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adenovirus infections“

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Abstract

Adenovirus infections in immunocompromised patients constitute a clinical problem and a frequent cause of fatal outcomes in allogeneic stem cell transplantation. Treatment options for adenovirus infections are still unsatisfactory and alternative treatment strategies are highly needed.

In the first part of presented studies, I investigated if delivery of the herpes simplex virus-encoded thymidine kinase gene (HSV-TK) into and selective expression within adenovirus-infected cells can render such cells amenable to anti-adenoviral treatment with the prodrug ganciclovir (GCV). GCV is exclusively converted into its active form by HSV-TK. Restriction of HSV-TK expression to wild-type (wt) adenovirus infected cells was achieved by transcription of the gene from the adenovirus 5 (Ad5) E4 promoter whose full activity is dependent on the presence of the adenoviral E1A protein. In order to guarantee high transduction rates and targeted delivery into typical adenoviral host cells, the entire expression cassette was transferred into a replication-deficient adenoviral vector lacking the E1A gene. I was able to show that E4 promoter-mediated HSV-TK background expression was sufficiently low to prevent cytotoxicity in the presence of low and therapeutically relevant GCV concentrations in cells not infected with wt Ad5. However, expression was several-fold increased in wt Ad5-infected cells and treatment with low levels of GCV efficiently inhibited wt Ad5 DNA replication. Genome copy numbers and output of infectious particles were reduced by up to >99.99% and cell viability was greatly increased.

In a separate project I was involved in the investigation of the potential of RNA interference (RNAi) for the inhibition of adenoviruses. I was able to demonstrate that siRNAs designed to silence the adenoviral genes coding for the E1A, preterminal protein (pTP), the DNA polymerase, IVa2, hexon and the protease gene are capable of inhibiting adenoviruses *in vitro*. Furthermore, I was also able to prove that artificial microRNAs (amiRNAs), expressed from replication-deficient adenoviral vectors are able to inhibit adenovirus replication. Both, HSV-TK/GCV and miRNAs influence the adenoviral DNA replication.

I have also investigated, if additional silencing of adenoviral genes involved in the replication of the viral genome leads to additive inhibitory effects. The

amiRNA targeting pTP, whose expression was controlled from a tetracycline-regulated CMV promoter, was incorporated into a replication-deficient adenoviral vector which additionally allows for the simultaneous expression of the HSV-TK gene from the adenoviral E4 promoter. I was able to show that the integration of the two conceptual approaches, HSV-TK/GCV and RNAi, further increased the efficacy in adenovirus inhibition.

In view of constantly rising numbers of transplant recipients and consequently increasing numbers of life-threatening adenovirus infections the findings are expected to aid in the development of alternative strategies to combat adenovirus infections.

Zusammenfassung

Adenovirale Infektionen von immunsupprimierten Patienten stellen ein großes klinisches Problem dar, und sind eine häufige Ursache für tödliche Folgen nach allogenen Stammzelltransplantationen. Da Behandlungsmöglichkeiten gegen adenovirale Infektionen immer noch nicht ausreichend verfügbar sind, besteht der dringende Bedarf an alternativen Behandlungsstrategien.

Im ersten Teil der hier vorgestellten Studien war ich in ein Projekt eingebunden, das untersuchte ob die selektive Expression des Herpes simplex Virus Thymidinkinasegens (HSV-TK) in Adenovirus-infizierten Zellen, diese für eine Behandlung mit dem Pro-Pharmakon Ganciclovir (GCV) empfänglich macht. GCV wird nur durch HSV-TK in seine aktive Form umgewandelt. Eine Expression von HSV-TK ausschließlich in Adenovirus-Wildtyp-infizierten Zellen wurde erreicht, indem dieses Gen unter die Kontrolle des Adenovirus 5 (Ad5) E4 Promotors gebracht wurde. Dieser Promotor wird nur aktiv, wenn das adenovirale Protein E1A vorhanden ist.

Um hohe Transduktionsraten und gezielten Transfer in für adenovirale Infektionen typische Zellen zu garantieren, wurde die gesamte Expressionskassette in einen replikationsdefizienten, adenoviralen Vektor transferiert, dem das E1A Gen fehlt. Wir konnten zeigen, dass die Hintergrundexpression durch den E4-Promotor niedrig genug war, um Zytotoxizität in der Gegenwart von niedrigen, therapeutisch relevanten, GCV-Konzentrationen in Zellen, die nicht mit Ad5 infiziert waren, zu verhindern.

Dennoch war die Expression in Adenovirus-infizierten Zellen um das 7-fache erhöht, und eine Behandlung mit geringen Mengen GCV konnte die Ad5 DNA Replikation effizient inhibieren. Die Genomkopiezahlen und die Produktion von infektiösen Viruspartikeln konnten um bis zu 99% reduziert werden. Weiters führte dies zu einer Erhöhung der Zellviabilität.

In Zuge eines anderen Projekts untersuchte ich das Potential von RNA Interferenz (RNAi), zur Inhibierung von Adenoviren. Ich konnte zeigen, dass siRNAs, die die adenoviralen Transkripte für E1A, das preterminale Protein (pTP), die DNA Polymerase, IVa2, Hexon und Protease degradieren, Adenoviren *in vitro* hemmen können. Des Weiteren konnte ich zeigen, dass artifizielle miRNAs

(amiRNAs), die von einem replikationsdefizienten adenoviral Vektor exprimiert werden, die adenovirale Replikation inhibieren können.

Da der HSV-TK Ansatz auf das Blockieren der DNA Replikation limitiert ist, habe ich auch untersucht, ob gleichzeitiges Stilllegen von adenoviralen Genen, die an der Replikation beteiligt sind, zu einem additiven, inhibitorischen Effekt führt.

Die gegen pTP gerichtete, amiRNA, deren Expression unter der Kontrolle eines Tetrazyklin-regulierten CMV Promotors stand, wurde in einen replikationsdefizienten adenoviralen Vektor transferiert, der gleichzeitig die Expression des HSV-TK Gens ermöglichte, das unter der Kontrolle des adenoviralen E4 Promotors stand. Ich konnte zeigen, dass das Zusammenführen der zwei Ansätze, HSV-TK/GCV und RNAi, die Effizienz der Adenovirus-Inhibierung noch weiter steigern kann.

Da die Zahl an Transplantationspatienten ständig ansteigt und damit verbunden auch vermehrt lebensbedrohliche Adenovirusinfektionen auftreten, können die vorliegenden Daten dazu beitragen, alternative Strategien zu entwickeln, um adenovirale Infektionen erfolgreich zu behandeln.

Aims of the present thesis

Because of the raising number of immunosuppressed patients and unsatisfactory anti-adenoviral treatment options, the development of alternative treatment strategies is highly needed. Some clinically used agents for the treatment of adenovirus infections, such as cidofovir, have limited activity or unwanted side effects such as nephrotoxicity and are still under investigation. The development of novel treatment possibilities would decrease adenovirus-mediated mortality.

In the first part of the presented thesis, the selective expression of the herpes simplex virus thymidine kinase gene (HSV-TK) in adenovirus-infected cells in conjunction with treatment with the antiherpetic prodrug ganciclovir (GCV) for the inhibition of adenoviruses was investigated. Selective expression of HSV-TK in virus-infected cells was thought to be achievable by placing the gene under control of the adenoviral E4 promoter, whose activity is dependent on the presence of the adenoviral E1A gene product provided by an infecting wt virus. Upon induction of HSV-TK expression the prodrug GCV should be converted into its toxic form, consequently leading to a blockage of DNA synthesis and an inhibition of adenovirus multiplication.

In a second approach, siRNA- and artificial miRNA-based silencing of adenoviral genes was investigated for its capacity to inhibit adenovirus infection. A central aim was to investigate which adenoviral processes can serve as targets for RNAi-mediated adenovirus inhibition. Therefore, a set of siRNAs targeting the E1A, DNA polymerase, pTP, IVa2, hexon, and protease mRNA, were designed and analyzed. It was also aimed to express the best-performing siRNAs (E1A, pTP and DNA polymerase) in the form of miRNAs with full complementarity to their target sequence, and to investigate the inhibitory effect *in vitro*. An adenoviral vector system harboring best-performing artificial miRNAs (amiRNAs) was generated. To enable the efficient delivery of amiRNA expression cassettes into cells, and for an envisioned *in vivo* application, this delivery system was based on replication-deficient adenoviral vectors lacking the E1A gene. It had to be assured that amiRNA expression, which would otherwise inhibit the replication of the

recombinant vector, is shut off in packaging cells. Because of that, amiRNA expression was regulated by a tetracycline-based inducible system.

Replication-deficient adenoviral vectors for the delivery of genes into adenovirus-infected cells offer a series of advantages over other vector systems: (a) delivery of the transgene into exactly those cells which are also the preliminary target for wt adenoviruses, (b) amplification of the vector in cells infected with the wt virus and consequently, increased copy numbers of the “therapeutic gene”, and (c) elimination upon recovery of the immune system.

In a third approach, a combinatorial approach, was aimed to identify which viral processes have to be blocked by RNA interference in conjunction with selective HSV-TK expression/GCV treatment to achieve a maximized additive inhibitory effect on adenovirus multiplication. It was expected that a combination of siRNA treatment and HSV-TK expression/GCV treatment has additive effects in terms of inhibiting adenovirus replication. It was also aimed to generate a vector system for combined HSV-TK/amiRNA expression that allows for effective inhibition of adenoviruses.

1. Virus evolution and pathogenesis

Evolution of viruses generally requires changes in the genetic composition of viral DNA or RNA over time. Unfortunately, there is no reliable experimental approach which can recreate an early scenario of virus evolution. However, using virus genomics-based approaches for the analysis of the organization and nucleotide composition of viral genomes and their hosts have been developed.

It is thought that some viruses are the descendents of primitive RNA or RNA-like replicons that preceded cellular forms (Eigen M., 1979, Domingo E., 2001). It is also possible that early virus evolution involved not only sequence of RNA but also of protein, or RNA, DNA and protein (Gesteland R. F., 1999).

Viruses may also be the result of the degeneration of microbes, and the genome composition of some complex DNA viruses supports this suggestion. There is also some believe that certain viruses are originating from cellular DNA or RNA that evolved to embody autonomous replication and an extracellular step in their replication cycle. Current genomics favours the theory that viruses are as ancient as cells with which they have shared functional modules (Mount D. *et al.*, 2004).

Comparison of different virus families and genera showed some phylogenetic relationships between them. These comparative studies are based on genomic nucleotide sequences, genome organization and replication, phenotypic traits, and viral proteins structure.

There are a few categories related to viral pathogenesis. The first one considers essential gene mutations that influence virus replication in the host. The second art of mutations impair virulence with no altering normal virus replication. Mutations of cellular genes can prevent virion adsorption and consequently prevent viral entry into the cell, or they can occur in enhancer elements and change the transcription of the virus. Some viruses produce proteins that alter the composition of the major histocompatibility complex proteins (MHC-I and MHC-II), which present short peptides on the cell surface that can be recognized by T-cells. Many viruses change the function or expression of MHC proteins (Fields Bernard N., 2007).

There are also RNA viruses whose maturation proceeds only after specific proteolytic cleavage of their structural proteins. Sometimes maturation is performed by viral proteases or in some cases by cellular proteases, with a specific amino acid

sequence required for proper cleavage. Altering this sequence can impair the spread of the virus in a host. A wide variety of polymorphisms or mutations in the host can result in modulated resistance or virulence of the virus. The host mechanisms that minimize viral diseases after infection are certainly major topics in viral pathogenesis (Fields Bernard N., 2007).

1.2. Adenovirus infections

Virus infections in immunocompromised patients have become major clinical problems that have escalated during the AIDS pandemic (Marie L. Talashek, Anna M. Tichy, 2007). Many different viruses, most prominently cytomegaloviruses (CMV) and adenoviruses have been the cause of systemic infections with significant rates of fatal outcomes in the patients. Among hematopoietic stem cell transplant recipients with disseminated disease the reported mortality rates are very high, reaching nearly 80% in some reports (Hale *et al.*, 1999, Howard *et al.*, 1999).

Adenovirus infections have been reported in as many as one-fifth of bone marrow transplant (BMT) recipients and patients with acquired immunodeficiency syndrome (AIDS). The relative contributions of primary infections versus reactivations from latency in immunocompromised patients remain unclear (Donald R. Carrigan *et al.*, 1997). Compared with adult BMT recipients, pediatric BMT recipients appear to be infected by adenovirus more frequently and earlier in the post-transplant period (Carrigan *et al.*, 1997).

Adenoviruses are the cause of an estimated 5–10% of febrile illnesses in children worldwide (Fox *et al.*, 1997). Adenoviruses cause a wide variety of clinical syndromes, including respiratory illness, enteritis, hepatitis, hemorrhagic cystitis, nephritis, conjunctivitis, and meningoencephalitis (Ruuskanen *et al.*, 1997). However, adenovirus infection can also be subclinical.

Risk factors for clinical adenovirus disease include the number of sites from which the virus is isolated and, in BMT recipients, the presence of moderate to severe acute graft-versus-host disease (Carrigan *et al.*, 1997).

However in neonates and immunosuppressed patients, including patients with acquired immunodeficiency syndrome (AIDS), adenoviruses can cause fatal infections (Fields Bernard N., 2007).

Adenovirus infection results in antibody formation that is protective against exogenous reintroduction of the same adenovirus type (Horwitz, 2001).

For gene therapies that use adenovirus as a vector, a pre-existing infection can lead to a lower efficacy and sometimes to severe immunological reactions (Mary Miu Yee Waye, 2010).

1.3. Molecular biology of adenoviruses

1.3.1. Adenovirus structure and basic properties

Human adenoviruses are non-enveloped, linear, double-stranded (ds) DNA viruses, first isolated in 1953 from tonsils and adenoids. They comprise over 60 distinct serotypes which have been classified into seven subgroups (A to G).

The virion consists of an outer protein shell, known as capsid, containing 240 hexon and 12 penton proteins as the main components. The protein coat (80-90 nm in diameter) is surrounding the inner nucleoprotein core. The pentons are made up of penton base and fiber that is required for attachment of adenovirus to the cell surface.

Human adenoviruses belong to the family of *Adenoviridae* with two main genera: *Mastadenovirus* and *Adenovirus*. Over 100 Adenovirus family members have been identified. They can infect a variety of mammals, birds, reptiles and amphibians (Harrach B., Benkö M., 2011; Berk A. J., 2007).

There are three major Ad5 capsid proteins (II, III and IV) and five minor polypeptides (IIIa, IVa2, VI, VIII and IX). The virus DNA is affiliated with three basic proteins: histone-like core protein VII that is organized in complex with 12 spherical

nucleoprotein projections, polypeptide Mu (a 79 amino acid precursor protein) and the core protein V that has the ability to directly or indirectly to interact with the capsid.

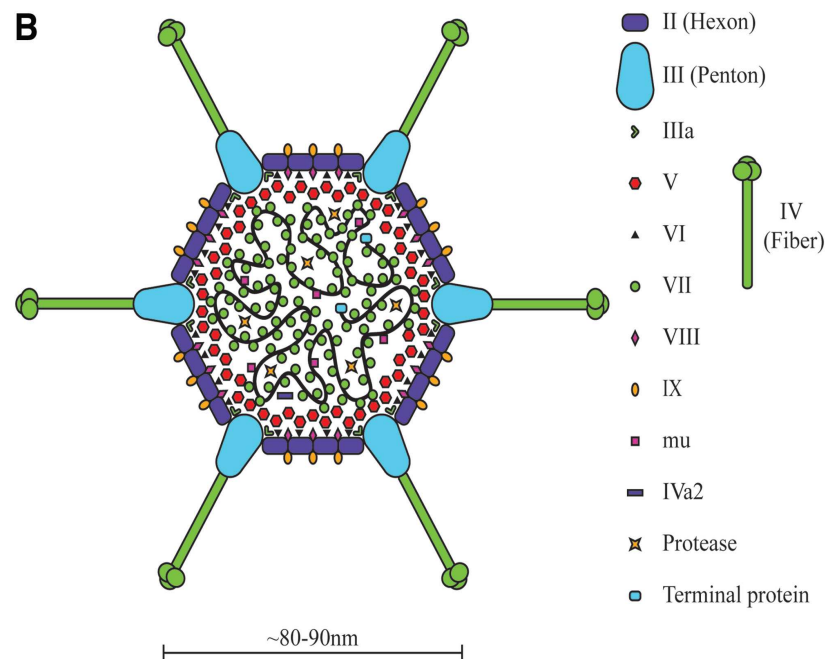


Figure 1. Schematic representation of the adenovirus virion (Andrea N. Giberson, 2011).

1.4. Virus attachment, entry and replicative cycle

The adenoviral fiber proteins are responsible for the attachment of the virion to the host's cell membrane. The adenovirus fibers bind to the Coxsackie-Adenovirus receptor (CAR), which also serves as the receptor for Coxsackie virus. Thereafter, the attached virus heads to clathrin coated pits and the formed receptosomes become internalized. It means adenovirus is internalized by receptor-mediated endocytosis and evades degradation by escaping from the early endosome (Leopold P. L., 1998). Consequently, the receptosome pH-value decreases and influences the properties of the virion surface, causing uncoating. The viral DNA migrates through the cytoplasm via microtubules. It is wrapped with protein VII and eventually takes the way through the nuclear pores into the nucleus. In the nucleus, the viral DNA is converted into a DNA histone complex.

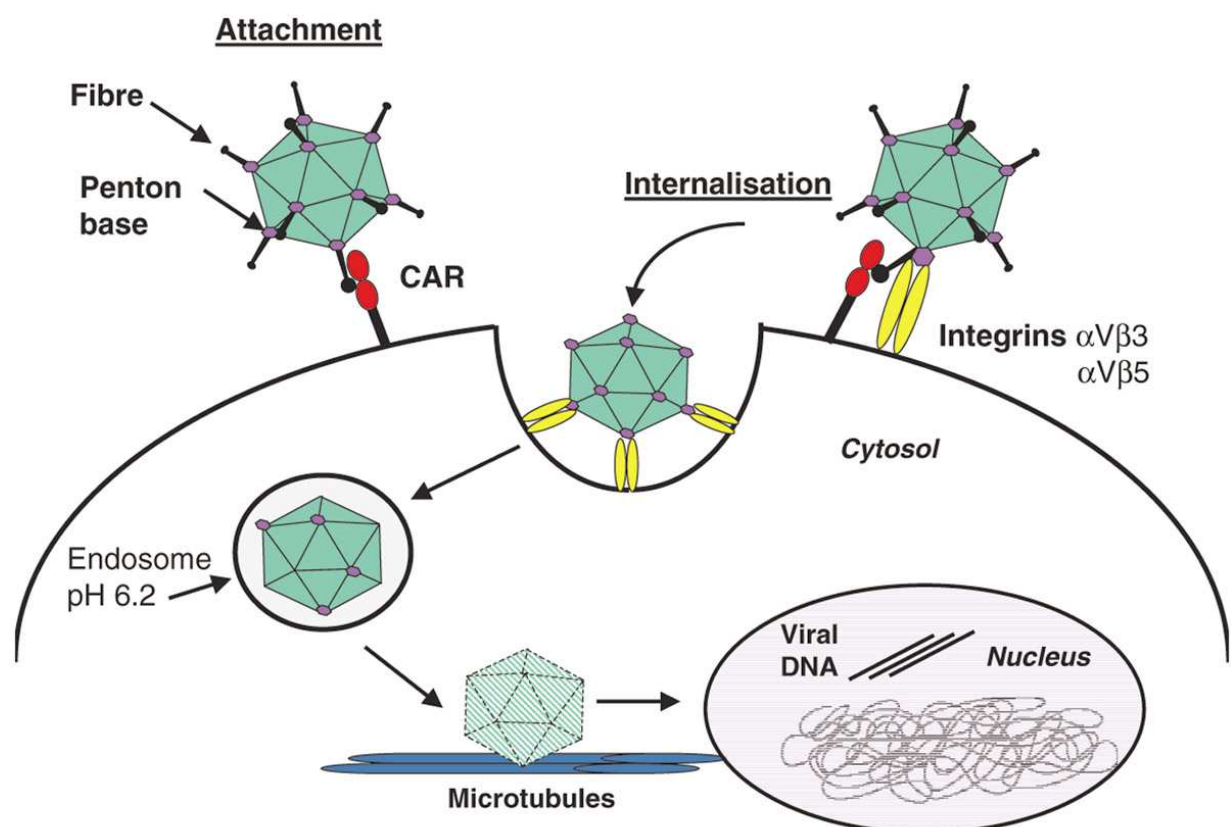


Figure 2. Schematic representation of adenoviral attachment and internalisation. CAR, Coxsackie virus and adenovirus receptor (Horwood *et al.*, 2002).

1.5. Genome organisation

1.5.1. Activation of early viral genes

The adenovirus genome (35-36 kb in size) contains 100-140 bp long inverted terminal repeats (ITRs). Both strands of the adenovirus DNA possess genes in a series of overlapping transcription units. Infection is divided into early and late phase of adenovirus gene expression. The structure proteins are synthesized during the late phase which starts immediately after the onset of DNA replication.

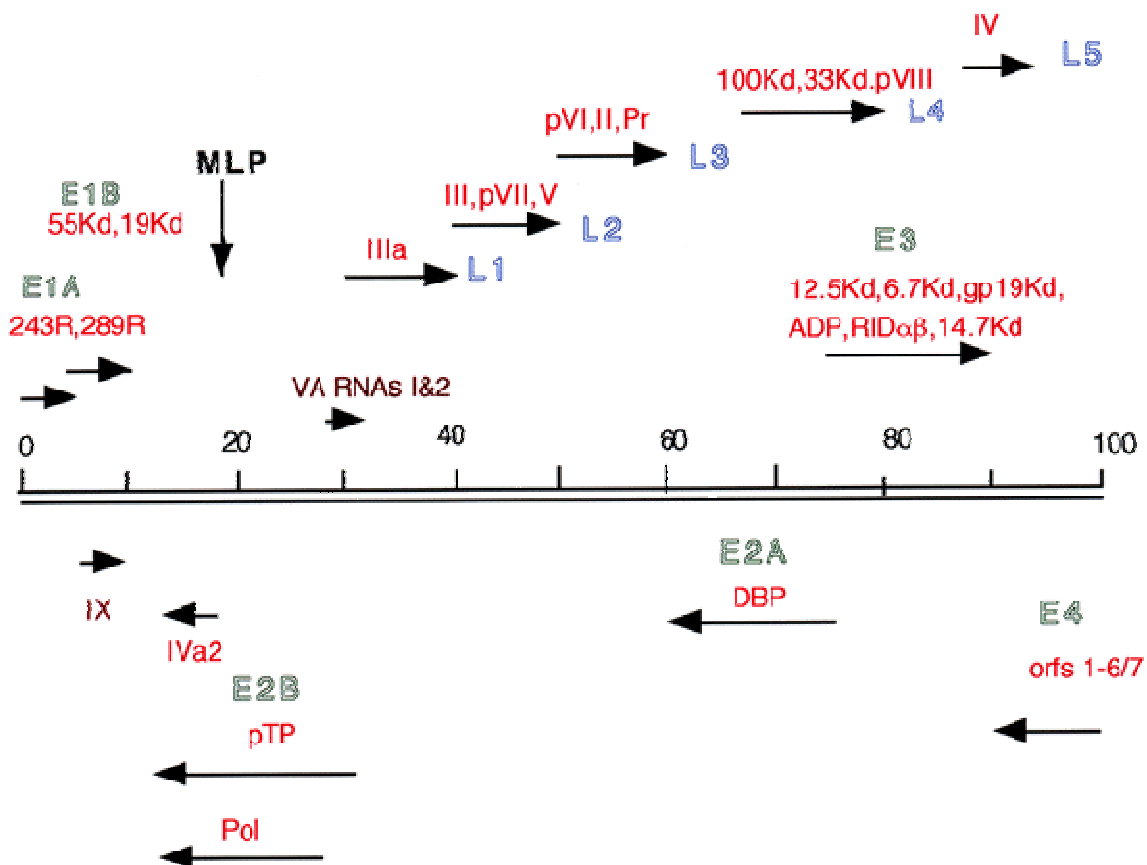


Figure 3. Transcription of the adenovirus genome (Russell *et al.*, 2000). The early transcripts are outlined in green, the late in blue. Arrows indicate the direction of transcription. The gene locations of the VA RNAs are denoted in brown. MLP, major late promoter.

There are six early transcription units (E1A, E1B, E2A, E2B, E3, and E4) and the first ones to be activated are E1A and E1B located in the early region 1 (E1). The E1A gene products directly or indirectly (by interfering with the regulation of several cellular genes) lead to the transactivation of other adenoviral promoters, consequently permitting the efficient expression of further transcription units (Liu & Green, 1994).

The replication of the linear adenovirus DNA is dependent on three adenoviral proteins: the preterminal protein (pTP), the DNA polymerase, and the DNA binding protein (DBP) (de Jong *et al.*, 2003). Besides creating dsDNA for packaging into capsids (accomplished with the help of the IVa2 protein) (Zhang & Imperiale, 2003), replication of the adenoviral genome is essential for activation of late gene expression, from the major late promoter (MLP). However, upregulation of late gene expression additionally involves the IVa2 protein (Tribouley *et al.*, 1994). It results in the synthesis of gene products mainly constituting structural components of the virion or being involved in virion assembly. Maturation of the virion requires the action of the adenovirus protease (Webster & Kemp, 1993). This protein, which is an integral part of the mature virion, is also required for disassembly of the virion upon virus entry and consequently for the release of the viral DNA (Greber *et al.*, 1996).

The E1A region is crucial for the expression of adenoviral genes. Its major product is the 13S mRNA, whose gene product is involved in the regulation of a wide range of cellular genes. The E1A proteins are also involved in transactivation of other early transcription units.

The E2 region is divided into two separate units: E2A (codes for the DNA binding protein (DBP)) and E2B (codes for the precursor terminal protein (pTP) and the DNA polymerase). All three are essential for DNA replication.

Away other proteins, the E3 region is nonessential in tissue culture and because of that frequently deleted in adenoviral vectors.

However, the E3 region contains genes which are important to modulate the immune response of the host. Via the MHC-I pathway, an infected cell presents virus antigens on its cell surface, which are subsequently recognized by cytotoxic T-lymphocytes.

The E3 region encodes a 19 kDa protein that can bind to the MHC-I heavy chain. This binding prevents loading of peptides onto MHC-I by tapasin proteins and the release of MHC-I from endoplasmic reticulum, and consequently impairs antigen presentation on the cell surface.

It means that the E3 gene products play very important role in viral pathogenesis. It was reported that deletion of an E3-encoded 19-kDa glycoprotein (gp19kDa), results in a virus that produced significantly more extensive pneumonia than its parental virus. However, deletion of the 3' portion of E3, which codes for 10.4-, 7.5-, 14.5-, and 14.7-kDa proteins, resulted the mutants which did not increase the extent of pneumonia, instead they affected the nature of inflammatory response (Harold S. Ginsberg *et al.*, 1989)

The E4 region encodes polypeptides with diverse functions: regulation of transcription, apoptosis, and DNA repair. The results of many studies showed that the E4 gene products can independently enhance viral DNA replication, viral late protein synthesis, the shut off of host cell protein synthesis, and the production of infectious virus (Min-Mei H., Patrick H., 1989).

A general, interesting feature of adenovirus gene expression is the extensive processing of viral RNAs. Many adenoviral primary transcripts give the rise to multiple mRNAs that are differentiated by alternative splicing, or by the use of alternative poly (A) sites. These processing events were detected by heteroduplex analysis, nuclease protection mapping and complementary DNA (cDNA) cloning, and sequencing. 5'ends were localized by sequencing of capped oligonucleotides isolated from viral mRNA (Shenk T., 2001)

1.5.2. Activation of late gene expression

The major adenoviral late coding region comprises a single large transcription unit of ~ 28kb in length (Evans R. M., 1977; Nevins J. R., 1978). Due to alternative splicing and differential polyadenylation this transcript gives rise to a minimum of twenty different mRNAs, being grouped into five families (L1 to L5) (Shenk T., 2001).

All of these transcripts are generated from the major late promoter (MLP). It has been shown that the MLP is also active at the early time points of virus gene

expression, but its activity increases by several hundred-fold in the late phase of infection (Shaw A. R., 1980). Transcription during the early phase terminates in the approximate middle of the genome and produces the mRNA of the L1 family encoding the 52/55K proteins (Akusjarvi G., 1981). Delayed activation of MLP is dependent on time-dependent, cis-acting modification of the adenovirus chromosome (Thomas G. P., 1980) and induction of a virus coded transcription factor (Young C. S., 2003).

Besides MLP activation, the accumulation of late mRNA also depends from RNA polymerase actions. All of adenovirus RNA polymerase II, except of the IX and IVa2 transcription units, give rise to two or more alternatively processed mRNAs (Fields Bernard N., 2007).

In HeLa cells infected at multiplicity of 10 plaque-forming units per cell, the early phase lasts for about six hours, after which viral DNA is first detected. The late phase of the cycle begins with expression of late transcription units. At the end of the cycle, about 10^4 progeny virus particles per cell are produced, along with the synthesis of virion proteins and DNA that are not assembled into virions (Green M., 1961).

In general, the adenovirus life cycle lasts between 24 and 36 hours, and in primary cells it can be even longer. It produces up to 10, 000 virions/cell in only one cycle. Eventually, the cells start to show cytopathic effects (CPEs) which are characterized by cell rounding, swelling and detaching from the culture surface of vessels, and an increased size of the nuclei (Kasel J. A., 1979).

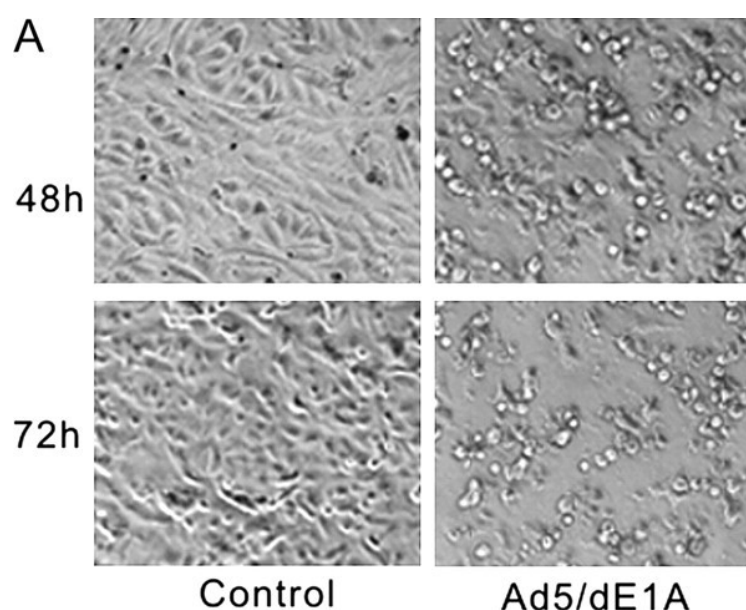


Figure 4. Viral replication in A549 cancer cells. Ad5/dE1A, an adenoviral mutant with a deletion of amino acids 121-129 in the E1A region CR2, was able to induce a cytopathic effect (CPE) in A549 cells, 48h and 72h after infection, when compared to non-infected cells as a control (Zigiang H., 2009).

1.6. Virus-associated non-coding RNAs

Genomic analysis of adenoviruses revealed that they encode one or two so-called virus-associated RNAs (VA-RNAs). Most human adenoviruses encode two VA RNA species, VA RNA I and VA RNA II, but 19% carry only a single VA RNA gene. The major structural features, the terminal stem, the apical stem-loop, and the central domain are conserved in all VA RNAs.

It is assumed that an ancestral single VA RNA gene underwent duplication during evolution, and it is speculated that this ancestral VA RNA gene originated from a cellular tRNA sequence (Ma Y., 1996).

Adenoviruses type 2 and type 5 encode two VA RNAs: VA RNA I and VA RNA II. The both of them are transcribed by RNA polymerase III.

Adenoviruses encode several gene products which counteract the cellular defence mechanisms against the virus. It is known, for example, that E1A and the

VA RNAs inhibit the cellular antiviral interferon response (Reich N. *et al.*, 1988). In the case of E1A, this action is performed through the conserved N-terminal region which binds directly to the signal transducer and activator of transcription 1 (STAT1) transcription factor that mediates IFN-stimulated transcription (Gutch M. J., 1991; Look D. C., 1998).

Deletion of VA RNA I gene in Ad5 virus mutant led to poor growth of the virus and to inefficient protein synthesis during the late phase of infection (Thimmappaya B., 1982). This poor growth is mainly attributable to the function of protein kinase receptor (PKR). In contrast to wt adenovirus, VA RNA mutants are not capable to block the PKR response.

In the last decade it was shown that human adenoviruses are able to inhibit RNA interference at late times of the infection cycle. A panel of adenovirus mutants defective in VA-RNA expression showed that VA RNA I and VA RNA II are functioning as suppressors of RNAi by interfering with the activity of Dicer (Anderson M.G., 2005).

In adenovirus type-5 approximately 80% of Ago2-containing RISC immunopurified from late-infected cells is associated with VA RNA-derived small RNAs (mivaRNAs) and VA RNA II, which is expressed at 20-fold lower levels compared to that of VA RNAI, appears to be the preferred substrate for Dicer and accounts for approximately 60% of all small RNAs in RISC. MivaRNAs have been shown to function as miRNAs, regulating translation of cellular mRNAs (Xu N. *et al.*, 2007).

The synthesis of the adenovirus E2A 72K DNA binding protein, involved in viral DNA replication, and the E3 19K glycoprotein, a membrane protein found associated with the major transplantation antigens in both humans and rodents, are stimulated by VA RNA (Svensson C., 1984).

1.7. Adenovirus vectors for gene transfer

Adenovirus vectors are commonly used in many clinical trials worldwide. About 26% of all vectors are adenoviral vectors. Adenovirus vectors enable very effective delivery of transgenes *in vitro* as well as *in vivo*.

In the last couple of years, the application of adenovirus-based vectors has doubled, indicating growing enthusiasm for the numerous positive characteristics of this gene transfer platform.

Adenovirus vectors can be easily and inexpensively produced to high titers and have a broad applicability for a wide range of clinical conditions, including both gene therapy and vaccine application (Sergey S. Seregin, 2010).

The first generation of adenoviral vectors had only the E1A early gene region deleted, rendering these vectors replication-deficient. For vector production, the E1 functions had to be provided in trans by a complementing producer cell line, such as the HEK293 cell line (Graham F. L., 1977).

First-generation vectors can have an additional deletion in E3 which is dispensable for viral replication in cell culture, yielding a total transgene capacity of these vectors of 8 kb. Major advantages of these vectors include that they can be produced in high titers, and that they transduce quiescent as well as proliferating cells (Volpers *et al.*, 2004). A disadvantage of the first generation vectors is the expression of other viral genes which can result in adaptive immune system responses (Vetrini F., 2010).

Deletion of the E2 and E4 early regions was characteristic for the second generation of adenoviral vectors, which led to lower expression of adenoviral genes, but did not preclude an immune reaction towards transduced tissues (Muruve D. A, 1999; Meulenbroek R. A, 2004).

Helper-dependent adenoviral vectors (HDA_d) or high-capacity adenovirus vectors (HCA_d) constitute the latest generation of adenoviral vectors. They contain only the packaging signal Ψ and the inverted terminal repeats (ITRs) as viral elements and can accommodate up to 36 kb of non-viral DNA. Thus, large cDNAs, longer tissue-specific or regulatable promoters, several expression cassettes or even small genomic loci can be inserted into these vectors (Volpers *et al.*, 2004).

All off these vectors are dependent on a helper virus for amplification. This feature makes them more complicate to handle, but they show great treatment potential (Vetrini F., 2010).

The lack of viral gene expression from these vectors has been shown to considerably reduce their toxicity and immunogenicity *in vivo*, and allow for long-term transgene expression *in vivo* (Morral N., 1998; Schiedner G., 1998).

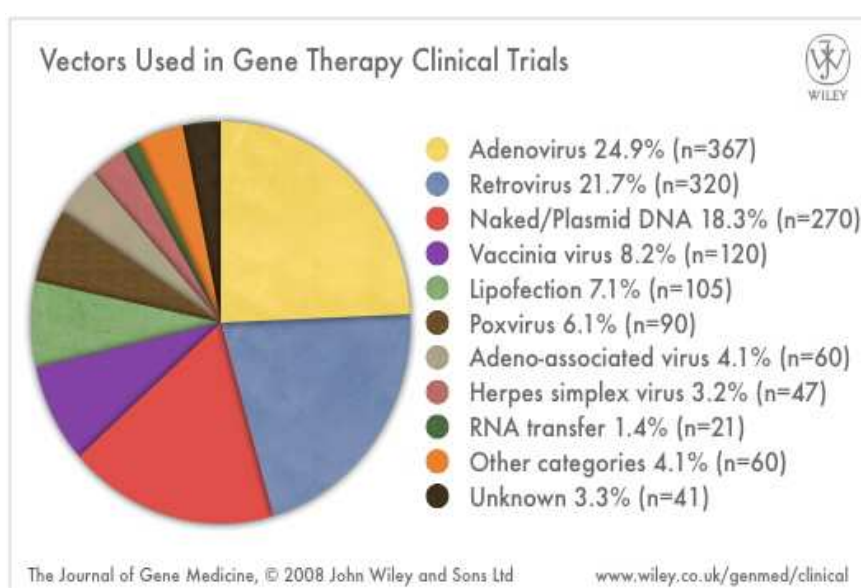


Figure 5. Adenovirus (Ad) vectors are the most widely used vectors in clinical trials (available at: <http://wiki.epfl.ch/gene-therapy/accident-adenoviral>).

1.8. Antiviral drugs

For many years virus diseases have been considered as intractable to selective antiviral chemotherapy because the replicative cycle of the virus was assumed to be too closely interwoven with normal cell metabolism so that any attempt to suppress virus reproduction would be doomed to kill (or severely harm) the uninfected cell as well (Erik de Clercq *et al.*, 2000).

In contrast to some antibiotics, antiviral drugs do not perish their pathogen; instead they prevent its multiplication.

Many antiviral drugs have been highly successful, saving lives and decreasing the morbidity. Examples of antiviral drugs are the anti-human immunodeficiency virus (HIV) drugs. They have been developed for the treatment of the HIV, where they have transformed a progressively fatal disease into a manageable condition in which is possible to inhibit virus replication and to extend a lifetime of the patients. In spite of this progress, there are still many other virus-related diseases that require the effective development of antiviral drugs. Each virus has its own array of specific proteins required for its replication and because of that only a few antiviral drugs developed against one virus can be active against other once.

Some viruses such as HIV or herpes viruses cause latent infections, and treating active infections does not cure the disease. Different viruses, and especially viruses infecting the respiratory tract, are causing similar symptoms making diagnosis more difficult. Because many viral diseases are quickly controlled by immune response, there is need to start very early with the treatment to be effective at all (Fields Bernard N., 2007).

There are many antiviral drugs that have been officially approved for the treatment of virus infections: zidovudine, didanosine, zalcitabine, stavudine, lamivudine, abacavir, nevirapine, delavirdine, efavirenz, saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, and lopinavir for the treatment of human immunodeficiency virus (HIV) infections; lamivudine also for the treatment of hepatitis B virus (HBV) infections; acyclovir, valaciclovir, penciclovir, famciclovir, idoxuridine, trifluridine, and brivudin for the treatment of herpes simplex virus (HSV) and/or varicella-zoster virus (VZV) infections; ganciclovir, foscarnet, cidofovir, and fomivirsen for the treatment of cytomegalovirus (CMV) infections; ribavirin for the treatment of

respiratory syncytial virus (RSV) infections and, in combination with interferon- α , for the treatment of hepatitis C virus (HCV) infections; amantadine and rimantadine for the treatment of influenza A virus infections; and finally, the neuraminidase inhibitors zanamivir and oseltamivir for the treatment of influenza A and B virus infections (Erik de Clercq *et al.*, 2000).

Typical targets for antiviral drugs are such as viral adsorption, penetration, uncoating, viral nucleic acid synthesis and viral protein synthesis (Erik de Clercq *et al.*, 2000).

In many cases there is enough specificity in virus polymerases to be used as an antiviral target. Some of those drugs which act as polymerase inhibitors can be very toxic for the cell and some are well tolerated (acyclovir, ganciclovir). Acyclovir and ganciclovir have to be phosphorylated before becoming active. Ganciclovir is ten times more effective against cytomegalovirus (CMV) than acyclovir. The required phosphorylation step is performed by a herpes virus-encoded kinase.

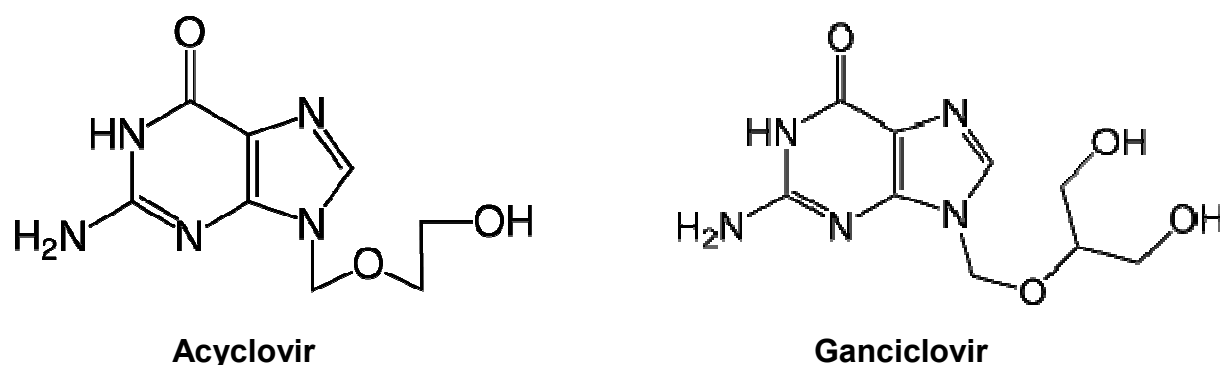


Figure 6. Acyclovir and ganciclovir, two antiviral agents.

Clinical reports about high mortality rates associated with adenovirus infections in transplant recipients show an urgent need for novel treatment options. For instance, small-scale studies have shown that ribavirin is only poorly effective against adenovirus infections, especially when therapy is initiated late in the course of an infection (Bordigoni, P., 2001; Gavin, P. J., 2002; Lankester, A. C., 2004).

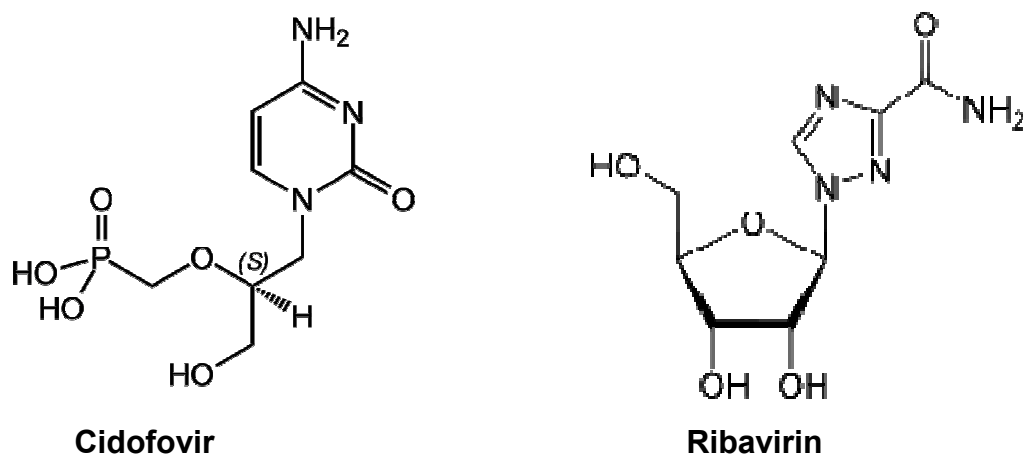


Figure 7. Cidofovir and ribavirin as first clinically used anti-adenovirus drugs.

CDV inhibits adenovirus replication. The monophosphate form is phosphorylated by cellular kinases, and the eventual triphosphate form competes with cellular dNTPs during DNA synthesis (Mary Miu Yee Waye, 2010).

Unfortunately, CDV displays significant cytotoxicity, especially nephrotoxicity.

1.9. Herpes virus encoded genes for the activation of antiviral prodrugs

Non-toxic prodrugs gain toxicity only upon activation by cellular or viral enzymes. A good example of an enzyme-prodrug system constitute the antiherpetic prodrugs ganciclovir (GCV), acyclovir (ACV), and brivudin (BVdU) that are only efficiently activated by thymidine kinases encoded by herpes viruses (Golankiewicz & Ostrowski, 2006). To be converted into the active drug all of them have to go through phosphorylation steps inside the cell. The first phosphorylation step is efficiently performed only by a thymidine kinase (TK) specifically encoded by herpes viruses (Elion *et al.*, 1977).

The resulting monophosphate form is further phosphorylated to the triphosphate form by cellular kinases (Miller & Miller, 1980, Miller & Miller, 1982) which competes with deoxyguanosine triphosphate (dGTP) for incorporation into nascent DNA strands and inhibits DNA polymerisation, consequently blocking DNA replication (Fillat *et al.*, 2003, Ilsley *et al.*, 1995). The efficient and selective phosphorylation by the viral thymidine kinase explains the success of these prodrugs in selectively affecting herpes virus-infected cells.

When experimentally introduced into and (selectively) expressed within (tumor) cells, the viral thymidine kinase gene renders these cells susceptible to prodrugs such as GCV. This fact is exploited in gene-directed enzyme prodrug therapy (GDEPT) approaches for the treatment of various cancers (Portsmouth *et al.*, 2007; Fillat *et al.*, 2003). While allowing the generation of high concentrations of the cytotoxic compound directly within the tumor, general systemic toxicity is avoided or decreased.

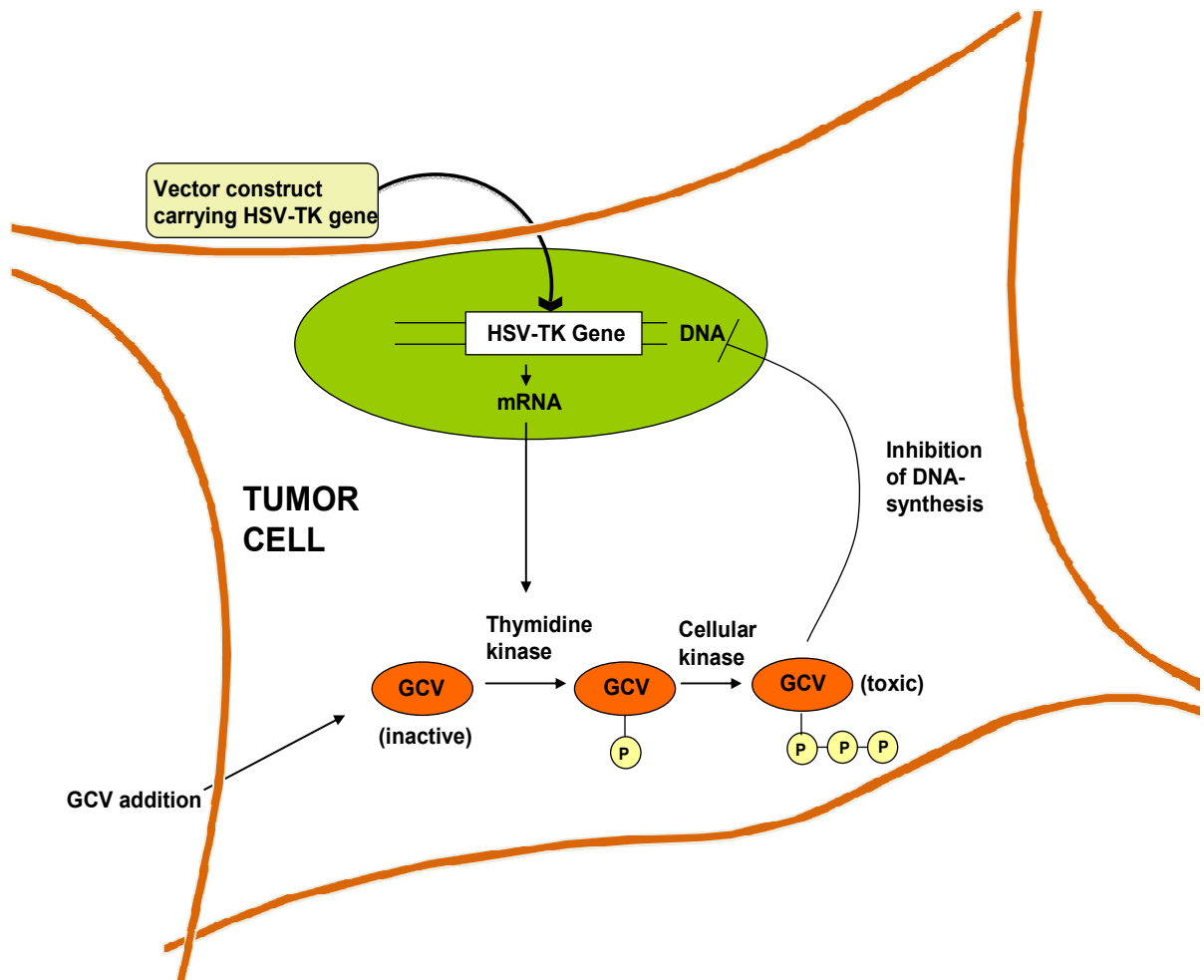


Figure 8. Schematic representation of the herpes simplex virus thymidine kinase (HSV-TK)/Ganciclovir (GCV) system. GDEPT principle.

The herpes simplex virus HSV-TK-based enzyme-prodrug system for cancer therapy was used for the first proof of principle of GDEPT (Moolten, 1986) and has since become the by far most intensively evaluated one. It has been found to be safe and has given encouraging results in several clinical trials (Portsmouth *et al.*, 2007 and references therein). For example, administration of a first-generation adenoviral vector carrying the (HSV-TK) gene followed by systemic treatment with GCV had no toxic side effects in patients with advanced hepatocellular carcinoma (HCC). This was also true for patients with impaired liver function (Sangro B., 2010).

Gene-directed enzyme prodrug therapy (GDEPT) systems		
Enzyme	Prodrug	Metabolite
Cytochrome P450	Cyclophosphamide	4-Hydroxycyclophosphamide
Carboxypeptidase G2	4-[(2-Chloroethyl)(2-mesyloxyethyl) amino]benzoyl-L-glutamic acid (CMDA)	4-[(2-Chloroethyl)(2-mesyloxyethyl) amino]benzoic acid (CMBA)
Carboxylesterase	Irinotecan (CPT-11)	7-Ethyl-10-hydroxy-camptothecin
Herpes simplex virus thymidine kinase (HSV-TK)	Ganciclovir	Ganciclovir triphosphate (GCV-3P)
Cytosine deaminase	5-Fluorocytosine	5-Fluorouracil
Nitroreductase 1	5-Aziridiny-2,4-dinitrobenzamide (CB1954)	5-(Aziridin-1-yl)-4-hydroxylamino- 2-nitrobenzamide

Tabel 1. Currently used gene-directed enzyme prodrug therapy (GDEPT) systems. Sources: Fillat, C. et al. (2003); Danks, M.K. and Potter, P.M. (2004); Niculescu-Duvaz, D. et al. (2003); O., Kingsman, S. and Naylor, S. (2002); Brown, N.L. and Lemoine, N.R. (2004); Denny, W.A. (2002).

Herpes simplex virus thymidine kinase and ganciclovir system are mostly used in gene directed enzyme prodrug therapy (GDEPT) (Moolten F. L., 1990; Smythe W. R., 1994). GCV, a derivative of the nucleoside analogue acyclovir, can be phosphorylated by HSV-TK to form GCV triphosphate, which causes DNA chain termination and cell death. The selective killing of HSV-TK transduced cells is based on the fact that the viral TK is almost 1000-fold more efficient than the mammalian TK at phosphorylating GCV. One of the major obstacles to successful gene therapy for cancer is the difficulty of transducing a high fraction of a cell population (Marc R. Wygoda, 1997). This limitation is partially relieved by the so-called bystander effect

of certain drugs. In field of gene therapy this term means the killing of several types of tumor cells by targeting only one type of cell (Eric J. H. *et al.*, 2003).

It was also demonstrated that HSV-TK transduced cells, having efficient gap junction communication, were significantly protected by bystander cells from drug-induced cytotoxicity (Marc R. W. *et al.*, 1997).

Different to some other GDEPT systems, the functionality of the HSV-TK system is restricted to cells undergoing DNA replication, thus per se restricting cytotoxic effects to cycling cells. One of the drawbacks of HSV-TK/GCV in cancer gene therapy is the limited bystander effect (Fig. 9) on neighbouring tumor cells, which is a result of the inability of GCV-ppp (in contrast to GCV) to pass freely through cellular membranes. Instead, intercellular transport of GCV-ppp requires cell-to-cell contact via gap junctions (Mesnil M., 2000).

However, in human osteosarcoma cells transfected with herpes simplex virus thymidine kinase, GCV demonstrated highly potent anti-adenovirus activity, suggesting that the efficacy of ganciclovir against adenovirus is limited by inefficient phosphorylation in adenovirus-infected cells. However, adenovirus can in principle be made susceptible to GCV by providing the infected cell with the herpes simplex virus-encoded thymidine kinase (Naesence L., 2004).

This makes a concept of enzyme/prodrug therapy to adenovirus infections conceivable.

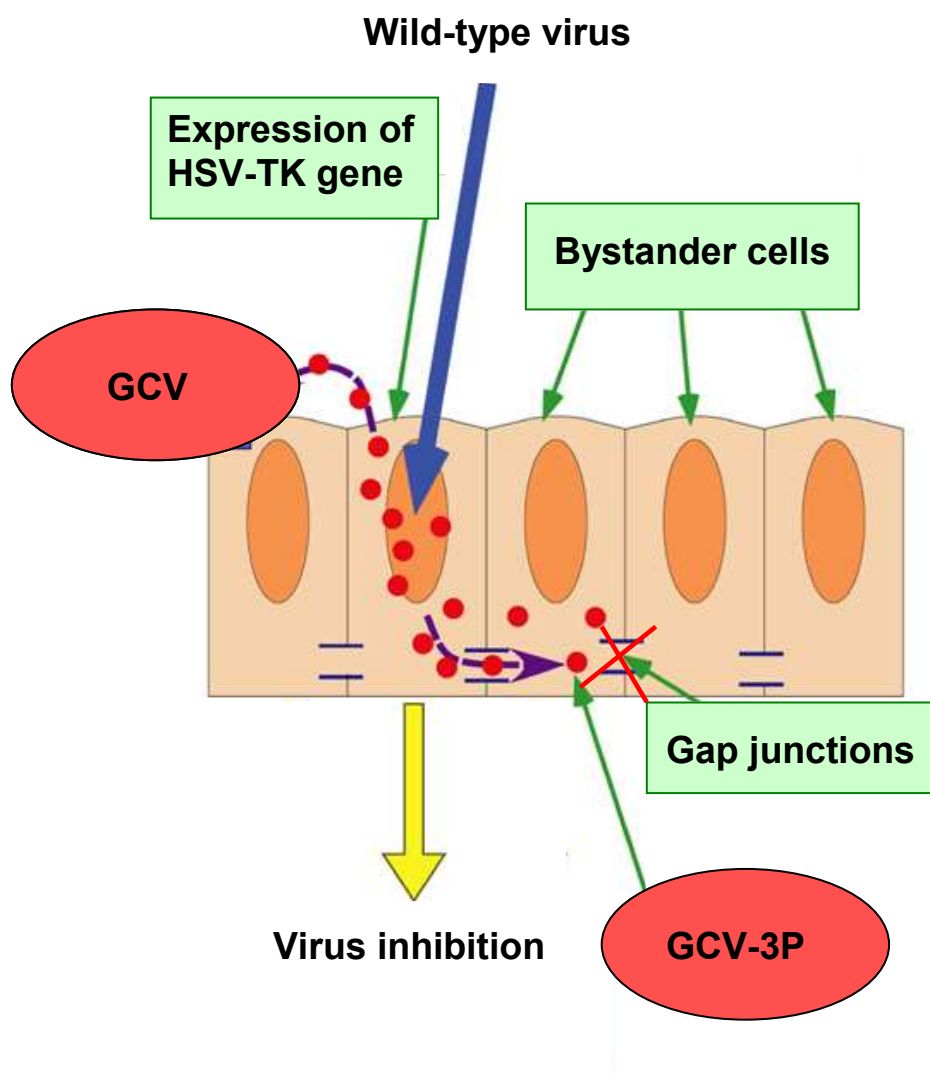


Figure 9. Limited bystander effect of the HSV-TK/GCV system.

2. RNA interference (RNAi) as a therapeutic tool

For a long time RNA was considered only to be an intermediate product between DNA and protein. The discovery that double-stranded RNAs (dsRNAs) in *Caenorhabditis elegans* can trigger the silencing of complementary messenger RNA (mRNA) sequence, led to the birth of RNAi-based tools to knock down gene expression (Castanotto D., 2009).

The RNAi pathway is described in figure 10. Small interfering (si)RNAs, short hairpin (sh)RNAs, and artificial micro (ami)RNAs have become powerful tools for the knockdown of mammalian and viral genes (Lopez-Fraga *et al.*, 2009).

SiRNAs can be synthesized as dsRNAs (of ~21 to 25 nt) and can be transfected directly into cells where they mediate a transient knock-down of the expression of corresponding target genes. Alternatively, siRNAs can be generated inside a cell by expression of synthetic shRNA genes from Pol III promoters. ShRNAs are eventually processed into siRNAs by the endogenous enzyme Dicer. In both cases, siRNAs enter the RNA-induced silencing complex (RISC) where they guide the cleavage of complementary mRNAs.

There are several strategies for harnessing RNAi pathways for therapy. Recombinant inhibitory RNAs are designed to mimic primary miRNAs (pri-miRNAs) (in the case of amiRNAs or exogenous miRNAs) or precursor miRNAs (pre-miRNAs) (in the case of short hairpin RNAs (shRNAs)), whereas chemically synthesized RNA oligonucleotides are designed to mimic Dicer products or substrates (Beverly L., 2011).

The main differences between siRNAs and amiRNAs or shRNAs are the ways of delivery and durability of gene silencing.

Endogenous miRNAs are short ~22 nt long RNAs that are generated from long, sometimes polycistronic primary RNAs (pri-miRNAs) (Brodersen & Voinnet, 2009).

Synthesized from Pol II promoters, pri-miRNAs are first processed by the nuclear endonuclease Drosha, which releases a shorter stem-loop miRNA precursor (pre-miRNA). This stem-loop structure (resembled by the synthetic shRNAs) is further processed by Dicer into the mature miRNA. MiRNAs can either inhibit translation through binding to partially complementary mRNAs, guide Argonaute

(proteins which have catalytic role in RNAi)-dependent degradation of mRNAs when perfectly complementary to those mRNAs, or lead to the degradation of mRNAs as a consequence of accelerated deadenylation (Brodersen & Voinnet, 2009).

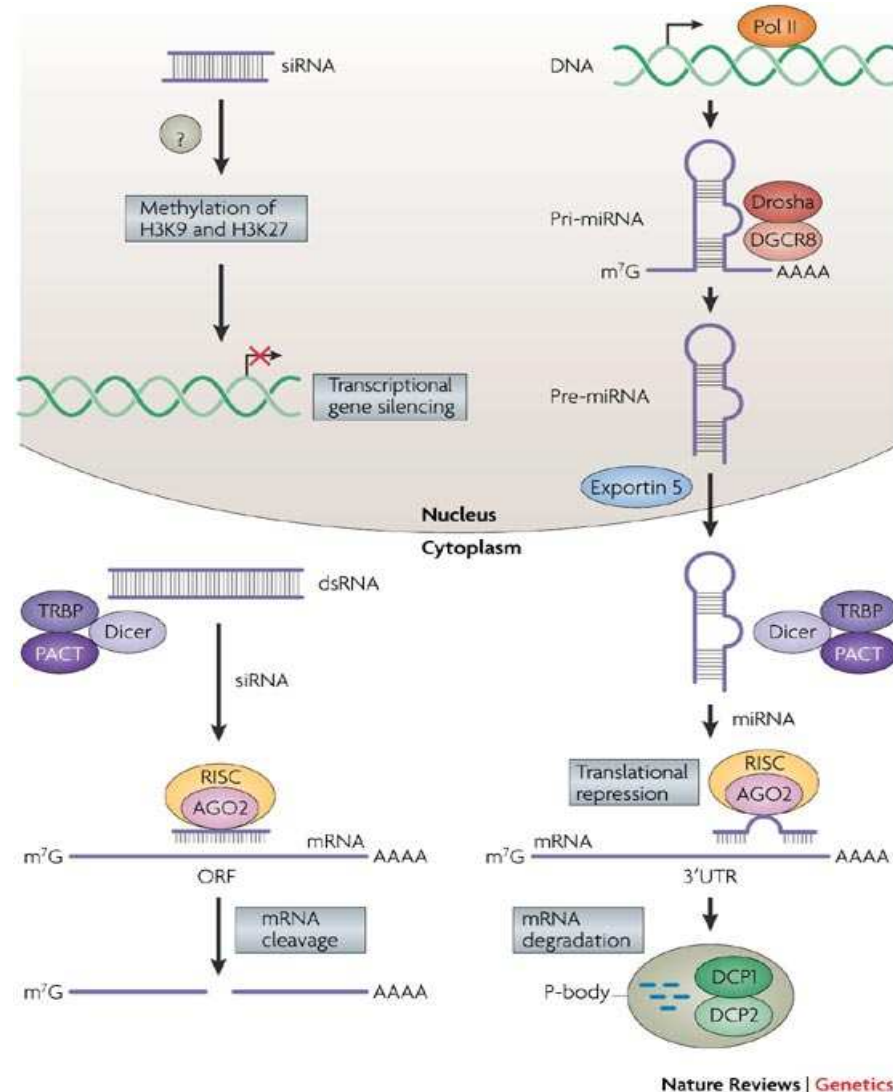


Figure 10. Mechanisms of RNAi in mammalian cells. As shown in the pathway at the bottom left, cytoplasmic double-stranded RNAs (dsRNAs) are processed by a complex consisting of Dicer, TAR RNA-binding protein (TRBP) and protein activator of protein kinase PKR (PACT) into small interfering RNAs (siRNAs), which are loaded into Argonaute 2 (AGO2) and the RNA-induced silencing complex (RISC). The siRNA guide strand recognizes target sites to direct mRNA cleavage, which is carried out by the catalytic domain of AGO2. siRNAs complementary to promoter regions direct transcriptional gene silencing in the nucleus through chromatin changes involving histone methylation (top left); the precise molecular details of this pathway

in mammalian cells are currently unclear. As shown in the pathway on the right, endogenously encoded primary miRNA transcripts (pri-miRNAs) are transcribed by RNA polymerase II (Pol II) and initially processed by Drosha–DGCR8 (DiGeorge syndrome critical region gene 8) to generate precursor miRNAs (pre-miRNAs). These precursors are exported to the cytoplasm by exportin 5 and subsequently bind to the Dicer–TRBP–PACT complex, which processes the pre-miRNA for loading into AGO2 and RISC. The mature miRNA recognizes target sites in the 3' untranslated region (3' UTR) of mRNAs to direct translational inhibition and mRNA degradation in processing (P)-bodies that contain the decapping enzymes DCP1 and DCP2.

H3K9; histone 3 lysine 9; H3K27, histone 3 lysine 27; m⁷G, 7-methylguanylate; ORF, open reading frame (Daniel H. *et al.*, 2007).

Naturally occurring, human miR-30 or murine miR-155 miRNA precursors have been modified by exchanging the original stem-loops with synthetic stem-loops that will later on constitute the synthetic siRNAs (Stegmeier *et al.*, 2005, Dickins *et al.*, 2005, Zeng *et al.*, 2005, Zeng *et al.*, 2002). These artificial introns give rise to amiRNAs which can potentially be used for the treatment of a variety of diseases.

This miRNA-type of expression can employ the entire spectrum of Pol II promoters, thus permitting cell type- or tissue-specific gene targeting.

RNAi has become a promising therapeutic tool for knocking down disease-related cellular or viral genes. Silencing of viral genes has been shown to successfully inhibit a number of mammalian viruses (Lopez-Fraga *et al.*, 2008). Most RNAi approaches for the inhibition of viruses are aimed at inhibiting the further spreading of an infection rather than curing a cell from an acute viral infection, although this was shown to be achievable, albeit with lower efficacy (Yuan *et al.*, 2005).

2.1. Artificial miRNAs (amiRNAs)

Several studies in human cells have shown that amiRNA can function as siRNA. Substituting the stem sequences of the mir-30 precursor with unrelated base-paired sequences, the novel miRNAs can be produced in human cells. This finding suggests that the sequence of the precursor stem does not contribute to mature miRNA production; it is plausible that some natural miRNA precursors, such as mir-30, have a relaxed requirement for their stem sequences and thus might be particularly suitable as “vectors” for novel miRNA production (Zeng Y., 2002). About 50% of known miRNAs are clustered, implying that many miRNAs are transcribed as polycistronic pri-miRNAs and produced from a single transcription unit (Kim V. N., 2006).

AmiRNAs are important tool in RNA interference technology and are commonly applied in gene therapy and gene function studies. Tao Hu and colleagues were able to show the nine expression strategies in which they used construct plasmid vectors expressing amiRNA (amiR-Fluc) against firefly luciferase (Fluc). They have also constructed multi-amiRNA expression vectors containing up to four different amiRNAs. They observed that the relative position of an amiRNAs in the multi-amiRNA expression vectors had no apparent influence on its RNAi activity (Tao Hu *et al.*, 2010)

AmiRNA-based RNA interference has also been used to inhibit viruses such as HIV-1. HIV-1 can escape from RNAi-mediated gene silencing by accumulating mutations in the target sequence (Ying P. L., 2008).

By inserting multiple, different, anti-HIV hairpins into the miRNA polycistron, it could be demonstrated that the occurrence of escape mutants could be prevented, thus assuring a prolonged inhibition of HIV-1 replication. However, the problem with escape mutants arises primarily in the case of RNA viruses which display a generally higher mutation rate compared to DNA viruses (Ying P. L., 2008).

2.2. Vector systems for RNAi

There are two strategies for the delivery of RNA interference reagents: (i) transient RNA interference and (ii) stable or inducible RNA interference. Transiently transfected siRNAs are produced by methods which include chemical synthesis, *in vitro* transcription and recombinant Dicer/*E. coli* RNase II digestion of long dsRNAs. ShRNAs and amiRNAs are expressed from plasmids and viral vectors. The shRNA/amiRNA expression cassettes can also be stably integrated into the genome of target cells, transcribed intranuclearly and processed into siRNAs by Dicer in the cytosol (Yan M., 2007).

Many vector systems have been designed for RNA interference studies. The ones that were developed were plasmid vectors that express shRNAs, controlled by polymerase III promoters. However, these vectors have some limitations such as the fact that only a single shRNA can be expressed from a single RNA polymerase III promoter. In a way to inhibit more than one gene it is required to employ multiple promoters or vectors (Yu J.Y., 2003; Jazag A., 2005).

Regulated expression from RNA polymerase III promoters is mostly more complicated as the expression derived by RNA polymerase II promoter (Kwan-Ho C., 2006).

There are RNA interference vectors that contain a synthetic siRNA or miRNA, expressed from a synthetic stem-loop precursor which is nevertheless based on the miR-30 miRNA precursor (Zang Y., 2005).

Even if a lot of has been made in the use of RNA interference, there are some obstacles, which have to be overcome. A major problem is the issue of delivery *in vivo*. Viral vector systems, such as retroviral or lentiviral vectors were developed to efficiently deliver siRNAs into mammalian cells (Brummelkamp T. R., 2002; Robinson D. A., 2003).

However, these systems have weaknesses such as lack of satisfactory tissue or organ specificity. In some case there is a danger of malignant transformation which may occur upon insertion of the vector into the human DNA.

Efficient delivery of siRNAs into the cells was also achieved by using the recombinant adenovirus vectors (James R.B. *et al.*, 2004). These vectors show a wide tissue tropism and malignant transformation is no issue with these vectors.

2.3. Conditional expression systems for RNA interference

The inability to adjust levels of suppression can impose limitations in the analysis of genes essential for cell survival, cell cycle regulation, and cell development, and suppression of a gene for longer periods may result in nonphysiological responses. This problem can be circumvented by generating inducible regulation of RNAi in mammalian cells. The two most widely used inducible mammalian systems use tetracycline- or ecdysone-responsive transcriptional elements (Sunita G., 2003).

Gossen has first described a tetracycline inducible system for RNA interference studies, containing a transcriptional transactivator that contains a VP16 activation domain fused to a mutant Tet repressor from *E. coli*. For the specific binding to the promoter, transactivator requires binding on tetracycline derivatives such as doxycycline (Gossen M., 1995).

By performing tightly regulated Trp53 knockdown using a tetracycline-based system, it has been also shown that tumors can be induced in mice and demonstrating that such systems can also be functional *in vivo* (Ross A. D., 2005).

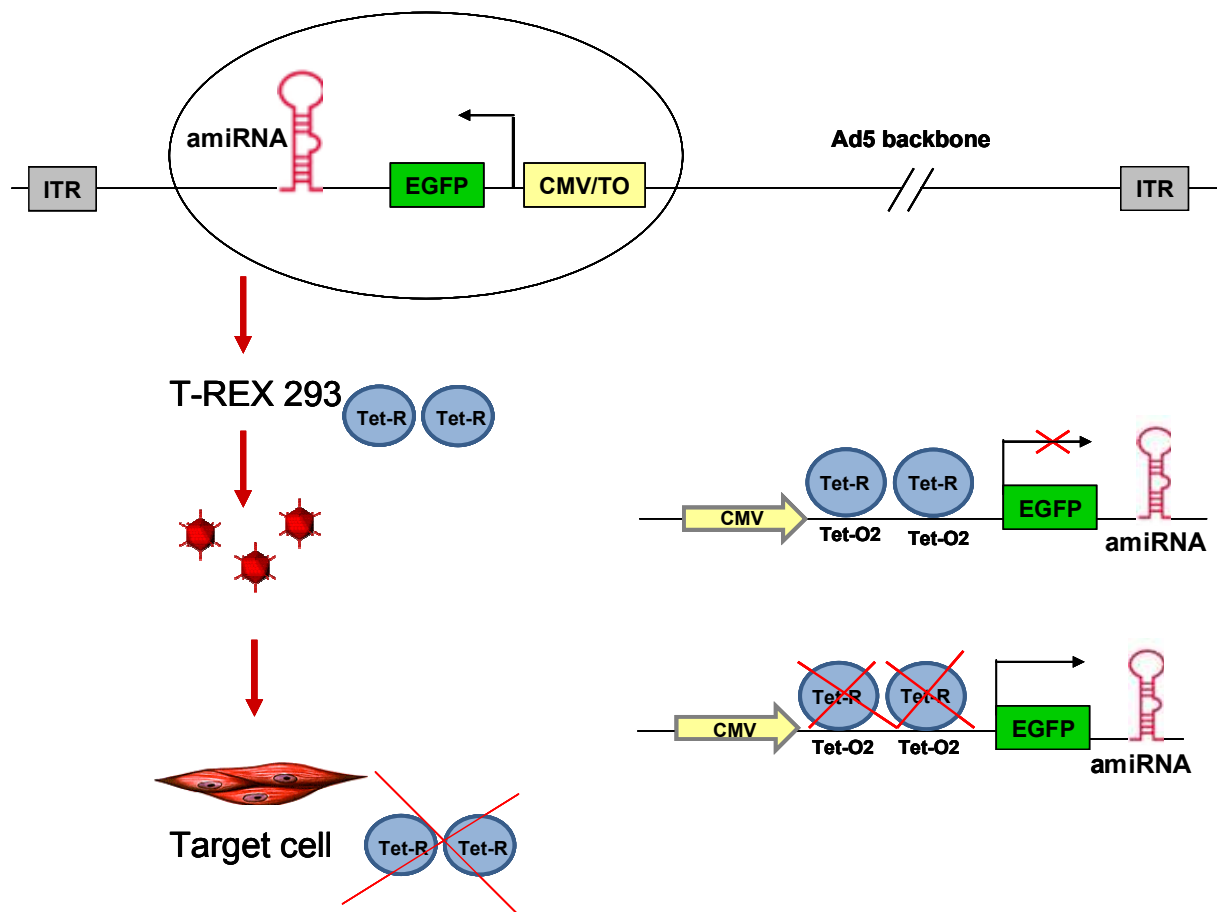


Figure 11. Tetracycline-dependent amiRNA expression. An amiRNA cassette-carrying adenovirus vector is transfected into T-REX 293 cells which produce a tetracycline repressor (Tet-R). Tet-R binds to the tetracycline responsive elements (Tet-O2) within the tetracycline-inducible CMV promoter and blocks the expression of the amiRNA cassette. However, after addition of tetracycline, which binds to the Tet-R, the Tet-R can no longer bind to Tet-O2, and amiRNA expression can take place. The expression of EGFP reporter gene will take place together with amiRNA expression in above described system and can be used for control of amiRNA expression by flow cytometry analysis.

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III. Specific topics

Author's contribution

I hereby declare that I have significantly contributed to the realization of the studies in the thesis presented here.

In the **first part** (*Targeted expression of herpes simplex virus thymidine kinase in adenovirus-infected cells reduces virus titers upon treatment with ganciclovir in vitro*), I participated in the study design and carried out most of the experimental work shown in Fig. 2., Fig. 4., Fig. 6., Fig. 9., Fig. 10. and Suppl. Fig. 1.. I was involved in data analysis and interpretation of results. The manuscript was written together with the co-authors.

In the **second part** (*Inhibition of adenovirus multiplication by short interfering RNAs directly or indirectly targeting the viral DNA replication machinery*), I participated in the study design and interpretation of results, performed MTS-assay experiments (Fig. 8. and Supplementary Fig. 6.), and helped in the optimization of the cell culture conditions. I was also involved in data analysis and writing of the manuscript.

In **third part** (*An adenoviral vector-based expression and delivery system for the inhibition of wild-type adenovirus replication by artificial microRNAs*) I participated in the study design and carried out most of the experiments (Fig. 1., Fig. 2., Fig. 4., Fig. 6., Fig. 7., Fig. 8., Fig. 9., Fig. 10., Fig. 11., Fig. 12., Suppl. Fig. 1., Suppl. Table 1.). I was involved in data analysis and interpretation of results. The manuscript was written together with the co-authors.

In the **fourth part** (*A combined approach for inhibition of adenoviruses*) all presented data were produced by myself.

Wien, 24.09.2012


Mag. Mirza Ibrišimović

1. First part

**TARGETED EXPRESSION OF HERPES SIMPLEX VIRUS THYMIDINE KINASE IN
ADENOVIRUS-INFECTED CELLS REDUCES TITERS UPON TREATMENT WITH
GANCICLOVIR *IN VITRO***

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Targeted expression of herpes simplex virus thymidine kinase in adenovirus-infected cells reduces virus titers upon treatment with ganciclovir *in vitro*

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Abstract

Background Adenoviruses are a frequent cause of life-threatening infections in immunocompromised patients. Available therapeutics still cannot completely prevent fatal outcomes. By contrast, herpes viruses are well treatable with prodrugs such as ganciclovir (GCV), which are selectively activated in virus-infected cells by virus-encoded thymidine kinases. This effective group of prodrugs is not applicable to adenoviruses and other DNA viruses because they lack those kinases.

Methods To render adenoviruses amenable to GCV treatment, we generated an adenoviral vector-based delivery system for targeted expression of herpes simplex virus thymidine kinase (HSV-TK) in wild-type adenovirus 5 (wt Ad5)-infected cells. HSV-TK expression was largely restricted to wt virus-infected cells by transcription of the gene from the Ad5 E4 promoter. Its activity is dependent on the adenoviral E1A gene product which is not produced by the vector but is only provided in cells infected with wt adenovirus. The anti-adenoviral effect of HSV-TK expression and concomitant treatment with GCV was assessed *in vitro* in four different cell lines or primary cells.

Results E4 promoter-mediated HSV-TK background expression was sufficiently low to prevent cytotoxicity in the presence of low-levels GCV in cells not infected with wt Ad5. However, expression was several-fold increased in wt Ad5-infected cells and treatment with low levels of GCV efficiently inhibited wt Ad5 DNA replication. Genome copy numbers and output of infectious particles were reduced by up to > 99.99% and cell viability was greatly increased.

Conclusions We extended the concept of enzyme/prodrug therapy to adenovirus infections by selectively sensitizing adenovirus-infected cells to treatment with GCV. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords Adenovirus infection; ganciclovir; gene therapy; HSV-TK

Introduction

In the immunocompetent host, human adenoviruses are responsible for mostly self-limiting respiratory, intestinal and ocular infections with predominant occurrence in children [1]. By contrast, adenovirus infections in immunocompromised patients, such as solid organ transplant but particularly allogeneic stem cell transplant recipients, often cause much more severe manifestations (e.g. hemorrhagic cystitis, nephritis, pneumonia, hepatitis, enterocolitis and disseminated disease) [2–4], which may be life-threatening or even lethal. Among hematopoietic stem

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cell transplant recipients disseminated disease can lead to mortality rates as high as 80% [5–9].

Several agents, including ribavirin and cidofovir, have been evaluated for anti-adenoviral activity, revealing convincing clinical efficacy only for cidofovir. Although cidofovir is currently the most effective anti-adenoviral drug available, its limited activity is unable to prevent significant mortality rates of invasive adenoviral infection [1,10–13]. Effective responses or complete adenovirus clearance may only be achievable upon lymphocyte recovery with re-establishment of a specific immune response [10,14,15]. Derivatives of cidofovir with potentially higher anti-adenoviral activity and/or lower nephrotoxicity have been developed, although they are currently still under investigation [16,17].

Cytomegaloviruses (members of the herpes virus group) are another major threat to immunocompromised patients. However, they can effectively be controlled with antitherpetic prodrugs such as ganciclovir (GCV), aciclovir, brivudin and derivatives [18–21]. The reason for the success of these prodrugs is their much more efficient phosphorylation by herpes virus-encoded thymidine kinases (TKs) compared to cellular TKs [22]. This (rate limiting) phosphorylation step and two subsequent phosphorylation steps mediated by cellular TKs eventually convert the nontoxic prodrugs into their active forms, such as GCV triphosphate (GCV-ppp) [23]. The final toxic derivative competes with natural deoxynucleoside triphosphates (e.g. dGTP in the case of GCV-ppp) for incorporation into nascent DNA strands, thereby inhibiting DNA polymerization and ultimately blocking viral (and cellular) DNA replication [24,25]. Other DNA viruses, including adenoviruses, lack a TK gene of the herpes virus type and are therefore resistant to prodrugs of the GCV group.

When experimentally introduced into and expressed within cells, the viral TK gene renders transduced cells susceptible to prodrugs such as GCV. This principle is exploited in gene-directed enzyme prodrug therapy (GDEPT) employed for the killing of cancer cells [24,26,27]. Although several other GDEPT systems for cancer gene therapy have also been developed, the HSV-TK/GCV system is by far the most intensively evaluated [24].

In principle, wild-type (wt) adenovirus DNA replication is susceptible to GCV-ppp-mediated inhibition provided that infected cells are armed with the HSV-TK gene [28,29]. However, for an extension of the HSV-TK/GCV-based GDEPT concept to the inhibition of adenovirus infections, the targeted delivery of HSV-TK into or selective expression within adenovirus-infected cells is a prerequisite. Full activity of most adenoviral promoters is dependent on the viral E1A gene product whose synthesis is the first step during adenovirus gene expression. E1A influences the expression of several cellular genes or interferes with cellular factors, which in turn causes the transactivation of other adenoviral promoters [30,31]. The activity of these promoters is dependent on the presence of the E1A gene products, thus making them to conceivable candidates for targeted expression of HSV-TK in wt adenovirus-infected cells.

In the present study, we evaluated the potential of a series of adenoviral promoters for selective expression of the HSV-

TK gene via a replication-incompetent adenoviral vector in cells infected with wt adenovirus 5 (Ad5). When inserted into the deleted E1 region of a replication-deficient adenoviral vector, some promoters such as the E4 promoter revealed low background activity in cells not infected with wt Ad5, as demonstrated by reporter gene assays. Background expression of the HSV-TK gene incorporated into this region and controlled by the E4 promoter was sufficiently low to largely prevent cytotoxicity in the presence of low levels of GCV in cells not infected with wt Ad5. However, upon full activation of the promoter in the presence of wt Ad5, HSV-TK levels increased to levels leading to the pronounced inhibition of wt virus replication upon treatment with GCV. A dramatic decrease in the production of infectious virus progeny was achieved, which was also reflected by significantly increased cell viability in adenovirus-infected cultures. Taken together, we extended the concept of expression-targeted HSV-TK/GCV GDEPT to adenovirus infections and provided proof-of-principle for its functionality *in vitro*.

Materials and methods

Cell culture

All of the cells used in the present study were maintained in Dulbecco's modified Eagle's medium (DMEM) with stabilized glutamine (PAA) supplemented with 10% fetal bovine serum (FBS; PAA): HEK293 (human embryonic kidney; ATCC CRL-1573), A549 (human epithelial lung carcinoma; ATCC CCL-185), HeLa (human epithelial carcinoma; ATCC CCL-2), Hep3B (human hepatocellular carcinoma; ATCC HB-8064), HepG2 (human hepatocellular carcinoma; ATCC HB-8065), SW480 (human colon carcinoma; ATCC CCL-228), HCT116 (human colorectal carcinoma; ATCC CCL-247), T-47D (human mammary gland ductal carcinoma; ATCC HTB-133), RD-ES (Ewing's sarcoma; ATCC HTB-166), IMR-90 (normal human embryonic lung fibroblasts; ATCC CCL-186), MRC-5 (normal human lung fibroblasts; ATCC CCL-171), primary human neonatal dermal fibroblasts (ATCC PCS-201-010), primary normal human kidney cells (kindly provided by Regina Grillari, University of Natural Resources and Applied Life Sciences, Vienna, Austria), primary human myometrium stroma cells (kindly provided by Iveta Yotova, Medical University of Vienna, Austria), HUVEC (primary human uterine microvascular endothelial cells; kindly provided by Iveta Yotova, Medical University of Vienna, Austria) were cultivated in endothelial cell culture medium (Technoclone). HUVEC (primary human umbilical vein endothelial cells; C2517A; Lonza, Allendale, NJ, USA) were grown in supplemented endothelial growth medium (Lonza EGM[®]-2 BulletKit[®]). Human adipose tissue-derived mesenchymal stem cells (kindly provided by Regina Grillari, University of Natural Resources and Applied Life Sciences, Vienna, Austria) were maintained in DMEM/Ham's-F12 (dilution 1:1) (PAA), 4 mM L-glutamine, 10% FBS (PAA), 1 ng/ml FGF and hFOB 1.19 (human fetal

osteoblasts; ATCC CRL-11372) were cultivated in DMEM/Ham's-F12 (dilution 1:1) (PAA), 2.5 mM L-glutamine, 10% FBS (PAA). All cell culture media were supplemented with penicillin/streptomycin (PAA) and cells were generally maintained in a humidified 5% CO₂ atmosphere at 37°C.

Vector constructions

Adenoviral vectors were constructed by first creating plasmid entry vectors harboring the various expression cassettes that were subsequently moved into an adenoviral vector by site-specific recombination using the Gateway® recombination system (Invitrogen, Carlsbad, CA, USA). Construction of entry vectors: for the construction of the enhanced green fluorescent protein (EGFP) reporter vectors, a fragment harboring the EGFP gene including the downstream SV40 poly(A) signal was amplified by polymerase chain reaction (PCR) from pEGFP-N1 (Clontech, Palo Alto, CA, USA) with primers pEGFP-f1 and pEGFP-r2 (all primers are listed in Table 1), restricted with *Xmn*I and *Xho*I and inserted into the respective sites of pENTR4 (Invitrogen). The E2B promoter sequence was amplified by PCR from wt Ad5 using primers E2p-f2 and E2p-r2, cut with *Sac*II and *Age*I and inserted into the *Sac*II and *Age*I sites of pENTR-EGFP1, giving rise to plasmid pENTR-EGFP-E2. To bring the expression cassette into the desired inverse orientation for recombination with the adenoviral destination vector during a later construction step, the entire cassette was inverted by excision with *Sal*I and *Xho*I and reinsertion it into the same sites. *Escherichia coli* clones were screened for plasmids harboring the cassette in the inverse orientation. The derivative of pENTR-EGFP-E2 containing the cassette in the inverse orientation was designated pEE2i. pENTR-EGFP-CMV containing the CMV promoter instead of the E2B promoter and its derivative pECMV containing the expression cassette in the inverse orientation were created

analogously to pENTR-EGFP-E2 and pEE2i, respectively. The CMV promoter fragment was amplified by PCR from pEGFP-N1 with primers CMV-f1 and CMV-r1. Entry vectors containing promoters other than the CMV and E2B promoters were constructed by replacing the CMV promoter-harboring *Age*I-*Sac*II fragment of pECMV with *Age*I/*Sac*II-restricted PCR fragments containing the respective promoter sequences. E3, E4, IVa2, IX and major late promoter sequences were amplified from pAd/PL-DEST (Invitrogen) or wt Ad5 (E3 promoter) by PCR using primers E3p-f1/E3p-r1, E4p-f1/E4p-r1, IVa2p-f1/IVa2p-r1, pIXp-f1/pIXp-r1, and MLP-f1/MLP-r1, respectively. The resulting entry vectors were designated pEE3 (E3 promoter), pEE4 (E4 promoter), pEIVa2 (IVa2 promoter), pEIX (IX promoter) and pEMLP (major late promoter). For the construction of entry vectors carrying the HS4 insulator element upstream of the expression cassettes the HS4 insulator sequence was excised from pSLJCa (provided by André Lieber, University of Washington, Seattle, WA, USA) as an *Xba*I-*Xba*I fragment, blunted with T4 DNA polymerase, and inserted into the *Eco*RV sites of pEE2i, pEE3 and pEE4, respectively, in two possible orientations, each giving rise to vectors pEE2i_9/pEE2i_1, pEE3_3/pEE3_7 and pEE4_3/pEE4_7, respectively. To create entry vectors additionally carrying the HS4 insulator element downstream of the expression cassettes, the blunted *Xba*I-*Xba*I HS4 insulator fragment was inserted into the *Xmn*I sites of those vectors. The resulting vectors were designated pEE2i_9.2, pEE2i_9.3, pEE2i_1.10, pEE2i_1.2 (E2 promoter); pEE3_3.1, pEE3_3.5, pEE3_7.1, pEE3_7.3 (E3 promoter); and pEE4_9.2, pEE4_9.3, pEE4_1.10, pEE4_1.2 (E4 promoter), respectively. To create HSV-TK-harboring entry vectors, a HSV1-originating TK gene fragment was amplified from vector pcDNA3NeoTK (provided by Christine Hohenadl, University of Veterinary Medicine, Vienna, Austria), restricted with *Age*I and *Not*I and inserted into the respective sites of pECMV, pEE2i, pEE3, pEE4, pEIVa2, pEIX and pEMLP, respectively, giving rise to vectors pECMV_TK,

Table 1. Primers and probes

Primer/probe	Sequence
pEGFP-f1	5'-CTCAAGGAACCAATTCTGCAGTCGACGGTAC-3'
pEGFP-r2	5'-TACAATCTCGAGCTTAAGATACATTGATGAGTTGG-3'
CMV-f1	5'-ACCGCCCCGCGGTAGTTATTAATAGTAATCAATTACGG-3'
CMV-r1	5'-CGACTGACCGGTTTCAAGCTTGAAGCTCGA-3'
E2p-f2	5'-GACAGGTGCTGGCCGCGGGTGTGGCCGCTGGAG-3'
E2p-r2	5'-AGCTTCGGCGCAACCGGTAAGACGCGGAGGCTCTTTC-3'
E3p-f1	5'-CAACGGAATCCGCGGCCACCGAAACCGAATTC-3'
E3p-r1	5'-TCTGAAATGACCGGTCCGGACGGAGACCAAG-3'
E4p-f1	5'-AGATCTTGACCGCGGGTACGTAAGTTTATAG-3'
E4p-r1	5'-TGGTAAACACCGGTCGACACGGCACCAGCTC-3'
IVa2p-f1	5'-CACCCCTTCGCGGCCCTTCAGGAACAC-3'
IVa2p-r1	5'-GCGGTGTTACCGGTCTCTCTGATAGAAAC-3'
MLP-f1	5'-TGGGAGGGGCGCGGTCTGTGCTCACTAG-3'
MLP-r1	5'-CACCGCGGGACCGGTGAATATCAATCTCTCTC-3'
pIXp-f1	5'-ACCATGAAGCCGCGGAAGGTGCTGAGGTACGATG-3'
pIXp-r1	5'-CGAGTTGGTACCGGTGCGGCGGCGGCTGCTG-3'
HSV1-TK-f1	5'-GCTAACTAGACCGGTCACTGCTTACTGGCTTATCG-3'
HSV1-TK-r1	5'-TAGACTCGAGCGGCCGCACTGTGCTGGATAT-3'
AdC-E1A-for	5'-GACGGCCCCGGAAGATC-3'
AdC-E1A-rev	5'-TCCTGCACCGCCAACATT3-3'
AdC-E1A-p	5'-CGAGGAGGCGGTTTCGCAGA-3'

pEE2i_TK, pEE3_TK, pEE4_TK, pEIVa2_TK, pEIX_TK and pEMLP_TK, respectively.

Construction of adenoviral vectors: the expression cassettes of all entry vectors were inserted into the deleted E1 region of pAd/PL-DEST (Invitrogen) by site-specific recombination via sites flanking the expression cassettes using LR Clonase™ II in accordance with the manufacturer's instructions (Invitrogen). All created adenoviral vectors are depicted in Figure 1. Restriction enzymes and DNA-modifying enzymes were purchased from Fermentas (Glen Burnie, MD, USA) or New England Biolabs (Beverly, MA, USA). PCR reactions were performed with *Pwo* DNA polymerase obtained from Roche Diagnostics (Basel, Switzerland).

Nucleic acid extractions

Circular plasmid DNA was extracted with QIAprep® Spin Miniprep Kits (Qiagen, Valencia, CA, USA), EasyPrep® Pro Plasmid Miniprep Kits (Biozym, Scientific GmbH, Oldendorf, Germany) or HiSpeed® Plasmid Midi Kits (Qiagen). PCR products were purified with a QIAquick® PCR Purification Kit (Qiagen) and linearized adenoviral vector DNA was purified on EasyPrep® Pro Plasmid Miniprep columns (Biozym).

Virus production and titer determination

Recombinant viral vector DNA isolated from *E. coli* was cut with *PacI* to expose the ends of the inverted terminal repeats. 2×10^6 HEK293 cells were seeded into six-well plates and, 24 h after seeding, cells were transfected with 10 µg of *PacI*-digested, linearized, purified adenoviral plasmid DNA using Lipofectamin 2000 in accordance with the manufacturer's instructions (Invitrogen). The day after, medium was removed and, after another 24 h, cells were transferred into fresh T75 flasks. Cells were maintained until approximately 80% of cytopathic effect was observed. Cells and cell supernatants were harvested and subjected to three freeze-thawing cycles to liberate adenovirus particles. Cell debris was removed by centrifugation for 15 min at $845 \times g$. Crude virus suspensions were filtered through 0.45 µm filters and stored at -80°C . Purification of virus particles was performed using Vivapure® AdenoPACK™ 20 columns (Sartorius, Gottingen, Germany) or by standard CsCl density gradient ultracentrifugation. Infectious virus particle titers of recombinant and wt Ad5 were determined on HEK293 cells by 50% tissue culture infective dose (TCID₅₀) assays. Titers of wt Ad5 present in mixed virus suspensions containing both wt and recombinant virus as obtained in co-infection experiments were determined on A549 cells.

Determination of gene expression rates

For experiments with subconfluent cultures, appropriate numbers of cells were seeded into the wells of 96-well or 48-well plates to achieve an approximate 50%

confluency 24 h after seeding. For experiments with confluent cultures, higher numbers of cells were seeded to obtain 100% confluency the day after. Additionally, those cells that were amenable to contact inhibition were cultivated for an additional 3–4 days after having reached confluency. Cells were infected with recombinant adenoviruses expressing either the EGFP or the HSV-TK gene at multiplicities of infection (MOI) of 100 (all fibroblasts: MRC-5, IMR-90, primary fibroblasts) or 50 TCID₅₀/cell (all other cells). Twenty-four after infection, cells were washed once with fresh cell culture medium. Four hours after washing, cells were superinfected with wt Ad5 at MOIs of 500 (MRC-5, IMR-90, primary fibroblasts) or 200 TCID₅₀/cell (all other cells) or were mock-superinfected. All infection experiments were performed in triplicates. EGFP expression rates were determined by fluorescence-activated cell sorting (FACS) analysis. Generally, FACS measurements were performed 48 h after infection with wt Ad5 with the exception of HS4 insulator evaluation experiments for which FACS analysis was conducted 24 h post-infection. Briefly, cells were harvested by trypsinization, resuspended in normal cell culture medium and pelleted by centrifugation at $135 \times g$ for 5 min. Thereafter, cells were washed once with phosphate-buffered saline (PBS) and fixed with 1% formaldehyde in PBS. Samples were analyzed with a FACS Calibur analyser (Becton-Dickinson Biosciences, Franklin Lakes, NJ, USA) and percentages of fluorescent cells and mean fluorescence intensities were calculated. HSV-TK expression was measured by western blot analysis: cells were pelleted, washed once with cold PBS and subsequently lysed on ice for 15 min in a buffer containing 20 mM Tris-HCl, pH 7.5, 400 mM NaCl, 0.5% NP-40, 0.3% Triton X-100 and a cocktail of protease inhibitors (complete, EDTA-free; Roche Diagnostics) at the recommended concentration. Lysates were cleared from cell debris by centrifugation at $16\,000 \times g$ for 15 min at 4°C and protein concentrations were determined using a Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Some 30 µg (A549, MRC-5) or 15 µg (HUVEC) total protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on NuPAGE® Novex 10% Bis-Tris gels (Invitrogen) and transferred to nitrocellulose membranes (Whatman, Dassel, Germany). Membranes were stained with Ponceau S to visualize protein concentration and subsequently blocked with blocking reagent (Roche Diagnostics). HSV-TK was detected with a primary goat anti-HSV1-TK antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a secondary fluorescent donkey anti-goat antibody (Li-Cor Biosciences, Lincoln, NE, USA). Fluorescence was monitored using a LI-COR Odyssey detection system (Li-Cor Biosciences).

Virus inhibition experiments

Infections of A549, HUVEC and MRC-5 cells with recombinant HSV-TK-expressing recombinant adenoviruses were performed essentially as described for the EGFP and HSV-TK expression experiments. Infection experiments were carried out in triplicate with confluent cells seeded into 96-well plates. Cells were infected with

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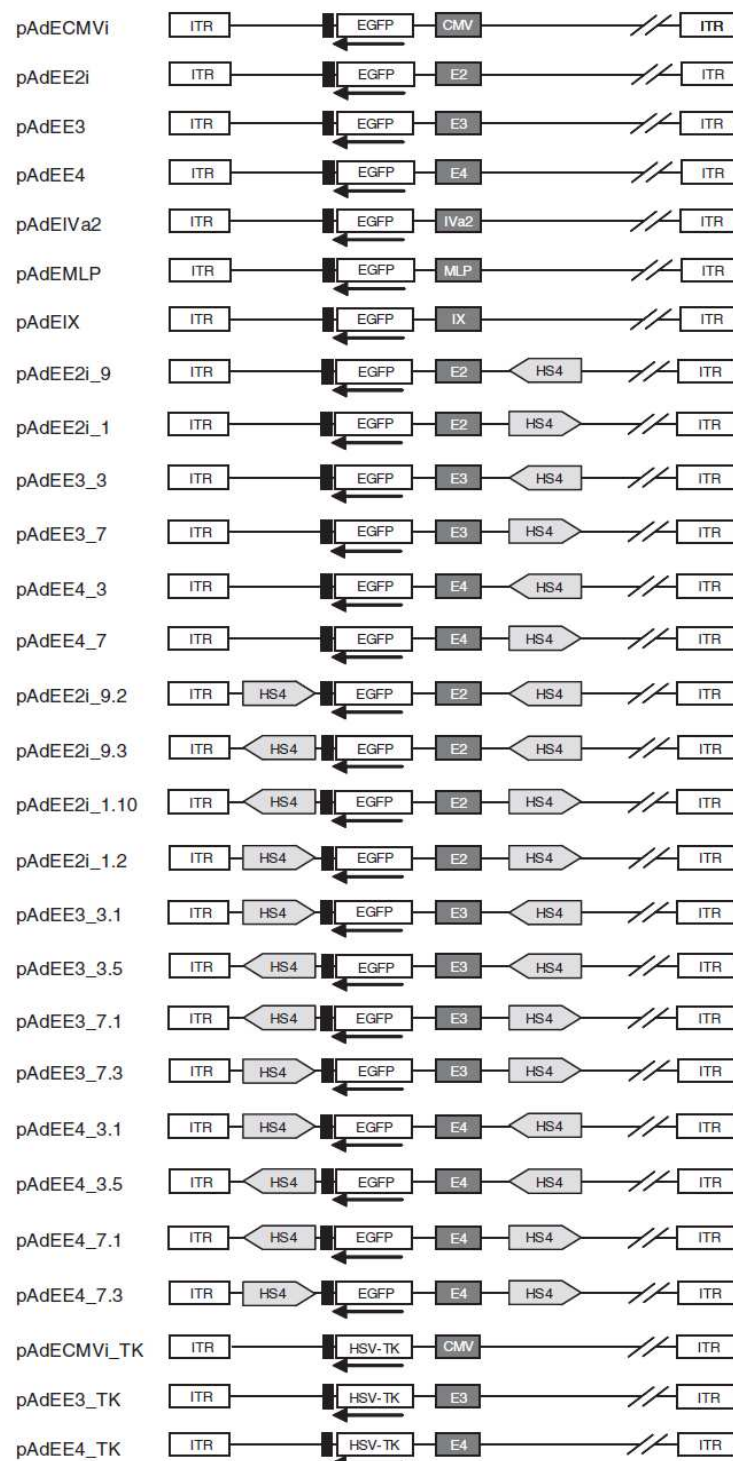


Figure 1. Schematic representation of adenoviral vectors. All vectors are based on the Ad5-derived vector pAd/PL-DEST (Invitrogen). Expression cassettes were inserted into the deleted E1 region of the parental vector in antisense orientation with respect to the left inverted terminal repeat (ITR). Expression cassettes consist of the indicated EGFP or HSV-TK genes, the indicated promoters (dark grey boxes) and a SV40 poly(A) sequence (black boxes). The direction of transcription of the transgenes is indicated with arrows beneath the boxes representing the genes. HS4 insulator elements and their orientations are indicated by shaded arrows.

recombinant viruses at a MOI of 100 TCID₅₀/cell and incubated for 24 h. Twenty-four post-infection, cells were washed once with fresh cell culture medium, cultivated for an additional 4 h and subsequently superinfected with 300 TCID₅₀ units of wt Ad5. Additionally, GCV (InvivoGen) or cidofovir (CDV; InvivoGen, Vienna, Austria) was added to each well in different concentrations. Plates were incubated for 0, 2, 4 and 6 days without change of medium before freezing at -80°C . Crude virus suspensions were obtained by freeze-thawing the plates thrice and removal of cell debris by centrifugation for 15 min at $740 \times g$.

Determination of genome copy numbers

Wt Ad5 genome copy numbers were determined by real-time quantitative PCR (qPCR) on a LightCycler[®] 480 (Roche Applied Science/Roche Diagnostics Austria GmbH, Vienna, Austria) using primers AdC-E1A-forward, AdC-E1A-reverse and probe AdC-E1A (Table 1) directed against the E1A region of Ad5. Quantifications were directly performed on dilutions of crude virus suspensions in duplicate. Fluorescence signals were analyzed using the LightCycler[®] 480 software, version 1.5. Ad5 genome copy numbers were calculated by using serial dilutions of an Ad5 reference DNA serving as a standard. All samples were analyzed in duplicate.

Cell viability

The experimental setup for the determination of the viability of cells infected with recombinant adenoviruses and superinfected with wt Ad5 were as described for the other virus inhibition experiments. For the determination of HSV-TK background expression-related cytotoxicity, the experiments were carried out in essentially the same way, with the exception that superinfection with wt Ad5 was omitted. All infection experiments were carried out in triplicate or quadruplicate. Viability of the cultures was determined 6 days after the addition of GCV using an MTS assay (CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay) in accordance with the manufacturer's instructions (Promega, Madison, WI, USA). Absorbance was determined at 490 nm.

Results

Adenovirus promoters inserted into the deleted E1 region of an adenoviral vector display different activity in wt Ad 5-infected cells

For the proof principle that selective expression of HSV-TK in wt adenovirus-infected cells can confer susceptibility to antiherpetic prodrugs *in vitro*, we created a delivery system for HSV-TK based on a replication-deficient adenoviral vector lacking the E1 and E3 genes. To restrict HSV-TK expression to cells infected with wt adenovirus,

we aimed at expressing the HSV-TK gene from selected adenoviral promoters. As a result of the dependence on the E1A gene products, those promoters were expected to be fully activated only in the concomitant presence of wt adenovirus in the same cell. We intended to insert the promoters into the deleted E1 region of a common E1/E3-deleted adenoviral vector, assuming that at least some of them would retain their functionality even in this unnatural location. Thus, we generated a set of replication-deficient Ad5-derived vectors based on pAd/PL-DEST (Invitrogen) that harbor Ad5-derived E2B, E3, E4, IVa2, IX and major late promoter (MLP) sequences in the deleted E1 region. To evaluate the performance of the promoters on a cell-to-cell basis and to simultaneously assess infectabilities of cells, an EGFP reporter gene was inserted downstream of the them, giving rise to vectors pAdEE2i, pAdEE3, pAdEE4, pAdEIVa2, pAdEMPL and pAdEIX (Figure 1). A control vector (pAdECMV) carrying the strong CMV promoter, which should be active under all conditions, was constructed as well and served as a measure for EGFP expression and infectability of cells. For construction details, see the Materials and methods.

The vectors were used to infect subconfluent and confluent A549 human lung cancer epithelial cells at a MOI of 50 TCID₅₀/cell followed by superinfection with wt Ad5 or mock-superinfection 24 h post-infection with the recombinant viruses. For superinfection with wt Ad5, we used relatively high MOIs of 200 TCID₅₀/cell because these MOIs ensured infection rates of almost 100% as determined in the preceding experiments. EGFP expression levels were measured by FACS analysis 48 h post-infection with wt Ad5 (Figure 2A). In almost all cases, superinfection of the cells with Ad5 (black bars) led to an increase in EGFP expression. However, the overall expression rates in superinfected cells differed dramatically depending on the promoters used: the IVa2, pIX and major late promoters permitted only low-level EGFP expression. The E2B and E4 promoters showed overall similar and reasonably high activities upon Ad5 superinfection and low activities in the absence of wt Ad5. The E3 promoter led to significantly higher EGFP expression rates, although at the cost of a relatively high background expression in the absence of wt Ad5. We repeated these experiments with MRC-5 normal human lung fibroblasts (Figure 2B) and primary HUVEC (Figure 2C) and received comparable results. Again, the IVa2, pIX and major late promoters performed poorly and the overall E2B and E4 promoter activity patterns in Ad5-superinfected cells were comparable to those obtained with A549 cells. For all cells and experiments taken together, the E2B and E4 promoters showed comparable induction rates of approximately 11- and nine-fold, respectively. The E3 promoter performed slightly differently in MRC-5 and HUVEC cells compared to A549 cells because the background expression in these was significantly lower. However, high background expression mediated by the E3 promoter was consistently observed for A549 cells.

Based on these results, we narrowed our candidate promoters down to E2B, E3 and E4. Because background expression and the inducibility of the promoters may be

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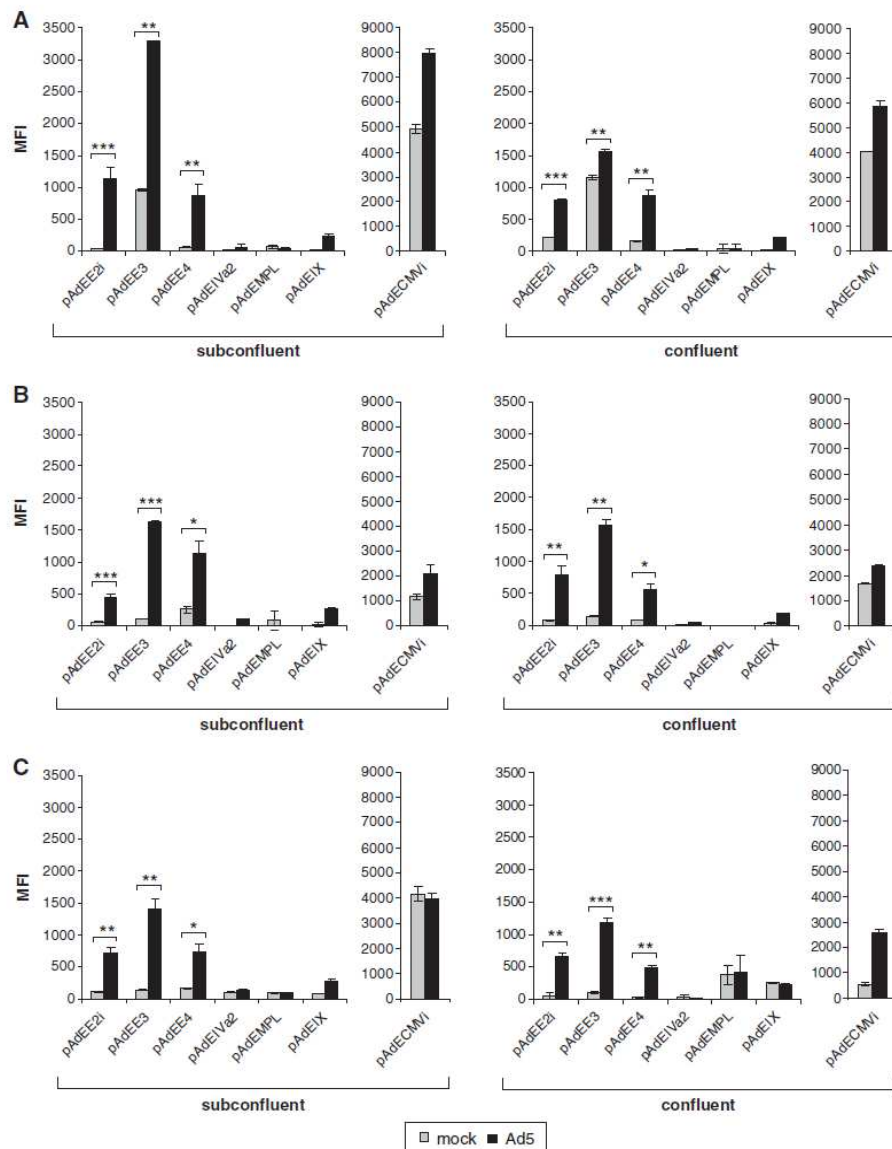


Figure 2. EGFP expression from various adenoviral promoters in either the absence or the presence of superinfecting wt Ad5. Subconfluent and confluent A549 (A), MRC-5 (B) and HUVEC cells (C) were infected with recombinant EGFP expression vectors pAdEE2i (E2B promoter), pAdEE3 (E3 promoter), pAdEE4 (E4 promoter), pAdEIVa2 (IVa2 promoter), pAdEMPL (major late promoter) and pAdEIX (pIX promoter) or as a control with the CMV promoter-harboring vector pAdECMV1 at a MOI of 50 (A549, HUVEC) or 100 (MRC-5) TCID₅₀/cell. Twenty-four hours after infection with the recombinant vectors, cells were either superinfected with wt Ad5 (black bars) at a MOI of 200 (A549, HUVEC) or 500 (MRC-5) TCID₅₀/cell or were mock-superinfected (grey bars). Forty-eight hours post-infection with wt Ad5, cells were analyzed for EGFP expression by FACS analysis and mean fluorescence intensities (MFIs) were calculated (mean $\pm$ SD; $n = 3$). *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

influenced by surrounding vector sequences such as viral enhancers [32,33], we investigated whether shielding of the promoters, particularly of the E3 promoter, by insulator elements was beneficial in terms of background expression. Thus, a sequence derived from the chicken β -globin locus (HS4) that has been reported to function as an insulator [34] was inserted upstream or both up- and downstream of the expression cassettes in all possible orientations (Figure 1). The entire set of vectors including

the parental vectors lacking the HS4 element was used to infect confluent A549 cells because these had given rise to highest background expression from the E3 promoter.

Infected cells were superinfected with Ad5 as before and FACS analysis was carried out 24 h post-infection with Ad5 (Figure 3). Because EGFP expression was measured at an earlier time point compared to before, overall EGFP levels were lower in this experiment. In the cases of promoters E2B and E3, the insertion of the HS4 insulator

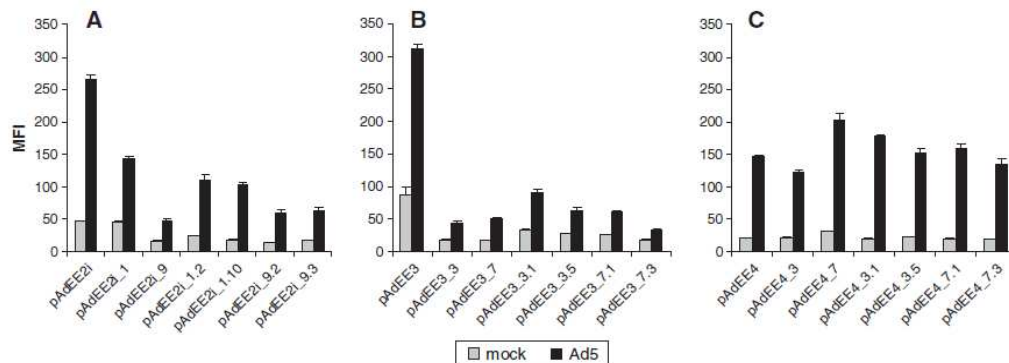


Figure 3. Effect of the HS4 insulator element on EGFP expression from the E2B (A), E3 (B) and E4 (C) promoter. Confluent A549 cells were infected with the indicated recombinant vectors at a MOI of 50 TCID₅₀/cell. Twenty-four hours after infection, cells were either superinfected with wt Ad5 (black bars) at a MOI of 200 TCID₅₀/cell or mock-superinfected (grey bars). Twenty-four hours post-infection with wt Ad5, cells were analyzed for EGFP expression by FACS analysis and mean fluorescence intensities (MFIs) were calculated (mean $\pm$ SD; $n = 3$).

sequences led to an overall reduction in background expression in the absence of wt Ad5 compared to vectors pAdEE2i and pAdEE3 lacking the insulator sequences (Figures 3A and 3B). No further reduction was observed for the E4 promoter (Figure 3C). However, the decrease in background expression by the HS4 elements in the cases of the E2B and E3 promoter-harboring vectors was accompanied by an overall dramatic reduction of gene expression in the presence of wt Ad5. Because the insertion of the HS4 element did not increase the performance of the E3 promoter in terms of background expression, we eliminated the E3 promoter as a possible candidate for HSV-TK expression. Because the HS4 insulator decreased the expression levels from the E2B promoter in wt Ad5-infected cells but did not increase the induction rates, we also excluded the vectors harboring the HS4-shielded E2B promoter. The best performing vectors turned out to be the ones containing the E2B promoter but lacking the HS4 insulator or the E4 promoter with or without a flanking HS4 sequence. In side-by-side experiments, the E2B and E4 promoters lacking a flanking HS4 element had given comparable results (Figure 2). However, because the E4 promoter appeared to be more robust, because it was not subject to deregulation upon insertion of flanking elements such as HS4, we selected this promoter in its HS4-lacking version for subsequent investigations.

Expression from the E4 promoter is significantly enhanced in various cell types in the presence of wt Ad5

We intended to validate the performance of the E4 promoter by testing 14 additional cell types, with half of them representing primary cells (Figure 4). Not unexpectedly, the overall expression rates differed between cell types. The range of background expression was approximately the same as that observed for the cells tested before and the increase in EGFP expression upon superinfection with wt Ad5 was on average 8.5-fold with no

significant overall difference between subconfluent and confluent cells. There was, however, a slight tendency towards higher induction rates in normal cells (approximately ten-fold) compared to cancer cell lines (approximately seven-fold). Based on these results, we concluded that the background activity of the E4 promoter, when used to drive HSV-TK expression, may be sufficiently low to prevent cytotoxic effects in cells not infected with wt Ad5, at least at low concentrations of GCV. On the other hand, we assumed that the activity of the promoter in wt virus-infected cells might be sufficiently high to permit inhibition of wt adenovirus replication. Thus, we employed the E4 promoter for HSV-TK expression in all subsequent experiments.

E4 promoter-mediated background expression is low enough to largely prevent cytotoxicity at low concentrations of GCV

We replaced the EGFP gene in pAdEE4 with the TK gene of HSV1 to create pAdEE4_TK (Figure 1). We also created analogous control vectors in which HSV-TK expression was under control of the E3 or CMV promoter (vectors pAdEE3_TK and pAdCMV-TK, respectively; Figure 1). A549, HUVEC and MRC-5 cells were infected with the vectors and subsequently superinfected or mock-superinfected with wt Ad5 as previously carried out. HSV-TK expression levels 48 h post-infection with Ad5 were determined by western blotting using an anti-HSV-TK antibody (Figure 5). As expected, CMV promoter-mediated HSV-TK expression was high in all cell types, regardless of whether or not cells were superinfected with wt Ad5. Expression from the E3 promoter in MRC-5 cells and HUVEC was only detectable upon wt Ad5 superinfection (Figure 5B and 5C) but was also pronounced in A549 cells in the absence of wt virus (Figure 5A), reflecting the FACS results obtained with the corresponding EGFP vector (Figure 2A). HSV-TK expression, when controlled by the E4 promoter, was measurable in all cell types upon wt Ad5 superinfection, although it was

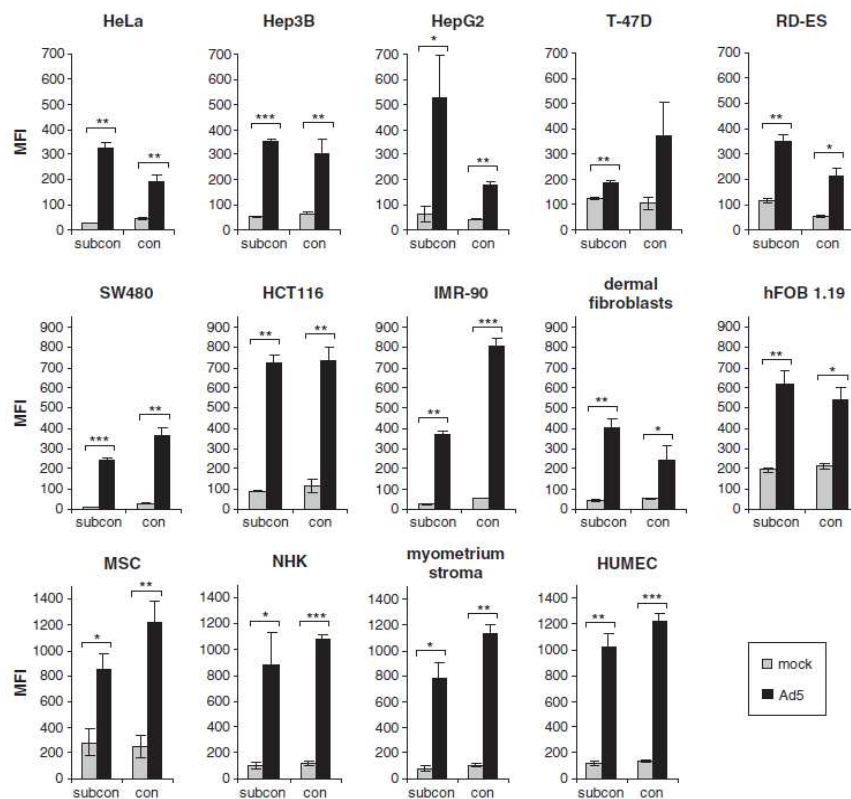


Figure 4. EGFP expression rates from the E4 promoter in the absence or presence of superinfecting wt Ad5 in various cells. Cells tested were: HeLa (human epithelial carcinoma), Hep3B and HepG2 (human hepatocellular carcinoma), T-47D (human mammary gland ductal carcinoma), RD-ES (Ewing's sarcoma), SW480 (human colon carcinoma), HCT116 (human colorectal carcinoma), IMR-90 (normal human embryonic lung fibroblasts), primary human neonatal fibroblasts, hFOB 1.19 (normal human fetal osteoblasts), mesenchymal stem cells from adipose tissue (MSC), primary normal human kidney cells (NHK), primary human myometrium stroma cells, and primary human uterine microvascular endothelial cells (HUVEC). Subconfluent (subcon) or confluent (con) cultures of the indicated cells were infected with pAdEE4 at a MOI of 100 (IMR-90, primary fibroblasts) or 50 TCID₅₀/cell (all other cells). Twenty-four hours after infection, cells were either superinfected with wt Ad5 (black bars) at a MOI of 500 (IMR-90, primary fibroblasts) or 200 TCID₅₀/cell (all other cells) or were mock-superinfected (grey bars). EGFP expression levels were determined by FACS analysis 48 h post-infection with wt Ad5 and mean fluorescence intensities (MFIs) were calculated (mean $\pm$ SD; $n = 3$). *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

undetectable in mock-superinfected cells at least under western blot conditions.

Because high background activity of the E4 promoter and consequently high levels of HSV-TK would inevitably result in cytotoxicity upon treatment with GCV even in cells not infected with wt adenovirus, we determined the impact of E4 promoter-mediated HSV-TK background expression on cell viability in the presence of GCV but absence of wt Ad5. Subconfluent and confluent A549 cells were infected with pAdEE4-TK or pAdEE4 at a MOI of 100 TCID₅₀/cell or were mock-infected. 24 h post-infection, GCV was added in increasing concentrations ranging from 0 and 750 μ M and the cells were cultivated for an additional 6 days without a change of medium. At the end of the incubation period, cellular metabolic activity as an indicator of cell viability was determined using an MTS assay (Figure 6A). Viability of both subconfluent and confluent mock-infected cells or of cells infected with the EGFP control vector was nearly unaffected even at the highest GCV concentrations. E4

promoter-related background expression of HSV-TK had no effect on the viability of confluent cells up to a GCV concentration of 1.2 μ M and a slight effect at 6 μ M but became more pronounced at concentrations of 30 μ M and higher. Confluent cultures tended to tolerate higher GCV concentrations. This pattern was consistently observed in repeated experiments with A549 cells but was also largely recapitulated in experiments with MRC-5 cells, HUVEC and primary fibroblasts (Figure 6A). In all cases, cell viability was not or at most insignificantly compromised at GCV concentrations of up to 1.2 μ M but started to decrease at concentrations of 6 μ M or higher. Interestingly, HUVEC reacted extremely sensitively to GCV even in the absence of HSV-TK expression. In general, the overall picture was similar for subconfluent and confluent cells. However, cells that had truly been contact-inhibited for prolonged times displayed significantly higher resistance to GCV as demonstrated for MRC-5 cells (Figure 6B). In any case, the fact that even dividing cells were not negatively affected by HSV-TK background

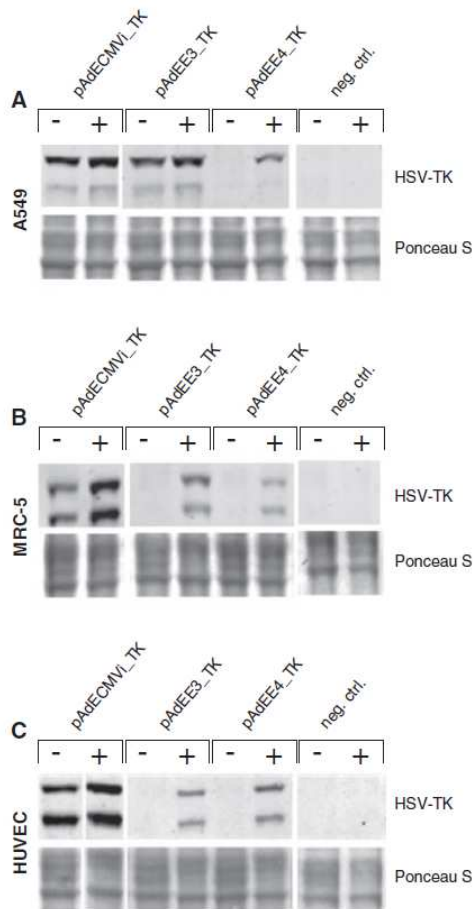


Figure 5. Selective HSV-TK expression from the E4 promoter in wt Ad5-superinfected cells. A549 (A), MRC-5 (B) and HUVEC cells (C) were infected with the recombinant, E4 promoter-harboring, HSV-TK expression vector pAdEE4_TK at a MOI of 50 (A549, HUVEC) or 100 (MRC-5) TCID₅₀/cell. As a control, cells were also infected with the analogous vectors pAdECMV1_TK and pAdEE3_TK harboring the HSV-TK gene under control of the CMV and E3 promoter, respectively. Twenty-four hours after infection, cells were superinfected with wt Ad5 (indicated with a '+') at a MOI of 200 (A549, HUVEC) or 500 (MRC-5) TCID₅₀/cell or were mock-superinfected (indicated with a '-'). Cells not infected with recombinant virus served as negative controls. Forty-eight hours after Ad5 infection, cells were harvested and equal amounts of protein were subjected to western blot analysis for detection of HSV-TK. Ponceau S-stained membranes confirming equal loading of samples are depicted beneath the western blot panels.

expression at GCV concentrations up to 1.2 μ M prompted us to investigate whether such low concentrations of GCV would be sufficient to inhibit adenovirus replication.

Adenovirus vector-mediated HSV-TK expression inhibits wt Ad5 upon treatment with GCV

A549 cells were infected with pAdEE4_TK or with the analogous EGFP expression vector pAdEE4 as a control

at a MOI of 100 TCID₅₀/cell. Twenty-four later, cultures were superinfected with 300 TCID₅₀ units of wt Ad5 (MOI 0.01). This rather low MOI was chosen to allow for monitoring of wt Ad5 spreading over a prolonged time span. Cells were concomitantly treated with 1.2 μ M GCV and cultivated for an additional 6 days without change of medium. At time points 0, 2, 4 and 6 days post-infection with wt Ad5, samples were collected and subjected to real-time qPCR to specifically determine wt Ad5 genome copy numbers. Primers and probe were directed against a part of the E1A gene present in wt Ad5 but absent in the recombinant viruses. In the absence of GCV, Ad5 genome copy numbers in both pAdEE4- and pAdEE4_TK-infected A549 cultures increased by approximately four orders of magnitude over the 6-day period (Figure 7A). However, in the presence of 1.2 μ M GCV, wt Ad5 genome copy numbers in HSV-TK-expressing cells were reduced by 2.4 orders of magnitude (99.6%) compared to cells not expressing HSV-TK. An inhibitory effect was visible already two days post-infection as a decrease of genome copy numbers by 1.9 orders of magnitude (98.7%) and was consistently observed in similar infection experiments with A549 cells.

We also repeated the experiments with HUVEC (Figure 7C) and obtained comparable inhibition rates of 1.9/2.7/2.9 orders of magnitude (98.8/99.8/99.9%) for time points 2, 4 and 6 days post-infection. An inhibitory effect was also observed for contact-inhibited MRC-5 cells that served as a model for cells promoting Ad5 replication rather poorly: over the 6-day period of the experiments virus titers increased by slightly more than one order of magnitude at best (Figure 7B). Six days post-infection, Ad5 genome copy numbers in cultures of HSV-TK expressing cells were decreased by 0.7 orders of magnitude compared to cultures of cells lacking HSV-TK expression, translating into a reduction rate of 80.9%. In contact-inhibited primary fibroblasts, which produced intermediate amounts of virus, HSV-TK expression/GCV treatment led to a reduction of wt Ad5 genome copy numbers by 1.6 orders of magnitude (97.2%) six days post-infection with wt Ad5 (Figure 7D).

The overall inhibitory effect of HSV-TK/GCV was not only evident as a decrease of Ad5 genome copy numbers, but also as an even more pronounced reduction in infectious virus progeny. Wt Ad5 titers of cultures of HSV-TK-expressing confluent A549 cells treated with 1.2 μ M GCV were reduced by 1.9 and 4.7 orders of magnitude (98.8% and >99.99%) at time points 2 and 6 days post-infection, respectively, compared to control cultures not treated with GCV (Figure 8A). Virtually the same result was obtained for HUVEC cells (Figure 8C) and wt Ad5 titers of MRC-5 cells were reduced by 0.5 and 1.3 orders of magnitude (67.6% and 94.4%) 2 and 6 days post-infection, respectively (Figure 8B). In primary fibroblasts, HSV-TK expression/GCV treatment led to a reduction of wt Ad5 titers by 1.4 orders of magnitude (96.3%) 6 days post-infection with wt Ad5 (Figure 8D).

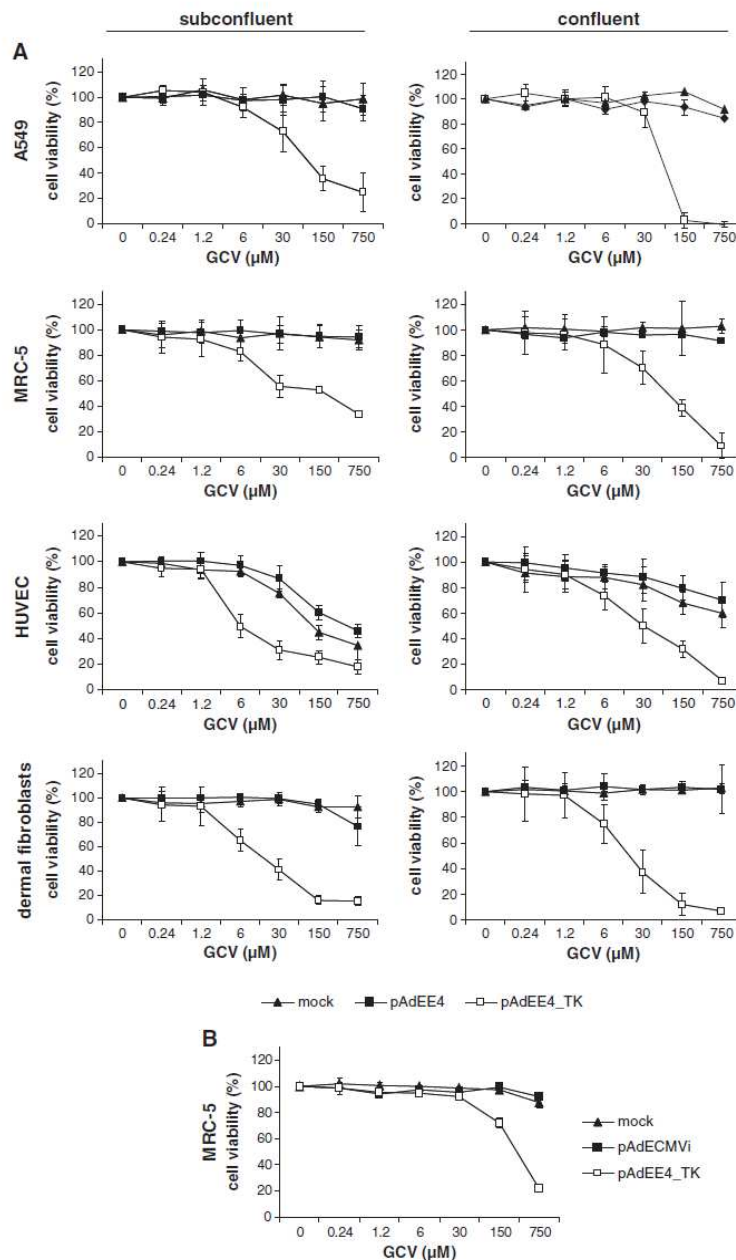


Figure 6. E4 promoter background activity is sufficiently low to largely prevent cytotoxicity in the presence of low concentrations of GCV. (A) Subconfluent and confluent A549 cells, MRC-5 cells, HUVEC and primary fibroblasts were infected with the adenoviral HSV-TK expression vector pAdEE4_TK or the analogous adenoviral EGFP expression vector pAdEE4 at a MOI of 100 TCID₅₀/cell, or were mock-infected. Cells were treated with serial dilutions of GCV ranging from 0 to 750 μM. After 6 days of incubation without change of medium, cells were subjected to MTS assay analysis. Cell viabilities at increasing concentrations of GCV were calculated in relation to viabilities of the respective cells that had not been treated with GCV (mean ± SD; *n* = 4). (B) Contact-inhibited MRC-5 cells were infected with the HSV-TK expression vector pAdEE4_TK or the control vector pAdECMV expressing EGFP from the CMV promoter at a MOI of 100 TCID₅₀/cell, or were mock-infected. Experimental setup and calculations otherwise as in (A) (mean ± SD; *n* = 3).

HSV-TK expression/GCV treatment was not only effective when the cells were infected with the recombinant virus prior to wt Ad5 infection, but also when the recombinant virus was added to the cultures simultaneously with

wt Ad5 or 5 or 10 h after infection with wt Ad5 (Figures 9A to 9C). Wt Ad5 genome copy numbers were comparably reduced in all cases (compare Figure 9A to 9C with Figure 7D).

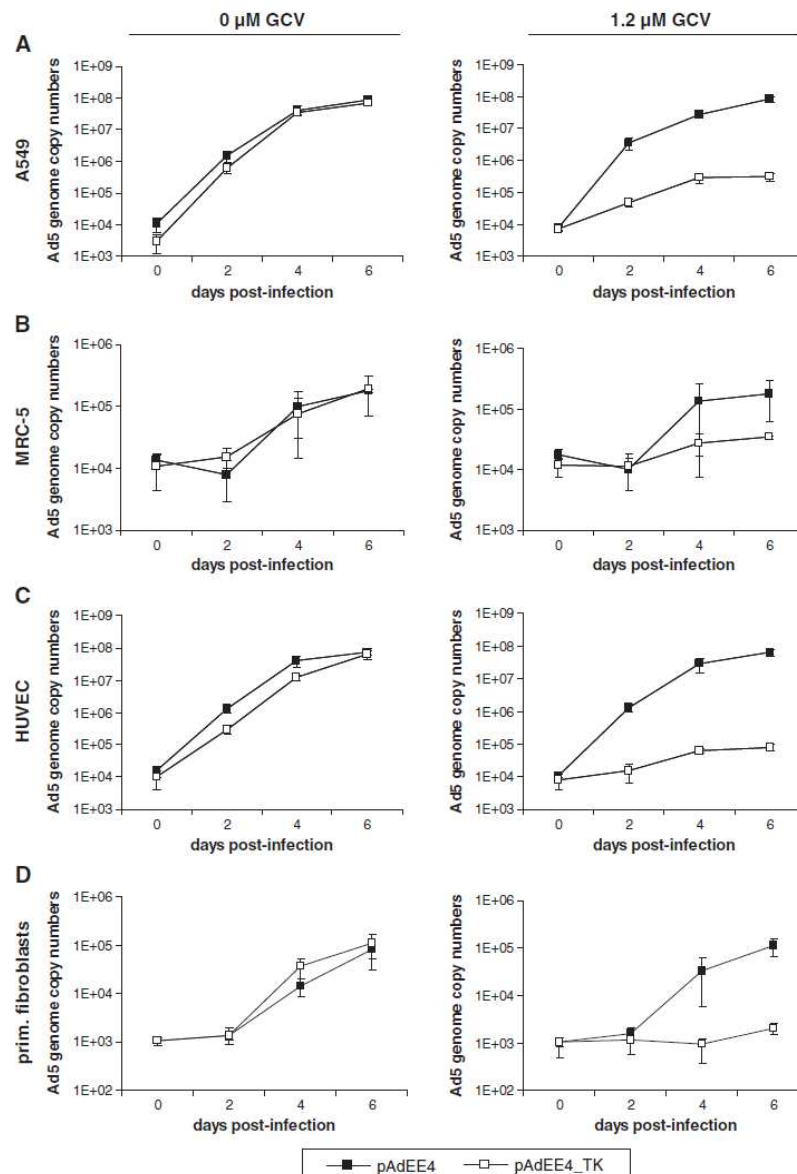


Figure 7. E4 promoter-mediated HSV-TK expression/GCV treatment inhibits wt Ad5 replication. A549 cells (A), MRC-5 cells (B), HUVEC (C) and primary fibroblasts (D) were infected with the recombinant, adenoviral HSV-TK expression vector pAdEE4_TK or the analogous EGFP expression vector pAdEE4 at a MOI of 100 TCID₅₀/cell. Twenty-four hours after infection, cells were superinfected with 300 TCID₅₀ units of wt Ad5 and treated with 1.2 μ M GCV. Ad5 genome copy numbers (mean $\pm$ SD; $n = 3$) at days 0, 2, 4 and 6 post-infection with wt Ad5 were determined by real-time qPCR with E1A-specific primers/probe using a standard curve for Ad5. Real-time qPCR quantification was performed in duplicate.

HSV-TK expression/GCV treatment improves cell viability in wt Ad5-infected cultures

The anti-adenoviral effect of HSV-TK expression/GCV treatment was also anticipated to become manifest as an overall increase in cell viability of wt Ad5-infected cultures. Thus, we repeated the virus inhibition experiments with A549 cells and primary fibroblasts and performed

MTS assays 6 days post-infection with wt Ad5 (Figure 10). We also increased the MOIs for wt Ad5 to 40, 60 and 80 TCID₅₀/cell to obtain more pronounced and better measurable cytopathic effects. Infection of the A549 cultures with the EGFP expression vector pAdEE4 did not rescue the cultures from wt Ad5-mediated cytotoxicity at all, regardless of the absence or presence of 1.2 μ M GCV, and cell viability was barely measurable (Figure 10A). Similarly, HSV-TK expression via pAdEE4_TK in the absence

Inhibition of adenovirus by HSV-TK/GCV

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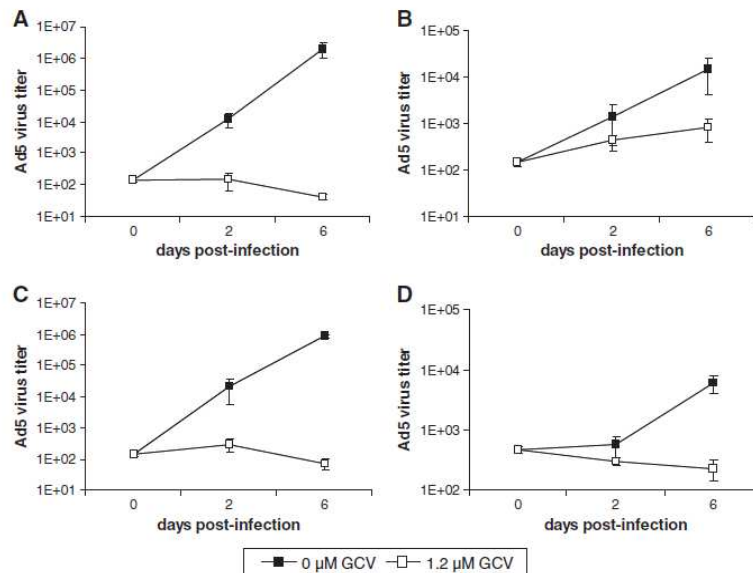


Figure 8. E4 promoter-mediated HSV-TK expression/GCV treatment reduces infectious virus progeny. Virus suspensions from the same virus inhibition experiments shown in Figure 7 were analyzed for numbers of infectious Ad5 particles. Virus suspensions were derived from A549 cells (A), MRC-5 cells (B) HUVEC (C) and primary fibroblasts (D) infected with pAdEE4_TK and superinfected with wt Ad5 and treated with 0 μ M and 1.2 μ M GCV, respectively. Numbers of infectious wt Ad5 particles of triplicate infection experiments were determined on A549 cells by TCID₅₀ assays (mean $\pm$ SD; $n = 3$).

of GCV did not result in any increase in cell viability. However, HSV-TK expression via pAdEE4_TK in the presence of 1.2 μ M GCV dramatically increased the cell viability. Although HSV-TK expression/GCV treatment was also unable to completely restore cell viability to levels obtained for uninfected cultures, cell viability was increased 18.3-fold (93.8% points) on average compared to cells infected with the EGFP control vector and treated with GCV, thus demonstrating a protective effect on cultures infected with wt Ad5 *in vitro*. The same effect was obtained with cultures of primary fibroblasts, which, however, were generally less sensitive towards wt Ad5 infection and, consequently, displayed higher overall cell viability even when only infected with the EGFP control vector or in the absence of GCV (Figure 10B).

Discussion

In the present study, we describe a HSV-TK/GCV-based GDEPT concept that is not aimed at the elimination of cancer cells but at the inhibition of adenoviruses. By contrast to some other GDEPT systems, the functionality of the HSV-TK system is restricted to cells undergoing DNA replication, thus *per se* restricting cytotoxic effects to cycling cells or cells infected with replicating adenovirus in the envisioned therapeutic setting. One of the drawbacks of HSV-TK/GCV in cancer gene therapy is the limited bystander effect on neighboring tumor cells, which is a result of the inability of GCV-ppp (in contrast to GCV) to pass freely through cellular membranes. Instead,

intercellular transport of GCV-ppp requires cell-to-cell contact via gap junctions [35]. However, this characteristic would be beneficial in a therapeutic anti-adenoviral scenario because the cytotoxic effect would be restricted to virus-infected cells and possibly to their immediate neighbors. Because gap junctions between infected and uninfected cells are likely resolved in the course of an infection, such an unwanted bystander effect is questionable at all.

Because targeting HSV-TK expression to infected cells is a prerequisite for anti-adenoviral GDEPT, we evaluated a series of Ad5 promoters with known dependence on adenovirus E1A for: (i) background activity in uninfected cells and (ii) enhanced activity in infected cells. Because copies of the promoters were inserted into the deleted E1 region of a replication-deficient adenoviral vector, expression from and inducibility of the promoters in this artificial setting were expected to be different from the natural situation. However, we figured that at least some of the promoters would largely retain their functions. Of the tested promoters, three (E2B, E3 and E4) displayed considerable activity upon superinfection with wt Ad5 and two of those (E2B and E4) showed low background activity. The E4 promoter that was identified as the best candidate displayed a relatively low background activity in all tested cell types tested and permitted increased gene expression upon superinfection with wt Ad5 (8.6-fold on average). The expression cassettes were inserted in antisense orientation with respect to the left ITR because this orientation had been reported to result in less undesired interferences of inserted promoters with sequences located within the left ITR [33]. Additional

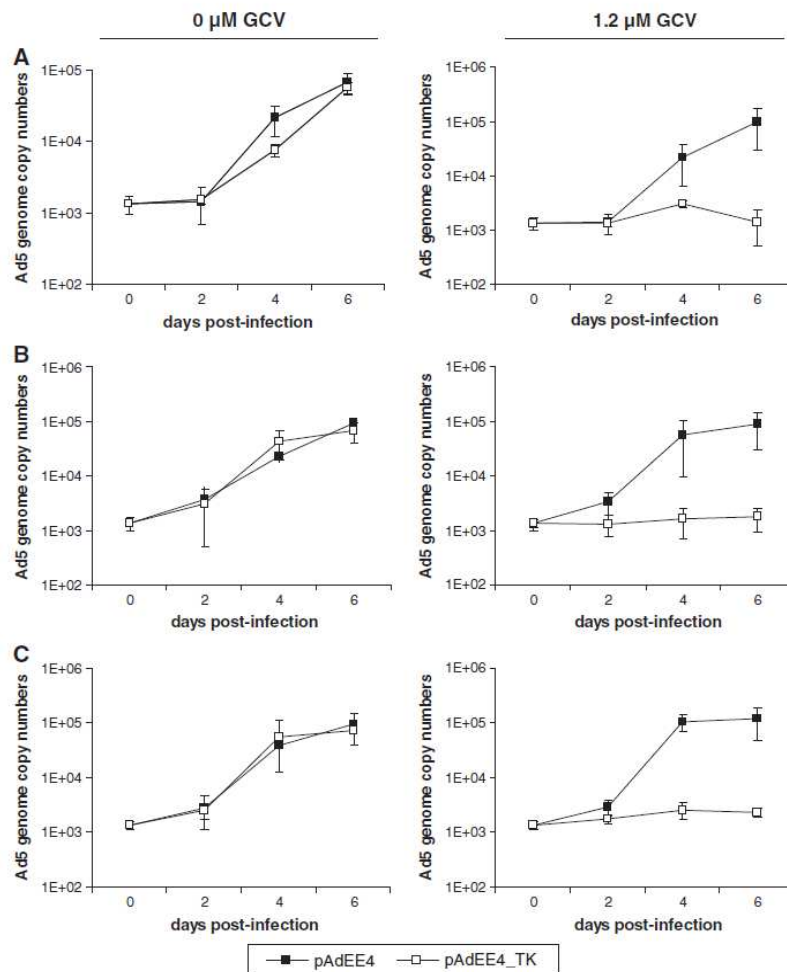


Figure 9. E4 promoter-mediated HSV-TK expression/GCV treatment inhibits wt Ad5 replication in cells already infected with wt Ad5. Dividing primary fibroblasts were co-infected with 300 TCID₅₀ units of wt Ad5 and the recombinant, adenoviral HSV-TK expression vector pAdEE4_TK. Infection with pAdEE4_TK was performed either concomitantly with wt Ad5 (A), 5 h after infection with wt Ad5 (B) or 10 h after infection with wt Ad5 (C). Ad5 genome copy numbers (mean $\pm$ SD; $n = 3$) at days 0, 2, 4 and 6 post-infection with wt Ad5 were determined by real-time qPCR with E1A-specific primers/probe using a standard curve for Ad5. Real-time qPCR quantification was performed in duplicate.

flanking of the promoters with HS4 insulators that are occasionally used for the shielding of promoters in adenoviral vectors [33,36,37] led only to an overall decrease of E2B and E3 promoter activities (and no effect on E4 promoter activity) but did not further increase the ratio in EGFP expression between Ad5-infected and uninfected cells. An insertion of the HSV-TK gene behind (preferentially early or delayed-early) adenoviral promoters at their natural positions within the genome may be an option to further increase inducibility rates.

The measured increases in EGFP or HSV-TK levels upon superinfection were not solely a result of increased promoter activities but have to be seen as the cumulative effects of elevated promoter activities and an increase in gene copy numbers as a result of induction of viral vector replication by the wt virus. Indeed, this anticipated increase in gene copy numbers upon encounter with the

wt virus was a rationale for delivering the HSV-TK gene via an adenoviral vector. It was clear, however, that GCV would also affect the replication of the recombinant vector. Nevertheless, even low-level replication of the vector was expected to contribute to the overall inhibitory effect on wt virus replication.

E4 promoter-driven HSV-TK background expression in A549, MRC-5, HUVEC and primary fibroblasts did not decrease cell viabilities in the cultures up to a GCV concentration of 1.2 μM or slightly higher (Figure 6) and truly contact-inhibited MRC-5 cells were resistant to GCV concentrations up to 30 μM (Figure 6B), which is consistent with the inhibitory effect of GCV-ppp on DNA replication. However, because cycling cells would also encounter the recombinant virus in an envisioned therapeutic setting, subsequent virus inhibition experiments were performed with 1.2 μM GCV. This concentration is also therapeutically

Inhibition of adenovirus by HSV-TK/GCV

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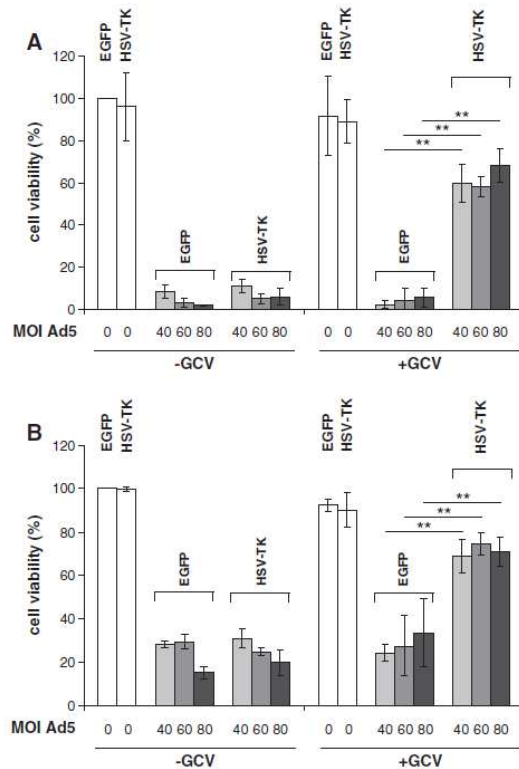


Figure 10. E4 promoter-mediated HSV-TK expression/GCV treatment increases cell viabilities in wt Ad5-infected cultures. A549 cells (A) or primary fibroblasts (B) were either infected with the EGFP expression vector pAdEE4 (indicated as EGFP in the figure) or the HSV-TK expression vector pAdEE4_TK (indicated as HSV-TK) at a MOI of 100 TCID₅₀/cell. Twenty-four hours post-infection with the recombinant vectors, cells were additionally infected with wt Ad5 at MOIs of 40, 60 and 80 TCID₅₀/cell or were mock-infected. Concomitantly, cells were treated/not treated with 1.2 μ M GCV and cultivated for an additional six days without change of medium. MTS assays were performed six days post-infection with wt Ad5. Cell viabilities (mean $\pm$ SD; $n = 3$) were calculated in relation to viabilities of cells infected with pAdEE4, mock-superinfected with wt Ad5 and not treated with GCV, which were arbitrarily set to 100%. ** $p < 0.01$.

relevant because it corresponds to levels measured in the serum of patients after treatment with typical doses of intravenous or oral GCV [38–40].

E4 promoter-mediated expression of HSV-TK and concomitant treatment of cells with 1.2 μ M GCV decreased the genome copy numbers of superinfecting wt Ad5 by up to 2.9 orders of magnitude (99.9%) depending on the cell type (Figure 7), demonstrating potent inhibition of viral DNA replication. This effect was not a result of nonspecific inhibition by GCV because Ad5 genome copy numbers in cells expressing EGFP instead of HSV-TK were not decreased (compare left and right panels in Figure 7). Moreover, data from side-by-side cell viability experiments in which Ad5 superinfection was omitted ruled out the possibility that the anti-adenoviral effect was only attributable to HSV-TK/GCV-mediated cell killing, which

might have compromised virus replication and spreading (data not shown).

The actual output in infectious virus from HSV-TK expressing cells was even more dramatically decreased in all cell types, regardless of whether they were strongly promoting (A549, HUVEC), or to a lower extent (MRC-5, primary fibroblasts), viral replication. Inhibition rates measured 6 days after infection ranged from 94.4% to 99.99% depending on the cell type (Figure 8). The differences in the inhibition rates obtained with the two read-out systems reflect the fact that qPCR detects not only full-length viral DNA, but also truncated, nonfunctional DNA as it emerges as a consequence of GCV-ppp-mediated premature termination of viral DNA synthesis. Thus, qPCR-based measurement of genome copy numbers slightly underestimates the inhibitory effect. Because GCV treatment was not only effective when the recombinant virus was allowed to infect the cells prior to wt Ad5 infection, but also when added to the cultures simultaneously with wt Ad5 or 5 to 10 h post-infection with wt Ad5 (Figure 9), attenuation of virus replication may be feasible even in already infected cells.

Nevertheless, virus inhibition was not complete because wt Ad5 titers did not drop to zero. The inhibitory effect of HSV-TK expression and treatment with 1.2 μ M GCV could not further be increased by combined treatment with increasing amounts of the anti-adenoviral drug cidofovir (see Supporting information, Figure 1). Consequently, combined treatment with cidofovir at the highest concentration of 30 μ M, which corresponds to peak serum concentrations obtained after intravenous administration of typical doses of cidofovir [41], and decreasing concentrations of GCV did not cause further virus inhibition either, nor did it result in inhibition rates reaching those achieved with 1.2 μ M GCV alone (see Supporting information, Figure 1).

Residual low-level production of wt virus explains why infected cultures could not completely be rescued from the viral infection (Figure 10). However, cell viability in these cultures was still significantly increased compared to control cultures infected with the adenoviral EGFP expression vector. Complete cure of cells from infection with a wt virus cannot be expected because significant intracellular amounts of HSV-TK are required for the blockage of DNA replication and such levels are not available immediately after infection of a cell with wt virus. However, the release of infectious virus from such cells can be minimized, thereby inhibiting further virus spreading.

It has been demonstrated that virus amplification obviously starts in the intestine before disseminating to other organs [13,42]. Hence, the concept presented here may allow at least partial protection of unaffected tissues from excessive virus infection and subsequent organ failure in the envisioned therapeutic application. Moreover, topical delivery to specific organs such as the lung, which is a common target of adenoviruses after lung transplantation [43–46], is conceivable.

Clearly, the success of such an approach depends on efficient delivery of the HSV-TK vector. The fact that the

adenoviral vector and the wt virus share the same cell and organ tropism may facilitate the delivery of HSV-TK into cells preferentially infected by the wt virus. Because adenoviruses of species C (including Ad5) have been determined as the predominant cause of severe disseminated disease in most geographical areas [47,48], Ad5-based vectors displaying the same tropism may be well suited. Furthermore, in the envisioned clinical application, the vector/wt virus system should be 'self-balancing': the vector would be expected not to replicate and spread in the absence of wt virus. However, as long as wt virus would be generated, the recombinant vector would be amplified as well, thus ensuring a continuous supply. Conversely, upon recovery of the immune system, clearance of both wt virus and recombinant virus would be expected.

Taken together we have provided proof-of-principle for the inhibition of wt Ad5 replication *in vitro* by means of a HSV-TK/GCV-based GDEPT strategy that largely restricts HSV-TK expression to wt virus-infected cells. Although our approach is unlikely to completely eradicate adenovirus infections, it may render patients able to survive until the immune system has recovered sufficiently to control infection. It will be interesting to see how the presented strategy will perform in a subsequent study in an animal model *in vivo*.

Acknowledgements

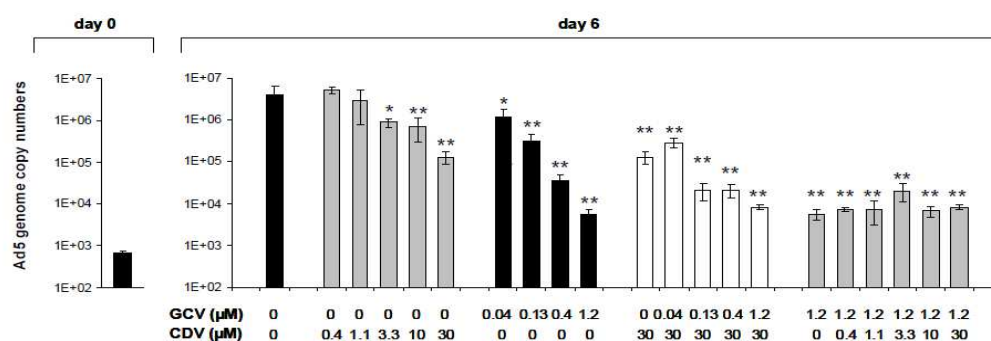
This work was supported by grant 12814 of the Austrian National Bank. The authors declare that there are no conflicts of interest.

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Supplementary Figure 1. Combined GCV and cidofovir (CDV) treatment does not further increase the attenuation of wt Ad5 replication. Primary fibroblasts were infected with the recombinant, adenoviral HSV-TK expression vector pAdEE4_TK at an MOI of 100 TCID₅₀/cell. 24 h after infection, cells were superinfected with 300 TCID₅₀ units of wt Ad5 and treated with GCV and/or CDV at the indicated concentrations. Ad5 genome copy numbers of infected cells from triplicate infection experiments (mean ± SD; n = 3) at days 0 and 6 post-infection with wt Ad5 were determined by real-time qPCR with E1A-specific primers/probe using a standard curve for Ad5. Real-time qPCR quantification was performed in duplicates, each. *p<0.05, **p<0.01.

2. Second part

INHIBITION OF ADENOVIRUS MULTIPLICATION BY SHORT INTERFERING RNAs DIRECTLY OR INDIRECTLY TARGETING THE VIRAL DNA REPLICATION MACHINERY

Doris Kneidinger, **Mirza Ibrišimović**, Thomas Lion, Reinhard Klein

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Inhibition of adenovirus multiplication by short interfering RNAs directly or indirectly targeting the viral DNA replication machinery

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ABSTRACT

Human adenoviruses are a common threat to immunocompromised patients, e.g., HIV-positive individuals or solid-organ and, in particular, allogeneic stem cell transplant recipients. Antiviral drugs have a limited effect on adenoviruses, and existing treatment modalities often fail to prevent fatal outcome. Silencing of viral genes by short interfering RNAs (siRNAs) holds a great promise in the treatment of viral infections. The aim of the present study was to identify adenoviral candidate targets for RNA interference-mediated inhibition of adenoviral replication. We investigated the impact of silencing of a set of early, middle, and late viral genes on the replication of adenovirus 5 *in vitro*. Adenovirus replication was inhibited by siRNAs directed against the adenoviral E1A, DNA polymerase, preterminal protein (pTP), IVa2, hexon, and protease genes. Silencing of early and middle genes was more effective in inhibiting adenovirus multiplication than was silencing of late genes. A siRNA directed against the viral DNA polymerase mRNA decreased viral genome copy numbers and infectious virus progeny by several orders of magnitude. Since silencing of any of the early genes directly or indirectly affected viral DNA synthesis, our data suggest that reducing viral genome copy numbers is a more promising strategy for the treatment of adenoviral infections than is reducing the numbers of proteins necessary for capsid generation. Thus, adenoviral DNA replication was identified as a key target for RNAi-mediated inhibition of adenovirus multiplication. In addition, the E1A transcripts emerged as a second important target, because its knock-down markedly improved the viability of cells at late stages of infection.

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1. Introduction

Human adenoviruses are double-stranded DNA (dsDNA) viruses associated with a wide range of human diseases. They are mainly responsible for self-limiting respiratory and intestinal infections, and predominantly affect children and young adults (Lenaerts et al., 2008). However, more severe manifestations, including hemorrhagic cystitis, nephritis, pneumonia, hepatitis, enterocolitis, and disseminated disease, are observed in immunocompromised patients, such as solid-organ and, in particular, allogeneic stem cell transplant recipients (Echavarría, 2008; Ison, 2006; Kojaoglanian et al., 2003). These manifestations can be life-threatening or even lethal. In the case of disseminated disease, mortality rates as high as 80% have been reported (Blanke et al., 1995; Hale et al., 1999; Howard et al., 1999; Lion et al., 2003; Munoz et al., 1998). Severe manifestations are most commonly associated with serotypes from species B and C (Kojaoglanian et al., 2003), with a high prevalence

of species C in certain geographical areas (Ebner et al., 2006; Lion et al., 2003, 2010).

In the immunocompetent host, a severe manifestation of adenovirus infection is epidemic keratoconjunctivitis (EKC). This is predominantly associated with serotypes 8, 19, and 37 (all belonging to species D), is highly contagious, and can have severe consequences on visual acuity (Gordon et al., 1996). Besides, EKC is generally associated with significant morbidity, which results in considerable economic losses.

The most common agents for treating adenovirus infections are ribavirin and cidofovir. However, apparent clinical efficacy has been demonstrated only for cidofovir. Although cidofovir is widely used, its activity is limited and insufficient to completely prevent fatal outcomes among hematopoietic stem cell transplant recipients (Lenaerts et al., 2008; Lindemans et al., 2010; Ljungman et al., 2003; Symeonidis et al., 2007; Yusuf et al., 2006). Furthermore, concomitant recovery of the immune system may be necessary for complete adenovirus clearance (Chakrabarti et al., 2002; Heemskerk et al., 2005; Lindemans et al., 2010). Cidofovir displays significant nephrotoxicity and limited bioavailability, and this has prompted the development of improved derivatives. However, the effectiveness of these compounds is still under evaluation

Abbreviations: RNAi, RNA interference; siRNA, small interfering RNA.

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(Hartline et al., 2005; Paolino et al., 2011). Thus, in view of the growing numbers of immunosuppressed patients, the development of alternative anti-adenovirus treatment options is required to decrease adenovirus-mediated mortality among immunocompromised patients, and also to decrease economic losses caused by milder forms of adenovirus-related disease.

RNA interference (RNAi) is a post-transcriptional mechanism of gene silencing conserved among eukaryotic cells (Carthew and Sontheimer, 2009; Ghildiyal and Zamore, 2009; Huntzinger and Izaurralde, 2011; Hutvagner and Simard, 2008; Kawamata and Tomari, 2010). It is mediated through small double-stranded RNAs (dsRNAs), of ~21–25 nt in length, which guide the RNA-induced silencing complex (RISC) to the respective target mRNAs (Fire et al., 1998). Depending on the degree of complementarity between the so-called antisense (or guide) strand of the dsRNA and target mRNA, RNAi can bring about the cleavage of the mRNA (in the case of full or nearly full complementarity), accelerated degradation (as a consequence of deadenylation), or translational repression. Following the discovery that the introduction of synthetic small interfering RNAs (siRNAs) into cells can trigger RNAi (Elbashir et al., 2001), this mechanism was rapidly harnessed as a tool to silence disease-associated human, and also viral genes (Davidson and McCray, 2011). Since then, siRNA-mediated silencing of viral genes has been employed to inhibit the replication of a variety of DNA and RNA viruses, *in vitro* and also *in vivo* (Arbuthnot, 2010; Haasnoot et al., 2007; Zhou and Rossi, 2011).

Adenoviruses contain a linear dsDNA genome, ~36 kb long. The first gene to be expressed during the infection cycle is E1A. This gene has a central role, because it reprograms the cell in a way that promotes efficient virus replication (Berk, 2005; Pelka et al., 2008; Zhao et al., 2003). Deletion of E1A renders adenoviruses replication deficient. E1A expression ultimately leads to the activation of other early and late promoters and triggers the onset of viral DNA replication. Viral DNA replication is dependent on three viral proteins: the viral DNA polymerase; the preterminal protein (pTP); and the DNA-binding protein (DBP) (de Jong et al., 2003). Besides creating dsDNAs for packaging into capsids (accomplished with the help of the IVa2 protein) (Zhang and Imperiale, 2003), replication of the adenoviral genome activates the expression of other viral genes, e.g., IVa2 (Flint, 1986; Iftode and Flint, 2004) and genes transcribed from the major late promoter (MLP) (Shaw and Ziff, 1980). Upregulation of major late (ML) gene expression also involves the IVa2 protein (Tribouley et al., 1994), and results in the synthesis of gene products that primarily constitute structural components of the virion or are involved in its assembly. The major component of the capsid is the hexon protein (Russell, 2009). Capsid maturation requires the action of adenovirus protease, also called adenain (Webster et al., 1989). This protein, which is an integral part of the mature virion, is also required for disassembly of the virion upon virus entry, and consequently for release of the viral DNA (Greber et al., 1996).

In vitro silencing of adenoviral genes by siRNAs has been demonstrated for an adenovirus (Ad) 11 strain (2K2/507/KNIH; species B; isolated in Korea) (Chung et al., 2007), and also for a mutant strain of Ad5 (species C) lacking the E1B and E3 genes (Eckstein et al., 2010). In the case of Ad11, siRNAs directed against E1A were reported to result in an overall reduction of plaque-forming capacity. For the Ad5 mutant strain, siRNAs targeting the E1A, IVa2, and hexon mRNAs were evaluated, and the IVa2 mRNA-targeting siRNA was reported to most efficiently decrease virus production. A protective effect on cell viability was observed only when the IVa2 mRNA-targeting siRNA was combined with an E1A mRNA-directed siRNA and administered at high concentration. The Ad5 mutant virus used represented a rather artificial test system, in that it lacked the E1B genes which, when present, prevent premature cell death, thereby prolonging virus replication and promoting viral

late mRNA export from the nucleus (Blackford and Grand, 2009; Flint and Gonzalez, 2003; Subramanian et al., 1995; Woo and Berk, 2007). Together with the fact that the E1A gene was expressed from an artificial minimal CMV promoter autoactivated by E1A (Fechner et al., 2003), these differences from the wild-type virus make it somewhat difficult to accurately assess the potential of siRNA-mediated adenovirus gene silencing as a strategy for inhibiting adenovirus multiplication.

Here, we investigated the impact of siRNA-mediated adenovirus gene silencing on the replication of wild-type adenovirus. We expanded the panel of potential adenoviral targets, by evaluating siRNAs directed against the Ad5 E1A, DNA polymerase, pTP, IVa2, hexon, and protease mRNAs. Based on our *in vitro* results, we propose that the adenoviral mRNAs originating from genes which are essential for viral DNA replication (i.e., the DNA polymerase and pTP (and potentially the DBP) genes are promising targets for RNAi-mediated inhibition of adenovirus multiplication. Moreover, we demonstrate that highly potent E1A mRNA-directed siRNAs, which are also able to inhibit virus replication (albeit to a lesser extent than the DNA polymerase mRNA-directed siRNA), are capable of concomitantly delaying cell death, without the need for combination with other siRNAs. This distinct mode of inhibition may be exploited *in vivo* for siRNA-mediated attenuation of virus release and, consequently, virus spread.

2. Materials and methods

2.1. Cell culture and virus production

HEK293 (human embryonic kidney; ATCC CRL-1573) and A549 (human epithelial lung carcinoma; ATCC CCL-185) cells were cultivated in Dulbecco's Modified Eagles Medium (DMEM) with stabilized glutamine (PAA Laboratories, Pasching, Austria) supplemented with 10% fetal bovine serum (FBS; PAA Laboratories) in a humidified 5% CO₂ atmosphere at 37 °C. Ad1 (ATCC VR-1), Ad2 (ATCC VR-846), and Ad6 (ATCC-VR6), were amplified in A549 cells; Ad5 (ATCC VR-5) was amplified in HEK293 cells. Virus purifications were performed by standard CsCl density gradient ultracentrifugation. Infectious virus particle titers were determined on A549 cells by 50% tissue culture infective dose (TCID₅₀) assays.

2.2. Vector construction

For the construction of vectors employed in dual-luciferase assays, parts of the Ad5 genome were amplified by PCR using primers specific for E1A (E1A-f1 5'-CGACACCGGTTTAAACATGAGACA TATTATCTGCCAC-3' and E1A-r1 5'-CAACTCATTGTTTAAACAAAGG CGTTAACCA-3'; annealing temperature [*T*_a]: 50 °C), DNA polymerase (Pol-f1 5'-ACTCATATGGCCTTGCTCAAGCTCACCGGGC-3' and Pol-r1 5'-ACTAGATCTACGGCATCTCGATCCAGCATATC-3'; *T*_a: 55 °C), pTP (pTP-f3 5'-CTTTTGACGGTCTCGAGCGTCAACGATTGCGC-3' and pTP-r3 5'-GTGTCCTTGGATGCGGCGGCTAAAAGCGGTGACGCGGG-3'; *T*_a: 65 °C), IVa2 (IVa2-f1 5'-CACC GGCTCGTTTAAACAGAGGGCGAAG AC-3' and IVa2-r1 5'-AAACATAAAGTTTAAACAGACTCTGTTTGGAT-3'; *T*_a: 50 °C), hexon (Hex-f1 5'-CCGCTTCTCGAGATGGCTACCCCTTC GATGATG-3' and Hex-r1 5'-TGTTGCGCGCGCTTATGTTTGCGG TTGCGCGG-3'; *T*_a: 57 °C), and protease (Prot-f1 5'-CAAGCAACAGTTT AAACAGCTGCCGCCATGG-3' and Prot-r1 5'-AAATAAGTTTAAACGCC TTTATTGAAAGTGCTC-3'; *T*_a: 50 °C). The PCR reactions were performed in a total volume of 50 µL containing 10x PCR buffer (PqLab), 400 µM dNTPs, 1 µM of each primer, 4 mM MgSO₄ and 2.5 U of Pwo-DNA-Polymerase (PqLab). The cycling parameters consisted of a total of 30 cycles of denaturing at 95 °C for 1 min, followed by annealing at the appropriate temperature for 1 min and extension at 72 °C for 2 min. The PCR products were subjected

to agarose gel electrophoresis, stained with ethidium bromide, and visualized on a UV transilluminator. The PCR fragments were inserted into the *PmeI* site (E1A, IVa2, protease fragments), *XhoI* and *NotI* sites (pTP, hexon), or *NdeI* and *BglII* sites (DNA polymerase) of psiCHECK-2 (Promega, Mannheim, Germany), all located within the 3' UTR of the *Renilla* luciferase gene. The resulting vectors were named psiCHECK-E1A, psiCHECK-pol, psiCHECK-pTP, psiCHECK-IVa2, and psiCHECK-hex. Restriction enzymes and DNA-modifying enzymes were purchased from Fermentas (St. Leon-Rot, Germany) or New England Biolabs (Frankfurt am Main, Germany). PCR reactions were performed with *Pwo* DNA polymerase obtained from Roche Diagnostics (Vienna, Austria).

2.3. Nucleic acid extraction

Circular plasmid DNA was extracted with QIAprep® Spin Miniprep Kits (QIAGEN, Hilden, Germany), EasyPrep® Pro Plasmid Miniprep Kits (Biozym, Oldendorf, Germany), or HiSpeed® Plasmid Midi Kits (QIAGEN). PCR products were purified with a QIAquick® PCR Purification Kit (QIAGEN). Adenoviral DNA was isolated from cells using a QIAamp DNA Blood Mini Kit (QIAGEN). Total RNA was isolated using an RNeasy® Mini Kit (QIAGEN).

2.4. siRNAs

With the exception of pTP-si1, pTP-si2, pTP-si3, and pTP-si4, all siRNAs (Table 1) were obtained from Invitrogen (LifeTechnologies Austria, Vienna, Austria). They represented 25-mer, blunt-ended siRNAs carrying the Invitrogen "Stealth" modification. Due to the type of chemical modification, only the antisense strand can participate in RNAi, thus avoiding not only unwanted, sense strand-mediated, off-target effects but also preventing any possible interference of the sense strand with adenoviral transcripts generated from the opposite viral DNA strand not intended to be targeted. Besides, this type of modification (frequently present in similar versions in commercial siRNAs) can increase the intracellular half-life of siRNAs and reduce their cytotoxicity. The pTP-si1 to pTP-si4 siRNAs (obtained from Ambion/LifeTechnologies Austria, Vienna, Austria) were 21-mer, unmodified siRNAs carrying two nucleotide (nt) TT overhangs at their 3' ends and were also included in our experiments. As negative controls, two distinct universal non-targeting siRNAs (Invitrogen, Ambion), matching the type of design of the respective targeting siRNAs, were employed. siRNAs were designed using the Invitrogen BLOCK-IT™ RNAi Designer or Dharmacon siDESIGN tools and target site accessibility, as calculated by RNAxs (<http://rna.tbi.univie.ac.at/cgi-bin/RNAxs>), was taken into account.

2.5. Dual-luciferase assay-based screening for functional siRNAs

1.4e+05 HEK293 and 3e+04 A549 cells were seeded into the wells of 96-well plates, and reverse transfected with 50 ng of individual dual-luciferase reporter vectors and 30 nM targeting or non-targeting control siRNA using Lipofectamine 2000 (Invitrogen/LifeTechnologies Austria, Vienna, Austria). Briefly, for each well 0.5 µL Lipofectamine 2000 was diluted with 24.5 µL OptiMEM medium (Invitrogen/LifeTechnologies Austria, Vienna, Austria), and after 5 min of incubation, 25 µL diluted Lipofectamine 2000 was mixed with 25 µL of a specific siRNA/reporter vector mix (diluted in OptiMEM). After 20 min of incubation, the mixes were pipetted directly into the wells of a 96-well plate and freshly harvested cells were added. After 24 h of incubation, medium was exchanged and cells were incubated for another 24 h. Culture conditions were as described above. Firefly and *Renilla* luciferase activities were determined at 48 h post-transfection using the Dual-Glo luciferase assay (Promega), according to the manufacturer's instructions.

Briefly, 75 µL of Dual-Glo Reagent was added to cells grown in 75 µL medium, and after 10 min of incubation at room temperature, firefly luciferase activity was measured. Next, one volume of Dual-Glo Stop & Glo reagent was added to each well, plates were incubated for an additional 10 min at room temperature, and eventually, *Renilla* luciferase activity was determined. Luminescence was measured on a Wallac Victor 1420 Multilabel Counter (Perkin Elmer Austria, Brunn am Gebirge, Austria). Knockdown rates were calculated by normalizing *Renilla* luciferase activities to firefly luciferase activities, and comparing dual-luciferase ratios between targeting and non-targeting control siRNAs.

2.6. Determination of mRNA levels

1.25e+05 A549 cells were seeded into the wells of 24-well plates and reverse transfected with siRNAs at a concentration of 10 nM. Volumes of transfection mixes were adjusted to the 24-well plate format. Briefly, for each well 1 µL Lipofectamine 2000 was diluted with 49 µL OptiMEM medium (Invitrogen/LifeTechnologies Austria, Vienna, Austria), and after 5 min of incubation, 50 µL diluted Lipofectamine 2000 was mixed with 50 µL of a specific siRNA diluted in OptiMEM. Transfection conditions were otherwise as described under 2.5. After 24 h of incubation, medium was exchanged and cells were infected with Ad5 at an multiplicity of infection (MOI) of 0.01 TCID₅₀/cell, and total RNA was isolated at 24 h post-infection using an RNeasy Mini® Kit (QIAGEN). Residual DNA was removed with RQ1 DNase (Promega), and reverse transcription was carried out using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Expression levels of the E1A-12S, E1A-13S, DNA polymerase, pTP, IVa2, hexon, and protease genes were determined by TaqMan real-time quantitative PCR (qPCR), using the LightCycler 480 Probes master mix (Roche Diagnostics) and primer/probe sets specific for E1A-13S (E1A 289R-cDNA-f1 5'-GCATGTTTGTCTACAGTCCTGTGTC-3', E1A 289R-cDNA-r1 5'-GGCGTCTCAGGATAGCAGGC-3', and E1A 289R-cDNA-p1 5'-AGGCTCCGTTCTGGCTCGGG-3'), E1A-12S (E1A 12S-cDNA-f1 5'-AGGATGAAGAGGGTCTGTGTC-3', E1A 289R-cDNA-r1 5'-GGCGTCTCAGGATAGCAGGC-3', and E1A 289R-cDNA-p1 5'-AGGCTCCGTTCTGGCTCGGG-3'), DNA polymerase (Pol-cDNA-f1 5'-ATGGCTTGGCTCAAGCTC-3', Pol-cDNA-r1 5'-GCGTAGGTGCTGGCGCAAC-3', and Pol-cDNA-p1 5'-CGCCTCTGCGTGAAGACGACGG-3'), pTP (pTP-cDNA-f2 5'-AAACCAACGCTCGGTGCC-3', pTP-cDNA-r2 5'-GGAGCGGTTCCAGATGTT-3', and pTP-cDNA-p2 5'-CGCGCGCAATCGTTGACCTG-3'), IVa2 (IVa2-cDNA-f1 5'-GGAAACAGAGGGCGAAGA-3', IVa2-cDNA-r1 5'-AGGGTCTCTGCTGAGCTAGTC-3', and IVa2-cDNA-p1 5'-CTTGAGGCTGGTCTGCTGGT-3'), hexon (Hex-cDNA-f1 5'-CAGTCACAGTCGCAAGAGAGC-3', Hex-cDNA-r1 5'-AGGTACTCCGAGGCGTCTG-3', and Hex-cDNA-p1 5'-ACCACTGCGCATCATCAAGGG-3'), and protease (Prot-cDNA-f1 5'-TCACATCGCAAGCTTTGACG-3', Prot-cDNA-r1 5'-GCGGCAGCTGTTGTTGATG-3', and Prot-cDNA-p1 5'-CCGAGAAGGGCGTGGCGAGGTA-3'). The specificity of the primers employed (i.e., covered exon-exon junctions) enabled them to discriminate between overlapping transcripts or transcripts originating from the (+) or (−) strand, respectively. Ad5 gene expression levels were normalized to GAPDH expression levels, which were previously proven to remain unchanged upon Ad5 infection under the selected experimental conditions. GAPDH expression was determined with the primer/probe set GAPDH-f1 5'-TGCACCACCAACTGCTTAGC-3', GAPDH-r1 5'-GGCATGGACTGTGTCATGAG-3', and GAPDH-p1 5'-CCTGGCCAAGTTCATCATGACAACTT-3'. All qPCR assays were set up in 96-well plates and contained 1× LightCycler 480 Probes master mix (Roche Diagnostics, Vienna, Austria), 500 nM of forward and reverse primers, each, 100 nM of probe, and 1 µL of cDNA in a total volume of 20 µL. For the detection of GAPDH cDNA, 300 nM of forward and reverse primers, each, and 1 µL of 1:10 diluted template cDNA were used.

Table 1
siRNA sequences.

Name	Sequence (sense/antisense)	Target site in Ad5 ^a	Target	Serotype specificity ^b
E1A-si1	5'-AACGAGGAGGCGUUCGAGAUUU-3' 5'-AAAUUCGCGAAACCGCCUCCUGUU-3'	731–755	E1A	1, 2, 5, 6
E1A-si2	5'-CAGGAAGGGAUUGACUACUCACUU-3' 5'-AAGUGAGUAAAGUCAAUCCCUUCUG-3'	782–806	E1A	1, 5
E1A-si3	5'-CCUGUGUCUAGAGAAUGCAAUAGUA-3' 5'-UACUAAUUGCAUUCUCUAGACACAGG-3'	1333–1357	E1A	2, 5, 6
E1A-si3_scr	5'-CCUUGUCGAGAAAGUACUAAUGGUA-3' 5'-UACCAUUAUAGUACUUUCUCGACAAGG-3'	–	–	–
Pol-si1	5'-CAAGACCGCUUACCUUACUUGCA-3' 5'-UGCAGUAGAAGGUAAGCGGUCUUG-3'	7782–7806	DNA pol	1, 2, 5, 6
Pol-si2	5'-CAACGUCUCCAGCGUCCAAACCAUA-3' 5'-UAUGGUUGGACGCGUAGAGACGUUG-3'	6928–6952	DNA pol	1, 2, 5, 6
Pol-si3	5'-TGUCUCAGAGUGGUCGAGUUUUA-3' 5'-UAGAAACUCGACCAUCUGAGACA-3'	5818–5842	DNA pol	5
Pol-si4	5'-CGUCUCCAGCGUCCACCAUAUUA-3' 3' UGAUAUGGUUGGACGCGUAGAGACG-3'	6925–6949	DNA pol	1, 2, 5, 6
Pol-si5	5'-CAGCGUCCAAACUUAUUAUCCAAU-3' 5'-AGUUGGUAUUAUGGUUGGACGUG-3'	6918–6942	DNA pol	2, 5, 6
Pol-si6	5'-GCGUCCAAACUUAUUAUCCAAU-3' 5'-UGAGUUGGUAUUAUGGUUGGACG-3'	6916–6940	DNA pol	2, 5, 6
Pol-si2_scr	5'-CAACUUCGAGCGUACCAACGCAUA-3' 5'-UAUUGCGUGGAGGCGCGGAAAGUUG-3'	–	–	–
IVa2-si1	5'-ACAUGCGAGUCAGGACAUUUA-3' 5'-UUAAGCAUGUCCUGACUCGCAUGU-3'	5013–5037	IVa2	5
IVa2-si2	5'-AAAUACAGUCCAAGAUCAUUAU-3' 5'-AUGAGAUCAUUGGACUUAUUU-3'	4410–4434	IVa2	1, 2, 5, 6
IVa2-si3	5'-CAUCCAGCUUAACCGCUUUGUAAA-3' 5'-UUUACAAGCGUUUAGCUGGGAUG-3'	4368–4392	IVa2	1, 2, 5, 6
Hex-si1	5'-UAGAAACUCGACCAUCUGAGACA-3' 5'-AUUUUAUACAGAAUAAGGCGCUGC-3'	5818–5842	Hexon	5
Hex-si2	5'-GAGAAUUAUUGGCGCAACAUUAU-3' 5'-AUAGAUUGUUGGCCAUUAGUUCUC-3'	19776–19800	Hexon	5
Hex-si3	5'-GCCUCAGAAUUCUUUGCCAUUAAA-3' 5'-UUUAAUGGCAAGAACUUUGAGGC-3'	20527–20551	Hexon	5
Hex-si4	5'-CCGUCAGUGGUGGAUUAUUA-3' 5'-UUUAGUAUUAUUAUUAUUAUUA-3'	21247–21271	Hexon	5
Prot-si1	5'-GAGCAGGAACUGAAGCCAUUGUCA-3' 5'-UGACAAUGGCUUUCAGUUCUUGUC-3'	21745–21769	Protease	2, 5, 6
Prot-si2	5'-AAGAUUCUUGUUGGCGCAUUAUU-3' 5'-AAAUUUGGCCCAACCAAGAUCUU-3'	21770–21794	Protease	2, 5, 6
Prot-si3	5'-UCAAGCAGGUUUAUUAUUAUUA-3' 5'-UACUAAACUGUAAACUGCUUGA-3'	21972–21995	Protease	1, 2, 5, 6
pTP-si1	5'-CCGCCUUAUUUAUUUAUUAU-3' 5'-AUGUAAUUAAGUAGGCGGT-3'	9805–9823	pTP	1, 2, 5, 6
pTP-si2	5'-GAGGAGAUUGAAGAAGAAGTT-3' 5'-CUUCUUUUAUUAUUAUUAUUA-3'	9359–9377	pTP	1, 2, 5, 6
pTP-si3	5'-GGUAGAAAGGCUCAUGCAATT-3' 5'-UUGCAUGAGCCUUUCUACCTT-3'	9945–9963	pTP	1, 2, 5, 6
pTP-si4	5'-CGAAAUUGAUUCUGUGCAATT-3' 5'-UUCGACAGAAUUAUUAUUAUUA-3'	8796–8814	pTP	1, 2, 5, 6
pTP-si5	5'-GACUACGUUUUGAUCGAGGGCUU-3' 5'-AAGCCUUCGAGUCAAUUAUUAUUA-3'	10289–10313	pTP	5
pTP-si6	5'-CGGUAGAAAGGCUCAUGCAAGACUA-3' 5'-UAGUUCUUGCAUGAGCCUUUCUACCG-3'	9940–9964	pTP	1, 2, 5, 6
pTP-si7	5'-ACUACCUUCUUUUCAGCGCCUGCGAAA-3' 5'-UUUCGAGGCGCUGAAAGAGGUAGU-3'	9103–9127	pTP	1, 2, 5, 6
pTP-si8	5'-GAAAUUGAUUCUGUCGAACUCUUU-3' 5'-AAGAGAGUUCGACAGAAUCAAUUUC-3'	8789–8813	pTP	1, 2, 5, 6

^a GenBank ID: AC_000008.^b Serotypes displaying 100% target site complementarity.

per reaction. All assays were performed in duplicates using a LightCycler 480 system (Roche Diagnostics, Vienna, Austria) with the following cycling parameters: heating to 95 °C for 1 min followed by 50 cycles at 95 °C for 15 s and 60 °C for 1 min. Data were analyzed using the LightCycler 480 software. Control included with every assay consisted of a 'no template control' (no DNA added).

2.7. Determination of adenovirus genome copy numbers

3e+04 A549 cells were seeded into the wells of 96-well plates, and reverse transfected with siRNAs at concentrations ranging from 0.04 nM to 30 nM. Transfection conditions were as described under 2.5., except that reporter plasmid DNA was omitted. After 24 h, cells were infected with Ad1, Ad2, Ad5, or Ad6 at an MOI of 0.01 TCID₅₀/cell. Samples were collected at 2, 4, and 6 days post-infection. Viral DNA was isolated using a QIAamp DNA Blood Mini Kit (QIAGEN). Ad5 genome copy numbers were determined by qPCR, using the following TaqMan primer/probe set directed against the viral E1A region: E1A-fwd 5'-GACGGCCCCGAAGATC-3', E1A-rev 5'-TCCTGCACCGCAACATT-3', and E1A-p 5'-CGAGGA GGCGGTTTCGAGA-3'. The setup of qPCR assays and the cycling parameters were the same as described above. For each reaction, 1 µL of isolated DNA was used. Adenovirus genome copy numbers were calculated by using serial dilutions of an adenoviral reference DNA as a standard.

2.8. Determination of numbers of infectious virus particles

To liberate the viruses from the cells, 96-well plates containing cells and viruses were subjected to three freeze–thaw cycles. Crude lysates were cleared by centrifugation of the plates for 15 min at 2800 rpm. The numbers of infectious virions were determined on A549 cells by TCID₅₀ assays.

2.9. Cell viability

The experimental setup for the determination of the viability of infected cells was as described for other virus inhibition experiments, except that A549 cells were infected at higher MOIs of 2 TCID₅₀/cell, 4 TCID₅₀/cell, or 6 TCID₅₀/cell. Metabolic activity as a measure of cell viability was determined at 6 days post-infection by performing an MTS assay (CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay), according to the manufacturer's instructions (Promega). Absorbance was determined at 490 nm on a Wallac Victor 1420 Multilabel Counter (Perkin Elmer).

2.10. Statistical analysis

All the data are expressed as mean ± standard deviation (SD). To test for statistical significance, one-way ANOVA corrected with Bonferroni's post hoc test was applied. A *p* value of <0.05 was considered statistically significant.

3. Results

3.1. Identification of best-performing siRNAs directed against E1A, DNA polymerase, pTP, IVa2, hexon, and protease

To analyze which adenoviral processes may constitute useful targets for RNAi-mediated inhibition of adenovirus multiplication, we designed a set of siRNAs targeting the E1A, DNA polymerase, pTP, IVa2, hexon, and protease mRNAs (Table 1). E1A siRNAs were designed to target E1A-12S and also E1A-13S splice isoforms. With the exception of pTP-si1 to pTP-si4, all siRNAs were 25-mer, blunt-ended siRNAs carrying the Invitrogen "Stealth" modification. pTP-

si1 to pTP-si4 were 21-mer, unmodified siRNAs carrying 2 nt TT overhangs at their 3' ends. To screen for the best-performing siRNA of each group, we employed a dual-luciferase assay-based system. The respective target sequences were individually inserted into the 3' UTR of a plasmid-located *Renilla* luciferase gene. The DNA polymerase, pTP, IVa2, hexon, and protease siRNAs, together with the respective reporter vectors, were used to co-transfect HEK293 cells. Knockdown of *Renilla* luciferase expression in relation to the expression of a firefly luciferase gene located on the same plasmid was determined in dual-luciferase assays. The silencing capacity of the E1A siRNAs was assessed in A549 cells because promoter activities of the reporter vectors turned out to be altered upon silencing of the endogenous E1A gene present in HEK293 cells (Graham et al., 1977) (data not shown). For all target mRNAs, we identified siRNAs enabling a knockdown of ≥78% at a concentration of 30 nM (Fig. 1). The best-performing siRNAs of each group, i.e., pTP-si8, Pol-si2, Hex-si2, E1A-si3, IVa2-si2, and Prot-si1, were selected for further characterization.

3.2. SiRNAs decrease mRNA levels directly or indirectly

The dual-luciferase assay-based screening system was employed to select the best-performing siRNAs of each group. Next, we investigated whether the selected siRNAs were able to knock-down gene expression during an adenovirus infection of A549 cells. Cells were transfected with the siRNAs at a concentration of 10 nM, and then infected with Ad5 at an MOI of 0.01 TCID₅₀/cell. Target mRNA levels were determined by RT-qPCR, using primers specific for the individual mRNAs (Fig. 2A). The highest silencing rates (93–97%) were observed for the E1A-, DNA polymerase-, pTP-, and IVa2-targeting siRNAs. Silencing of the hexon and protease genes was less pronounced (79–87%). Except for the difference in residual hexon and pTP mRNA levels, the differences between hexon or protease mRNA levels and those of all other early genes were statistically significant.

As the pTP, DNA polymerase, and IVa2 mRNAs share a common 3' part (Supplementary Fig. 1), and the DNA polymerase target site is also part of the pTP mRNA, IVa2- and DNA polymerase-directed siRNAs were therefore expected to concomitantly silence pTP/DNA polymerase/IVa2 and pTP/DNA polymerase, respectively. Furthermore, siRNA-mediated silencing of early genes was expected indirectly to affect the expression of those middle or late genes for which expression is known to depend on early viral gene products. Thus, we also determined the effect of the E1A-, pTP-, DNA polymerase-, and IVa2-targeting siRNAs on the expression of the other genes. Silencing of E1A resulted in a marked reduction in the expression of all other genes (Fig. 2B). This can be attributed to the central role of E1A in activating the expression of downstream genes.

Silencing of the E1A gene actually resulted in a greater reduction in the expression of hexon and protease than did direct silencing of these genes by the hexon and protease siRNAs (compare Fig. 2A and B). As expected, the DNA polymerase-directed siRNA also silenced the pTP gene, and indirectly also affected the expression of all other genes (Fig. 2C). This finding is in accordance with the dependency of IVa2 and ML transcription on the replication of the adenoviral genome, for which DNA polymerase expression is mandatory (Flint, 1986; Ifthode and Flint, 2004; Shaw and Ziff, 1980). The same holds true for silencing of pTP (Fig. 2D), which is also essential for virus DNA replication, and consequently activation of transcription from the other promoters. Although the pTP siRNA target site is absent from DNA polymerase mRNA, pTP silencing also decreased DNA polymerase mRNA levels, albeit to a lesser extent than DNA polymerase silencing did. This reduction can be attributed to the inhibition of DNA replication by the pTP siRNA, and consequently decreased DNA polymerase gene copy

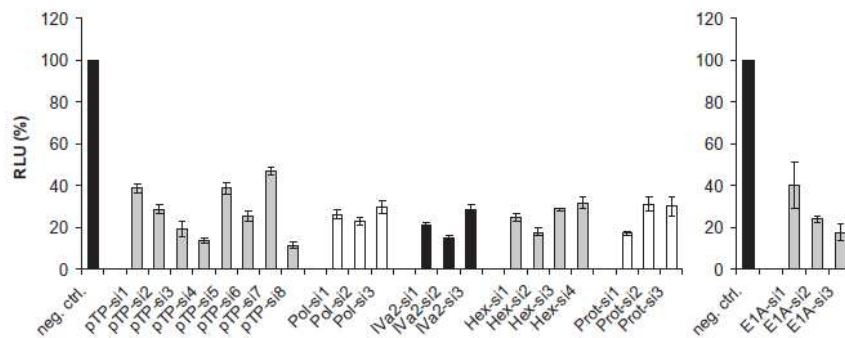


Fig. 1. Screening for functional siRNAs. siRNAs and their respective dual-luciferase target vectors were used to co-transfect HEK293 cells (DNA polymerase, pTP, IVa2, hexon, and protease siRNAs) or A549 cells (E1A siRNAs). A non-targeting control siRNA served as a negative control (neg. ctrl.). All siRNAs were employed at a concentration of 30 nM. *Renilla* luciferase activities in relation to firefly luciferase activities were determined 48 h post-transfection. Relative light units (RLU; mean $\pm$ SD, $n = 3$) in comparison to a non-targeting control siRNA are shown. Differences between neg. ctrl. siRNA and targeting siRNAs were statistically significant in all cases ($p < 0.05$).

numbers. As expected, the IVa2 siRNA led to a reduction not only in IVa2, but also in pTP and DNA polymerase mRNA levels (Fig. 2E). Since transcription from the MLP is highly activated by the IVa2 protein (Tribouley et al., 1994), ML transcript levels were also indirectly decreased.

3.3. siRNAs decrease Ad5 genome copy numbers, and also the output of infectious virus progeny

In order to investigate the gene silencing effect of the individual siRNAs on adenovirus replication, A549 cells were transfected with the siRNAs at a concentration of 10 nM and infected as before. At 2 days post-infection, Ad5 genome copy numbers were determined by qPCR, using primers directed against the E1A gene (Fig. 3A). With the exception of the hexon and protease siRNAs, all siRNAs effectively inhibited adenovirus replication. The highest inhibition rate was achieved with the DNA polymerase siRNA, which decreased Ad5 genome copy numbers on average by approximately 2.5 orders of magnitude (99.6%). The failure of the hexon and protease siRNAs to decrease virus genome copy numbers was not surprising, because a reduction in hexon and protease levels is not expected to affect viral DNA replication.

Next, we evaluated the performance of those siRNAs that were expected directly or indirectly to affect the output of viral DNA (i.e., E1A, DNA polymerase, pTP, and IVa2 siRNAs) in a time-course experiment spanning 6 days in which Ad5 was allowed to spread throughout the cultures (Fig. 3B). As expected, viral genome copy numbers were also decreased at later time points. We repeated the experiments with higher siRNA concentrations (30 nM and 90 nM) and obtained comparable results (data not shown). The inhibition rate at late time points may be generally underestimated; although the cells were infected with Ad5 at a low MOI of 0.01 TCID₅₀/cell, the high burst size of adenovirus rapidly leads to infection of the entire culture. This prevents an exponential increase in virus multiplication at later time points, in those cultures in which replication is not attenuated by siRNAs.

The impact of siRNAs on viral processes other than DNA replication is not fully elucidated by the measurement of virus genome copy numbers. Thus, we also determined the output of infectious virus progeny. Cells were transfected and infected as described above, and the numbers of infectious virus particles at 48 h post-infection were determined (Fig. 4). We observed that hexon and protease siRNAs inhibited the production of infectious virus progeny by approximately 1.3 and 0.8 orders of magnitude (94.9% and 83.1%), respectively. However, the other siRNAs led to an even

higher decrease in virus titers of up to 2.8 orders of magnitude (99.8%).

Taken together, our data indicate that silencing of early or intermediate genes seems to be more effective in terms of reducing the output of viral DNA, and also the number of infectious virus progeny, than is silencing of late genes. Computational calculation of the target site accessibility of the DNA polymerase siRNA, using the RNAx software tool, suggested high accessibility of the entire region embedding the Pol-si2 target site. Target site accessibility has been reported to correlate with high effectiveness of siRNAs (Tafer et al., 2008; Westerhout and Berkhout, 2007). Thus, we speculated that siRNAs capable of binding to target sites in the immediate vicinity of, or overlapping, the target site of the Pol-si2 siRNA may allow similar or even better knockdown of DNA polymerase gene expression than Pol-si2. Thus we designed three more such siRNAs (Fig. 5A). However, none of them proved superior to the Pol-si2 siRNA (Fig. 5B). The functionality of Pol-si2 was also validated by comparing its activity not only to that of a universal non-targeting control siRNA but also to that of a scrambled version. No change in the inhibition rate was observed (Supplementary Fig. 2). The inhibitory effect of Pol-si2 was also shown to be dose-dependent (Fig. 6). The silencing capacity of low siRNA concentrations may even be underestimated in some experiments; in control experiments employing fluorescence-labeled siRNAs, the transfection efficiency decreased significantly at concentrations of <5 nM (data not shown). Thus, low siRNA concentrations do not truly reflect the silencing capacity, because significant numbers of cells contain no siRNA.

The target sequence of the DNA polymerase siRNA is also present in the mRNAs of the other members of adenovirus species C (i.e., Ad1, Ad2, and Ad6), all of which commonly account for life-threatening disseminated adenovirus disease. Consequently, the inhibitory effect of the DNA polymerase siRNA was not restricted to Ad5. Replication of Ad1, Ad2, and Ad6 was also efficiently inhibited (Supplementary Fig. 3).

3.4. Treatment with individual siRNAs or combinations of siRNAs results in a comparable decrease in the numbers of infectious virus particles

Given the dependency of intermediate or late adenoviral gene expression on certain early viral gene products, simultaneous silencing of different adenoviral genes may have synergistic effects on the inhibition of virus multiplication. We therefore performed virus inhibition experiments using combinations of siRNAs. In all

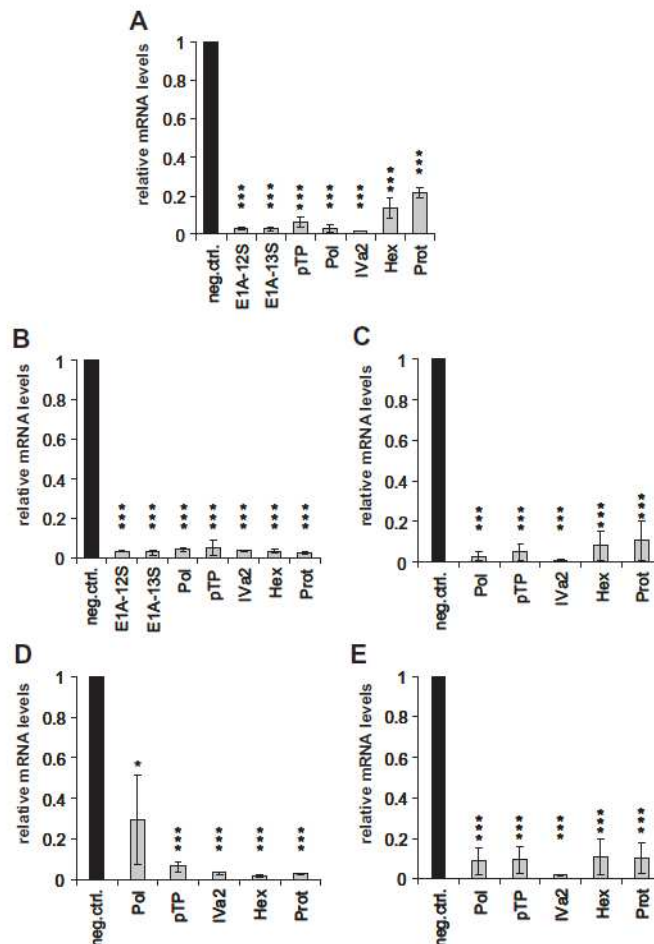


Fig. 2. siRNAs decrease mRNA levels directly and indirectly. A549 cells were transfected with siRNAs directed against the E1A, DNA polymerase (Pol), pTP, IVa2, hexon (Hex), and protease (Prot) genes, or a non-targeting control siRNA (neg. ctrl.) at a concentration of 10 nM, and then infected with Ad5 at an MOI of 0.01 TCID₅₀/cell. Levels of direct targets (A) or indirect targets such as the E1A (B), DNA polymerase (C), pTP (D), and IVa2 (E) mRNAs were determined by RT-qPCR. Relative mRNA levels in comparison to a non-targeting siRNA are shown. Values represent mean $\pm$ SD of three independent experiments, each performed in triplicate. For each experiment, real-time qPCR quantification was performed in duplicate. * $p < 0.05$, *** $p < 0.001$.

of these experiments, we used a total siRNA concentration of 10 nM, i.e., combined siRNAs were employed at a concentration of 5 nM each. As a control, cells were transfected with the individual siRNAs at a concentration of 10 nM. To correct for potential saturation effects (e.g., during transfection and/or RISC loading of siRNAs), cells were also transfected with a combination of 5 nM individual targeting siRNA and 5 nM non-targeting control siRNA. The numbers of infectious virus particles were determined at 48 h post-infection by TCID₅₀ assay (Fig. 7). As shown in Fig. 7B, the superior anti-adenoviral effect mediated by the DNA polymerase siRNA was not enhanced by simultaneous targeting of those mRNAs whose generation depends on the function of the DNA polymerase, e.g., the IVa2 or hexon genes. Similarly, combined E1A and DNA polymerase silencing did not further decrease virus titers (Fig. 7A). The same held true for all other siRNA combinations. In general, combining a highly effective siRNA with a less well-performing siRNA led to an intermediate inhibition rate, or

an inhibition rate equal to the one caused by the individual better-performing siRNA. Moreover, the anti-adenoviral effect of an individual siRNA was not reduced by halving its concentration upon combination with an equal concentration of non-targeting negative control siRNA.

We speculated that possible synergistic effects may have been undetectable, because the cells were harvested at a relatively early time point (48 h post-infection). However, they might become detectable at later time points, when the virus was allowed to spread throughout the culture. We hypothesized that combinations comprising the E1A siRNA on the one hand, and siRNAs targeting mRNAs originating from other early/middle genes on the other, would be most likely to cause a synergistic effect. We therefore repeated the virus inhibition experiment using the respective siRNA combinations, and determined Ad5 genome copy numbers at 6 days post-infection. However, we did not detect any synergistic effects at this late time point (Supplementary Fig. 4). We also

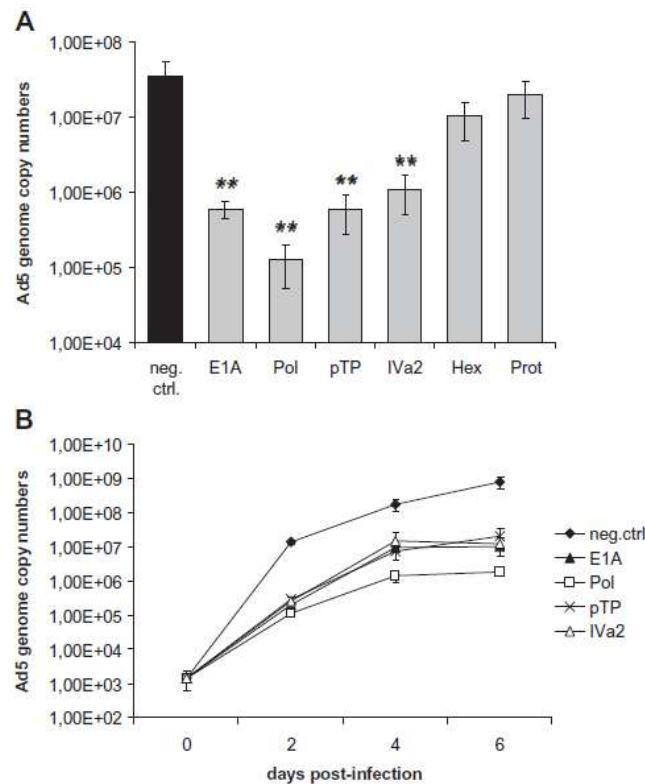


Fig. 3. Impact of siRNAs on viral DNA replication and virus spreading. (A) A549 cells were transfected with siRNAs directed against the E1A, DNA polymerase (Pol), pTP, IVa2, hexon (Hex), and protease (Prot) genes, or a non-targeting control siRNA (neg. ctrl.) at a concentration of 10 nM, and then infected with Ad5 at an MOI of 0.01 TCID₅₀/cell. Virus genome copy numbers were determined at 48 h post-infection by qPCR, using E1A-specific primers. Values represent mean $\pm$ SD of three independent experiments, each performed in triplicate. For each experiment, real-time qPCR quantification was performed in duplicate. * $p < 0.001$. (B) Long-term infection of A549 cells with Ad5 at an MOI of 0.01 TCID₅₀/cell, and treatment with 10 nM of the indicated siRNAs. Virus genome copy numbers were determined at time points 0, 2, 4, and 6 days post-infection by qPCR. Representative data from at least three independent experiments, each performed in triplicates, are shown (mean $\pm$ SD; $n = 3$). For each experiment, real-time qPCR quantification was performed in duplicate. ** $p < 0.01$.

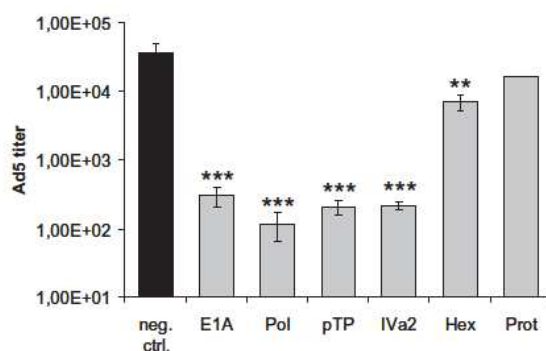


Fig. 4. siRNAs decrease the numbers of infectious virus particles. A549 cells were transfected with siRNAs directed against the E1A, DNA polymerase (Pol), pTP, IVa2, hexon (Hex), and protease (Prot) genes, or a non-targeting control siRNA (neg. ctrl.) at a concentration of 10 nM, and then infected with Ad5 at an MOI of 0.01 TCID₅₀/cell. Numbers of infectious virus particles at 48 h post-infection were determined on A549 cells by TCID₅₀ assays. Representative data from three independent experiments, each performed in triplicate, are shown (mean $\pm$ SD; $n = 3$). ** $p < 0.01$, *** $p < 0.001$.

repeated the experiment using lower concentrations of siRNAs. Although there was a slight trend toward somewhat increased inhibition for some combinations, none of these differences were statistically significant, and under no conditions did any combinations of siRNAs result in a higher inhibition rate than the inhibition rate caused by Pol-si2 when applied alone (Supplementary Fig. 5).

3.5. siRNAs increase the viability of infected cells to different extents

Next, we quantitatively assessed the impact of Ad5 gene silencing on the viability of infected cultures. We transfected A549 cells with the siRNAs at a concentration of 10 nM as before, and then infected them with Ad5 at a higher MOI (4 TCID₅₀/cell) to ensure pronounced cell killing. We determined the metabolic activity as a measure of cell viability at 6 days post-infection, by means of an MTS assay (Fig. 8). As expected, the siRNAs, although greatly decreasing the output of virus progeny, were not capable of preventing already infected cells from cell death. This was also clearly deducible from experiments in which the overall appearance of infected cultures was assessed by crystal violet staining (data not shown).

However, all siRNAs were capable of prolonging cell survival, albeit to different extents. This protective effect was most pro-

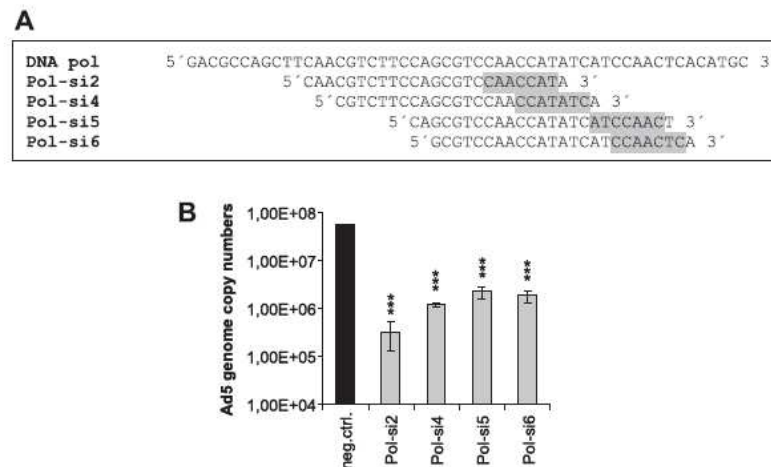


Fig. 5. Differential inhibition of Ad5 replication by DNA polymerase siRNAs binding in the immediate vicinity of, or overlapping, the Pol-si2 target sequence. (A) Region of the DNA polymerase open reading frame (indicated as DNA pol) targeted by siRNAs Pol-si2, Pol-si4, Pol-si5, and Pol-si6. The DNA sequences corresponding to the individual siRNA target sites on the target mRNAs are given below. The nucleotides corresponding to the seed regions of the respective siRNAs are shaded in grey. (B) A549 cells were transfected with the viral DNA polymerase-directed siRNAs or a non-targeting control siRNA (neg. ctrl.) at a concentration of 10 nM, and then infected with Ad5 at an MOI of 0.01 TCID₅₀/cell. Virus genome copy numbers from triplicate infections (mean $\pm$ SD; $n = 3$) were determined at 48 h post-infection by qPCR using E1A-specific primers. *** $p < 0.001$.

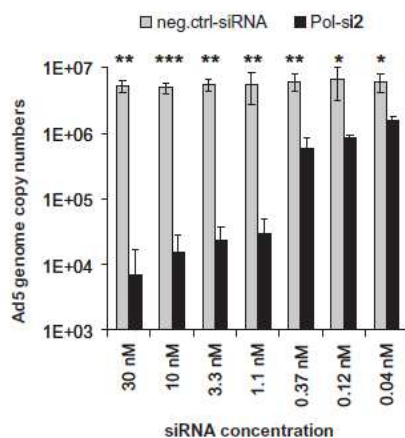


Fig. 6. Dose-dependent decrease in Ad5 genome copy numbers mediated by the DNA polymerase siRNA. A549 cells were transfected with the DNA polymerase siRNA or a non-targeting control siRNA (neg. ctrl.) in decreasing concentrations as indicated, and then infected with Ad5 at an MOI of 0.01 TCID₅₀/cell. Virus genome copy numbers from triplicate infections (mean $\pm$ SD; $n = 3$) were determined at 48 h post-infection by qPCR, using E1A-specific primers. For each experiment, real-time qPCR quantification was performed in duplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

nounced for cells transfected with the E1A siRNA. Although such cells displayed severe cytopathic effects and were already partially detached from the culture vessels, the culture viability was remarkably high (>80%) at 6 days post-infection. We repeated the experiment using lower and higher MOIs (2 TCID₅₀/cell and 6 TCID₅₀/cell, respectively) and obtained comparable results with a tendency towards higher and lower protection at decreased and increased MOIs, respectively (data not shown). The observed protective effect of the E1A siRNA could not be attributed to a possible unspecific general increase in cellular metabolic activity, because neither the E1A siRNA nor any of the other siRNAs altered the viability of uninfected cells (Supplementary Fig. 6). Thus, although the E1A siRNA did not inhibit the output of infectious virus progeny as

efficiently as did the DNA polymerase siRNA, it enhanced the viability of infected cells and kept them alive for a prolonged time period.

4. Discussion

In the present study, we evaluated a larger panel of potential targets, and also determined the inhibitory effect of siRNAs on wild-type adenovirus. SiRNAs directed against the E1A, DNA polymerase, pTP, and IVa2 transcripts were all capable of efficiently silencing the respective genes in the course of an adenovirus infection. By contrast, although having displayed a comparable silencing capacity in luciferase reporter assays, the hexon- and protease-directed siRNAs, showed only a limited capacity to reduce the number of ML transcripts. This observation can be attributed to the markedly higher amounts of hexon and protease mRNAs generated from the particularly strong MLP, in comparison with the mRNA levels of the other genes. This high number of MLP-derived late mRNAs may become even more problematic in RNAi-based attempts to inhibit adenovirus multiplication, because the virus-associated RNAs (VA-RNAs) I and II (non-coding RNAs produced in low amounts during the early stages of infection, but in vast amounts at later time points) appear to counteract RNAi. This effect is thought to be partially caused by the incorporation into and saturation of the RISC by VA-RNA subfragments, which behave like miRNAs (Andersson et al., 2005). Thus, siRNA-mediated inhibition of adenovirus gene expression during the early stages of infection may generally be more beneficial than inhibition of late-stage gene expression. In this regard, inhibition of viral DNA replication may be particularly advantageous, because a decrease in viral genome copy numbers should significantly lower VA-RNA gene copy numbers. In the present study, we observed such an indirect effect when measuring pTP mRNA levels following knockdown of viral DNA polymerase expression. Although not a direct target of the DNA polymerase siRNA, the pTP mRNA levels dropped significantly as a consequence of reduced genome (and pTP gene) copy numbers (Fig. 2D).

Effective knockdown of hexon gene expression may be even more complicated, because hexon mRNA-directed siRNAs target not only the hexon, but also the pVI mRNA. This is caused by the

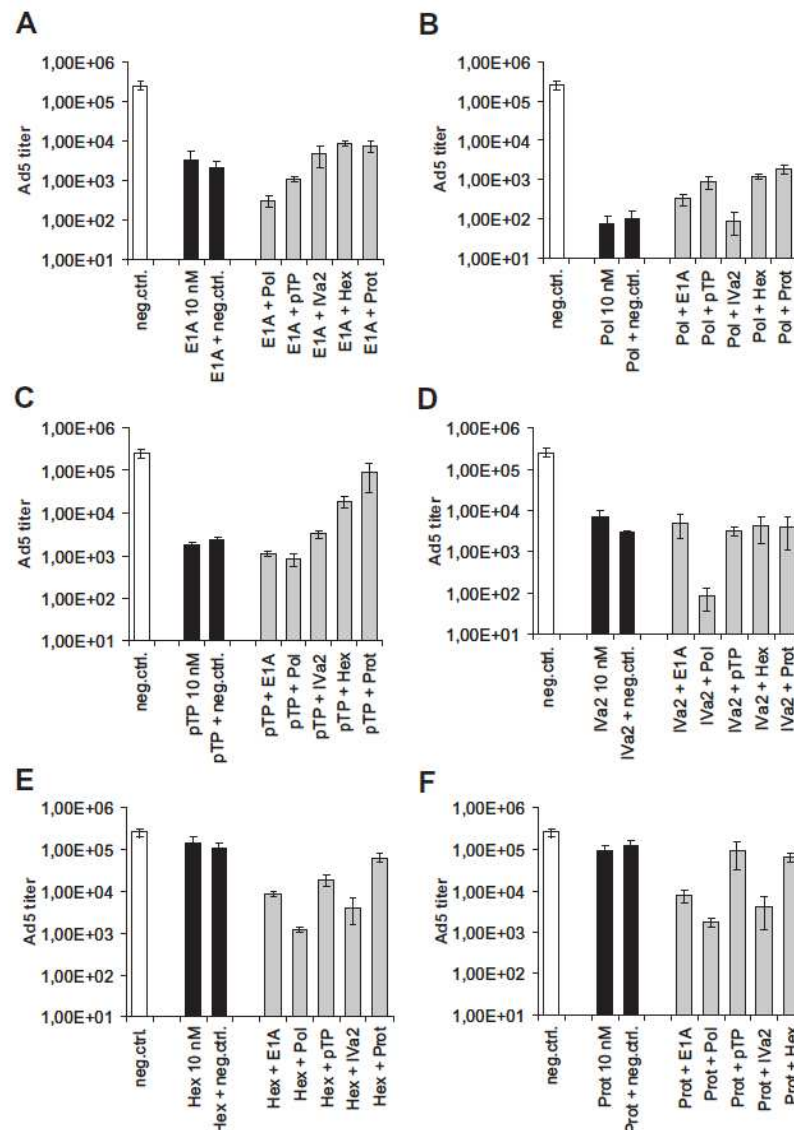


Fig. 7. Effect of combinatorial silencing of Ad5 genes on virus output. A549 cells were transfected with the indicated siRNAs either alone or in combination. siRNA combinations for E1A (A), DNA polymerase (B), pTP (C), IVa2 (D), hexon (E), and protease (F) are depicted. For all transfections, the total concentration of siRNA was 10 nM. For combinations, each siRNA was employed at a concentration of 5 nM. As a control, cells were transfected with the individual siRNAs alone at a concentration of 10 nM, or as a mix of 5 nM targeting siRNA and 5 nM non-targeting negative control siRNA. Subsequently, cells were infected with Ad5 at an MOI of 0.01 TCID₅₀/cell, and cells and supernatants were harvested at 48 h post-infection. Numbers of infectious Ad5 particles of triplicate infections were determined on A549 cells by TCID₅₀ assays (mean $\pm$ SD; $n = 3$).

presence of the hexon-encoding sequence downstream of the pVI open reading frame on all pVI transcripts. Thus, hexon mRNA-targeting siRNAs may be partially sequestered away from their actual target by the pVI mRNA, thereby becoming limiting in hexon silencing. The same holds true for the protease siRNA (which concomitantly silences all other genes of the L3 region, i.e., pVI and hexon), the IVa2 siRNA (which additionally binds to the DNA polymerase and pTP mRNAs), and the DNA polymerase siRNA (which

concomitantly silences the pTP gene). However, the mRNA levels of these genes, especially those coding for DNA polymerase and pTP, are far lower than those produced by the MLP, and siRNAs may less easily become limiting. Hexon gene silencing was previously demonstrated to be as effective in inhibiting adenovirus multiplication as was silencing of the early E1A gene (Eckstein et al., 2010). This may be attributed to the fact that the mutant virus used was deficient in the E1B-55K gene. E1B-55K has been reported to

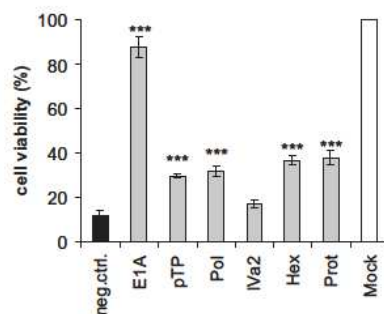


Fig. 8. siRNAs increase the viability of infected cells. A549 cells were transfected with siRNAs directed against the E1A, DNA polymerase (Pol), pTP, IVa2, hexon (Hex), and protease (Prot) genes, or a non-targeting control siRNA (neg. ctrl.) at a concentration of 10 nM, and then infected with Ad5 at an MOI of 4 TCID₅₀/cell. An MTS assay was performed at 6 days post-infection. The viability of cells from triplicate infection experiments (mean $\pm$ SD; $n = 3$) was calculated in relation to the viability of mock-infected cells. *** $p < 0.001$.

promote the export of MLP-derived transcripts from the nucleus (Woo and Berk, 2007). Thus, and consequently, lower amounts of ML mRNAs may accumulate in the cytoplasm of cells infected with this mutant virus.

In the present study, we speculated that silencing of early rather than late adenoviral genes would be more effective in inhibiting adenovirus multiplication. We observed that indirect inhibition of hexon and protease gene expression by silencing of genes for which expression activates ML transcription was more effective than was direct targeting of the hexon and protease transcripts (Fig. 2B–E). Importantly, this included E1A silencing. It was previously reported that E1A promotes adenoviral DNA replication, even when present at very low concentrations (Hitt and Graham, 1990). The rather disappointing anti-adenoviral effect obtained with an E1A-directed siRNA (Eckstein et al., 2010) was ascribed to this fact. In the present study, the E1A siRNA employed was obviously potent enough efficiently to decrease not only the E1A mRNA levels, but also, indirectly, the mRNA levels of E1A downstream targets such as the DNA polymerase, pTP, IVa2, hexon, and protease genes (Fig. 2B). Consequently, E1A silencing markedly inhibited the synthesis of viral DNA, and also the generation of infectious virus progeny (Figs. 3 and 4).

The E1A siRNA also substantially improved the viability of the infected cultures, as measured by MTS assay (Fig. 8). This effect was not immediately evident by visual inspection of the cultures, because the siRNA failed to prevent certain adenovirus-related cytopathic effects. The increase in cell viability may be derived from prevention of the well-known toxic effects caused by the main E1A splice isoforms, which eventually drive cells into apoptosis (Cuconati et al., 2002; Lowe and Ruley, 1993; White, 2001). Cell viability was only moderately improved upon silencing of the other early genes. This contradicts a possible indirect E1A siRNA-mediated protective effect (which may occur following blockage of viral DNA replication), and a consequent decrease in the copy numbers of other genes, such as the adenovirus death protein (ADP) gene, which is required for efficient cell lysis and virus release (Tollefson et al., 1996). The inability of the E1A siRNA used by Eckstein et al. (2010) to increase cell viability may also be partially related to the absence of the anti-apoptotic E1B genes from the mutant virus employed.

A reduction in infectious virus progeny was also achievable by knockdown of IVa2 gene expression. However, the fact that IVa2-directed siRNAs silenced not only the IVa2 gene, but also the DNA polymerase and pTP genes, makes it impossible to distinguish

whether the main inhibitory effect was caused by blockage of IVa2-mediated viral processes (i.e., activation of late gene expression or DNA packaging), or by inhibition of viral DNA synthesis. The other 2 siRNAs targeting the viral DNA replication machinery (i.e., the pTP and DNA polymerase genes) were among the most effective in inhibiting adenovirus multiplication. This finding does not exclude IVa2-mediated viral processes as potential targets for RNAi-mediated intervention, but clearly establishes adenoviral DNA replication as a key target for the inhibition of adenovirus multiplication.

Combinatorial targeting of different viral transcripts has occasionally been reported to lead to synergistic effects (Chen et al., 2005; ter Brake et al., 2006). In the present study, combinatorial targeting of different adenoviral transcripts did not further decrease virion production. This observation is in accordance with similar findings of Eckstein et al. (2010). It is possible that, in some cases, targeting of 2 distinct transcripts may be redundant. For example, it is conceivable that reducing hexon protein, and also viral genome numbers, is of no additional benefit, because the output of DNA-containing virions will remain unchanged regardless of whether high or low amounts of structural proteins are produced. Nevertheless, synergistic effects are conceivable for other combinations. At least at high siRNA concentrations, competitive effects during lipofection or saturation of RISC are conceivable reasons for the failure to observe synergistic effects. To correct for these, we compared the inhibitory effects of combined siRNAs to those of individual siRNAs, and also to individual siRNAs combined with non-targeting negative control siRNA. Throughout these experiments, we maintained a constant concentration of each siRNA, regardless of whether it was combined with another targeting siRNA. However, we did not observe any synergistic effects. The repeatedly observed failure to produce synergistic effects upon combining siRNAs has been suspected to be attributable to the competition between siRNAs for RISC loading (Castanotto et al., 2007; Formstecher et al., 2006; Koller et al., 2006). It is possible that some of the siRNAs employed in the present study were more efficiently incorporated into the RISC, and were therefore able to outcompete the others.

Animal studies will eventually reveal how efficiently the siRNAs selected in this study can inhibit adenovirus multiplication *in vivo*. Delivery of siRNAs into living organisms is much more challenging than delivery into cells *in vitro*. However, a number of delivery vehicles have been developed over the past years which have continuously improved the delivery rates *in vivo* (Rettig and Behlke, 2011), and RNAi has successfully been applied to condemn virus replication *in vivo* (Arbuthnot, 2010; Haasnoot et al., 2007; Zhou and Rossi, 2011). The results reported here may also help to generate viral vectors for the efficient expression and delivery of anti-adenoviral siRNAs in the form of shRNAs or artificial miRNAs, a potential alternative way of eliciting anti-adenoviral RNAi in infected cells.

5. Conclusion

Taken together, our data indicate that: (i) highly potent siRNAs are able to inhibit adenovirus multiplication, making them attractive anti-adenoviral drug candidates; (ii) silencing of early adenoviral genes may be more beneficial than silencing of late genes; (iii) silencing of certain early genes can indirectly reduce late gene products more efficiently, or at least as well as, direct silencing of the late genes; (iv) adenoviral infections may be more effectively treated by reduction of adenoviral DNA than by reduction of the proteinaceous components of the virion; (v) the adenoviral DNA replication machinery, and in particular the DNA polymerase gene, constitutes a key target for RNAi-mediated inhibition of adenovirus

multiplication; and (vi) silencing of the E1A gene (although less effective than silencing of the DNA polymerase gene in preventing the generation of virus progeny) should not be excluded as a potential strategy, because it may impair virus spread *in vivo*, by prolonging the survival of infected cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.antiviral.2012.03.011>.

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3. Third part

**AN ADENOVIRAL VECTOR-BASED EXPRESSION AND DELIVERY SYSTEM
FOR THE INHIBITION OF WILD-TYPE ADENOVIRUS REPLICATION BY
ARTIFICIAL microRNAs**

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Submitted to Antiviral Research

An adenoviral vector-based expression and delivery system for the inhibition of wild-type adenovirus replication by artificial microRNAs

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Abstract

Human adenoviruses are rarely associated with life-threatening infections in healthy individuals. However, immunocompromised patients, and particularly allogeneic hematopoietic stem cell transplant recipients, are at high risk of developing disseminated and potentially fatal disease. The efficacy of commonly used drugs to treat adenovirus infections (*i.e.*, cidofovir in most cases) is limited, and alternative treatment options are needed. Artificial microRNAs (amiRNAs) are a class of synthetic RNAs resembling cellular miRNAs, and, similar to their natural relatives, can mediate the knockdown of endogenous gene expression. This process, termed RNA interference, can be harnessed to target and potentially silence both cellular and viral genes. In this study, we designed amiRNAs directed against adenoviral E1A, DNA polymerase, and preterminal protein (pTP) mRNAs in order to inhibit adenoviral replication *in vitro*. For the expression of amiRNA-encoding sequences, we utilized replication-deficient adenoviral vectors. In cells co-infected with both the recombinant vectors and the wild-type (wt) adenovirus, one particular amiRNA that was directed against the pTP mRNA was capable of decreasing the output of infectious wt virus progeny by 2.6 orders of magnitude. This inhibition rate could be achieved by concatemerizing amiRNA-encoding sequences to allow for high intracellular amiRNA concentrations. Because superinfecting wt virus induces the replication and amplification of the recombinant adenoviral vector, amiRNA concentrations were increased in cells infected with wt adenovirus. Furthermore, a combination of amiRNA expression and treatment of infected cells with cidofovir resulted in additive effects that manifested as a total reduction of infectious virus progeny by greater than 3 orders of magnitude.

1. Introduction

Virus infections in immunocompromised individuals represent a major clinical problem that has escalated during the AIDS pandemic and is currently fueled by the permanently increasing number of organ and hematopoietic stem cell transplantations. Among others, human adenoviruses (Echavarria, 2008; Ison, 2006; Kojaoghlanian et al., 2003), belonging to the group of double-stranded (ds) DNA viruses, are a major cause of systemic infections with significant mortality rates in immunocompromised patients (Blanke et al., 1995; Hale et al., 1999; Howard et al., 1999; Lion et al., 2003; Munoz et al., 1998). Severe manifestations are mostly caused by adenoviruses belonging to species B and C (Kojaoghlanian et al., 2003), with a predominance of species C members reported in certain studies (Ebner et al., 2006; Lion et al., 2003; Lion et al., 2010). To date, the only drug with convincing anti-adenoviral activity *in vivo* is cidofovir (CDV). However, CDV is neither capable of fully preventing fatal outcomes in all instances (Lenaerts et al., 2008; Lindemans et al., 2010; Ljungman et al., 2003; Symeonidis et al., 2007; Yusuf et al., 2006), nor thought to be able to completely clear adenovirus infections without the concomitant re-establishment of the immune system (Chakrabarti et al., 2002; Heemskerk et al., 2005; Lindemans et al., 2010). Moreover, it displays significant nephrotoxicity and limited bioavailability. Derivatives of CDV have been developed, but are still under investigation (Hartline et al., 2005; Paolino et al., 2011). Thus, there is a need for the development of alternative drugs or even alternative treatment strategies. RNA interference (RNAi) is a post-transcriptional cellular process that results in gene silencing (Carthew and Sontheimer, 2009; Ghildiyal and Zamore, 2009; Huntzinger and Izaurralde, 2011; Hutvagner and Simard, 2008; Kawamata and Tomari, 2010). It

is triggered by short (~21–25 nt) dsRNAs displaying partial or complete complementarity to their target mRNAs. MicroRNAs (miRNAs) are members of this group of small RNAs. Their precursors, primary miRNAs (pri-miRNAs), are generated in the nucleus by transcription from (predominantly) RNA polymerase II promoters, from either stand-alone genes, clusters of miRNA-encoding sequences, or introns. These pri-miRNAs are processed by Drosha/DGCR8 into 60–70 nt precursor miRNAs (pre-miRNAs) (Cullen, 2004), which are subsequently exported from the nucleus by exportin-5 in a Ran-GTP-dependent manner (Yi et al., 2003). Pre-miRNAs are further processed into mature miRNAs by the ribonuclease-III enzyme Dicer (Cullen, 2004). The so-called guide strand of the mature miRNA is loaded into the RNA-induced silencing complex (RISC) to give rise to the mature miRISC (Sontheimer, 2005), a complex that mediates the recognition of the target mRNA by the guide strand of the miRNA (Fire et al., 1998). Depending on the degree of complementarity between these 2 RNA molecules, this interaction can result in the cleavage of the mRNA (in the case of full or nearly full complementarity), deadenylation (followed by increased degradation), and/or translational repression (Huntzinger and Izaurralde, 2011).

RNAi is not only induced by endogenous miRNAs, but can also be artificially triggered by the introduction of synthetic short interfering RNAs (siRNAs) of ~21–25 nt in length into living cells (Elbashir et al., 2001). These siRNAs bypass the processing of endogenous pri- and pre-miRNA by Drosha and Dicer, and are directly incorporated into RISC. Alternatively, artificial siRNAs can also be generated inside a cell by the expression of pre-miRNA-mimicking short hairpin RNAs (shRNAs) from polymerase III promoters; these shRNAs are subsequently processed into siRNAs by Dicer (Burnett and Rossi, 2012). Yet another method of producing artificial siRNAs is

expression in the form of artificial miRNAs (amiRNAs) from (mostly) RNA polymerase II promoters (first demonstrated for mammalian cells by (Zeng et al., 2002)). They resemble natural miRNAs, *i.e.*, they have to be processed by Drosha and Dicer before they are incorporated into RISC. For proper pri-miRNA/pre-miRNA processing, the sequence giving rise to the artificial shRNA hairpin loop is embedded into sequences originating from natural, human, or nonhuman pri-miRNAs, such as miR-30 (Zeng et al., 2002), miR-155 (Chung et al., 2006), miR-31 (Ely et al., 2008), miR-122 (Ely et al., 2008), miR-106b (Aagaard et al., 2008), miR-17-92 (Liu et al., 2008), miR-19a (Seidl and Ryan, 2011), miR-21 (Yue et al., 2010), or mir-126 (Chen et al., 2011). Currently, most amiRNA expression cassettes are based on human miR-30 and murine miR-155 backbone sequences.

Following the discovery that artificial siRNAs can induce RNAi when introduced into cells (Elbashir et al., 2001), this principle has quickly been adopted as a tool to knock down the expression of disease-associated genes or to inhibit viral gene expression (Davidson and McCray, 2011). RNAi-based approaches have since successfully been employed to inhibit the replication of a wide range of mammalian viruses, both *in vitro* and *in vivo* (Arbuthnot, 2011; Haasnoot et al., 2007; Zhou and Rossi, 2011). We and others have recently demonstrated the successful *in vitro* inhibition of the replication of wild-type (wt) adenovirus (Ad) serotypes 1, 2, 5, and 6 (all belonging to species C and representing a main cause of severe adenovirus-related disease) (Kneidinger et al., 2012) and a mutated version of Ad5 lacking the E1B and E3 genes (Eckstein et al., 2010). The inhibition of an Ad 11 strain (2K2/507/KNIH; species B; isolated in Korea) has also been described (Chung et al., 2007). In all cases, inhibition of virus replication was mediated by exogenously added

siRNAs, and mRNAs originating from viral genes directly or indirectly associated with viral DNA replication were identified as key targets (Kneidinger et al., 2012).

Although *in vivo* siRNA delivery has continuously been improved over the last years (Rettig and Behlke, 2012), it still represents a major challenge. In particular, targeted delivery into certain cell types or organs has proven tricky. In the past, viral vectors have frequently and successfully been employed for the delivery of protein-encoding DNA sequences into living organisms. Consequently, they have also been adopted for the delivery of shRNAs and amiRNAs (Liu and Berkhout, 2011; Mowa et al., 2010; Raoul et al., 2006). Depending on the type of target cell, organ, or delivery route, they may still outperform nonviral delivery systems in certain instances. Adenoviral vectors have been used for a long time to deliver DNA sequences into living organisms (Goncalves and de Vries, 2006). Since they display the same cell tropism as wt adenoviruses (when belonging to the same adenoviral species), they deliver transgenic DNA into exactly those cells that represent the main targets of their wt counterparts. Thus, adenoviral vectors may constitute a particularly attractive tool for the delivery of anti-adenoviral shRNAs or amiRNAs.

Consequently, in the present study, we generated a series of replication-deficient adenoviral amiRNA expression vectors for the silencing of selected Ad5 genes and investigated whether these amiRNAs are capable of efficiently inhibiting the replication of wt Ad5 upon co-infection of a cell with the recombinant vector. The amiRNAs were designed to recognize those mRNAs that had been identified as candidate targets in our previous study (Kneidinger et al., 2012), *i.e.*, mRNAs encoding the viral E1A protein, a key regulator of the infection cycle (Pelka et al., 2008), the preterminal protein (pTP), and the viral DNA polymerase, both essential for viral DNA replication (de Jong et al., 2003). Here, we present data demonstrating

the efficient silencing of the wt Ad5 pTP gene upon infection with amiRNA expression vectors. Moreover, we demonstrate an increase in the knockdown rate upon concatemerization of amiRNA-encoding sequences, and we show that amiRNA expression is strongly boosted in wt Ad5 infected cells, a prerequisite for the efficient targeting of high numbers of viral transcripts. Taken together, our data indicate that amiRNA-mediated knockdown of wt Ad5 gene expression significantly inhibits viral DNA replication and efficiently decreases the output of infectious virus progeny *in vitro*.

2. Materials and methods

2.1. Cell culture, virus production, and titer determination

HEK 293 (human embryonic kidney; ATCC CRL-1573), A549 (human epithelial lung carcinoma; ATCC CCL-185), HeLa (human epithelial carcinoma; ATCC CCL-2), SW480 (human colon carcinoma; ATCC CCL-228), RD-ES (Ewing's sarcoma; ATCC HTB-166), and T-REx-293 cells (Life Technologies Austria, Vienna, Austria) were cultivated in Dulbecco's Modified Eagles Medium (DMEM) with stabilized glutamine (PAA Laboratories, Pasching, Austria) and supplemented with 10% fetal bovine serum (FBS; PAA Laboratories) in a humidified 5% CO₂ atmosphere at 37°C. Recombinant adenoviral vectors expressing Ad5-directed amiRNAs were amplified in T-REx-293 cells. All other adenoviral vectors and wt Ad5 (ATCC VR-5) were amplified in HEK 293 cells. Titers of infectious adenoviruses expressing amiRNAs were determined on T-REx-293 cells by 50% tissue culture infective dose (TCID₅₀) assays. Titers of wt Ad5 present in mixed virus suspensions

containing both wt and recombinant virus as obtained in co-infection experiments were determined on A549 cells using the same method. All other TCID₅₀ assays were performed with HEK 293 cells.

2.2 Vector constructions

The vectors employed in dual-luciferase assays for the screening of Ad5-directed amiRNAs have been described elsewhere (Kneidinger et al. 2012). The dual-luciferase target vector used for the determination of *Renilla* luciferase gene silencing in Ad5-infected cells was constructed as follows: a part of the modified coding region of the firefly (*Photinus pyralis*) luciferase open reading frame (ORF) representing the target sequence for the corresponding amiRNA was amplified by PCR with primers Fluc-f2 (5'-ATAAGGCTATCTCGAGATACGCCCTGGTTCC-3') and Fluc-r2 (5'-AATGTCGTTCGCGGCCGCAACTGCAACTCCGAT-3') from vector pGL3 (Promega, Mannheim, Germany). This fragment was restricted with *Xho*I and *Not*I and inserted into the corresponding sites located within the 3'UTR of the *Renilla* luciferase gene present on plasmid psiCHECK-2 (Promega, Mannheim, Germany). From the resulting vector (psiCHECK-FLuc2), a *Bgl*II-*Bam*HI fragment comprising both the firefly and *Renilla* luciferase expression cassettes was transferred into pENTR4 (Life Technologies Austria, Vienna, Austria) that had been restricted with *Xmn*I and *Eco*RV. From the resulting vector (pENTR-Luc), the entire expression cassette was eventually moved into the deleted E1 region of the adenoviral vector pAd/PL-DEST (Life Technologies Austria, Vienna, Austria) giving rise to vector pAd-Luc-as (Fig. 1). This final transfer was mediated by employing Life Technologies' Gateway technology, *i.e.*, by site-specific recombination between sequences flanking

the expression cassette on pENTR-Luc and the corresponding sequences located on the adenoviral vector. The recombination reaction was performed according to the instructions of the manufacturer (Life Technologies Austria, Vienna, Austria). The adenoviral vector expressing the amiRNA directed against the target sequence present in the 3'UTR of the *Renilla* gene on pAd-Luc-as was constructed in a similar way by transferring the enhanced green fluorescence protein (EGFP)/amiRNA expression cassette of plasmid pcDNA6.2-GW/EmGFP-miR-luc (Life Technologies Austria, Vienna, Austria) into pAd/CMV/V5-DEST™ via site-specific recombination as before. A corresponding adenoviral vector carrying a negative control amiRNA cassette (originating from pcDNA6.2-GW/EmGFP-miR-neg; Life Technologies Austria, Vienna, Austria) was constructed analogously. The resulting adenoviral vectors were named pAd-Fluc-mi1 and pAd-mi-, respectively (Fig. 1).

Construction of amiRNA expression vectors for the targeting of adenoviral mRNAs: amiRNAs were designed using Life Technologie's BLOCK-iT™ RNAi Designer and target site accessibility, as calculated by RNAs (http://rna.tbi.univie.ac.at/cgi-bin/RNAs), was taken into account. The annealed, double-stranded (ds), oligonucleotides (Supplementary Table 1) supposed to give rise to pre-miRNA hairpins (Fig. 2) contained 4 nucleotide (nt), 5' overhangs. Via these overhangs, the oligonucleotides were inserted into the pre-cut plasmid vector pcDNA6.2-GW/EmGFP-miR (Life Technologies Austria, Vienna, Austria) giving rise to amiRNA expression vectors for E1A silencing (pmiRE-E1A-mi1 to -mi4), Ad5 DNA polymerase silencing (pmiRE-Pol-mi1 to -mi7), and pTP silencing (pmiRE-pTP-mi1 to -mi5). In these vectors, the pri-miRNAs are located in the 3'UTR of an EGFP gene. Both the EGFP gene and the pri-mRNAs are co-expressed from a constitutive CMV promoter/enhancer. The analogous vector pcDNA6.2-GW/EmGFP-miR-neg

(Life Technologies Austria, Vienna, Austria) harboring a universal, negative control amiRNA in the 3'UTR of the EGFP gene served as a negative control.

Concatemerization of amiRNA-encoding sequences: the fragment supposed to be added to the existing copy of the amiRNA-encoding sequence was excised from the respective pcDNA6.2-GW/EmGFP-miR-based vector with *Sall* and *Bgl*II. The vector already harboring one copy was restricted with *Sall* and *Bam*HI, and the second copy was inserted into those sites. Further fragments containing single copies or multiple copies were added analogously by excision/insertion using the same restriction enzymes. Concatemerization of pTP-mi5- and the negative amiRNA-encoding sequences gave rise to vectors pmiRE-pTP-mi5x2, pmiRE-pTP-mi5x3, pmiRE-pTP-mi5x6 and pmiREx2, pmiREx3, pmiREx6, respectively.

Construction of plasmid vectors for doxycycline-controlled EGFP/amiRNA expression: this series of vectors is based on pENTR4 (Life Technologies Austria, Vienna, Austria) and contains a fragment comprising a CMV promoter/enhancer followed by a 2xTetO₂ tetracyclin repressor binding site, a multiple cloning site, and a BGH poly(A) site between the *Xmn*I and *Xho*I sites of the pENTR4 backbone. This fragment was obtained by PCR from pcDNA4/TO (Life Technologies Austria, Vienna, Austria) using primers CMV-TO-f1 (5'-TTGCATTTTGAATCTGCTTAGGGTTAGG-3') and BGHpA-r2 (5'-CCCAGCGAATTCTTTCCGCCTCAGAAG-3'). The *Bcl*I site located between the promoter/operator region and the BGH poly(A) site was subsequently used for the insertion of the individual EGFP/miRNA cassettes. These cassettes were amplified from the corresponding pcDNA6.2-GW/EmGFP-miR-based vectors lacking the tetracyclin repressor binding sites by PCR using primers pmiRE-f2 (5'-CAAAAATGATCACTTTAAAACCATGGTGAGC-3') and pmiRE-r2 (5'-AAGCTGTGATCAGATATCTCGAGTGCGGC-3'). The resulting EGFP/miRNA

expression vectors were termed pTO-mi- (carrying the negative control miRNA), pTO-E1A-mi1 (carrying amiRNA E1A-mi1), pTO-Pol-mi4 and pTO-Pol-mi7 (carrying the DNA polymerase-targeting amiRNAs Pol-mi4 and Pol-mi7, respectively), and pTO-pTP-mi5 (carrying the pTP-targeting amiRNA pTP-mi5). Versions of pTO-mi- carrying 2, 3, or 6 copies of the negative control miRNA-encoding sequence were generated in an analogous way and were named pTO-mi-x2, pTO-mi-x3, and pTO-mi-x6. Versions of pTO-pTP-mi5 carrying 2, 3, or 6 copies of the pTP-mi5-encoding sequence were termed pTO-pTP-mi5x2, pTO-pTP-mi5x3, and pTO-pTP-mi5x6.

Construction of adenoviral amiRNA expression vectors: eventually, the expression cassettes present in the pENTR4-based plasmid vectors were transferred into pAd/PL-DEST (Life Technologies Austria, Vienna, Austria) by site-specific recombination between sequences flanking the expression cassette and the corresponding respective sequences located on the adenoviral vector as described above. All resulting adenoviral vectors are depicted in Fig. 1. Restriction enzymes and DNA-modifying enzymes were purchased from Fermentas (St. Leon-Rot, Germany) or New England Biolabs (Frankfurt am Main, Germany). PCR reactions were performed with *Pwo* DNA polymerase obtained from Roche Diagnostics (Vienna, Austria) or PEQLAB (Erlangen, Germany).

2.3. Nucleic acid extraction

Circular plasmid DNA was extracted with an EasyPrep Pro Plasmid Miniprep Kit (Biozym, Oldendorf, Germany), or a HiSpeed Plasmid Midi Kit (QIAGEN, Hilden, Germany). PCR products were purified with a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany), and adenoviral DNA was isolated with a QIAamp DNA

Blood Mini Kit (QIAGEN, Hilden, Germany). Total RNA was extracted using a standard acid phenol/choloroform method.

2.4. Dual-luciferase assays

For amiRNA screens 1.2×10^5 HEK 293 or 1×10^5 HeLa cells were seeded into the wells of 96-well plates and reverse transfected with 100 ng of individual dual-luciferase reporter vectors and 200 ng of amiRNA expression vector using Lipofectamine 2000 (Life Technologies Austria, Vienna, Austria). For each well 0.5 μ L Lipofectamine 2000 was diluted with 24.5 μ L OptiMEM medium (Life Technologies Austria, Vienna, Austria), and after 5 min of incubation, 25 μ L diluted Lipofectamine 2000 was mixed with 25 μ L of plasmid DNA diluted in OptiMEM. After 20 minutes of incubation, the mixes were pipetted directly into the wells of a 96-well plate and freshly harvested cells were added. After 24h of incubation, the medium was exchanged, and the cells were incubated for another 24h. Firefly and *Renilla* luciferase activities were determined at 48 h post-transfection using the Dual-Glo luciferase assay (Promega, Mannheim, Germany), according to the manufacturer's instructions. Luminescence was measured on a Wallac Victor 1420 Multilabel Counter (Perkin Elmer Austria, Brunn am Gebirge, Austria). Knockdown rates were calculated by normalizing *Renilla* luciferase activities to firefly luciferase activities, and comparing dual-luciferase ratios between the targeting amiRNAs and the non-targeting negative control amiRNA.

Knockdown experiments in which the firefly luciferase-specific amiRNA was employed were performed as follows: 1.5×10^5 HEK 293 cells or 2×10^4 A549 cells were seeded into the wells of a 96-well plate. 24 h thereafter, the cells were co-

infected with pAd-Luc-as at a multiplicity of infection (MOI) of 1 TCID₅₀/cell and either pAd-FLuc-mi1 or pAd-mi-, each at an MOI of 10 TCID₅₀/cell. In the case of A549 cells, the cells were additionally co-infected with wt Ad5 at an MOI of 100 TCID₅₀/cell. Alternatively, 2e+04 A549, 1.6e+05 HEK 293, 1.6e+05 SW480, or 1e+04 RD-ES cells seeded into 96-well plates were infected with wt Ad5 at an MOI of 100 TCID₅₀/cell, and 1 h after infection, cells were co-transfected with 100 ng of the target vector psiCHECK-FLuc2 and increasing amounts (25-200 ng) of the amiRNA expression vector pcDNA6.2-GW/EmGFP-miR-luc or its corresponding negative control vector pcDNA6.2-GW/EmGFP-miR-neg. *Renilla* luciferase activities in relation to firefly luciferase activities were determined 24 h or 48 h post-infection as described above. Experiments in which the effect of chaining of amiRNA-encoding sequences present on plasmid vectors was investigated were carried out essentially in the same way except that 50 ng of amiRNA expression vector and 50 or 100 ng target vector was used for co-transfections. Analogous experiments with adenoviral vectors were carried out by first transfecting T-REx-293 cells with 100 ng of psiCHECK-pTP followed by infection with adenoviral miRNA expression vectors at an MOI of 30 TCID₅₀/cell and treatment of the cells with or without 1 µg/ml doxycycline. Luciferase activities were determined 24 h post-infection as before.

2.5. Determination of amiRNA and mRNA levels

Total RNA was isolated from cells using a standard acid phenol/chloroform extraction method and residual DNA was removed with TURBO™ DNase (Life Technologies Austria, Vienna, Austria). pTP-mi5 levels were determined with a custom-designed TaqMan small RNA assay (proprietary to Life Technologies Austria,

Vienna, Austria) according to the instructions of the manufacturer. For the quantitation of mRNAs, total RNA was first reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies Austria, Vienna, Austria) and subsequently analyzed by real-time quantitative PCR (qPCR) using a LightCycler 480 Probes master mix (Roche Diagnostics, Vienna, Austria) and primer/probe sets specific for GAPDH (GAPDH-f1 5'-TGCACCACCAACTGCTTAGC-3', GAPDH-r1 5'-GGCATGGACTGTGGTCATGAG-3', GAPDH-p1 5'-CCTGGCCAAGGTCATCCATGACAACCTT-3'), or Ad5 pTP (pTP-cDNA-f2 5'-AAACCAACGCTCGGTGCC-3', pTP-cDNA-r2 5'-GGACGCGGTTCCAGATGTT-3', pTP-cDNA-p2 5'-CGCGCGCAATCGTTGACGCT-3'). All qPCRs were performed in duplicates on a LightCycler 480 platform (Roche Applied Science, Vienna, Austria).

2.6. Virus replication experiments

3e+04 A549 cells were seeded into the wells of a 96-well plate and infected with the recombinant adenoviruses at an MOI of 100 TCID₅₀/cell. 24 h later, cells were superinfected with wt Ad5 at an MOI of 0.01. If required, CDV was added to each well in concentrations ranging from 0 to 30 µM. The plates were incubated for 0, 2, 4, or 6 days without change of medium before freezing at -80°C. Crude virus suspensions were obtained by freeze-thawing the plates thrice and removal of cell debris by centrifugation for 15 min at 2800 rpm. The replication rate of recombinant adenoviruses carrying different numbers of amiRNA-encoding sequences was assessed by infecting 1e+05 T-REx-293 cells with the vectors at an MOI of 0.1 TCID₅₀/cell. AmiRNA expression was induced by addition of 1 µg/ml doxycycline to

the medium and cells were allowed to grow for an additional 48 h. Crude lysates were prepared as described above.

2.7. Determination of adenovirus genome copy numbers

Wt Ad5 DNA levels were determined by qPCR using the following TaqMan primer/probe set directed against the viral E1A gene (E1A-fwd 5'-GACGGCCCCCGAAGATC-3', E1A-rev 5'-TCCTGCACCGCCAACATT-3', and E1A-p 5'-CGAGGAGGCGGTTTCGCAGA-3'). Adenovirus genome copy numbers were calculated by using serial dilutions of an adenoviral reference DNA as a standard. DNA levels of amiRNA-expressing recombinant viruses were determined using a TaqMan primer/probe set specific for the adenoviral hexon gene (hexon-fwd 5'-CACTCATATTTCTTACATGCCCACTATT-3', hexon-rev 5'-GGCCTGTTGGGCATAGATTG-3', hexon-probe 5'-AGGAAGGTAACCTCACGAGAACTAATGGGCCA-3'). Otherwise, qPCR conditions were as described above.

2.8. FACS analysis

EGFP expression rates were determined by FACS analysis. Cells infected with EGFP-expressing adenoviruses were harvested by trypsinization, resuspended in normal cell culture medium, and pelleted by centrifugation at 1200 rpm for 5 min. Thereafter, cells were washed once with phosphate buffered saline (PBS) and fixed with 1% formaldehyde in PBS. Samples were analyzed with a FACS Calibur analyzer

(Becton Dickinson, Heidelberg, Germany) and percentages of fluorescent cells and mean fluorescence intensities (MFIs) were calculated.

2.9. Statistical analysis

All the data are expressed as mean $\pm$ standard deviation (SD). To test for statistical significance, one-way ANOVA corrected with Bonferroni's post-hoc test was applied. A p value of < 0.05 was considered statistically significant.

3. Results

3.1. Artificial miRNAs can mediate the knockdown of gene expression in adenovirus-infected cells

At late stages of infection, adenoviruses produce high amounts of the noncoding virus-associated RNAs (VA RNAs) I and II, respectively. These RNAs are at least partially processed into functional miRNAs (mivaRNAs), and their production has been reported to inhibit cellular RNAi (Andersson et al., 2005; Lu and Cullen, 2004). This inhibition is thought to be mediated by the saturation of the cellular RNAi machinery at different levels (*i.e.*, cleavage of pri-miRNAs by Drosha, export of pre-miRNAs by exportin-5, processing by Dicer, and loading into RISC). Such an inhibition of RNAi in adenovirus-infected cells may prevent successful knockdown of adenoviral gene expression by adenovirus-targeting amiRNAs. Thus, we first investigated whether expression of an amiRNA with proven activity at reasonably high levels could mediate efficient RNAi in adenovirus-infected cells. We made use of

a plasmid vector (pcDNA6.2-GW/EmGFP-miR-luc) that produces an amiRNA from the 3'UTR of a transcript coding for EGFP. This amiRNA was directed against the mRNA of the luciferase gene of a humanized firefly variant, and the guide strand displayed 100% complementary to its target sequence, thus leading to the cleavage of its target RNA in an siRNA-like fashion. A corresponding vector (pcDNA6.2-GW/EmGFP-miR-neg) carrying a universal, nontargeting, negative control miRNA was employed as well. We inserted the corresponding luciferase miRNA target sequence into the 3'UTR of a *Renilla* luciferase gene located on a distinct plasmid vector which, for internal normalization, also harbored a firefly luciferase gene (with a sequence distinct from the one against which the amiRNA was directed). This vector was named psiCHECK-FLuc2. Since our goal was to deliver amiRNAs into target cells via adenoviral vectors, we moved the expression cassettes for the targeting and nontargeting amiRNAs into the deleted E1 region of a replication-deficient, Ad5-based vector, giving rise to the adenoviral miRNA expression vectors pAd-FLuc-mi1 and pAd-mi1-, respectively (Fig. 1). A corresponding adenoviral target vector (pAd-Luc-as; Fig. 1) carrying the dual-luciferase expression cassette, which included the amiRNA target site, was constructed in an analogous way.

When we co-infected A549 cells with the adenoviral target vector and its corresponding amiRNA expression vector, we observed an efficient knockdown of *Renilla* luciferase gene expression (> 90%) at 24 and 48 h post infection when compared to the artificial negative control miRNA vector. This knockdown rate was not changed upon concomitant infection of the cells with high numbers of wt Ad5 (MOI = 100; Fig. 3A). This high amount of wt virus was chosen to assure high-level production of VA RNAs. We repeated the experiments with HEK 293 cells and observed similarly efficient knockdown rates of approximately 90% (Fig. 3B). In this

experimental setting, co-infection with wt Ad5 was omitted because the presence of the E1 gene in the genome of HEK 293 cells promotes the replication of replication-deficient adenoviral vectors in this cell line, consequently enhancing the production of high amounts of VA RNAs in the absence of wt adenovirus. We also repeated the experiments in a slightly different way by expressing the amiRNA and its target gene from their respective nonviral plasmid vectors in wt Ad5-infected A549, HEK 293, SW480, and RD-ES cells and observed comparable knockdown rates (data not shown). There was, however, a slight tendency (albeit not statistically significant in all cases) toward a slightly less pronounced knockdown rate in A549 cells at 48 h post infection in the presence of wt Ad5. This effect likely reflected the observations made by Andersson et al. (2005) and Lu and Cullen (2004). No such decrease in gene expression knockdown was detectable at 24 h post infection.

In any case, the data indicated that it is feasible to efficiently knock down the expression of a gene carried by a replicating adenovirus via an amiRNA provided by a second, co-infecting adenovirus with no decrease in the knockdown rate at least at 24 and 48 h post infection. Considering that all amiRNAs we intended to design were supposed to target early viral processes and should thus be able to execute their functions, these results encouraged us to continue with the actual development of adenovirus-directed amiRNAs.

3.2. Prevention of amiRNA expression in packaging cells

Adenovirus-directed amiRNAs, when expressed from adenoviral vectors that carry the corresponding target sequence, would inevitably impair the amplification of these vectors in packaging cells, such as HEK 293 cells, consequently leading to

poor virus titers. Thus, we needed to assure that amiRNA expression is abolished in these packaging cells. To this end, we generated an adenoviral expression system in which the expression of amiRNAs (encoded by sequences located in the 3'UTR of the EGFP gene, as above) is driven by a tetracycline (Tet) repressor-controlled CMV promoter containing binding sites for 2 Tet repressor homodimers downstream of its TATA box. Thus, this promoter was repressed in cells expressing the Tet repressor and active only in the presence of tetracycline or in cells lacking the repressor, such as the target cells into which the vectors would be delivered.

This expression cassette was moved into the adenoviral vector as before, and the adenoviral vectors were amplified and packaged in T-REx-293 cells, a derivative of HEK 293 cells harboring the Tet repressor. Since artificial pri-miRNAs are generated from longer transcripts encoding EGFP in their 5' region, EGFP expression was used as a measure for the repression of pri-miRNA expression in the absence of doxycycline in T-REx-293 cells. FACS analysis of EGFP expression revealed that transcription from the CMV promoter is heavily reduced in the repressed state (*i.e.*, in the absence of doxycycline), as exemplified for the adenoviral vector pAd-mi- in Fig. 4. These data demonstrated that the controllable system (although not completely tight) was also functional when incorporated into adenoviral vectors and importantly, upon replication of these vectors. EGFP expression from this viral vector-located expression cassette was high upon addition of doxycycline, comparable to the expression rate typically achievable with analogous vectors containing a constitutively active version of the CMV promoter (data not shown).

3.3. Construction of adenovirus-directed amiRNA expression vectors

All amiRNAs were designed to be first expressed as pri-miRNAs from the (nonviral) miRNA expression vector pcDNA6.2-GW/EmGFP-miR. In this vector context, amiRNA hairpins are embedded in the flanking sequences of the murine mmu-miR-155 miRNA. The pri-miRNA-encoding sequences are incorporated in the 3'UTR of a *Renilla* luciferase gene and are concomitantly expressed with the EGFP gene from a common, constitutive CMV promoter. AmiRNA-containing transcripts can then be generated and processed in the same way as naturally occurring pri-miRNAs/pre-miRNAs. However, the inserted sequences were designed to match their target sequences completely and were therefore expected to lead to the degradation of their target mRNAs.

Based on our results obtained with adenovirus-directed siRNAs, we designed amiRNAs directed against E1A, DNA polymerase, and pTP mRNAs of Ad5, which had previously been identified as promising targets (Kneidinger et al., 2012). For each target mRNA, at least 4 different amiRNAs were designed (Fig. 2), and the respective oligonucleotides containing the sequences of the pre-miRNA hairpins (Supplementary Table 1) were cloned into pcDNA 6.2-GW/EmGFP-miR giving rise to the plasmid expression vectors pmiRE-E1A-mi1 to -mi4, pmiRE-Pol-mi1 to -mi7, and pmiRE-pTP-mi1 to -mi5. A vector (pcDNA6.2-GW/EmGFP-miR-neg) encoding a universal, nontargeting amiRNA served as a reference for all other amiRNA expression vectors, thus allowing for comparison between groups of amiRNA expression vectors (*i.e.*, amiRNA expression vectors for the targeting of distinct adenoviral transcripts).

To select the most efficient amiRNAs, we employed the same dual-luciferase-based reporter system as described above. We first tested each group of amiRNAs (*i.e.*, groups targeting either the E1A, DNA polymerase, or pTP mRNAs) individually in combination with reporter plasmid vectors harboring the respective target sequences in the 3'UTR of the *Renilla* luciferase mRNA (Fig. 3A–C). Finally, we compared amiRNAs selected from each group (*i.e.*, E1A-mi3, Pol-mi4 and Pol-mi7, and pTP-mi5) side-by-side (Fig. 3D). The obtained knockdown rates were similar for all selected amiRNAs. Because the transfection rates were well below 100% in these experiments (but were identical for different vectors), as determined by parallel FACS experiments in which EGFP expression was measured (data not shown), the absolute knockdown rates were rather low. Thus, the knockdown rates observed in these experiments did not reflect the true capacities of the tested amiRNAs. For targeting of the DNA polymerase mRNA, we selected 2 distinct amiRNAs: Pol-mi7, which showed the highest knockdown rate, and Pol-mi4, which performed slightly worse, but contained the same seed sequence as Pol-si2, the most potent siRNA identified through our previous study (Kneidinger et al., 2012).

Next, we modified the expression system of the selected vectors by bringing the EGFP/amiRNA cassettes under the control of the tetracycline repressor-regulated CMV promoter and subsequently transferred these expression cassettes into the deleted E1 region of the Ad5-based replication-deficient adenoviral vector already employed for the experiments described in section 3.1. The resulting adenoviral vectors pAdTO-mi-, pAdTO-E1A-mi3, pAdTO-Pol-mi4, pAdTO-Pol-mi7, and pAdTO-pTP-mi5 are depicted in Fig. 1.

Since the dual-luciferase assay system represents an artificial set-up, the efficacy of amiRNAs must be properly evaluated in the biological context. To this end, we

infected T-REx-293 cells (which propagate the replication of otherwise replication-deficient adenoviral vectors lacking the E1 genes) with the individual adenoviral amiRNA expression vectors. The cells were cultivated in the presence of doxycycline to allow for amiRNA expression, which, in turn, was expected to lead to the attenuation of viral DNA replication in cases of highly efficient amiRNAs. Finally, we determined viral genome copy numbers for the time point 2 days post infection by real-time qPCR using a primer/probe set directed against the adenoviral hexon gene. As shown in Fig. 6, expression of E1A-mi3, Pol-mi4, and Pol-mi7 did not cause a significant reduction in viral genome copy numbers. The only amiRNA that was able to decrease the amplification of its own vector significantly was pTP-mi5. In this case, the copy number of the vector was decreased to 26.9%. Thus, we selected the pTP-mi5 expression vector for further optimization.

3.4. Concatemerization of pTP-mi5-encoding sequences increases pTP-mi5 levels

It has been reported that expression of shRNA or amiRNA hairpins as tandem copies can enhance knockdown efficacies. Consequently, we generated vectors in which the pTP-mi5 pre-mRNA hairpins were concatemerized. We first constructed additional pcDNA 6.2-GW/EmGFP-miR-based plasmid vectors containing 2, 3, or 6 copies of pTP-mi5-encoding sequences in the 3'UTR of the EGFP gene (vectors pmiRE-pTP-mi5x2, pmiRE-pTP-mi5x3, and pmiRE-pTP-mi5x6) and the respective negative control vectors carrying a corresponding number of negative control amiRNA hairpins (vectors pmiREx2, pmiREx3, and pmiREx6). Transfection of HEK 293 cells with pTP-mi5-encoding vectors revealed that the amount of mature pTP-mi5 increased with rising copy numbers in the constructs (Fig. 7A). The gain in the

amount of pTP-mi5 present in the cells ranged from 6.8-fold (2 copies) to 20.3-fold (6 copies). Not surprisingly, there was an inverse correlation with EGFP expression: increased numbers of hairpins present in the 3'UTR of the EGFP gene led to decreased EGFP levels (Fig. 7B). This effect was not only evident for the pTP-mi5-encoding constructs but also for constructs encoding the negative control amiRNA. The observed decrease was likely due to enhanced processing of the primary transcripts by Drosha with increased amiRNA hairpin copy numbers, accelerated degradation of the processed forms due to lack of a 3' poly(A) tail after Drosha cleavage, or decreased translation.

3.5. Concatemerization of pTP-mi5-encoding sequences increases the knockdown rate

To determine whether elevated levels of pTP-mi5 produced by pmiRE-pTP-mi5x6 in comparison to pmiRE-pTP-mi5 had a positive effect on the knockdown rate, we performed dual-luciferase-based knockdown experiments as before. Knockdown of *Renilla* luciferase expression in HEK 293 cells transfected with the target vector psiCHECK-pTP was significantly increased upon raising the copy number of pTP-mi5 from 1 to 6 (Fig. 8A), revealing the expected positive correlation between amiRNA levels and knockdown capacities.

Next, we modified these plasmid vectors by replacing the constitutive CMV promoter with the tetracycline-regulated CMV promoter and subsequently converted those intermediate vectors into adenoviral vectors as before. The final set of adenoviral vectors (Fig. 1) contained 1, 2, 3, or 6 copies of the pTP-mi5-encoding sequence (vectors pAdTO-pTP-mi5, pAdTO-pTP-mi5x2, pAdTO-pTP-mi5x3, and

pAdTO-pTP-mi5x6), or a corresponding number of copies of the sequence encoding the negative control amiRNA (vectors pAdTO-mi-, pAdTO-mi-x2, pAdTO-mi-x3, and pAdTO-mi-x6). We evaluated this set of vectors by again performing dual-luciferase assays; briefly, we transfected T-REx-293 cells with the pTP-mi5 target vector psiCHECK-pTP and subsequently infected those cells with the adenoviral vectors at an MOI of 30 TCID₅₀/cell. The cells were cultivated in the presence of doxycycline for an additional 24 h to allow for the expression of amiRNA before determining luciferase activities. As shown in Fig. 8B, *Renilla* luciferase expression showed a steady decrease with increasing copy numbers of pTP-mi5-encoding sequences present on the vectors. This indicated that the amiRNA expression cassette giving rise to highest number of pTP-mi5 hairpins was the most effective when incorporated into the adenoviral vector backbone.

The positive effect of incorporating 6 copies of pTP-mi5 hairpins was also reflected by the increased inhibition of viral vector amplification in T-REx-293 cells when the cells were cultivated in the presence of doxycycline, *i.e.*, upon derepression of EGFP and pTP-mi5 expression (Fig. 9). No such effect was observed for vectors encoding the negative control amiRNA, indicating that the decrease in vector copy number was specifically related to pTP-mi5 expression and not to the treatment of the cells with doxycycline. Viral DNA synthesis was decreased by 0.9 orders of magnitude (86.2%) for the vector containing 1 copy of the pTP-mi5 hairpin. There was no significant difference in the inhibition rate when the copy number was raised to 2 or 3. However, doubling the copy number further from 3 to 6 generated a markedly increased inhibitory effect on vector amplification. Here, viral DNA synthesis was decreased by 1.6 orders of magnitude (97.6%) compared to the negative control vector. We also monitored the amplification kinetics of the vector

containing 6 copies of the pTP-mi5-encoding sequence over a 6-day period and found a pronounced decrease in vector copy numbers also at later time points in the presence of doxycycline (Supplementary Fig. 1).

Because all experiments clearly suggested that chaining of pTP-mi5 hairpins was a prerequisite for the successful inhibition of adenovirus replication, all subsequent experiments were performed with the vector carrying 6 copies of pTP-mi5.

3.6. Levels of amiRNAs, when expressed from a replication-deficient adenoviral vector, are increased in wt adenovirus-infected cells

Replication-deficient adenoviral vectors were chosen for the expression of amiRNAs based on the assumption that net levels of amiRNA should increase upon exposure of the recombinant vector to wt adenovirus in co-infected cells. Provided the amiRNA was not capable of completely blocking viral DNA replication, amiRNA gene copy numbers should increase upon onset of replication of the recombinant vector, which should be induced by E1A generated by the co-infecting wt adenovirus. Indeed, we found pTP-mi5 levels increased by ~6-fold in A549 cells co-infected with wt Ad5 (Fig. 10A).

To determine whether and to what extent pTP-mi5 inhibited the expression of pTP during virus replication, we co-infected A549 cells with the adenoviral pTP-mi5 expression vector pAdTO-pTP-mi5x6 or its corresponding negative control amiRNA expression vector pAdTO-mi-x6. Subsequently, we superinfected the cells with wt Ad5 and determined pTP mRNA levels at 24 h post infection with wt Ad5 by RT-

qPCR. As shown in Fig. 10B, pTP-mi5 expression decreased pTP mRNA levels by nearly 80% compared to the negative control amiRNA.

3.7. Adenoviral vector-based expression of pTP-mi5 inhibits the replication of wt Ad5

To finally investigate whether pTP-mi5 was capable of inhibiting the replication of wt Ad5, we co-infected A549 cells with pAdTO-pTP-mi5x6 or the negative control vector pAdTO-mi-x6 and wt Ad5. To assure that all cells were infected by the recombinant vectors, we used rather high MOIs of 100 TCID₅₀/cell and infected the cells with the recombinant vectors 24 h prior to infection with wt Ad5. Wt Ad5 genome copy numbers were determined at 0, 2, 4, and 6 days post-infection by real-time qPCR using a primer/probe set directed against a part of the E1A gene. As shown in Fig. 11A, wt Ad5 DNA levels were decreased by 1.24, 1.21, and 1.77 orders of magnitude (94.2%, 93.8%, and 98.4%) on days 2, 4, and 6, respectively, in cells expressing pTP-mi5, as compared to cells expressing the negative control amiRNA. The negative control amiRNA itself did not significantly inhibit wt Ad5 replication.

As a consequence of the inhibition of viral DNA synthesis, the generation of infectious wt Ad5 progeny was also heavily inhibited. The number of infectious wt Ad5 virions as determined by TCID₅₀ analysis using A549 cells as indicator cells (which permitted the specific detection of wt Ad5 replication) was decreased by 2.6 orders of magnitude (99.8%) in cultures co-infected with the pTP-mi5-expressing vector compared to control cultures expressing the negative control amiRNA (Fig. 11B).

3.8. AmiRNA expression and concomitant treatment with CDV results in an additive inhibitory effect

The inhibitory effect of pTP-mi5 when expressed from and delivered with a replication-deficient adenoviral vector was very pronounced, but not complete. Thus, we investigated whether knockdown of pTP expression by pTP-mi5 and concomitant treatment of infected cells with CDV may result in additive inhibitory effects. To this end, we co-infected A549 cells as before and treated them with therapeutically relevant concentrations of CDV. The highest dose of CDV (30 μ M) corresponded to *in vivo* peak serum concentrations typically measured after intravenous administration (Cundy, 1999). We assessed the inhibition of wt Ad5 replication by determining wt Ad5 genome copy numbers at time points 2 and 6 days post infection (Fig. 12 A and B). In our experimental setting, adenoviral vector-mediated expression of pTP-mi5 was generally more effective in inhibiting wt Ad5 replication than was treatment with CDV. However, the inhibitory effect of pTP-mi5 could clearly be further increased by concomitant treatment of the cells with CDV. pTP-mi5 expression alone decreased wt Ad5 genome copy numbers by 1.2 orders of magnitude (94.2%) at day 2 post-infection and by 1.8 orders of magnitude (98.4%) at day 6 post-infection when compared to the negative control amiRNA. However, concomitant treatment of the cells with 30 μ M CDV decreased wt Ad5 genome copy numbers by 2.2 orders of magnitude (99.3%) at day 2 and by 2.5 orders of magnitude (99.7%) at day 6. This clear additive effect also manifested as a further drop in the output of infectious virus progeny; concomitant treatment with 30 μ M CDV decreased the titer of wt Ad5 by another 0.6 orders of magnitude and resulted in a total reduction rate of 3.2 orders of magnitude (99.9%).

4. Discussion

We have previously demonstrated that exogenously added siRNAs are able to efficiently knock down adenoviral gene expression and to inhibit wt adenoviral multiplication *in vitro* (Kneidinger et al., 2012). This inhibition of virus multiplication was efficient even though it had to take place in the presence of the miRNA-resembling mivaRNAs encoded by the virus. These RNAs are thought to inhibit cellular RNAi by competing with endogenous miRNAs and their precursors at the levels of Dicer and Drosha processing, export from the nucleus, and RISC loading (Andersson et al., 2005; Lu and Cullen, 2004). The functionality of anti-adenoviral siRNAs may be explained by the fact that mivaRNAs reach high levels only at very late stages of infection. Inhibiting adenovirus replication prevents the generation of these high levels by reducing the gene copy number of the VA-RNAs from which the mivaRNAs are generated. This scenario fits well with the function of the most promising target genes identified in our previous study (Kneidinger et al., 2012); all of them are directly or indirectly involved in viral DNA replication. Moreover, in contrast to miRNAs, exogenous siRNAs are not dependent on any processing steps and hence avoid the bottlenecks of Drosha- and Dicer-mediated processing, as well as exportin-5-mediated nuclear export. However, the functionality of amiRNAs, which we intended to evaluate for the inhibition of adenoviral replication in this study, was much more questionable. In contrast to exogenous siRNAs, amiRNA precursors cannot avoid the potential bottlenecks created by the mivaRNAs. We estimated the performance of amiRNAs during the first 48 h of adenovirus infection as being especially critical, because viral DNA replication – the viral process which we intended to target – largely takes place within this time frame. However, we found

that amiRNA function was not affected during these stages of adenovirus infection when the amiRNA was delivered via adenoviral vectors (Fig. 3). These data therefore justified the evaluation of amiRNAs as tools for the knockdown of adenoviral gene expression.

Based on the data obtained from our previous study (Kneidinger et al., 2012), we designed amiRNAs directed against E1A 13S and 12S, pTP, and DNA polymerase mRNAs, all of which had been identified as promising targets. The design of amiRNAs follows slightly different rules compared to those required for the design of 25-nt-long, blunt-ended siRNAs. Although we designed certain amiRNAs (*i.e.*, pTP-mi5 and Pol-mi4) to contain the same seed sequences as their successful siRNA relatives, these amiRNAs did not necessarily represent the most efficient amiRNAs (see Pol-mi4), indicating that it was not always feasible to automatically convert an effective siRNA into a potent amiRNA. This may be due to the different lengths of amiRNAs and siRNAs, their different types of ends (*i.e.*, blunt ends in the case of siRNAs and 2-nt 3' overhangs in the case of amiRNAs), and the lack of any chemical modifications within amiRNAs.

While all selected amiRNAs performed similarly in the dual-luciferase-based test system, there was a clear difference when they were tested in the context of an adenovirus infection. Here, only the pTP mRNA-directed amiRNA, but not amiRNAs directed against E1A and DNA polymerase mRNAs, were able to significantly inhibit the replication of the adenoviral vectors by which they were encoded. These different behaviors in the 2 test systems may be explained by differences in the secondary structures of the target mRNAs or different occupancies of the mRNAs by RNA-binding proteins.

Concatemerization of identical amiRNA-encoding sequences has been shown to increase knockdown rates (Chung et al., 2006; Wu et al., 2011). Consequently, we concatemerized pTP-mi5-encoding sequences to increase the inhibition of adenoviral replication. While inhibition of the replication of the vector carrying the pTP-mi5 expression cassette was limited to 0.9 orders of magnitude (86.2%) when only one copy was present, increasing the copy number from 1 to 6 resulted in a decrease of viral genome copy number by 1.6 orders of magnitude (97.6%; Fig. 9). This effect correlated with an increase in pTP-mi5 levels (Fig. 7A). However, the increase in the amount of mature amiRNA was disproportionately higher compared to the increase in the number of hairpins present on primary transcripts. This effect may be related to an observation made by others when placing a pre-amiRNA hairpin onto a miRNA polycistron: when combined with other amiRNA hairpins, the silencing capacity of the individual amiRNA was increased (Liu et al., 2008).

Interestingly, increasing the number of hairpins from 1 to 3 did not result in statistically significant improvement of the knockdown efficiency (Fig. 9). However, doubling the number from 3 to 6 clearly increased the knockdown efficiency, maybe suggesting some sort of threshold inherent to the particular target gene/amiRNA expression systems. Expression of pTP-mi5 from the adenoviral vector inhibited the replication of superinfecting wt Ad5 by approximately 1.2 orders of magnitude (94%) at 2 days post infection with wt Ad5. This inhibitory effect was also evident by the suppression of infectious wt Ad5 progeny output by 2.6 orders of magnitude (99.8%). Although we used a low MOI of 0.01 TCID₅₀/cell for wt Ad5 to allow for monitoring of virus spreading within the cultures, the high burst size of adenovirus quickly led to infection of the entire culture. Consequently, the exponential increase in virus

multiplication at later time points was disproportionately prevented in cultures in which replication was not attenuated by amiRNAs. Thus, regardless of the readout system, the pTP-mi5-mediated inhibition rate at late time points (4 or 6 days post infection) was probably underestimated.

One anticipated advantage of using a replication-deficient adenoviral vector for the expression of amiRNAs was based on the expectation that superinfection of a cell with wt Ad5 should induce the replication of the adenoviral amiRNA expression vector (under the assumption that residual viral DNA replication would take place even in the presence of the amiRNA). Consequently, copy numbers of the amiRNA vector and the amiRNA itself should increase in wt adenovirus-infected cells. Indeed, upon superinfection with wt Ad5, the amount of pTP-mi5 was about 6-fold higher than in mock-infected cells (Fig. 10A). Thus, this expression system is to some extent, self-regulating, *i.e.*, residual replication of wt Ad5 in the presence of amiRNA expression triggers the production of recombinant virus progeny. This particular feature of the expression system may represent an advantage in a therapeutic scenario *in vivo* by maintaining the recombinant vector in the system. Recombinant virus progeny may spread to neighboring cells to elicit its (at least partial) protective effect and potentially slow down wt adenovirus spreading.

Because the concentration of pTP-mi5 was obviously critical for obtaining an adequate inhibitory effect, we abandoned the option of combining amiRNAs directed against different target mRNAs to potentially obtain synergistic effects. Instead, we focused on maximizing the intracellular amount of pTP-mi5. This decision was also based on the fact that no synergistic effects had been observed in our previous study (Kneidinger et al., 2012) or in another study by Eckstein et al. (2010). However, we also investigated whether a combined knockdown of pTP gene expression by pTP-

mi5 and treatment of infected cells with CDV resulted in additive inhibitory effects. CDV is currently the only drug with proven activity against adenoviruses (Lenaerts et al., 2008; Lindemans et al., 2010; Ljungman et al., 2003; Symeonidis et al., 2007; Yusuf et al., 2006). It belongs to the class of nucleoside analogs and inhibits viral DNA synthesis (Cundy, 1999). Thus, both CDV and pTP-mi5 target the same viral process, namely viral DNA replication. However, while pTP-mi5 decreases the number of functional protein complexes that have to be formed for efficient initiation of viral DNA synthesis, CDV acts downstream of this step by preventing DNA polymerization. Thus, it was conceivable that a combination of both mechanisms may result in additive inhibitory effects; while pTP-mi5 would in a first step limit the number of available DNA replication complexes, CDV would in a second step inhibit residual DNA synthesis that could not be prevented by the amiRNA. Indeed, a combination of pTP-mi5 expression and treatment with CDV resulted in a further decrease of wt Ad5 genome copy numbers and infectious virus progeny by an additional 1 and 0.6 orders of magnitude, respectively, at 2 days post infection with wt Ad5 (Fig. 12A and C). The combined treatment resulted in a total reduction of viral DNA and infectious virus progeny at 2 days post infection by 2.5 and 3.2 orders of magnitude, respectively.

The delivery of amiRNAs, shRNAs, or siRNAs into living organisms is a challenging task. Based on the development of a plethora of different delivery vehicles, nonviral delivery methods have constantly been improved (Rettig and Behlke, 2012). This progress has permitted the successful inhibition of virus replication *in vivo* (Arbuthnot, 2010; Haasnoot et al., 2007; Zhou and Rossi, 2011). However, the *in vivo* transduction rates achievable with nonviral delivery vehicles are still far from perfect. In this regard, the delivery of anti-adenoviral amiRNAs, as

presented here via a replication-deficient adenoviral vector, may have several unique advantages. For example, it may allow for the amplification of amiRNA expression cassette copy numbers upon exposure to the wt virus and theoretically ensure a constant supply of recombinant vector as long as wt adenovirus is present. Moreover, based on the shared organ tropism of the adenoviral vector and its wt counterpart, this type of delivery may also permit the directing of amiRNAs predominantly to those cells that are also the preferred targets of the wt virus (at least for recombinant adenoviruses and wt adenoviruses that use the same cell surface receptors). Which of the 2 RNAi-based approaches, *i.e.*, silencing of adenoviral gene expression by siRNAs, such as the ones presented in our previous study (Kneidinger et al., 2012), or by amiRNAs expressed from and delivered by adenoviral vectors (this study), provide a greater probability to permit efficient inhibition of adenovirus multiplication *in vivo* will eventually have to be clarified in animal studies.

5. Conclusion

Taken together, our data indicate that (i) adenoviral vector-based delivery and expression of amiRNAs can mediate significant gene expression knockdown in cells infected with wt adenovirus; (ii) targeting of adenoviral pTP mRNA by amiRNA can inhibit the replication of wt adenovirus *in vitro*; (iii) efficient inhibition requires a sufficiently high intracellular concentration of amiRNA, which can be achieved by concatemerization of amiRNA hairpins in primary transcripts; (iv) the intracellular amiRNA concentration can be further increased upon the encounter of the recombinant vector with its co-infecting wt counterpart; and (v) amiRNA expression in

cells infected by wt virus and their concomitant treatment with CDV can result in additive inhibitory effects.

Acknowledgments

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Conflicts of interest

The authors declare no conflict of interest.

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6. Figures and tables

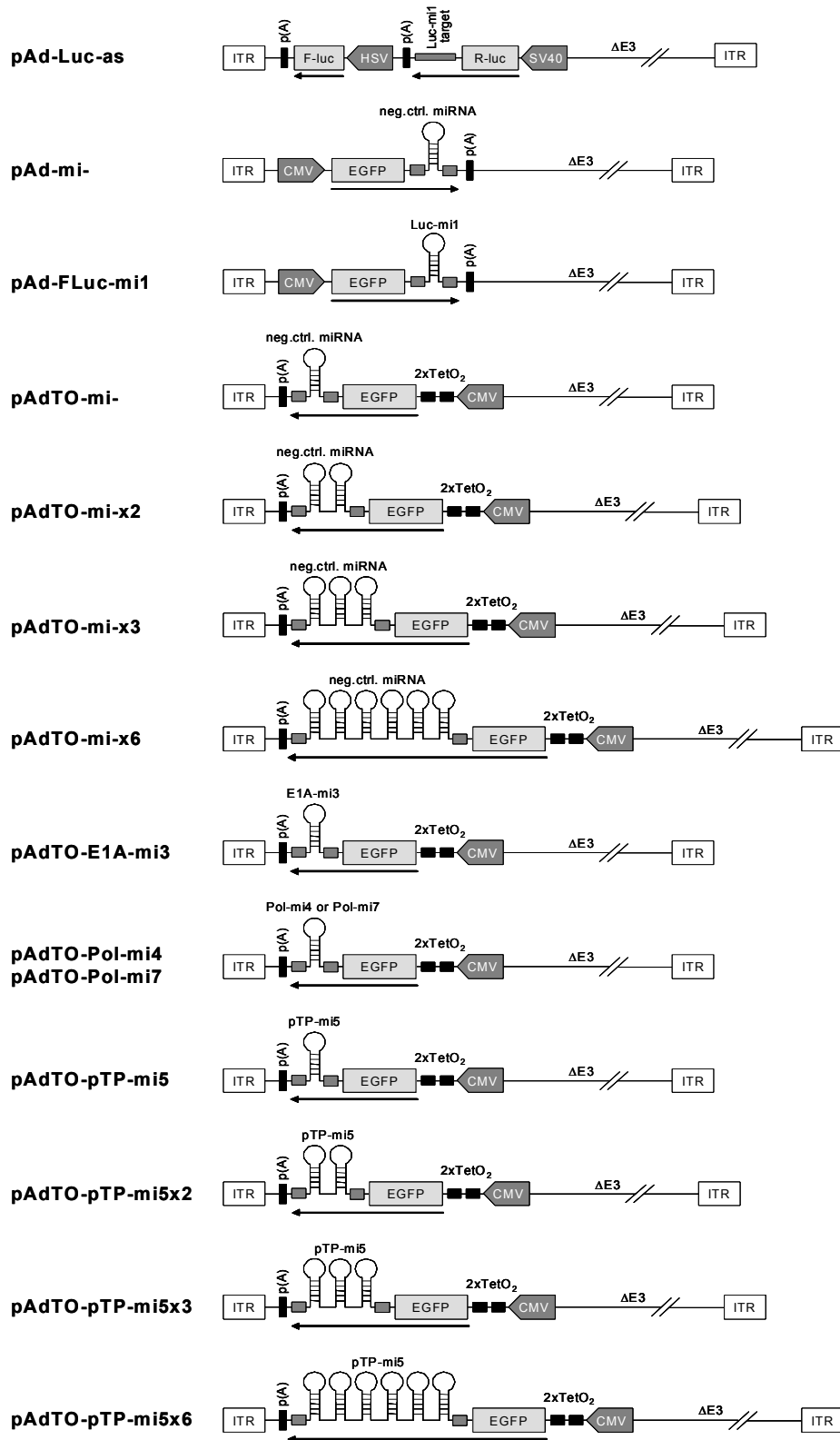


Fig. 1. Schematic representation of adenoviral vectors. All vectors were based on the Ad5-derived vectors pAd/CMV/V5-DEST[™] or pAd/PL-DEST[™] (both from Life Technologies) and lack the E1 and E3 genes. Expression cassettes were inserted into the deleted E1 region in sense or antisense orientation with respect to the left inverted terminal repeat (ITR). Expression cassettes contained the indicated open reading frames (light grey) for firefly luciferase (F-Luc), *Renilla* luciferase (R-Luc), or enhanced green fluorescent protein (EGFP). The target sequence for amiRNA Luc-mi1 within the 3'UTR of the *Renilla* luciferase gene is indicated by a small, dark grey box. Gene expression was driven by the herpes simplex virus 1 thymidine kinase promoter (indicated as HSV), simian virus 40 early promoter (SV40), or cytomegalovirus promoter (CMV). Promoters are indicated as boxed grey arrows. The 2 copies of tetracycline repressor-binding sequences are indicated as 2xTetO₂. Mmu-miR-155-derived sequences flanking the hairpins that give rise to mature amiRNAs are indicated as small, dark grey boxes. Polyadenylation sequences are indicated as p(A).

E1A-mi1	5' -UG <u>AU</u> uuuggcc CUGUUUCUUCGUA AACACCUCGU a 3' -AGGACAAAGAAGCCAU--UUGUGGAGCAgucaguc
E1A-mi2	5' -UG <u>AC</u> uuuggcc CUGUUCUCUAGAC AGGUGAUGUGU a 3' -AGGACAAGAGAUCUG--UCCACUACACAgucaguc
E1A-mi3	5' -UG <u>CU</u> uuuggcc CUGUAUUGCAUUCU AGACACAGGU a 3' -AGGACAUAACGUAAGA--UCUGUGUCCAgucaguc
E1A-mi4	5' -UG <u>AC</u> uuuggcc CUGAUCACAGGUUAC CUUAUGGU a 3' -AGGACUAGUGUCCAAUG--GAAUACCAgucaguc
Pol-mi1	5' -UG <u>GU</u> uuuggcc CUGUUAAAGUCCUC AGGUGAGCGU a 3' -AGGACAAUUAAGGAG--UCCACUCGCAgucaguc
Pol-mi2	5' -UG <u>CA</u> uuuggcc CUGUGUUGUGGCCCA AUGUAAAGU a 3' -AGGACACAACACGGGU--UACAUUUCAgucaguc
Pol-mi3	5' -UG <u>UG</u> uuuggcc CUGAUAAAGUUGCGUG AUGCGGGU a 3' -AGGACUAAUUAACGCAC--UACGCCCAgucaguc
Pol-mi4	5' -UG <u>GC</u> uuuggcc CUGUAUGGUUGGAC UGGAAGACGU a 3' -AGGACAUACCAACCUG--ACCUUCUGCAgucaguc
Pol-mi5	5' -UG <u>GA</u> uuuggcc CUGUGGACGCUGGAA CGUUGAAGU a 3' -AGGACACCUGCGACCUU--GCAACUUCAgucaguc
Pol-mi6	5' -UG <u>GA</u> uuuggcc CUGUGAUUGGUUG CGCUGGAAGU a 3' -AGGACACUUAUCCAAC--GCGACCUUCAgucaguc
Pol-mi7	5' -UG <u>GT</u> uuuggcc CUGTTGGATGATATG TGGACGCGU a 3' -AGGACAACCTACTATAC--ACCTGCGCAgucaguc
pTP-mi1	5' -UG <u>UU</u> uuuggcc CUGUAACGGACCAG AACGGUCUGU a 3' -AGGACAUUGCCUGGUC--UUGCCAGACAgucaguc
pTP-mi2	5' -UG <u>CU</u> uuuggcc CUGAUCUCUUGCAU AUCGCUGCGU a 3' -AGGACUAGAGAACGUA--UAGCGACGCAgucaguc
pTP-mi3	5' -UG <u>AU</u> uuuggcc CUGUGAACUGCUCG CUCUCCUGU a 3' -AGGACACUUGACGAGC--GAGAAGGACAgucaguc
pTP-mi4	5' -UG <u>UG</u> uuuggcc CUGAUCAAUUUCGG UCGUUGACGU a 3' -AGGACACUUGACGAGC--GAGAAGGACAgucaguc
pTP-mi5	5' -UG <u>AC</u> uuuggcc CUGAAGAGAGUUCG AGAAUCAAGU a 3' -AGGACUUCUCUCAAGC--UAGAUUUCAgucaguc

Fig. 2

Fig. 2. Predicted stem-loop structures giving rise to adenovirus-directed amiRNAs. The amiRNAs were designed to target E1A-13S and E1A-12S mRNAs (E1A-mi1 to E1A-mi4), DNA polymerase mRNA (Pol-mi1 to Pol-mi7), or pTP mRNA (pTP-mi1 to pTP-mi5). All pre-amiRNAs were based on the murine mmu-miR-155 pre-miRNA and contain 2-nt central mismatches. The sequences giving rise to the mature antisense strands are underlined.

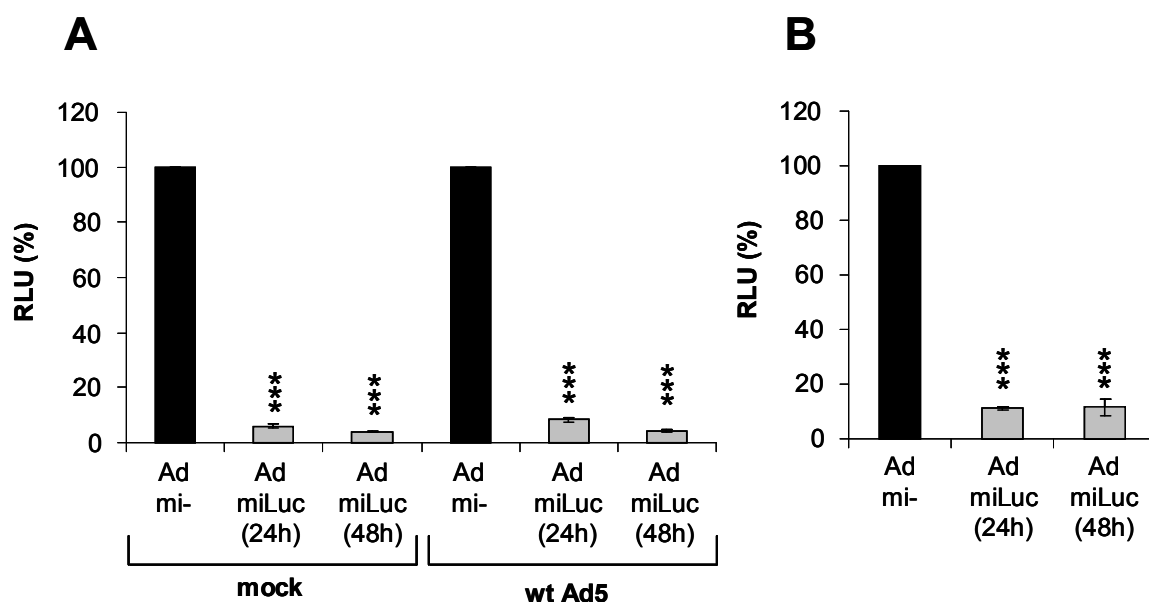


Fig. 3. Artificial miRNA-mediated RNAi in cells infected with wt Ad5 or packaging cells promoting the replication of the adenoviral miRNA expression vectors. A. A549 cells were co-infected with the adenoviral target vector pAd-Luc-as carrying an miRNA target sequence in the 3'UTR of a *Renilla* luciferase gene at an MOI of 1 TCID₅₀/cell and the adenoviral vector pAd-FLuc-mi1, which produces an artificial miRNA directed against the target sequence of the target vector at an MOI of 10 TCID₅₀/cell. A corresponding adenoviral vector (pAd-mi-) producing an artificial, nontargeting, amiRNA was used as a control. Concomitantly, cells were infected with

wt Ad5 at an MOI of 100 TCID₅₀/cell or were mock-infected with wt Ad5. *Renilla* luciferase activities in relation to firefly luciferase activities were determined at 24 and 48 h post infection. B. Co-infection of HEK 293 cells. Experimental setup as in A, except that superinfection with wt Ad5 was omitted. Data represent relative light units (RLU; mean $\pm$ SD) in comparison to the nontargeting artificial control miRNA from representative triplicate infection experiments. *** $p < 0.001$.

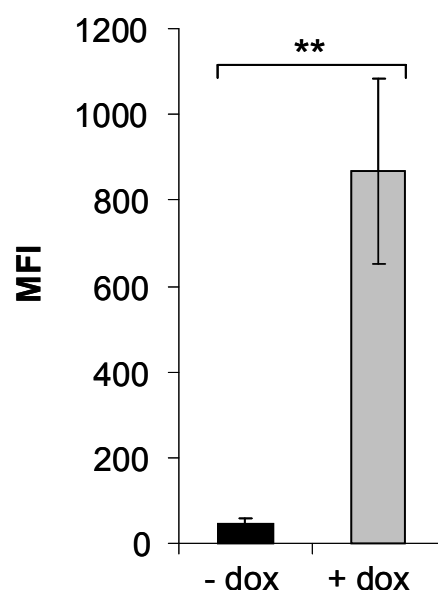


Fig. 4. Expression of the EGFP/miRNA cassette from a tetracycline repressor-controlled CMV promoter is heavily decreased in adenovirus packaging cells in the absence of doxycycline. T-REx-293 cells were transduced with the adenoviral vector pAdTO- and incubated with or without doxycycline. Twenty-four hours later, the cells were analyzed for EGFP expression by FACS analysis, and mean fluorescence intensities (MFIs) were calculated. Data represent the mean values of 3 independent experiments, each performed in triplicate (mean $\pm$ SD; $n = 3$). ** $p < 0.01$.

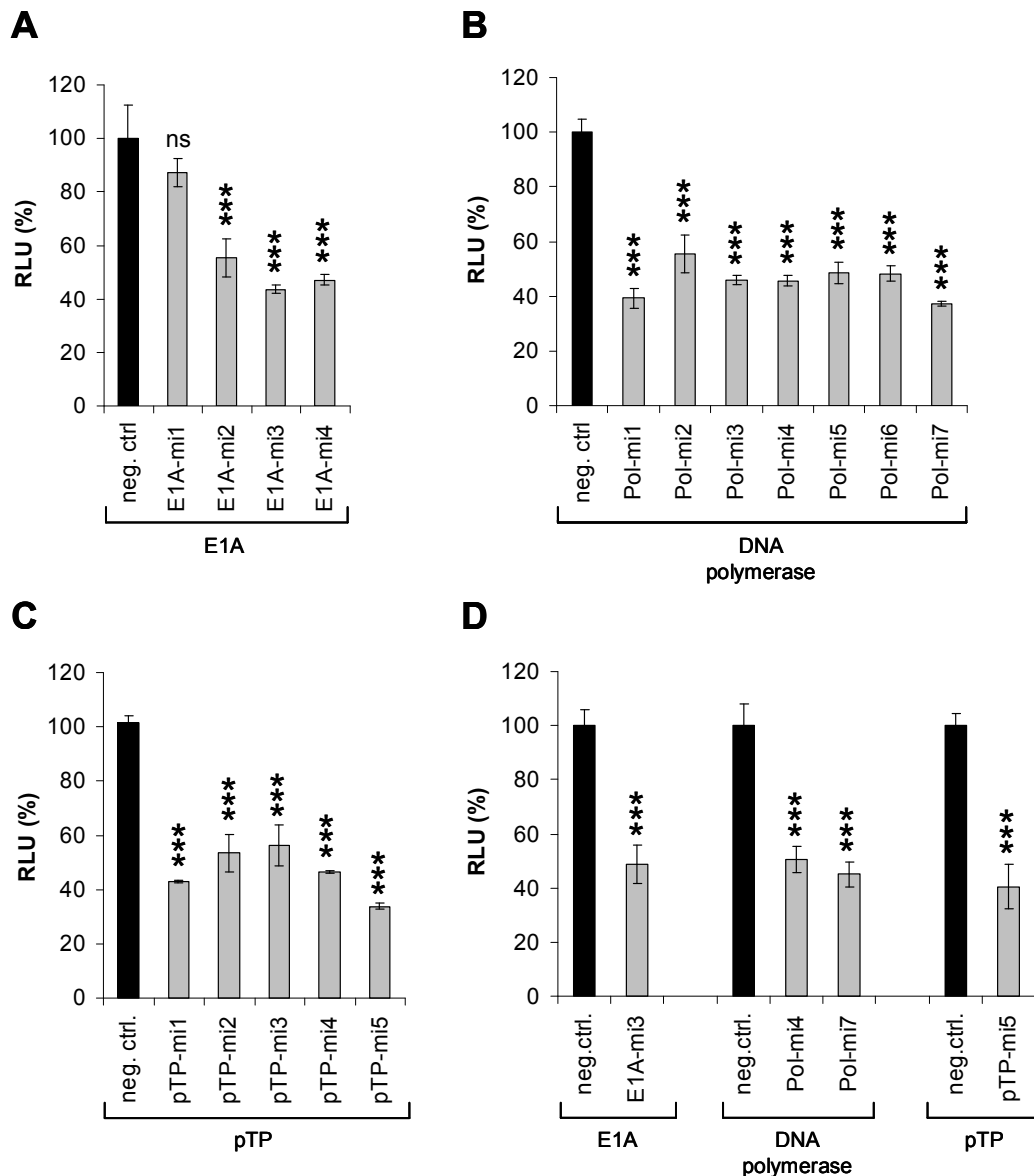


Fig. 5. Screening for functional amiRNAs. Plasmid-based amiRNA expression vectors and their respective dual-luciferase target vectors were used to cotransfect HeLa cells (A, D) or HEK293 cells (B, C). Each group of artificial miRNAs, *i.e.*, those directed against E1A (A), DNA polymerase (B), or pTP (C) target sequences, were first evaluated individually, and selected candidates were eventually evaluated side-by-side in a second round (D) by determining *Renilla* luciferase activities in relation to

firefly luciferase activities. A plasmid vector expressing a universal nontargeting amiRNA served as a negative control (neg. ctrl.) in all experiments. Relative light units (RLU; mean $\pm$ SD, $n = 3$) in comparison to the nontargeting artificial control miRNA are shown. *** $p < 0.001$; ns: not significant.

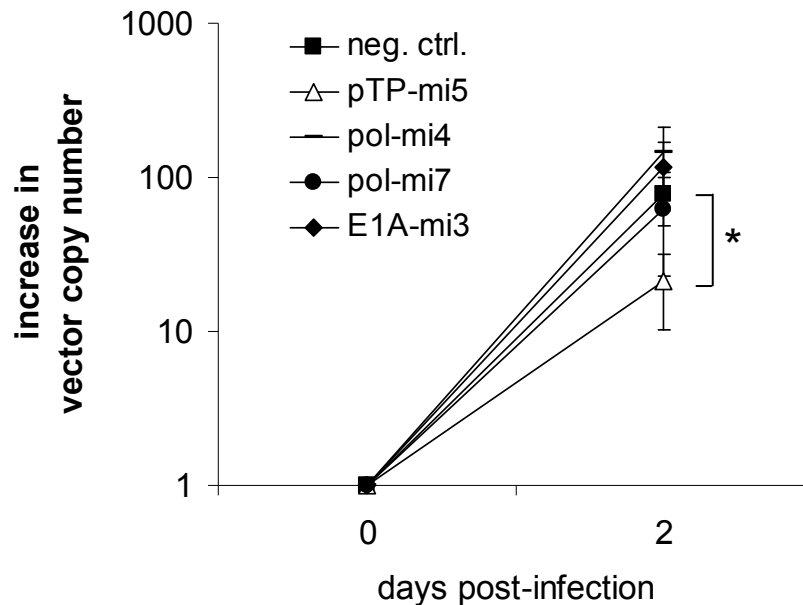


Fig. 6. Replication of adenoviral amiRNA expression vectors is inhibited in packaging cells upon amiRNA expression. T-REx-293 cells were infected with the amiRNA expression vectors pAdTO-mi-, pAdTO-E1A-mi3, pAdTO-Pol-mi4, and pAdTO-Pol-mi7 at an MOI of 0.1 TCID₅₀/cell, and amiRNA expression was induced by addition of doxycycline. Copy numbers of the vectors were determined for time points 0 and 2 days post infection by qPCR using a TaqMan primer/probe set specific for the Ad5 hexon gene. Fold-increases of vector copy numbers were calculated for all vectors. Data represent the means $\pm$ SD of 3 independent experiments, each performed in triplicate. * $p < 0.05$.

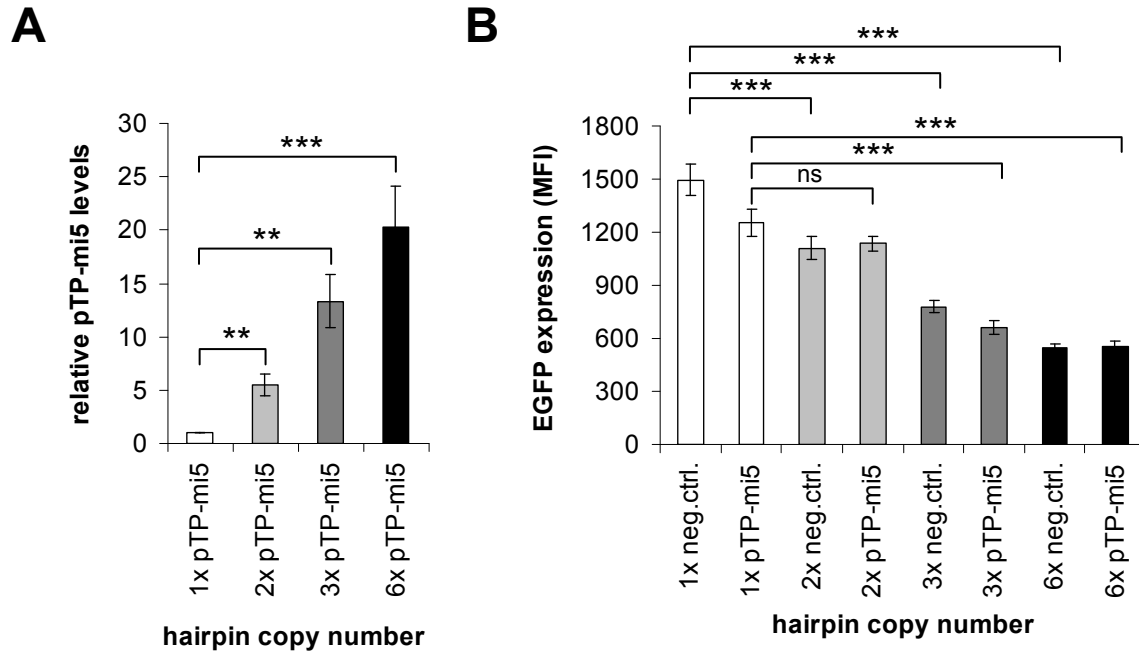


Fig. 7. Concatemerization of amiRNA-encoding sequences increases amiRNA levels and decreases EGFP expression. HEK293 cells were transfected with identical amounts of plasmid expression vectors carrying 1–6 copies of pTP-mi5 (*i.e.*, vectors pmiRE-pTP-mi5, pmiRE-pTP-mi5x2, pmiRE-pTP-mi5x3, and pmiRE-pTP-mi5x6) or the corresponding negative control amiRNA (*i.e.*, vectors pcDNA6.2-GW/EmGFP-miR-neg, pmiREx2, pmiREx3, and pmiREx6) in the 3'UTR of the EGFP gene. Forty-eight hours after transfection, pTP-mi5 levels were measured by RT-qPCR (A), and EGFP expression was determined by FACS analysis (B). Means of relative pTP-mi5 levels and mean fluorescent intensities (MFIs) from 3 independent transfections (mean $\pm$ SD, $n = 3$) are shown. * $p < 0.05$, ** $p < 0.01$, ns: not significant.

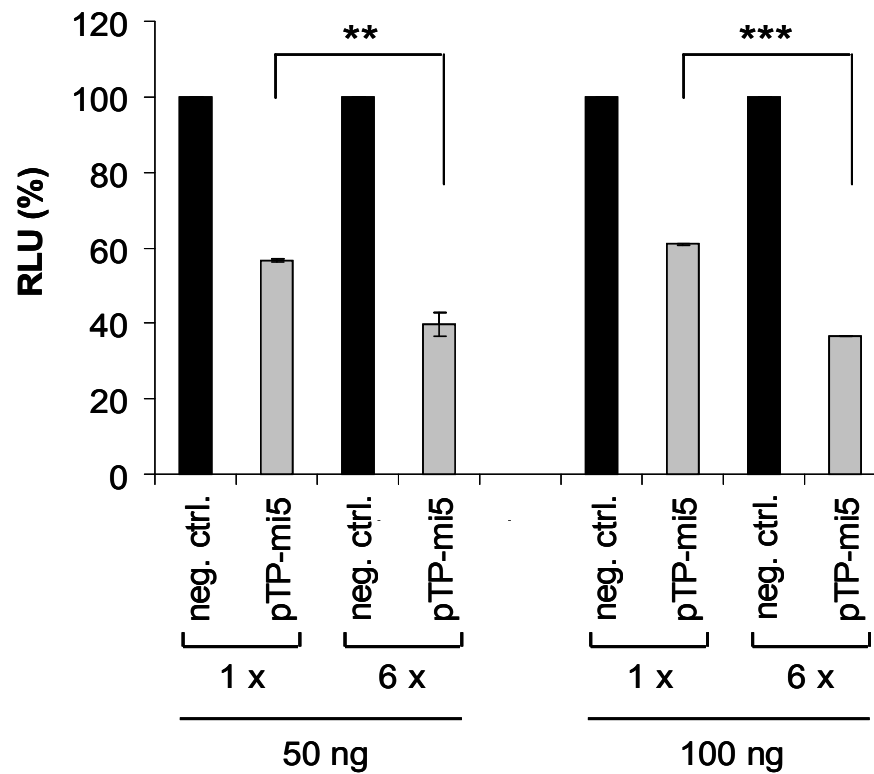
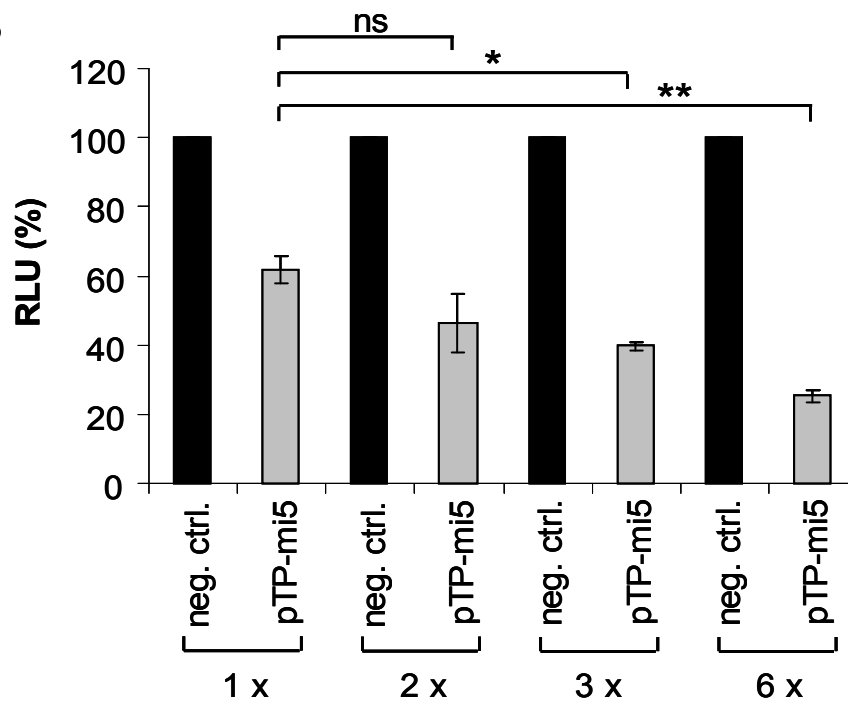
A**B**

Fig. 8. Concatemeration of pTP-mi5 hairpins increases the knockdown rate. A. HEK 293 cells were transfected with 50 ng of the plasmid vectors pmiRE-pTP-mi5

and pmiRE-pTP-mi5x6 carrying 1 or 6 copies of the sequence encoding pTP-mi5, respectively, and either 50 or 100 ng of the dual-luciferase reporter vector psiCHECK-pTP harboring the target sequence of pTP-mi5. The vectors pmiRE and pmiREx6, carrying 1 or 6 copies of a sequence encoding the nontargeting amiRNA, served as negative controls (neg. ctrl.). Numbers of hairpins for amiRNAs in the individual vectors are indicated as 1 x and 6 x, respectively. *Renilla* luciferase activities in relation to firefly luciferase activities were determined 24 h post-transfection. Relative light units (RLU) obtained with the vectors carrying pTP-mi5 in relation to their respective negative control vectors are shown. Data represent the means $\pm$ SD of 2 independent experiments, each performed in triplicate. B. HEK 293 cells were transfected with 100 ng of psiCHECK-pTP. Twenty-four hours after transfection, the cells were infected with the adenoviral vectors pAdTO-pTP-mi5, pAdTO-pTP-mi5x2, pAdTO-pTP-mi5x3, or pAdTO-pTP-mi5x6 expressing pTP-mi5 or pAdTO-mi-, pAdTO-mi-x2, pAdTO-mi-x3, or pAdTO-mi-x6 expressing the negative control amiRNA at an MOI of 30 TCID₅₀/cell. The copy number of the amiRNA hairpins is indicated beneath the figure. *Renilla* luciferase activities in relation to firefly luciferase activities were determined 24 h post infection. Relative light units (RLU) obtained with the vectors carrying pTP-mi5 in relation to their respective negative control vectors are shown. Data represent the means $\pm$ SD of 2 independent experiments, each performed in triplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns: not significant.

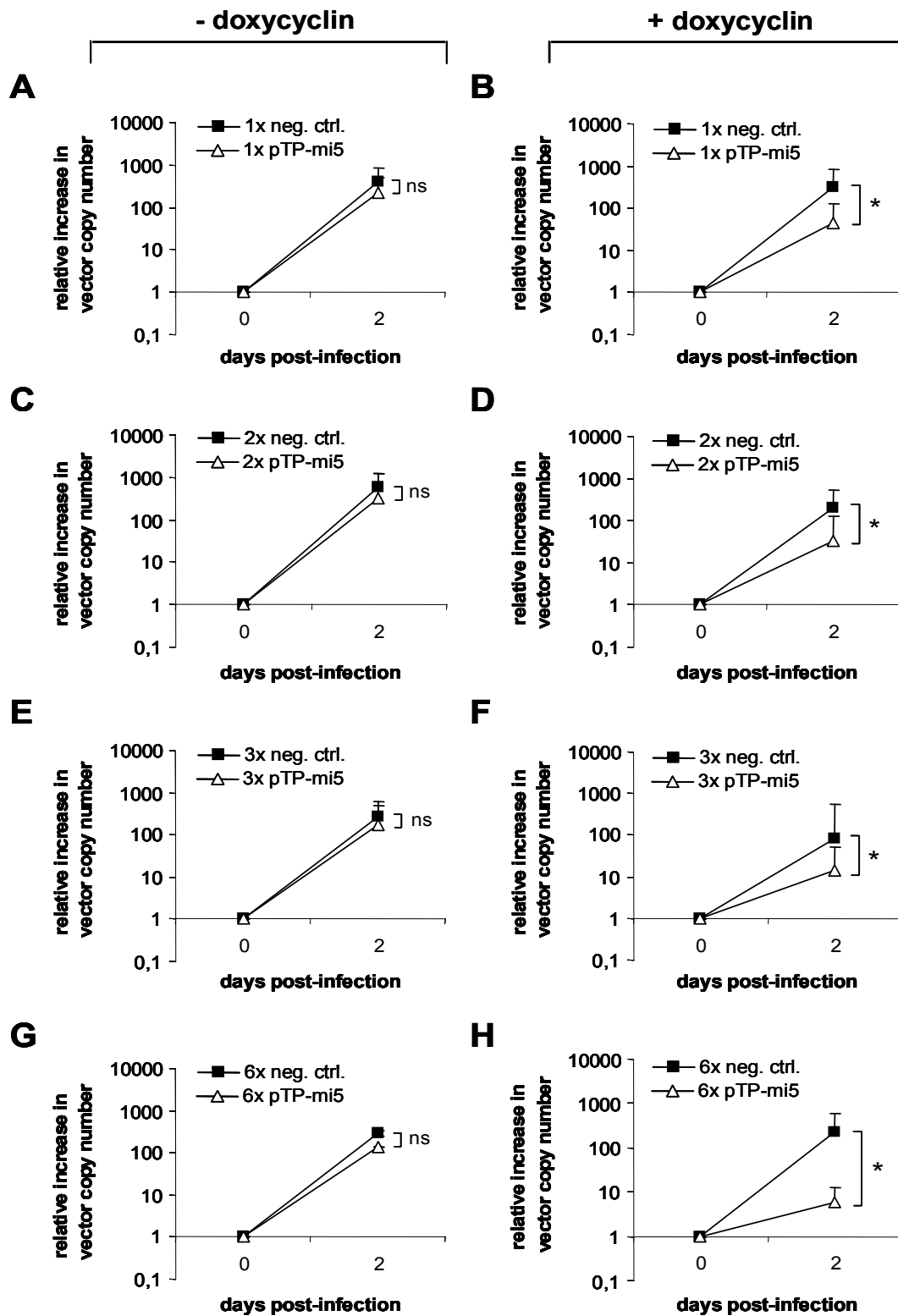


Fig. 9. Concatemerization of pTP-mi5 hairpins increases the inhibition of adenoviral pTP-mi5 expression vectors in packaging cells. T-REx-293 cells were infected with

the adenoviral pTP-mi5 expression vectors pAdTO-pTP-mi5, pAdTO-pTP-mi5x2, pAdTO-pTP-mi5x3, or pAdTO-pTP-mi5x6 or their respective negative control vectors pAdTO-mi-, pAdTO-mi-x2, pAdTO-mi-x3, or pAdTO-mi-x6 at an MOI of 0.1 TCID₅₀/cell. The vectors carried amiRNA-encoding sequences with copy numbers increasing from 1 (A, B) to 2 (C, D), 3 (E, F), or 6 (G, H). The infected cells were cultivated in the absence (A, C, E, G) or presence (B, D, F, H) of doxycycline. Copy numbers of the vectors were determined for time points 0 and 2 days post infection by qPCR using a TaqMan primer/probe set specific for the Ad5 hexon gene. Fold-increases in vector copy numbers were calculated for all vectors. Data represent the means $\pm$ SD of 3 independent experiments, each performed in triplicate. * $p < 0.05$, ns: not significant.

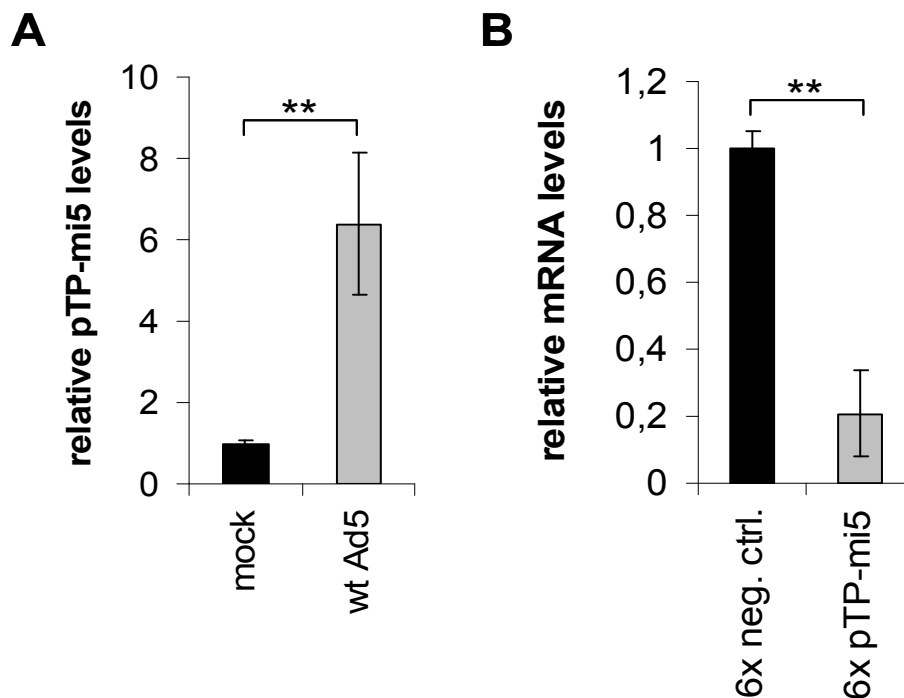


Fig. 10. Adenoviral vector-mediated expression of pTP-mi5 increased upon superinfection with wt Ad5 and led to knockdown of pTP expression. A549 cells were infected with the adenoviral pTP-mi5 expression vector pAdTO-mi-x6 or its

respective negative control vector pAdTO-mi-x6 at an MOI of 100 TCID₅₀/cell. Twenty-four hours later, cells were superinfected with wt Ad5 at an MOI of 100 TCID₅₀/cell or were mock-superinfected. Another 24 h later, cells were analyzed for relative pTP-mi5 levels (A) and relative pTP mRNA levels (B) by RT-qPCR. Data represent the means of 3 independent infections (mean $\pm$ SD; n = 3). ***p* < 0.01.

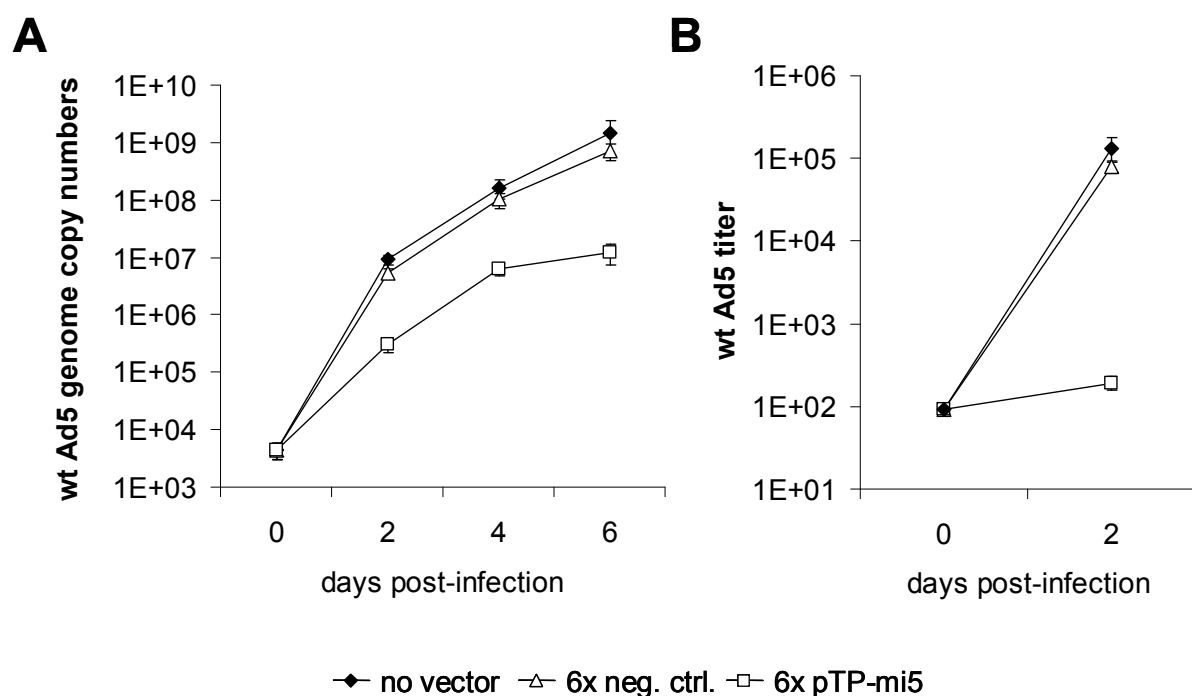


Fig. 11. Adenoviral vector-mediated expression of pTP-mi5 decreases the replication of wt Ad5. A549 cells were infected with the adenoviral pTP-mi5 expression vector pAdTO-pTP-mi5x6 (indicated as 6x pTP-mi5) or its respective negative control vector pAdTO-mi-x6 (indicated as neg. ctrl.) at an MOI of 100 TCID₅₀/cell or were mock-infected (indicated as no vector). Twenty-four hours after infection, cells were superinfected with wt Ad5 at an MOI of 0.01 TCID₅₀/cell. Cells were then cultivated for an additional 6 days. A. Genome copy numbers of wt Ad5 were determined for time points 0, 2, 4, and 6 days post infection with wt Ad5 by qPCR using a

primer/probe set specific for the E1A gene present only on wt Ad5. Data represent the means $\pm$ SD of 3 independent experiments, each performed in triplicate. B. Output of infectious virus progeny for time point 2 days post infection was determined by TCID₅₀ assays. Data represent the means $\pm$ SD of 3 independent experiments, each performed in triplicate.

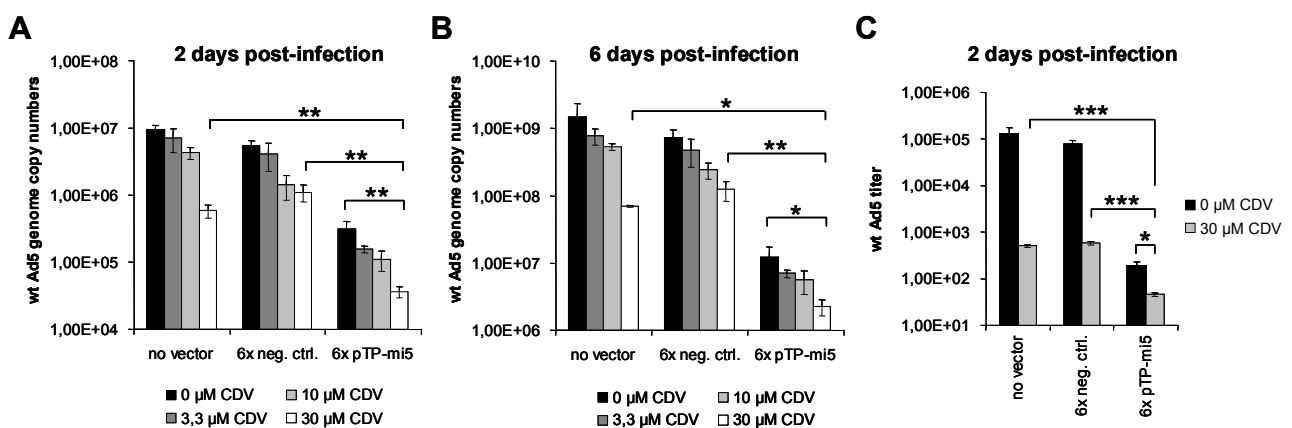
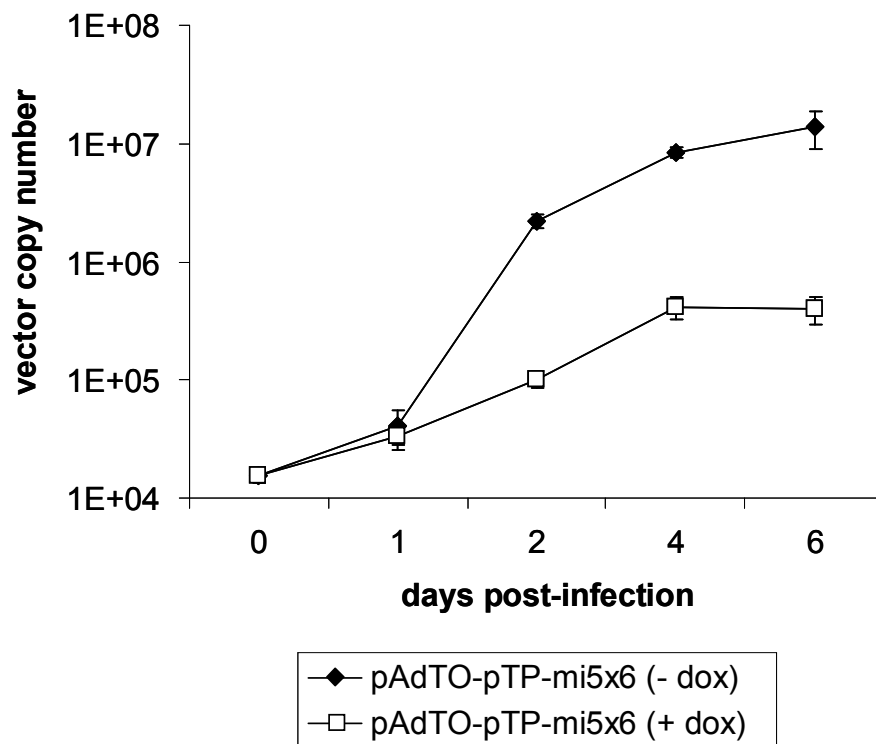


Fig. 12. Adenoviral vector-mediated expression of pTP-mi5 and concomitant treatment with CDV has an additive effect on the inhibition of wt Ad5 replication. A549 cells infected with pAdTO-pTP-mi5x6 at an MOI of 100 TCID₅₀/cell were superinfected with wt Ad5 at an MOI of 0.01 TCID₅₀/cell and were concomitantly treated with 0, 3.3, 10, or 30 μM CDV. A. Two days and 6 days after infection with wt Ad5, genome copy numbers of wt Ad5 were determined by qPCR using a primer/probe set specific for the E1A gene present only on wt Ad5. Data represent the means $\pm$ SD of 3 independent experiments, each performed in triplicate. B. Output of infectious virus progeny for time point 2 days post infection was determined by TCID₅₀ assays. Data represent the means $\pm$ SD of 3 independent experiments, each performed in triplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



Supplementary Fig. 1. The replication rate of the adenoviral pTP-mi5 expression vector is decreased in packaging cells when pTP-mi5 expression is permitted. T-REx-293 cells were infected with the adenoviral pTP-mi5 expression vector pAdTO-pTP-mi5x6 at an MOI of 0.1 TCID₅₀/cell. The infected cells were cultivated in the absence (- dox) or presence of doxycycline (+ dox). Amplification of the vector was monitored by determining vector copy numbers at time points 0, 2, 4, and 6 days post infection (mean $\pm$ SD, n = 3) by qPCR using a TaqMan primer/probe set specific for the Ad5 hexon gene.

Supplementary Table 1

Oligonucleotides used for the generation of Ad5-directed artificial miRNAs ¹⁾

Name	Sequence (sense strand/antisense strand oligonucleotides giving rise to pri-miRNA hairpins)	Target site Ad5 ²⁾	Target
E1A-mi1a E1A-mi1b	5'-TGCTGTTTCTTCGGTAATAACACCTCGTTTTGGCCACTGACTGACGAGGTGTTACCGAAGAAA-3' 5'-CCTGTTTCTTCGGTAAACACCTCGTCAGTCAGTGGCCAAAACGAGGTGTTATTACCGAAGAAAC-3'	582-602	E1A
E1A-mi2a E1A-mi2b	5'-TGCTGTTCTCTAGACACAGGTGATGTGTTTTGGCCACTGACTGACACATCACCTGTCTAGAGAA-3' 5'-CCTGTTCTCTAGACAGGTGATGTGTCAGTCAGTGGCCAAAACACATCACCTGTGTCTAGAGAAC-3'	1327-1347	E1A
E1A-mi3a E1A-mi3b	5'-TGCTGTATTGCATTCTCTAGACACAGGTGTTTTGGCCACTGACTGACCTGTGTCTAGAATGCAATA-3' 5'-CCTGTATTGCATTCTAGACACAGGTGATCAGTCAGTGGCCAAAACCTGTGTCTAGAGAATGCAATAC-3'	1334-1354	E1A
E1A-mi4a E1A-mi4b	5'-TGCTGATCACAGGTTTACACCTTATGTTTTGGCCACTGACTGACCATAAGGTAAACCTGTGAT-3' 5'-CCTGATCACAGGTTTACCTTATGGTCAGTCAGTGGCCAAAACCATAAGGTGTAACCTGTGATC-3'	1541-1561	E1A
Pol-mi1a Pol-mi1b	5'-TGCTGTTAAGTTCTCTAGGTGAGCGTTTTGGCCACTGACTGACGCTCACCTGAGGAACTTAA-3' 5'-CCTGTAAAGTTCTCAGGTGAGCGTCAGTCAGTGGCCAAAACGCTCACCTACGAGGAACTTAAC-3'	7604-7624	DNA pol
Pol-mi2a Pol-mi2b	5'-TGCTGTGTTGTGGCCCAATGTAAAGTTTTGGCCACTGACTGACTTTACATTGGGCCACAACA-3' 5'-CCTGTGTTGTGGCCCAATGTAAAGTCAGTCAGTGGCCAAAACCTTACATTGTGGGCCACAACAC-3'	7542-7562	DNA pol
Pol-mi3a Pol-mi3b	5'-TGCTGATAAAGTTGCGTGTGATGCGGGTTTTGGCCACTGACTGACCCGCATCACGCAACTTTAT-3' 5'-CCTGATAAAGTTGCGTGATGCGGGTCAGTCAGTGGCCAAAACCGCATCACACGCAACTTTATC-3'	7451-7471	DNA pol
Pol-mi4a Pol-mi4-b	5'-TGCTGTATGGTTGGACGCTGGAAGACGTTTTGGCCACTGACTGACGTCTTCCAGTCCAACCATA-3' 5'-CCTGTATGGTTGGACTGGAAGACGTCAGTCAGTGGCCAAAACGCTTCCAGCGTCCAACCATAC-3'	6928-6948	DNA pol
Pol-mi5a Pol-mi5b	5'-TGCTGTGGACGCTGGAAGACGTTGAAGTTTTGGCCACTGACTGACTTCAACGTTCCAGCGTCCA-3' 5'-CCTGTGGACGCTGGAACGTTGAAGTCAGTCAGTGGCCAAAACCTCAACGCTTCCAGCGTCCAC-3'	6934-6954	DNA pol
Pol-mi6a Pol-mi6b	5'-TGCTGTGATATGGTTGGACGCTGGAAGTTTTGGCCACTGACTGACTTCCAGCGCAACCATATCA-3' 5'-CCTGTGATATGGTTGCGCTGGAAGTCAGTCAGTGGCCAAAACCTCCAGCGTCCAACCATATCAC-3'	6925-6945	DNA pol
Pol-mi7a	5'-TGCTGTGGATGATATGGTTGGACGCGTTTTGGCCACTGACTGACGCGTCCACATATCATCCAA-3'	6920-6940	DNA pol

4. Fourth part

A COMBINED APPROACH FOR THE INHIBITION OF ADENOVIRUSES

1. Aim of the study

In the first part of my work I was able to selectively express the herpes simplex virus thymidine kinase (HSV-TK) gene in cells infected with wt adenoviruses. This selective expression was achieved by placing the gene under control of the adenoviral E4 promoter whose activity is dependent on the presence of the adenoviral E1A protein. This gene product is only provided by wild-type adenovirus. Upon treatment with the anti-herpetic prodrug ganciclovir (GCV), which is converted into its active form by HSV-TK, adenovirus replication could be inhibited, and the output of infectious virus progeny was decreased by several orders of magnitude *in vitro*.

In the second part of the project in which I was involved, adenovirus inhibition could be inhibited by siRNAs and amiRNAs directed against the pTP and/or DNA polymerase mRNAs.

By designing amiRNAs and targeting several viral processes, it was shown that a pTP mRNA-directed amiRNA was able to significantly inhibit the generation of virus progeny. This could be of great benefit, since the pTP is at least to some extent conserved.

Although both approaches (HSV-TK/GCV and RNAi) led to a dramatic inhibition of adenovirus replication, neither of them was able to completely block the generation of virus progeny. Thus, in another approach I integrated the two concepts to investigate potential additive inhibitory effects in the inhibition of adenoviral DNA replication.

For the efficient delivery of HSV-TK/amiRNA expression cassettes into cells and for an *in vivo* application, a replication-deficient adenovirus vector was constructed. To this end, an amiRNA targeting the pTP mRNA was expressed from a strong constitutive promoter (*i.e.*, the CMV promoter), and the HSV-TK gene was expressed from the adenoviral E4 promoter. Both expression units were incorporated

into the same vector. For the efficient production of these recombinant adenovirus vectors, it was very important to assure that amiRNA expression is shut off in packaging cells. Because of that, a tetracycline-inducible system for amiRNA expression was generated.

Combining the HSV-TK/amiRNA or HSV-TK/siRNA concepts was expected to lead to an increased inhibition of adenovirus replication.

2. Materials and methods

2.1. Cell culture and virus amplification

HEK 293 (human embryonic kidney; ATCC CRL-1573), A549 (human epithelial lung carcinoma; ATCC CCL-185), and T-REx-293 cells (Life Technologies Austria, Vienna, Austria) were cultivated in Dulbecco's Modified Eagles Medium (DMEM) with stabilized glutamine (PAA Laboratories, Pasching, Austria) and supplemented with 10% fetal bovine serum (FBS; PAA Laboratories) in a humidified 5% CO₂ atmosphere at 37°C. Recombinant adenoviral vectors expressing Ad5-directed amiRNAs alone or in combination with HSV-TK gene were amplified in T-REx-293 cells. Titers of infectious adenoviruses expressing amiRNA or amiRNA/HSV-TK were determined on T-REx-293 cells by 50% tissue culture infective dose (TCID₅₀) assays.

2.2. Vector construction

The construction of adenoviral vectors pAdEE4 and pAdEE4_TK was already described in a previous study (Ibrišimović *et al.*, 2012) (Figure 1A.). The entry vector from the above mentioned study (pEE4_TK) was used for the construction of plasmid vectors for doxycycline-controlled EGFP/amiRNA expression. The *Bcl*I site which located between the promoter/operator region and the BGH poly (A) site was used for the insertion of the individual EGFP/amiRNA cassettes (pTP-amiRNA and negative control-amiRNA) (Fig. 4.). The adenoviral vectors for the combinatorial approach are also based on the Ad5-derived vector pAd/PL-DEST (Life Technologies).

2.3. Nucleic acid extraction

For the extraction of circular plasmid DNA an EasyPrep Pro Plasmid Miniprep Kit (Biozym, Oldendorf, Germany), or a HiSpeed Plasmid Midi Kit (QIAGEN, Hilden, Germany) were used. Purification of PCR products was performed with a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany), and adenoviral DNA was isolated with a QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany).

2.4. Determination of adenovirus genome copy numbers

Determination of wt Ad5 DNA levels was performed by qPCR using the following TaqMan primer/probe set directed against the viral E1A gene (E1A-fwd 5'-GACGGCCCCCGAAGATC-3', E1A-rev 5'-TCCTGCACCGCCAACATT-3', and E1A-p 5'-CGAGGAGGCGGTTTCGCAGA-3'). Adenovirus genome copy numbers were determined by using serial dilutions of an adenoviral reference DNA as a standard.

2.5. FACS analysis

For determination of the EGFP expression FACS analysis was employed. First, the cells were infected with EGFP-expressing adenoviruses and then harvested by trypsinization. After they were resuspended in normal cell culture medium, the cells were pelleted by centrifugation at 1200 rpm for 5 min. Thereafter, cells were washed once with phosphate buffered saline (PBS) and fixed with 1% formaldehyde in PBS. FACS Calibur analyzer (Becton Dickinson, Heidelberg, Germany) was used for analysis of the samples and percentages of fluorescent cells and mean fluorescence intensities (MFIs) were determined.

2.6. Primers and probes

pEGFP-f1	5'-CTCAAGGAACCAATTCTGCAGTCGACGGTAC-3'
pEGFP-r2	5'-TACAATCTCGAGCTTAAGATACATTGATGAGTTTGG-3'
HSV1-TK-f1	5'-GCTAACTAGACCGGTCACTGCTTACTGGCTTATCG-3'
HSV1-TK-r1	5'-TAGACTCGAGCGGCCGCGCCACTGTGCTGGATAT-3'
AdC-E1A-for	5'-GACGGCCCCCGAAGATC-3'
AdC-E1A-rev	5'-TCCTGCACCGCCAACATT-3'
AdC-E1A-p	5'-CGAGGAGGCGGTTTCGCAGA-3'
pmiRE-f1	5'-CTTTAAACCATGGTGAGCAAGG-3'
pmiRE-r1	5'-GCTGGGGAAAGATTTCTCGAGTGCGGCCAGA-3'
BGHpA-f1	5'-AGTGGCGGCCGCTCGAGT-3'
BGHpA-r2	5'-CCCAGCGAATTCTTTCCGCCTCAGAAG-3'
pTP-mi5a	5'-TGCTGAAGAGAGTTTCGACAGAATCAAGTTTTGGCCACT GACTGACTTGATTCTCGAACTCTCTT-3'
pTP-mi5b	5'-CCTGAAGAGAGTTTCGAGAATCAAGTCAGTCAGTGGCC AAACTTGATTCTGTGCGAACTCTCTTC-3'
pTP-siRNA	5'-GAAAUUGAUUCUGUCGAACUCUCUU-3' 5'-AAGAGAGUUCGACAGAAUCAAUUUC-3'
Pol-siRNA	5'-CAACGUCUUCAGCGUCCAACCAUA-3' 5'-UAUGGUUGGACGCUGGAAGACGUUG-3'
IVa2-siRNA	5'-AAAUACAGUCCAAGAUGCAUCUCAU-3' 5'-AUGAGAUGCAUCUUGGACUGUAUUU-3'
Hex-siRNA	5'-GAGAACUAAUGGGCCAACAAUCUAU-3' 5'-AUAGAUUGUUGGCCCAUUAGUUCUC-3'
Prot-siRNA	5'-GAGCAGGAACUGAAAGCCAUUGUCA-3' 5'-UGACAAUGGCUUUCAGUUCCUGCUC-3'

3. Results and discussion

3. 1. Inhibition of adenoviruses by combined GCV/siRNA treatment

In order to demonstrate, that combined RNAi/HSV-TK/GCV treatment can lead to additive effects, the siRNAs and amiRNAs silencing different adenoviral genes were employed.

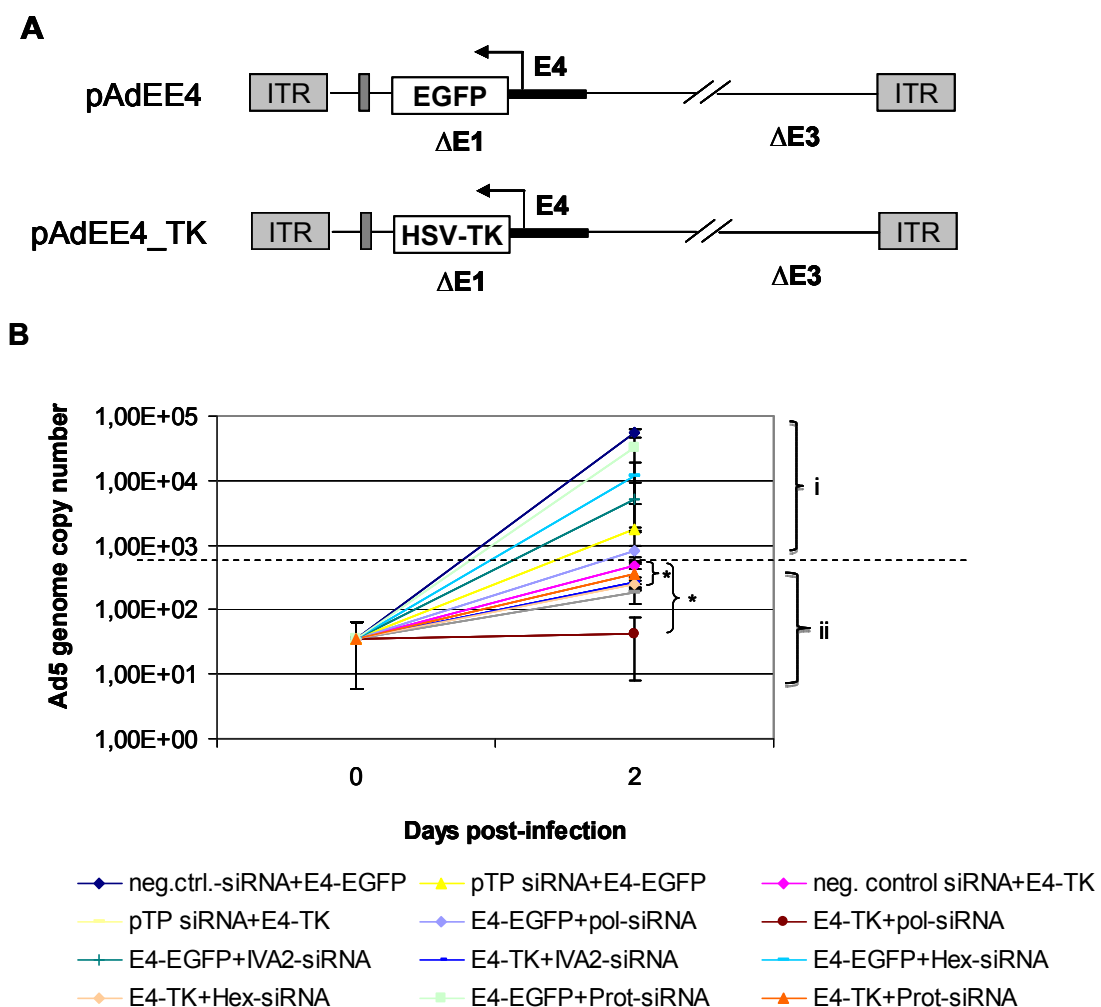


Figure 1. (A) Vector map for pAdEE4 and pAdEE4_TK. The expression cassettes (EGFP or HSV-TK genes) were inserted into the E1-deleted region of the parental vector in antisense orientation with respect to the left inverted terminal repeat (ITR). (B) Combination of E4 promoter-mediated HSV-TK/GCV and siRNAs increase inhibition of wt Ad5 replication. A549 cells were transfected with siRNAs directed

against the DNA polymerase (Pol), pTP, IVa2, hexon (Hex), and protease (Prot) genes, or a non-targeting control siRNA (neg. ctrl.) at a concentration of 30 nM. 24 h after transfection the cells were infected with the recombinant adenovirus vector (i) pADEE4 (E4-EGFP) and (ii) pAdEE4_TK (E4-TK). 48 h after transfection the cells were infected with Ad5 at an MOI of 300 TCID₅₀/cell and treated with 1,2 µM GCV. Virus genome copy numbers were determined at time points 0 and 2 days post-infection by qPCR. For each experiment, real-time qPCR quantification was performed in duplicate. * $p < 0,05$.

An Ad5-based replication-deficient adenoviral vector (pAdEE4_TK) lacking the E1 and E3 regions that allows selective expression of the HSV-TK gene from the Ad5-derived E4 promoter in wt Ad5-infected cells had already been constructed during the previous project (Ibrišimović *et al.*, 2012).

In order to investigate additive effects, the A549 cell line was infected with the recombinant vector or an analogous control vector containing the EGFP instead of the HSV-TK gene (Fig. 1A.).

Additionally, infected cells were transfected with siRNAs designed to silence the adenoviral DNA polymerase, pTP, IVa2, hexon, and viral protease genes, and a non-targeting control siRNA, respectively. Chemically modified siRNAs were used to assure incorporation of only the guide strands into RISC.

A549 cells infected with pAdEE4_TK or pAdEGFP vector and transfected with siRNAs were superinfected with a laboratory strain of wild-type Ad5. Thereafter, the cells were treated with low concentration of GCV (1.2 µM) and were further cultivated for an additional two (Fig. 1B.) or six days (Fig. 3.) to allow for virus spreading. This concentration has been found in the course of the previous project to efficiently inhibit Ad5 replication and is in the range of what has been detected in the plasma of patients after treatment with typical doses of GCV (Asano-Mori *et al.*, 2006).

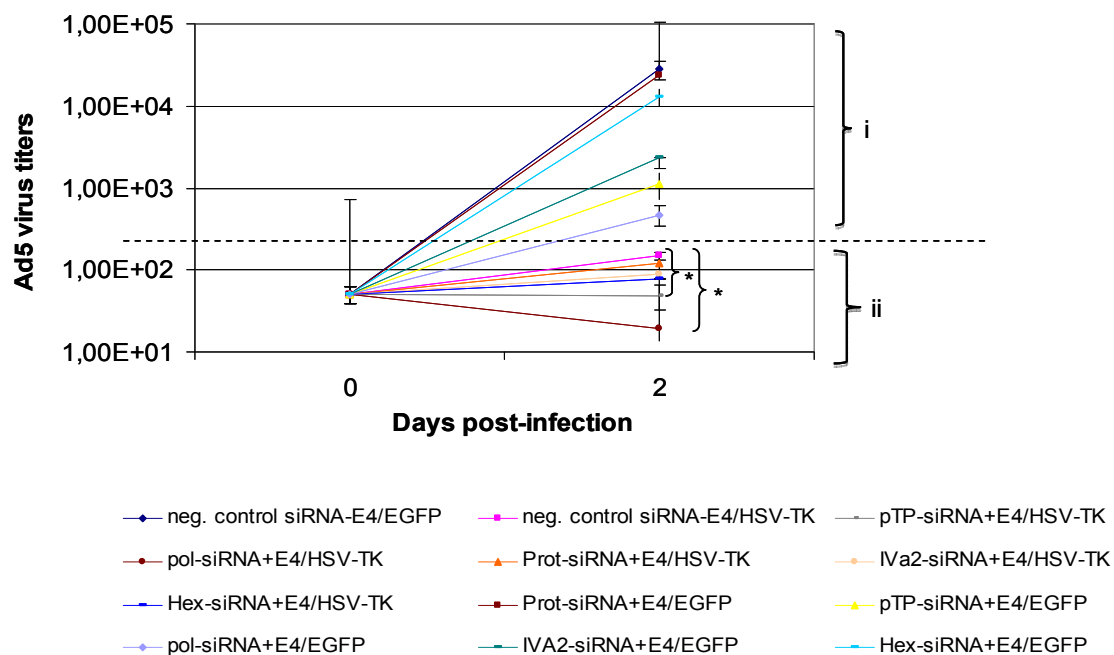


Figure 2. A combination of E4 promoter-mediated HSV-TK expression/GCV treatment and siRNAs treatment reduces infectious virus progeny. A549 cells were transfected with siRNAs directed against the DNA polymerase (Pol), pTP, IVa2, hexon (Hex), and protease (Prot) genes, or a non-targeting control siRNA (neg. ctrl.) at a concentration of 30 nM. 24 h after transfection the cells were infected with the recombinant adenovirus vectors (i) pADEE4 (E4-EGFP) and (ii) pAdEE4_TK (E4-TK). 48 h after transfection the cells were infected with Ad5 at an MOI of 300 TCID₅₀/cell and treated with 1,2 μ M GCV. Numbers of infectious wt Ad5 particles of triplicate infection experiments were determined on A549 cells by TCID₅₀ assays (mean_{SD}; n=3). * $p < 0,05$.

The genome copy numbers of Ad5 progeny viruses were determined by real-time qPCR using E1A-specific primers/probe, and virus titers of cells expressing/not expressing HSV-TK were compared to those additionally transfected with siRNAs (Fig. 1. and Fig. 3.).

I was able to demonstrate that targeting the processes of the viral infection cycle by siRNAs adds to the overall inhibitory effect of HSV-TK expression/GCV treatment. Viral DNA synthesis was significantly reduced for pTP-siRNA and pol-siRNA transfected cells after HSV-TK/GCV treatment when compared to HSV-TK/GCV treatment. This decrease ranged from 0,4 (61,5%) (for pTP-siRNA) to 1,1 (91,2%) (for pol-siRNA) orders of magnitude (Fig.1). This tendency remained the same after six days of infection (Fig. 3).

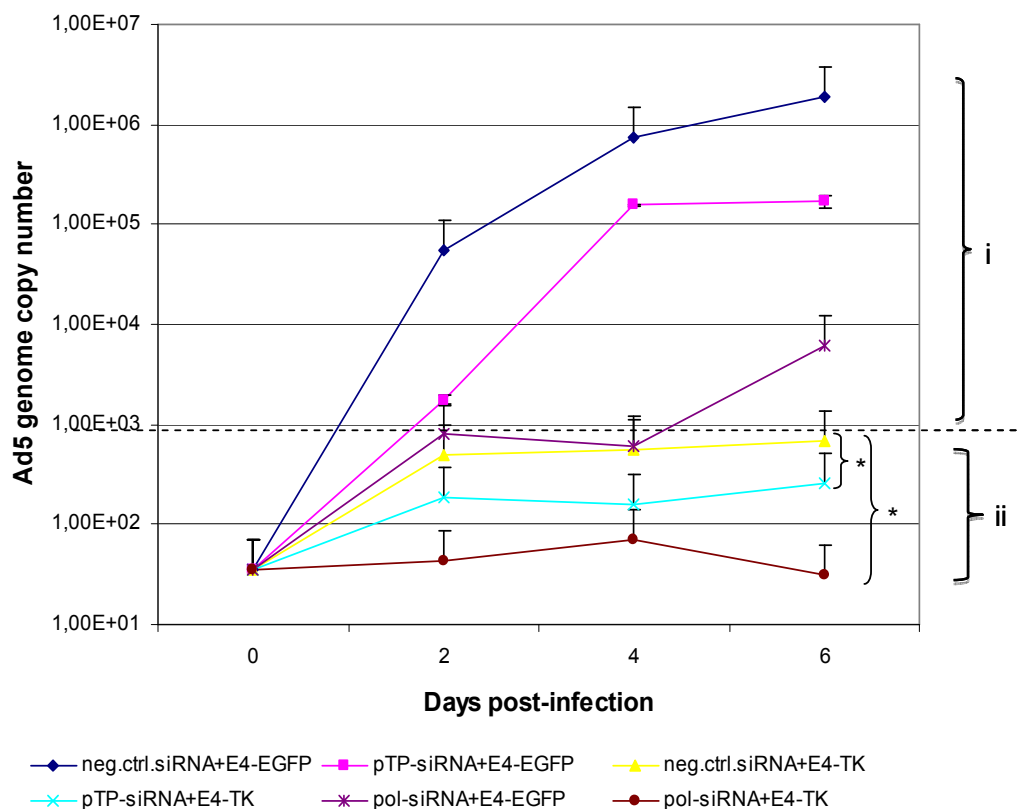


Figure 3. A combination of E4 promoter-mediated HSV-TK/GCV treatment and siRNA-mediated gene silencing increase the inhibition of wt Ad5 replication. A549 cells were transfected with siRNAs directed against the DNA polymerase (Pol), and pTP genes, or a non-targeting control siRNA (neg. ctrl.) at a concentration of 30 nM. 24 h after transfection the cells were infected with recombinant adenovirus vector (i) pADEE4 (E4-EGFP) and (ii) pAdEE4_TK (E4-TK). 48 h after transfection the cells were infected with Ad5 at an MOI of 300 TCID₅₀/cell and treated with 1,2 μ M GCV. Virus genome copy numbers were determined at time points 0, 2, 4 and 6 days post-

infection by qPCR. For each experiment, real-time qPCR quantification was performed in duplicate. * $p < 0,05$.

Exact numbers of infectious viruses, in the same experimental setup as described above, were determined by an end-point dilution-based method (Fig. 2). Adenovirus infectious progeny was significantly reduced two days post-infection with wt Ad5, ranging from 0,7 orders (80,2%) for the pTP-siRNA/HSV-TK approach to 0,9 orders of magnitude (87,2%) for the pol-siRNA/HSV-TK approach after treatment with 1,2 μ M GCV (Fig. 2.).

Taken together, the integration of the two conceptual approaches led to a further remarkable improvement of the concepts already successfully tested during the previous projects and led to a further drop in virus titers.

3.2. Combined GCV treatment and amiRNA expression for the inhibition of adenoviruses

AmiRNAs were designed to target the adenoviral pTP, DNA polymerase, and E1A mRNAs. The designed amiRNAs exhibit full complementarity to their target sequences, which consequently leads to their degradation of the target mRNAs.

The construction of amiRNA expression cassettes employed vector pcDNA6.2-GW/EmGFP-miR (Life Technologies) which provides the amiRNA flanking sequences derived from the murine miR-155 pri-miRNA gene, thus permitting the processing of the amiRNA in the way of a natural pri-/pre-miRNA. The pri-miRNA cassette itself is situated in the 3'UTR of an EGFP gene, which is under control of a CMV promoter.

The E4 adenoviral promoter has been chosen for the expression of HSV-TK gene because of its relatively low background expression in many tested human cells (Ibrišimović *et al.*, 2012), and increased gene expression upon coinfection with wt Ad5. In contrast to the HSV-TK expression cassette, the expression of amiRNAs was controlled by a constitutive CMV promoter. Because the sequence of amiRNAs

confers specificity in term of targeting only adenovirus-infected cells, selective expression of amiRNAs in adenovirus-infected cells was not a prerequisite.

Vectors construction

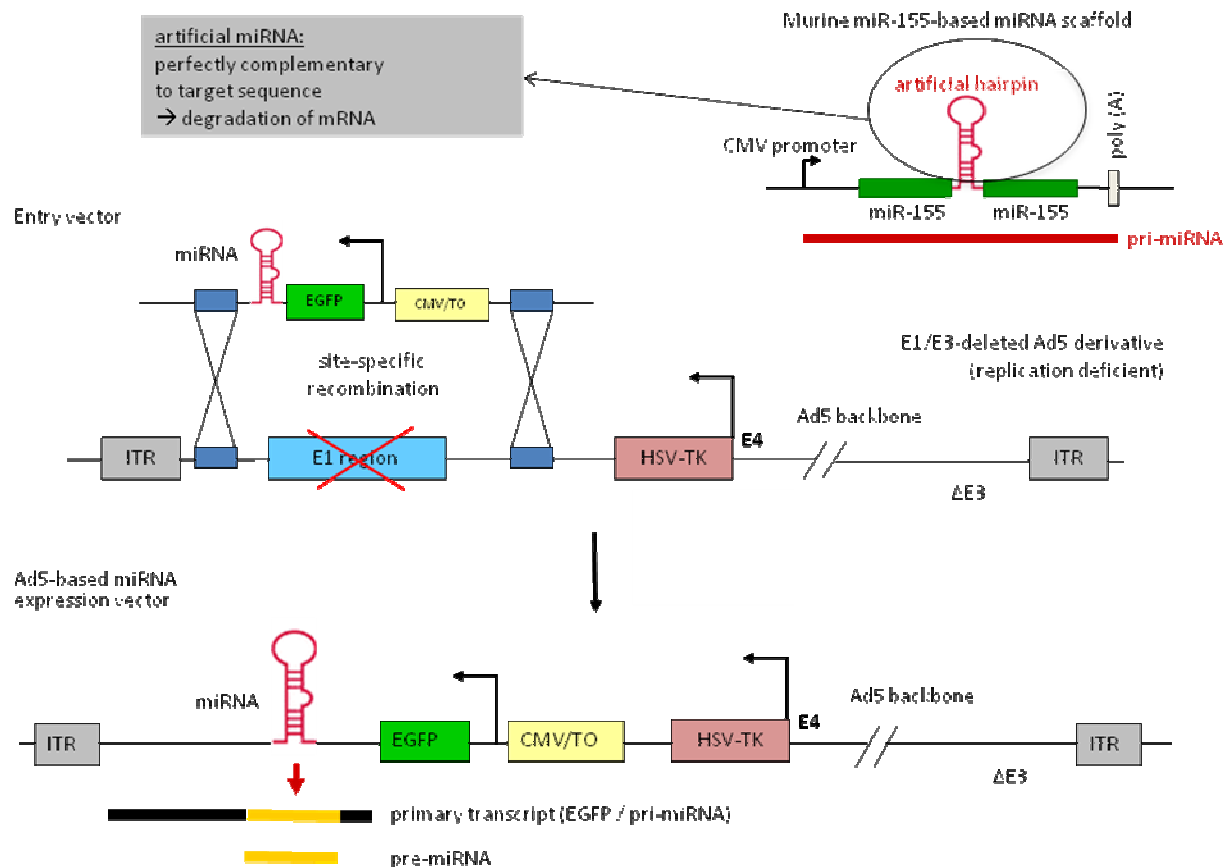


Figure 4. Schematic representation of adenoviral vectors for the combinatorial approach. Both vectors are based on the Ad5-derived vector pAd/PL-DEST (Life Technologies). The expression cassettes were inserted into the deleted E1 region of the parental vector in antisense orientation with respect to the left inverted terminal repeat (ITR). The expression cassettes comprise: (1) the E4 promoter derived HSV-TK gene and (2) the CMV promoter and the EGFP/amiRNA genes (negative control amiRNA or pTP-amiRNA).

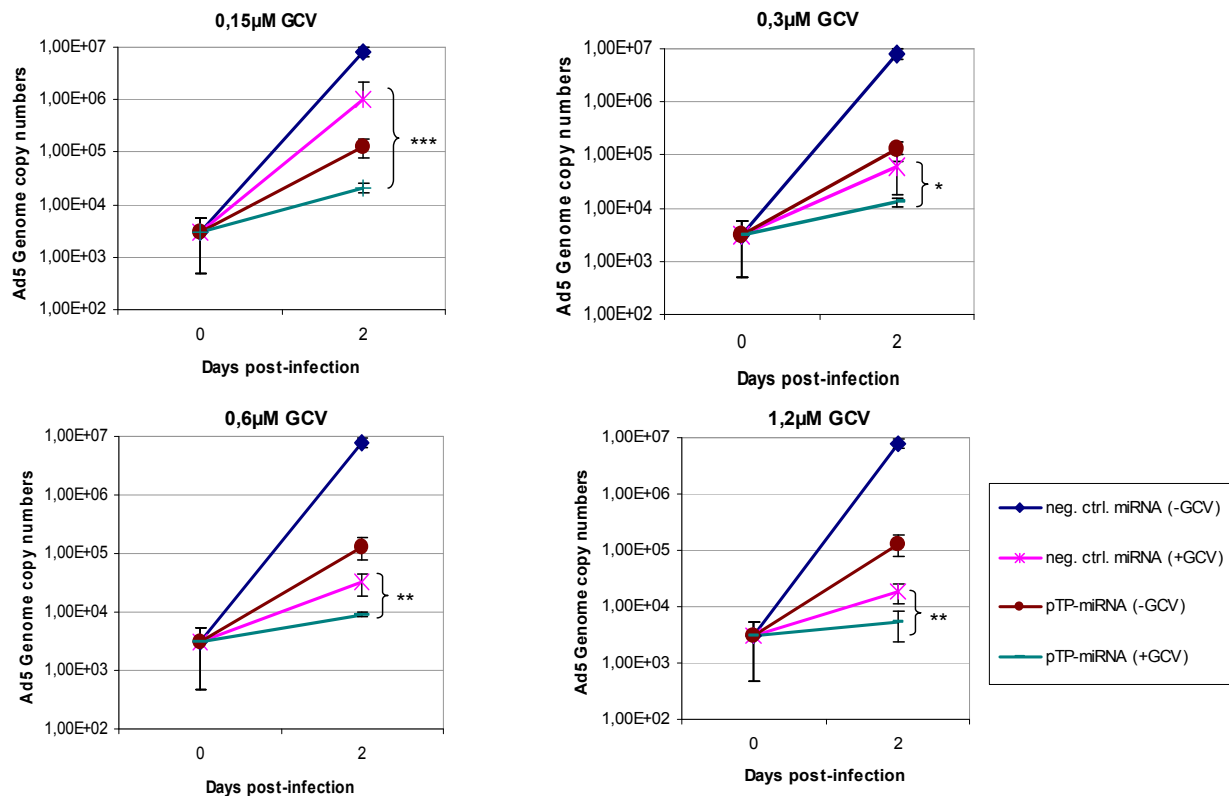


Figure 5. AmiRNA-mediated virus inhibition: E4 promoter-mediated HSV-TK expression/GCV treatment and pTP-miRNA expression inhibit wt Ad5 replication. A549 cells were infected with the recombinant, adenoviral HSV-TK/amiRNA expression vector at an MOI of 100 TCID₅₀/cell. 24 h after infection, cells were superinfected with 400 TCID₅₀ units of wt Ad5 and treated with four different GCV concentrations (A) 0,15 μM; (B) 0,3 μM; (C) 0,6 μM and (D) 1,2 μM. Ad5 genome copy numbers (mean ± SD; n = 3) at days 0 and 2 post-infection with wt Ad5 were determined by real-time qPCR with E1A-specific primers/probe using a standard curve for Ad5. Real-time qPCR quantification was performed in duplicates, each. *p < 0,05, **p < 0,001, ***p < 0,0001.

The expression cassettes containing the EGFP/amiRNA genes under control of the tetracycline-dependent CMV promoter was subsequently inserted into the adenoviral vector pAdEE4_TK already containing the HSV-TK gene under control of the adenoviral E4 promoter. I used this vector (pAdHSV-TK/pTP-amiRNA) and a control vector (pAdHSV-TK/neg.ctrl.-amiRNA) to infect A549 cells at an MOI of 100

TCID₅₀/cell. 24 h after infection, cells were superinfected with 400 TCID₅₀ units of wt Ad5 and treated with four different GCV concentrations (A) 0,15 μ M; (B) 0,3 μ M; (C) 0,6 μ M and (D) 1,2 μ M (Fig. 5.) or only with 1,2 μ M GCV (Fig. 6.). Ad5 genome copy numbers at days 0 and 2 post-infection (Fig. 5) or at days 0, 2, 4, and 6 post-infection (Fig. 6.) with wt Ad5 were determined by real-time qPCR.

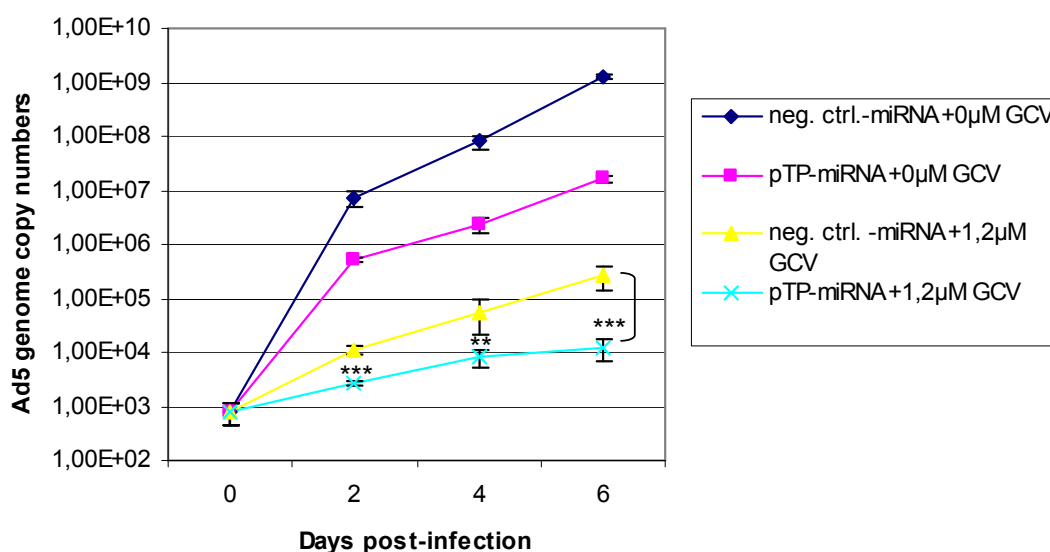


Figure 6. Inhibition of virus replication in A549 cells: E4 promoter-mediated HSV-TK expression/GCV treatment and pTP-amiRNA expression inhibit wt Ad5 replication. A549 cells were infected with the recombinant, adenoviral HSV-TK/amiRNA expression vector at an MOI of 100 TCID₅₀/cell. 24 h after infection, cells were superinfected with 400 TCID₅₀ units of wt Ad5 and treated with 1,2 μ M GCV. Ad5 genome copy numbers (mean $\pm$ SD; n = 3) at days 0, 2, 4 and 6 post-infection with wt Ad5 were determined by real-time qPCR with E1A-specific primers/probe using a standard curve for Ad5. Real-time qPCR quantification was performed in duplicates, each. **p < 0,001, ***p < 0,0001.

Approximately the same additive inhibitory effect on wt Ad5 replication was observed for GCV concentrations even lower than 1,2 μ M, reducing the adenovirus genome copy numbers from 0,54 (71,2%) to 1,22 (93,9%) orders of magnitude when compared to the inhibitory effect of the HSV-TK/GCV concept alone (Fig. 5.). After six days an inhibition of wt Ad5 by 5,04 (99,99%) orders of magnitude was detectable. In contrast to HSV-TK/1,2 μ M GCV treatment alone, reduced genome copy numbers by only 3,7 (99,98%) orders of magnitude (Fig. 6.).

The HSV-TK concept alone is limited to the blockage of viral DNA replication. I was able to show that additional silencing of the viral genes involved in replication of the viral genome leads to additive inhibitory effects, and that the blockage of viral DNA replication by pTP- and pol-siRNAs or pTP-amiRNAs in combination with HSV-TK expression/GCV treatment was able to decrease wt virus titers even more.

Conclusion

Because of the lack of effective anti-adenoviral therapeutics, human adenoviruses are a frequent cause for life-threatening infections in immunocompromised patients (HIV-positive individuals or patients undergoing solid organ or allogeneic stem cell transplantation). Since the mortality rates associated with adenovirus infections are very high, the development of alternative treatment strategies is highly required.

In my first approach, I contributed to a project in which the HSV-TK gene was selectively expressed in cells infected with adenoviruses. This selective expression was achieved by placing the gene under control of the adenoviral E4 promoter, whose activity is dependent on the presence of the adenoviral E1A gene product. HSV-TK is able to convert non-toxic prodrugs such as GCV, into their toxic forms, consequently blocking DNA replication. GCV is commonly used for the successful treatment of human herpes virus infections. By selective expression of HSV-TK and concomitant treatment with GCV I was able to inhibit Ad5 replication and decrease Ad5 virus titers by several orders of magnitude *in vitro*.

In a distinct project I was involved in RNAi-based knockdown of adenovirus genes by siRNAs or amiRNAs. It turned out that siRNAs directed against E1A, pTP, DNA polymerase and IVa2 transcripts efficiently silenced the respective target genes during adenovirus infection. Although demonstrating a similar silencing capacity in luciferase reporter system, the hexon- and protease siRNAs showed limited inhibition of adenovirus replication during an infection. With regard to these findings it can be assumed that silencing of early viral genes may be more beneficial than silencing of late viral genes, and that the reduction of adenoviral DNA is central to the inhibition of adenovirus replication.

After having identified the best gene targets, amiRNAs directed against the E1A or pTP and DNA polymerase mRNAs, were designed respectively.

In order to guarantee efficient delivery of amiRNA-expression cassettes into cells, also for an envisioned *in vivo* application, a delivery system based on replication-deficient adenoviral vectors (lacking the E1A gene) was generated. In order to be able to efficiently produce these recombinant viruses, it had to be assured

that amiRNA expression, which would inhibit the replication of the recombinant vector, is deactivated in packaging cells. Thus, a tetracycline-inducible system for amiRNA expression was generated.

I was able to show that only an amiRNA directed against the pTP mRNA was able to significantly inhibit the replication of wt Ad5, and that the reduction rates of adenovirus progeny were even higher after concatemerization of up to six pTP-amiRNA hairpins in primary transcripts.

However, although both methods, the HSV-TK/GCV and the RNAi, dramatically inhibited adenovirus replication, neither of them was able to completely block the generation of virus progeny. The incomplete blockage of adenovirus multiplication was not due to insufficient delivery rates, as average transduction rates of > 90% could be achieved. Complete blockage of virus inhibition could not be achieved by this approach because the HSV-TK is not available immediately after wt Ad5 infection. Sufficiently high levels of HSV-TK have to be first built up once wt Ad5 has infected the cell. Thus, to overcome this limitation, I tried to additionally provide the cells with siRNAs or a miRNAs, which are present in the cell from the beginning of an infection with wt Ad5.

I was able to demonstrate that when employing the siRNA- and amiRNA-based strategies to silence a panel of adenoviral genes together with HSV-TK expression/GCV treatment, there was an additive inhibitory effect on adenovirus multiplication. These results suggest that combinatorial approaches may be beneficial for the control of adenoviral infections. Combinations may also include conventional drugs such as cidofovir (combining amiRNA expression with CDV treatment).

In general, the results generated in the projects in which I was involved may open up a novel therapeutic scenario for the treatment of adenovirus infections to decrease adenovirus-mediated morbidity and mortality in patients.

Curriculum Vitae

Surname: Ibrišimović
Firstname: Mirza
Date of birth: January 4th, 1984
Place of birth: Brčko
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Education

September 1st, 2009: PhD in Molecular Biology at St. Anna Children's Cancer Research Institute, Vienna, in the lab of Univ.-Prof. DDr. Thomas Lion, under the supervision of Dr. Reinhard Klein (Department for Molecular Microbiology), with the title: *"Alternative approaches for the treatment of adenovirus infections"*.

- October 1st, 2008: Start of my Diploma thesis under supervision of Univ. Prof. Dr. Fritz Pittner (Department for Biochemistry and Molecular Cell Biology; University of Vienna): *„Evaluation of novel, nanotechnological biosensor-chips for monitoring of bacterial spoilage: Evaluation of sensor sensitivity and correlation with currently used microbiological testing methods and their improvement”*.
- June 10th, 2009: Successful completion of diploma exam in molecular biology.
- March 1st, 2005: Start of my studies in Molecular Biology at University of Vienna (major subjects: genetics, biochemistry, and molecular medicine).
- Juni 2003: Grammar School final examination.
- 1999-2003: Grammar School „Vaso Pelagić“, Brčko, Bosnia and Herzegovina.
- 01.-07.2002: Abroad semester in USA (Ironwood High School in Phoenix, Arizona).
- 1996-1999: Elementary School „Arif Dervišević“, Prutače, Bosnia and Herzegovina.
- 1993-1996: Elementary School „Hamid Berbić“, Gornji Rahić, Bosnia and Herzegovina.
- 1992-1993: Elementary School, Maribor, Slovenia.
- 1991 -1992: Elementary School „Nasto Nakić“, Brčko, Bosnia and Herzegovina.

Courses

- June-August 2008: Elective Laboratory Course (Molecular Medicine)-Part 1 and Part 2: “*Molecular mechanisms involved in the transport of VLDL from the egg yolk to the embryo proper; Localisation and cloning of LRP-380; R/O Genotyping*”, under supervision of Univ. Prof. Dr. Marcela Hermann (Department of Medical Biochemistry, Division Molecular Genetics; University of Vienna).
- March-April 2008: Advanced Course in Developmental Biology: “*Metal Ion Homeostasis in Cells and Organelles; The gating mechanisms in the CorA Mg^{2+} - Channel*”, under supervision of Univ. Prof. Dr. Rudolf Schweyen (Department of Genetics; University of Vienna).
- October-November 2007: Advanced Course in Biochemistry “*Optimization of Meat Fresh Sensors on Gold Nanoparticle Basis*”, under supervision of Univ. Prof. Dr. Fritz Pittner (Department for Biochemistry and Molecular Cell Biology; University of Vienna).

Work Experience

Since October 1st, 2008: Assistant in Laboratory Course C + Advanced Laboratory Course in Biochemistry (Protein Biochemistry) and assistant in Biochemistry Laboratory Course for Chemists - Basic Techniques; Department for Biochemistry and Molecular Cell Biology; University of Vienna.

Publications

- „Targeted expression of herpes simplex virus thymidine kinase in adenovirus-infected cells reduces virus titers upon treatment with ganciclovir *in vitro*”; **Ibrišimović M**, Nagl U, Kneidinger D, Rauch M, Lion T, Klein R; Journal of Gene Medicine, 2012.
- „An adenoviral vector-based expression and delivery system for the inhibition of wild-type adenovirus replication by artificial microRNAs”; **Ibrišimović M**, Kneidinger D, Lion T, Klein R; Submitted to Antiviral Research.

- „Inhibition of adenovirus multiplication by short interfering RNAs directly or indirectly targeting the viral DNA replication machinery”;
*Doris Kneidinger, **Mirza Ibrišimović**, Thomas Lion, Reinhard Klein*
Antiviral Research, 2012.
- „Biomimetic PLGA sensor: proof of principle and application”; *N. Ibrišimović, **M. Ibrišimović**, M. Barth, U. Bohrn*, Chemical Monthly 141:125- 130, 2010.
- „Biomimetic sensor chip monitoring real-time food degradation: correlating chemical deterioration with microbiological status”; *N. Ibrišimović, **M. Ibrišimović**, M. Barth, U. Bohrn, F. Pittner*, Chemical Monthly 140: 941-945, 2009.

Presentations

- Presentations at the university in several courses.
- Publications oral talks at the Children’s Cancer Research Institute (on a regular basis).
- Poster presentation at the ÖGMBT annual Meeting 2010.
- Poster presentation at the 10th International Adenovirus Meeting 2012; Umeå, Sweden.

Patents and Inventions

July, 2009: Patent: „ Device comprising a polymer layer and a reflecting layer”;
EP2278300

February, 2009: „Test-Tool for Food Microbiology” invention.

April, 2009: „Test-Tool for Food Microbiology”- license has been given to
Sony DADC company from Salzburg.

Other Qualifications

Languages:

Bosnian, Croatian, Serbian: native languages

Englisch: fluent spoken and written

German: fluent spoken and written

Excellent computer skills.

Soft Skills

- Fast introduction in foreign methods and software
- Team player
- Very good organization and coordination
- Loyalty and preciseness

