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„Human Adenovirus in the Context of RNA Interference – Interaction and Treatment Options“

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The single biggest threat to man's
continued dominance on the planet is a
virus.

-Joshua Lederberg-

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ABSTRACT

Human adenoviruses are a common threat to immunocompromised patients, like HIV-positive individuals or hematopoietic stem cell transplant and bone marrow transplant recipients. Since the antiviral drugs used for the treatment of adenoviral infections have limited effects, these infections can become quite severe and even life threatening with mortality rates up to 80%. Thus, there is the urgent need for alternative treatment options.

The aim of the here presented studies was to provide new strategies to overcome adenoviral infections and their possible fatal outcomes.

In the first approach I used the concept of RNA interference (RNAi) to inhibit adenovirus 5 multiplication *in vitro*. First of all, I wanted to identify a suitable viral target gene or process for this approach. Thus, I designed several different siRNAs targeting adenoviral genes essential for viral replication or virion assembly. These target genes include E1A, DNA polymerase, the preterminal protein, IVa2, the capsid component hexon and the virus protease.

Silencing of early and middle genes was significantly more effective in inhibiting adenovirus multiplication than was silencing of late genes. The most effective downregulation was exhibited by a siRNA directed against the viral DNA polymerase; this siRNA 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 affects 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, E1A emerged as a second important target, because its knockdown markedly improved the viability of cells at late stages of infection.

In a separate approach I was involved in the investigation of the potency of artificial miRNAs targeting E1A, DNA polymerase or pTP, to inhibit adenovirus multiplication. Since the artificial miRNAs were cloned into a replication-deficient adenoviral vector we generated a delivery system that may allow targeted delivery into typical adenovirus host cells. We could show that the pTP-specific artificial miRNA has the highest potency to inhibit adenovirus replication *in vitro*.

In a further project we used the concept of gene-directed enzyme prodrug therapy to inhibit adenoviral infections *in vitro*. Therefore, we investigated the potency of the

prodrug ganciclovir (GCV) that is converted into its active form by the Herpes virus simplex thymidine kinase (HSV-TK). To guarantee that only adenovirus-infected cells produce HSV-TK, this gene was expressed from a replication-deficient adenovirus vector lacking the E1A gene and HSV-TK expression was under the control of the adenoviral E4 promoter. Our data indicated that the adenoviral E4 promoter is suitable for our approach due to sufficiently low background expression in non-infected cells, but high expression in AdV-infected cells. Treatment with low levels of GCV efficiently inhibited Ad5-wt DNA replication. Genome copy numbers and output of infectious particles were reduced by up to >99% and cell viability was greatly increased.

In the last part of the here presented theses we were interested if there is an interaction between adenovirus encoded miRNAs (mivaRNAs) and cellular target mRNAs. To answer this question a RIP-chip assay was established revealing 20 potential cellular target genes for adenoviral mivaRNAs involved in cell metabolism, signaling or immune response. These target genes were further validated with bioinformatical tools and molecular biological methods to gain more insight into the effect of the mivaRNAs on cellular gene regulation during an adenoviral infection. It is essential to better understand the interaction of the virus with its host to identify potential treatment options.

ZUSAMMENFASSUNG

Humane Adenoviren sind häufig eine Bedrohung für immunsupprimierte Patienten, wie etwa HIV-positive Menschen oder hämatopoietische Stammzelltransplantations- oder Knochenmarktransplantationspatienten. Da antivirale Medikamente, die für die Behandlung von adenovirale Infektionen verwendet werden, nur limitierte Effekte erzielen, können diese Infektionen schwerwiegende Folgen haben und sogar lebensbedrohlich werden, mit Sterberaten bis zu 80%. Daher ist es äußerst wichtig, neue Therapiemethoden zu entwickeln.

Das Ziel der hier vorgestellten Arbeit war neue Strategien aufzuzeigen, um adenovirale Infektionen und deren fatale Folgen zu verhindern.

Im ersten Ansatz verwendete ich das Konzept der RNA Interferenz (RNAi), um Adenovirus 5 Vermehrung *in vitro* zu inhibieren. Zuerst wollte ich für diesen Ansatz geeignete virale Targets oder Prozesse identifizieren. Daher designte ich siRNAs, die Gene erkennen, die für die virale Replikation oder den Aufbau des Virions zuständig sind. Diese Gene umfassen E1A, DNA Polymerase, das pre-terminale protein (pTP), IVa2, die Capsidkomponente Hexon und die virale Protease.

Stilllegung von frühen oder mittleren Genen war signifikant effektiver in der Inhibierung der Adenvirusvermehrung als die Stilllegung von späten Genen. Die effizienteste Reduktion konnte mit einer siRNA erzielt werden, die gegen die DNA Polymerase gerichtet ist; diese siRNA verringerte die viralen Genomkopiezahl und die infektiösen Viruspartikel um mehrere Zehnerpotenzen. Da die Stilllegung von jedem frühen Gen entweder direkt oder indirekt die virale DNA Synthese beeinflusst, weisen unsere Daten darauf hin, dass die Reduzierung von viralen Genomkopiezahlen ein vielversprechenderer Ansatz ist für die Behandlung von adenoviralen Infektionen als die Reduzierung von Proteinen, die für die Capsidbildung notwendig sind. Somit wurde die adenovirale DNA Replikation als Schlüsselziel für die RNAi-basierende Inhibierung von Adenovirusvermehrung identifiziert. Zusätzlich zeigte sich E1A als zweites wichtiges Zielgen, da dessen Knockdown deutlich zu einer Verbesserung der Viabilität von Zellen in späten Phasen der Infektion führt.

In einem separaten Ansatz war ich beteiligt an der Untersuchung der Wirksamkeit von artifiziellen miRNAs, die das E1A-, das DNA Polymerase- oder das pTP-Gen erkennen, die adenovirale Vermehrung zu inhibieren. Da diese miRNAs in einen replikationsdefizienten adenoviralen Vektor kloniert worden sind, generierten wir ein System, das gezielte Expression der miRNAs in AdV-infizierten Zellen ermöglicht. Wir

konnten zeigen, dass die pTP-spezifische artifizielle miRNA das höchste Potential zeigt, adenovirale Replikation *in vitro* zu inhibieren.

In einem weiteren Projekt nützten wir das Konzept von Gen-gerichteter Enzym Prodrug Therapie um adenovirale Infektionen *in vitro* zu bekämpfen. Daher untersuchten wir die Wirksamkeit der Prodrug Ganciclovir (GCV), welche durch die Herpesvirus simplex Thymidinkinase (HSV-TK) in die aktive Form konvertiert wird. Um zu garantieren, dass ausschließlich adenovirus-infizierte Zellen HSV-TK produzieren, wurde dieses Gen von einem replikationsdefizienten adenoviralen Vektor exprimiert, dem die E1A Region fehlte und die HSV-TK Expression war unter der Kontrolle des adenoviralen E4 Promotors. Unsere Daten weisen darauf hin, dass der adenovirale E4 Promotor für diesen Ansatz gut geeignet ist, da es geringe Background-Expression in nicht-infizierten Zellen gibt, aber eine hohe Expression in AdV-infizierten Zellen. Die Behandlung mit geringen Mengen an GCV konnte effizient die Ad5 DNA Replikation inhibieren. Die Genomkopiezahlen und die Menge an infektiösen Partikeln konnte um bis >99% reduziert werden und die Zellviabilität wurde stark erhöht.

Im letzten Teil der hier präsentierten Dissertation untersuchten wir, ob es eine Interaktion zwischen Adenovirus-kodierten miRNAs (mivaRNAs) und zellulären Ziel-mRNAs gibt. Um diese Frage zu beantworten, wurde ein RIP-Chip Assay etabliert, der 20 potentielle zelluläre Zielgene für adenovirale mivaRNAs aufzeigte, die in Zellmetabolismus, Signaling oder Immunreaktionen involviert sind. Diese Targetgene wurden mit bioinformatischen Tools und molekularbiologischen Methoden validiert, um mehr Einblick in den Effekt von mivaRNAs auf zelluläre Genregulation während einer adenoviralen Infektion zu bekommen. Es ist essentiell, die Interaktion zwischen dem Virus mit seinem Wirt besser zu verstehen, um mögliche Therapiemethoden zu erkennen.

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I. INTRODUCTION

1. ADENOVIRIDAE

Adenoviruses (AdVs) belong to the family *Adenoviridae* that can be further divided into the genera *Aviadenovirus*, *Mastadenovirus*, *Atadenovirus*, *Siadenovirus* and *Ichtadenovirus*. As shown in Tab. 1, adenoviruses can infect a broad range of vertebrate species. *Mastadenovirus* and *Aviadenovirus* originate from mammals or birds, respectively, while the genera *Atadenovirus* and *Siadenovirus* have a broader range of hosts. Each genus can be further divided into species or subgroups, which are named from the host and supplemented with letters of the alphabet (reviewed in [1]).

Table 1 | Adenovirus Classification [1]

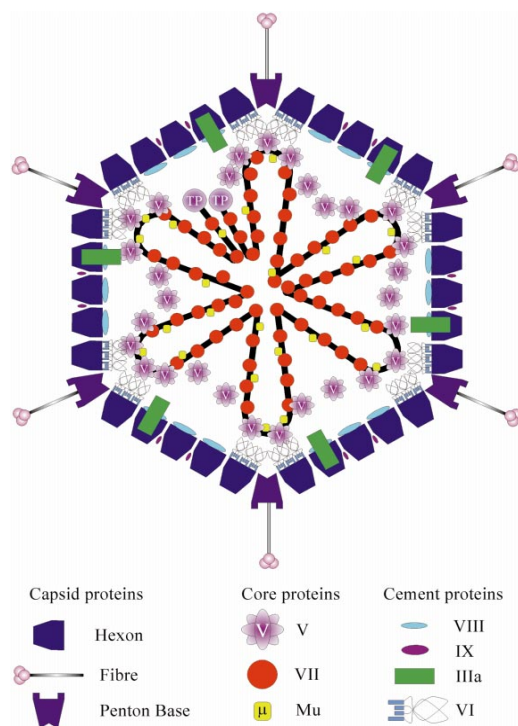
Genus	Species	Host range
<i>Aviadenovirus</i>	Fowl adenoviruses	Chicken, turkey, goose
	Goose adenoviruses	Pigeon, duck
<i>Mastadenovirus</i>	Human adenoviruses	Humans, simians, cattle
	Bovine adenoviruses	Sheep, goats, dogs
	Ovine adenoviruses	Cats, tree shrews, pigs
	Simian adenoviruses	
<i>Atadenovirus</i>	Bovine adenovirus D	Cattle, goats, deer
	Black tailed deer adenovirus	Duck, possum
	Duck adenovirus	
	Possum adenovirus	
<i>Siadenovirus</i>	Frog adenovirus	Frogs, pheasants
	Turkey hemorrhagic enteritis virus	Turkey, duck
<i>Ichtadenovirus</i>	Fish adenovirus	

1.1. Human adenovirus

In the early 1950's the first human adenoviruses (hAdVs) were isolated from adenoid tissue [2, 3]. Human adenoviruses, belonging to the genus *Mastadenovirus*, comprise more than 60 serotypes that are clustered in 7 species (A to G) based mainly on immunological criteria. Different adenovirus species show diverse tissue tropisms (Tab. 2).

Human adenoviruses, like all members of the family *Adenoviridae*, are non-enveloped, lytic DNA viruses with a characteristic icosahedral morphology [4]. Mature virions are approximately 90 nm in diameter consisting of a DNA-protein core complex and a protein capsid shell. The viral capsid is built up by three major proteins: (i) 240 trimers of the hexon protein (pII), which is the most abundant coat protein; (ii) 12 pentamers of the homotrimeric fiber that consist of a carboxyterminal globular knob, a central shaft and an amino-terminal tail by which each fiber is anchored to (iii) a penton base (Fig. 1) [5].

Both, penton base and fiber are essential for the interaction between the viral capsid and the surface of a target host cell [6]. Minor coat proteins are the polypeptides



pIIIa, pVI, pVIII and pIX which are less well characterized and mainly act as capsid cement [7]. For the production of mature infectious viral particles the processing of some structural proteins is required. For that purpose adenoviruses encode their own protease [8, 9]. Precursor terminal protein (pTP), the polypeptides pV, pVII (major core protein) and pX (also known as μ) make up together with the viral DNA, the core of the virion. pVII binds the viral genome tightly forming a compact nucleoprotein complex [5], whereas pV links the viral chromosome to the virus capsid ("p" accounts for "precursor") (Fig. 1) [10].

Figure 1 | Human Adenovirus Structure [11]

The organization of the capsid and the cement components is shown. The location of the capsid and its minor components was defined by structural studies using cryo-electron microscopic and crystallographic techniques whereas the arrangement of the core proteins and the viral genome is rather conjectural. The nomenclature of the core and the cement proteins refers to their mature, cleaved forms.

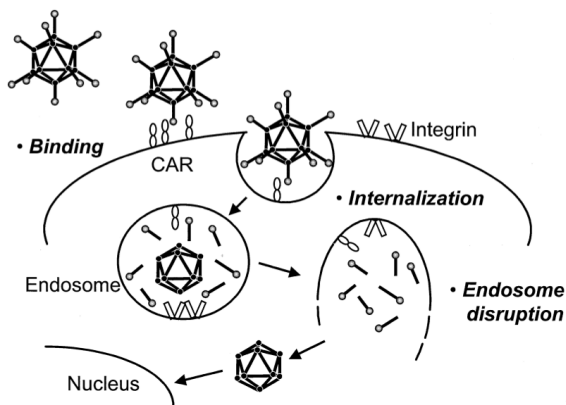
Table 2 | Human Adenovirus Diversity [12-14]

Species	Serotype	Tissue tropism [15]	Primary cellular receptor [15]
A	12, 18, 31, 61	Gastrointestinal tract	CAR
B	3, 7, 11, 14, 16, 21, 34, 35, 50, 55	Urinary tract, lung	CD46; CD80/86;X
C	1, 2, 5, 6, 57	Upper/lower respiratory tract	CAR; heparan sulfate proteoglycans
D	8, 9, 10, 13, 15, 17, 19, 20, 22, 23, 24, 25, 26, 27, 28, 29, 30, 32, 33, 36, 37, 38, 39, 42, 43, 44, 45, 46, 47, 48, 49, 51, 53, 54, 56, 58, 60, 62, 63, 64, 65, 67	Eye, Gastrointestinal tract	CAR; sialic acid; CD46
E	4	Respiratory tract	CAR
F	40, 41	Gastrointestinal tract	CAR
G	52	Gastrointestinal tract	

X refers to a yet unidentified glycoprotein

AdVs have linear, double-stranded DNA molecules ranging from 30 – 38 kbp in size with a terminal protein (TP) covalently attached to the 5' termini, which contains 103-nucleotide long inverted repeats (ITRs) [16]. Here, the viral origin of replication is embedded.

1.2. Classical adenovirus infection pathway¹

**Figure 2 | Adenovirus Infection Pathway [17]**

The entry of AdV into host cells is first initiated by a high affinity binding of the virus to the primary cellular receptor, CAR. The following internalization of the virus by receptor-mediated endocytosis is mediated via an interaction of Arg-Gly-Asp (RGD) peptide sequences in the penton base protein with secondary host cell integrins. After endosomal escape the virion translocates into the nucleus via nuclear pores.

¹ The term 'adenovirus' refers to as 'human adenovirus', from now on, if not mentioned explicitly.

The classical adenovirus infection pathway can be divided into five stages, including binding, entry, escape from endosomes, translocation and nuclear import (Fig. 2) [18].

1.2.1. Binding

The first step in an adenoviral infection of a host cell is a high affinity interaction between the adenovirus fiber knob and the specific cell surface receptor (Fig. 3). All human adenovirus species, with the exception of species B, use the Coxsackie virus and Adenovirus Receptor (CAR) (Tab. 2) [19, 20].

CAR is present on various cell types in different organs and its association with tight junctions explains its presumable role in cell adhesion [21]. Besides CAR, several other molecules have been described to function as adenoviral receptors, including CD46 [22], CD80/CD86 [23], sialic acid [24] and heparan sulfate proteoglycans [25].

In addition to a high affinity binding of the fiber knob, a secondary, lower affinity interaction takes place between the integrin binding arginine-glycine-aspartate (RGD) domain in the adenovirus penton base and cell surface integrins including $\alpha V\beta 3$, $\alpha V\beta 5$, $\alpha M\beta 2$ and $\alpha 5\beta 1$ [26-33]. In combination, both interactions lead to a cooperative, irreversible binding of the viral capsid to the cell surface [34].

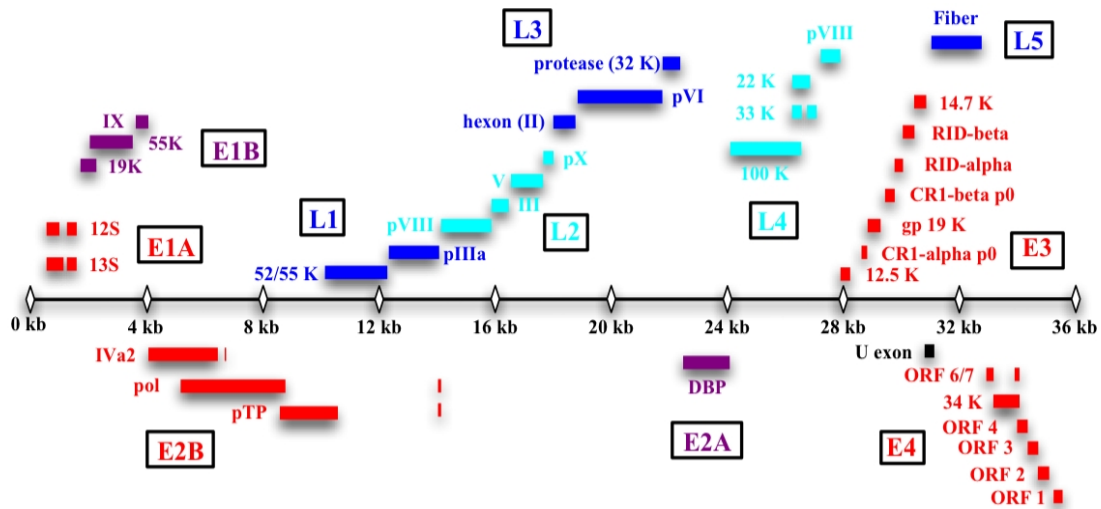
1.2.2. Entry, escape from endosome, translocation and nuclear import

After binding to the cell surface, the capsid enters the cell by a process termed receptor-mediated endocytosis. Upon endosomal acidification the capsid undergoes conformational changes leading to endosomal lysis and escape of the virions into the cytoplasm [35, 36]. Following, the partially dismantled capsid is translocated along microtubules towards the nucleopore complex [37-39]. Subsequently, further disassembly occurs and the viral genome is transferred into the nucleus (Fig. 2) [40].

1.3. Adenoviral replication

Once having entered the nucleus of a host cell, adenoviral replication starts. Importantly, the replication can be divided into an early (E) and late (L) phase (Fig. 4, 5). The host RNA polymerase II complex transcribes the whole AdV genome [41]. The only exception represent the small virus-associated (VA) RNAs I and II that are transcribed by the host RNA polymerase III [42].

A



B

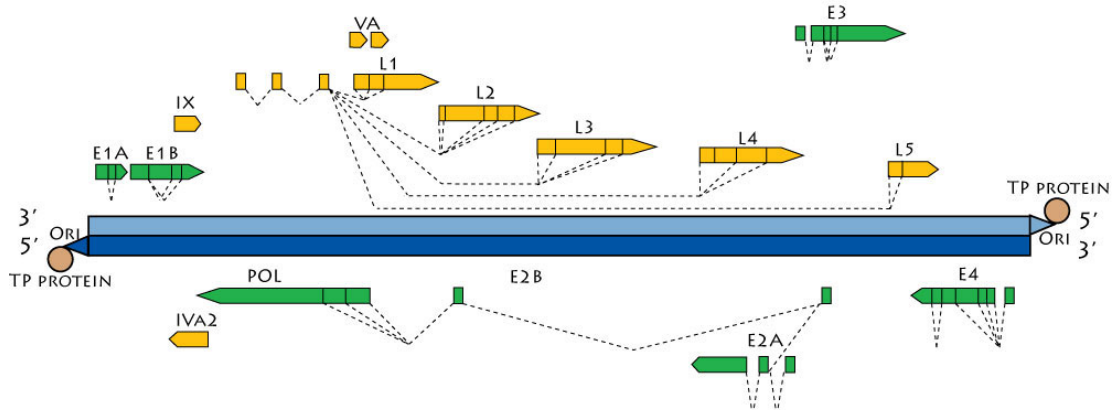


Figure 3 | Human Adenovirus 5 Genome [11], [43]

A. The scheme shows all adenoviral gene products from the early E1A, E1B, E2A, E2B, E3 and E4 promoters, and the L1 – 5 promoters. **B.** The schematic illustration of the adenoviral genome organization shows the complexity of the same. The early transcripts are outlined in green, the late transcripts in yellow. The fragmentation of the arrows depicts the splice variants of the transcript. The brown circles represent the terminal protein that is bound the 5' end of both strands.

1.3.1. The early phase of replication

During the early phase that can take 6-8 h in a permissive cell, the early viral transcription units (E1A, E1B, E2A, E3 and E4) become activated and lead to the production of early proteins, which then transactivate other viral transcription units and lead to the transcription and translation of the late genes. Besides, early viral proteins interfere with the host cell cycle in order to provide optimal viral replication and modulate the host's antiviral immune response (reviewed in [11]). In more detail, each early region encodes multiple messenger RNAs (mRNAs) that arise by differential

splicing (Fig. 5). First the, E1A and E1B-genes are transcribed. The proteins from these early regions regulate cellular genes and transactivate other early transcription units [44]. Interestingly, it was reported that these early products can transform rodent cells into an oncogenic phenotype [45]. Recently, Schreiner *et al.* could show for the first time that the capsid protein VI is necessary for efficient initiation of adenoviral gene expression by activating the E1A promoter and promoting initial expression of the E1A transactivator. This function of VI is mediated via a conserved PPxY motif, a conserved ubiquitin ligase interacting motif. Adenoviruses with mutated PPxY motifs showed reduced E1A transcription and E1A protein levels, resulting in decreased replication capacities. The authors suggest that PPxY dependent activation of the E1A promoter is mediated by the removal of Daxx (death domain associated protein) from the promoter, thereby leading to depression of E1A transcription [46].

Three other products generated during the early phase are essential for DNA replication: the E2B-encoded pTP, the E2A-encoded DNA binding protein (DBP) and the E2B-encoded viral DNA polymerase (Pol). In infected cells, pTP and Pol form heterodimers and, together with DBP and a core origin of replication, can maintain basal level of replication [47, 48].

The role of DBP during viral replication comprises DNA replication initiation and elongation. Additionally, DBP may be involved in unwinding of the duplex termini due to its ATP-independent helix-destabilizing activity [49] and it is involved in processes as DNA recombination and virus assembly [50].

pTP serves in its mature form TP as a structural component and covalently attached to the viral DNA termini [16] and may facilitate the circularization of the virus genome [51].

1.3.1.1. Adenoviral VA RNAs

As mentioned above AdV expresses either one or two short non-coding RNAs called virus-associated (VA) RNAs. VAI can be found in all adenovirus serotypes, while VAIL is only detectable in some of them. Both VA RNAs show comparable length: VAI is 157-160 nucleotides long whereas VAIL is 158-163 nucleotides long [52]. Significant sequence diversity between VA RNAs from different species and even serotypes was found. All VA RNAs share a conserved region in the 5' end and near the 3' terminus both of which are essential for VA RNA transcription initiation and termination. Additionally, highly conserved sequences of up to 4 nucleotides can be found throughout the VA gene, which are probably required for the maintenance of the VA RNA secondary structure (Fig. 6) [53, 54]. VAI and VAIL are also different concerning

their synthesis profile. While VAI synthesis starts during the early phase of infection and rapidly accelerates during the late phase eventually resulting in a very high numbers of VAI molecules (10^8 molecules/cell) [55, 56], VAIL is synthesized at high levels in the late phase, but accumulates at lower concentrations (10^7 molecules/cell). This observation can be explained by the fact that the VAI promoter is stronger than the VAIL promoter. Indeed, similar expression levels can be obtained when both VA RNAs are under the control of the same promoter [57].

In order to study the function of these VA RNAs, mainly adenovirus 2 (Ad2) and adenovirus 5 (Ad5) were used as a model. Deletion of either VAI alone or both VA RNAs in Ad2 leads to a 10-20-fold or even 60-fold decrease in viral growth, respectively [58-61]. However, inactivation of VAIL alone has only little impact on viral growth, suggesting that VAIL has non-essential functions but can compensate in part for the lack of VAI in VAI-mutated adenovirus [62]. Consequently, VA RNAs are thought to fulfill some sort of silencing suppressor function by interfering with the cellular RNA interference machinery. One indication for that theory is that VA RNAs have a stem-loop structure similar to that of precursor-microRNAs (pre-miRNA) and are subject to similar processing. Both, pre-miRNAs as well as VA RNAs use exportin 5 (Exp5) to enter the cytosol [63]. Specifically, VAI binding to Exp5 is very efficient and leads to the saturation of Exp5. This saturation leads to the inhibition of the export of cellular pre-miRNAs into the cytoplasm. The role of VAIL in Exp5 saturation remains unclear.

In line with that VA RNAs also interfere with later steps of the RNA interference pathway. It was also shown that VA RNAs could bind to Dicer and block its activity both *in vivo* and *in vitro*. VA RNAs act as RNA decoys and prevent Dicer from binding to cellular pre-miRNA substrates. Additionally, this was also described that VA RNAs saturate the RNA-induced silencing complex (RISC) by binding to Argonaute proteins (Ago) when present at very high amounts [64]. This interaction results in a reduced RISC activity *in vitro* [57]. It is worth mentioning that Ago saturation is not due to a direct effect of VA RNAs rather than Ago becomes saturated due to the high level of viral-derived micro-RNAs (miRNAs) that are generated by Dicer processing of VA RNAs. These miRNA-like processed forms are termed mivaRNAs.

Besides the interaction with components of the cellular RNA interference machinery another interesting aspect is the question whether mivaRNAs can directly target cellular transcripts. Recent data could show that mivaRNAs indeed inhibit the expression of genes containing a complementary sequence [57, 65, 66]. The fact that mivaRNAIL can associate with polyribosomes suggests a possible role of gene expression on the translational level [67]. Genes influenced by mivaRNAs play

important roles in cell signaling, cell growth or apoptosis, DNA transcription or repair and finally in RNA metabolism [68].

1.3.2. The late phase of the infection cycle

Compared to the early phase of the infection cycle, the late phase then is much more rapid, i.e., lasts for 4-6 h [11]. The synthesis of viral DNA results in the activation of the intermediate phase genes coding for pIX (stabilizes the viral capsid [69]) and IVa2 (attached to the virus DNA and involved in packaging of the DNA into the viral capsid [70-73]), the E2 late promoter and the major late promoter (MLP). MLP activity results in the transcription of nearly all late genes encoded on the regions L1-L5 (Fig. 4, 5). Comparable to early mRNAs, late mRNAs also originate from long primary transcripts that are processed into the mature transcripts by alternative polyadenylation and alternative splicing [74] (Fig. 5).

Late genes encode core proteins (pV, pVII, protease and μ), structural proteins (hexon, penton base, fiber, pIIIa, pVI and pVIII), and proteins required for encapsulation and maturation of infectious viral particles (pVI) (reviewed in [75]).

A cellular factor, whose titration is depend on virus replication, is involved in the regulation of MLP-specific transcription [76]. In order to prevent unscheduled MLP activation this factor blocks the synthesis of the MLP inducer protein IVa2 via binding to the IVa2 gene promoter of incoming adenoviral genomes [77].

The assembly of the virion takes place in the nucleus of the host cell and about 3 days after infection, virus progeny is released by cell lysis which is triggered by the adenoviral death protein [78]. The virus protease plays a crucial role during the maturation of the viral capsid by cleaving several capsid precursor proteins into their mature and functionally active forms [79].

1.4. Adenovirus and the immune system – persistence and latency

Adenoviral infections induce predominately cellular immunity mediated by natural killer cells (NKs) and monocytes. This leads to an interleukin-1 (IL-1), IL-6 and tumor necrosis factor (TNF) cytokine release as has been shown in the murine pneumonia model [80]. Higher levels of these cytokines in human sera are directly linked to more severe infections [81]. Additionally, antigen-specific T-cells, mainly CD4⁺ but, to a lower extend, also CD8⁺ are generated upon adenoviral infections [82]. High cross-reactivity in their T-cell response can be observed among different serotypes [83].

Adenoviruses produce proteins whose only functions are linked to the maintenance of the virus in a persistent state. Especially, DNA of Ad1, Ad2 and Ad5 of species C can be detected in tonsils and adenoids of children. Here they persist for years through low-grade replication mainly in T-lymphocytes [85].

Either due to immune elimination or to depletion of latent stores, detectable adenoviral DNA decreases with higher age. Interestingly, in peripheral blood of healthy individuals adenovirus DNA is absent or present at very low levels (1.7%) [86].)

It is known that E3-19K plays a dominant role in maintaining adenovirus in a latent status and to protect it from immune responses. This particular protein prevents the transport of class I major histocompatibility complex (MHC I) molecules loaded with

antigens to the cell surface. Thus, E3-19K blocks adenoviral antigen presentation and activation of specific cytotoxic T-cells [87]. On the one hand, E3-19K fulfills its functions through binding to the MHC class I heavy chain that leads to the retention of MHC class I in the endoplasmic reticulum [88, 89]. On the other hand, E3-19K prevents tapasin-mediated peptide loading onto MHC class I molecules (Fig. 7) [90, 91].

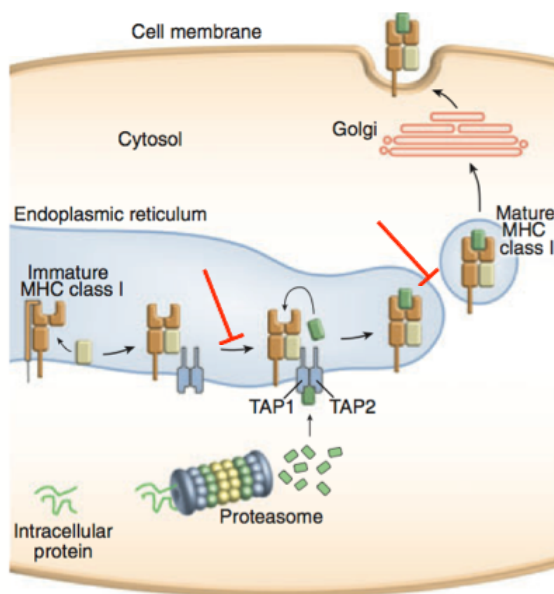


Figure 6 | Interference of E3-19K with Antigen Presentation ([92]

Red lines indicate the steps where Adv protein E3-19K interferes with MHC class I antigen processing and presentation. First, the binding of MHC class I – antigen complex to the TAP complex is inhibited. Second, the MHC class I – antigen complex retracts in the endoplasmic reticulum, no antigen presentation on the cell surface can occur.

1.5. Adenoviral infections and clinical manifestations

1.5.1. Immunocompetent individuals

Infections with human adenovirus are common, have a worldwide distribution, and occur throughout the year. These infections are frequent during childhood and remain, in most cases, unnoticed and without symptoms in immunocompetent individuals [93]. However, in young children, adenoviruses can cause viral gastroenteritis, and are responsible for ~5% of the acute respiratory illnesses and 10% of febrile illnesses [94]. Remarkably, military recruits are also particularly vulnerable to acute respiratory

disease (ARD) due to close living conditions and physical and hygienic challenges. Here, ARD, mainly caused by Ad4 and Ad7, is resulting in high morbidity and occasional mortality (reviewed in [95]).

Adenoviruses can also be the cause of ocular infections. While follicular conjunctivitis or pharyngeal conjunctival fever are relatively mild, epidemic keratoconjunctivitis (EKC) is a highly contagious and more serious disease. These infections, mainly caused by Ad8, Ad19 or Ad37, can harm the cornea and conjunctiva, with potential long-term consequences on visual acuity [96].

1.5.2. Immunocompromised patients

Table 3 | Adenovirus Infections in the Immunocompromised host [93]

Study authors (yr of publication)	Population (period)	% Adenovirus infection (no. of adenovirus-infected patients/total no. of patients)	% Adenovirus disease (no. of symptomatic patients/no. of infected patients)	% Adenovirus-related mortality (no. of deaths/no. of infected patients)	Types of specimens positive for adenovirus (methods)	Species or serotype(s)
Shields et al. (1985) [97]	BMT (1976-1982)	5 (51/1,051)	20 (10/51)	10 (5/51)	U, S, TH, BS (culture, histopathology)	1, 2, 5, 7, 11, 34, 35
Wasserman et al. (1988) [98]	BMT (allo and auto), C, TBI (1979-1986)	18 (17/96)	ND	6 (1/17)	U, S, TH, B (culture)	1, 2, 3, 4, 11, 12, 15
Ljungman et al. (1989) [99]	BMT (1987)	6 (5/78)	ND	20 (1/5)	TH (culture, IIF, histopathology)	34
Flomenberg et al. (1994) [100]	BMT (allo and auto), TCD, TBI (1987-1990)	21 (42/201); A, 14 (16/118); C, 31 (26/83)	31 (13/42)	17 (7/42)	S, TH, U, B (culture)	1, 2, 4, 5, 29, 35
Blanke et al. (1995) [101]	BMT (allo), TCD (1990-1992)	14 (10/74)	10 (1/10)	50 (5/10)	U, L, S, TH (culture)	1, 11, 12
Hale et al. (1999) [102]	BMT (auto and allo), C, TBI (1990-1994)	6 (13/206)	46 (6/13)	50 (7/13)	U, TH, S, L, B, LI (culture)	5, 7, 11
Howard et al. (1999) [103]	HSCT (allo and auto), TCD, TBI (1986-1997)	12 (64/532); A, 9 (35/405); C, 23 (29/127)	64 (41/64)	17 (11/64)	U, S, L, LI, BM, B, BR, NP (culture, histopathology)	ND
Venard et al. (2000) [104]	BMT (allo and auto), C, TCD (1995-1996)	20 (13/65)	61 (8/13)	70 (9/13)	U, S, TH, BAL, CO, SA (culture)	1, 2, 3
Hoffman et al. (2001) [105]	HSCT C(allo) (1998)	47 (17/36)	82 (14/17)	12 (2/17)	U, TH, CSF, B ND (culture)	
Bordignon et al. (2001) [106]	HSCT C(allo), TCD (1985-1999)	12 (35/303)	60 (21/35)	68 (15/22)	U, S, TH, CO (culture, EIA)	1, 2, 3, 5, 8, 31
Echavarria et al. (2001) [107]	HSCT (allo) (1985-1999)	12 (38/328)	45 (17/38)	18 (7/38)	U, TH, S (culture), serum (PCR)	1, 2, 5, 12/31
La Rosa et al. (2001) [108]	BMT, HSCT A(allo and auto) (1990-1998)	3 (85/2889)	89 (76/85)	23 (20/85)	U, NP, S, BAL, BM, CO (culture)	ND
Leruez-Ville et al. (2004) [109]	BMT, other immunocompromised A, C (2002)	18 (8/44)	ND	25 (2/8)	B, BM, NP, BAL, U, BS (IIF, culture, RT-PCR)	2, 5, 6, 12
Kampmann et al. (2005) [110]	HSCT C(allo), TCD (1999-2002)	41 (63/155)	ND	19 (5/26)	B, S (culture, PCR, EM, histopathology)	A, B, C
van Tol et al. (2005) [111]	HSCT C(allo) (1985-1999)	11 (37/328)	46 (17/37)	19 (7/37)	S, U, B, TH (culture, PCR)	ND
Yusuf et al. (2006) [112]	HSCT C (2001-2004)	32 (57/177)	75 (43/57)	2 (1/57)	PBMC (RT-PCR)	ND
Kroes et al. (2007) [113]	HSCT C (2001-2004)	40 (33/83)	29 (8/28)	30 (10/33)	S, U, TH (culture)	1, 2, 3, 5, 6, 7, 11, 21, 31 (36% multiple infection)
Kalpoe et al. (2007) [114]	HSCT A and C (2001-2005)	A, 5 (5/107); C, 14 (8/58)	A, 20 (1/5); C, 50 (4/8)	A, 20 (1/5); C, 38 (3/8)	B (RT-PCR)	ND

A, adults; allo, allogeneic; auto, autologous; B, blood; BAL, bronchoalveolar lavage; BM, bone marrow; BMT, bone marrow transplantation; BR, brain; BS, biopsy specimens; C, children; CO, conjunctiva; CSF, cerebrospinal fluid; EM, electron microscopy; HSCT, hematopoietic stem cell transplantation; IIF, indirect immunofluorescence; L, lung; LI, liver; NP, nasopharynx; ND, not determined/done; PBMC, peripheral blood mononuclear cells; RT-PCR, real-time PCR; S, stool; SA, saliva; TBI, total body irradiation; TCD, T-cell depletion; TH, throat; U, urine.

1.5.2.1. Adenoviruses in congenital immunodeficiency

In general, congenital immunodeficiency disorders are characterized by abnormalities in the development and maturation of cells of the immune system. Thus, patients suffering from congenital immunodeficiency disorders show an increased susceptibility to any kind of infection. A wide variety of different congenital immunodeficiencies was described so far [115]. In several illnesses linked to this kind of immune system disorder adenoviral infections causing severe outcomes were described, sometimes in the combination with secondary infections. The clinical manifestations are reaching from respiratory infections, infections of the lungs, liver or kidneys, pneumonia, bronchiolitis, hepatitis, or gastroenteritis, disseminated disease, and sometimes death. These kinds of diseases are related to the human adenovirus serotypes 1, 2, 3, 5, 7, 11, 31, 34, 35, and 41. Due to the lack of data the fatality rate of adenoviral infections in such patients could be suspected to be as high as 55%. [116-121].

1.5.2.2. Adenovirus in solid organ transplant recipients

Adenoviral infections can cause severe problems in solid organ transplant recipients mainly related to the transplanted organ. Diseases described in transplanted patients include pneumonia, hepatitis, nephritis, hemorrhagic cystitis, enteritis and disseminated disease [122]. For pediatric liver transplant recipients an incidence of infection ranges from 4 to 10%, with mortality rates up to 53% [82]. Adenoviral infections in such patients are usually caused by serotypes 1, 2 and 5 of adenovirus species C.

Besides, serotypes 7, 11, 34, and 35 from species B are the main cause of acute hemorrhagic cystitis and pneumonia in renal transplant recipients. The fatality rate for those infections is about 17%

An accurate monitoring of adenoviral infections and spread is essential in solid organ transplant recipients to overcome fatal outcomes like the above-mentioned diseases or graft loss [123].

1.5.2.3. Adenovirus in AIDS patients

Acquired immunodeficiency syndrome (AIDS) patients are also highly vulnerable to adenovirus infections with fatal outcomes. Illnesses described in adenovirus positive AIDS patients include pneumonia, hepatitis, meningoencephalitis, nephritis, and gastrointestinal and disseminated disease [124, 125]. Mostly, adenoviruses from species D could be detected in AIDS patients [126].

1.5.2.4. Adenovirus in acquired immunodeficiency – BMT and HSCT recipients

The frequency of adenoviral infections in either bone marrow transplant (BMT) or hematopoietic stem cell transplant (HSCT) recipients has increased in the last years. That might be due to enhanced awareness of these infections, improved methods for detection and aggressiveness of conditioning regimens [100, 127]. Depending on patient age, conditioning regimens, type of diagnostic method and its sensitivity, clinical sample analyzed, lack of strict criteria for defining infection and disease, the rate of adenovirus infection varies from 5 to 47% (Tab. 3).

Risk factors for adenoviral infections include (i) younger age (pediatric recipients are 2 to 3.5 times more likely than adults to become infected) [100, 103, 128], (ii) receiving a transplant from a matched unrelated or mismatched donor [102, 103, 108, 128-133], (iii) total body irradiation [102], (iv) presence of adenovirus antibody in the donor [133], (v) T-cell depletion, (vi) use of the anti-viral drug Campath *in vivo* (50-100 mg) or anti-thymocyte globulin *in vivo* and *ex vivo* [129, 130, 132], and low T-cell count after transplantation [111]. It is worth mentioning that in the tonsils of children of less than 9 years old larger amounts of adenovirus DNA from species C were detected compared to older children [85]. It is likely that younger children are more susceptible to adenoviral infection because of experiencing primary infections or reactivation [93].

Risk factors for adenoviral disease can be (i) immunosuppressive therapy [108, 129, 130], (ii) moderate-to-severe graft-versus-host disease [100, 108, 133], (iii) the number of sites where adenovirus can be detected [100, 103], (iv) detection of adenovirus in the peripheral blood and rising viral load in peripheral blood [129, 130, 132], (v) detection of rising viral load in stool [134], (vi) and lymphocytopenia. In the latest case, severe lymphocytopenia (<300 cells/mm³) is an indicator for the progression to disseminated disease, which often results in fatal outcomes [130].

The clinical manifestations in such patients range from upper or lower respiratory disease (interstitial pneumonitis), hepatitis, hemorrhagic cystitis [135] or nephritis to gastrointestinal disease including hemorrhagic colitis [98, 136]. The most common manifestation described is diarrhea [137]. In the worst case, the patients develop disseminated disease with mortality rates up to 80% [103].

Usually, adenovirus in BMT or HSCT patients can be detected within 100 days post-transplant with means ranging from 58 – 333 days. Some patients also show prolonged viral excretion [82].

1.5.3. Prevention and treatment options of adenoviral infections

1.5.3.1. Pharmacological treatment

So far, there is no specific treatment for adenoviral infections. It has been reported that some antiviral drugs could also clear adenoviral infections to some extent. Such antivirals include ganciclovir (GCV), vidarabine, ribavirin and cidofovir (Fig. 8) [105, 106, 138]. Both, ribavirin as well as cidofovir, were used in clinical studies treating immunocompromised patients with limited success [103, 138]. Importantly, the efficiency of an antiviral drug is closely related to early treatment and serotypes involved. For example, one study could show that only serotypes of species C were sensitive to ribavirin treatment *in vitro* [139].

A major issue about the existing studies is the lack of data concerning immune system recovery. Since it is thought that clearance of an adenoviral infection is very often more likely due to an efficient immune system recovery than to an antiviral effect of the administered drugs [111, 140]. Indeed, Heemskerk *et al.* could show that in patients with adenoviral infections the decrease of virus titer went hand in hand with the onset of humoral immune response, as titers of serotype-specific antibodies increased 8- to 16-fold [140].

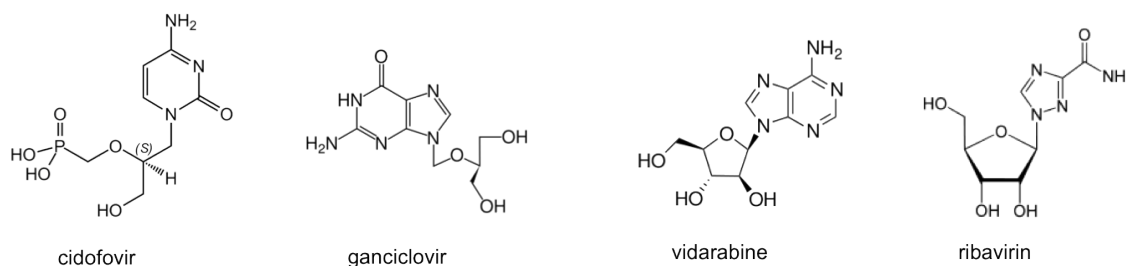


Figure 7 | Antiviral drugs used for Adenovirus Treatment [141]

Chemical structures of the antiviral agents cidofovir, ganciclovir, vidarabine and ribavirin.

1.5.3.2. Gene-directed enzyme prodrug therapy

Besides adenoviruses, cytomegaloviruses (CMV) (members of the herpes virus group) represent a major threat to immunocompromised patients. In contrast to the unsatisfactory efficiency of antiviral drugs like GCV in the context of adenovirus treatment, GCV is much more effective in controlling CMV infections. That is due to the fact, that prodrugs like GCV, aciclovir, brivudin and derivatives [142-145] are much more efficiently phosphorylated in to their active forms by herpes virus-encoded thymidine kinases (TKs) compared to cellular TKs [146]. This kind of mechanism can be used for an antiviral approach known as gene-directed enzyme prodrug therapy.

This approach is mainly used for the targeted killing of cancer cells by introducing a gene like the herpes simplex virus thymidine kinase (HSV-TK) into a cancer cell to make it susceptible for later treatment with a prodrug such as GCV. In the case of the HSV-TK – GCV system, HSV-TK leads to the metabolization of GCV into a monophosphorylated form that is further phosphorylated by cellular kinases into the toxic triphosphate form (GCV-ppp) (reviewed in [147]). Upon incorporation of GCV-ppp instead of natural deoxynucleoside triphosphates, like desoxyguanosintriphosphat (dGTP) (in the case of GCV) into the DNA of a replicating cell (or virus), DNA replication is blocked by premature strand termination, subsequently leading to cell death [148, 149].

Since it was already shown that adenovirus DNA replication is in principle a potential target for gene-directed enzyme prodrug therapy using the HSV-TK - GCV system [150, 151].

1.5.3.3. Preemptive adenovirus treatment

A very important aspect of adenoviral therapy represents the preemptive branch of treatment options. The permanent monitoring of adenovirus load and spread in risk patients could be a possibility to avoid fatal outcomes of such infections. One study could show that disseminated adenovirus disease was preceded by a period of asymptomatic viremia [132]. There was indeed already success in resolving adenoviral infections in asymptomatic patients by preemptive treatment with cidofovir [152] and an algorithm for adenovirus surveillance and preemptive therapy has been developed: on the first detection of an adenovirus isolate in a surveillance sample from urine, stool, or the throat, immunosuppression should be reduced. Preemptive antiviral therapy should be initiated if adenovirus-positive samples are obtained by PCR for blood, the patient has severe lymphocytopenia, or immunosuppression cannot be reduced or withdrawn [153].

1.5.3.4. T-cell therapy for adenovirus

Due to the fact that adenoviral complications in transplant recipients are mainly caused by inefficient recovery of virus-specific cellular immune responses, reconstitution of the host with *in vitro* generated and expanded virus-specific cytotoxic T-lymphocytes (CTLs) is a promising approach to prevent and treat adenoviral infections and associated disease. However, to successfully design such treatment methods a clear understanding of the effector processes that control viral infections and the mechanisms viruses use to evade such responses is required [13]. So far, several

studies could show effective prevention and treatment of diseases associated with CMV and Epstein-Barr virus (EBV) [154-159]. The efficiency of such a treatment is mainly based on the understanding of adenovirus T-cell immunity. Unfortunately little is known about immunodominant T-cell antigens [84, 160-163]. One CD4⁺ T-cell epitope has been mapped [84] and recent data indicate that the immediate early proteins E1A [164-166] and E2A [167, 168] are strong targets for specific T-cells. Since most of the data are based on experiments carried out in mice one has to be careful about the results. As adenovirus is a species-specific virus that is not or only to a low extent replicating or causing disease in mice, more investigation has to be done in appropriate model organisms.

Although it will be a long way until specific T-cell therapy will be the treatment of choice to overcome adenoviral infections in immunocompromised patients, there is a report providing proof of principle for this treatment approach: a 19-year-old man underwent a T-cell depleted 6/6 human leukocyte antigen (HLA)-matched related donor HSCT for T-cell lymphoblastic lymphoma. On day +42 post-transplantation, he developed hemorrhagic cystitis secondary to adenovirus infection. Since treatment with foscarnet was ineffective the patient was given a donor lymphocyte infusion (DLI) (10⁶ CD3 cells/kg) on day +61. After 5 weeks, the patient's haematuria cleared, and urine adenovirus cultures were negative by day +94 [169].

1.5.4. Adenovirus infection and animal models

In order to fully understand and treat adenoviral infections it is essential to develop appropriate animal models. So far, a number of rabbit and rodent models has been established. Mice and cotton rats are susceptible for intranasal inoculation with human adenovirus species B and C, resulting in pneumonia comparable to human disease [80, 170, 171]. Since replication of human adenovirus in these animal models is restricted to the synthesis of early proteins, better animal models have to be established for future investigations about anti-adenoviral therapeutics. For example, the efficiency of the 2',3'-dideoxynucleoside analogue ddC was confirmed in one AdV mouse pneumonia model [172].

For ocular adenoviral infections models were established in cotton rats and New Zealand rabbits [173-175].

Another promising animal model for studying adenoviral infections comprises the Syrian hamster. As described for the first time in 1968, adenovirus serotype 12 is able to replicate in the Nil-2 Syrian hamster cell line, and this model has been characterized in more detail during the last years [176].

1.6. Adenovirus-based vectors

Only a few years after the discovery of human adenoviruses the first treatment of acute respiratory disease caused by adenoviruses in military personal in the United States took place. For this purpose, formalin-inactivated HAdV-4 and HAdV-7 were used as vaccines to block ARD [2, 3]. Unfortunately, vaccination to prevent ARD was discontinued due to the discovery of simian virus 40 (SV40) in the vaccine stocks that were produced in monkey kidney cells. All attempts to eliminate these contaminations failed because the insidious SV40 and/or respective antigens originated from hybrid viruses carrying SV40 and hAdV DNA [177-179]. Interestingly, only the selection for these hybrid viruses allowed the production of the vaccine material in the first place since the SV40 large T antigen (T-Ag) releases the translational block of late HAdV transcripts in simian cells [180]. Besides, two types of HAdV-SV40 hybrid viral genomes could be identified: (i) the non-defective type (helper-independent) that contained small portions of SV40 DNA in regions that did not disrupt essential HAdV genes and (ii) the defective type (helper-dependent) that had insertions of large SV40 DNA segments or even several tandem copies of the SV40 DNA substituting genes needed for HAdV replication [181]. Further analysis of these different kinds of hybrid viruses and their functions formed the basis for subsequent development of HAdV-based vectors because it showed that (i) small foreign DNA segments could be inserted into HAdV genomes and be packaged into HAdV capsids without disrupting viral viability, setting the stage for the generation of helper-independent HAdV vectors, (ii) heterologous DNA could substitute large regions of the HAdV genome and be propagated in the presence of a *trans*-complementing helping provided that key HAdV *cis*-acting sequences remained intact establishing the conceptual framework for helper-dependent or high-capacity HAdV vectors and (iii) HAdVs could be used to ectopically express genes, such as T-Ag and to study their products [182, 183].

Nowadays, a broad range of different HAdV-based vectors is used for diverse properties. The most prominent adenoviral vectors are either deleted in early region 1, or in multiple early regions (reviewed in [11, 184]).

1.6.1. Applications of adenovirus vectors

The use of adenovirus vectors to deliver and express genes at high yields, especially *in vitro*, is very common and works very efficiently. Nevertheless, the use of such vectors *in vivo* is more challenging, most of all, because of the elimination of the vectors through the immune system. On the other hand, induction of a vigorous immune response can even be beneficial for cancer therapy approaches. In line with a better

understanding of the molecular and immunological factors that operate *in vivo*, great improvements have been made over the last few years in the construction of more effective vectors (reviewed in [11]).

1.6.1.1. Adenovirus vectors and cancer treatment

A lot of work is done on adenoviral vector development to use them for cancer treatment. The therapies, so far, can be divided into three groups. (i) tumor suppressors, (ii) oncolytic and sensitizing drug therapy and (iii) vaccines:

- (i) **Tumor suppressors.** Above all, mutations in the tumor suppressor gene p53 that lead to loss of function are a predominant cause of cancer development. Thus, many adenovirus vectors have been constructed for the expression of wild-type p53 to induce apoptosis in tumor cells [185]. So far, these vectors showed high efficiency in animal models with anaplastic thyroid cancer [186], human malignant gliomas [187, 188] and breast cancer [189]. A lot of work is done to use this approach for the treatment of lung, head, neck and liver cancers. One major issue of this approach is the directed and efficient targeting of the vectors used.
- (ii) **Oncolytic and sensitizing drug therapy.** In 1996, Bischoff *et al.* could show for the first time that an adenovirus that had a mutation in E1B-55k replicated selectively in p53-defective tumor cells [190]. Thus, this virus could function as an oncolytic virus. This finding resulted in the commercial development of the mutant Onyx-015 that is used in the treatment of some tumors [191], also in the combination with standard chemotherapy [192].

Another treatment method for the selective elimination of tumor cells is to deliver a prodrug enzyme via a vector into target cells. That is combined with the administration of a non-toxic drug that is converted into a cytotoxic agent by the prodrug enzyme [193]. A very prominent example for such an approach is the use of HSV-TK to convert the prodrug ganciclovir into its phosphorylated and activated form leading to termination of DNA synthesis [194].

The success of these systems is mostly dependent on the efficiency in specific targeting. This can be achieved by the use of tissue-specific promoters incorporated into the vectors [195]. Intensive investigation is done in that field to improve the effect of such vector-based treatment

methods. It is worth mentioning here that in contrast to most other approaches that employ adenovirus vectors, the retention of E1A genes is of great advantage, because of their ability to promote p53 transcription and to enhance the sensitivity of tumor cells to cytotoxic agents [196-198] and to radiation therapies [199].

- (iii) **Vaccines.** A quite promising approach in tumor therapy is to boost the host anti-tumor cell immunity that can be achieved by using vectors expressing immunomodulatory genes and/or tumor-specific antigens. Although a variety of different approaches result in tumor regression in animal models, problems appeared such as high toxicity when overexpressing cytokines such as IL-2 occurred. This was due to the immune response to the vectors and prevented longer-term anti-tumor responses [200].

An additional strategy is to use the ability of dendritic cells to express antigens: dendritic cells can be isolated from a patient and infected with adenoviral vectors expressing specific tumor antigens. These modulated dendritic cells can be readministered to the patient in combination with standard therapies [193].

Recent applications of adenoviral vectors comprise a variety of different diseases and autoimmune disorders as shown in Tab. 4.

Table 4 Recent Adenovirus Vector Applications [11]	
Site/disease	Transgene(s)
Tumors	
Prostate	p16 PKC Cytolytic virus Fas ligand
Colon	GM-CSF IL-2 CD suicide gene APC Lymphotoxin suicide
Cervix	p21 Papillomavirus p21
Ovary	Stomatostatin receptor Cytolytic virus IL-6
Endometrium	p53 p21
Gliomas	Caspase-3 Fas ligand
Carcinoembryonic antigen-producing cancer	HSV-tk
Other conditions	
Autoimmune diabetes	Adenovirus E3
Glycogen storage disease II	Glucosidase
CNS conditions	(via astrocytes)
Factor VIII deficiency	Factor VIII
OTC deficiency	OTC
Liver graft	Bcl-2
<i>Leishmania</i> infection	IL-12
Tay-Sachs disease	Hexosaminidase
Motor neurone disease	Neurotrophic factor
APC, Adenomatous polyposis coli; GM-CSF, granulocyte-macrophage colony-stimulating factor; PKC, protein kinase C.	

Although all these approaches seem to be very promising in treating different disease a lot of severe problems became obvious. First of all, the site-directed administration of adenoviral vectors, high enough expression levels, and the persistence of the transgene are quite challenging. Additionally, abolishment of the vectors by the host's immune system represents a major issue [201]. In contrast, the immune system is not only problematic for the persistence of the vectors, but it was also reported that adenoviral vectors could activate innate immunity with fatal outcomes [202, 203]. In 1999, a patient carrying a defect in the ornithine transcarbamylase (OTC) pathway died after adenovirus vector therapy due to developing a massive activation of innate immunity followed by systematic inflammation [204]. A possibility to overcome or at least reduce immunogenicity is the use of gutless vectors [205].

(Another very important considerations when using adenoviral vectors are the costs of scaling up, purification and production of these vectors [11].)

It becomes clear that investigations have to continue for a better understanding of the complexity of the interaction between adenoviral vectors and the host response. Nevertheless, adenoviral vectors comprise a very promising tool to improve alternative treatment methods for a variety of different diseases (reviewed in [11]).

2. RNA INTERFERENCE

2.1. The mechanism of RNA interference in mammalian cells

In 1998, Mello and Fire discovered a completely new way of gene regulation in *Caenorhabditis elegans*, namely the mechanism of RNA interference (RNAi) [206]. Soon after that, it could be shown that a similar regulatory pathway that is driven by either exogenous small interfering RNAs (siRNAs) [207] or endogenous microRNAs exists in mammalian cells. In contrast to siRNAs, miRNAs derive from imperfectly paired hairpin RNA structures encoded in the genome [208]. The RNAi pathway can result in direct sequence-specific cleavage of perfectly complementary mRNAs or in translational repression and transcript degradation for imperfectly complementary targets. Additionally, RNAi pathways can lead to direct transcriptional gene silencing (TGS) in the nucleus [209, 210].

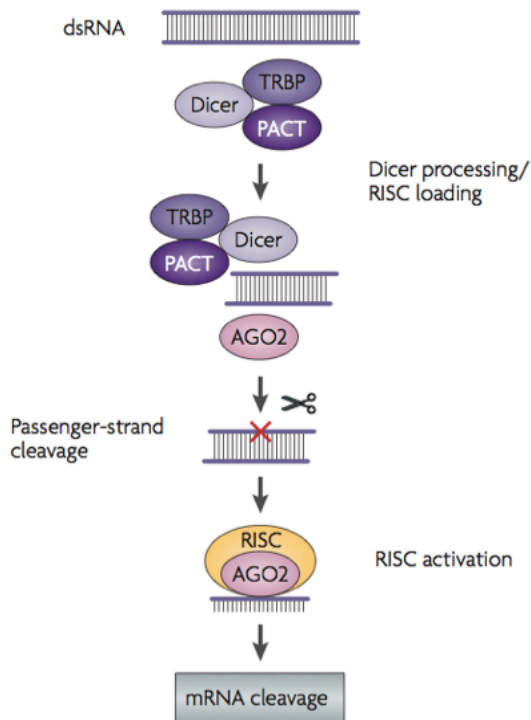
2.1.1. The small interfering RNA pathway

2.1.1.1. Exogenous siRNAs

Exogenous siRNAs are double stranded RNAs (guide/antisense strand + passenger/sense strand) of ~21-25 nucleotides (nt) in length [207] and show perfect or near-perfect sequence complementarity of the guide strand to their target mRNA. siRNAs mediate transcript cleavage and degradation in a process known as post-transcriptional gene silencing (PTGS) [211]. This pathway is known to exist in nematodes, insects and plants as innate immune response against viruses. Here, viral double-stranded RNA (dsRNA) molecules are processed by the RNase III enzyme Dicer into siRNAs that drive an RNAi response. Although the mechanism of Dicer-dependent siRNA processing is similar in mammalian cells, it remains unclear, whether mammalian cells use RNAi as defense mechanisms against antigens as well [212].

After the siRNA is processed by Dicer it contains 2 nt overhangs at the 3' ends and phosphate groups at the 5' ends [208, 214]. Important to mention here, Dicer can use linear dsRNA as well as hairpin-RNA substrates [215].

Following, the siRNA is incorporated into the RNA-induced silencing complex (RISC) [216]. Within RISC the cleavage of the target mRNA is mediated by the endonuclease Argonaute 2 (AGO2) [217]. It is thought that AGO2 is also responsible for RISC activation via cleaving the passenger or sense strand of the double-stranded siRNA [218, 219], generating the single-stranded guide or antisense strand that guides RISC



to complementary sequences in target mRNAs. Inside RISC, the guide strand is bound within the catalytic, RNase H-like PIWI domain of AGO2 at the 5' end [220] and a PIWI-Argonaute-Zwille (PAZ) domain that recognizes the siRNA 3' end [221]. The subsequent cleavage of the target mRNA occurs between bases 10 and 11 relative to the 5' end of the siRNA guide strand [222]. Further on, the cleaved mRNA is degraded by cellular exonucleases [223]. Besides, guide-strand activated RISC can undergo multiple rounds of mRNA cleavage mediating a robust PTGS response against the target gene (Fig. 11) [224].

Figure 8 | Mechanism of siRNA-mediated RNA interference in mammalian cells [213]

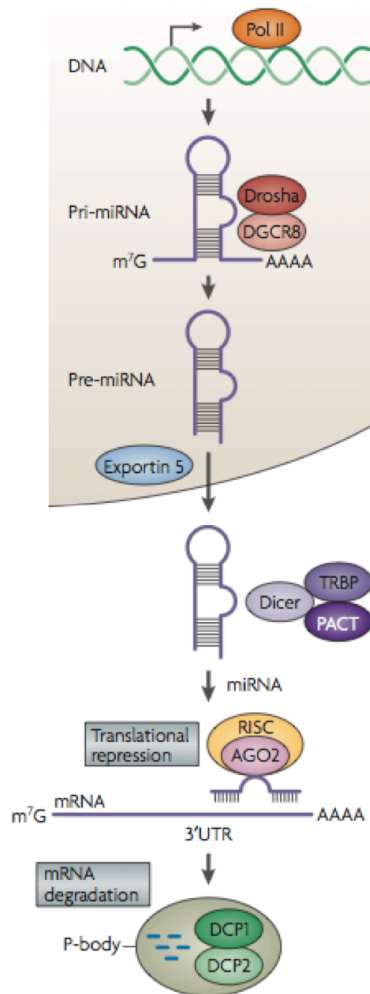
Exogenous siRNAs in the cytoplasm of a mammalian cell (here depicted as double-stranded (ds) RNA) are processed by a complex consisting of Dicer, TAR RNA-binding protein (TRBP) and protein activator of protein kinase PKR (PAKT) and further on loaded into Argonaute 2 (AGO2) which is a component of the RNA-induced silencing complex (RISC). Upon passenger strand cleavage the guide strand is directed to its target mRNA subsequently leading to the cleavage of the same.

2.1.1.2. Endogenous siRNAs

Besides the exogenous siRNAs described above, endogenous siRNAs have been discovered in various different organisms and can be further categorized in at least four classes:

- (i) **trans-acting siRNAs (tasiRNAs)** – small ~21 nucleotides long RNAs in plants; in *Arabidopsis thaliana* (*A. thaliana*), this small RNAs use components of the miRNA machinery to cleave their targets in *trans* [225, 226]
- (ii) **repeat-associated siRNAs (rasiRNAs)** – match sense and antisense sequence;
involved in transcriptional gene silencing in *Schizosaccharomyces pombe* and *A. thaliana* [227-230]
- (iii) **small-scan (scn)RNAs** – ~28 nucleotides long; found in *Tetrahymena thermophila* and might be involved in scanning DNA sequences in order to induce genome rearrangements [231]
- (iv) **Piwi-interacting (pi)RNAs** – ~26-31 nucleotides long and might be important for mammalian gametogenesis [232-236]; bind to MILI and MIWI proteins, a subgroup of Argonaute proteins that belong to the Piwi family; are essential for spermatogenesis in mice [237]

2.1.2. The microRNA pathway



Unlike siRNAs, miRNAs are endogenous RNAs that are involved in the regulation of gene expression regulation during cell development and differentiation [238]. miRNA genes are located in intergenic regions and introns, and in defined transcription units in both the sense and antisense orientations (26-28). miRNAs are usually transcribed by RNA polymerase II (Pol II) in the nucleus as long primary transcripts (pri-miRNAs) wherein the miRNA-encoding portions form imperfect stem-loop hairpin structures [239]. However, RNA polymerase III can also transcribe miRNA genes [240]. First, a nuclear protein complex called 'microprocessor' cleaves the hairpin loop.

This protein complex consists of the RNase III enzyme Drosha, the double-stranded RNA-binding protein of diGeorge syndrome critical region 8 gene (DGCR8), and a number of other proteins. Processing of the pri-miRNAs results in ~70 nucleotides long stem-loop structures known as precursor miRNAs (pre-miRNAs) [241].

Figure 9 | Mechanism of miRNA-mediated post-translational gene silencing [213]

Endogenously encoded miRNAs are mainly transcribed by RNA polymerase II (Pol II). A complex consisting of Drosha and diGeorge syndrome critical region 8 gene (DGCR8) processes the long primary-(pri)-miRNA resulting in the precursor-(pre)-miRNA. Further on, the pre-miRNA is translocated from the nucleus into the cytoplasm in an exportin 5-dependent manner. In the cytoplasm the pre-miRNA is processed into its mature form, the mature miRNA, by a Dicer – TAR RNA-binding protein (TRBP) – protein activator of protein kinase PKR (PACT) complex. Subsequently, the miRNA is incorporated into the RNA-induced silencing complex (RISC). Dependent on the grade of complementarity of the miRNA to its target mRNA, miRNA binding can lead to translational repression (no perfect complementarity) or to mRNA degradation in the P-bodies (perfect complementarity).

In the next step, the dsRNA-binding protein complex exportin 5 – RanGTP transports the pre-miRNA into the cytoplasm [242, 243] where it is processed by a protein complex containing Dicer and its dsRNA-binding protein partners, HIV-1 TAR RNA-binding protein (TRBP) [244] and protein activator of protein kinase PKR (PACT) [245], into the 22 nucleotides long mature miRNA. Eventually, it is loaded into RISC [246]. During the loading into RISC no cleavage of the passenger strand occurs. Instead, the passenger strand gets unwound and discarded. Sequence differences between the mature miRNA and its passenger strand prevent AGO2 mediated cleavage of the passenger strand [246].

Mature miRNAs have only partial sequence complementarity to the 3' untranslated regions (3' UTRs) of their target mRNAs [247, 248]. In contrast, the seed region of the miRNA (the first 2-7 or 2-8 nucleotides from the 5' end of the mature miRNA) must be perfectly complementary to the target sequence [249]. miRNAs induce translational repression, which often leads to mRNA degradation that takes place in cytoplasmic compartments known as processing bodies (P-bodies) [250]. In contrast, a miRNA that shares perfect complementarity to its target mRNA mediates RISC-dependent cleavage of the same (Fig. 12) [251]. Studying the interactions of miRNAs with their targets revealed different mechanisms of miRNA actions: (i) miRNAs could be found in the polyribosome fraction indicating a role in decreasing productive extensions of the polypeptide chain after initiation of translation [252, 253], (ii) miRNAs interfere with the recognition of the 5' cap of the mRNA to prevent translation initiation, (iii) miRNAs also destabilize mRNA via deadenylation of the poly-A tail and (iv) recruit the ribosome-inhibitory protein eIF6 [254-257]. A recent study could also show that under some distinct conditions, such as cell cycle arrest, miRNAs can also upregulate the translation of a target RNA [258].

2.1.3. microRNAs and disease

An immense number of studies revealed the very important role of miRNAs occurring in various kinds of human tissues and cells. Their presence is required for stem cell functions, germline development, in skeletal and cardiac muscle development and function, during hematopoiesis and immunity, and for a fully functional nervous system (reviewed in [259]).

Above all, modified miRNA functions seem to play important roles in cancer development. To mention one example, it was reported that miRNAs can indeed act as oncogenes by contributing to the transformed phenotype when expressed at elevated levels. miR-21 is one such miRNA that is overexpressed in glioblastoma multiforme,

cervical cancer, breast cancer, and many other solid tumors [260-262]. Increased expression of miR-21 contributes to decreased apoptosis in malignant cells [261]. Other miRNAs were reported to be involved in B-cell lymphomas, nasal-type NK/T-cell lymphoma, and in solid tumors (miR17-92) [263].

Apart from cancer development, miRNA functions are also connected to (i) heart diseases and (ii) infectious diseases.

- (i) miR-195 was found to cause frank cardiac hypertrophy in a mouse heart. miR-208 is involved in cardiac hypertrophy and upregulation of beta-myosin heavy chain that occurs in response to increased cardiac afterload. Additionally, miR-208 is required for the cardiac upregulation of beta-myosin heavy chain that occurs in the absence of normal thyroid hormone signaling [265, 266].
- (ii) It was known for a long time that viruses express short non-coding transcripts from their genomes. It seems that these short viral RNAs interfere with the host cell RNAi machinery in order to subvert or make use of the same. As mentioned above, adenoviruses express two short RNAs, VA-RNAs that are thought to interfere with the cellular RNAi pathway in order to fulfill some sort of silencing suppressor function. In line with that some other viruses were described to express short RNAs with the purpose to alter cell proliferation, apoptosis and immune signaling. It was reported that, for example, Epstein-Barr virus, Kaposi's sarcoma virus, cytomegalovirus and SV40 DNA tumor virus express miRNAs from their genomes (reviewed in [259]).

2.2. Therapeutic applications of RNA interference

Since the discovery of RNA interference by Fire *et al.* in 1998 ([206]), a large amount of work has been done to use and modulate this mechanism for clinical applications. RNAi based treatment methods may circumvent many target-related problems, such as the lack of specificity for a specific target [267, 268]. Additionally, undesired toxicity due to the lack of 'cancer-exclusive' inhibition of target proteins occurs. The success of monoclonal antibodies is often restricted by inaccessibility of the target protein [269-271].

Nevertheless, the successful development of RNAi-based therapeutics is still bearing several crucial issues. It is quite challenging to develop highly specific and efficient delivery methods, to assign systemic biodistribution and subcellular localization, to avoid potentially toxic off-target effects, or to overcome immunogenicity when not desired (Tab. 5) [272].

Table 5 | Challenges in Using RNAi-based therapeutics [272]

Challenges	Solutions
Biological barriers	
Intravascular degradation	Chemical modification and nanoparticle encapsulation
Tissue penetrance	Exploit abnormal angiogenesis and EPR effect
Intracellular delivery	Exploit receptor-mediated endocytosis
Intracellular trafficking	Incorporate biomaterials that enhance endosomal escape
Extracellular matrix	Optimize physico-chemical properties to escape immune and stromal interactions
Toxicities of RNAi	
Immune response to RNAi	Sequence optimization and oligonucleotide modifications
Carrier toxicity	Use of biodegradable, biocompatible and non-immunogenic materials
Off-target effects	Sequence optimization and oligonucleotide modifications
Oversaturation of RISC	Annotation of 'dose' effects on direct and indirect gene regulation
Tissue specificity	
Healthy versus cancer	Study of long-term RNAi side effects and exploit cancer features for targeting
Predictable delivery	Create carrier libraries annotating delivery efficacy based on healthy tissue and tumor type, and in silico modelling
Monitoring delivery	
Pharmacokinetics	Use of PEGylation or logic-embedded vectors
Pharmacodynamics	Use of bioluminescent imaging and novel biomarkers of delivery and response
Resistance	Study of altered processing machinery and SNP variants affecting RNAi
EPR, enhanced permeability and retention; PEG, polyethylene glycol; RISC, RNA-induced silencing complex; RNAi, RNA interference; SNP, single nucleotide polymorphism.	

Tab. 6 provides an overview about clinical trials on RNAi-based therapeutics.

Table 6 Clinical Trials on RNAi-based Therapeutics [213, 273]				
Disease	Stage	RNAi reagent	Delivery	Drug/Company
Ocular diseases				
Pachyonychia Congenita	Completed Phase I	siRNA	Injection	TD101/ Pachyonychia Congenita Project
AMD Choroidal Neovascularization	Completed Phase I, II	siRNA	Direct intravitreal injection	AGN211745/ Allergan; Sirna Therapeutics Inc.
Optic Atrophy Non-arteritic Anterior Ischemic Optic Neuropathy	Ongoing, Phase I	siRNA	Injection into the eye	QPI-1007/ Quark Pharmaceuticals
AMD	Withdrawn Phase III	siRNA	Direct intravitreal injection	Bevasiranib/ Opko Health, Inc.
Diabetic Macular Edema	Completed Phase II	siRNA	Injection into the eye	Bevasiranib/ Opko Health, Inc.
Ocular Pain Dry Eye	Completed Phase I	siRNA	Eye drops, topical administration	SYL1001/ Sylentis, S.A.
Glaucoma Ocular Hypertension	Phase I, II	siRNA	ophthalmic drops	SYL040012/ Sylentis, S.A.
Viral infections				
Hepatitis B and C	Preclinical stage	shRNA	Liganded nanoparticle	NucB1000/ Nucleonics; Intradigm
RSV	Phase IIb	siRNA	Aerosol	ALN-RSV/ Alnylam
HIV	Phase I	shRNA	Lentivirus	Benitec; City of Hope
Cancer				
Solid tumors	Phase I	shRNA	Nanoparticle	NCT01596374/ National University Hospital, Singapore
		siRNA		CALAA-01/ Calando Pharmaceuticals
		shRNA		FANG/ Gradalis, Inc.
Advanced cancer	Phase I	siRNA	Neutral Liposome	siRNA-EphA2-DOPC/ M.D. Anderson Cancer Center; Ovarian Cancer Research Fund
Metastatic Melanoma Absence of CNS Metastases	Phase I	siRNA	Proteasome siRNA and tumor antigen RNA-transfected dendritic cells	NCT00672542/ Duke University
Chronic Myeloid Leukemia	Completed	siRNA	SV40 vector	NCT00257647/ Hadassah Medical Organization
Advanced Solid Tumors	Phase I	siRNA	Direct intravitreal injection	Atu027/ Silence Therapeutics AG
Pancreatic Ductal Adenocarcinoma Pancreatic Cancer	Phase I	siRNA	biodegradable polymeric matrix	siG12D LODER/ Prof. Eithan Galun, Silenseed Ltd
Neuroblastoma	Completed	siRNA		NCT01075360/ National Taiwan University Hospital
Stage IIIC Ovarian Cancer	Phase II	shRNA	Injection	FANG™, Bevacizumab/ Gradalis, Inc.
Advanced Cancer Metastatic Cancer Solid Tumors	Phase I	shRNA	Intratumoral injection	pbi-shRNA STMN1 LP/ Gradalis, Inc.
Colorectal Cancer Pancreas Cancer Gastric Cancer Breast Cancer Ovarian Cancer (all with hepatic metastase)	Completed Phase I	siRNA	Lipid nanoparticles; intra arterial administration	TKM-080301/ National Institutes of Health Clinical Center
Other disease types				
ALS	Preclinical stage	siRNA		CytRx
Inflammatory diseases	Preclinical stage	siRNA	Peptide	Nastech
Delayed Graft Function Other Complication of Kidney Transplant	Phase I, II	siRNA	Injection	I5NP/ Quark Pharmaceuticals
Injury of Kidney Acute Renal Failure	Completed Phase I	siRNA	Injection	I5NP/ Quark Pharmaceuticals
Hypercholesterolemia	Terminated Phase I	siRNA	Liposome	PRO-040201/ Tekmira Pharmaceuticals Corporation

AMD, age-related macular degeneration; RSV, respiratory syncytial virus

2.2.1. The siRNA-based approach

In vitro, the most common way to use RNAi for gene silencing is to transfect cells with 21-25 nt siRNAs using commercially available transfection reagents. Another possibility is to use longer, ~27 nt siRNAs that serve as Dicer substrates and are processed into their mature form. These 'Dicer-ready siRNAs' showed in some cases higher silencing capacities when compared to their shorter versions [274, 275] For *in vivo* approaches additional packaging of siRNAs is required in most of the cases (Tab. 7).

2.2.2. The miRNA/shRNA-based approach

In contrast to directly deliver small interfering RNAs, it is also possible to design systems where artificial miRNAs or small hairpin-based RNAs (shRNAs) are directly expressed from a plasmid or viral/bacterial vector. shRNAs bear a hairpin loop structure and mimic pre-miRNAs, artificial miRNAs mimic pri-miRNAs. Therefore, artificial miRNAs are processed similarly to endogenous miRNAs. Although shRNAs seem to be analogs of pre-miRNAs, they often do not reflect Drosha cleavage products [276, 277]. It is crucial for them to contain the typical 3' dinucleotide overhang of pre-miRNAs that is essential for correct exportin 5-mediated transport from the nucleus into the cytoplasm. Impaired exportin 5 transport leads to accumulation of shRNAs in the nucleus and causes toxicity [278, 279].

Another issue using shRNAs can be their very high expression levels, because in most cases, their expression is under the control of very strong promoters. First, a saturation of exportin 5 can occur which can result in an impaired export of endogenous miRNAs into the cytoplasm. Additionally, other downstream components of the cellular RNAi machinery can be saturated and become no longer available for cellular miRNA processing. This can result in liver toxicity and fatal effects as has been observed in mice [278]. These problems may be overcome by overexpressing saturated components, like exportin 5 or Argonaute proteins, simultaneously to shRNA expression [280].

However, it seems to be more appropriate for therapeutic approaches to use weaker promoters [276, 281] for shRNAs, or artificial miRNAs to avoid negative side effects such as saturation of the RNAi machinery [279, 282-285]. Another advantage of artificial miRNAs is that they can be embedded in larger transcripts and are therefore more suited for RNA polymerase II-based expression systems. RNA polymerase II-based transcription provides the opportunity for tissue- or cell type-specific expression.

2.3. Delivery methods for RNA-interference-based therapeutics

2.3.1. siRNA delivery methods

Table 7 | si/shRNA Delivery Methods [213, 286, 287]

Method	Nucleic acid delivery	Advantages	Disadvantages
Non-viral delivery			
Cholesterol	siRNA	Systemic delivery, stable, non-immunogenic, proven <i>in vivo</i> delivery in non-human primates, potentially low cost	Non-selective delivery, possible liver toxicity if used repeatedly, not useful for delivery to most organs other than liver, very large doses required for efficiency in animal model testing
SNALP	siRNA	Systemic delivery, highly stable	Non-selective delivery
Antibodies	siRNA	Receptor-specific delivery, can use monoclonals or antibody fragments	Relatively complex formulation, costly to produce, repeated treatments might be necessary, possibly immunogenic
Aptamer	siRNA	Receptor-specific delivery, large scale manufacturing possible, can be backbone modified for stability <i>in vivo</i>	Large-scale sequence screening required, large relative to siRNA, repeated treatment might be necessary, need to be modified for enhanced circulation and pharmacodynamics, costly to manufacture
Nanoparticle	siRNA	Receptor-specific, self-assembling, specific targeting possible, can be synthesized in large scale, able to accommodate large amounts of siRNAs and can be engineered to escape endosome	Need to be conjugated to specific ligands for tissue specific delivery, costly manufacturing, repeated administration necessary
Viral delivery			
Lentivirus	RNA (shRNA produced)	Stable expression, transduces non-dividing cells	Gene-disruption risk, localized delivery
Adenovirus	dsDNA (shRNA produced)	Episomal, no insertional mutagenesis	Immunogenic, dose-dependent hepatotoxicity
AAV	ssDNA/dsDNA (shRNA produced)	Episomal, low genomic integration	Immunogenic, small vector capacity
Herpes simplex virus	ss/dsDNA	Episomal, lasting expression	Immunogenic

AAV, adeno-associated virus; dsDNA, double-stranded DNA; Fab, heavy-chain antibody fragment; shRNA, short hairpin RNA; SNALP, stable nucleic acid-liquid particles; ssDNA, single stranded DNA

Besides the appropriate design of siRNA for the use *in vivo*, the delivery strategy is the next challenging step. Cellular uptake of naked siRNA is very unlikely due to the negative charge and the size. Further on, uncomplexed siRNAs are very unstable in the circulation. The siRNAs are degraded by RNase A-type nucleases and are readily cleared by the kidney [288]. Thus, a broad range of delivery methods was developed to assure systemic delivery (Tab. 7). Additionally to the protection of the siRNA and the specific delivery, the appropriate administration strategy has to ensure two other steps, namely endocytosis and correct release from endosomes. A crucial step during

endocytosis of a siRNA is the correct release of the endosome to escape elimination via the Golgi network [287]. Some approaches use specially designed polymers to escape the endosome by the so-called 'proton-sponge effect' and direct membrane interaction of polycation and sulphoglucanes which enhance the escape [289]. Another possibility to avoid siRNA elimination is to create receptor ligands that express His-tags and enable the protein-siRNA complex to escape the endosome [290]. Stable nucleic acid liquid particles (SNALPs) are delivery vehicles for siRNAs that mediate endosomal escape via membrane fusion and subsequent release of the siRNA into the cytoplasm [287].

The most commonly used carriers for siRNAs comprise lipid-based carriers or cholesterol conjugates to the sense strand of the duplex (Fig. 12). An important feature of cholesterol-conjugated siRNAs is their efficient uptake by the liver, because these complexes are bound by low-density lipoprotein (LDL) in serum, and LDL uptake into the liver is robust [291]. Additionally, lipophilic siRNAs can bind high-density lipoprotein (HDL) resulting in specific targeting of tissues expressing HDL receptors, such as gut [291], kidney [292], vaginal epithelial cells [293], and oligodendrocytes in the brain [294].

A recent study demonstrate high delivery effectiveness of siRNA complexed into SNALPs in nonhuman primates. The reduction of the target gene was stable over two weeks after only one single delivery [295]. These data were confirmed by another study in nonhuman primates. The administration of siRNA-SNALPs complexes targeting three Ebola virus proteins led to the successfully protection from a lethal challenge of Ebola virus in nonhuman primates [296].

The right choice of a carrier system for siRNAs can permit a specific uptake by the desired tissues or cell types, e.g. cancer cells. Such carriers for tissue specific targeting include aptamers [297, 298], nanoparticles [299], antibodies [300-303], peptides and proteins [299, 304], and oligonucleotide agonists (Fig. 12) [305].

2.3.2. Viral vectors as delivery system

The most commonly used viral vectors for the delivery and expression of shRNAs or artificial miRNAs are lentivirus, adenovirus or adenovirus-associated viruses (AAV), and herpesviruses [306-310]. On the one hand, the genomes of adenoviral and AAV-based vectors show the advantage to stay episomal. Therefore, undesired insertions into the host genome are avoided. On the other hand, usage of these vectors is only suitable for slowly dividing target cells, because upon cell division the vectors will be lost. Since the capsid genes/proteins of both viruses can be modified, a specific cell

and tissue tropism can be achieved [311, 312]. Both types of vectors infect cells at multiple copies per cell which should be kept in mind when using shRNAs, as dosing may be amplified [277].

A major difference between adenovirus and AAV vectors lies in their packaging capacity that is 35 kb for adenovirus and only ~4.5 kb for AAV. Whereas most genes were removed from AAV vector genomes most adenoviral vectors comprise the whole adenovirus genome. Thus, an issue working with adenoviral vectors *in vivo* is the high risk to induce immune responses resulting in the elimination of the vectors [286].

Lentiviral vectors can also infect both, dividing and non-dividing cells. However, they can integrate into the host genome, and thereby can cause dramatic outcomes when activating oncogenes. To avoid this phenomenon, insertion-defective lentivirus vectors were designed [313].

Apart from all improvements that were worked over in the last years, there are still several challenges that limit the use of RNAi in the clinics. Some current issues for the use of RNAi-based therapeutics and possible solutions are listed in Tab. 7 ([272]).

2.3.2.1. RNAi-mediated off-target effects

During the design of RNAi molecules for further applications, a variety of factors has to be kept in mind to avoid possible toxic or unspecific off-target effects. Such off-target effects can be (i) specific or (ii) non-specific:

- (i) specific or miRNA-like off-target effects are due to the limited degree of siRNA/shRNA complementarity to non-targeted mRNAs and result in the unintended suppression of alternative targets

Pivotal features of siRNAs for the desired efficiency include the sequence and the length of the siRNA, the chemical nature of the silencing moiety, e.g. RNA with or without modified bases and sugars, and the nature of the 5' and 3' ends. Concerning the sequence of a siRNA, it was shown that within the seed region of the antisense strand (nucleotide 2-7 or 2-8) perfect target complementarity to the 3' UTR of the target mRNA has to be provided. If the seed region also perfectly matches other mRNA sequences apart from the desired gene, specific off-target effects might occur [314, 315]. Some distinct features of the seed region can induce the phenomenon of off-target effects. The presence of adenosine opposite to siRNA base 1 and of an adenosine or uridine opposite to siRNA base 9 enhance off-target mRNA repression. It was also shown that an increased AU content in the 3' end of the seed region could enhance off-target mRNA repression [316].

It has to be kept in mind that the antisense as well as the sense strand of a siRNA can induce off-target effects, because both strands can be taken up by RISC. RISC preferentially uses the strand with the lower thermodynamical stability in its 5' part as the guide strand [317, 318].

- (ii) non-specific off-target effects, such as toxicity or undesired immune responses caused by the siRNA or its delivery vehicle [314, 319, 320]

If a siRNA carries a 5' triphosphate, it is very likely that a type I interferon response (type I IFN response) is induced [213]. Blunt ended siRNAs can lead to an activation of cytoplasmic retinoic acid inducible gene 1 protein (RIG1 or DDX58) or INF production. These issues can be overcome by creating appropriate 5' and 3' ends (reviewed in [286]). Since siRNAs are double stranded RNAs they are recognized by the pattern recognition Toll-like receptors (TLRs) [321]. It was shown that siRNAs activate endosomal TLR3 signaling in a sequence-independent manner [322, 323]. Nevertheless, the major mechanism of siRNAs to trigger innate immune responses, is mediated via the endosomal TLRs, TLR7 and TLR8. TLR activation leads to the production of pro-inflammatory cytokines or IFN- α [324-326]. Interestingly, in contrast to TLR3, TLR7 and TLR8 recognize siRNAs in sequence-dependent manner. It seems that both TLRs preferentially recognize uridine (U)- and guanosine (G)-rich sequences, including UG-dinucleotides and the 5'-UGU-3' motif. [327, 328].

However, the activation of the immune system can also have advantages, for example, when it leads to a stimulation of dendritic cells to immunologically respond to cancer cells [329].

In contrast to siRNAs, shRNAs are more unlikely to introduce immune responses, since they are expressed in the nucleus and processed by the endogenous machinery and their 5' ends are less inflammatory [330-332].

Not only the RNAi molecule itself can cause undesired off-target effects. The second possibility to induce toxicity or immune responses is related to the delivery vehicle.

Another type of unspecific off-target effects comprises the saturation of the cellular RNAi machinery resulting in altered cell function (as discussed under 2.2.2.).

Although synthetic siRNAs have not to be processed in the nucleus and transported into the cytoplasm it was shown that they can saturate components of the RNAi machinery, thereby blocking RISC [333].

2.3.3. RNAi-based approaches to inhibit viral infections

Viral infections and diseases associated with them are serious public health, social and economic problems. There is the urgent need for more effective antiviral drugs and vaccines for some very dangerous viral infections including HIV, hepatitis B and C, severe acute respiratory syndrome (SARS), influenza or Ebola virus. Due to the high effectiveness, specificity and the easy use, RNAi-based antivirals represent very promising tools for inhibiting virus infections (reviewed in [384]). A high number of studies exists that demonstrates the inhibition of diverse viruses both *in vitro* and *in vivo*.

One of the first efficient applications of siRNAs to inhibit viral replication was a study by Gitlin *et al.* Here, siRNAs were potent in reducing viral RNA, protein and viral titers of poliovirus. This group used a mixture of different siRNAs targeting poliovirus to overcome the problem that viruses can escape siRNA functions through target sequence mutations (escape virus formation) [385].

An alternative approach to overcome the problem of target site mutations and ineffectiveness of siRNAs is the selection of highly conserved regions within the virus genome as target sites. There is a strong selective pressure against mutations in such highly conserved regions. Another advantage for the targeting of such regions may be the simultaneous inhibition of various serotypes, assuming that these regions are evolutionally conserved between different serotypes. The success of this approach was demonstrated by Kahana *et al.*, who efficiently inhibited the replication of foot-and-mouth disease virus (FMDV) [386-388].

Another possible use of siRNAs to inhibit viral infections is not to target viral RNAs, but to alter cellular functions that are essential for viral replication. One such approach was made in order to inhibit human immunodeficiency virus (HIV) replication. Since HIV shows very high mutation rates, it is nearly impossible to design siRNAs that effectively silence their targets over a long time period. Instead, the alternative approach was to target the entry receptor of HIV on host cells, namely CCR5. Although that approach was very promising and could fully inhibit HIV replication in macrophages, the inhibition in primary human microglia at 15 days post challenge with HIV was not complete [389]. However, a combination of targeting conserved regions of HIV on the one hand and the viral entry receptor on the other hand completely inhibited viral replication, even after 15 days of infection [390].

In contrast to the so far discussed RNA viruses, DNA viruses are more difficult to inhibit. In DNA viruses the inhibitory effect of siRNAs is dependent on the cleavage of

the viral mRNA. In order to fully prove reduced viral replication, it is essential to measure remaining viral DNA levels [391].

However, there is a tremendous number of studies and reports describing the potency of RNAi-based applications to inhibit viral infections in vitro or in vivo (reviewed in [384], [391], [392] and [393]).

Nevertheless, as already described above, the successful development of RNAi-based therapeutics is still a long way down, dealing with lots of different issues.

The potency of some RNAi-based antiviral therapeutics is currently evaluated in clinical trials (Tab. 6).

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II. AIMS OF THE THESIS

1. Using RNA interference as a tool to inhibit adenoviral multiplication

One focus of this thesis was to answer the question whether RNA interference could be a possible tool to inhibit adenoviral infections. For that purpose siRNAs and artificial miRNA were designed and validated targeting different adenoviral genes involved in different processes.

2. Using gene-directed enzyme prodrug therapy to block adenoviral replication

In this part of the thesis I was involved in a project that wanted to answer the question whether HSV-TK and ganciclovir can be used to treat adenoviral infections.

3. Identifying cellular target genes of adenoviral miRNAs

The aim of this part of the thesis was to validate potential target genes of adenoviral miRNAs to gain more insight into the interplay between adenovirus and cellular gene expression during an infection.

III. SPECIAL TOPICS

Author's contribution

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

In the **first 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 carried out most of the experimental work shown in Fig. 1, Fig. 2, Fig. 3, Fig. 4, Fig.5, Fig. 6, Fig. 7 and Suppl. Fig. 1, Suppl. Fig.2, Suppl. Fig.3, Suppl. Fig.4 and Suppl. Fig.5. I was involved in data analysis and interpretation of results.

In the **second 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 interpretation of results, performed endpoint dilution assay experiments (Fig. 8) and helped with the optimization of cell culture conditions.

In the **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, data analysis and interpretation of the results. I performed luciferase assay experiments shown in Fig. 5 and helped to clone the luciferase reporter constructs.

In the **fourth part** (*Identification of cellular targets for adenoviral miRNAs*) I performed experiments to validate potential candidate genes for miRNA-directed targeting of cellular genes identified in a RIP-Chip analysis. I was further involved in data analysis and interpretation.



Vienna, 2013

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

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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 (Echavarria, 2008; Ison, 2006; Kojaoghanian 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 (Kojaoghanian 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* (Arbutnot, 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'-CGACACCGGGTTAAACATGAGACA TATTATCTGCCAC-3' and E1A-r1 5'-CAACTCATTGTTAAACAAAGG CGTTAACCA-3'; annealing temperature [T_a]: 50 °C), DNA polymerase (Pol-f1 5'-ACTCATATGGCCTTGGCTCAAGCTCACC GGCG-3' and Pol-r1 5'-ACTAGATCTACGGCATCTCGATCCAGCATATC-3'; T_a: 55 °C), pTP (pTP-f3 5'-CTTTGACCGTCTCGAGCGTCAACGATTGCGC-3' and pTP-r3 5'-GTGTCCTTGGATGCGGCGGCTAAAAGCGGTGACGCGG-3'; T_a: 65 °C), IVa2 (IVa2-f1 5'-CACC GGCTCGTTAAACCAGAGGGCGAAG AC-3' and IVa2-r1 5'-AAACATAAAGTTTAAACCAGACTCTGTTGGAT-3'; T_a: 50 °C), hexon (Hex-f1 5'-CCGCTTCTCGAGATGGCTACCCCTTC GATGATG-3' and Hex-r1 5'-TGTTGCGCGGCGCTTATGTTGTGGCG TTGCGG-3'; T_a: 57 °C), and protease (Prot-f1 5'-CAAGCAACAGTTT AAACAGCTGCCGCATGG-3' and Prot-r1 5'-AAATAAGTTTAAACGCC TTTATTGAAAGTGTCTC-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 (Life Technologies 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/Life Technologies 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 RNAs (http://rna.tbi.univie.ac.at/cgi-bin/RNAs), 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/Life Technologies Austria, Vienna, Austria). Briefly, for each well 0.5 µL Lipofectamine 2000 was diluted with 24.5 µL OptiMEM medium (Invitrogen/Life Technologies 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/Life Technologies 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 a 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'-GCATGTTTGTCTACAGTCTCTGTGTC-3', E1A 289R-cDNA-r1 5'-GGCGTCTCAGGATAGCAGGC-3', and E1A 289R-cDNA-p1 5'-AGGCTCCGGTTCTGGCTCGGG-3'), E1A-12S (E1A 12S-cDNA-f1 5'-AGGATGAAGAGGGTCTGTGTC-3', E1A 289R-cDNA-r1 5'-GGCGTCTCAGGATAGCAGGC-3', and E1A 289R-cDNA-p1 5'-AGGCTCCGGTTCTGGCTCGGG-3'), DNA polymerase (Pol-cDNA-f1 5'-ATGGCCTTGGCTCAAGCTC-3', Pol-cDNA-r1 5'-GCGTAGGTTGCTGGCGAAC-3', and Pol-cDNA-p1 5'-CGCCTCTGCGTGAAGACACCG-3'), pTP (pTP-cDNA-f2 5'-AAACCAACGCTCGGTGCC-3', pTP-cDNA-r2 5'-GGACGCGGTTCCAGATGTT-3', and pTP-cDNA-p2 5'-CGCGCGCAATCGTTGACGCT-3'), IVa2 (IVa2-cDNA-f1 5'-GGAAACAGAGGGCGAAGA-3', IVa2-cDNA-r1 5'-AGGGTCTCTGTCAGCTAGTC-3', and IVa2-cDNA-p1 5'-CTTGAGGCTGGTCTGCTGGT-3'), hexon (Hex-cDNA-f1 5'-CAGTCACAGTCGCAAGAGGAGC-3', Hex-cDNA-r1 5'-AGGTACTCCGAGGCGTCTG-3', and Hex-cDNA-p1 5'-ACCACTCGCGCATCATCGAAGGG-3'), and protease (Prot-cDNA-f1 5'-TCACAGTCGCAAGTCTTTGACG-3', Prot-cDNA-r1 5'-GCGGCAGCTGTTGTTGATG-3', and Prot-cDNA-p1 5'-CCGAGAAGGGCGTGGCAGGTA-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'-TGACACCACTGCTTAGC-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'-AACGAGGAGGCGGUUUCGAGAUUU-3' 5'-AAAUUCUGCGAAACCGCCUCCUGUU-3'	731–755	E1A	1, 2, 5, 6
E1A-si2	5'-CAGGAAGGGAUUGACUACUCACUU-3' 5'-AAGUGAGUAAGUCAAUCCCUUCCUG-3'	782–806	E1A	1, 5
E1A-si3	5'-CCUGUGUCUAGAGAAUGCAAUAGUA-3' 5'-UACUAUUGCAUUCUCUAGACACAGG-3'	1333–1357	E1A	2, 5, 6
E1A-si3_scr	5'-CCUUGUCGAGAAAGUACUAAUGGUA-3' 5'-UACCAUUGAUACUUUCUGACAAGG-3'	–	–	–
Pol-si1	5'-CAAGACCCGCUUACCUUCUACUGCA-3' 5'-UGCAGUAGAAGGUAAGCGGGUUCUG-3'	7782–7806	DNA pol	1, 2, 5, 6
Pol-si2	5'-CAACGUCUCCAGCGUCCAACCAUA-3' 5'-UAUGGUUGGACGCUUGAAGACGUUG-3'	6928–6952	DNA pol	1, 2, 5, 6
Pol-si3	5'-TGUCUCAGAGUUGUCCGAGUUUCUA-3' 5'-UAGAAACUCGGACACUCUGAGACA-3'	5818–5842	DNA pol	5
Pol-si4	5'-CGUCUCCAGCGUCCAACCAUAUA-3' 3' UGAUAUGGUUGGACGCUUGAAGACG-3'	6925–6949	DNA pol	1, 2, 5, 6
Pol-si5	5'-CAGCGUCCAACCAUAUCCAACU-3' 5'-AGUUGGAUGAUUUGGUUGGACGUG-3'	6918–6942	DNA pol	2, 5, 6
Pol-si6	5'-GCGGUCCAACCAUAUCCAACUCA-3' 5'-UGAGUUGGAUGAUUUGGUUGGACGC-3'	6916–6940	DNA pol	2, 5, 6
Pol-si2_scr	5'-CAACUUUCCGAGCCUACACGCAUA-3' 5'-UAUGCGUGGUAGGCUUGGAAAGUUG-3'	–	–	–
IVa2-si1	5'-ACAUGCGAGUCAGGGACAUGCUUA-3' 5'-UUAAGCAUGUCCUGACUCGCAUGU-3'	5013–5037	IVa2	5
IVa2-si2	5'-AAAUACAGUCCAAGAUCAUCUCAU-3' 5'-AUGAGAUCAUCUUGGACUGUAUUU-3'	4410–4434	IVa2	1, 2, 5, 6
IVa2-si3	5'-CAUCCAGCUUAAACCGCUUUGUAAA-3' 5'-UUUACAAGCGGUUAAAGCUGGGAUG-3'	4368–4392	IVa2	1, 2, 5, 6
Hex-si1	5'-UAGAAACUCGGACACUCUGAGACA-3' 5'-AUUUUAUACCAUAUAAAGCGCCUGC-3'	5818–5842	Hexon	5
Hex-si2	5'-GAGAACUAAUGGGCCAACAUCUAU-3' 5'-AUAGAUUGUUGGCCCAUUAUUCUC-3'	19776–19800	Hexon	5
Hex-si3	5'-GCCUCAGAAGUUCUUUGCCAUAUAA-3' 5'-UUUAAUGGCAAGAACUUCUGAGGC-3'	20527–20551	Hexon	5
Hex-si4	5'-CCGUCAGGUGGUGGAUUAUUAUAA-3' 5'-UUUAGUAUUAUCCACCACGACGG-3'	21247–21271	Hexon	5
Prot-si1	5'-GAGCAGGAACUGAAAGCCAUUGUCA-3' 5'-UGACAAUGGCUUUCAGUUCUGCUC-3'	21745–21769	Protease	2, 5, 6
Prot-si2	5'-AAGAUUCUUGGUUGGGCCAUUUUU-3' 5'-AAAUUGGCCCAACCAAGAUUUU-3'	21770–21794	Protease	2, 5, 6
Prot-si3	5'-UCAAGCAGGUUUAACAGUUUGAGUA-3' 5'-UACUCAAAUGGUAAACUGCUUGA-3'	21972–21995	Protease	1, 2, 5, 6
pTP-si1	5'-CCGCCUACUUUAUUACAUTT-3' 5'-AUGUAAUUAAGUAGGCGGTT-3'	9805–9823	pTP	1, 2, 5, 6
pTP-si2	5'-GAGGAGAUUGAAGAAGAGTT-3' 5'-CUUCUUCUUAUUCUCCCTT-3'	9359–9377	pTP	1, 2, 5, 6
pTP-si3	5'-GGUAGAAAGGCUAUGCAATT-3' 5'-UUGCAUGAGCCUUUCUACCTT-3'	9945–9963	pTP	1, 2, 5, 6
pTP-si4	5'-CGAAAUUGAUUCUGUGCAATT-3' 5'-UUCGACAGAAUCAAUUCGTT-3'	8796–8814	pTP	1, 2, 5, 6
pTP-si5	5'-GACUACGUAAUUGACUGAGGGCUU-3' 5'-AAGCCUCGAGUCAAAUACGUAGUC-3'	10289–10313	pTP	5
pTP-si6	5'-CGGUAGAAAGGCUAUGCAAGACUA-3' 5'-UAGUCUUGCAUGAGCCUUUCUACCG-3'	9940–9964	pTP	1, 2, 5, 6
pTP-si7	5'-ACUACCUCUUUCAGCGCCUGCGAAA-3' 5'-UUUUCGAGGCGCUGAAAGAGGUAGU-3'	9103–9127	pTP	1, 2, 5, 6
pTP-si8	5'-GAAAUUGAUUCUGCAACUCUCUU-3' 5'-AAGAGAGUUCGACAGAAUCAAUUC-3'	8789–8813	pTP	1, 2, 5, 6

^aGenBank ID: AC_000008.

^bSerotypes 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'-TCCTGCACCGCCAACATT-3', and E1A-p 5'-CGAGGAGCGGTTTCGAGA-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; Iftode 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

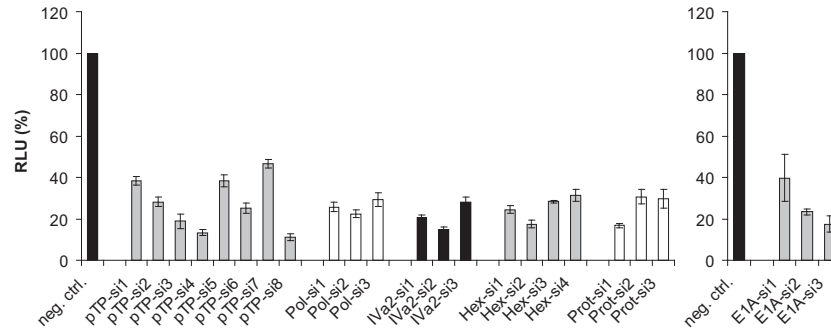


Fig. 1. Screening for functional siRNAs. siRNAs and their respective dual-luciferase target vectors were used to co-transfect HEK293 cells (DNA polymerase, pTP, IVa2, hexon, and protease siRNAs) or A549 cells (E1A siRNAs). A non-targeting control siRNA served as a negative control (neg. ctrl.). All siRNAs 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 RNAxs 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

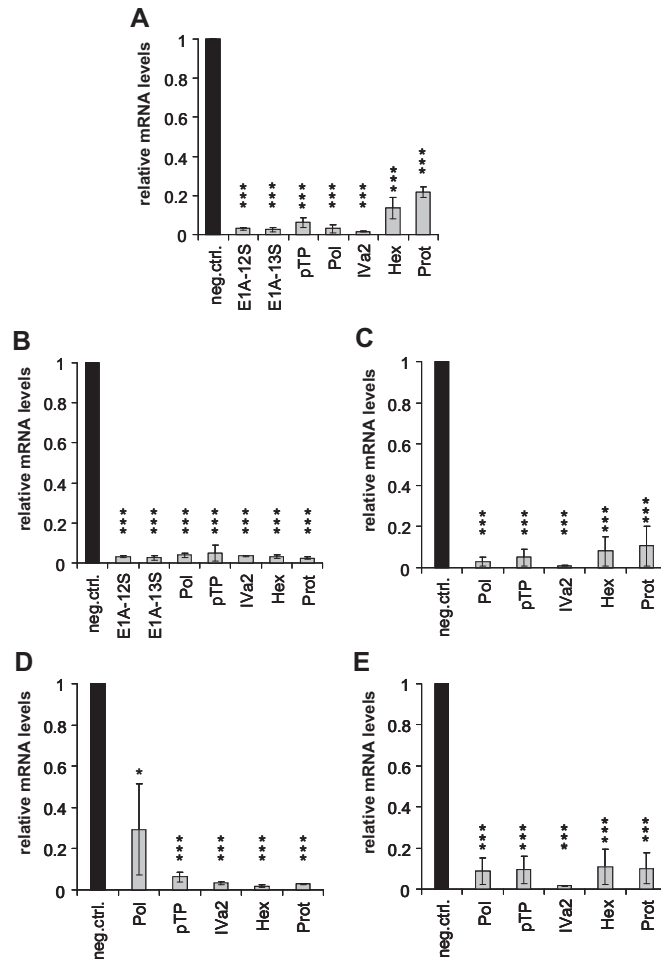


Fig. 2. siRNAs decrease mRNA levels directly and indirectly. A549 cells were transfected with siRNAs directed against the E1A, DNA polymerase (Pol), pTP, IVa2, hexon (Hex), and protease (Prot) genes, or a non-targeting control siRNA (neg. ctrl.) at a concentration of 10 nM, and then infected with Ad5 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

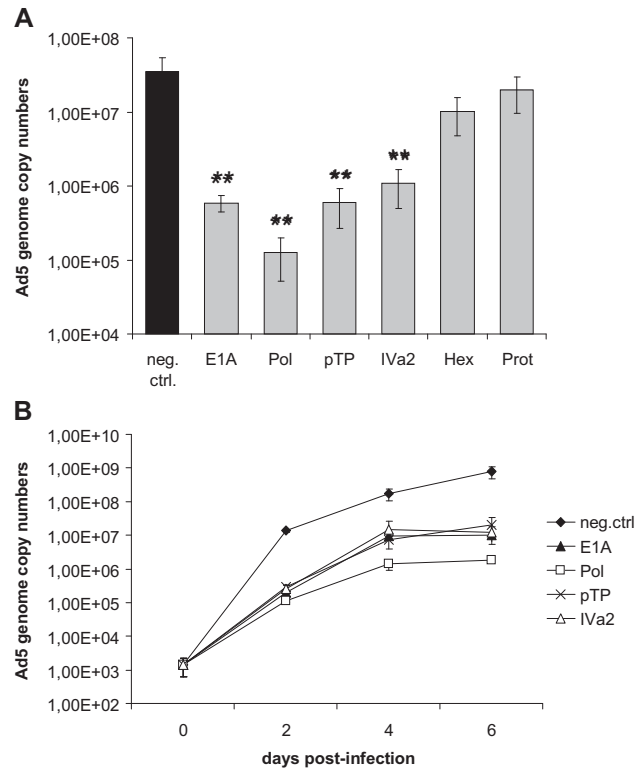


Fig. 3. Impact of siRNAs on viral DNA replication and virus spreading. (A) A549 cells were transfected with siRNAs directed against the E1A, DNA polymerase (Pol), pTP, IVa2, hexon (Hex), and protease (Prot) genes, or a non-targeting control siRNA (neg. ctrl.) at a concentration of 10 nM, and then infected 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. ** $p < 0.01$.

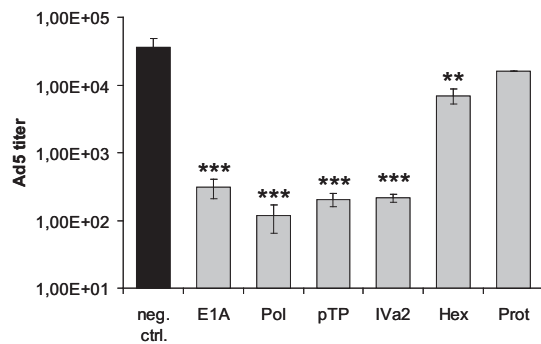


Fig. 4. siRNAs decrease the numbers of infectious virus particles. A549 cells were transfected with siRNAs directed against the E1A, DNA polymerase (Pol), pTP, IVa2, hexon (Hex), and protease (Prot) genes, or a non-targeting control siRNA (neg. ctrl.) at a concentration of 10 nM, and then infected with 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-

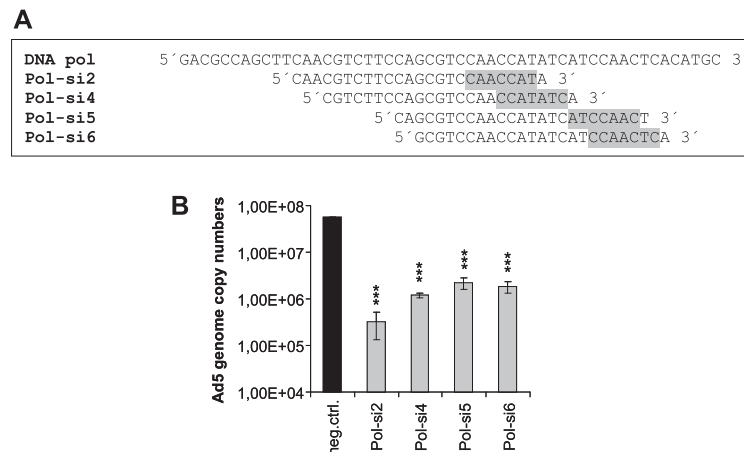


Fig. 5. Differential inhibition of Ad5 replication by DNA polymerase siRNAs binding in the immediate vicinity of, or overlapping, the Pol-si2 target sequence. (A) Region of the DNA polymerase open reading frame (indicated as DNA pol) targeted by siRNAs Pol-si2, Pol-si4, Pol-si5, and Pol-si6. The DNA 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. *** $p < 0.001$.

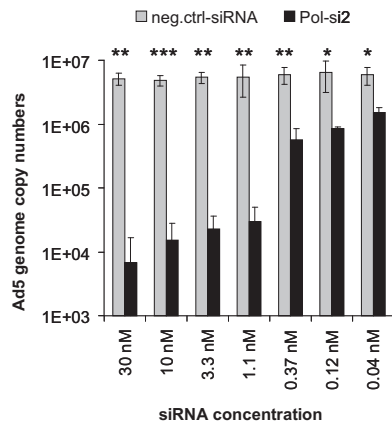


Fig. 6. Dose-dependent decrease in Ad5 genome copy numbers mediated by the DNA polymerase siRNA. A549 cells were transfected with the DNA polymerase siRNA or a non-targeting control siRNA (neg. ctrl.) in decreasing concentrations as indicated, and then infected with Ad5 at an MOI of 0.01 TCID₅₀/cell. 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

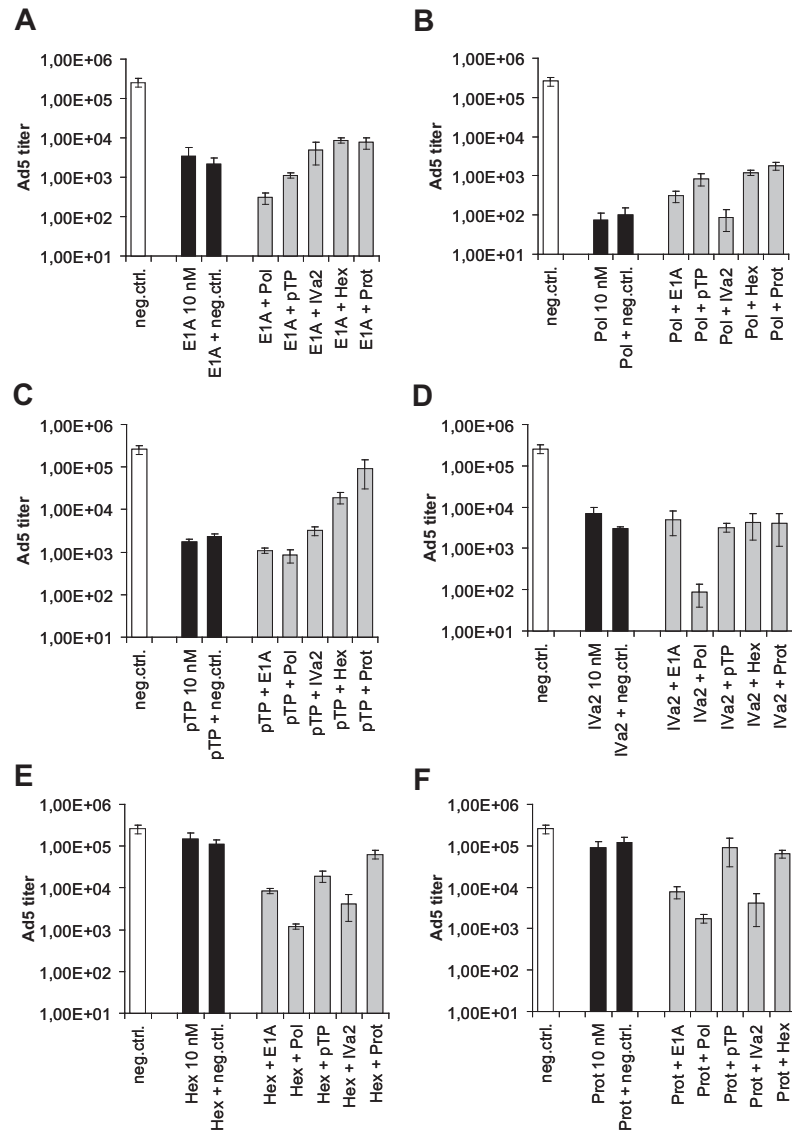


Fig. 7. Effect of combinatorial silencing of Ad5 genes on virus output. A549 cells were transfected with the indicated siRNAs either alone or in combination. SiRNA combinations for E1A (A), DNA polymerase (B), pTP (C), Iva2 (D), hexon (E), and protease (F) are depicted. For all transfections, the total 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

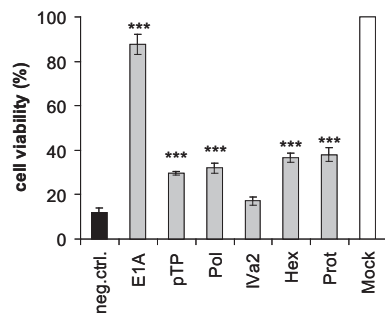


Fig. 8. siRNAs increase the viability of infected cells. A549 cells were transfected with siRNAs directed against the E1A, DNA polymerase (Pol), pTP, IVa2, hexon (Hex), and protease (Prot) genes, or a non-targeting control siRNA (neg. ctrl.) at a concentration of 10 nM, and then infected with Ad5 at 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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2. 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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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

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 system.

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'-TACAATCTCGAGCTTAAGATACATTGATGAGTTTGG-3'
CMV-f1	5'-ACCGCCCCGCGGTAGTTATTAATAGTAATCAATTACGG-3'
CMV-r1	5'-CGACTGACCGGTTCTGAAGCTTGAGCTCGA-3'
E2p-f2	5'-GACAGGTGCTGGCCGCGGTGTGGCCGCTGGAG-3'
E2p-r2	5'-AGCTTCGGCGCAACCGGTAAAGACGCGGAGGCTCTCTTC-3'
E3p-f1	5'-CAACGGAATCCGCGGCCACCGAAACCGAATTC-3'
E3p-r1	5'-TCTGAAATGACCGGTCCGGACGAGACCAAG-3'
E4p-f1	5'-AGATCTTGACCGCGGTGACGTAGGTTTATG-3'
E4p-r1	5'-TGGTAAACACCGGTGACACGCGCACCAGCTC-3'
IVa2p-f1	5'-CACCCCTTCGCGGCCCTTCCAGGAACAC-3'
IVa2p-r1	5'-GCGGTGTTACCGGTCTCTCTGATAGAAAC-3'
MLP-f1	5'-TGGGAGGGGCCGCGTCTGTGCTCCACTAG-3'
MLP-r1	5'-CACCGCGGGACCGGTGAATATCAATCCTCTC-3'
pIXp-f1	5'-ACCATGAAGCCGCGGAAGGTGCTGAGGTACGATG-3'
pIXp-r1	5'-CGAGTTGGTACCGGTGGCGGCGGCGGCTGCTG-3'
HSV1-TK-f1	5'-GCTAACTAGACCGGTCACTGCTTACTGGCTTATCG-3'
HSV1-TK-r1	5'-TAGACTCGAGCGCGCCCACTGTGCTGGATAT-3'
AdC-E1A-for	5'-GACGGCCCCGAAGATC-3'
AdC-E1A-rev	5'-TCTGCAACCGCAACATT3-3'
AdC-E1A-p	5'-CGAGGAGGCGGTTTCGAGA-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

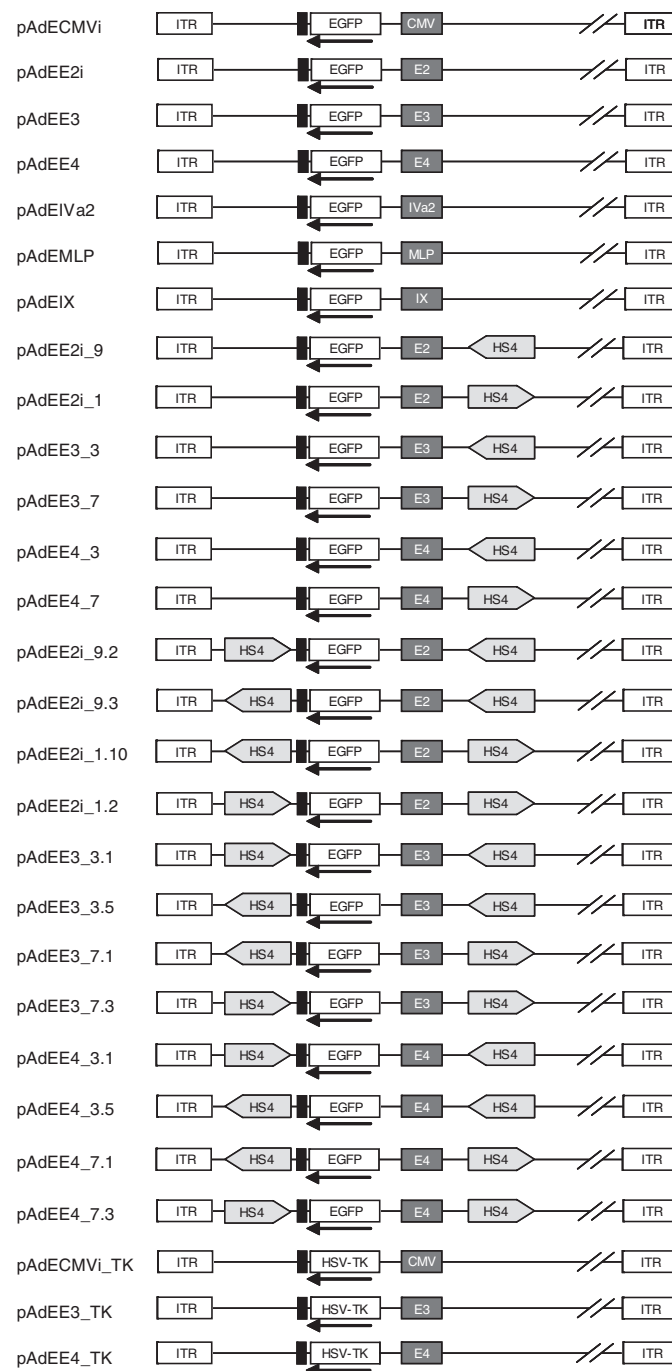


Figure 1. Schematic representation of adenoviral vectors. All vectors are based on the Ad5-derived vector pAd/PL-DEST (Invitrogen). Expression cassettes were inserted into the deleted E1 region of the parental vector in antisense orientation with respect to the left inverted terminal repeat (ITR). Expression 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, pAdEMLP and pAdEIX (Figure 1). A control vector (pAdECMVi) 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

Inhibition of adenovirus by HSV-TK/GCV

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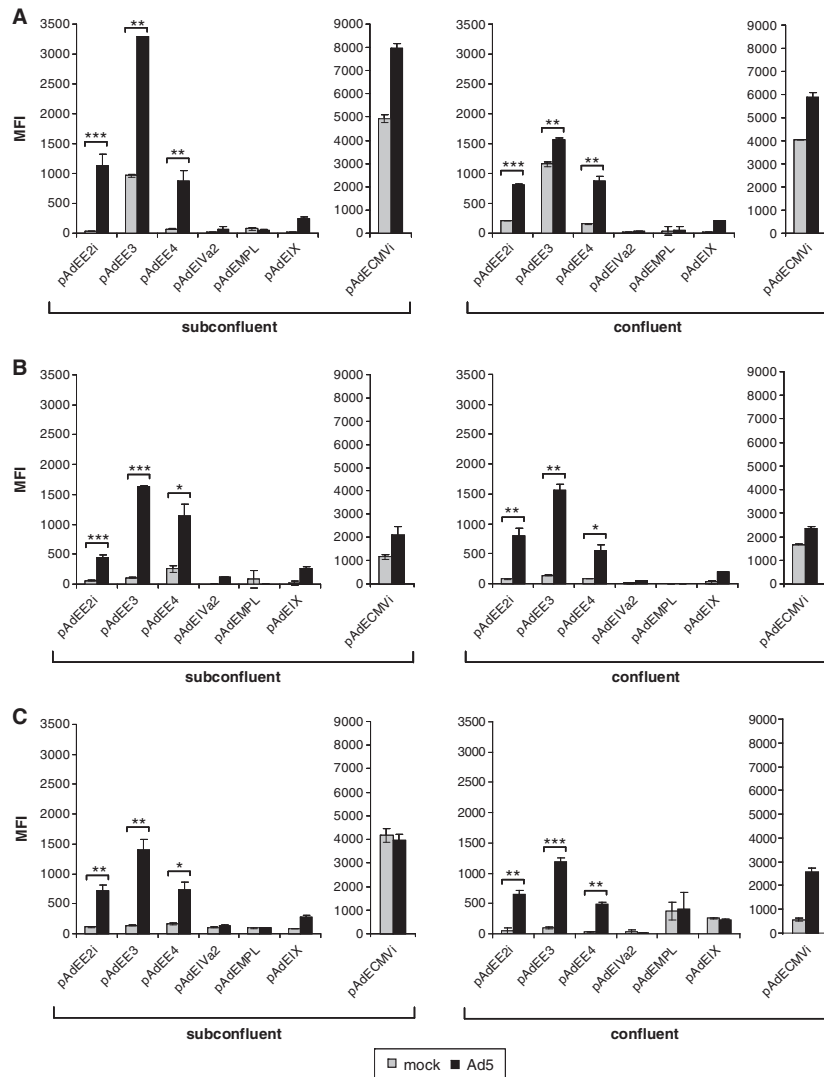


Figure 2. EGFP expression from various adenoviral promoters in either the absence or the presence of superinfecting wt Ad5. Subconfluent and confluent A549 (A), MRC-5 (B) and HUVEC cells (C) were infected with recombinant EGFP expression vectors pAdEE2i (E2B promoter), pAdEE3 (E3 promoter), pAdEE4 (E4 promoter), pAdEIVa2 (IVa2 promoter), pAdEMLP (major late promoter) and pAdEIX (pIX promoter) or as a control with the CMV promoter-harboring vector pAdECMV 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

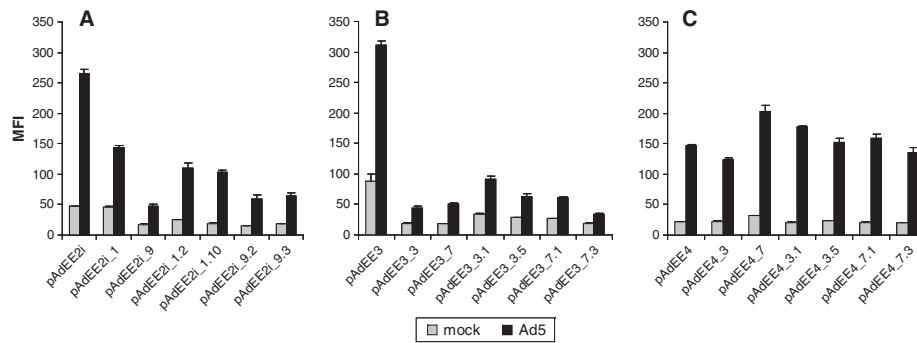


Figure 3. Effect of the HS4 insulator element on EGFP expression from the E2B (A), E3 (B) and E4 (C) promoter. Confluent A549 cells were infected with the indicated recombinant vectors at a MOI of 50 TCID₅₀/cell. Twenty-four hours after infection, cells were either superinfected with wt Ad5 (black bars) 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

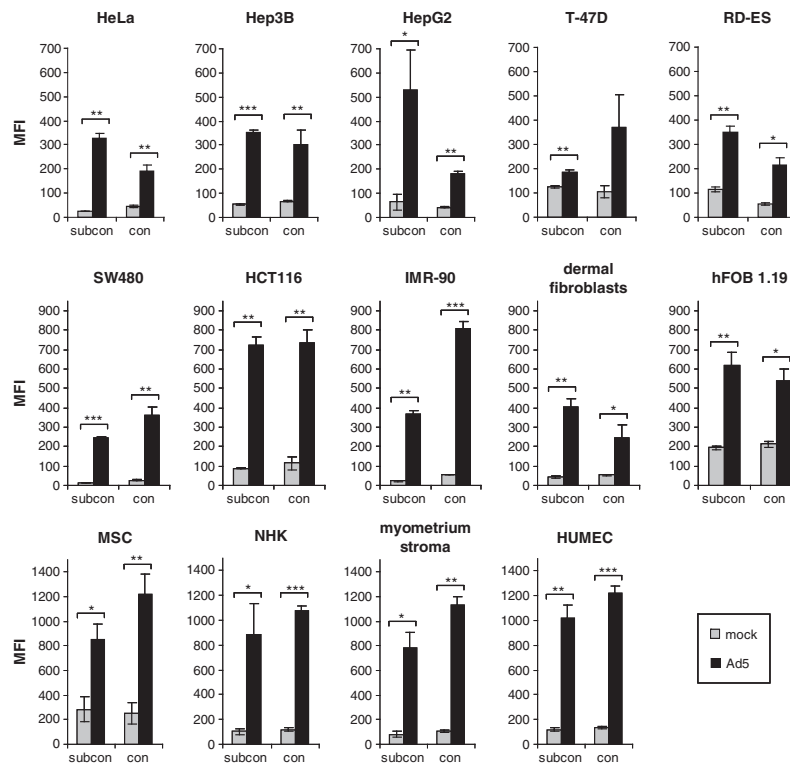


Figure 4. EGFP expression rates from the E4 promoter in the absence or presence of superinfecting wt Ad5 in various cells. Cells tested were: HeLa (human epithelial carcinoma), Hep3B and HepG2 (human hepatocellular carcinoma), T-47D (human mammary gland ductal carcinoma), RD-ES (Ewing's sarcoma), SW480 (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

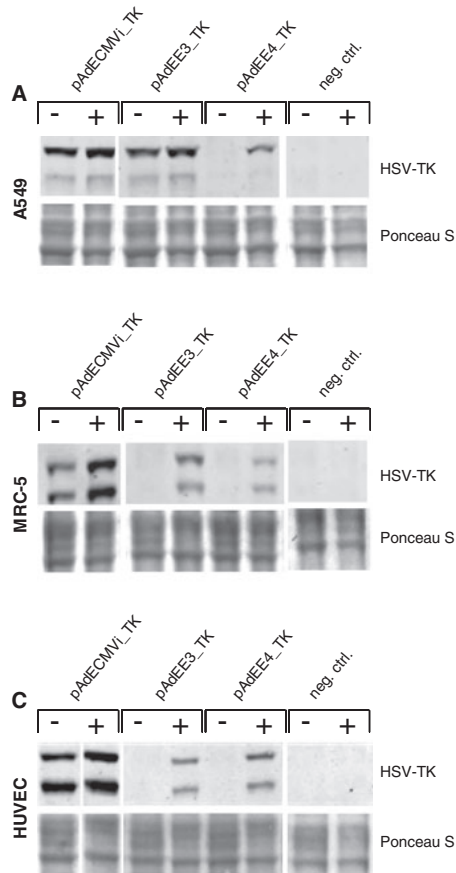


Figure 5. Selective HSV-TK expression from the E4 promoter in wt Ad5-superinfected cells. A549 (A), MRC-5 (B) and HUVEC cells (C) were infected with the recombinant, E4 promoter-harboring, HSV-TK expression vector pAdEE4_TK at a MOI of 50 (A549, HUVEC) or 100 (MRC-5) TCID₅₀/cell. As a control, cells 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).

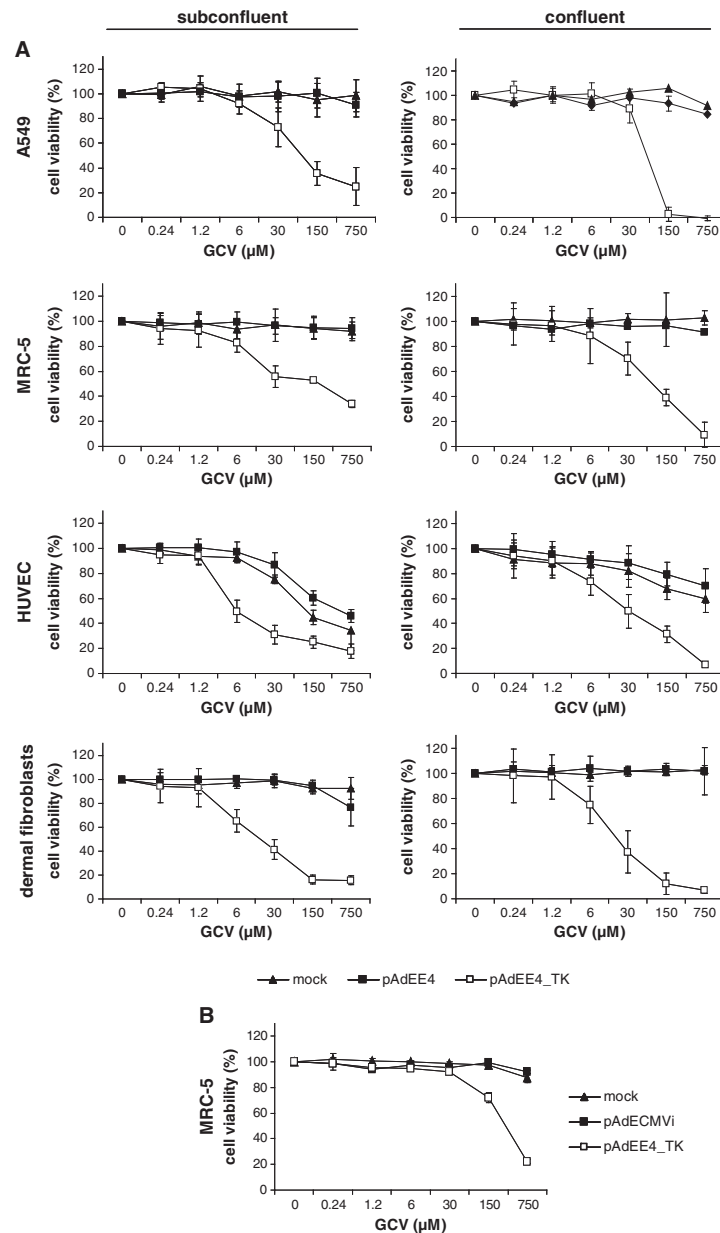


Figure 6. E4 promoter background activity is sufficiently low to largely prevent cytotoxicity in the presence of low concentrations of GCV. (A) Subconfluent and confluent A549 cells, MRC-5 cells, HUVEC and primary fibroblasts were infected with the adenoviral HSV-TK expression vector pAdEE4_TK or the 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).

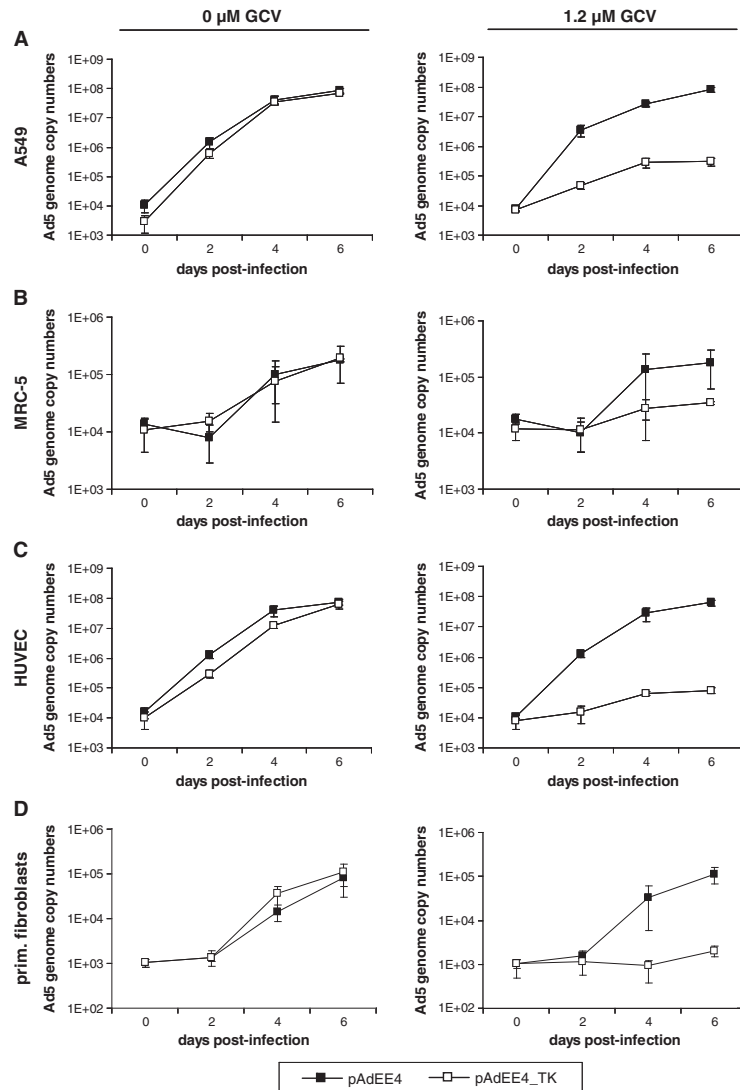


Figure 7. E4 promoter-mediated HSV-TK expression/GCV treatment inhibits wt Ad5 replication. A549 cells (A), MRC-5 cells (B), HUVEC (C) and primary fibroblasts (D) were infected with the recombinant, adenoviral HSV-TK expression vector pAdEE4_TK or the analogous EGFP expression vector pAdEE4 at a MOI of 100 TCID₅₀/cell. Twenty-four hours after infection, cells were superinfected with 300 TCID₅₀ units of wt Ad5 and treated with 1.2 μM 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 μM GCV, and cell viability was barely measurable (Figure 10A). Similarly, HSV-TK expression via pAdEE4_TK in the absence

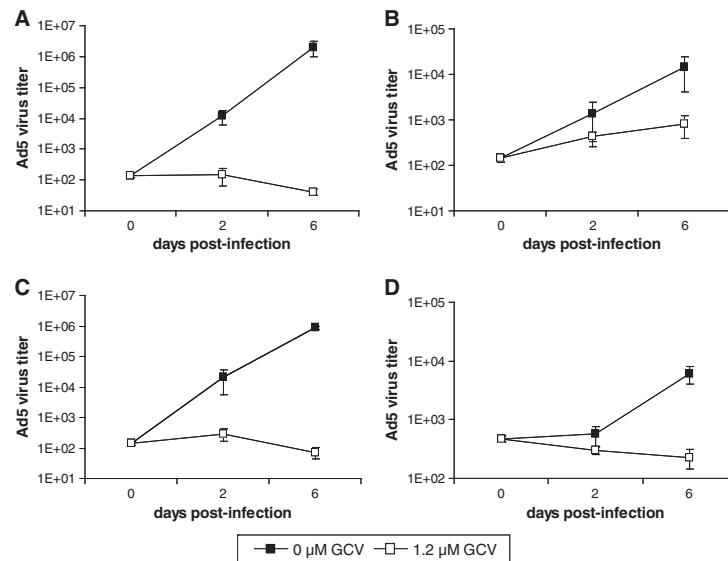


Figure 8. E4 promoter-mediated HSV-TK expression/GCV treatment reduces infectious virus progeny. Virus suspensions from the same virus inhibition experiments shown in Figure 7 were analyzed for numbers of infectious Ad5 particles. Virus suspensions were derived from A549 cells (A), MRC-5 cells (B) HUVEC (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

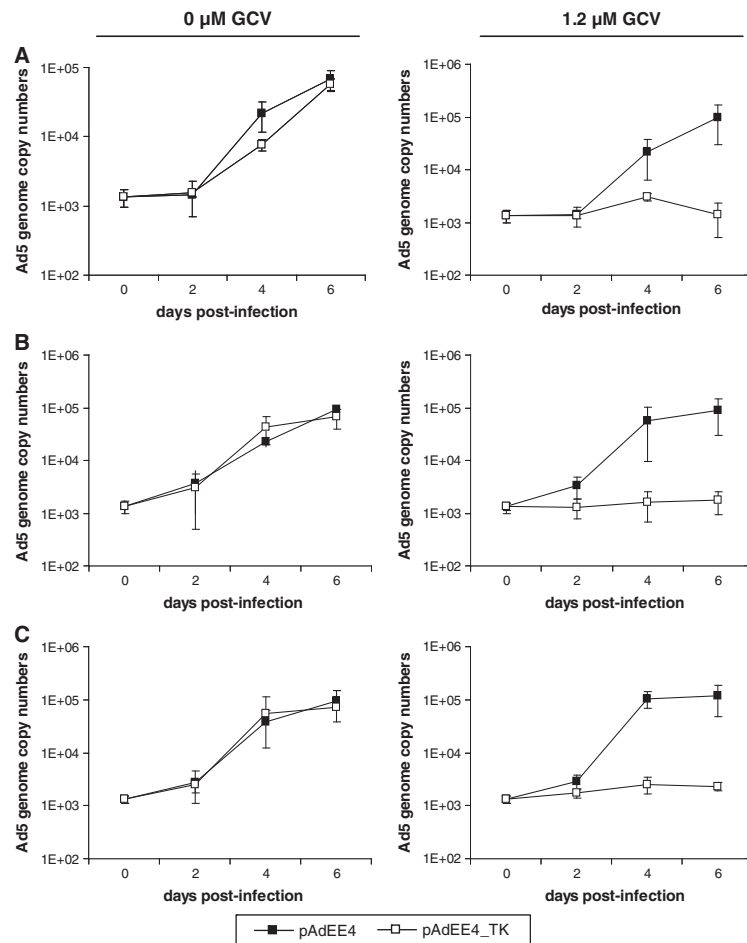


Figure 9. E4 promoter-mediated HSV-TK expression/GCV treatment inhibits wt Ad5 replication in cells already infected with wt Ad5. Dividing primary fibroblasts were co-infected with 300 TCID₅₀ units of wt Ad5 and the recombinant, adenoviral HSV-TK expression vector pAdEE4_TK. Infection with pAdEE4_TK 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 μ M or slightly higher (Figure 6) and truly contact-inhibited MRC-5 cells were resistant to GCV concentrations up to 30 μ M (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 μ M GCV. This concentration is also therapeutically

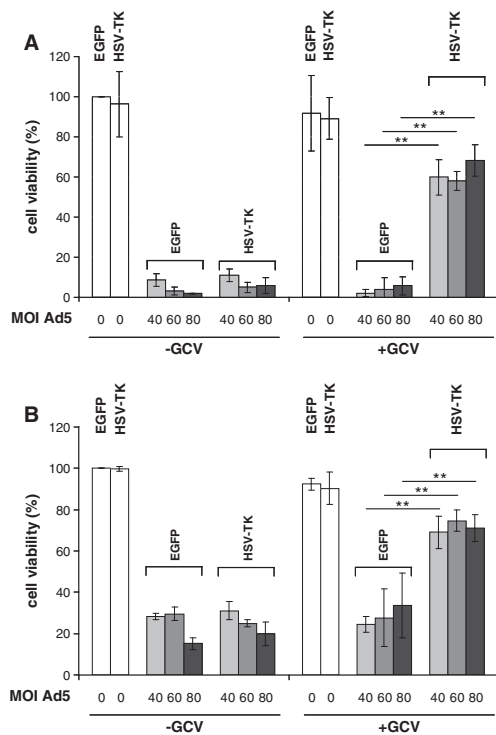


Figure 10. E4 promoter-mediated HSV-TK expression/GCV treatment increases cell viabilities in wt Ad5-infected cultures. A549 cells (A) or primary fibroblasts (B) were either infected with the EGFP expression vector pAdEE4 (indicated as EGFP in the figure) or the HSV-TK expression vector pAdEE4_TK (indicated 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*.

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3. AN ADENOVIRAL VECTOR-BASED EXPRESSION AND DELIVERY SYSTEM FOR THE INHIBITION OF WILD-TYPE ADENOVIRUS REPLICATION BY ARTIFICIAL MICRORNAS

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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 transduced with the recombinant vectors and infected with 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 concatemering 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.

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1. Introduction

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 such as hematopoietic stem cell transplant recipients (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, 2010). To date, the only drug with convincing anti-adenoviral activity *in vivo* is cidofovir (CDV).

However, regarding the treatment of adenoviral infections in immunocompromised patients, 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 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 (Fire et al., 1998). MicroRNAs (miRNAs) are members of this group of small RNAs. Their precursors, primary miRNAs (pri-miRNAs), are processed by Drosha/DGCR8 into 60–70 nt precursor miRNAs (pre-miRNAs) (Cullen, 2004), that are subsequently exported from the nucleus by Exportin-5 (Yi et al., 2003), and eventually processed into mature miRNAs by the ribonuclease-III enzyme Dicer (Cullen, 2004). The so-called guide strand is loaded into the RNA-induced silencing complex (RISC) (Sontheimer, 2005), where it mediates the cleavage or deadenylation of its target mRNA, or leads to translational repression (Huntzinger and Izaurralde, 2011).

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RNAi 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). This is either mediated by synthetic short interfering RNAs (siRNAs) that are directly incorporated into RISC (Elbashir et al., 2001), short hairpin shRNAs that resemble pre-miRNAs (Burnett and Rossi, 2012), or artificial miRNAs (amiRNAs) that are analogs of pri-miRNAs (Zeng et al., 2002). RNAi-mediated inhibition of viral replication has been demonstrated for a wide range of viruses, both *in vitro* and *in vivo* (Arbuthnot, 2010; 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 transduction 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 transduction 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 combined transduction/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'-ATAA GGCTATCTCGAGATACGCCCTGGTCC-3') and Fluc-r2 (5'-AATGT CGTTCGCGGCCCACTGCAACTCCGAT-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 Ad-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 Ad-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 Ad-Fluc-mi1 and Ad-mi-, respectively (Fig. 1).

Construction of amiRNA expression vectors for the targeting of adenoviral mRNAs: amiRNAs were designed using Life Technologies' 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.

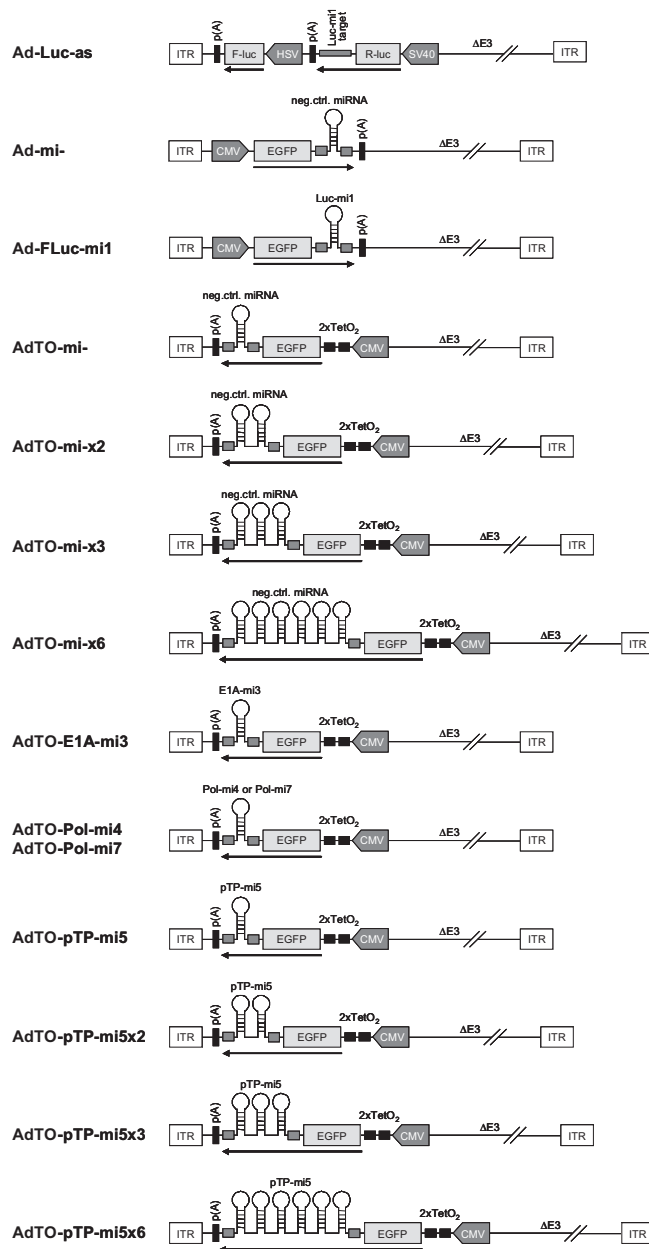


Fig. 1. Schematic representation of adenoviral vectors. All vectors were based on the Ad5-derived vectors pAd/CMV/V5-DEST™ or pAd/PL-DEST™ (both from Life Technologies) and lack the E1 and E3 genes. Expression cassettes were inserted into the deleted E1 region in sense or antisense orientation with respect 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 p(A).

Via these overhangs, the oligonucleotides were inserted into the pre-cut plasmid vector pcDNA6.2-GW/EmGFP-miR (Life Technolo-

gies Austria, Vienna, Austria) giving rise to amiRNA expression vectors for E1A silencing (pmiRE-E1A-mi1 to -mi4), Ad5 DNA

E1A-mi1	5' -UG CUGUUUCUUCGGUA 3' -AGGACAAAGAACCAU--	<u>AU</u> <u>AACACCUCGU</u>	uuuggcc a
E1A-mi2	5' -UG CUGUUCUCUAGAC 3' -AGGACAAAGAUUCUG--	<u>AC</u> <u>AGGUGAUGUGU</u>	uuuggcc a
E1A-mi3	5' -UG CUGUAUUGCAUUCU 3' -AGGACAAAGAACCAU--	<u>CU</u> <u>AGACACAGGU</u>	uuuggcc a
E1A-mi4	5' -UG CUGAUCACAGGUUAC 3' -AGGACUAGUGUCCAAUG--	<u>AC</u> <u>CUUAUGGU</u>	uuuggcc a
Pol-mi1	5' -UG CUGUUAAGUUCUC 3' -AGGACAAUUCAGGAG--	<u>GU</u> <u>AGGUGAGCGU</u>	uuuggcc a
Pol-mi2	5' -UG CUGUGUUGUGGCCCA 3' -AGGACACACACCGGGU--	<u>CA</u> <u>AUGUAAAGU</u>	uuuggcc a
Pol-mi3	5' -UG CUGAUAAGUUGCGUG 3' -AGGACUUAUUCACGCAC--	<u>UG</u> <u>AUGCGGGU</u>	uuuggcc a
Pol-mi4	5' -UG CUGUAUGGUUGGAC 3' -AGGACUACCAACUCUG--	<u>GC</u> <u>UGGAAGACGU</u>	uuuggcc a
Pol-mi5	5' -UG CUGUGGACGUGGAA 3' -AGGACACUUGGACCUU--	<u>GA</u> <u>CGUUGAAGU</u>	uuuggcc a
Pol-mi6	5' -UG CUGUGAUAUGGUUG 3' -AGGACACUUAACCAAC--	<u>GA</u> <u>CGCUGGAAGU</u>	uuuggcc a
Pol-mi7	5' -UG CUGTTGGATGATATG 3' -AGGACAACTACTATAC--	<u>GT</u> <u>TGGACGCGU</u>	uuuggcc a
pTP-mi1	5' -UG CUGUAACGGACCA 3' -AGGACAUUGCCUGGUC--	<u>UU</u> <u>AACGGUCUGU</u>	uuuggcc a
pTP-mi2	5' -UG CUGAUCUCUUGCAU 3' -AGGACUAGAGAACGUA--	<u>CU</u> <u>AUCGUCGCGU</u>	uuuggcc a
pTP-mi3	5' -UG CUGUGAACUGUCUG 3' -AGGACACUUGACGAGC--	<u>AU</u> <u>CUCUCCUGU</u>	uuuggcc a
pTP-mi4	5' -UG CUGAUCAAUUUCGG 3' -AGGACACUUGACGAGC--	<u>UG</u> <u>UCGUUGACGU</u>	uuuggcc a
pTP-mi5	5' -UG CUGAAGAGAGUUCG 3' -AGGACUUCUCUACAGC--	<u>AC</u> <u>AGAAUCAAGU</u>	uuuggcc a

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.

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-miRNAs 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 *BglII*. The vector already harboring one copy was restricted with *Sall* and *BamHI*, 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 *XmnI* and *XhoI* 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'-TTGCATTTCGAATCTGCTTAGGTTAGG-3') and BGHPA-r2 (5'-CCCAGCGAATTCTTCGCTCAGAAG-3'). The *BclI* site located between the promoter/operator region and the BGH poly(A) site was subsequently used for the insertion of the individual EGFP/amiRNA 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'-CAAAAATGATCACTTTAAACCATGGTGAGC-3') and pmiRE-r2 (5'-AAGCTGTGATCAGATATCTCGAGTCGGC-3'). The resulting EGFP/amiRNA expression vectors were termed pTO-mi- (carrying the negative control miRNA), pTO-E1A-mi3 (carrying amiRNA E1A-mi3), 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/chloroform 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 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, the medium was exchanged, and the cells were incubated for another 24 h. 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. Twenty-four hours thereafter, the cells were transduced with Ad-Luc-as at a multiplicity of infection (MOI) of 1 TCID₅₀/cell and either Ad-FLuc-mi1 or Ad-mi-, each at an MOI of 10 TCID₅₀/cell. In the case of A549 cells, the cells were additionally infected with wt Ad5 at an MOI of 100 TCID₅₀/cell. Alternatively, 2×10^4 A549, 1.6×10^5 HEK 293, 1.6×10^5 SW480, or 1×10^4 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 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 transduction with adenoviral miRNA expression vectors at an MOI of 30 TCID₅₀/cell and treatment of the cells with or without 1 μ g/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 Tech-

nologies 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'-TGCACCACCACTGCTTAGC-3', GAPDH-r1 5'-GGCATGGACTGTGGTCATGAG-3', GAPDH-p1 5'-CCTGGCCAAGGTCATCCATGACAACCT-3'), or Ad5 pTP (pTP-cDNA-f2 5'-AAACCAACGCTCGGTGCC-3', pTP-cDNA-r2 5'-GGACGCGTTCAGATGTT-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

3×10^4 A549 cells were seeded into the wells of a 96-well plate and transduced with the recombinant adenoviruses at an MOI of 100 TCID₅₀/cell. Twenty-four hours later, cells were infected with wt Ad5 at an MOI of 0.01. If required, CDV was added to each well in concentrations ranging from 0 to 30 μ M. 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 1×10^5 T-REx-293 cells with the vectors at an MOI of 0.1 TCID₅₀/cell. AmiRNA expression was induced by addition of 1 μ g/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'-GACGGCCCCGAAGATC-3', E1A-rev 5'-TCCTGCACCGCCACATT-3', and E1A-p 5'-CGAGGAGGCGGTTTCGAGA-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'-CACTCATATTCTTACATGCCACTATT-3', hexon-rev 5'-GGCTGTGGGCATAGATTG-3', hexon-probe 5'-AGGAAGGTA ACTACGAGAACTAATGGGCCA -3'). Otherwise, qPCR conditions were as described above.

2.8. FACS analysis

EGFP expression rates were determined by FACS analysis. Cells transduced 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). 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, non-targeting, 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 non-targeting amiRNAs into the deleted E1 region of a replication-deficient, Ad5-based vector, giving rise to the adenoviral miRNA expression vectors Ad-FLuc-mi1 and Ad-mi1-, respectively (Fig. 1). A corresponding adenoviral target vector (Ad-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-transduced 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 after transduction 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, 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 Ad-mi- in Fig. 4. These data demonstrated that the controllable system 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/

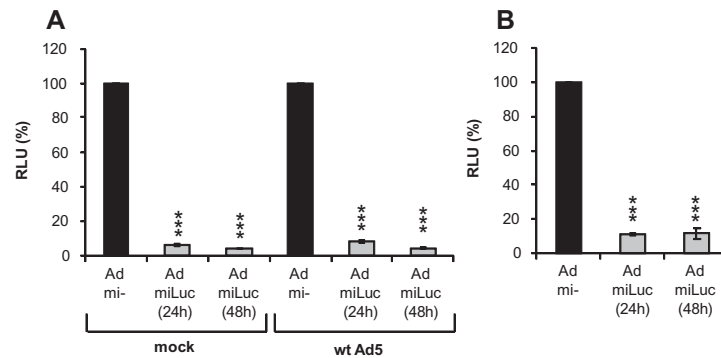


Fig. 3. Artificial miRNA-mediated RNAi in cells infected with wt Ad5 or packaging cells promoting the replication of the adenoviral miRNA expression vectors. A. A549 cells were co-transduced with the adenoviral target vector Ad-Luc-as carrying an miRNA target sequence in the 3'UTR of a *Renilla* luciferase gene at an MOI of 1 TCID₅₀/cell and the adenoviral vector Ad-FLuc-mi1, which produces an amiRNA directed against the target sequence of the target vector at an MOI of 10 TCID₅₀/cell. A corresponding adenoviral vector (Ad-mi-) producing an artificial, non-targeting, 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 set-up as in A, except that superinfection with wt Ad5 was omitted. Data represent relative light units (RLU; mean $\pm$ SD) in comparison to the non-targeting artificial control miRNA from representative triplicate infection experiments. *** $p < 0.001$.

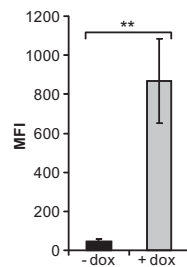


Fig. 4. Expression of the EGFP/miRNA cassette from a tetracycline repressor-controlled CMV promoter is heavily decreased in adenovirus packaging cells in the absence of doxycycline. T-REx-293 cells were transduced with the adenoviral vector AdTO- and incubated with or without doxycycline. Twenty-four 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; $n = 3$). ** $p < 0.01$.

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, non-targeting 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. 5A–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. 5D). 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 amiR-

NAs. 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 AdTO-mi-, AdTO-E1A-mi3, AdTO-Pol-mi4, AdTO-Pol-mi7, and AdTO-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 transduced 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 (Chung et al., 2006; Wu et al., 2011). 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-

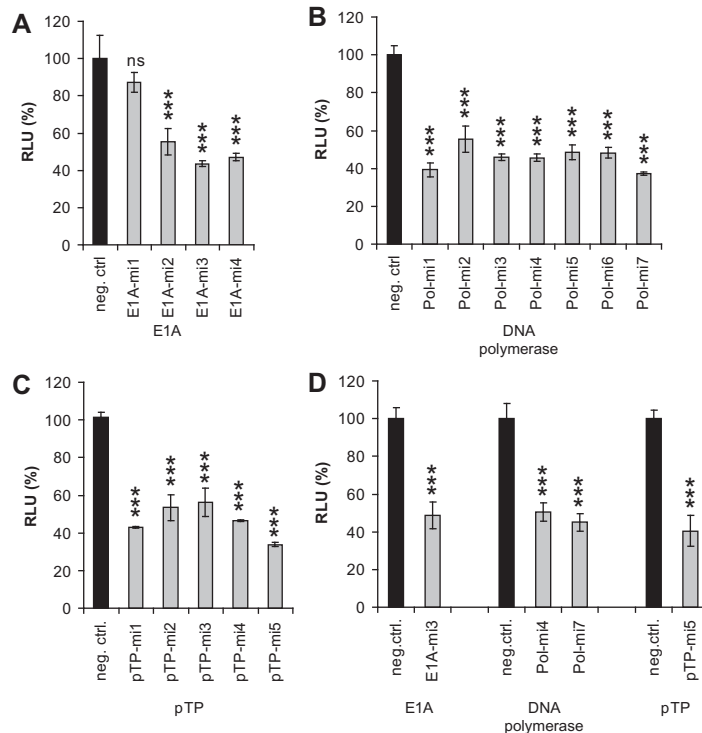


Fig. 5. Screening for functional amiRNAs. Plasmid-based amiRNA expression vectors and their respective dual-luciferase target vectors were used to cotransfect HeLa cells (A, D) or HEK293 cells (B, C). Each group of artificial miRNAs, i.e., those directed against E1A (A), DNA polymerase (B), or pTP (C) 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 non-targeting amiRNA served as a negative control (neg. ctrl.) in all experiments. Relative light units (RLU; mean $\pm$ SD, $n = 3$) in comparison to the non-targeting artificial control miRNA are shown. *** $p < 0.001$; ns: not significant.

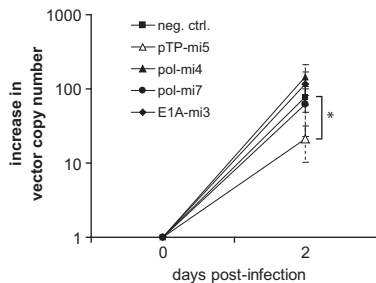


Fig. 6. Replication of adenoviral amiRNA expression vectors is inhibited in packaging cells upon amiRNA expression. T-REx-293 cells were transduced with the amiRNA expression vectors AdTO-mi- (indicated as neg. ctrl.), AdTO-E1A-mi3 (indicated as E1A-mi3), AdTO-Pol-mi4 (indicated as Pol-mi4), AdTO-Pol-mi7 (indicated as Pol-mi7), and AdTO-pTP-mi5 (indicated as pTP-mi5) 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. * $p < 0.05$.

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-encod-

ing 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

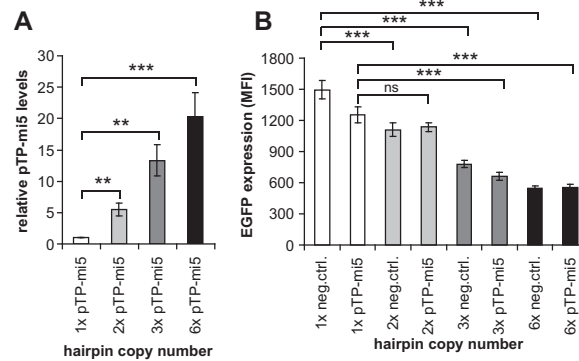


Fig. 7. Concatemerization of amiRNA-encoding sequences increases amiRNA levels and decreases EGFP expression. HEK293 cells were transfected with identical amounts of plasmid expression vectors carrying 1–6 copies of pTP-mi5 (i.e., vectors pmiRE-pTP-mi5, pmiRE-pTP-mi5x2, pmiRE-pTP-mi5x3, and pmiRE-pTP-mi5x6) 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.

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 AdTO-pTP-mi5, AdTO-pTP-mi5x2, AdTO-pTP-mi5x3, and AdTO-pTP-mi5x6), or a corresponding number of copies of the sequence encoding the negative control amiRNA (vectors AdTO-mi-, AdTO-mi-x2, AdTO-mi-x3, and AdTO-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 transduced 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 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 infected with wt Ad5 (Fig. 10A).

To determine whether and to what extent pTP-mi5 inhibited the expression of pTP during virus replication, we transduced A549 cells with the adenoviral pTP-mi5 expression vector AdTO-pTP-mi5x6 or its corresponding negative control amiRNA expression vector AdTO-mi-x6. Subsequently, we infected 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 transduced A549 cells with AdTO-pTP-mi5x6 or the negative control vector AdTO-mi-x6 and infected them with wt Ad5. To assure that all cells were transduced with the recombinant vectors, we used rather high MOIs of 100 TCID₅₀/cell and transduced 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.

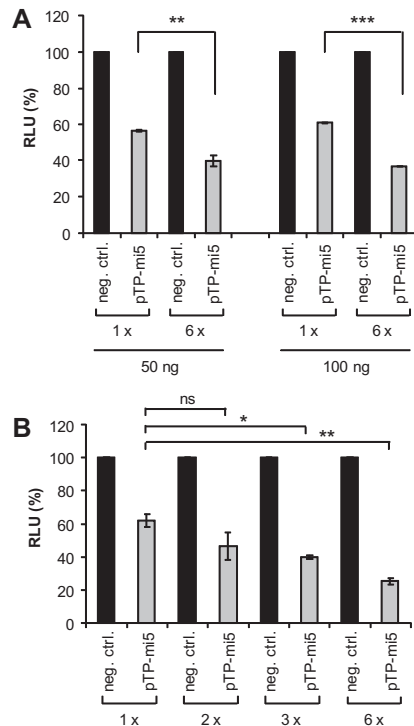


Fig. 8. Concatemerization of pTP-mi5 hairpins increases the knockdown rate. A. HEK 293 cells were transfected with 50 ng of the plasmid vectors pmiRE-pTP-mi5 and pmiRE-pTP-mi5x6 carrying 1 or 6 copies of the sequence encoding pTP-mi5, respectively, and either 50 or 100 ng of the dual-luciferase reporter 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 non-targeting 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 transfected with the adenoviral vectors AdTO-pTP-mi5, AdTO-pTP-mi5x2, AdTO-pTP-mi5x3, or AdTO-pTP-mi5x6 expressing pTP-mi5 or AdTO-mi-, AdTO-mi-x2, AdTO-mi-x3, or AdTO-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.

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 transduced with the pTP-mi5-expressing vector compared to control cultures expressing the negative control amiRNA (Fig. 11B).

The amiRNA-mediated inhibitory effect on wt Ad5 DNA replication was also revealed when the cells were infected with wt Ad5 at higher MOIs of up to 100 TCID₅₀/cell (Supplementary Fig. 2). No differences were observed for MOIs ranging between 0.01 and 1 (Supplementary Fig. 2A–C). At higher MOIs of 10 and 100 (Supplementary Fig. 2D and E) there was a tendency toward a decrease in the difference between wt Ad5 genome copy numbers of cells

transduced with the pTP-mi5 amiRNA expression vector and its negative control vector. However, this decrease was not due to a diminished inhibition of wt Ad5 DNA replication by the amiRNA, because the slope from day 0 to day 2 was comparable for pTP-mi5-expressing cells regardless of which MOI was used for wt Ad5 infection. The observed effect was rather due to a decrease in the gain of wt Ad5 DNA from day 0 to day 2 when cells were infected with wt Ad5 at high MOIs (compare slopes for cells not transduced with any recombinant vector or with the amiRNA control vector at different MOIs).

The inhibitory effect described above was revealed with cells that had been transduced with the recombinant amiRNA expression vector 24 h prior to infection with wt Ad5. However, an inhibitory effect on wt Ad5 replication was also observed when cells were transduced with the pTP-mi5 expression vector only 6 h prior to, concomitant with, or 6 h after infection with wt Ad5 (Supplementary Fig. 3). Wt Ad5 replication was inhibited at all MOIs. However, we observed a tendency toward a slightly decreased inhibition rate when cells were infected with wt Ad5 prior to transduction with the recombinant vectors and when low MOIs were used for wt Ad5 infection (compare slopes for cells transduced with the pTP-mi5 expression vector in panels A, B, and C at wt Ad5 MOIs of 0.01–1).

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 transduced and 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. 12A 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 (Fig. 12C); 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

In contrast to anti-adenoviral siRNAs such as the ones used in our previous study (Kneidinger et al., 2012), the generation of anti-adenoviral amiRNAs is dependent on intracellular processing steps which may be disturbed in adenovirus-infected cells due to the saturation of several components of the RNAi pathway by miRNAs (Andersson et al., 2005; Lu and Cullen, 2004). We estimated the performance of amiRNAs during the first 48 h of

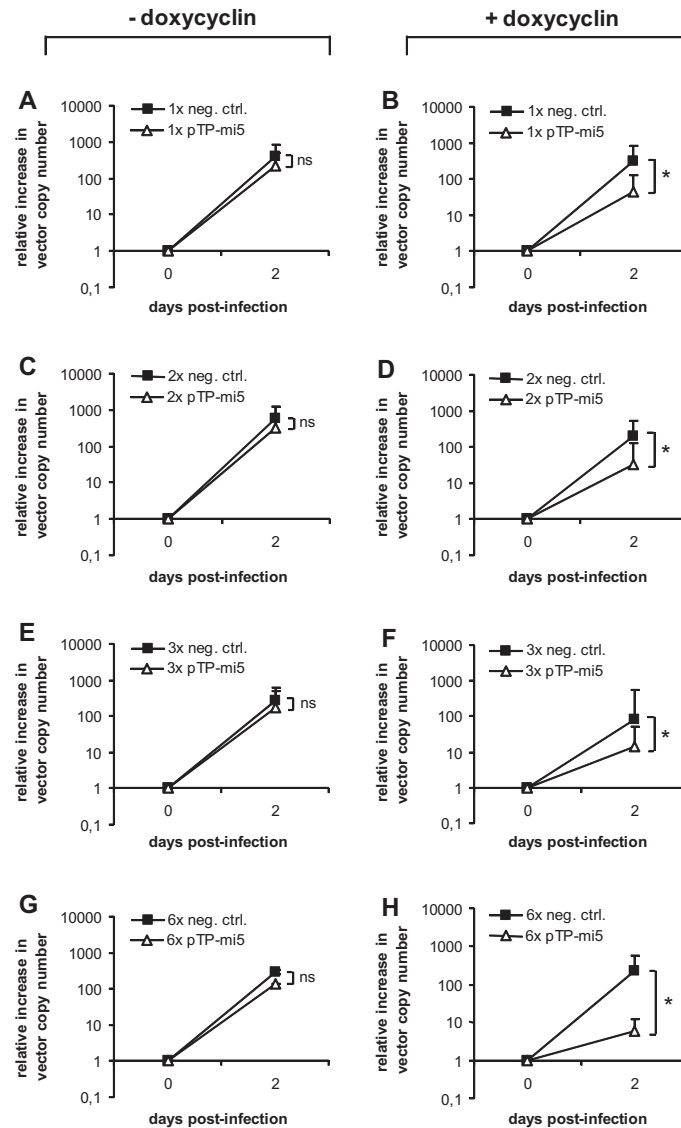


Fig. 9. Concatemerization of pTP-mi5 hairpins increases the inhibition of adenoviral pTP-mi5 expression vectors in packaging cells. T-REx-293 cells were transduced with the adenoviral pTP-mi5 expression vectors AdTO-pTP-mi5, AdTO-pTP-mi5x2, AdTO-pTP-mi5x3, or AdTO-pTP-mi5x6 or their respective negative control vectors AdTO-mi-, AdTO-mi-x2, AdTO-mi-x3, or AdTO-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 transduced 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.

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 an adenoviral vector (Fig. 3). This is likely due to the fact that miRNAs reach high levels only at very late stages of infection, and pTP mRNA-tar-

geting amiRNAs prevent the otherwise steady increase in VA-RNA gene copy numbers after the onset of viral DNA replication.

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 used in our previous study (Kneidinger et al., 2012), these

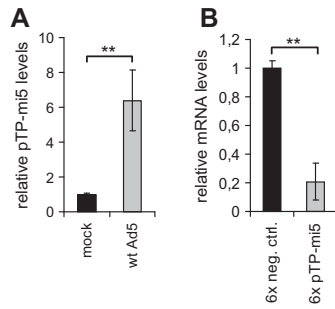


Fig. 10. Adenoviral vector-mediated expression of pTP-mi5 increased upon super-infection with wt Ad5 and led to knockdown of pTP expression. A549 cells were transduced with the adenoviral pTP-mi5 expression vector AdTO-mi-x6 or its respective negative control vector AdTO-mi-x6 at an MOI of 100 TCID₅₀/cell. Twenty-four hours later, cells were infected with wt Ad5 at an MOI of 100 TCID₅₀/cell or were mock-infected. 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; $n = 3$). ** $p < 0.01$.

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.

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

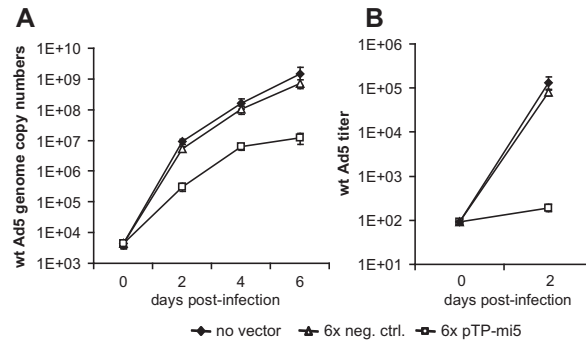


Fig. 11. Adenoviral vector-mediated expression of pTP-mi5 decreases the replication of wt Ad5. A549 cells were transduced with the adenoviral pTP-mi5 expression vector AdTO-pTP-mi5x6 (indicated as 6x pTP-mi5) or its respective negative control vector AdTO-mi-x6 (indicated as neg. ctrl.) at an MOI of 100 TCID₅₀/cell or were mock-transduced (indicated as no vector). Twenty-four hours after infection, cells were infected 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.

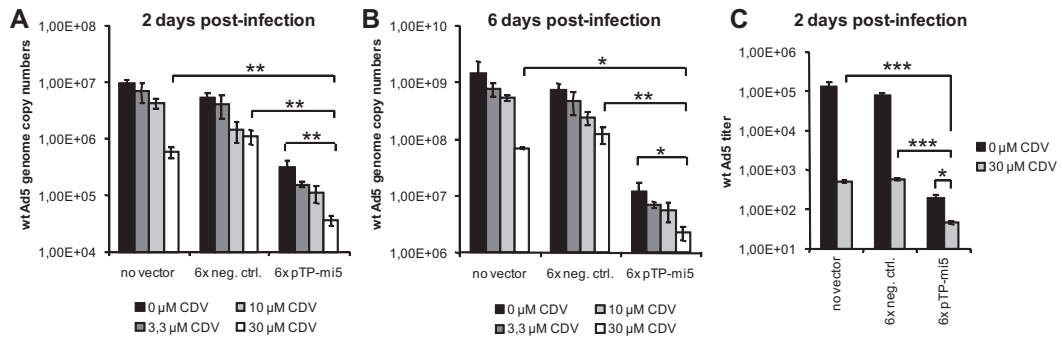


Fig. 12. Adenoviral vector-mediated expression of pTP-mi5 and concomitant treatment with CDV has an additive effect on the inhibition of wt Ad5 replication. A549 cells transduced with AdTO-pTP-mi5x6 at an MOI of 100 TCID₅₀/cell were infected with wt Ad5 at an MOI of 0.01 TCID₅₀/cell and were concomitantly treated with 0, 3.3, 10, or 30 μ M CDV. Two days (A) and 6 days after infection with wt Ad5 (B), 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. C. 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. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

observation made by others when placing a pre-miRNA hairpin onto a miRNA polycistron: when combined with other miRNA hairpins, the silencing capacity of the individual miRNA was increased (Liu et al., 2008).

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 in most experiments 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 miRNAs. Thus, regardless of the readout system, the pTP-mi5-mediated inhibition rate at late time points (4 or 6 days post-infection) is probably underestimated.

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, as a nucleoside analog, acts downstream of this step by preventing DNA polymerization (Cundy, 1999). 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 miRNA. 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 delivery of miRNAs, 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 but are still far from perfect (Rettig and Behlke, 2012). In this regard, the delivery of anti-adenoviral miRNAs, via a replication-deficient adenoviral vector, may have several unique advantages. For example, it may allow for the amplification of miRNA expression cassette copy numbers upon exposure of the recombinant virus to the wt virus as demonstrated in our *in vitro* experiments (Fig. 10) 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 miRNAs predominantly to those cells that are also the preferred targets of the wt virus.

It may be argued that treating a virus infection with a vector derived from the very same virus may generally be dangerous. For example, recombination events between the wt virus and the recombinant virus are conceivable, which may result in the generation of a replication competent virus. This potential problem could generally be circumvented by the generation of “gutted” versions of the vectors presented here. Such gutted adenoviral vectors lack all parts of the viral genome except for the 5′ and 3′ inverted terminal repeats and the packaging signal (Ψ) required for replication and DNA packaging, respectively (Alba et al., 2005). In general, due to the presence of the inhibitory miRNA sequences on the vector, a virus emerging from a recombination between the recombinant virus and the wt virus would be attenuated in its replication. At the same time, such a recombination event would likely render the “donor” wt virus replication-deficient. Thus, the generation of a virus that is more dangerous than the parent wt virus seems unlikely. In any case, this issue would have to be addressed in animal studies.

Such animal studies are also needed to eventually clarify, 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 miRNAs expressed from and delivered by adenoviral vectors (this study), provide a greater probability to permit efficient inhibition of adenovirus multiplication *in vivo*.

5. Conclusion

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

Acknowledgments

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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.2012.10.008>.

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4. CELLULAR TARGETS OF ADENOVIRUS-ENCODED miRNAS

4.1. Rational of the study

In this part of the thesis the focus changes from RNA interference as a tool to inhibit adenoviral infections to the question whether there is an interaction between adenovirus encoded miRNAs and cellular mRNAs and what kind of interaction can that be (for more detail see part 1.3.1.1. in the Introduction). So far, not much is known about the function of the adenoviral miRNAs. It is described that these miRNAs saturate the cellular RNAi machinery at different stages (saturation of Dicer, Exportin 5 and RISC) to interfere with cellular gene expression in a way that is beneficial for the virus [1], [2]. Further on it was described that the apical part (terminal loop) of miRNA VAI functions as a decoy for the double stranded RNA activated protein kinase R (PKR) and inactivates it. Activation of PKR would otherwise lead to an inhibition of protein synthesis by phosphorylating the translation initiation factor eIF2 and thus induces an anti-viral response (reviewed in [3]).

Aparicio et al. used bioinformatics, proteomics and genetics to identify potential cellular targets of adenoviral miRNAs. They found a broad range of potential target genes involved in cell signaling, cell growth and apoptosis, DNA transcription and repair, and in RNA metabolism [4].

The aim of this project was to investigate if miRNAs are really produced just to saturate the cellular RNAi machinery or if there are specific cellular mRNA targets for these adenovirus-encoded miRNAs and what their functions are. Answering this question can give more insight into adenoviral features like how can they produce a latent infection, or how do latent adenoviruses get reactivated. Moreover, a better understanding of the host-pathogen interaction can reveal possible therapeutic options.

4.2. The miRNA mimics

To investigate the functions of the adenovirus-encoded miRNAs, two siRNAs were designed mimicking 2 isoforms of miRNAs derived from VA RNA I (focus on VA RNA I is based on preliminary experiments that had shown higher targeting capacity of VA RNA I compared to VA RNA II). It has been described that depending on the processing of the miRNAs by RNA polymerase III and the enzyme Dicer several miRNA isoforms exist that differ in start positions, length and seed sequences. The predominant versions of the miRNAs are cleaved by Dicer at nucleotide position 137 and 138 [5]. Since miRNA mimics starting at 137, compared to those starting at 138, showed higher prevalence in RNA sequencing data sets (data not shown) we focused

on mivaRNAs starting at position 137. In addition, RNA polymerase III can start the transcription at different nucleotides leading to mivaRNAs of different length. Depending on the start nucleotide, which can either be an A or a G, respective mivaRNA mimics were designed and named mivaRI(A)-137 or mivaRI(G)-137 [6].

Importantly, the seed sequence of the right arms of both version, the A and the G version, are identical whereas the seed sequences of the left arms are different.

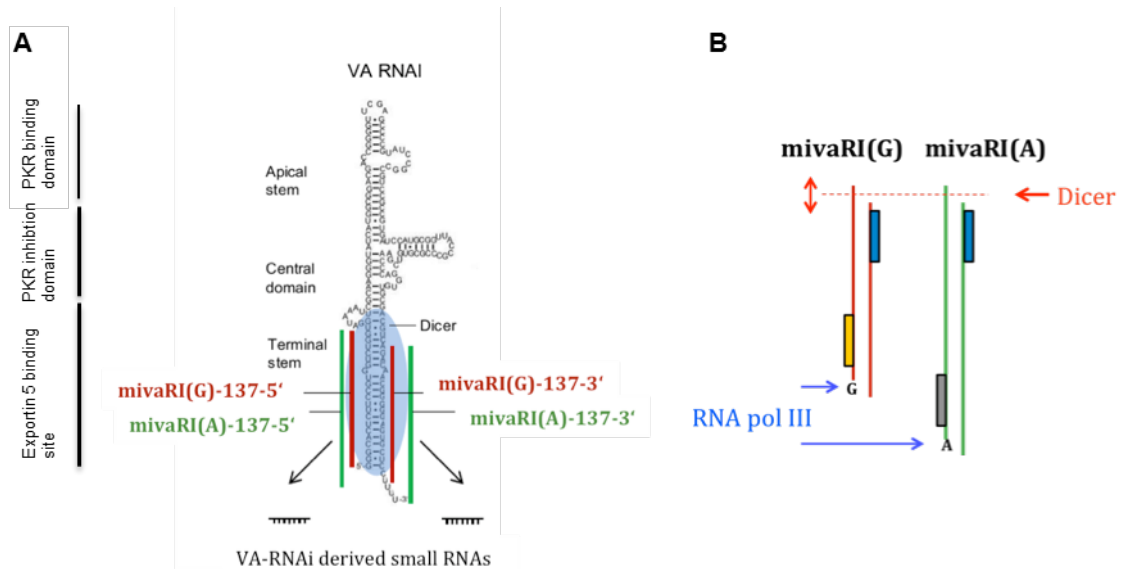


Figure 1 | Structure of the mivaRNA mimics mivaRI(A)-137 and mivaRI(G)-137

Depending on the processing of the adenovirus-encoded VA-RNAI two mivaRNA mimics were designed, named mivaRI(A)-137 and mivaRI(G)-137. On the one hand, RNA polymerase III can start the transcription of the mivaRNAs at different nucleotides that can either be an A or a G. On the other hand, Dicer processes the opposite end of the mivaRNAs and can either cut at nucleotide position 137 or 138. Thus, we designed two mivaRNA mimics with a focus on the version starting at the nucleotide position 137, which is slightly more prevalent in Ad-infected cells. **A.** The red lines represent the single strands of the mivaRI(G)-137 mimic, the green lines those of the mivaRI(A)-137 mimic. The black lines indicate the Exportin 5 binding site (terminal stem), the PKR inhibition domain (central domain) and the PKR binding domain (apical stem) (adapted from [2]). **B.** Schematic drawings of mivaRI(A)-137 and mivaRI(G)-137. The blue boxes show the identical seed sequence of the right arm of the mivaRNAs. The yellow and gray boxes represent the seed sequences of the left arms of the mivaRNAs, which are different.

That means, if the right arm of both miRNAs functions as guide strand and is incorporated into RISC, both miRNA mimics can bind to the same target sequences. In contrast, if the left strand of the miRNAs fulfills the function of the targeting guide strand miRNA(A)-137 and miRNA(G)-137 can bind to different targets, due to their different seed sequences (Fig. 1). Therefore, depending on the silencing capacities of both miRNA mimics, it should be feasible to identify which strand preferentially functions as the guide strand. If both miRNA mimics show similar reductions of their targets, this would indicate that the right strand is the predominant guide strand. If a difference in the reducing potencies of both miRNA mimics is measurable, this would suggest that the left arms are preferentially incorporated into RISC and function as guide strands.

4.2.1. Target mRNA identification by RIP-Chip assay

To identify potential target mRNAs of adenovirus-encoded miRNAs, a RIP-Chip assay was established by Bellutti F, MSc in our lab [7]. Therefore, A549 cells were transfected with miRNA-mimics or infected with adenovirus 5. As a control, cells were transfected with a negative control siRNA or remained non-infected, respectively. Following, RISCs were immunoprecipitated (RISC IP) using an antibody binding all four Argonaute proteins (anti-AGO1-4), and an IgG-antibody served as isotype control. From the immunoprecipitated RISC fraction mRNA was isolated and subjected to microarray analysis to identify RISC-associated cellular mRNAs (RIP-Chip) (Fig. 2). Fig. 3 shows a heat-map analysis depicting a comparison of mRNA levels from cells transfected with either negative control siRNA or miRNA mimics. Genes identified to be enriched in miRNA transfected cells compared to control cells were used for further validation experiments.

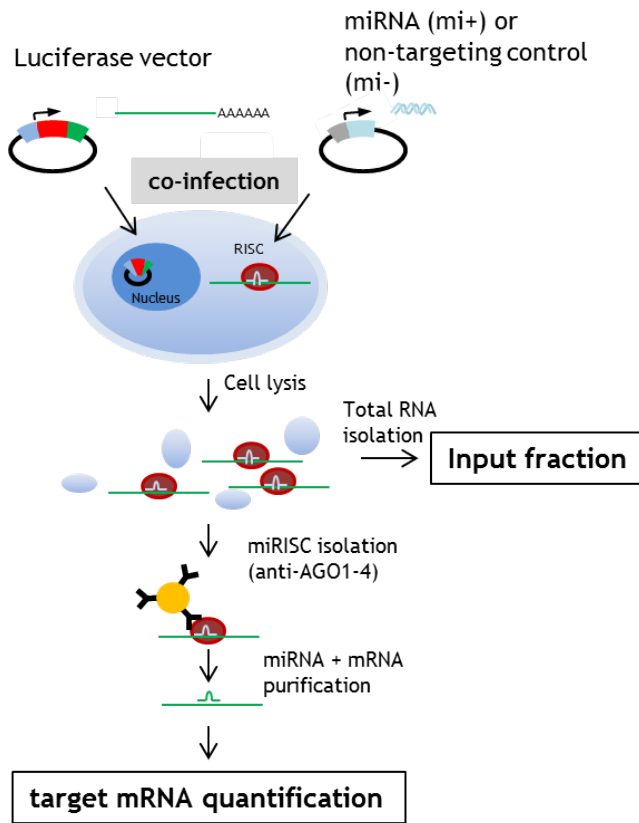


Figure 2 | Establishment of the RISC-immunoprecipitation method (taken from[7])

To establish and proof that the RISC IP is suitable for our approach, HEK293 cells were co-infected with, first, an adenoviral vector carrying the *Renilla* luciferase gene (pAd-Luc-as, R. Klein, unpublished) and a second adenoviral vector carrying an artificial miRNA specifically targeting the 3'UTR of the *Renilla* luciferase mRNA (mi+; pAd-FLuc-mi1, R. Klein, unpublished). Another adenoviral vector expressing a non-targeting miRNA gene (mi-; pAd-mi-, R. Klein, unpublished) was used as a control. 24h after infection, the cells were lysed and RISCs were isolated using antibodies detecting all four Argonaute proteins (anti-AGO1-4). Total RNA from lysed cells served as input fraction. Further on, RNA was isolated from the AGO1-4 immunoprecipitated fraction. This fraction contains the target sequence of the miRNA that was incorporated into RISC together with the specific miRNA. The mRNA was quantified with qPCR.

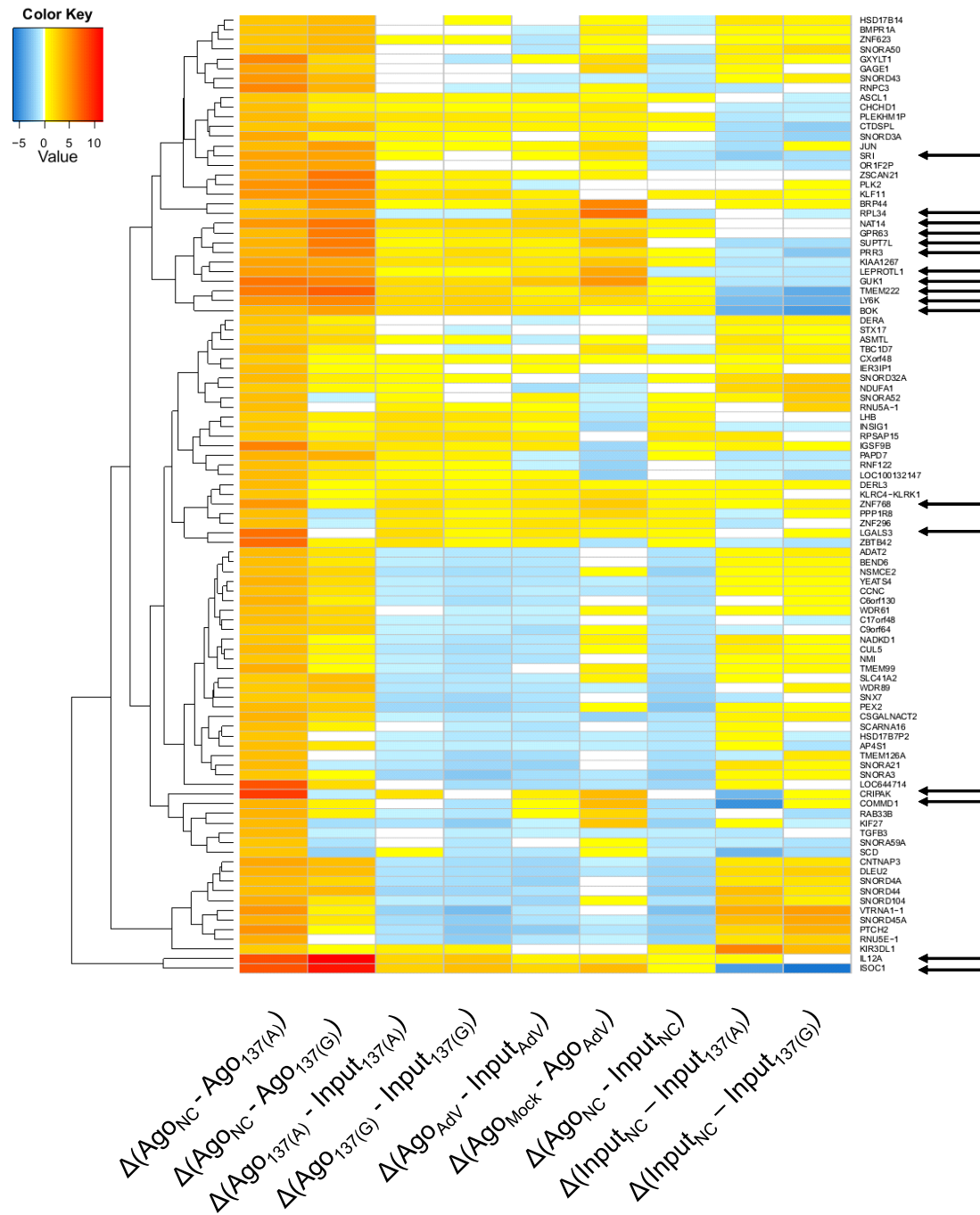


Figure 3 | Heatmap showing differences (Δ) between microarray groups [7].

This heatmap displays the differences in mRNA levels from immunoprecipitated RISCs (Ago) from samples transfected with either miRNA mimics ($\text{Ago}_{137(\text{A})}$ and $\text{Ago}_{137(\text{G})}$) or with the negative control siRNA (Ago_{NC}). In addition, mRNA levels obtained from immunoprecipitated RISCs of adenovirus 5 infected cells (Ago_{Adv}) were compared to non-infected cells (Ago_{Mock}). As further control, input fractions were compared to their corresponding RISC fractions. The arrows indicate the transcripts that were used for further analysis.

Tab. 1 and Tab. 2 show a list of target genes identified by RIP-Chip analysis and selected for further validation. Their enrichment in RISC after mivaRI(A)-137 or mivaRI(G)-137 transfection compared to control conditions or an adenoviral infection (AdV) is depicted.

COMMD1 was included in this validation analysis, because it was one of the top ranked genes in the TargetScan analysis (Tab. 3).

Most of the identified genes showed similar enrichment levels for both mivaRNA mimics. CRIPAK, COMMD1, ZNF768; GXYLT1 are mRNAs that are higher enriched in RISC fractions from cells transfected with mivaRI(A)-137. That might indicate that the CRIPAK mRNA is a target of the left arm of the mivaRI(A) version, because the right arms of the A and the G mivaRNA isoform share the same seed sequence.

Table 1 | Putative targets of mivaRI(A)-137 identified by RIP-Chip [7]

Genes were sorted according to the enrichment following mivaRI(A)-137 transfection

Name	Ago NC - Ago 137 (A)	Ago Mock - Ago AdV
CRIPAK	3.89	8.51
IL-12A	3.56	2.43
ISOC1	3.39	8.75
LGALS3	2.83	2.04
GUK1	2.69	18.51
TMEM222	2.6	4.5
GXYLT1	2.39	3.43
NAT14	2.14	3.05
LY6K	2.13	3.56
ZNF768	2.11	4.99
LEPROTL1	2.1	12.13
SRI	1.99	3.03
PRR3	1.79	2.83
COMMD1	1.77	7.94
SUPT7L	1.74	8.75
GPR63	1.72	5.7
BOK	1.72	1.52
RPL34	1.66	56.1
BRP44	1.53	29.45
COMMD9	1.51	3.1
SMAGP	1.43	4.23

Table 2 | Putative targets of mivaRI(G)-137 identified by RIP-Chip [7]

Genes were sorted according to the enrichment following mivaRI(G)-137 transfection

Name	Ago NC - Ago 137 (G)	Ago Mock - Ago AdV
IL-12A	5.66	2.43
ISOC1	4.76	8.75
TMEM222	3.01	4.5
NAT14	2.58	3.05
GPR63	2.55	5.7
SUPT7L	2.5	8.75
GUK1	2.43	18.51
PRR3	2.41	2.83
LY6K	2.3	3.56
COMMD9	2.23	3.1
BRP44	2.16	29.45
LEPROTL1	2.03	12.13
BOK	1.93	1.52
SRI	1.92	3.03
RPL34	1.79	56.1
SMAGP	1.79	4.23
GXYLT1	1.42	3.43
COMMD1	1.17	7.94
ZNF768	1.13	4.99
LGALS3	1.01	2.04
CRIPAK	0.91	8.51

In addition to the RIP-Chip analysis targets were also compared to predicted target sites identified by a TargetScan analysis (Tab. 3). For that analysis seed sequences of the distinct miRNAs were used as input and target sites were divided into sites located within the UTR or the coding sequence of a target mRNA. Since for some targets multiple hits were identified, the minimum score was used as a criterion to predict the targeting efficiency.

Table 3 | Predicted target sites on transcripts identified by RIP-Chip [7]

Name	minimal score cds	hitCount cds	minimal score utr	hitCount utr	Putative targets
BOK	-0.49	1	-0.47	1	mival137(A)-3'; mival137(G)-3'
COMMD1	-0.63	3	-0.62	1	mival137(A)-5'
COMMD9	-0.12	5	-	-	mival137(A)-3'; mival137(G)-3'
CRIPAK	-0.33	129	-	-	mival137(A)-5'; mival137(G)-5'
GPR63	-0.19	3	-	-	mival137(A)-3'; mival137(G)-3'
GUK1	-0.23	5	-	-	mival137(G)-5'
IL-12A	-0.44	5	-0.45	1	mival137(A)-3'; mival137(G)-3'
LEPROTL1	-0.24	6	-0.08	1	mival137(A)-5'; mival137(G)-5'
LGALS3	-0.22	3	-	-	mival137(A)-5'; mival137(G)-5'
LY6K	-0.46	9	-0.35	3	mival137(A)-3'; mival137(G)-3'
PRR3	-0.29	119	-0.19	4	mival137(A)-3'; mival137(G)-3'; mival(A)137-5'
RPL34	-0.16	5	-	-	mival137(A)-3'; mival137(G)-3'; mival(G)137-5'
SMAGP	-0.18	7	-	-	mival137(A)-3'; mival(A)137-5'
SRI	-0.38	3	-0.18	2	mival137(A)-3'; mival137(G)-3'
SUPT7L	-0.24	9	-0.22	2	mival137(A)-5'
TMEM222	-0.32	23	-0.32	4	mival137(A)-3'; mival137(G)-3'; mival(G)137-5'
ZNF768	-0.31	4	-	-	mival137(A)-5'
ISOC1	-	-	-	-	-
NAT14	-	-	-	-	-

cds, coding sequence; utr, untranslated region;

In addition to the so far selected target genes I also included COMMD7 to complete the COMMD group for further validation. Tab. 8 summarizes our potential target genes and their known functions.

4.3. Materials and methods

4.3.1. 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. Ad5 (ATCC VR-5) was amplified in HEK293 cells that were grown in cell culture flasks until they were 80% confluent. Upon showing cytopathic effects, supernatants and cells were harvested and subjected to three freeze (liquid nitrogen) – thawing (37°C water bath) cycles to lyse the cells. Cells plus supernatant were centrifuged for 15 min at 3000 rpm to pellet cell debris. The supernatant was filtered through a 0.2µm filter to obtain an adenovirus crude stock. Infectious virus particle titers were determined on 293 cells by 50% tissue culture infective dose (TCID₅₀) assays.

4.3.2. Vector construction

4.3.2.1. Luciferase reporter vector construction

For the construction of luciferase reporter vectors to validate putative target genes, the TargetScan algorithm was used to predict putative target sites within the different genes. These target sites, either lying within the 3'UTR, the 5'UTR, or the coding sequence (CDS) of the genes, were inserted into the 3'UTR (3'UTR and CDS sites) or the 5'UTR (5'UTR sites), respectively, of the *Renilla* luciferase gene into the vector psiCHECK-2 (Promega, Mannheim, Germany).

For the construction of the reporter vectors psiCHECK-IL12A, psiCHECK-RPL34 and psiCHECK-SRI, parts of the cDNA sequences were amplified by PCR using primers for IL-12A, SRI and RPL34 cDNA listed in Tab. 4.

Table 4 | Primer sequences used for InFusion cloning

Target	Forward primer (sequence 5' → 3')	Reverse primer (sequence 5' → 3')
IL-12A	CCCGGGAATTCGTTTATTCGGGCA GTGACTATTGA	GGCCGCTCTAGGTTTTCAGATGCT TTCATGATTACC
RPL34	CCCGGGAATTCGTTTTTCTTCCTC TTCCGGGG	GGCCGCTCTAGGTTTGCCCTGCTG ACATGTTTCTT
SRI	CCCGGGAATTCGTTTATTCTAATGC TGGCTTGT	GGCCGCTCTAGGTTTCTTAGAGAAA ATGTTTTATTTTC

The PCR fragments were inserted into the PmeI site of psiCHECK-2 all located within the 3' UTR of the Renilla luciferase gene using the InFusion cloning kit from Clontech (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France).

Table 5 | Luciferase reporter vectors – target sites within the 3'UTR

Reporter vector name	Accession number target gene (mRNA)	Nucleotide position
psiCHECK-BOK	NM_032515.4	15282 - 15406
psiCHECK-BRP44	NM_001143674.2	1051 - 1603
psiCHECK-COMMD1	NM_152516.2	585 - 712
psiCHECK-COMMD7	NM_053041.2	1221 - 1880
psiCHECK-COMMD9	NM_014186.3	41 - 224
psiCHECK-GUK1-1	NW_004078000.1	2 - 150
psiCHECK-GUK1-2	NM_001159390.1	48 - 138
psiCHECK-GXYLT1	NM_173601.1	4281 - 6976
psiCHECK-IL-12A	NM_000882.3	933 - 1340
psiCHECK-ISOC1	NM_016048.2	1261 - 1627
psiCHECK-LY6K-1	NM_017527.3	488 - 633
psiCHECK-LY6K-2	NM_017527.3	841 - 1051
psiCHECK-NAT14	NM_020378.3	739 - 1350
psiCHECK-PRR3-1	NM_025263.3	981 - 1117
psiCHECK-PRR3-2	NM_025263.3	1541 - 1733
psiCHECK-RPL34	NM_000995.3	5 - 274
psiCHECK-SMAGP	NM_001031628.1	36- 439
psiCHECK-SRI	NM_003130.3	1671 - 2016
psiCHECK-SUPT7L	NM_014860.1	1750 - 3211
psiCHECK-TMEM222	NM_032125.2	771 - 1630
psiCHECK-ZNF768	NM_024671.3	1151 - 1610
psiCHECK-CRIPAK	NM_175918	2924 - 3701

Luciferase reporter vectors – target sites within the 5'UTR

Reporter vector name	Accession number target gene (mRNA)	Nucleotide position
psiCHECK-GPR63	NM_030784.2	1 - 225
psiCHECK-GXYLT1	NM_173601.1	1 - 225
psiCHECK-LEPROTL1	NM_015344.2	1 - 107
psiCHECK-LGALS3	NM_002306.3	1 - 88
psiCHECK-NAT14	NM_020378.3	2 - 117
psiCHECK-RPL34	NM_000995.3	1 - 44
psiCHECK-SMAGP	NM_001031628.1	1 - 201
psiCHECK-SUPT7L	NM_014860.1	30 - 141

For the construction of psiCHECK-CRIPAK, a pGEX-5X-2-CRIPAK vector was used containing the cDNA fragment of the complete open reading frame of CRIPAK (gladly provided by Talukder et al. [8]). To obtain the CRIPAK sequence the vector was cut with BamHI. This fragment was gel eluted, blunted, and purified with the PCR purification kit from Qiagen (Hilden, Germany). The CRIPAK sequence was subsequently inserted into the psiCHECK-2 vector that was linearized with PmeI.

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 HotStarTaq DNA Polymerase obtained from Qiagen (Hilden, Germany).

The other reporter vectors were synthesized by Biomatik (Ontario, Canada). The sequences were inserted into either the 3'UTR via the SgfI/PmeI sites or the 5'UTR via the AatI/NheI sites of the *Renilla* luciferase gene of the psiCHECK-2. The coordinates of the cloned sequences are shown in Tab. 5.

4.3.3. Synthesis and transfection of mivaRNA mimics

Single-stranded RNA oligonucleotides (complementary sense and antisense sequence of the respective mimic; Tab. 6) were purchased from RiboTask (Odense, Denmark). Oligonucleotides were annealed to produce dsRNAs. Therefore, 20µM of the corresponding single-strands were combined in a 75µL reaction containing 50mM Tris (pH 7.5) and 100nM NaCl in nuclease-free H₂O. The solution was incubated for 2 min at 95°C and allowed to slowly cool down to room temperature for 1 hour. The double-stranded mivaRNA mimics were analysed by gel electrophoresis on a 4% agarose gel and stored at -80°C.

Table 6 | Sequences of the RNA oligonucleotides used for producing mivaRNA mimics

RNA oligo name	Sequence (5' to 3')
mivaRI(A)-137-5'	AGCGGGCACUCUCCGUGGUCUGG
mivaRI(A)-137-3'	AGACAACGGGGGAGUGCUCCUUUU
mivaRI(G)-137-5'	GGGCACUCUCCGUGGUCUGG
mivaRI(G)-137-3'	AGACAACGGGGGAGUGCUCCU

4.3.4. Luciferase reporter gene assay

4.3.4.1. mivaRNA mimics

1.2e+05 HEK293 cells were seeded into the wells of 96-well plates, and co-transfected with 50ng of individual dual-luciferase reporter vectors and 100nM mivaRNA mimic or non-targeting control siRNA (Ambion/Life Technologies Austria, Vienna, Austria). Transfections were performed using Lipofectamine 2000 (Invitrogen/Life Technologies Austria, Vienna, Austria). For each well, 0.75µL Lipofectamine 2000 were diluted with 24.25µL OptiMEM medium (Invitrogen/Life Technologies Austria, Vienna, Austria) and incubated for 5 min. In the next step, 25µL of a specific siRNA/reporter vector mix (diluted in OptiMEM) were added. Mixes were incubated for 20 min and pipetted directly into the wells of a 96-well plate and freshly harvested cells were added. After 24h, medium was exchanged with fresh medium.

Firefly and *Renilla* luciferase activities were determined at 48h post-transfection using the Dual-Glo luciferase assay (Promega, Mannheim, Germany), according to the manufacturer's instructions. Luminescence was measured on an EnSpire® Multimode Plate Reader (PerkinElmer, Brunn am Gebirge, Österreich).

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.

4.3.4.2. pAdVantage vector

The experimental setup remains the same as described above. Instead of transfecting the cells with mivaRNA mimics, 200ng of pAdVantage vector (Promega, Mannheim, Germany) were transfected in combination with the luciferase reporter vectors.

Firefly and *Renilla* luciferase activities were determined at 48h post-transfection using the Dual-Glo luciferase assay (Promega, Mannheim, Germany), according to the manufacturer's instructions. Luminescence was measured on an EnSpire® Multimode Plate Reader (PerkinElmer, Brunn am Gebirge, Österreich).

Knockdown rates were calculated by normalizing *Renilla* luciferase activities to firefly luciferase activities, and comparing dual-luciferase ratios between cells transfected with or without pAdVantage vectors and the corresponding reporter vector. In a further step, these reactions were normalized to cells transfected with the psiCHECK-2 vector carrying no target sequence.

4.3.5. Nucleic acid extraction

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

4.3.6. cDNA synthesis and determination of mRNA levels

1e+05 A549 cells were seeded into the wells of 24-well plates, and transfected with mivaRNA mimics or a non-targeting control siRNA (Ambion/Life Technologies Austria, Vienna, Austria) at a concentration of 100nM using Lipofectamine 2000 (Invitrogen/Life Technologies Austria, Vienna, Austria). For each well, 1µL Lipofectamine 2000 was diluted with 49µL OptiMEM medium (Invitrogen/Life Technologies Austria, Vienna, Austria) and incubated for 5 min. In the next step, 50µL of a specific mivaRNA mimic (diluted in OptiMEM) were added. Mixes were incubated for 20 min and pipetted directly into the wells of a 24-well plate and freshly harvested cells were added. After 24h, medium was exchanged with fresh medium.

Total RNA was isolated at 48h post-transfection using an RNeasy Mini® Kit (QIAGEN, Hilden, Germany). Residual DNA was removed with RQ1 DNase (Promega, Mannheim, Germany), and reverse transcription was carried out using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems/Life Technologies Austria, Vienna, Austria). All qPCR reactions were performed in duplicates, with the iQ™ SYBR® Green Supermix (Bio-Rad Laboratories Ges.m.b.H., Vienna, Austria) on a LightCycler 480 system (Roche Applied Science, Vienna, Austria). For each reaction 600nM of each primer was combined with 2µL 1:10 diluted cDNA in a reaction volume of 20µL. Thermocycling conditions consisted of a pre-denaturation step at 95°C, followed by 50 cycles of amplification: denaturation 10 sec at 95°C, annealing for 10 sec at 63°C, and elongation for 15 sec at 72°C. Primer sequences are listed in Tab. 7. Cellular target gene expression levels were normalized to GAPDH expression levels.

Table 7 | Primers used for RT-qPCR to validate microarray data

Primer name	Sequence (5' → 3')	Primer name	Sequence (5' → 3')
CRIPAK-f1	GAGTCAGACGCTGTTACCGT	COMMD1-f1	GCTACGGAGCCAGCTATATCC
CRIPAK-r1	ACGTTATCTCGAGGTGCAGAG	COMMD1-r1	GGTTTGAGCAGTCAAGAATGCC
IL-12A-f1	TGCTCCAGAAGGCCAGACAA	COMMD7-f1	TCTCTGAATTTGCCACCACCA
IL-12A-r1	AAACAGGCCTCCACTGTGCT	COMMD7-r1	GCACCATTGGAACCAGAAGG
ISOC1-f1	CCGAGGAGTCGAGGTTTAC	COMMD9-f1	CGAGGCAATTCTGGCTCTCTT
ISOC1-r1	TATGATCCCGGTTTCGAGCGA	COMMD9-r1	GGCTTCGGTTCTCCAAGTAGAC
TMEM222-f1	TGCTCTACGGGAAGTACGTCA	COMMD9-f4	GCTCAAGGCCTCCTCGAAA
TMEM222-r1	CCGGAGGTTAAAGACCAGGC	COMMD9-r4	CTGGAGCAGTTCTCTGCCT
GPR63-f1	CCGTAGGAAACCCCGACCTG	NAT14-f1	GCGGGAGATGAGGGAAGATG
GPR63-r1	GCCTGGTAGCCTGGATTGGT	NAT14-r1	GTTTTCCGTGTCTTTCACGC
SUPT7L-f1	GGAGGAACCTGTGAGCGACA	SMAGP-f1	ACCAGCCTCCTGACTACTCC
SUPT7L-r1	CCCAAGCACTCCCATAGGCA	SMAGP-r1	CTGCAATGAGTGCTGTGCTG
GUK1-f1	GTATGGCACGAGCAAGGTG	BOK-f1	CAGTCTGAGCCTGTGGTGAC
GUK1-r1	GCGGCTGCACAGAGATGTAG	BOK-r1	GCATACAGGGACACCACCTT
GUK1-f4	TAGCATCTCCTCGAACAGTGG	LGALS3-f1	TGGTGCCTCGCATGCTGATA
GUK1-r4	TGGCGGCATAATCTCCTCCT	LGALS3-r1	GCGTGGGTTAAAGTGAAGGC
PRR3-f1	CCTCGAAGGCTCAAAAGCTG	RPL34-f1	TGGTGGTTCCATGTGTGCTAA
PRR3-r1	CGGATTTGTCTTCCATAACCTGG	RPL34-r1	GCTTGTGCCTTCAACACTTTC
LY6K-f1	ACCTGACTGCGAGACAACGA	ZNF768-f1	AGGATGTGCAGAGTTCTGACG
LY6K-r1	TGGGTTCTGGCACTCGAAAGT	ZNF768-r1	TCGACTTCATAGTCCCCACTG
LEPROTL1-f1	TTTGATGCTTGGATGTGCCCT	GAPDH-f1	TGCACCACCAACTGCTTAGC
LEPROTL1-r1	GCCCGTTGTAAGAAAGATGGC	GAPDH-r1	GGCATGGACTGTGGTCATGAG
SRI-f1	CAGAAGGCCCTGACAACAATG		
SRI-r1	GGCGATGTAGTCGTCAAGG		

4.3.7. Statistical analysis

All 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.

4.4. Results

4.4.1. Target gene mRNA validation

To validate the so far identified target genes (Tab. 8), A549 cells were transfected with the VA-RNA mimics miva137(A) and miva137(G) and mRNA levels of the targets were measured by RT-qPCR. Results for each individual target are shown in Fig. 4. mRNA levels were normalized to GAPDH mRNA levels, and normalized values of cells transfected with a mivaRNA mimic and the control siRNA were compared. For some genes similar mRNA reduction rates were obtained (up to 80% compared to the control

conditions) with both miRNA mimics (BRP44, COMMD7, COMMD9, GXYLT1, RPL34 and TMEM222) whereas COMMD1 and SRI led to higher reduction rates when miRNA(A)-137 mimic was used. The target genes BOK, GPR63, GUK, ISOC1, LEPROTL1, LY6K, PRR3, SMAGP and SUPT7L reached higher mRNA level reduction with the miRNA(G)-137 mimic.

Some of the cellular targets (CRIPAK, IL-12A, LGALS3, NAT14 and ZNF768) showed no reduction upon miRNA mimic transfection. The failure to detect a reduction of mRNA levels of those genes may either be due to an artificial interaction of the miRNAs and the mRNAs in the RIP-Chip assay, or may indicate a post-transcriptional downregulation on the protein level but not on the RNA level.

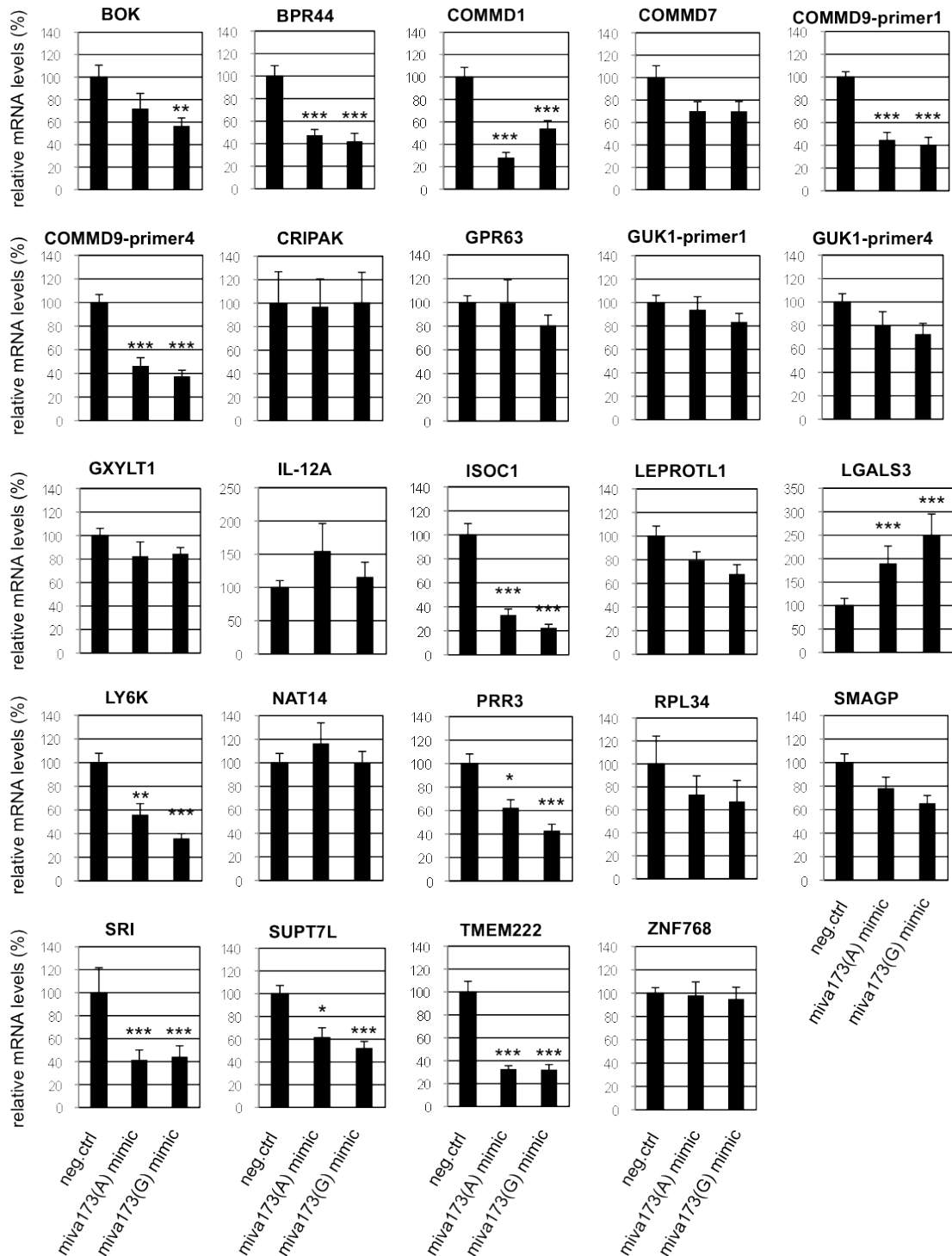


Figure 4 | mRNA level reduction of target genes after transfecting cells with mivaRNA mimics.

A549 cells were transfected with mivaRNA mimics and mRNA levels were measured 48h after transfection by RT-qPCR. mRNA levels of target genes were normalized to GAPDH levels and to a non-targeting negative control siRNA. mRNA levels were shown as % compared to the negative control siRNA. For the targets COMMD9 and GUK1 two primer pairs were used named primer1 and primer4. * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.005

4.4.2. Luciferase reporter gene assay

Dual-luciferase reporter gene assays were used to answer the question whether the miRNA mimics or the adenovirus-encoded miRNAs can mediate Ago2-guided target mRNA reduction. For that purpose reporter constructs were engineered basing on the psiCHECKTM-2 vector, which carries two luciferase genes, the firefly luciferase and the *Renilla* luciferase gene. Transfecting cells with these reporter vectors results in the production of luciferase proteins whose luminescence is measurable.

The target sequences of the identified target genes were cloned either into the 3'UTR of the *Renilla* luciferase gene of the reporter vector or the 5'UTR of the *Renilla* luciferase gene, depending on the naturally occurring position of the target sequence for each target gene. The resulting fusion mRNA should be degraded upon binding of miRNAs leading to a decreased *Renilla* luciferase activity.

HEK293 cells were co-transfected with the reporter vectors and either the miRNA mimics mivaRI(A)-137 and mivaRI(G)-137 or with the pAdVantage vector that produces the adenovirus-encoded miRNAs (Fig. 5). Firefly and *Renilla* luciferase signals were measured to validate the ability of either the miRNA mimics or the virally encoded miRNAs to destruct the target mRNAs and lead to translational inhibition.

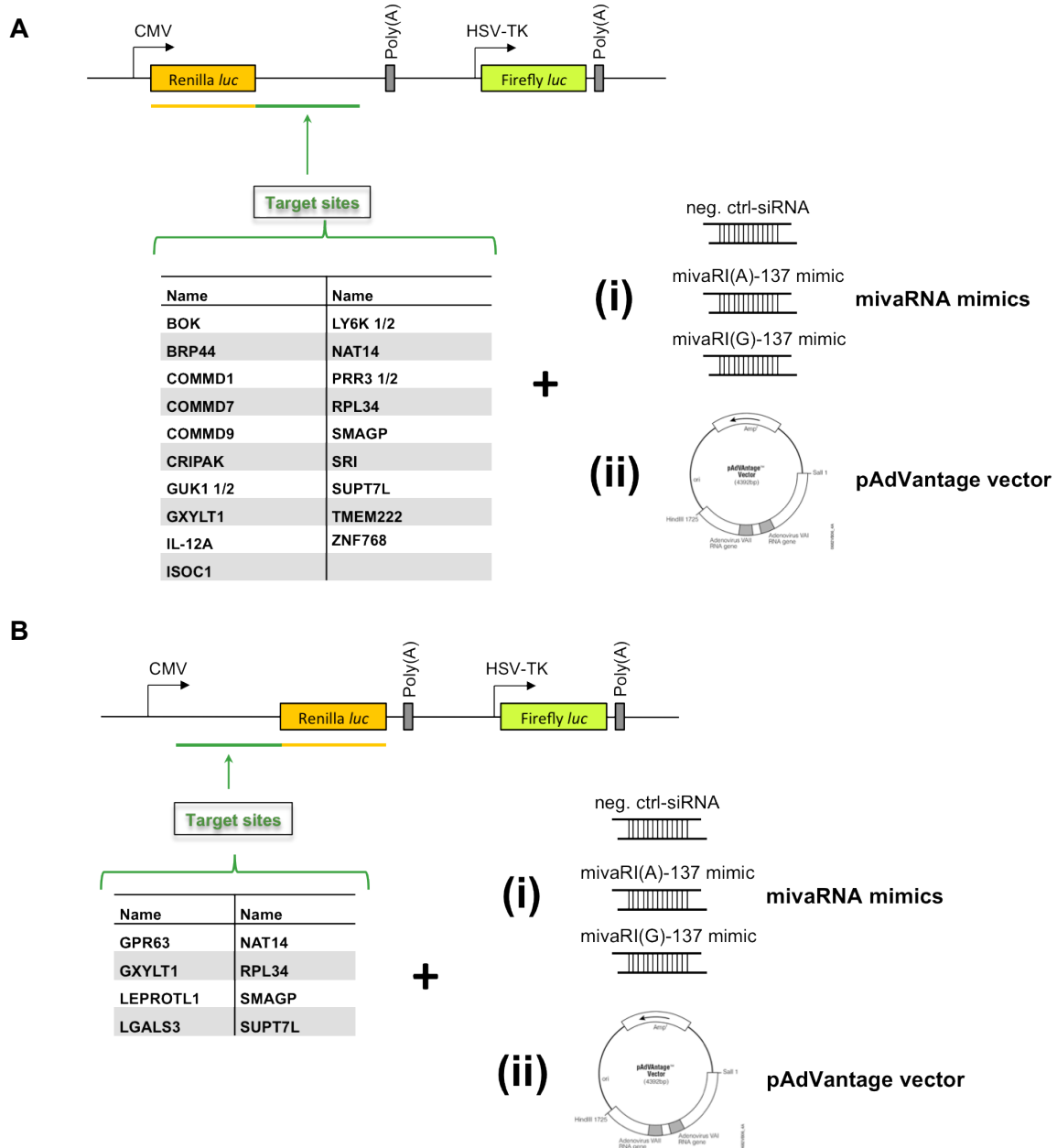


Figure 5 | Cloning strategies for dual-luciferase reporter vectors

For the dual-luciferase reporter gene assay the psiCHECKTM-2 vector was used as backbone that carries the firefly luciferase gene under the control of the CMV promoter, and the *Renilla* luciferase gene under the control of the HSV promoter. Within the *Renilla* luciferase gene the target sequences were inserted either into the 3'UTR or the 5'UTR resulting in a fusion mRNA. Binding of the mivaRNA mimics or the virally encoded miRNAs to these target sequences will lead to a measurable reduction in *Renilla* luciferase activity.

HEK293 cells were co-transfected with appropriate reporter gene vectors and with either (i) mivaRNA mimics mivaRI(A)-137 or mivaRI(G)-137 or (ii) the pAdVantage vector expressing the VA-RNAs I and II. **A.** Target sequences naturally occurring in the 3'UTR or CDS of the target gene were cloned into the 3'UTR of the *Renilla* luciferase gene. **B.** Target sequences lying within the 5'UTR of the target gene were cloned into the 5'UTR of the *Renilla* luciferase gene.

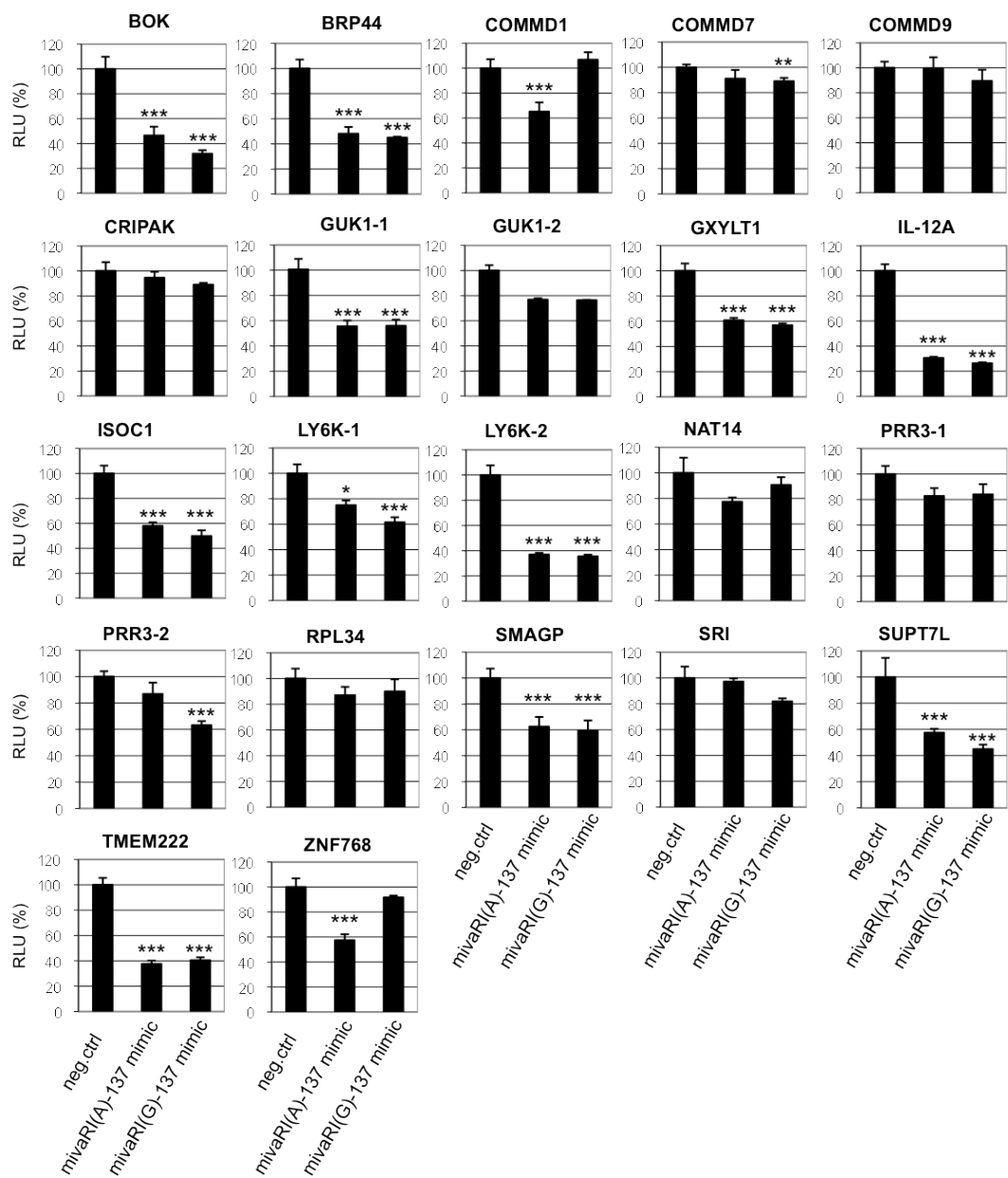
4.4.2.1. Target mRNA reduction using mivaRNA mimics

A dual-luciferase reporter gene assay was used to validate mivaRNA mimics for their potency to regulate Ago-2 mediated destruction of the so far identified putative mRNA targets. Therefore, HEK293 cells were co-transfected with reporter vectors carrying the target sequences within the *Renilla* luciferase gene, and with the mivaRNA mimic mivaRI(A)-137 or mivaRI(G)-137. A non-targeting siRNA served as negative control. As shown in Fig. 6, the mivaRNA mimic transfection led to reduced *Renilla* luciferase activities for the target sequences within the 3'UTR of the *Renilla* luciferase gene to different extents. The highest reduction rates could be observed for reporter vectors carrying the target sequences of IL-12A, BOK, BRP44, LY6K-2 and TMEM222 with reducing capacities ranging from 60-80%. COMMD1, NAT14 and ZNF768 were the only target sequences that resulted in higher reduction rates when transfecting the mivaRI(A)-137 mimic. This is partly in accordance with the micro array data showing higher mRNA enrichments for COMMD1 and ZNF768 in cells transfected with mivaRI(A)-137 compared to cells transfected with mivaRI(G)-137. Although CRIPAK was highly enriched in the microarray data set, targeting by the respective mivaRNA did not result in a high reduction rate. All the other targets were equally decreased by the 2 mivaRNAs or showed a slightly better reduction by mivaRI(G)-137.

In general, only very slight or no reduction was measurable when transfecting cells with the reporter vectors carrying the target sequences within the 5'UTR of the *Renilla* luciferase gene. The only exception is GPR63 with 60% reduction upon transfection with the mivaRI(A)-137 mimic and 50% reduction upon transfection with the mivaRI(G)-137 mimic.

The fact that cloning of some sequences into the *Renilla* luciferase gene did not result in any change of *Renilla* luciferase levels may indicate that the suspected target region does not contain the actual target site, or, the interaction with the mivaRNA has no consequence on gene expression.

A



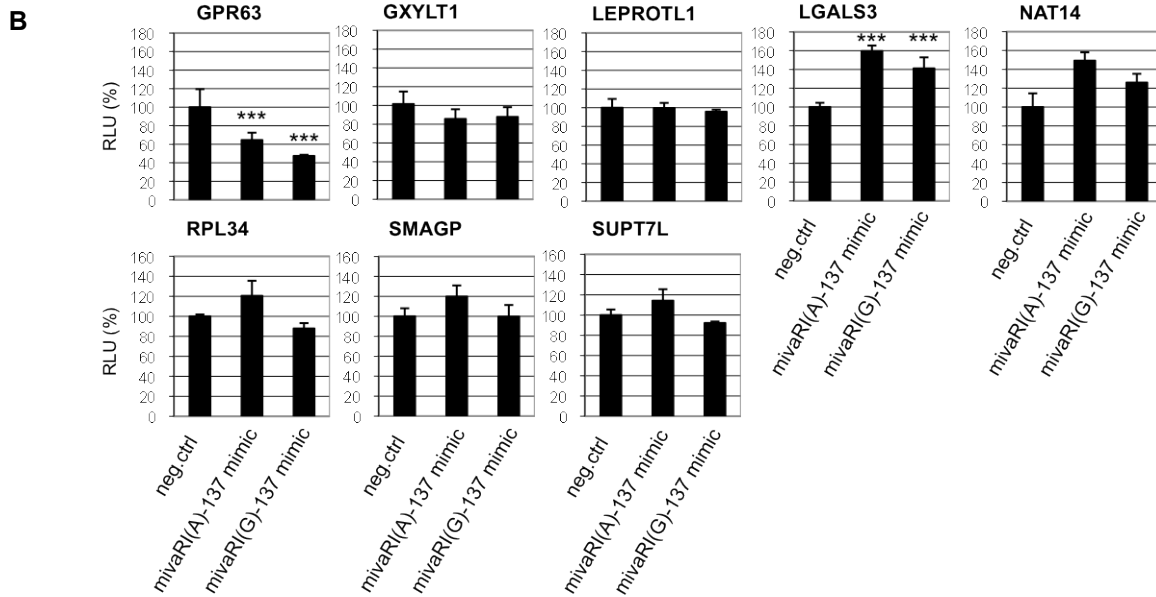


Figure 6 | Luciferase reporter gene assay of cells transfected with mivaRNA mimics

HEK293 cells were co-transfected with luciferase reporter vectors and mivaRNA mimics mivaRI(A)-137 and mivaRI(G)-137. 48h after transfection luciferase signals of firefly and *Renilla* luciferase were measured. The firefly luciferase signal served as internal control. A negative control siRNA served as off-target effect control. The *Renilla* luciferase signal was first normalized to the firefly luciferase values and second to the negative control siRNA. Luciferase activities are shown in %. **A.** Target sequences located within the 3'UTR of the 3'UTR of the *Renilla* luciferase gene. For some target genes more than one potential target sequence was found. Therefore, more than one reporter vector was generated as for GUK1-1/2 and LY6K-1/2. **B.** The predicted target sequences of cellular target genes were located within the 5'UTR and were thus cloned into the 5'UTR of the *Renilla* luciferase gene. For some targets, target sequences within both the 3'UTR and the 5'UTR could be identified. Therefore, two different types of reporter constructs were engineered such as in the case of genes GXYLT1, NAT14, RPL34, SMAGP and SUPT7L. RLU – relative luciferase units. * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.005

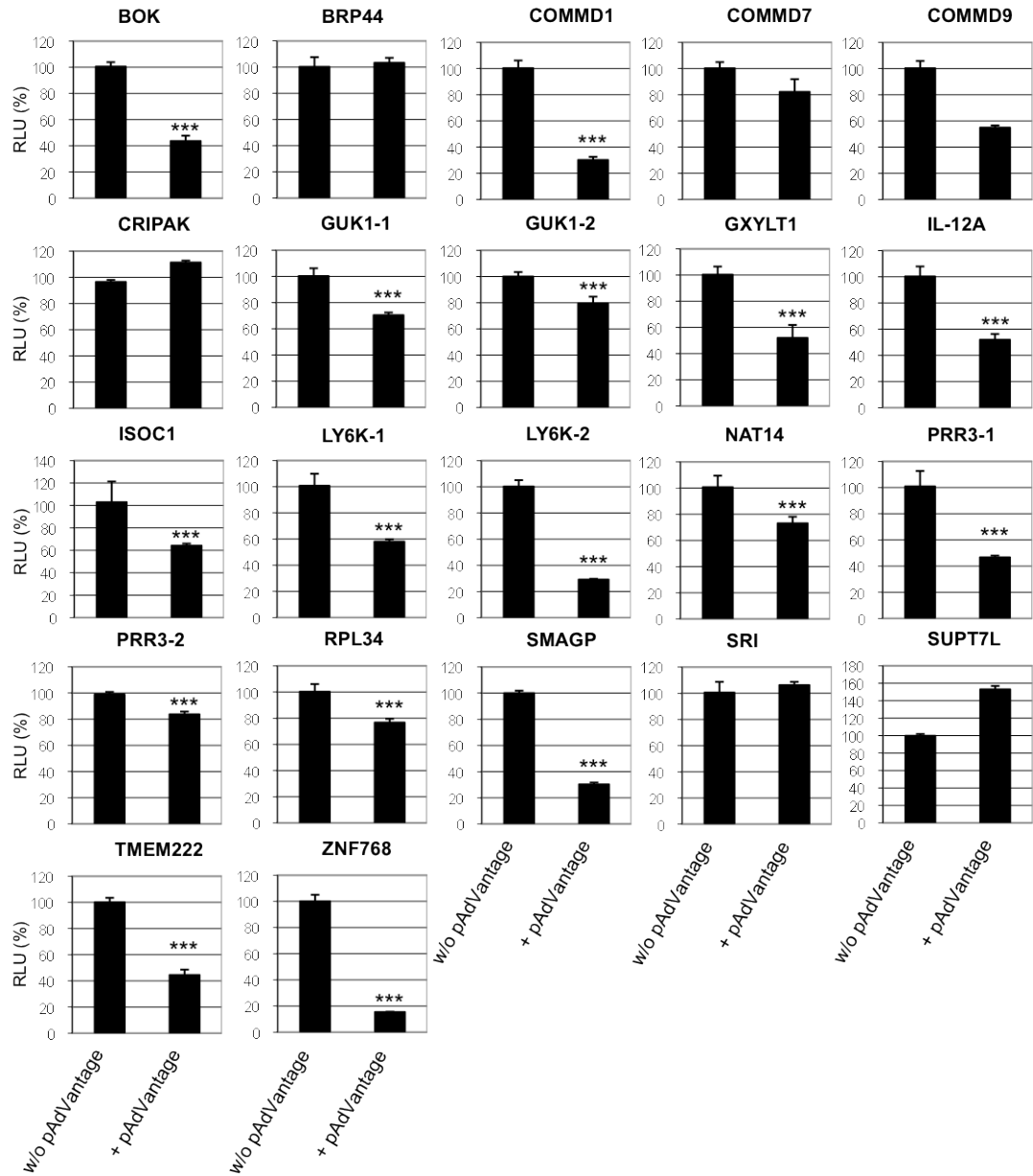
4.4.2.2. Target gene reduction using the vector pAdVantage

To simulate a naturally occurring adenovirus infection and the expression and procession of mivaRNAs instead of transfecting cells with the mivaRNA mimics, cells were co-transfected with the reporter vectors and the pAdVantage vector that expresses VA-RNAI and VA-RNAII. As negative control served on the one hand cells only transfected with the corresponding reporter vectors but not with the pAdVantage vector, and on the other hand cells co-transfected with psiCHECKTM-2 vectors carrying no target sequence and the pAdVantage vector. Results of the luciferase reporter gene assay are shown in Fig. 7.

For targets whose target sequences lie within the 3'UTR, the highest reduction rates in the *Renilla* luciferase signal could be achieved for COMMD1, SMAGP and ZNF768 with reduced luciferase activities of up to 80%. For BRP44, CRIPAK, SRI and SUPT7L no decrease in the *Renilla* luciferase activity could be measured. In this experimental setup, when I employed the pAdVantage vector instead of mivaRNA mimics, a reduction in *Renilla* luciferase activities could also be seen for target sequences lying within the 5'UTR. For the constructs carrying the target sequences of GPR63, GXYLT1 and SUPT7L luciferase activities were reduced by up to 60% compared to control conditions without pAdVantage vector.

The reason why the pAdVantage vector caused higher inhibition compared to the mivaRNA mimics in certain cases may be related to the fact that additional mivaRNA isoforms are generated by pAdVantage. Some of them may have higher targeting potency compared to the subset of mivaRNA mimics, which I employed. In addition, it can also not be ruled out that targeting by VA RNA II-derived mivaRNA plays a role.

A



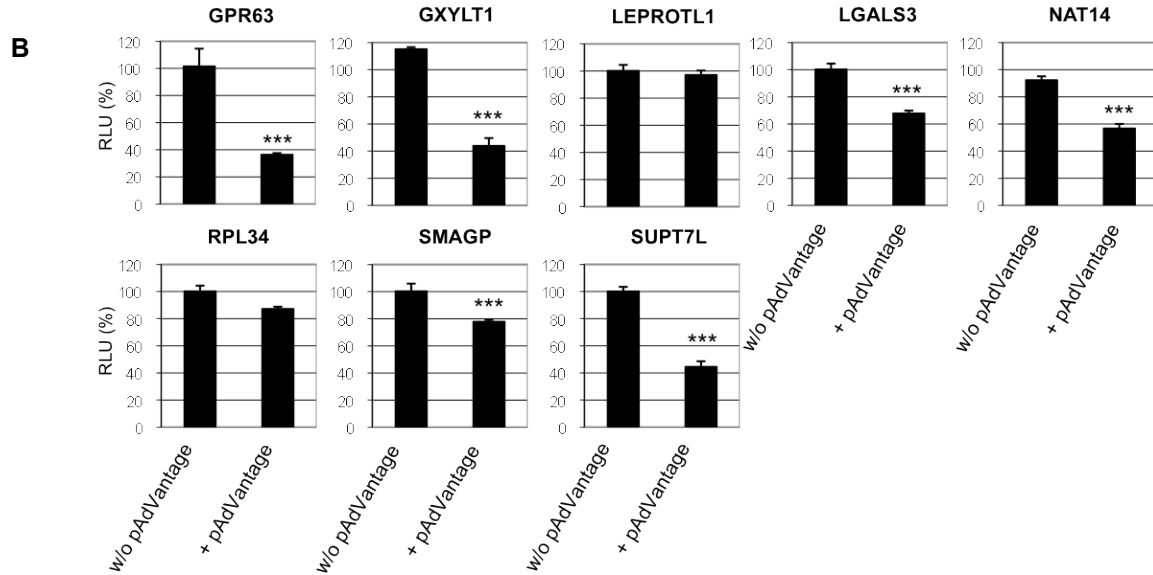


Figure 7 | Luciferase reporter gene assay after co-transfection of cells with pAdVantage

HEK293 cells were co-transfected with reporter vectors and the pAdVantage vector. 48h after transfection luciferase signals of the Firefly luciferase and the *Renilla* luciferases were measured. Cells transfected with the reporter vectors but not with pAdVantage vector served as control. Cells transfected with luciferase reporter vectors carrying no target sequence constituted another control. *Renilla* luciferase values were normalized to cells transfected with the reporter vector carrying no target sequence and to cells transfected with the same reporter vector but not transfected with pAdVantage vector. Luciferase activities are shown in %. **A.** Target sequences cloned into the 3'UTR of the *Renilla* luciferase gene. For some target genes more than one potential target sequences was found. Therefore, more than one reporter vector was designed such as for GUK1-1/2 and LY6K-1/2. **B.** Target sequences cloned into the 5'UTR of the *Renilla* luciferase gene. For some targets sequences could be identified within the 3'UTR and the 5'UTR. Therefore, both types of reporter constructs were generated (GXYLT1, NAT14, RPL34, SMAGP and SUPT7L). RLU – relative luciferase units. * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.005

4.5. Discussion

The major aim of this project was to answer the question whether adenoviral miRNAs specifically target cellular genes. For that purpose a RIP-Chip assay was established allowing the identification of mRNAs enriched in RISC during either an Ad5 infection or in cells transfected with miRNA mimics. Based on preliminary data, we focused on siRNAs mimicking miR(A)-137 and miR(G)-137 isoforms deriving from VA RNA I. Additionally, we used TargetScan prediction to identify putative target genes and compared those genes to the genes found decreased in the RIP-Chip analysis. We summarized these results and came up with 22 potential miRNA target genes (Tab. 8). The potency of miRNAs to downregulate the found target genes was further validated using reporter gene assay and measuring mRNA levels in cells transfected with miRNA mimics. Most genes analyzed showed decreased mRNA levels upon miRNA transfection with reduction rates up to 80%. Surprisingly, the analysis of CRIPAK, IL-12A, LGALS3, NAT14 and ZFN mRNAs revealed no reduction in mRNA levels, although these genes were clearly enriched in RISC fractions of Ad5 infected or miRNA transfected cells. An explanation can be that a regulation of the target genes happens on a translation level, not on the mRNA level. To clarify that, further experiments have to be performed analyzing the protein levels of these targets. Further on it can be possible that other miRNA isoforms besides miR(A)-137 or miR(G)-137 are binding to these target mRNAs. It can also not be excluded that the RIP-Chip analysis provides false-positive results. For example, NAT14 was not identified as putative target gene in the TargetScan prediction.

In another validation experiment we cloned predicted target sites of each gene into a dual-luciferase reporter vector to measure whether miRNAs potentially bind and decrease the putative target genes. Depending on the location of the predicted binding sites we cloned either the 3'UTR or the CDS of the distinct genes into the 3'UTR of the *Renilla* luciferase gene or the 5'UTR of these genes into the 5'UTR of the *Renilla* luciferase gene. We performed luciferase reporter gene assays co-transfecting cells with the reporter vectors together with either the miRNA mimics or the pAdVantage vector that is expressing VA RNA I and VA RNA II. The miRNA mimics could efficiently destruct most of the target mRNAs except for CRIPAK, COMMD7, COMMD9 and RPL34 which were only very slightly or not at all downregulated. Further on it is very important to notice that COMMD1 and ZNF are the only targets that were higher decreased upon miR(A)-137 transfection compared to miR(G)-137 transfection what is in accordance with the enrichment levels revealed in the RIP-Chip analysis. In contrast, PRR3 is stronger inhibited in the reporter gene assay upon miR(G)-137

transfection what can also be confirmed by RIP-Chip analysis. However, for most of the targets both miRNA mimics showed comparable reduction rates. The strongest target decreases upon miRNA mimic transfection were achieved for BOK, BRP44, IL-12A, LY6K and TMEM222 with reduction rates up to 80%. It is important mentioning that it cannot be guaranteed that for all target genes the target sites with the strongest affinity for miRNAs were cloned. It is also possible that some of the genes have more than one target site or have different target sequences with diverse binding capacities for other miRNA isoforms besides the tested ones.

Transfecting cells with the pAdVantage vector overall led to similar results as obtained for the miRNA mimics. COMMD9, SUPT7L (3'UTR and 5'UTR target site), GXYLT1 (5'UTR target site), LGALS3 (5'UTR target site), NAT14 (5'UTR target site) and SMAGP (5'UTR target site) revealed reductions only upon transfection with pAdVantage, not upon transfection with miRNA(A)-137 or miRNA(G)-137 mimic. That can be due to the fact that pAdVantage expresses VA I and VA II, and all their different isoforms. So it is possible that additional miRNAs interact with these target sites and lead to a reduction in the reporter gene assay that could not be found when only using the two distinct miRNA mimics.

Comparing all validation experiments we could identify BOK, COMMD1, ISOC1, LY6K (also described as potential target in [4]), SMAGP, SUPT7L and TMEM222 as putative targets of adenoviral miRNAs.

Data obtained from the TargetScan analysis could obviously not identify all potential targets thus it is essential to do additional validation experiments to clarify which predicted targets are true targets in an adenovirus infection situation.

To fully elucidate the function of the miRNAs on cellular target genes further validation experiments are needed, especially on the protein level. That could reveal the role of miRNAs on IL-12A regulation, for example. IL-12A mRNA was the highest enriched target mRNA in the RIP-Chip analysis of miRNA(G)-137 transfected cells and second highest enriched in miRNA(A)-137 transfected cells. Although, no mRNA level reduction was measurable in miRNA transfected cells. In contrast, the reporter gene assays confirmed that IL-12A is a putative target of miRNAs. To clarify the mechanism behind this regulation more experiments on the functional and protein level of IL-12A are needed. IL-12A is one subunit of IL-12 consisting of IL-12A (p35) and IL-12B (p40). Since activation of IL-12 leads to the production of IFN- γ and TNF- α and is involved in the differentiation of naïve T-cells into Th1 cells (reviewed in [9]) it would be beneficial for the adenovirus to inhibit IL-12 activation to overcome an immune response.

Another highly enriched target is CRIPAK. However a downregulation of this gene could neither be achieved on mRNA level nor in the reporter gene assays.

In general we could show that RIP-Chip analysis provides highly reliable target prediction for the interaction of adenoviral miRNAs with cellular mRNAs.

Table 8 | Functions of potential target genes of miRNAs [10], [11]

Target gene	Function
IL-12A	Growth factor for activated T and NK cells; enhances the lytic activity of NK/lymphokine-activated Killer cells, and stimulates the production of IFN-gamma by resting PBMC
BOK	Involved in apoptosis
BRP44	Mediates the uptake of pyruvate into mitochondria
COMMD1	Involved in proteasomal degradation; down-regulates NF-kappa-B activity and SOD1 activity
COMMD7	Associates with the NF-kappa-B complex and suppresses its transcriptional activity
COMMD9	Belongs to the COMMD family; involved in controlling Na ⁺ homeostasis [12]
CRIPAK	Negative regulator of PAK1
GPR63	Orphan receptor
GUK1	Essential for recycling GMP and indirectly, cGMP
GXYLT1	Glycosyltransferase which elongates the O-linked glucose attached to EGF-like repeats in the extracellular domain of Notch proteins by catalyzing the addition of xylose
ISOC1	Involved in physiological processes, e.g. lipid or steroid metabolism, sex determination, calcium transduction or cell proliferation [13]
LEPROTL1	Negatively regulates growth hormone receptor cell surface expression in liver
LGALS3	Galactose-specific lectin which binds IgE; required for terminal differentiation of columnar epithelial cells during early embryogenesis; acts as a pre-mRNA splicing factor; involved in acute inflammatory responses including neutrophil activation and adhesion, chemoattraction of monocytes; macrophages, opsonization of apoptotic neutrophils, and activation of mast cells
LY6K	Involved in cell growth
NAT14	Probable acetyltransferase that binds the 5'-GGACTACAG-3' sequence of coproporphyrinogen oxidase promoter
PRR3	Involved in cell-cell adhesion
RPL34	RNA binding; structural constituent of ribosome
SMAGP	Involved in epithelial cell-cell contacts
SRI	Calcium-binding protein that modulates excitation-contraction coupling in the heart; modulates the activity of RYR2 calcium channels
SUPT7L	Transcription coactivator; contributes to histone acetyltransferase activity
TMEM222	Transmembrane protein
ZNF768	Involved in transcriptional regulation

4.6. References

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IV. CONCLUSION

Human adenoviruses represent a common threat to immunocompromised patients like hematopoietic stem cell or bone marrow transplant recipients, or HIV positive individuals. Due to the lack of effective antiviral drugs against adenoviruses the outcomes of such infections can be severe and in some cases with fatal outcomes. Thus, there is the urgent need for alternative treatment options to inhibit adenoviral multiplication.

In the present thesis I presented alternative different approaches to provide proof of principle for several treatment opportunities.

In the first part, I focused on the use of siRNAs to inhibit adenovirus 5 infections *in vitro*. To identify a suitable adenoviral several siRNAs targeting different genes involved in DNA replication, transcription initiation and capsid generation, were evaluated for their down regulating capacities. Interfering with viral DNA replication turned out to be a key target for the efficient decrease of adenovirus progeny. Inhibition rates about 2 log steps could be achieved with the use of siRNAs targeting the DNA pol transcript. In general, targeting E1A or pTP also led to high down regulation of adenovirus RNA and DNA levels. So I could conclude that genes involved in DNA replication serve as more suitable targets for a siRNA-based inhibiting of adenoviral infections *in vitro* than do genes that are involved in the capsid generation, like the hexon or the viral protease gene. The DNA polymerase specific siRNA was also effective in inhibiting the other members of the adenovirus C subgroup. Further on, targeting of the viral E1A transcript resulted in higher cell viabilities. One pitfall of this approach is the need to deliver high amounts of siRNAs to overcome adenoviral infections *in vivo*.

In the second part we used artificial miRNAs to interfere with adenovirus 5 replication *in vitro*. Based on the results found in the siRNA project we focused on miRNAs targeting genes involved in adenovirus DNA replication. As delivery system for the miRNAs we chose a replication deficient adenovirus vector to provide an adenovirus-specific delivery system. In this approach, the miRNA targeting the pTP transcript showed the highest inhibition capacity of adenovirus multiplication *in vitro* with blocking rates comparable to those of the specific siRNAs. The inhibition rates could even be enhanced after concatemerization of up to six pTP- amiRNA hairpins in primary transcripts. The advantage of the miRNA-based approach may be the delivery of miRNAs into predominantly those cells that constitute the host cells for adenovirus wild-type adenoviruses.

In the third part, I was involved in the investigation whether gene-directed enzyme prodrug therapy can be used to inhibit adenovirus 5 multiplication *in vitro*. For that purpose, a replication deficient adenovirus vector was constructed that carries the HSV-TK gene under the control of the adenovirus E4 promoter. HSV-TK is able to convert the prodrug ganciclovir into its active form that further interferes with adenovirus replication by blocking it. Given that HSV-TK expression is under the control of an adenovirus promoter, we could achieve targeted expression of HSV-TK in adenovirus-infected cells. It is absolutely essential to limit HSV-TK expression to adenovirus-infected cells since ganciclovir can cause severe side effects when uncontrolled activated in healthy cells. By using an adenovirus vector we utilized the tropism of the vector to only infect cells that comprise common host cells for adenoviruses. This approach was very successful in inhibiting adenoviral multiplication *in vitro* by decreasing virus progeny by several orders of magnitude.

Taken together we could depict different approaches to overcome adenoviral infections *in vitro*. As a next step it is essential to investigate the treatment potency of these methods *in vivo* to further evaluate their therapy potential. Overall we provide new treatment strategies to combat life-threatening adenoviral infections.

In the fourth part of the here present thesis I wanted to gain more insight into the interplay between the adenovirus encoded miRNAs and cellular targets. So far it is thought that the miRNA VAI and VAI1 are mainly expressed to saturate the cellular RNAi machinery. Since we thought that cellular targets of these miRNAs should exist, we established a RIP-chip assay to identify such possible targets. A combination of this approach together with bioinformatical target site prediction provided 20 potential target genes involved in cell metabolism or immune response. Further on, we investigated which versions of VAI are predominant and show the highest inhibition capacities. We used miRNA mimics representing the versions miVAI(A)-137 or miVAI(G)-137 for further analysis.

My part in this project was to further validate these targets with luciferase reporter gene assays and to investigate if miVAI(A)-137 and miVAI(G)-137 cause a decrease in the mRNA levels of the respective target genes. I could show that many of the identified targets are indeed regulated by miRNAs. The determination of the biological consequences would constitute the next logical step.

Overall, this thesis could provide a deep insight into the possible treatment options of RNAi-based approaches to overcome adenoviral infections.

Besides, a microarray analysis and further validation experiments revealed interactions between adenovirus-encoded miRNAs and cellular target mRNAs.

V. ABBREVIATIONS

3' UTRs	3' untranslated regions
5' UTRs	5' untranslated regions
A. thaliana	Arabidopsis thaliana
AAV	adenovirus-associated viruses
Ad2	adenovirus 2
Ad5	adenovirus 5
AdVs	adenoviruses
Ago	Argonaute proteins
AGO2	Argonaute 2
AIDS	Acquired immunodeficiency syndrome
ARD	acute respiratory disease
BMT	bone marrow transplant
CAR	coxsackie virus and Adenovirus Receptor
cGMP	Cyclic guanosin monophosphat
CMV	cytomegaloviruses
CTLs	cytotoxic T-lymphocytes
DBP	DNA binding protein
DGCR8	diGeorge syndrome critical region 8 gene
dsRNA	double-stranded RNA
E	early
EBV	Epstein-Barr virus
EGF	Epidermal growth factor
EKC	epidemic keratoconjunctivitis
Exp5	exportin 5
FMDV	foot-and-mouth disease virus
G	guanosine
GCV	ganciclovir
GMP	Guanosin monophosphat
hAdVs	human adenoviruses
HDL	high-density lipoprotein
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HSCT	hematopoietic stem cell transplant
HSV-TK	herpes simplex virus thymidine kinase
IL-1	interleukin-1
INF	interferon
ITRs	inverted repeats

Abbreviations

L	late
LDL	low-density lipoprotein
MHC I	class I major histocompatibility complex
miRNAs	micro-RNAs
MLP	major late promoter
mRNAs	messenger RNAs
NKs	natural killer cells
Nt	nucleotides
OTC	ornithine transcarbamylase
P-bodies	processing bodies
PACT	protein activator of protein kinase PKR
PAZ	PIWI-Argonaute-Zwille
PBMC	Peripheral blood monocyte
PKR	protein kinase R
pi	Piwi-interacting
Pol	DNA polymerase
Pol II	polymerase II
Pre-miRNA	precursor-microRNAs
pre-miRNAs	precursor miRNAs
Pri-miRNA	primary transcripts
PTGS	post-transcriptional gene silencing
pTP	preterminal protein
rasiRNA	repeat-associated siRNAs
RGD	arginine-glycine-aspartate
RIG1	retinoic acid inducible gene 1 protein
RISC	RNA-induced silencing complex
RNAi	RNA interference
SARS	severe acute respiratory syndrome
scn	small-scan
shRNAs	small hairpin-based RNAs
siRNAs	small interfering RNAs
SNALPs	Stable nucleic acid liquid particles
SOD1	Superoxide dismutase 1
SV40	simian virus 40
T-Ag	T antigen
TGS	transcriptional gene silencing
TKs	thymidine kinases
TLRs	Toll-like receptors
TNF	tumor necrosis factor
TP	terminal protein

Abbreviations

TRBP	TAR RNA-binding protein
U	uridine
VA	virus-associated

VI. CURRICULUM VITAE

Curriculum Vitae Mag. rer. nat. Doris Kneidinger

Personal Information

Address: Arndtstraße 42-44/20, 1120 Vienna
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Date of birth: 1st of October 1984
Place of birth: Linz
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Work Experience

since 06/2013 **Labdia Labordiagnostik GmbH, Department of Biology of leukaemias**
Technician
MRD diagnostic

10/2009 – 05/2013 **St. Anna Children's Cancer Research Institute, Department of Molecular Microbiology**
PhD student
supervisor: Reinhard Klein, PhD
(group leader Prof. Thomas Lion, MD, PhD)

Title of PhD Thesis:

RNA Interference-mediated Inhibition of Adenoviral Infections

02/2008 – 03/2009 **Medical University of Vienna, Institute for Immunology**
Diploma Student
supervisor: Prof. Dr. Herbert Strobl

Title of Diploma Thesis:

Molecular Analysis of Dendritic Cell Development during Hematopoiesis

10/2005 – 08/2007 **Medical University of Vienna, Department of Cranio- Maxillofacial and Oral Surgery**
Staff scientist
supervisor: Prof. DDr. Dritan Turhani

Bone tissue engineering as a promising approach for the treatment of defective or lost bone in the maxillofacial region

Education

10/2003 – 10/2009 **Diploma study of biology at the University of Vienna**

- Major field of studies: genetics and microbiology with a special focus on immunology and virology
- Final diploma exam 10/2009: graduation with honors

09/1995 – 07/2003 **High School Körnerstraße, Linz, Austria**

- Final examination, June 2003

Skills and Qualifications

Practical experience

- Cell culture maintenance of cell lines and primary cells (differentiation of CD34⁺ cord blood cells into specialized immune cells), immunofluorescence, FACS, cytotoxicity assays, luciferase assays, transfections
- Virus retroviral transfections, work with adenoviral vectors (cloning, transfections), work with adenovirus wild type (amplification, purification, titer determination, infection)
- Bacteria cloning, transformation, plasmid purification
- DNA, RNA PCR purification, cDNA transcription, reverse transcriptase PCR, real time PCR (Roche, Biorad)
- Protein purification, Western Blot, co-immunoprecipitation
- siRNA/miRNA design, cloning, transfections

Languages

- German native speaker
- English excellent command
- French basic command

Computer skills

- Operating systems Microsoft Windows, Apple Macintosh
- Software MS Office
Adobe CS3 (Illustrator, Photoshop)
scientific data bases
statistical software (Graphpad prism, SPSS)

Talks and presentations

- Poster presentation at the 10th Annual Adenovirus Meeting, 2012 in Umeå, Sweden (price for best poster)
- Poster presentation at the 8th YSA PhD Symposium, 2010 in Vienna, Austria
- Poster presentation at the 2nd ÖGMBT annual meeting, 2010 in Vienna, Austria
- Progress reports and journal club presentations at the St. Anna Children's Cancer Research Institute

Publication list

- **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, 08/2012)

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

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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.**

Ibrišimović M., Nagl U., **Kneidinger D.**, Rauch M., Lion T., Klein R.
Journal of Gene Medicine. 2012

- **Reciprocal role of GATA-1 and vitamin D receptor in human myeloid dendritic cell differentiation.**

Göbel F., Taschner S., Jurkin J., Konradi S., Vaculik C., Richter S., **Kneidinger D.**, Mühlbacher C., Bieglmayer C., Elbe-Bürger A., Strobl H.
Blood. 2009

- **Aryl hydrocarbon receptor activation inhibits in vitro differentiation of human monocytes and Langerhans dendritic cells**

Platzer B., Richter S., **Kneidinger D.**, Waltenberger D., Woisetschläger M., Strobl H.
Journal of Immunology. 2009

- **Expression patterns of Ets2 protein correlate with bone-specific proteins in cell-seeded three-dimensional bone constructs.**

Wanschitz F., Stein E., Sutter W., **Kneidinger D.**, Smolik K., Watzinger F., Turhani D.
Cells Tissues Organs. 2007