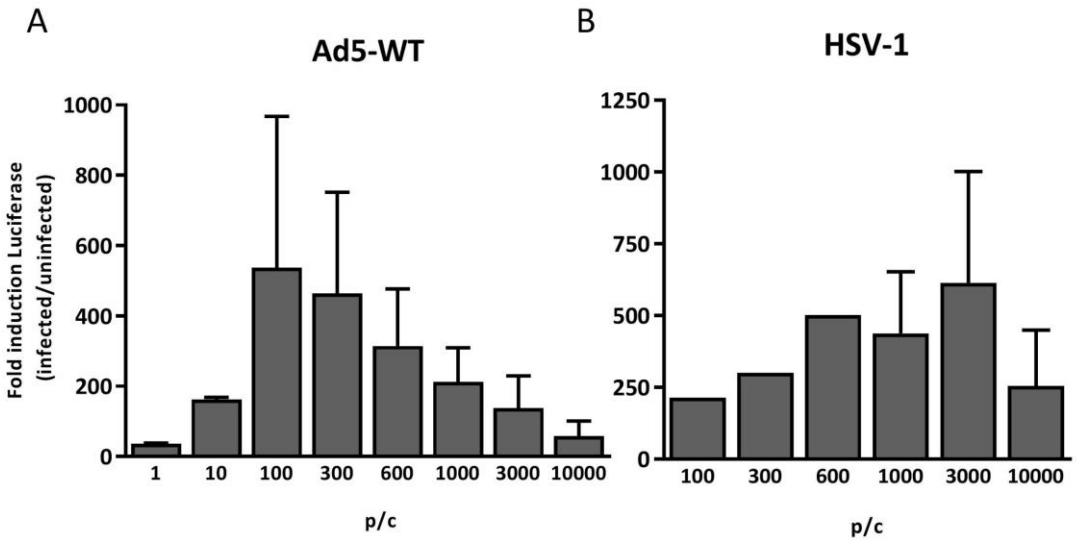


Figure 23: Transduction of 293A cells by replicon containing recombinant AAV particles.
293A cells were transduced with the indicated number of replicon-containing recombinant AAV particles (rAAV2-REP-GLuc) per cell (p/c) and 6 h later infected with (A) Ad5-WT at MOI 10, (B) HSV-1 at MOI 0.1, or kept non-infected. 48 h p.i. GLuc induction was measured and compared to values of transduced but non-infected cells. Shown are data from three independent experiments, done in triplicates; the error-bars represent means ± SD;

We observed an infection-dependent induction of the replicon gene expression of at least 10-fold under all conditions tested. A transduction dose-dependent increase of the GLuc reporter gene expression after Ad5-WT infection was also detectable up to the amount of 100 rAAV2-REP-GLuc per cell. Interestingly, transduction with higher particle numbers resulted in a proportional decrease of the signal. Nevertheless, the 537-fold induction after HAd5 infection, compared to non-infected but transduced cells, was the highest induction we could observe in our study up to this point for Ads. In case of HSV-1 infection, the induction also increased dose-dependently in the low-dose settings. In contrast to Ad5-WT, HSV-1 infection resulted in a plateau of GLuc induction after transduction with 1000 p/c. With an induction of 613-fold following HSV-1 infection, the rAAV2-REP-GLuc also showed a better inducibility of GLuc compared to previously tested delivery methods of the AAV replicon vector. The subsequent decrease or constant signal with increasing amounts of rAAV2-REP-GLuc particles after Ad5 and HSV-1 infections indicated a potential toxicity of AAV transduction at high dose. The big variation of induction levels also indicated suboptimal conditions for the replicon response upon transduction of 293 cells.

Nevertheless, the reporter gene of the rAAV2-REP-GLuc was better inducible in 293A cells than in any other experiment before. Therefore, we decided to test other cell lines and viruses for

their ability to induce the AAV replicon response after delivery by rAAV particle transduction. For each cell line and virus, we tested multiple conditions of transduction, infection, and collection of the supernatants to draw a comprehensive conclusion of the applicability of the AAV replicon system.

Hence, A549, Vero and HFF cells were seeded onto 96-well plates at a density of 3 x 10^4/ well and transduced with different doses of the rAAV2-REP-GLuc right after seeding. After 6 hours, the cells were infected with Ad5-WT at MOI 10, with HSV-1 and HSV-2 at MOI 0.1 and with HCMV at MOI 1. The supernatants of infected and non-infected cells were collected for analysis at different time points after the respective virus infections.

In the rAAV2-REP-GLuc transduced A549 cells, the replicon encoded reporter gene expression was induced by infection with Ad5-WT at MOI 10 to an unexpectedly high value of 13500-fold compared to non-infected cells 48 h p.i. This result was in sharp contrast to the low transduction efficacy we observed in this cell line after transduction of the constitutive GFP expressing rAAV2-REP-GLuc in the previous experiment (chapter 4.2.4.1). In addition, the reporter gene induction directly correlated with the number of AAV replicon containing rAAV particles without showing a drop at higher doses (Figure 24A). HSV-1 was able to significantly induce the transgene expression 54-fold in transduced Vero cells with 600 p/c after 24 h p.i. Similar to A549 cells after Ad5-WT infection, this cell line showed a transduction dose-dependent increase of the GLuc induction. The induction also increased linearly by time, showing an about 7-fold increase from 24 h p.i. to 48 h p.i. The highest induction, 2800-fold compared to non-infected transduced Vero cells, was observed on day 2 after transduction of 10000 particles and infection with HSV-1 (Figure 24B).

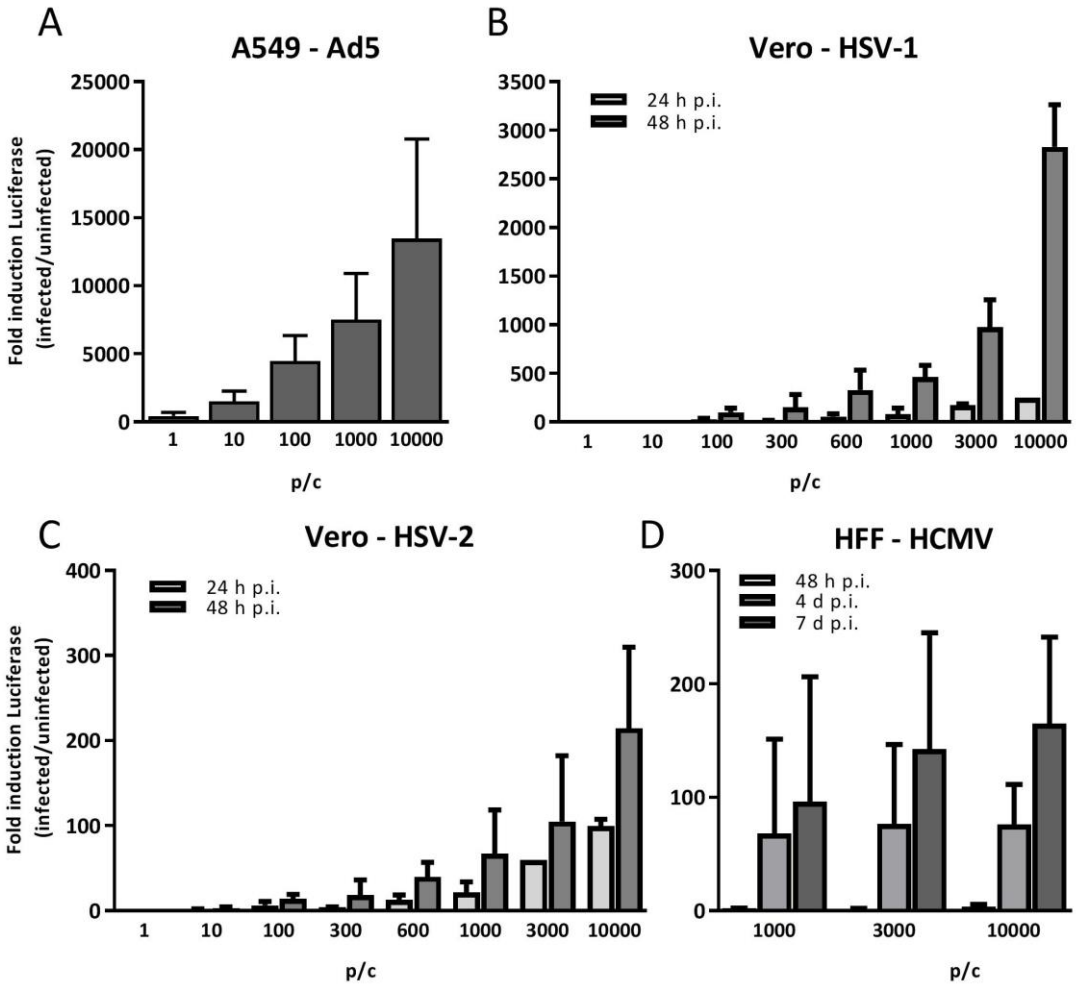


Figure 24: Induction of AAV replicon after transduction.
(A) A549 cells were transduced with indicated dose (p/c) of rAAV2-REP-GLuc and 6 h later infected with Ad5-WT at MOI 10. 48 h p.i. supernatants were collected, GLuc induction measured and compared to those of transduced but non-infected A549 cells. (B +C) Vero cells were transduced with indicated rAAV2-REP-GLuc doses (p/c) and infected with (B) HSV-1 or (C) HSV-2 at MOI 0.1 6 h later. Supernatants were collected at 24- and 48 h p.i. and induction were calculated as above. (D) HFF cells were transduced with 1000, 3000 and 10000 p/c of rAAV2-REP-GLuc and 6 h later infected with HCMV at MOI 1. Supernatants were collected after 48 h p.i., 4- and 7 d p.i. GLuc activity was analyzed by bioluminescence and induction was calculated. Data represents three independent experiments, done in triplicates; the error-bars represent means ± SD;

In previous experiments we could not induce the AAV replicon system significantly with two other herpesviruses: HSV-2 and HCMV. The stability of the transduced rAAV2-REP-GLuc motivated us to re-test the inducibility of the AAV replicon after infection with these viruses. The supernatants of HSV-2 infected Vero cells were therefore analyzed after 24 and 48 hours and the supernatants of HCMV infected HFF cells at 48 h p.i., 4- and 7 d p.i., taking the longer replication time of HCMV in comparison with Ad5 or α-herpesvirinae into account. As a result, both viruses were able to significantly induce the AAV replicon system after transduction of

the corresponding cell line. HSV-2 showed an induction of 21-fold 24 h p.i. after transduction with 1000 p/c and a 100-fold induction with 10000 p/c. Two days after infection, the GLuc induction increased to 215-fold at 10000 particles of rAAV2-REP-GLuc per cell. In case of HCMV, hardly any induction could be seen at 48 h p.i. 4 p.i., we measured a 76-fold increase at transduction levels of 10000 p/c compared to non-infected but transduced HFF cells. The effect was even more significant 7 days after infection, when the induction of the transgene was 165-fold compared to non-infected cells. Interestingly the level of induction after transduction of either 1000 or 10000 particles was only moderately increased (77- and 76-fold at 1000 p/c and 143- and 165-fold at 10000 p/c). Yet, the deviation between the two independent experiments, each performed in technical triplicates, was very high.

The results revealed that the nature of target cell line plays an important role in both the inducibility and the reproducibility of the AAV replicon induction. High level inducibility, significant dose response, and good reproducibility were observed upon transduction of A549 cells and infection with Ad5 as well as transduction of Vero cells and infection with HSV-1 and 2. In contrast, 293A cells were not optimal using the same viruses for induction of AAV replicon containing rAAV particles. Further optimization is also needed for replicon induction by HCMV infection in transduced HFF cells. Nevertheless, with the ability of transducing the AAV replicon vector via a viral vector in more cell lines than before, the efficacy of the replicon induction increased significantly for all tested viruses. Furthermore, with the new delivery method, a significant induction of the AAV replicon system was observed for the first time after HSV-2 and HCMV infection.

4.3. Applications

The different delivery systems of the AAV replicon offer several possible applications: We tested the AAV replicon vector for its applicability for *trans*-complementation, since this is a feasible method to investigate essential genes [214]. Furthermore, we established a suitable AAV replicon-based resistance test in order to analyze the application of the AAV replicon system in the field of diagnostic. Additionally, we developed a new screening platform for antiviral inhibitors using the AAV replicon vector. For the first time, this allows high-throughput tests of inhibitors against genetically unmodified large DNA viruses.

4.3.1. *Trans*-complementation of HAd5 late protein pVI by the AAV replicon vector

For analyzing the *trans*-complementation potential of the AAV replicon system, we selected pVI, an essential late viral gene of HAd5, as a target. pVI is important for the activation of Ad genomes during the entry phase, for the endosomal escape, for the nuclear transport of Ad particles and it is an allosteric activator for the viral encoded cysteine protease [162]. In addition, pVI is toxic in isolated expression and therefore trans-complementing cell lines were not reported so far. Previously the pVI functions were studied in the same laboratory that provided the required tools to perform a proof-of-principle experiment for trans-complementation using AAV replicon [288].

Adenovirus late proteins such as pVI are expressed only after the onset of viral DNA replication. For the expression of the toxic Ad5 *pVI* in *trans*, the AAV replicon system seemed to be a plausible alternative, since the expression of the transgene in the AAV replicon vector is dependent on the early functions of Ad. Without helper virus infection the reporter genes were practically silent in all our previous experiments. The early phase of Ad replication therefore is a prerequisite for viral DNA replication, onset of Ad late gene expression, and induction of p40 controlled transgene expression in the AAV replicon.

For the *trans*-complementation of pVI deletion of Ad5 with the AAV replicon vector, cloning of *pVI* as transgene into the pAV1-Hyg vector was necessary. Furthermore, an Ad5 mutant lacking the *pVI* gene had to be constructed as well. In theory, upon infection with the Ad5 mutant, the AAV replicon vector with *pVI* as transgene should be induced. Expression of the

essential pV gene could then complement the missing gene for production of the virions in the replicon containing cells.

4.3.1.1. Construction of a HAd5ΔpVI mutant and the pIV expressing AAV replicon

The deletion of the protein pVI would lead to an Ad5 mutant which is not able to spread. To test whether a deletion of the essential pVI gene can be *trans*-complemented and reverse this defect in *trans*, we constructed the viral mutant Ad5Δ*pVI*. We generated the genomic constructs using the E1-E3 deleted Ad5 BAC, namely the pBA5-FRT, as a basis. This BAC can be genetically tagged by site specific recombination at its FRT site. Additionally, the E1-E3 deleted viruses derived from this BAC should possess WT-like growth properties in 293 and 911 cells.

To mark the genome before deleting the *pVI* gene, we introduced a mCherry expression cassette (Che) using the Flp/FRT recombination system with pBA5-FRT as an acceptor and pO6-A5-mCherry as donor plasmid leading to pBA5-Che. The correct insertion of the marker gene was checked by restriction digest and verified by sequencing the insertion sites. We deleted the *pVI* gene from the pBA5-FRT and inserted a zeocin resistance cassette by homologous recombination in E. coli SW102 cells. For this approach the selection marker was flanked on both ends with sequences homologous to those in the region exactly up and down-stream of the *pVI* gene.

We verified the replacement of *pVI* to a selection marker, leading to pBA5-ΔpVI-Che, by fragment length polymorphism, comparing the original pBA5-FRT BAC and the pBA5-FRT-Che BAC (Figure 25). Finally, the constructs were verified by Sanger sequencing at the site of the mutagenesis (see chapter 3.2.9).

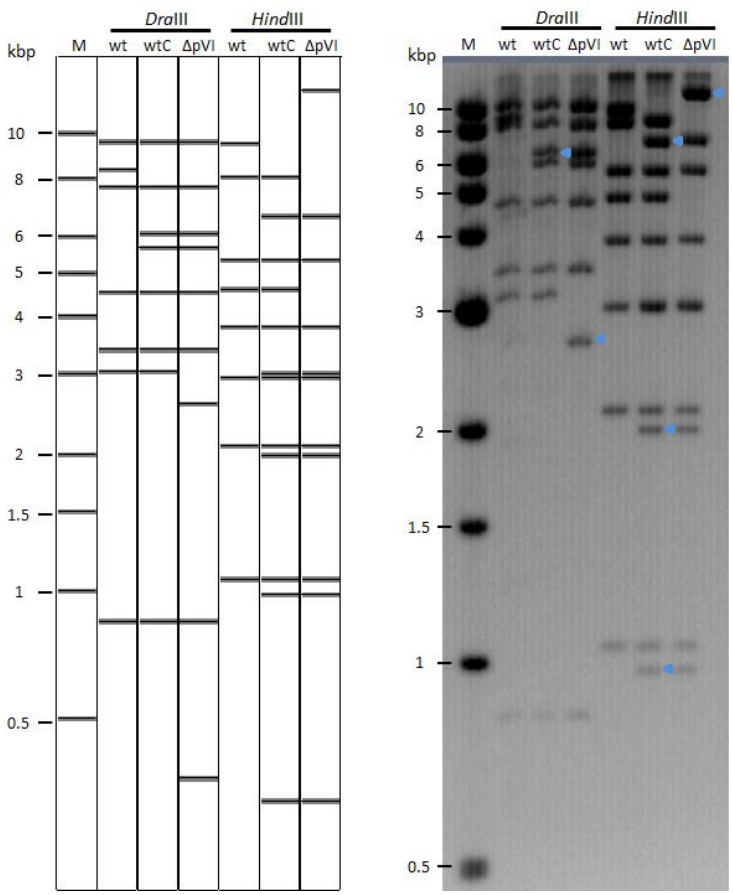


Figure 25: Construction of a genetically marked pVI deletion mutant of Ad5 genome by BAC technology.

Specific changes in the restriction pattern after insertion of the mCherry marker and deletion of the pVI gene were tested by restriction analysis. BAC DNA of Ad5-WT (wt or pBA5-FRT), pBA5-Che (wtC), and pBA5-ΔpVI-Che (ΔpVI) were digested with *Dra*III and *Hind*III (right panel). E.g. cleavage of Ad5-WT with *Dra*III resulted in fragments of 9.5, 8.3, 7.7, 4.5, 3.4, 3 and 0.85 kbp. Flip-in reaction of Che to pBA5-Che resulted in the loss of the fragment at 8.3 kbp but addition of 6 and 5.7 kbp. Deletion and replacement of pVI gene leaded to the addition of the fragments of 2.6 and 0.3 kbp (the last one is not visible anymore) and in the deletion of 3 kbp (additional fragments are indicated with blue arrowheads). These results were further confirmed by *Hind*III restriction. Here, the loss of fragment 9.4 kbp and the addition of fragments 6.6, 2.9, 1.9, 1 and 0.2 and 0.07 kbp (latter two were not visible) demonstrated the difference between wt and wtC. Whereas the difference between wtC and ΔpVI could be displayed by the addition of fragment at 12.5 kbp and loss of fragment at 4.6 kbp.

In addition to the mutated pBA5-ΔpVI-Che BAC, we also constructed a corresponding AAV replicon. For that purpose, we replaced the pAV1-GLuc-Hyg replicon vector (GLuc ORF) with the Ad5 pVI ORF, thus in direct control of the p40 promoter. This construct was named pAV1-pVI-Hyg (see chapter 3.2).

4.3.1.2. Assessment of the AAV replicon system for trans-complementation

In order to trans-complement the deleted pVI protein of the mutated Ad5 BAC during virus rescue, sub-confluent 293A cells were transfected in a 6-well plate at first with either pAV1-pVI-Hyg or with pAV1-GLuc-Hyg. The replicon vector with GLuc served here for two purposes: as a control for potential inhibitory effects of the AAV replication, and to exclude the negative effects of the transfection process on the reconstitution of Ad5. Three days after replicon transfection, the cells were re-transfected with either the mutated pBA5-ΔpVI-Che or with the wt pBA5-Che BACs. Then the cells were harvested five days after the BAC transfection according to the standard rescue protocol for the Ad5 BACs. We subsequently re-suspended the harvested cells in normal media and lysed them by three freeze and thaw cycles in order to release reconstituted viruses. Finally, the lysates were cleared from cell debris by centrifugation. A new batch of 293A cells was transfected with either pAV1-pVI-Hyg or with pAV1-GLuc-Hyg replicons again. 72 hours after transfection, this batch was treated with the cleared lysates of the rescue cultures and viral plaque formation was examined in the course of the next seven days in order to quantify the efficiency of the virus rescue.

Due to the high variability of BAC transfection efficiency, this experiment was repeated six times. All results are summarized in Table 4.3.1.2.1. We did not observe any trans-complementation of the pVI deletion for the pVI gene deleted pBA5-ΔpVI-Che (ΔpVI-BAC) by the pAV1-pVI-Hyg replicon (pVI-R) and even the wt BAC, pBA5-Che, failed to be rescued in replicon transfected cells. In half of the experiments, a rescue of the wt BAC was observed despite the presence of pVI-R transfection but not in presence of pAV-GLuc-Hyg (GLuc-R), independent of the amount of the AAV replicon vector DNA transfected into the cells.

Growing of the rescued wt Ad5 BAC, pBA5-Che, happened independently of the presence of pre-transfected or non-pre-transfected cells. Altogether, the number of plaques varied significantly between experiments.

Table 4.3.1.2.1: Rescue after trans-complementing pVI gene in mutated Ad5 BAC.

Transfer of rescued virus with cells:	WT BAC	WT BAC + pVI-R	WT BAC + GLuc-R	pBA5-ΔpVI-Che	pBA5-ΔpVI-Che + pVI-R	pBA5-ΔpVI-Che + GLuc-R
non-pre-transfected	16.7 ± 15.9*	0.8 ± 1	0	0	0	0
pre-transfected with GLuc Replicon	12.8 ± 19.8	1.0 ± 2	0	0	0	0
pre-transfected with pVI Replicon	12.2 ± 15.1	0.8 ± 1.2	0	0	0	0

*This table shows the mean and standard deviation of counted plaques from three independent experiments.

To exclude an inhibitory effect by pre-transfection of the AAV replicon vectors on the virus reconstitution process, a recombinant AAV2 vector was packaged with pAV1-pVI-Hyg (named rAAV2-R-pVI). Here, 293A cells were transduced with the rAAV-R-pVI, containing the essential *pVI* gene for *trans*-complementation using 500 rAAV p/c. 5 days later, cell lysates were prepared and, to test virus rescue, the lysates of wt BAC and the mutated BAC were transferred to two groups of cells, one treated and one not treated with rAAV2-R-pVI using the same schedule as for the BAC transfection.

Unfortunately, the pVI trans-complementation did not function upon transduction of the pVI containing rAAV replicon vector. The influence of the recombinant AAV replicon vector (rAAV2-R-pVI) transduction after transfection of the pBA5-ΔpVI-Che BAC vector was the same as observed after transfection of the AAV replicon vector and resulted in no viral progeny (Table 4.3.1.2.2) in three independent experiments.

In contrast, we were able to rescue the wt BAC Ad5-Che independent of the presence of the recombinant AAV replicon vector. We even had to dilute the viral lysate and repeat the second part of the latter two experiments because of very high plaque numbers. Since trans-complementing pVI of the mutated Ad5 BAC failed, we did not aim to investigate the reason for the high amount of plaques.

Table 4.3.1.2.2: Rescue after trans-complementing pVI with rAAV2-R-pVI in mutated Ad5 BAC.

	Ad5-Che +rAAV2-R-pVI			pBA5-ΔpVI-Che+rAAV2-R-pVI		
Transfer of rescued virus with cells:	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
non-pre-transduced	152*	1880	1270	0	0	0
pre-transduced with rAAV2-R-pVI	158	640	1860	0	0	0

*counted plaques.

To test a potential effect of the timing of the AAV replicon transduction, the transfection of the BAC DNAs was performed one day before transduction, on the same day, and two days

after the transduction of the 293A cells. As before, plaques were counted after seven days. Independent of the transduction time point, the trans-complementation of pVI was not successful after transducing 293A cells with the rAAV2-R-pVI vector. Nevertheless, independent of timing, the inhibitory effect of the replicon transfection on Ad-WT rescue was not observed by transduction of the replicons as rAAV particle.

Overall, independent of the AAV replicon delivery to the cells, we were not able to observe trans-complementation of the pVI gene of the Ad deletion mutant. Transfection of the AAV replicon vector showed a toxic effect on virus rescue even independently of the pVI gene. This toxic effect was not observed if the AAV replicon vector was delivered packaged in a recombinant AAV2 vector. Nevertheless, the genetic defect of the pVI deletion in the viral mutant could not be rescued in the course of this work.

4.3.2. AAV replicon system for diagnostic approaches

For patients suffering from a drug-resistant HSV infection, a timely decision on switching to an effective antiviral therapy plays an important role in therapy success. Successful antiviral therapy of herpesvirus infections in immunocompromised patients, such as transplant recipients, newborns, leukemia patients, and patients with AIDS, are dependent on availability of phenotypic or genotypic drug resistance tests. The prevention of aggravation of the disease in these patients, by either increasing the dose of intravenous ACV administration or switching to another drug is very important. Concerning phenotypic methods, the “gold standard” for drug resistance testing for HSV isolates is still the plaque reduction assay (PRA), which is very labor and time intensive [123]. With tailoring the AAV replicon system as a quantitative reporter of viral growth in an antiviral drug resistance test, we aimed to establish a fast, reliable, easy to handle alternative to the PRA.

The major potential to analyze the infectivity of herpes- or adenovirus samples relies on the ability of the AAV replicon vector to express a readily quantifiable reporter gene product upon infection. This reporter gene can be detected directly, in contrast to the indirect assay, which is needed to test the infectivity itself. At the same time, the absence of the reporter signal must reliably indicate the absence of infection or virus spread and both the positive and the negative results need to be highly quantitatively reproducible.

4.3.2.1. Establishment of the AAV replicon-based drug resistance test for HSV-1 based on the LE2D8 cell line

For testing the general ability of the AAV replicon system to respond to a drug treatment, we first examined the impact of the widely used drug ACV to an ACV sensitive HSV-1 laboratory strain by the induction of the replicon encoded reporter gene. Important to notice at this point is that ACV is highly specifically inhibiting HSV DNA metabolism [96]. Therefore, an influence of ACV on the replication of the AAV based replicon system itself is not to be expected, since the AAV genome replication is entirely dependent on host DNA polymerases [253].

For a reliable diagnostic assay, reproducibility of the measurements is very important. To proof this, the infection of the AAV replicon transformed stable cell line LE2D8 was chosen for the assay. To this end, LE2D8 cells were propagated in presence of hygromycin selection and frozen in a large batch with the aim of using a freshly thawed LE2D8 each time to avoid signal loss due to potential instabilities during passaging. Hence, freshly thawed LE2D8 cells were seeded after one passage on 96-well plates and infected with different doses of WT HSV-1. The doses ranged from 0.1 to 0.001 MOI in order to test the induction of the replicon encoded expression of GLuc at different time points. We aimed to identify the lowest viral dose still inducing a significant reporter signal within a reasonably short time after infection.

Infection of LE2D8 cells with HSV-1 at MOI 0.1 showed a constant induction of the bioluminescence signal of 97- to 125-fold over a period of 2 – 6 days. In contrast we measured no induction of infection at MOI 0.001 at day 2 to day 4, and an induction of 21-fold after 6 days post infection. The bioluminescence signals after infection at MOI 0.005 to MOI 0.075 showed constantly increasing values, indicating an increase of viral spread over time.

To test the effect of ACV on HSV-1 viral replication, we chose infection at MOI 0.035 with an induction of the bioluminescence signal by 55-fold after 3 days. Using this low initial viral dose ensures that the final signal strength will be a result of significant virus spread, providing ideal conditions for the inhibitors of the virus replication to affect the final replicon signal. For testing the effect of the ACV treatment on the induction of the AAV replicon in LE2D8 after HSV-1 infection, the cells were seeded as described above and treated with ACV at concentrations ranging from 0.22 to 426 µM or kept non-treated directly after seeding. 4 h after ACV treatment the cells were infected with HSV-1 at MOI 0.035 or kept non-infected. 48 h p.i. the bioluminescence was measured in the supernatants of infected- and non-infected

cells. The bioluminescent signals of ACV-treated or n.t. but infected cells were compared to non-treated and non-infected cells and the induction was calculated for each individual reaction. Then, the induction of GLuc expression of non-treated cells infected with HSV-1 was defined as 100% and the induction of the ACV treated cells was normalized to the induction observed non-treated cells and expressed as % non-treated. (Figure 26).

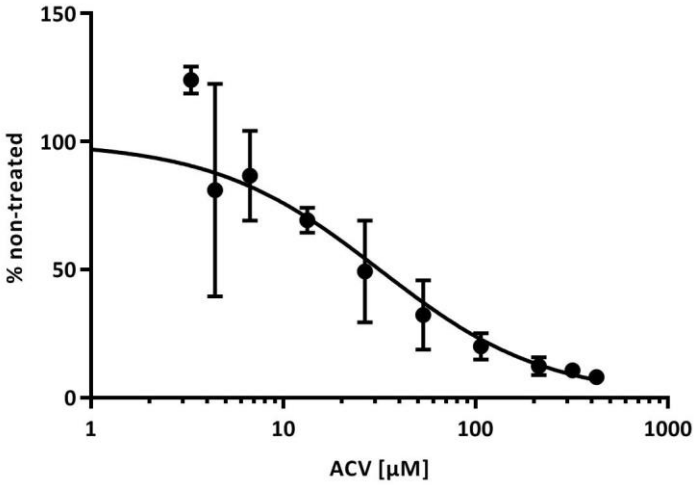


Figure 26: ACV dose-response to HSV-1 replication measured by the Replicon assay.
LE2D8 stable AAV replicon cell line was treated with indicated concentrations of ACV or kept non-treated (n.t.) and infected with HSV-1 at a MOI of 0.035 or mock infected. 48 h p.i. Supernatants were collected and analyzed. GLuc induction values of infected cells were compared to non-infected cells. The fold activity of the n.t. cells was set to 100 % and activities of ACV treated cells was depicted as % of non-treated values. Data from three independent experiments are shown; the error-bars represent means ± SD;

In summary, we observed a clear decrease of HSV-1 induced reporter gene expression upon treatment with increasing concentrations of ACV. The half maximal inhibitory dose (IC_{50}) to reduce the HSV-1 infection-dependent induction of GLuc expression was calculated to be 32 µM. This value is much higher than IC_{50}s described in the literature: depending on the assay conditions and the cell line in use the half inhibitory concentration of ACV should not exceed 8.9 µM [289].

Since ACV treatment clearly inhibited the replicon induction by an ACV sensitive strain, we wanted to test the replicon response of a drug-resistant HSV-1 strain in the presence of ACV. To this end, the LE2D8 cells were seeded and treated as above. Then, the cells were either infected with the WT laboratory strain HSV-1 (ACV sens) or with a known ACV resistant clinical isolate HSV-1 strain (ACV res) at a MOI of 0.035. ACV res is used as resistant control strain for

phenotypic resistance tests at the Clinical Virology Laboratory of the Max von Pettenkofer-Institute. Experiment were performed as described above (Figure 27).

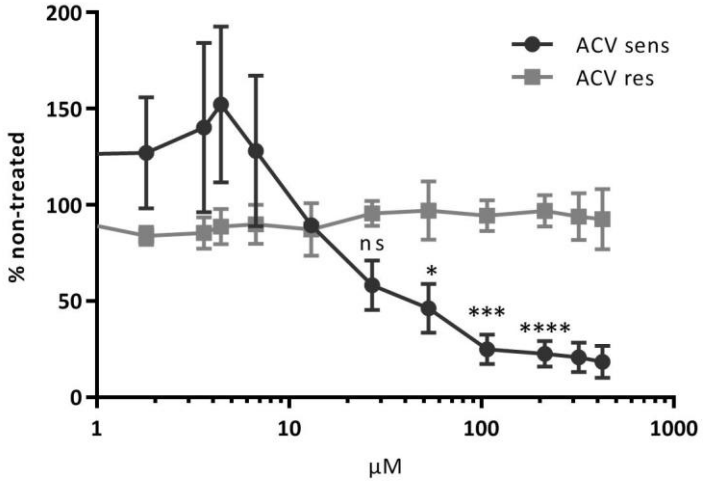


Figure 27: HSV-1 resistance to ACV can be evaluated with LE2D8 cells.
The LE2D8 cells were treated with indicated concentrations of ACV and infected with either the ACV sensitive WT HSV-1 strain (ACV sens) or with the isolated ACV resistant HSV-1 strain (ACV res). After 48 h p.i. Supernatants were collected and bioluminescence assay of GLuc activity was performed. Depicted is the induction of the bioluminescence signal in the ACV treated cell relative to non-treated cells (100%). Data from three independent experiments are shown; the error-bars represent means ± SD;

The WT strain in these experiments was inhibited at a higher IC_{50} of 74 µM by ACV compared to the last experiment. Nevertheless, the ACV res strain induced the same replicon response in presence of all tested concentrations of ACV. The ACV res strain could therefore be clearly distinguished from the ACV sens strain starting at an ACV concentration of 53 µM. Interestingly, the replicon response after ACV sens infection is even increasing after treatment at lower inhibitor concentrations. It seems that the AAV replicon response has an advantage if the inducing virus is not fully inhibited. The prolonged survival of the infected cells seems to allow a higher marker expression after AAV replicon induction.

To verify that the differences in the replicon response observed under ACV treatment were indeed reflecting differences in HSV-1 replication, we tested the HSV-1 production after infection of LE2D8 cells with either the WT laboratory strain HSV-1 (ACV sens) and the ACV resistant HSV-1 strain (ACV res) by endpoint dilution assay. LE2D8 cells were seeded and treated with ACV as above. Supernatants were collected 48 h p.i. and the infectious HSV-1

loads in each supernatant sample were determined by limiting dilution (see chapter 3.5.5). The result of one representative experiment is depicted in Figure 28.

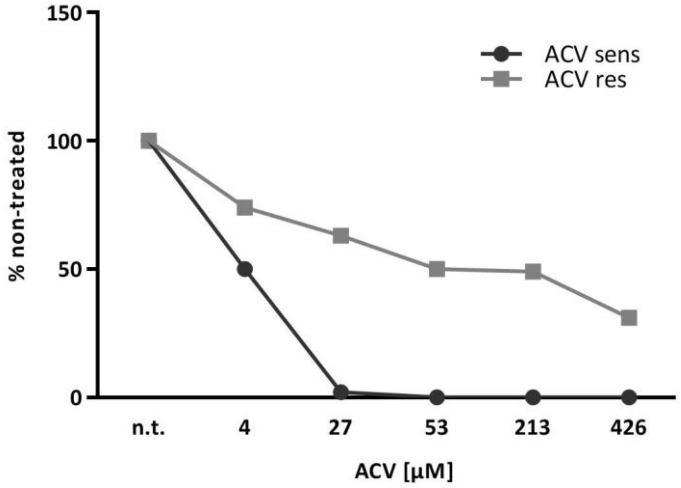


Figure 28: The production of infectious particles after infection of LE2D8 cells in the presence of ACV was dependent on the ACV sensitivity of the HSV-1 strain.
The LE2D8 cells were infected with either the ACV sensitive WT HSV-1 strain (ACV sens) or with the isolated ACV resistant HSV-1 strain (ACV res) using MOI of 0.035. Treatment with indicated concentrations of ACV (4.4 - 426 µM) followed. 48 h p.i. Supernatants were collected, and endpoint dilution-assays performed. TCDI50 values per ml were calculated, depicted are normalized values in % of the virus production in n.t. cells.

In infected cells, a treatment with 4.4 µM ACV resulted in more than 50% reduction of WT HSV-1 virus production compared to non-treated cells. In contrast, the resistant HSV-1 strain showed a reduction of 50% in infectious virus particle production only at a concentration of 53 µM ACV. Generally, the assay on virus production appeared to be more sensitive to ACV treatment compared to the replicon response in stable transfected cells.

All HSV-helper functions for AAV replication are expressed early after infection while release of virus particles is the last event in the HSV-1 life cycle. Apparently, infection of the cell and early gene expression, naturally required for the production of infectious particles, is sufficient to induce replicon response after the ACV targeted DNA replication. Thereby, the replicon assay detects the difference in virus spread whereas the end point dilution assay shows the virus production in infected cells. However, both assays were clearly revealing the difference in the ACV resistance of the two HSV-1 strains in our experiments, with the replicon-based response yielding more reliable results.

4.3.2.2. Resistance test of clinical HSV-1 isolates based on the replicon response in LE2D8 cells

To be useful in everyday practice, a resistance test should be robust enough to tolerate differences in probe quality, which are inherent in-patient derived probes. The above tests were performed with well characterized HSV-1 strains using quantified virus stocks. To challenge the robustness of the newly developed replicon-based resistance test, we selected seven ACV resistant and seven ACV sensitive HSV-1 strains. All strains were obtained by virus isolation directly from clinical samples during daily praxis of Virological Diagnostic Laboratory of the Institute of Virology of the University Medical Center Freiburg. The virus isolates were propagated on Vero cells according to the standard HSV-1 isolation protocol until the primary cultures showed 80-100% CPE. Afterwards, according to the normal diagnostic protocol, the supernatants were collected, aliquoted and stored at -80°C. All isolates used in this test were typed by indirect immunofluorescence assay and found to be HSV-1. Their ACV resistance was diagnosed using plaque reduction assay (PRA). LE2D8 cells with a defined low passage number were seeded on 96-well plates and then either treated with 0.8, 4, 20, 100 and 500 µM ACV in order to produce a dilution series used in the standard PRA or left untreated. 6 h later the cultures were infected with the HSV-1 culture supernatants at dilutions of 1:10, 1:100 and 1:1000. 2 days after infection the bioluminescence was measured, and the relative induction of the AAV replicon encoded reporter was calculated as before (see 4.3.2.1).

Table 4.3.2.2.1 summarizes the relative induction compared to non-infected LE2D8 cells and shows the bioluminescence signals of ACV resistant and ACV sensitive clinical HSV-1 isolates tested with a dilution of 1:10, 48 h after infection of the LE2D8 cells.

Table 4.3.2.2.1: Resistance test of clinical HSV-1 isolates with LE2D8 cells.

ACV sens	# 824670	# 842369	#842913	#854014	# 838500	# 847890	# 848759
n.t.	79.8*	123.5	51.6	48.3	36.7	72.3	51.3
0,8 µM	173.1	151.5	97.2	76.1	57.8	129.7	76.7
4 µM	97.4	59.1	68.7	54.3	69.3	83.3	71.4
20 µM	25.5	10.9	17.4	17.5	22.8	14.9	28.8
100 µM	13.6	4.4	5.0	5.3	7.4	4.3	5.7
500 µM	12.3	3.4	3.9	3.7	3.5	3.8	4.2

*fold induction of RLU values from infected compared to non-infected LE2D8 cells.

ACV res	# 852044	#846206	#861747	# 845708	# 854437	# 860929	# 845531
n.t.	196.1	81.0	34.0	44.3	42.3	16.3	63.3
0,8 µM	204.5	93.3	30.3	47.0	40.0	22.8	44.5
4 µM	198.1	96.1	35.9	68.6	51.0	24.3	54.2
20 µM	187.7	119.1	21.3	85.8	57.4	19.9	47.2
100 µM	311.8	136.1	27.3	68.0	42.4	17.0	101.2
500 µM	426.2	37.0	18.9	21.7	31.8	13.5	133.4

The cumulative results obtained by the infection with 1:10 diluted supernatants are depicted in Figure 29.

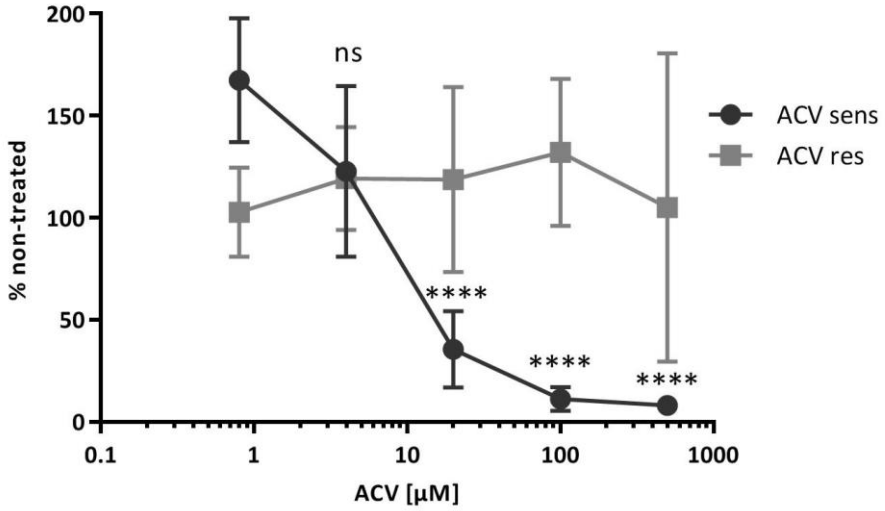


Figure 29: LE2D8 resistance test in the diagnostic context.
LE2D8 was treated with indicated concentrations of ACV and infected with 14 different patient derived HSV-1 isolates using a 1:10 dilution of propagated viruses. Supernatants were collected 48 h p.i. and GLuc assay was performed. Fold induction of treated compared to non-treated cells was calculated and then the fold activity of the non-treated cells was set to 100% and the activity of the ACV treated cells was depicted as % of the non-treated values (****: $p < 0.0001$, ns: $p > 0.05$, Two-Way-ANOVA, depicted are means ± SD).

At a 1:10 dilution of the viral isolates, the bioluminescence signal induced by the infection of the non-treated LE2D8 cells was in average 66-fold for sensitive strains and 68-fold for resistant strains, which indicated the robustness of the assay after accounting the passage number of the stable cell line. In general, the GLuc induction of all sensitive HSV-1 isolates decreased with increasing amounts of ACV concentrations. In sharp contrast to the sensitive HSV-1 isolates, the resistant HSV-1 isolates showed a different pattern. The IC_{50} of ACV for all sensitive HSV-1 isolates was 25 µM whereas the mean IC_{50} value for the resistant HSV-1 isolates was not obtainable due to the lack of the useful regression curve for many strains. The resistant viral isolates induced a similar replicon response than the non-treated cells in the presence of almost all ACV concentration. Two isolates showed no reduction at any concentration, but the replicon response even increased up to over 200% compared to n.t. values. This increase of the replicon response after treatment of the cells with suboptimal inhibitory concentration of ACV was also evident after infection with ACV sensitive strains demonstrated as a peak at 0.4 µM in the cumulative curve. In summary, detection of ACV resistance for all HSV-1 strains could be indicated by the sustained replicon response at high

ACV concentration after 2 days of the assay start. In comparison the PRA took 5 to 7 days until results could be evaluated.

4.3.2.3. Establishment of an AAV replicon-based drug resistance test for HSV-based rAAV transduction

Considering that the AAV replicon packaged in recombinant viral particles, in comparison with the stable AAV replicon vector carrying cell line LE2D8, was able to be induced not only by HSV-1 but also HSV-2 we evaluated the applicability of rAAV2-REP-GLuc for drug-resistance testing. We also hoped that the transduction would allow us to find the optimal cell line for the assay and that this would lead to IC_{50} values mirroring the results from PRA.

Therefore, we choose Vero cells which were efficiently transduced by rAAV (see chapter 4.2.4.1). First, we wanted to test the replicon response upon rAAV2-REP-GLuc transduction using ACV-sensitive standard HSV-1 and HSV-2 strains. For that purpose, Vero cells were transduced with 600 p/c of rAAV2-REP-GLuc directly after seeding 3 x 10^4 cells/well in 96-well plate. The transduced cells were treated with 0.8, 4, 20, 100 and 500 µM ACV or left untreated right after seeding. 6 h later they were infected with either HSV-1 or HSV-2 at MOI of 0.035. After 48 h p.i., we measured the induction of the replicon encoded GLuc expression bioluminescence assays (Figure 30).

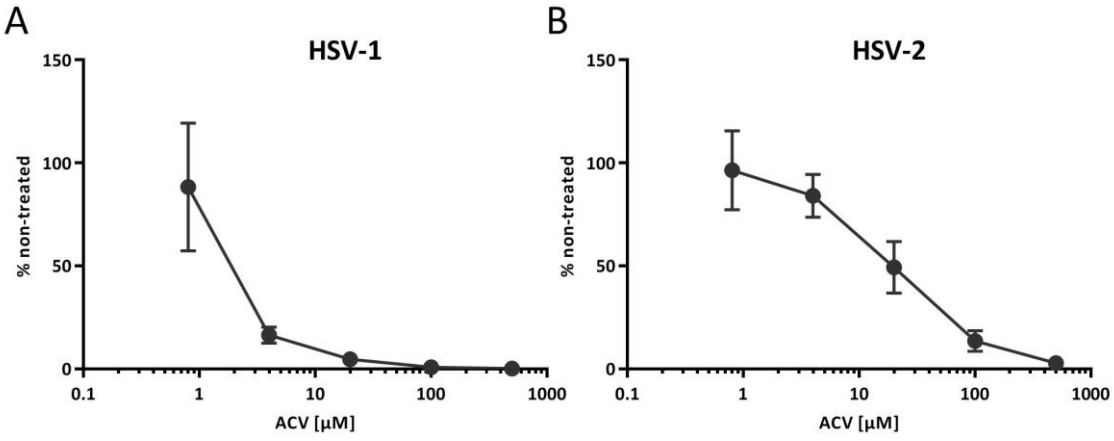


Figure 30: ACV susceptibility testing after HSV-1 and HSV-2 infection with rAAV replicon.
Recombinant AAV replicon vector transduced Vero cells were treated at indicated concentrations of ACV and infected with (A) HSV-1 and (B) HSV-2 at a MOI of 0.035. Supernatants were collected after 48 h p.i. and induction of the replicon encoded reporter gene in presence of ACV is depicted as % of the response of the non-treated cells. Data from three independent experiments are shown; the error-bars represent means ± SD;

The replicon inductions of the ACV treated samples were normalized to the non-treated samples, which were set to 100%. In general, both HSV-1 and HSV-2 induced a measurable replicon response upon infection, which decreased by increasing amounts of ACV. The HSV-1 induced replicon response showed an IC_{50} of 1.87 µM (Figure 30A), whereas for HSV-2 (Figure 30B) we observed an IC_{50} of 19.1 µM towards ACV. The results of both viruses indicate a more sensitive assay system if transduction of the AAV Replicon vector is used for delivery. Furthermore, as observed before, HSV-1 was more sensitive to ACV treatment than HSV-2.

Summarizing these results, transduction by AAV particles provides a useful tool to test ACV resistance with the AAV replicon system. The calculated IC_{50} values for HSV-1 correspond to the published in vitro inhibitory concentrations of ACV tested by PRA. The IC_{50} value for HSV-2 in contrast is 2-fold higher compared to the concentrations published in the literature.

4.3.2.4. ACV resistance test of clinical HSV isolates using recombinant AAV-based replicon assay

For setting up a transduced replicon-based resistance test for clinical isolates, we reconsidered some conditions based on the preliminary test described before. We decided to increase the rAAV2-REP-GLuc particles from 600 p/c to 1500 p/c to gain higher induction levels after HSV-2 induction. We used the set of 10 clinical HSV-1 isolates, which we had evaluated with the LE2D8 cell line-based tests before. In addition, we selected 4 sensitive and 3 resistant HSV-2 isolates, which were also derived from the Virological Diagnostic Unit in Freiburg and were treated and diagnosed the same way as the HSV-1 isolates. Vero cells were seeded in a 96-well plate, transduced with 1500 p/c of rAAV2-REP-GLuc and incubated overnight. We decided to prolong the incubation after transduction to increase the efficiency of the replicon delivery, as longer incubation after transduction resulted in higher transgene expression in the pilot assays (see chapter 4.2.4). On the next day, the transduced cells were treated either with 0.8, 4, 20, 100, 500 µM ACV or left untreated and infected right after the ACV treatment with HSV isolate, containing culture supernatants at 1:10 and 1:100 dilution (as before for the cell line tests). The supernatants were collected 24 and 48 h p.i. Then the light emission in the supernatants were measured with a plate luminometer and analyzed as described before. The detailed fold inductions of the RLU results obtained for the HSV-1 tests are listed in Table 4.3.2.4.1 the cumulative analysis of the relative values is depicted in Figure 31.

Table 4.3.2.4.1: Resistance test of clinical HSV-1 isolates using the replicon containing rAAV particles.

ACV sens	# 842369		# 842913		# 838500		# 847890		# 848759	
	24 h p.i.	48 h p.i.	24 h p.i.	48 h p.i.	24 h p.i.	48 h	24 h p.i.	48 h p.i.	24 h p.i.	48 h
n.t.	159.4	417.9	103.8	315.4	97.4	236.0	180.2	116.7	159.5	161.4
0.8 µM	158.6	556.2	103.7	437.9	115.4	336.2	177.5	206.8	199.6	260.5
4 µM	79.7	496.1	55.2	349.0	78.7	309.4	33.0	57.7	75.9	135.0
20 µM	4.9	50.5	4.5	42.9	22.6	188.2	2.1	1.2	2.7	7.0
100 µM	1.7	3.1	2.0	3.8	1.8	7.8	1.6	0.8	1.4	1.2
500 µM	1.6	3.3	2.0	4.0	1.6	2.7	1.7	0.7	1.5	1.2

ACV res	# 846206		# 861747		# 845708		# 854437		# 860929	
	24 h p.i.	48 h p.i.	24 h p.i.	48 h p.i.	24 h p.i.	48 h p.i.	24 h p.i.	48 h p.i.	24 h p.i.	48 h p.i.
n.t.	129.5	97.2	16.9	200.8	111.7	92.3	57.1	75.7	22.8	113.7
0.8 µM	145.5	104.7	19.2	235.6	141.8	117.9	48.1	84.5	33.9	171.1
4 µM	162.7	127.8	18.5	218.3	149.2	129.6	44.5	76.4	28.5	166.8
20 µM	160.4	126.3	17.1	212.7	173.3	159.0	28.6	56.5	21.1	157.2
100 µM	180.2	152.4	15.6	198.0	122.1	144.3	7.6	23.4	19.9	153.3
500 µM	107.4	103.8	8.8	81.6	18.8	42.7	44.5	64.3	8.7	24.9

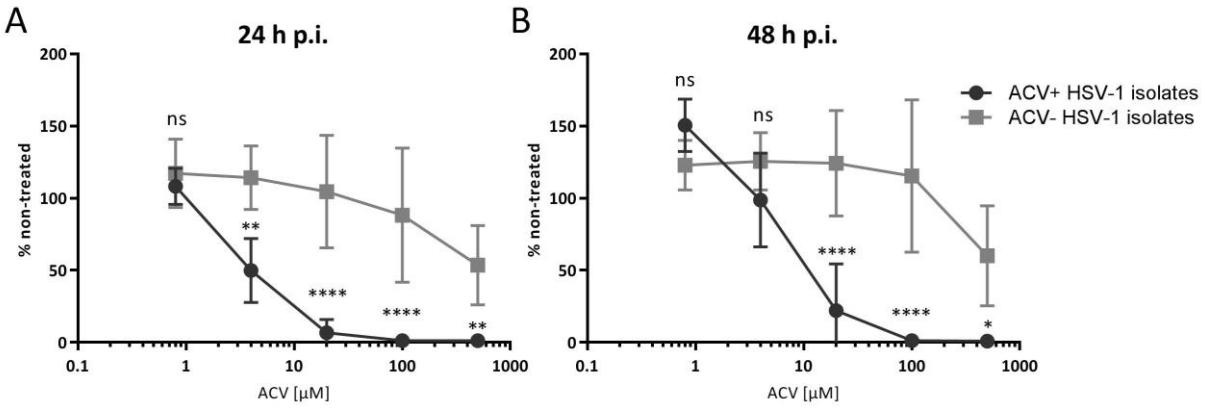


Figure 31: Replicon containing rAAV particles for resistance testing of HSV-1.
Vero cells were seeded and transduced with rAAV2-REP-GLuc using 1500 p/c. After incubation time of 24 h the cells were treated with indicated concentrations of ACV and infected with 10 different patient derived HSV-1 isolates using a 1:10 dilution of propagated viruses. Supernatants were collected after 24 and 48 h p.i. and GLuc assay was performed. Fold induction of treated compared to non-treated cells was calculated and then the fold activity of the non-treated cells was set to 100 % and the activities of the ACV treated cells were depicted as % of the non-treated values. (****: $p < 0.0001$, ns: $p > 0.05$, Two-Way-ANOVA, depicted are means ± SD).

After an induction time of 24 h, the IC_{50} of all sensitive strains of HSV-1 towards ACV was 4.6 µM and increased to 14.6 µM after an induction time of 48 h p.i. Contrary to that, the resistant HSV-1 strains showed IC_{50} values of 633 µM after 24 h and 1197 µM after 48 h. A significant differentiation between sensitive and resistant HSV-1 strains could therefore be made earlier than with the LE2D8 cell line and even showed a higher sensitivity towards ACV. Interestingly, the sensitivity of the assay using the rAAV2-REP-GLuc replicon system decreased after 48 h. It

is possible, however, that this effect is caused by prolonged spreading of the virus. The increased sensitivity of the system 24 h p.i. was also observed after infection with resistant strains.

In contrast to the LE2D8 cell line, HSV-2 is able to strongly induce the AAV replicon after transduction of the recombinant particle. Therefore, we tested the AAV replicon containing rAAV particles for the applicability of testing resistance in HSV-2 clinical isolates. The setup of the experiment was the same as for testing HSV-1 isolates. Altogether four sensitive and two resistant HSV-2 viral isolates that were previously tested could be selected for AAV replicon-based differentiation based on their resistance to ACV within 48 h. The detailed fold inductions of the RLU results obtained for the HSV-2 with 1:10 diluted supernatants are listed in Table 4.3.2.4.2, the cumulative analysis of the relative values is depicted in Figure 32.

Table 4.3.2.4.2: Resistance test of clinical HSV-2 isolates with rAAV particles.

ACV sens	# 17-168	# 17-604	# 17-742	# 18-650
0.8 µM	183.6	108.7	132.8	98.7
4 µM	31.9	31.9	42.6	33.9
20 µM	15.1	14.4	20.2	5.4
100 µM	2.9	2.8	14.4	2.0
500 µM	1.3	2.5	12.1	1.0

ACV res	# 18-161	# 18-553
0.8 µM	126.4	130.5
4 µM	92.1	104.2
20 µM	62.3	67.6
100 µM	56.5	31.5
500 µM	34.4	19.6

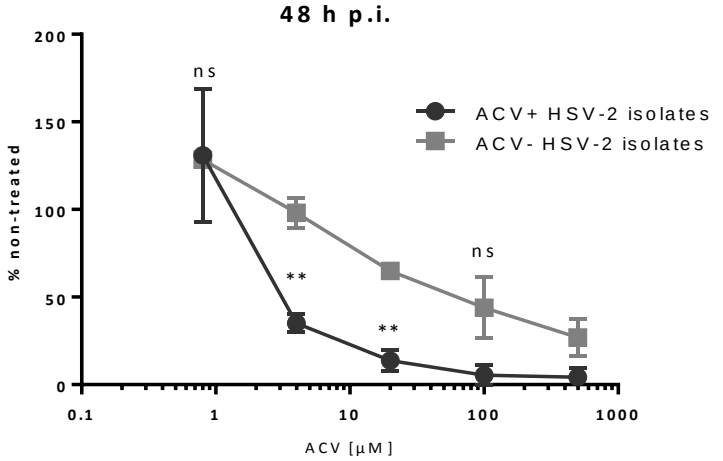


Figure 32: Replicon containing rAAV particles for resistance testing of HSV-2.
Vero cells were seeded and transduced with rAAV2-REP-GLuc using 1500 p/c. After an incubation time of 24 h, the cells were treated with indicated concentrations of ACV and infected with 6 different patient derived HSV-2 isolates using a 1:10 dilution of propagated viruses. Supernatants were collected after 48 h. and GLuc assays were performed. Fold induction of treated versus non-treated cells was calculated and then the fold activity of the non-treated cells was set to 100 % and the activities of the ACV treated cells were depicted as % of the non-treated values. (**: $p < 0.01$, ns: $p > 0.05$, Two-Way-ANOVA, depicted are means ± SD).

The results show a clear differentiation between sensitive and resistant HSV-2 isolates after 48 h. The significance was calculated for a very low number of strains and is therefore only an indication. The IC_{50} values of the sensitive strains were between 3.2 and 4 µM in contrast to the IC_{50} values of the resistant strains (130 µM and 53 µM). Therefore, we determined a cut-off at a concentration of 20 µM ACV for the differentiation between ACV resistant and sensitive HSV-2 strains.

The validity of the AAV replicon-based resistance test for HSV-2 was considerable weaker that it was observed for HSV-1. This is maybe due to lower induction levels using HSV-2 and can be optimized, for example, by further increasing the replicon load.

All in all, the AAV replicon response was able to discriminate between infections with ACV-sensitive and -resistant HSV-1 shown within one or two days and HSV-2 within two days post infection. Therefore, the transduction-based assay is now prepared for testing its applicability in retrospective and prospective studies in diagnostic settings.

4.3.3. Screening for new antivirals by the AAV replicon-based assay

Our data so far showed that the AAV replicon system is useful for testing the resistance of HSV to the known inhibitor ACV. In this setting, the replicon-based assay was robust enough to respond reliably to infection with minimally characterized clinical isolates with different infectivity. Moreover, the same replicon was inducible with HSV, Ad, and HCMV infections upon transduction into the respective permissive cell lines. All in all, these features of the AAV replicon suggest that it may be a valuable and unique tool for screening of new antivirals. The assay for drug resistance or susceptibility can be carried out in multi-well format. The measurement requires one liquid handling step and can be automated. Most importantly, the AAV replicon-based assay does not need any genetic modification on the virus or the cell line of interest. Consequently, clinical isolates and virus strains with limited laboratory adaptation can be subjected to screening approaches. This feature is especially valuable in testing low passage, not well-established herpesvirus strains, which undergoes substantial genetic changes upon laboratory passages, and genetic manipulation.

To test this possibility, we set up a pilot study using A549 cells for testing of Ad, as well as Vero cells for HSV-1 testing in a 96-well plate format. The before tested ACV was dissolved in water and diluted in culture medium before use. As the compound libraries are normally provided as highly concentrated stocks dissolved in DMSO, we first tested the effect of DMSO on the induction of the AAV replicon.

For this, Vero, A549 and HFF cells were transduced with 1000 p/c of the replicon containing recombinant AAV particle, rAAV2-REP-GLuc, after seeding. Then, infection with HSV-1 at a MOI of 0.1 followed after 6 h in parallel with the treatment of the cells with different concentrations of DMSO, ranging from 0.1 to 6%. Two days later the induction of GLuc was measured in a bioluminescence assay. The results showed that the use of DMSO of up to 1% did not influence the results significantly in all cells tested (see Figure 33).

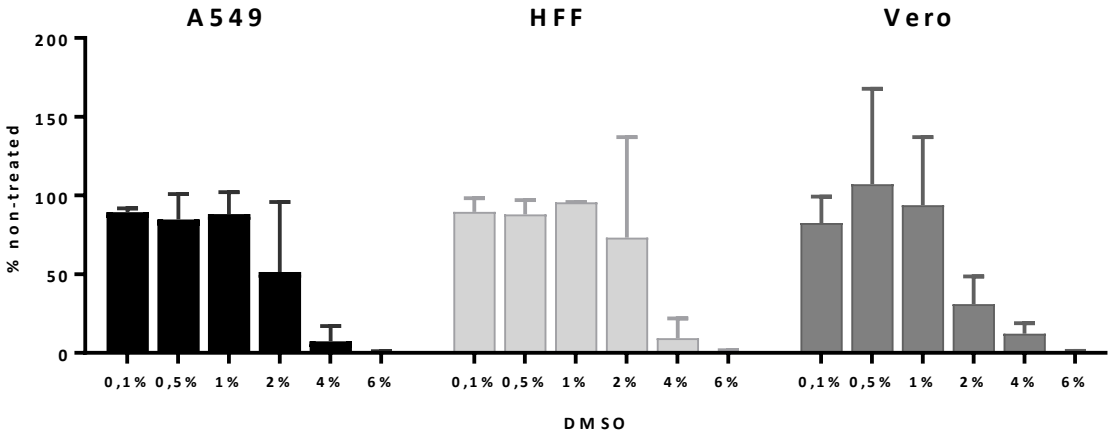


Figure 33: DMSO toxicity test of different cell lines.
HFF, Vero and A549 cells were transduced with rAAV2-REP-GLuc using 1000 p/c. Then the cells were infected with HSV-1 at a MOI of 0.1 or kept mock infected and treated additionally with different concentrations of DMSO of 0%, 0.1%, 0.5%, 1%, 2%, 4% and 6%. 48 h p.i. Supernatants were collected, GLuc induction values were analyzed and values of infected cells were compared to non-infected cells. The fold activity of the n.t. cells was set to 100% and activities of ACV treated cells were depicted as % of non-treated values.

As discussed before, there is an urgent need for new antiviral drugs against HSV-1 and against human Ad. With regard to this challenge, the next step in this study was to test the applicability of the AAV replicon test for drug discovery. For this, a set of heterocyclic small molecular kinase inhibitors with known in vitro and in vivo toxicity profiles were provided by Origenis GmbH (Martinsried, Germany).

In order to run the pilot screen, 22 potential inhibitor compounds, deriving from different series of compound families, were selected. Additionally, an apoptosis-inducing drug called staurosporine [290] was included as inhibitory control as well as a published herpesvirus inhibitor. The compounds were labeled “ORI-SIRI-01” to “ORI-SIRI-24” in the first round of experiments. In the reproducing second round they were labeled “ORI-SIRI-25” to “ORI-SIRI-48” in a randomized order to guarantee blind testing. To receive a good overview of the inhibition spectrum, both HSV-1 and Ad5 were tested for their inhibition by the different compounds. To accomplish this, rAAV1/2-REP-GLuc, a chimeric rAAV particle was produced. This particle showed almost as much transduction efficacy as rAAV2 and in addition yielded better production titers in our pre-experiments. Experiments to test the rAAV1/2-REP-GLuc demonstrated no difference compared to rAAV2-REP-GLuc (data not shown). For testing the different kinase-inhibitors, Vero and A549 cells were seeded on 96-well plates at a density of 10^4 cells pro well. To receive comparable luciferase induction values with both viruses Vero

cells were then transduced with 500 p/c of rAAV1/2-REP-GLuc whereas A549 were transduced with a lower number of 10 p/c. The transduced cells were incubated for 6 h in a final volume of 100 µl/well. The cells were treated with 4 and 40 µM of each compound in presence of 4% DMSO, which was applied by multichannel pipetting in 50 µl, resulting in a DMSO concentration of 1% in total. Known inhibitors in two concentrations were included as positive controls in order to have immediate and known controls of the experiment. For Vero cells, 80 and 2000 µM ACV were added into a volume of 50 µl. For Ad5, 80 and 800 µM of Cidofovir (CDV) were also added in a volume of 50 µl. These treatments resulted in a final concentration of 1 or 10 µM of each kinase inhibitors: 20 and 500 µM ACV, 20 and 200 µM CDV, and 1% DMSO in the respective wells. CDV inhibits Ad5 in vitro at an IC_{50} of 25 µM [291]. In addition, to generate DMSO treated cells for determining the infection induced luciferase production without any inhibition, normal medium was added to n.t. cells and 4% DMSO. Immediately after application of the inhibitors, Vero cells were infected with HSV-1 at a MOI of 0.035 and A549 cells with Ad5-WT using a MOI of 10. As established in former experiments, the GLuc activity was measured after 48 h p.i. in the supernatants. The induction of the GLuc activity was calculated by comparison of the values derived from infected and non-infected values.

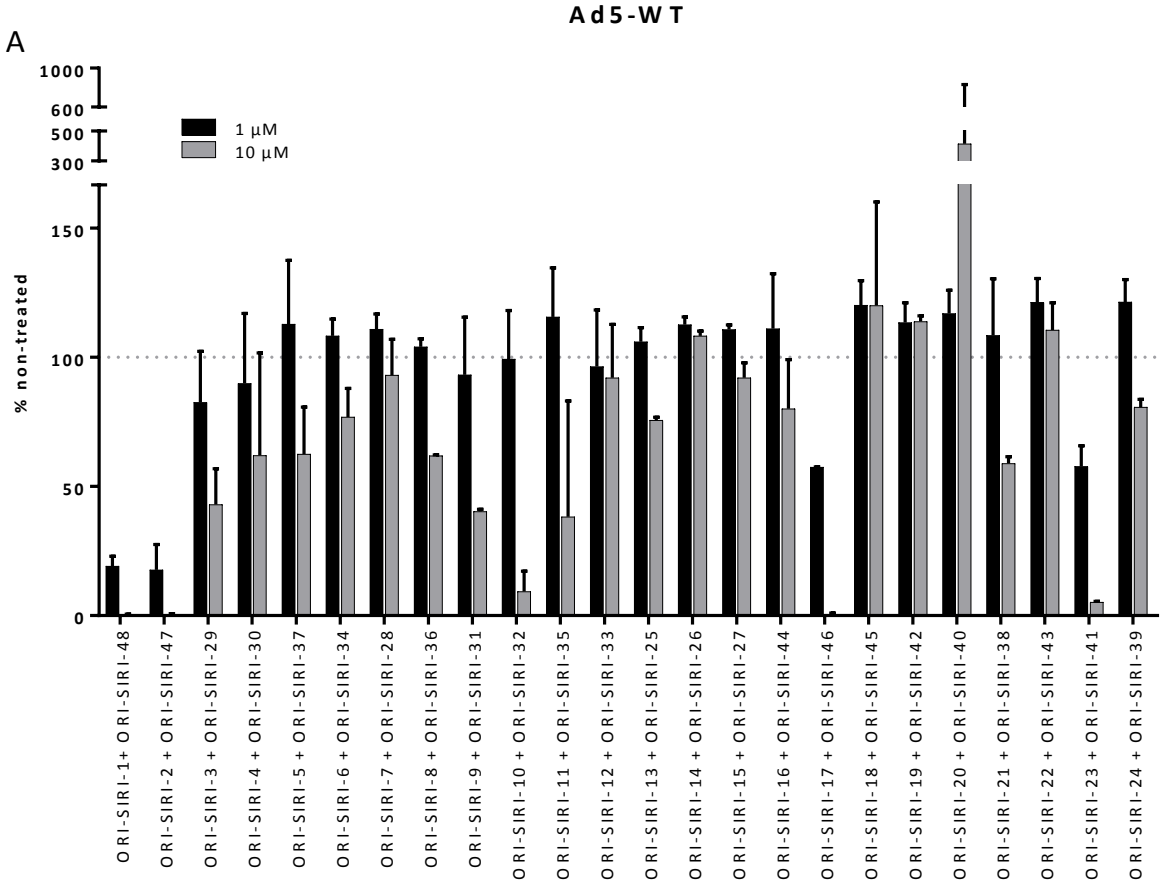
In order to compare the primary and secondary experiment, the compound specific inhibition of the GLuc induction was calculated. The GLuc induction after infection in the absence of inhibitors were defined as 100% and the induction values were calculated from the compound treated cells as % of the non-drug treated cells (Figure 34).

The results of the internal control with approved drugs showed that CDV treatment of Ad5 and ACV treatment of HSV-1 leaded to an inhibition of the signal in case of both experiments (Table 4.3.3.1). The AAV replicon screening system was capable of detecting the two control inhibitors (ORI-SIRI-1, ORI-SIRI-2 in the primary screen and ORI-SIRI-47 and ORI-SIRI-48 in the secondary screen) in the reproduced screening with either virus tested. The internal controls therefore verified the functionality of the established cell-based assay.

Since we only tested compounds at a concentration of 1 µM and 10 µM, a scientific statement concerning the dose response cannot be made. Nevertheless, results of testing Ad indicated a more reliable response comparing the two experiments. Only one compound (ORI-SIRI-20) showed a 540% higher induction after treatment with 10 µM compared to treatment with 1 µM concentration, without reproducibility in the second screen (Table 4.3.3.1). In contrast,

for HSV-1 10 different compounds showed an increase in induction (more than 20%) after treatment with higher compound concentrations whereas only half of these results were reproducible. Generally, the peak-out, defined as showing a 50% higher induction than the DMSO control, are not reproducible, since 7 out of 9 compounds showing in either virus a peak-out could not be observed in the second experiment.

Generally, for Ad more inhibitory compounds were found: Three new compounds showed at least a 50% inhibition at the higher compound concentration and two compounds even inhibited the Ad activity to over 90%. In contrast, HSV-1 inhibition to over 90% could only be observed for one compound (ORI-SIRI-17 in primary and ORI-SIRI-46 in secondary screening). This compound demonstrated promising results for both viruses. A compound exclusively inhibiting HSV-1 could not be found in this application test for inhibitor screening.



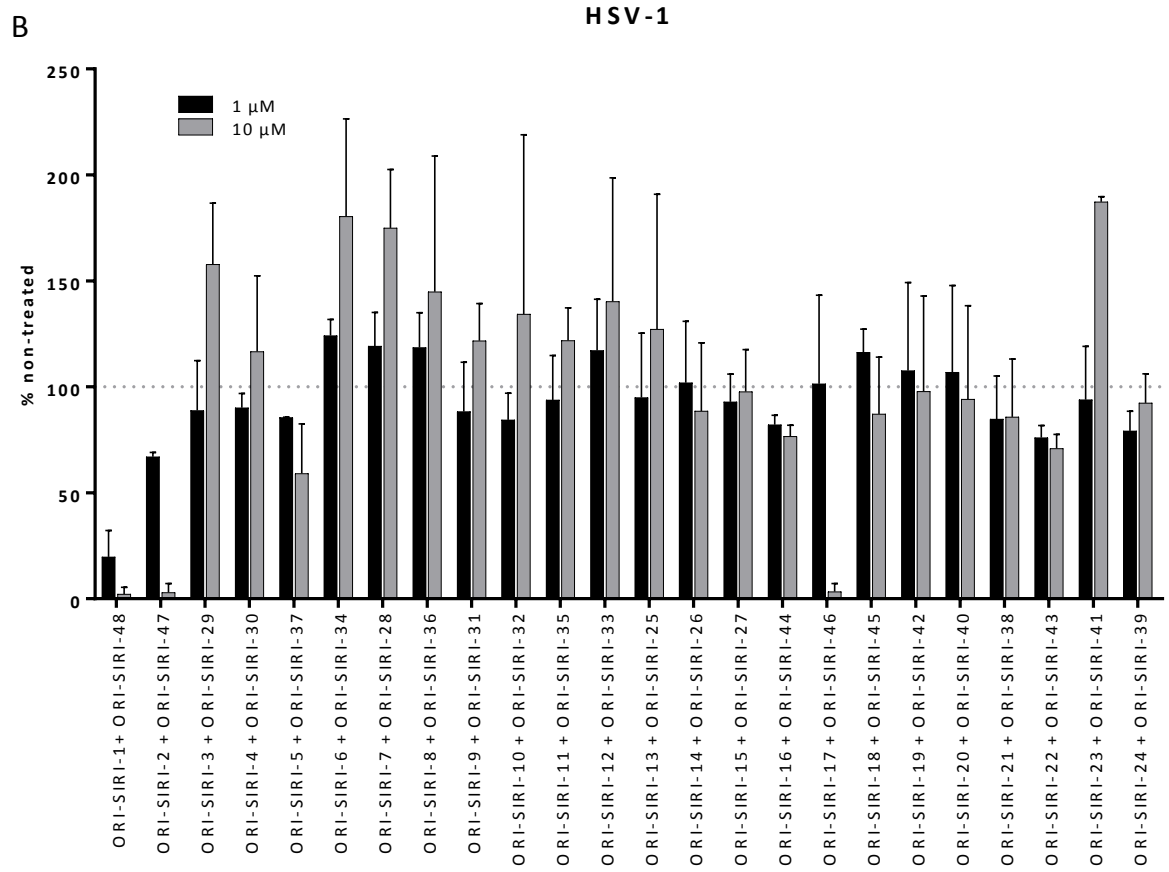


Figure 34: AAV replicon-based drug susceptibility assay for testing potential HSV-1 and Ad5 inhibitors.

Average inhibition by of the corresponding compounds (Cmpd) from the primary and secondary experiment are shown (% non-treated).

A549 cells (A) were transduced with 10 p/c of rAAV1/2-REP-GLuc replicon and infected with Ad5 -WT at a MOI of 10 whereas Vero cells (B) were transduced with 500 p/c of rAAV1/2-REP-GLuc and infected with HSV-1 at a MOI of 0.035. Addition of 1 and 10 µM of the undisclosed Cmpd followed next to no treatment (n.t.) or treatment with 1% DMSO without Cmpd. Supernatants of Vero and A549 cells were collected 48 h p.i. and fold induction GLuc were calculated by comparing RLU values of infected and non-infected cells. Inhibition of both viruses by each Cmpd was calculated by setting the induction of the GLuc expression of the non-drug treated cells as 100% and activities of treated cells were depicted as % of non-treated values.

Table 4.3.3.1: Comparison of compounds tested twice with the AAV replicon drug susceptibility test.

Ad5-WT					HSV-1				
	primary screen		secondary screen		[µM]	primary screen		secondary screen	
[µM]	Cmpd	fold induction	Cmpd	fold induction	[µM]	Cmpd	fold induction	Cmpd	fold induction
	1 % DMSO	311.0	1% DMSO	571.0		1 % DMSO	105.0	1% DMSO	32.0
20	CDV	246.0	CDV	7.7	20	ACV	8.5	ACV	2.3
200		1.0		1.0	500		1.9		1.7
1	ORI-SIRI-01	68.1	ORI-SIRI-48	95.3	1	ORI-SIRI-01	11.5	ORI-SIRI-48	9.9
10		1.5		1.3	10		4.7		1.0
1	ORI-SIRI-02	76.5	ORI-SIRI-47	62.4	1	ORI-SIRI-02	68.9	ORI-SIRI-47	22.4
10		1.9		1.4	10		6.2		1.0
1	ORI-SIRI-03	213.2	ORI-SIRI-29	551.7	1	ORI-SIRI-03	75.8	ORI-SIRI-29	33.9
10		103.2		302.2	10		144.4		56.7
1	ORI-SIRI-04	220.2	ORI-SIRI-30	623.3	1	ORI-SIRI-04	89.6	ORI-SIRI-30	30.6
10		105.4		515.1	10		96.1		45.3
1	ORI-SIRI-05	296.4	ORI-SIRI-37	744.6	1	ORI-SIRI-05	89.9	ORI-SIRI-37	27.7
10		234.5		284.0	10		79.4		14.3
1	ORI-SIRI-06	351.1	ORI-SIRI-34	592.7	1	ORI-SIRI-06	136.0	ORI-SIRI-34	38.1
10		263.5		395.5	10		223.6		47.2
1	ORI-SIRI-07	357.7	ORI-SIRI-28	610.3	1	ORI-SIRI-07	137.0	ORI-SIRI-28	34.7
10		320.0		477.0	10		204.2		49.6
1	ORI-SIRI-08	330.4	ORI-SIRI-36	581.7	1	ORI-SIRI-08	112.5	ORI-SIRI-36	41.7
10		191.7		355.5	10		104.7		60.4
1	ORI-SIRI-09	240.5	ORI-SIRI-31	622.9	1	ORI-SIRI-09	75.4	ORI-SIRI-31	33.7
10		123.6		234.6	10		140.9		35.1
1	ORI-SIRI-10	267.4	ORI-SIRI-32	643.6	1	ORI-SIRI-10	79.2	ORI-SIRI-32	30.2
10		46.4		22.4	10		78.3		61.7
1	ORI-SIRI-11	317.6	ORI-SIRI-35	737.2	1	ORI-SIRI-11	83.1	ORI-SIRI-35	34.9
10		217.5		38.3	10		116.5		42.5
1	ORI-SIRI-12	251.3	ORI-SIRI-33	639.6	1	ORI-SIRI-12	141.0	ORI-SIRI-33	32.2
10		240.9		609.6	10		190.6		32.0
1	ORI-SIRI-13	317.4	ORI-SIRI-25	628.1	1	ORI-SIRI-13	77.2	ORI-SIRI-25	37.4
10		232.9		437.5	10		86.3		54.8
1	ORI-SIRI-14	343.8	ORI-SIRI-26	655.6	1	ORI-SIRI-14	85.6	ORI-SIRI-26	39.3
10		332.9		626.4	10		69.1		35.8
1	ORI-SIRI-15	340.2	ORI-SIRI-27	640.2	1	ORI-SIRI-15	87.9	ORI-SIRI-27	32.9
10		273.5		549.9	10		88.0		35.9
1	ORI-SIRI-16	298.4	ORI-SIRI-44	720.6	1	ORI-SIRI-16	82.9	ORI-SIRI-44	27.7
10		207.4		534.8	10		76.6		26.1
1	ORI-SIRI-17	178.5	ORI-SIRI-46	329.2	1	ORI-SIRI-17	137.5	ORI-SIRI-46	23.5
10		2.6		1.6	10		6.3		1.3
1	ORI-SIRI-18	352.9	ORI-SIRI-45	737.2	1	ORI-SIRI-18	130.2	ORI-SIRI-45	34.9
10		285.2		847.9	10		111.4		22.3
1	ORI-SIRI-19	336.1	ORI-SIRI-42	679.2	1	ORI-SIRI-19	143.9	ORI-SIRI-42	25.4
10		349.1		659.4	10		136.2		21.6
1	ORI-SIRI-20	344.2	ORI-SIRI-40	704.8	1	ORI-SIRI-20	142.6	ORI-SIRI-40	25.3
10		2204.0		697.4	10		131.7		20.7
1	ORI-SIRI-21	289.3	ORI-SIRI-38	708.5	1	ORI-SIRI-21	73.9	ORI-SIRI-38	32.0
10		188.9		326.8	10		69.9		33.8
1	ORI-SIRI-22	356.8	ORI-SIRI-43	730.5	1	ORI-SIRI-22	75.5	ORI-SIRI-43	26.0
10		320.6		674.6	10		69.7		24.6
1	ORI-SIRI-23	197.3	ORI-SIRI-41	298.3	1	ORI-SIRI-23	79.9	ORI-SIRI-41	35.9
10		17.0		29.2	10		198.5		59.0
1	ORI-SIRI-24	358.5	ORI-SIRI-39	728.6	1	ORI-SIRI-24	76.3	ORI-SIRI-39	27.8
10		244.6		473.6	10		86.9		32.9

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5. Discussion

There is an increased risk in immune-compromised patients infected with herpesviruses or adenoviruses, for development of resistant virus strains especially after long-term therapy against nearly all antiviral drugs in clinical use [17]. Most of anti-α-herpesvirus compounds are targeting the viral DNA replication, either indirectly by targeting the thymidine kinase (TK) or directly by targeting the viral DNA polymerase. The choice of taking an alternative drug is very limited and it often goes along with severe side effects. In case of adenoviruses there is only one approved drug in clinical use. Especially immune compromised children are of high risk to be affected by invasive and disseminated infections by Ad. This is the reason why, on the one hand, effective and fast assays are needed to test drug resistance of herpesviruses and, on the other hand, new effective drugs with alternative viral targets are urgently required for treatment of adeno- and herpesvirus infections.

In this work, we reported the development of a new AAV-based replicon vector that is suitable for detection of DNA virus infections. Using this assay, we established a new phenotypic assay for testing HSV resistance and set up a platform, which allows screening for new antiviral compounds targeting wild-type adeno- and herpesviruses.

5.1. Advantages and disadvantages of taking AAV as basis for the AAV replicon vector

5.1.1. Comparison of the AAV replicon to RNA replicon vectors

The RNA replicase-sensor reporter systems used assaying replication of different RNA viruses, can be paralleled with the AAV replicon system which responds to DNA virus infection. Phenotypic tests for the evaluation of new drug candidates in a high-throughput format can be conducted with either using the RNA replicons for RNA viruses [292] as well as for the AAV replicon vector system as we reported here. Replicons are essentially live cell reporter systems, which are induced by infectious viral particles. This represents a more attractive system in certain applications than genome detection-based technologies like PCR or serological-based antigen detection-based assays. Furthermore, in the field of RNA virus research, live cell reporter systems, which are dependent on virus replication, play an

important role for the viral phenotypical monitoring of host infectious diseases [213]. In case of HCV for example, chimeric shuttle replicon vectors carrying all possible single and double variants of mutations are used to assess the consequences in response to increasing concentrations of antiviral therapy and the level of resistance in comparison to wild-type HCV. As a consequence, this knowledge can be used for future genotypic resistance analysis [293]. Due to the small genome size of RNA viruses the information of single and multiple mutations of RNA viruses leading to drug susceptibility are easily available for the genotypic analysis. Concerning resources and time, the phenotypic testing by AAV-based replicon assay may become superior to genotyping, due to the larger genome size of the activating DNA viruses.

One of the major approaches of either positive- or negative-strand RNA replicons which are unable to produce infectious progeny, but can be propagated by trans-complementation, is the development of vaccines [211]. RNA virus replicons with the gene of interest will be abortively replicated and transcribed after delivery inducing an immune response by mimicking WT virus replication. In contrast, the AAV replicons, we constructed are not capable even of an abortive replication cycle by their own. Therefore, it is very unlikely that using the AAV replicon vectors, carrying Rep in addition to the *trans* gene, will induce better immune response as standard recombinant AAV-based recombinant vaccines.

Alternatively, replicons can provide a trans-complementation for propagation of attenuated or replication-incompetent recombinant vaccines [214]. We have tested the trans-complementation ability of our AAV replicon system and found that the replicon cannot trans-complement defective Ad genomes. In same settings, we even observed that an inhibitory effect at the level of virus rescue from wild-type recombinant genomes, showing that the AAV replicon in its present form is not suited for such an application.

5.1.2. Comparison of the heterologous AAV replicon to the homologous DNA virus replicon system

To our knowledge, until now only one DNA virus replicon was reported (Mohr et al., 2012) which is based on a homologous ori and reacted to murine cytomegalovirus (MCMV) infection in *trans* [214]. Although the MCMV ori based replicon vector also is a DNA virus inducible expression system, a number of differences between the AAV based and the MCMV based replicon vectors are important to indicate.

First, in case of the MCMV based replicon vector, the MCMV oriLyt is the key factor for transgene expression. The activation of the replicon oriLyt is very specifically dependent on the MCMV DNA replication machinery. No other tested virus within the herpesvirus family was able to induce this replicon vector. The here described AAV replicon system utilizes the replication system of the AAV and is induced by replication of different helper viruses. Therefore, the AAV replicon carries a heterologous origin of replication and, thus, the recombination between the replicon and the inducing virus is not possible by homologous recombination, which provides a favorable biosafety profile. In addition, the AAV replicon is activated by infection with many different helper viruses. The inducibility of the same AAV replicon by HSV-1, HSV-2 and HCMV and all Ad serotypes tested so far represents a great advantage towards the MCMV replicon vector or potential other replicons constructed in the same way, because it allows one standardized setting for testing different viruses. For example, as we have shown in the pilot screen for kinase inhibitor-based antivirals (chapter 4.3.3) both HSV-1 and Ad could be tested in parallel in high throughput.

Secondly, the inducibility of the MCMV-based replicon vector required chromatin silencing of the basal expression of the transgenes. In addition, it was only responsive in an episomal state. After integration into the host cell chromosome, the MCMV replicon lost its inducibility by MCMV infection. In contrast, the AAV-based replicon was readily inducible upon transient transfection and upon rAAV transduction indicating that chromatin silencing does not play a major role in its control. However, similar to the MCMV replicon, the extrachromosomal AAV replicons showed better induction than the stable cell lines carrying most likely integrated or chromatinized replicon copies. One additional remarkable difference between the two DNA virus replicon systems is their basal activity in transient settings, which may explain their dissimilar inducibility. While in case of the AAV replicon vector, the basal transgene expression without infection was very low and often under detection limit, the MCMV replicon vector showed very high basal expression levels of the marker gene without induction. This was silenced 16 weeks after the establishment of stable transfectants. The episomal and therefore circular state of the MCMV replicon vector was very instrumental for a response to MCMV infection although it is known that herpesviruses in general are able to replicate via circular and linear intermediates [294]. The MCMV replicon vector however failed to respond to WT MCMV infection after integration into the host chromosome. The reason for this could not be clarified by now. On the contrary, here it has been shown that the AAV replicon vector could

efficiently start from circular, linear, and most likely also integrated intermediates as described for AAV in the literature [295] [237]. Dependent on the use, the AAV replicon vector could be delivered either by transient transfection, stably integrated, or packaged into a recombinant AAV vector. Altogether, these features make the AAV replicon the most versatile replicon system described so far including the RNA virus replicons as well.

5.1.3. The versatility of the AAV replicon system

For establishing the AAV replicon system, it was very convenient to use Ad because we were able to compare the results after a wild-type Ad5 virus infection and the viral mutant Ad5-Che transduction, lacking essential genes which are only complemented in 293A and 911 cells. Because of differences concerning the productive replication ability of the viruses in different cell lines, we could test via the usage of transient transfection whether the induction of the AAV replicon system was dependent on a full productive cycle of the helper virus. Interestingly, there was no signal expression in U2-OS cells after infection with the viral mutant because of the lack of viral essential gene complementation in contrast to the wild-type virus which showed induction of the signal in all cell types tested. Together with the results of the qPCR of the GLuc gene, we could therefore assume that the induction of the AAV replicon vector is in fact dependent on a full viral replication cycle of the helper virus.

In the course of exploring the AAV replicon system, we have chosen the chemical transfection delivery system FuGENE® HD as transfection reagent showing the highest and most stable results. In principle, transient transfection with non-viral delivery systems like the nonliposomal FuGENE® HD reagent is suitable for a number of cell lines although relative inefficiency and cytotoxicity is an issue [296]. Nevertheless, HSV-1 and HSV-2 inducibility of the AAV replicon system could be successfully validated in Vero cells, whereas different human Ad serotypes were verified for helper functions in 293A cells. This cell line is derived from human embryonic kidney cells. Due to easy culture conditions, good transfection efficiencies, and reproducible infection conditions for Ad, HSV-1 and also HSV-2, 293A cells were chosen as model cell line for all basic experiments. However, this delivery system restricted, for example, the testing of HCMV induction of the AAV replicon in primary HFF cells or made the delivery of the AAV replicon system to hepatocarcinoma cell lines for testing its HBV inducibility impossible because of the lag time of cell differentiation after transfection prior infection.

The construction of the stable cell line LE2D8 was also based on 293A cells because of the indicated advantages. However, the induction rate of LE2D8 was not as strong as the transient system. In general, the stable cell line was very useful for the establishment of different assays in the course of this work because it was possible to standardize it for the comparison of different studies and the AAV replicon vector was equally distributed while being transduced in all cells. However, since it was very time-consuming and extensive work to pick one stable cell clone, a translation of the system to different cell lines is not recommended. Nevertheless, the transduction of the replicon resulted in better results in all tested cell lines in terms of inducibility and also showed similar reproducibility as the stable cell line-based assays.

Until now, the AAV replicon is the only replicon system, which functions independently of transfection or stable cell lines. We tested this possibility in order to circumvent transfection inefficiencies shown by some target cells as well as disadvantages of the fixed cell type by the stable AAV replicon cell line. The endogenous regions of the AAV replicon vector together with the ITRs were used as *cis*-elements whereas addition of a separate construct coding for rep and cap genes provided the structural proteins. These constructs together with the adenovirus helper factors delivered in a separate plasmid enabled the production of recombinant AAV particles packaging the AAV replicon vector.

For each target cell type, there are recommended serotypes of AAV and therefore the respective rAAV can be chosen for the appropriate application [297]. This feature increased the delivery of the recombinant AAV replicon vector into specific cell lines, which were barely transfectable before, such as A549 and HFF cells. For choosing the serotype of the rAAV, transduction efficacies of different types were tested. Since rAAV2 showed the best results in case of the cell lines planned for application, this serotype was further chosen for AAV replicon packaging. A549 cell line is a human lung adenocarcinoma cell line which is very susceptible for adenovirus infections but hardly transfectable. With the newly established rAAV2-based replicon vector the induction rate after human Ad5 wild-type infection was 230-fold higher in A549 cells compared to 293A cells treated with the same conditions.

Furthermore, the transduction of Vero cells with rAAV2-replicon vector and infection with HSV-1 leaded to a 2800-fold induction of the marker gene GLuc compared to non-infected cells and therefore there was a greater sensitivity compared to the transient or the stable system. Moreover, using this delivery system of the AAV replicon vector for Vero cells, the

HSV-2 induction became significantly higher, enabling the establishment of a phenotypical HSV-2 antiviral resistance test.

Using the AAV replicon vector packaged into a recombinant AAV particle, we were able to transduce HFF cells and therefore testing HCMV as inducer for the AAV replicon system. Primary HFF cells are derived from human foreskin and the combination of inefficient transfection ability and a slowly replicating virus like HCMV leaded to inappropriate experimental conditions. However, transduction of the rAAV2-replicon vector and infection with HCMV resulted in 110-fold induction of a marker gene compared to non-infected cells after an incubation time of 7 days. Differentiation between HCMV-infected and non-infected cells could already be detected after 4 days.

5.1.4. Comparison to other virus-inducible reporters

There are several conditional or inducible systems for the transcriptional regulation of reporter genes, consisting of a regulatory element, a responsive unit which is linked to a transgene and an external inducer. The most prominent inducible transgenic systems are the tetracycline-controlled transcriptional activation system (Tet-ON/Tet-OFF) [298] and the streptogramin-based gene regulation system (PipOFF/PipON) [299]. In case of the Tet-ON/Tet-OFF system the external inducer is tetracycline and in case of the PipOFF/PipON system it is streptogramin. The AAV replicon system in contrary was induced by an incoming viral infection.

The most important requirement for trigger-inducible transgene expression technologies in general is that the system upon induction provides high-level transgene expression, whereas under repressed conditions and in the absence of an inducer the expression should be suppressed completely or at least elicit only low basal expression. Such technologies are especially interesting for precise delivery of protein therapeutics via viral vectors in clinically relevant cell phenotypes. In case of small-molecule-inducible systems a high compatibility of the transfer system to the specific aimed tissue is extremely important to limit negative effects of not affected cells. In principle, the AAV replicon vector would possess the ability for clinical applications against herpes-, adeno- and possibly even papillomavirus-infected cells in vivo. The latter infection is especially interesting due to the fact that papillomavirus infections can cause cervical cancer [300]. Because of these prerequisites it may be possible to insert an

antiviral compound as transgene in the AAV replicon vector and package it into an AAV serotype particle for targeting a specific tissue. After delivery of the AAV replicon vector into the targeted tissue cells, the activation of the compound expression would only occur after infection of the same cell, leading to an inhibition of the incoming virus. This would prevent induction of transgene expression in a non-affected cell. However, before the AAV replicon system could be used in this manner, the rep genes in the AAV replicon vector would have to be modified in order to prevent integration of the AAV replicon vector into the host cell chromosome. Without this safety process an application in vivo is inconceivable.

5.2. AAV Replicon vector for testing drug resistance

5.2.1. Advantages and disadvantages of AAV replicon-based test compared to classical PRA

The most important phenotypic test used during the last decades which is standardized by the Clinical and Laboratory Standards Institute for the use of susceptibility validations of clinical HSV-1 and HSV-2 strains is the plaque reduction assay (PRA) (detailed description in chapter 1.2.2.8) [123]. There are a number of disadvantages concerning the PRA which led to the invention of other tests. But due to the fact that in general phenotypic tests are critical in terms of standardization, there are only limited numbers approved by the FDA and the PRA remains the ‘gold standard’. The standardized protocol of the PRA includes a lot of different technical steps and is therefore very costly in terms of labor. The other very significant disadvantage of this phenotypic test is the time which is needed to proof whether a patient-derived viral isolate is resistant to certain drugs or not. Because of laboratory routine issue, the assay takes 5-7 days before a final conclusion about the further therapy decision can be made. Furthermore, the manual counting of the plaques needs an experienced person and results are highly dependent on the individual opinion of the respective executive technician.

In contrast, the AAV replicon-based susceptibility assay, established in this study, demonstrated a number of advantages over the PRA. First of all, the different working steps could be limited to seeding of the cells in a 96-well plate format and direct transduction with the recombinant AAV replicon vector. After 6 hours incubation time, the 1:100 diluted pre-cultured virus as well as different concentrations of the respective antiviral drug to be tested were added to the wells without any further treatment and incubated for 24- or respective 48

hours. Then 20 µl of the cell supernatants were transferred directly to a white assay plate and the GLuc could be measured. To quantitate drug resistance or susceptibility to drugs, the IC_{50} values could be calculated based on the inhibition of the virus-dependent inductions of the luciferase expression.

Since the results of the AAV replicon-based resistance tests were measurable via the chemiluminescent reagent GLuc, the standardization and comparison between experiments can be provided qualitatively.

In critical cases of HSV-infected patients, therapy decisions have to be made as quick as possible [301]. Not only can the treatment of the patient start earlier after the AAV replicon-based susceptibility assay compared to the PRA but also the economical factor of time is reduced to limited technical aspects, reduced working power and time needed for finishing the assay.

Before using the standardized PRA protocol, the patient-derived viral isolate has to be propagated in cell culture to produce a standardized viral inocula. For assaying clinical strains with the AAV replicon system, the step of pre-culturing of viral material was done as well. In this case, patient-derived samples were never tested directly. Since the pre-culturing needs 1-3 days it would be interesting in the future to test whether this step may be skipped. On the other side, standardization of the number of viral particles may be an important requirement for quantitative analysis. It was published by van der Beek et al. [130] that without pre-culturing the cells for a short period of time, viral replication was not detectable. They explain this by a possible adoption step of the virus to cell culture conditions which may be a key factor. Interestingly, they also tested the influence of pre-culturing on the existing mutations by PCR and they did not observe differentiations due to selection pressure after 48 h. However, in case of PRA the subpopulation of ACV-resistant viruses has to be at least 20 % to give an IC_{50} of ≥ 2 µg/mL (8.9 µM) as threshold for resistance [289] [115] [302]. In general the IC_{50} of antiviral resistance to ACV or penciclovir has been proposed as > 10-fold higher compared to the sensitive control strain [303].

Using the LE2D8 stable AAV replicon cell line we were able to differentiate between 7 sensitive and 7 resistant HSV-1 strains using a 1:10 dilution of the viral pre-culture 48 h post infection. The mean IC_{50} values for HSV-1 ACV sensitive isolates was 25 µM, whereas the IC_{50}s of HSV-1 resistant strains were not calculable because no sigmoidal curve could be observed after

treatment with different doses of ACV. Although the induction levels of the stable cell line were generally low, the response of the AAV replicon cell line to the treatment with ACV after infection with the clinical isolates enabled a differentiation between sensitive and resistant strains. In case of RLU values, the sensitive strains were 4 times less responsive compared to resistant strains. Here, the sensitivity of PRA standards could not be provided. Furthermore, the induction after HSV-2 infection was in general too low for an establishment of a susceptibility assay for this serotype.

The low sensitivity towards HSV infection could be overcome by a higher induction rate of the AAV replicon vector after delivery into cells via transduction. Packaging of the AAV replicon vector into a recombinant AAV particle allowed a great increase of sensitivity towards susceptibility testing. We calculated an IC_{50} of 1.87 µM for HSV-1 WT strain and 19.1 µM for HSV-2 WT strain 48 hours after infection using the rAAV replicon system.

With this improvement on hands, significant results were obtained for virus susceptibility testing, even after using a higher dilution of the respective pre-cultured virus (1:100). Furthermore, significant differentiation between sensitive and resistant strains could be observed after a much shorter period of time of only 24 h. In conclusion, the possibility of transducing the AAV replicon vector enabled the establishment of a diagnostic test for HSV drug resistance, providing answers on antiviral susceptibility within one day after pre-culturing the virus.

PRA for testing the susceptibility of HCMV in general is not performed due to the long pre-culturing time necessary for this slowly replicating herpesvirus serotype (> 4 weeks) [304]. The actual time of the PRA takes 7 to 10 days which results in a test period of approximately 4-6 weeks [305]. There are proposed cutoffs for the susceptibility to GCV, CDV and FOS (6, 2 and 400 µM respectively) [306] [307]. However, since the results are dependent on the respective laboratory, the IC_{50} value for resistance can be generally interpreted as ≥ 2-fold compared to the reference strain [305]. Therefore, phenotypic testing systems for HCMV are very challenging and restricted to specialist diagnostic labs. Using the AAV based replicon, it may be possible to establish a test, similar what we have established for HSV here, which would be less demanding and therefore easily applicable in standard laboratories.

5.2.2. Advantages and disadvantages of AAV replicon compared to other methods

Direct analysis of patient-derived viral isolates by PCR-based methods allows fast identification of mutations connected to antiviral resistance and is technically easy to perform. The distinction whether an analyzed mutation indeed leads to antiviral resistance of the respective virus or whether it simply belongs to a natural inter-strain variation has to be pre–validated by a phenotypic assay. The comparison with mutations described in the literature is fundamental for the analysis and interpretation process.

Genotyping HSV-1 and HSV-2 is concentrating mainly on mutations within the thymidine kinase gene (U_L23) or the DNA polymerase encoding gene (U_L39) [308]. For sequencing of the TK gene, patient material can directly be amplified and sequenced whereas analysis of the pol gene needs to be performed on previously cultured viral stocks (18). Therefore, the overall time for susceptibility testing via sequencing methods still lasts 3-4 days.

Clearly for HSV tests, the AAV replicon-based system is competitive with the genotypic-based methods. It does not require more time and is applicable without further knowledge on resistance-associated mutations.

In general, laboratory testing of HCMV resistance to antiviral drugs is based on genotypic diagnostic. Mutations especially in the viral UL97 kinase and the UL54 DNA polymerase gene are described [304]. A genotypic test for HCMV lasts 3-4 days, provided sufficient material is available. Here, the AAV replicon probably will not be a competitive alternative for genotyping, since the in vitro culture of HCMV also is time-consuming. However, the replicon-based resistance test may allow the generation of a reliable database for resistance-associated mutations, allowing HCMV resistance test in a broader range of laboratories.

There are a number of phenotypic assays to detect HSV which were developed to overcome the problems associated with the PRA. Methods which are evaluating the antigen expression after virus multiplication in vitro are based on enzyme-linked immunosorbent assays (ELISAs) like the sandwich ELISA or the microplate *in situ* ELISA called MISE [124] [125] [126]. For plaque autoradiography in the MISE assay, the activity of viral thymidine of virally infected and drug treated cells were measured by the addition of [^{125}I]iododeoxycytidine 72 h post infection [125]. Other established methods are based on DNA hybridization assays using radioactivity

48 h after incubation with different drug concentrations [127] or based on measuring HSV-1 gC expression in infected cells stained with an appropriate antibody by FACS [128]. The FACS-based method is very labor-intensive due to several washing steps and the results can be observed only 72 h after the pre-cultured virus is recovered.

A similar method like PRA is the assay with an inducible Vero cell line which responds to HSV infection with β-Galactosidase expression. 10-fold dilutions of non-titrated HSV samples are used to determine histochemical stained blue plaques after 48 h [129]. Although plaques are better visualized, and the assay time is shorter than in case of PRA, the method is still labor-intensive and has no objective endpoint.

A recently developed method is a combined assay using cell culture and nucleic assay detection and is named DNA reduction assay. Pre-cultured clinical samples are cultured in presence of antivirals and after only 24 h the increase of the viral DNA load in the infected cells is measured with quantitative real-time PCR whereas the cell count and lysis are corrected by beta-globin PCR [130]. This assay is fast, however, very instrumentation-intensive and expensive.

All these tests cannot satisfy the requirements of sensitivity, time consumption, laboratory technical efforts, standardization procedure, and cost effectiveness at the same time. This is the reason why the PRA assay - or at least variations of the PRA - still belongs to the methods of choice in specialized laboratories. Yet, the AAV replicon system, as we show here provided an improved alternative by exhibiting a high sensitivity which was comparable to the standard PRA test. Additionally, in the AAV replicon-based assay, the time needed for the performance of the test was reduced to a minimum due to the decreased technical working steps, which also led to reduced costs because less personal expenses have to be calculated. Furthermore, the assay can be standardized very well due to gathering quantitative data.

5.3. AAV replicon vector for testing new inhibitors

5.3.1. Why new inhibitors are needed

HSV infections cause clinically mild manifestations with chronically recurrent labial and genital herpes. Although these painful lesions are not life-threatening, patients can suffer from stress going along each infection which can lead to psychosocial problems [309]. Moreover,

especially for neonates and immunosuppressed individuals HSV-1 and HSV-2 is a common cause for devastating clinical consequences. For instance HSV encephalitis, which is responsible for 20% of all cases of sporadic encephalitis [310] typically leads to coma within 6 days after the first neurological symptom if the disease is not treated in time. The incidence of herpes simplex encephalitis is 1 in 250,000 to 500,000 per year with a mortality rate of 20 - 30% if ACV therapy is started timely at an early stage of encephalitis [311] [312] [313]. The number of patients dying although receiving an ACV treatment is very high. Currently, new drugs aiming for the helicase-primase complex of HSV are under clinical development as well as new vaccines against HSV-2 [101]. Nevertheless, alternative drugs are urgently needed that target different sites of the virus than currently used drugs, having additionally a high oral bioavailability, an efficient delivery through the blood brain barrier, no, or at least, less toxic effects and a high efficacy.

In case of human Ad infections, 15% of upper- and 5% of lower respiratory tract inflammatory diseases in children are accounted to this virus and the course of disease is mainly mild and self-limiting in immunocompetent individuals. The situation is different in case of immunocompromised patients with an impaired immunological response, such as solid-organ transplant or hematopoietic stem cell recipients. In this case, an acute or a persistent infection can lead to high morbidity and even mortality. The clinical manifestation is highly dependent on the patient age, the virus serotype, the primary disease, and the tissue which is infected by Ad. Children have a 2 to 3.5 times higher risk of infections compared to adults [208] [141]. At present, there is no approved drug against human Ad infection. Only broad-spectrum antiviral agents can be used as an alternative with limited success. Therefore, patients at risk are dependent on decreasing the immunosuppression or treatment with adoptive immunotherapy using adenovirus-specific T cells. There is an urgent need for new potent anti-adenoviral drugs, or at least one, to be able to treat the severe cases of infections. New antiviral Ad compounds have to meet additionally the challenge that constantly new serotypes are recognized that may show difference in drug susceptibility.

5.3.2. Current testing systems

Screening of potential new drugs against viruses is a process that can be divided into several phases. The most important point is to have an in vitro and in vivo model for testing a large number of new target molecules. Fortunately, the HSV-1, HSV-2 and Ads, the mostly used

viruses in the course of this work, are in general suitable for in vitro analyses. In case of HSV, the in vivo models commonly used to study infections are mice, guinea pigs, rats and rabbits [314]. For human Ad there is a new Zealand white rabbit model for corneal infections of Ad-1, -2, -5, -6, -8 and -37 [315]. Human Ad5 can be tested intranasally or intravenously in different mouse models, in the pig and in the Syrian hamster (reviewed in [208]). The latter one is the most promising one and was used for testing the new antiviral candidate drug Brincidofovir [316] [317].

For screening of new compounds with antiviral activity, either biochemical or target-based assays are used for testing the direct effect on a target protein or a certain function such as the enzymatic assay published by McGrath *et al.* for testing inhibitors of the human Ad proteinase [318]. However, for evaluation studies of antiviral activity in the cell, phenotypic assays based on cell culture are preferred because a preconceived idea concerning the target site, at the stage of high throughput compound library testing is not necessary.

A widely used assay, which can be used for drug screening towards cell viability, is the 3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method, suitable for a 96-well format high throughput screening. Compared to the AAV replicon assay there are more steps to perform the MTT assay, it cannot be applied in many different cell lines and the sensitivity depends highly on different parameters [319] [320].

Another method to test the impact of certain compounds on Ad or HSV is the yield reduction assay. For HSV-1 for example the enzyme-linked virus inhibitor reporter assay (ELVIRA) is published [321] [322]. However, this method is restricted to a certain cell line and furthermore the implementation includes more working steps compared to the newly established AAV replicon system. Therefore, especially in case of Ad, it is mainly used to ensure already selected compounds [323]. The same is true for the plaque reduction assay, which is even more time and labor-intensive for Ad and for HSV [324]. Quantification after immunofluorescence with antibodies targeting virus specific antigens has the advantage of evaluating the antiviral activity of the compound at a defined step of the viral life cycle. However, the following quantification of fluorescent cells is again very time consuming and not convenient to be performed in high throughput conditions.

Other test systems used for screening in high throughput format are cell lines expressing a marker gene. For HSV-1 a strategy using a modified plaque reduction assay with a transgenic

cell line is published. The promoter of the HSV-1 UL39 gene is induced to express β-galactosidase upon HSV-1 infection [325]. In contrast, the AAV replicon assay is applicable in several tested cell lines including primary cells.

Genetically marked viruses expressing a reporter gene upon viral infections for the evaluation of viral infectivity are the most commonly used approaches for high throughput screenings of large compound libraries [326] [208]. Ad5-GFP or Ad16-GFP for example have been used for high throughput screenings based on labelled genes and modified PRAs [326].

The advantage of the AAV replicon system over labelled viruses is the ability to use it for wild-type viruses and clinically isolated strains. Especially concerning all the different relevant serotypes of human Ad, this issue is very relevant because the AAV replicon assay provides a direct testing of all in vitro growing Ad without the inconvenient construction of genetically labelled BAC-derived serotypes. Until now all alpha- and beta-herpesviruses tested responded to the AAV replicon system as well. Most important, viruses known to be resistant to certain drugs can be evaluated independent of the mutation leading to this resistance.

Yet, the newly developed AAV replicon-based screening assay was used to test a compound library of 24 candidates for their potential to inhibit Ad5 as well as HSV-1. Compounds that inhibited these viruses were analyzed via an absent induction signal of GLuc as transgene of the AAV replicon vector, which was transduced in the adopted cell line 4 h before adding the possible inhibitors together with the respective virus. The compounds were highly selected for their inhibitory effect on certain targets in the cell. Out of 24 small molecules 7 were able to inhibit Ad5 and 3 of these compounds were also able to inhibit HSV-1. Out of these 7 Ad and 3 HSV-1 inhibitors, one was the known apoptosis inducer staurosporin, and another one was a published kinase inhibitor and therefore both were used as internal controls of the assay system. For further validations of the compounds, toxicity was tested via an MTT-test in two different cell lines (A549 and Vero) using different concentrations (data not shown). The 3 compounds, inhibiting both viruses (including the two control inhibitors) showed a high toxicity profile of less than 80% viability in both cell lines. The two compounds that demonstrated 50% inhibition of Ad in contrast did not show any toxicity within those concentrations we used in the screening assay. For evaluation, if the inhibitory effect of those compounds does not derive from cell toxicity, the MTT test would have to be reproduced and other cell lines have to be tested with the replicon system as well. Since this screen was only

made with a limited data-set, the AAV replicon system could be further used to establish a high throughput screen for testing a greater compound library. Nevertheless, the first application of the AAV replicon-based screening system granted a first hint on compound groups to be more or less interesting for further studies.

However, in case of the AAV replicon compound screening it is possible, that the inhibitory effect of possible antiviral drugs roots on the inhibition of the AAV replicon vector itself. Therefore, it is necessary to further evaluate screened hits with methods for direct analysis of the targeted viral inhibition.

5.4. Concluding remarks

In this study a new form of a DNA based replicon vector that is inducible by infection with large DNA viruses combining the regulatory elements of the replication controlled late AAV gene with the transcription unit of an exchangeable gene of interest was established. The novel and major advantages of this new AAV based replicon over similar systems resides in the possibility to induce this replicon vector by productive helper viruses without the risk of recombinant homologous sequences to these viruses.

Several delivery systems of the AAV replicon vector were established for varying demands. The AAV replicon proved its functionality in context of all tested delivery approaches and remained responsive in multiple physical states of its genome demonstrating its superior versatility.

However, the viral delivery system of transducing the AAV replicon by an adeno-association virus particle appeared to be superior for most of the applications tested in this study allowing fast, easy to apply and quantitative marker of helper virus infections.

In this study, the construction of the AAV replicon containing recombinant AAV particle was the prerequisite for the establishment of two unique approaches towards diagnostic and drug development. The phenotypic susceptibility assay for HSV-1 and HSV-2 resistance testing was developed and already tested in a small cohort of 16 clinical isolates. The sensitivity of the AAV replicon assay was validated by PRA.

The second major application of the AAV replicon system, was a novel phenotypic screening system for the detection of new antivirals. The applicability of the system as sensitive, easy to handle and time-saving high-throughput assay was demonstrated in a primary screening test of potential inhibitors for human Ad and HSV-1 using conditions adopted for each virus. The antiviral effect of 5 compounds inhibiting Ad5 and 1 compound inhibiting HSV-1 could be detected as hits towards possible new antivirals against important DNA viruses. We believe that in future the AAV replicon can be adapted to detect replication of further viruses, such as VZV and HCMV, and other applications such as testing neutralization activity of antisera and trans-complementation, which can boost both clinical and basic research on large DNA viruses.

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Abbreviations

aa	Amino-acid
AAP	Assembly activating protein
AAV	Adeno-associated virus
ACV	Acyclovir
ACV res	Acyclovir resistant herpes simplex virus type 1
ACV sens	Acyclovir sensitive herpes simplex virus type 1
ACV-MP	Acyclovir-monophosphate
ACV-TP	Acyclovir -triphosphate
Ad	Human adenovirus
allo-HSCT	Hematopoietic stem cell transplantation
amp	Ampicillin
BACs	Bacterial artificial chromosomes
bp	Base pair
CAR	Primary coxsackie B virus and adenovirus receptor
CDV	Cidofovir Hydrate
Che	Expression cassette mCherry
cm	Chloramphenicol
Cmpd	Compound
CMV	Cytomegalovirus
CN	Copy number
CsCl	Cesium chloride
CTL	T-lymphocytes from donors
d p.i.	Days post infection
dCMP	Deoxycytidine monophosphate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxid
DRA	DNA reduction assay
DSG-2	Cadherin protein Desmoglein-2
E	Early genes
EBV	Epstein-Barr virus
EDTA	Ethylen-Diamin-Tetra-Acetic acid
EGFP	Enhanced green fluorescence protein
ELISA	Enzyme-linked immunosorbent assay
ELVIRA	Enzyme-linked virus inhibitor reporter assay
FACS	Fluorescence activated cell sorting analysis
FCS	Fetal calf serum
FDA	US Food and Drug Administration
F-factor	Fertility factor

GLuc	*Gaussia* luciferase
h p.i.	Hours after infection
h p.t.	Hours post transfection
h p.th.	Hours after thawing
HCF	Host cell factor
HCMV	Human cytomegalovirus
HFF	Human foreskin fibroblasts
HIV	Human immunodeficiency virus
HBV	Hepatitis B virus
HP	Helicase/primase complex
HPV	Human papillomavirus
HS	Heparin sulfate
HSV	Herpes simplex virus
HSV-1	Herpes simplex virus type 1
HSV-2	Herpes simplex virus type 2
HYG	Hygromycin cassette
IC_{50}	Half inhibitory dose
ICTV	International Committee on Taxonomy of Viruses
IE	Immediate-early genes
IFN	Type I interferons
IR	Internal repeat sequence
ITRs	Inverted terminal repeats
IVIG	Intravenous immunoglobulin therapy
kbp	Kilobase pair
kn	Kanamycin
KSHV	Kaposi's sarcoma-associated herpesvirus
L	Late genes
LATs	Latency-associated transcripts
LB	Lucia Broth medium
MCMV	Murine cytomegalovirus
MCS	Multiple cloning site
MOI	Multiplicity of infection
MT	Metallothionein I promoter
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
n.i.	Non-infected
n.t.	Non-treated
NCS	Newborne calf serum
NDP	Nucleoside diphosphate kinase
NEB	New England Biolabs
NF	Nuclear factor-1
NK	Natural killer cells
ns	Non-significant

Oct-1	Octamer-binding transcription factor-1
OD	Optical density
ORFs	Open reading frames
ORI	Origin of replication
oriLyt	Origin of lytic replication
oriS	Replication S
p.t.	Post transfection
p/c	Particles per cell
PAA	Phosphonoacetic acid
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDCs	Plasmacytoid dendritic cells
PEI	Polyethylenimine
PFA	Phosphonoformic acid or Foscarnet
POL	DNA polymerase
PRA	Plaque reduction assay
PrV	Pseudorabies virus
pTP	Pre-terminal protein
qPCR	Quantitative real-time PCR reaction
RBS	Rep-binding site
RFLP	Restriction fragment length polymorphism
RLU	Relative light unit
SD	Standard deviation
TE	Trypsin/EDTA Solution
TK	Thymidine kinase
TNF-α	Tumor necrosis factor alpha
TP	Terminal protein
TR	Terminal repeat sequence
TRS	Terminal resolution site
U_L	Unique segment long
U_S	Unique segment short
v/v	Volume per volume
VZV	Varicella zoster virus
w/v	Weight per volume
wt	Wild-type
WTBAC	Wild-type Bacterial Artificial Chromosome
zeo	Zeocin

Publications and posters

This thesis describes the work performed at the Max von Pettenkofer-Institut, in Munich between January 2013 and May 2017. Parts of this thesis were published, patented or presented at conferences.

Patents

01/2016 AAV-based conditional expression system (Co-inventor) - EP-patent application 16 15 3329.4. An in vitro system for fast detection of phenotypic resistant Herpes- and Adenoviruses and for discovering new antiviral drugs.

Posters

04/2016 "AAV based replicon technology for phenotypic detection of adeno- and herpesvirus infections", 26th Annual Meeting of the Society for Virology, Münster, Germany.

07/2016 "AAV based replicon technology for detection and quantification of herpesvirus replication and drug resistance", 41st Annual International Herpesvirus Workshop, Madison, USA.

11/2016 "Fast detection of drug-resistant herpesviruses using an AAV based replicon technology", DZIF Annual Meeting, Köln, Germany.

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