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Inhibition of adenovirus infections by siRNA-mediated silencing of early and late adenoviral gene functions

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ABSTRACT

Adenoviruses are pathological agents inducing mild respiratory and gastrointestinal infections. Under certain circumstances, for example in immunosuppressed patients, they induce severe infections of the liver, heart and lung, sometimes leading to death. Currently, adenoviral infections are treated by palliative care with no curative antiviral therapy yet available. Gene silencing by RNA interference (RNAi) has been shown to be a potent new therapeutic option for antiviral therapy. In the present study, we examined the potential of RNAi-mediated inhibition of adenovirus 5 infection by the use of small interfering (si)RNAs targeting both early (E1A) and late (hexon, IVa2) adenoviral genes. Several of the initially analyzed siRNAs directed against E1A, hexon and IVa2 showed a distinct antiviral activity. Among them, one siRNA for each gene was selected and used for the further comparative investigations of their efficiency to silence adenoviruses. Silencing of the late genes was more efficient in inhibiting adenoviral replication than comparable silencing of the E1A early gene. A combination strategy involving down-regulation of any two or all three of the targeted genes did not result in an enhanced inhibition of viral replication as compared to the single siRNA approaches targeting the late genes. However, protection against adenovirus-mediated cytotoxicity was substantially improved by combining siRNAs against either of the two late genes with the siRNA against the E1A early gene. Thus, an enhanced anti-adenoviral efficiency of RNAi-based inhibition strategies can be achieved by co-silencing of early and late adenoviral genes, with down regulation of the E1A as a crucial factor.

1. Introduction

Adenoviruses induce respiratory, gastrointestinal and conjunctival infections. In general, these infections are mild and are resolved within a few days. In some cases, however, such as in immunosuppressed patients, the virus surmounts primary barriers of the antiviral defence system of the host, leading to systemic infections. Severe organ diseases like hepatitis (Hough et al., 2005), myocarditis (Bowles et al., 2003) and pneumonia (Faden et al., 2005) can occur and in some cases patients die from fulminant progression of the disease (Faden et al., 2005; Hough et al., 2005). Despite some encouraging reports showing inhibition of adenovirus infection by interferon γ and β (Mistchenko et al., 1987; Kuhl et al., 2003), a specific antiviral therapy is currently not available.

A number of recent investigations demonstrated that small interfering (si) RNAs, which typically have a length of 21–23 bp, are potent molecular tools to silence target genes and inhibit viral infections (Elbashir et al., 2001). These siRNAs are the actual effector molecules in RNA interference (RNAi), a highly specific and evolutionary conserved mechanism of post-transcriptional silencing induced by double stranded RNAs, which was initially described for the nematode *Caenorhabditis elegans* (Fire et al., 1998). A protein complex referred as RNA-induced silencing complex (RISC) incorporates the antisense strand of the siRNA, whereas the complementary sense strand is degraded. Activated RISC then binds to the target RNA in a sequence-specific manner and induces its cleavage by the Argonaute 2 (AGO2) protein (for reviews see Castanotto and Rossi, 2009; Kurreck, 2009).

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

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Based on the universal principle of RNAi, several different strategies have already been employed to inhibit viral infections (for an overview, see Haasnoot et al., 2007). Accordingly, the genome of RNA viruses can be targeted directly by RNAi (Fechner et al., 2008; Rothe et al., 2009), whereas both RNA and DNA viruses can be inhibited at the level of transcription of viral genes (Li et al., 2005; Chung et al., 2007). Virus infection can also be inhibited indirectly through silencing of cellular receptors involved in viral attachment and entry (An et al., 2007; Fechner et al., 2007), or cellular genes involved as essential co-factors in the viral replication cycle (Murray et al., 2005).

The adenoviral infection cycle involves a complex pattern of interactions between early and late viral proteins and the host cell machinery. It can principally be divided into three phases. In the early phase, the virus enters the cell and the viral genome is uncoated and translocated into the nucleus, followed by transcription and translation of the early genes. These early events also modulate cellular functions such as cell cycle control and thus facilitate replication of the viral genome, which in turn is closely linked to transcription and translation of the late genes during the second phase of the viral life cycle. These processes ultimately result in the last phase, the nuclear assembly of the structural proteins and maturation and release of infectious virus (Russell, 2000). While the adenoviral E3 region is not necessary for virus replication *in vitro* and the E1B is dispensable as well (Bischoff et al., 1996; Fechner et al., 2003), other proteins such as the E1A early gene and the late IVa2 and hexon protein are absolutely essential for virus replication *in vitro* and *in vivo*.

E1A is the first viral component expressed in the nucleus after infection. The two major E1A proteins, 12S and 13S, modulate cellular metabolism to create suitable conditions for viral genome replication and expression of the other early genes (Nevins et al., 1988; Russell, 2000). Deletion or inactivation of the E1A gene completely abolishes the viral infection cycle (Biederer et al., 2002). The IVa2 protein binds to the adenoviral major late promoter in a sequence-specific manner and thereby acts as enhancer of transcription of late structural proteins (Tribouley et al., 1994). Furthermore, IVa2 is important for capsid assembly (Zhang and Imperiale, 2003) and also for encapsidation of the viral genome by binding to the packaging signal as a multimeric complex with the virus-encoded non-structural protein L1 52/55K (Gustin et al., 1996; Zhang et al., 2001; Tyler et al., 2007). Mutant viruses lacking IVa2 are unable to form viral capsids (Zhang and Imperiale, 2003). The hexon protein is the major protein of the virus capsid. It is expressed in the late phase of the virus replication cycle and is essential to form virus particles (Russell, 2000). Moreover, the hexon protein binds to the coagulation factor (F) X which represents the major mechanism for adenovirus uptake into hepatocytes from the bloodstream *in vivo* (Waddington et al., 2008).

In the present study, we investigated the antiviral potential of siRNAs directed against the E1A, IVa2 and hexon adenoviral genes. Inhibition of viral production by silencing the IVa2 and hexon genes with siRNAs was more efficient than silencing the E1A gene. While no synergistic effects on the inhibition of viral production by simultaneous targeting of two or three of these genes were observed, down-regulation of E1A was the decisive and most effective means of protecting the infected cells from adenovirus-induced cell lysis, but only in combination with silencing of a late gene.

2. Materials and methods

2.1. Adenoviruses

The replication competent adenoviral vector Ad5TRE-E1A (Fechner et al., 2003) is an E1B and E3 deleted adenovirus 5 mutant. The replication deficient Ad5CMVluc (Fechner et al., 1999) was described previously. This vector also harbours deletions of the E1 and E3 regions of the adenovirus 5 genome.

2.2. Generation of plasmids with siRNA target sequences

The siE1A, siIVa2 and siHexon target sequences were generated by annealing the following primer pairs:

5′-CGACA*CGGAGGTGTTATTACCGAA*T-3′
5′-CTAG*ATTCGGTAATAACACCTCCG*TGTCG-3′
for siE1A,
5′-CGACA*GTTAGTGATCCCAGAAATA*T-3′
5′-CTAG*ATATTTCTGGGATCACTAAC*TGTCG-3′
for siIVa2 and
5′-CGAAA*GCTAGAAAGTCAAGTGGAA*T-3′
5′-CTAG*ATTCCACTTGACTTTCTAGC*TTTCG-3′
for siHexon.

These duplexes contain *Nru*I and *Xba*I overhangs at their ends and were inserted into the *Nru*I/*Xba*I digested luciferase reporter plasmid pUFCMV$_{enh}$/MLC-260-Luc (Muller et al., 2008) (kindly provided by O. Müller, DKFZ, Heidelberg, Germany). With this approach, the target sequences were placed between the luciferase cDNA and the SV40 polyA signal. The resulting plasmids were termed pLuc-*TS*-siE1A, pLuc-*TS*-siIVa2 and pLuc-*TS*-siHexon, respectively. Correct sequences were confirmed by sequencing using an ABI 319 Genetic Analyzer (Applied Biosystems, Foster City CA, USA).

2.3. Cell cultures

HeLa (human cervical carcinoma) and HEK293 (human embryonal kidney) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Karlsruhe, Germany) supplemented with 10% FCS and 1% penicillin/streptomycin. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.4. siRNA and plasmid transfection

The siRNAs used for down regulation of adenoviral genes as well as the negative control non-targeting, commercially available siRNA (Table 1), which does not match any sequence present in the viral or human genome, were purchased from Qiagen (Qiagen, Hilden, Germany). For each gene analyzed four different siRNAs with putative high silencing efficiency were designed by use of the "HP OnGuard siRNA Design tool" (Qiagen). Twenty-four hours before transfection, 8×10^4 cells were seeded in 24 well plates with cells reaching a confluence of 60–80% the next day. The growth medium was then changed for medium without serum and antibiotics and cells were transfected with the respective siRNA or siRNA mixture by lipofection. To this end, siRNAs were diluted in 50 μl Opti-MEM and gently mixed. In parallel, 1 μl of Lipofectamine 2000 (Invitrogen GmbH, Karlsruhe, Germany) was diluted in 50 μl Opti-MEM and incubated for 5 min at room temperature. Subsequently the diluted siRNA was mixed with the Lipofectamine 2000 dilution. After 20 min incubation at room temperature, the solution was added drop-wise to the cells, followed by incubation at 37 °C for 4 h. Medium was then replaced by fresh medium with 10% FCS and 1% penicillin/streptomycin and cells were incubated for a further 32 h. For siRNA – plasmid co-transfection experiments, 50 ng of the firefly luciferase-expressing plasmid and 5 ng of the renilla luciferase expression vector phRL-TK (Promega GmbH, Mannheim, Germany) as an internal control were co-transfected with different concentrations of siRNAs using Lipofectamine 2000. Twenty-four hours

Table 1
siRNA sequences targeting E1A, IVa2 and hexon of adenovirus 5.

Name	Sequence sense antisense	Target sites in adenovirus 5	Target gene
siE1A_1	CCU UUG GAC UUG AGC UGU AdTdT UAC AGC UCA AGU CCA AAG GdTdT	1508–1528	E1A
siE1A_2	CUG UGU CUA GAG AAU GCA AdTdT UUG CAU UCU CUA GAC ACA GdGdT	1331–1352	E1A
siE1A_3	GGA UUG ACU UAC UCA CUU UdTdT AAA GUG AGU AAG UCA AUC CdCdT	787–807	E1A
siE1A_4	CGG AGG UGU UAU UAC CGA AdTdT UUC GGU AAU AAC ACC UCC GdTdG	578–598	E1A
siHexon_1	AGU GGU AUU GUA CAG UGA AdTdT UUC ACU GUA CAA UAC CAC UdTdT	19,686–19,708	Hexon
siHexon_2	CAC CUA AAU AUG CCG AUA AdTdT UUA UCG GCA UAU UUA GGU GdTdT	19,414–19,434	Hexon
siHexon_3	CUA AUG GGC CAA CAA UCU AdTdT UAG AUU GUU GGC CCA UUA GdTdT	19,779–19,799	Hexon
siHexon_4	GCU AGA AAG UCA AGU GGA AdTdT UUC CAC UUG ACU UUC UAG CdTdT	19,610–19,630	Hexon
siIVa2_1	CGC UUU GUA AAC ACU UAC AdTdT UGU AAG UGU UUA CAA AGC GdGdT	4260–4280	IVa2
siIVa2_2	GUU AGU GAU CCC AGA AAU AdTdT UAU UUC UGG GAU CAC UAA CdGdT	4648–4668	IVa2
siIVa2_3	GGA UAU GGC UGG GAA CAU AdTdT UAU GUU CCC AGC CAU AUC CdCdT	4441–4465	IVa2
siIVa2_4	CCA GCA GGA CCA GCC UCA AdTdT UUG AGG CUG GUC CUG CUG GdTdG	5484–5404	IVa2
Non-silencing siRNA	UUC UCC GAA CGU GUC ACG UdTdT ACG UGA CAC GUU CGG AGA AdTdT		

Note: Gene Bank (http://www.ncbi.nlm.nih.gov) number of adenovirus sequence used for selection of siRNAs was BK000408.

after transfection the medium was replaced by fresh medium and luciferase expression was determined 24 h later.

2.5. Trans-complementation assay

Cells were transfected with the siRNAs as described above followed by infection with a mixture of the replication competent adenovirus (Ad5TRE-E1A) and the reporter virus Ad5CMVluc. Medium was changed 4 h later. After 32 h cells were washed with PBS, treated with 200 μl of 0.25% trypsin and suspended in 300 μl medium containing 10% FCS. Cells were lysed by 4 freeze/thaw cycles and centrifuged to pellet the cellular debris. 9×10^4 to 10^5 HeLa cells were seeded in 24 well plates and infected with 400 μl of the supernatants 24 h later. After 3 h incubation 600 μl medium was added and luciferase activity was determined 24 h thereafter.

2.6. Indirect immunofluorescence

HeLa cells grown in 24 well plates were fixed and permeabilized with 1 ml of PBS solution containing 4% formaldehyde and 0.5% Triton X-100. After blocking in TBS buffer with 5% donkey serum and 0.1% Triton X-100, cells were incubated with mouse monoclonal antibody raised against the hexon antigen of adenovirus (Santa Cruz Biotechnology) at a dilution of 1:200 in blocking solution for 1 h. Cells were rinsed and incubated with goat anti-mouse secondary antibody Alexaflour594 (Invitrogen), respectively, at a dilution of 1:400 for 45 min. Cover slips were mounted using Dapi-Fluoromount-G™ (Southern Biotechnology Associates Inc., Birmingham, AL, USA). Fluorescence images were taken with Microscope Observer.D1 using the software AxioVision Rel.4.7 (Carl Zeiss MicroImaging GmbH, Jena, Germany).

2.7. Real-time RT-PCR

Total RNA was isolated with the TRIZOL Reagent (Invitrogen GmbH, Karlsruhe, Germany) according to the company's recommendations followed by DNaseI digestion (Peqlab, Erlangen, Germany). RNA was then reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Forster City, CA, U.S.A.). Expression levels of E1A, IVa2 and Hexon genes were determined by real-time PCR with the SYBR Green PCR Master Mix (Applied Biosystems Inc.) using oligonucleotide primers as follows:

E1A forward primer (FWD)
5′-CTTGGGTCCGGTTTCTATGC-3′
E1A reverse primer (REV)
5′-CCCGTATTCCTCCGGTGATA-3′
IVa2 FWD
5′-CGTCTCTGGGGTGGAGGTAG-3′
IVa2 REV
5′-TCCCAGCTTAACCGCTTTGT-3′
Hexon FWD
5′-CACATCCAGGTGCCTCAGAA-3′
Hexon REV
5′-AGGTGGCGTAAAGGCAAATG-3′
For normalization, human 18S rRNA was used:
18S rRNA FWD
5′-CCCCTCGATGCTCTTAGCTG-3′
18S rRNA REV
5′-TCGTCTTCGAACCTCCGACT-3′

PCR conditions were 95 °C for 3 min and 40 cycles of 95 °C for 15 s, 55 °C for 30 s, 72 °C for 30 s. PCR reactions were carried out in triplicate with the StepOnePlus Real-time PCR System (Applied Biosystems Inc.). Analysis was carried out using the $2^{-\Delta\Delta Ct}$ method.

2.8. Measurement of luciferase activity

Firefly luciferase activity of the adenoviral reporter vectors was measured with the Luciferase Reporter Gene Assay (Roche Diagnostics GmbH, Mannheim, Germany) according to the instructions of the manufacturer. In cells co-transfected with firefly and renilla luciferase expressing plasmids, the luciferase activity was

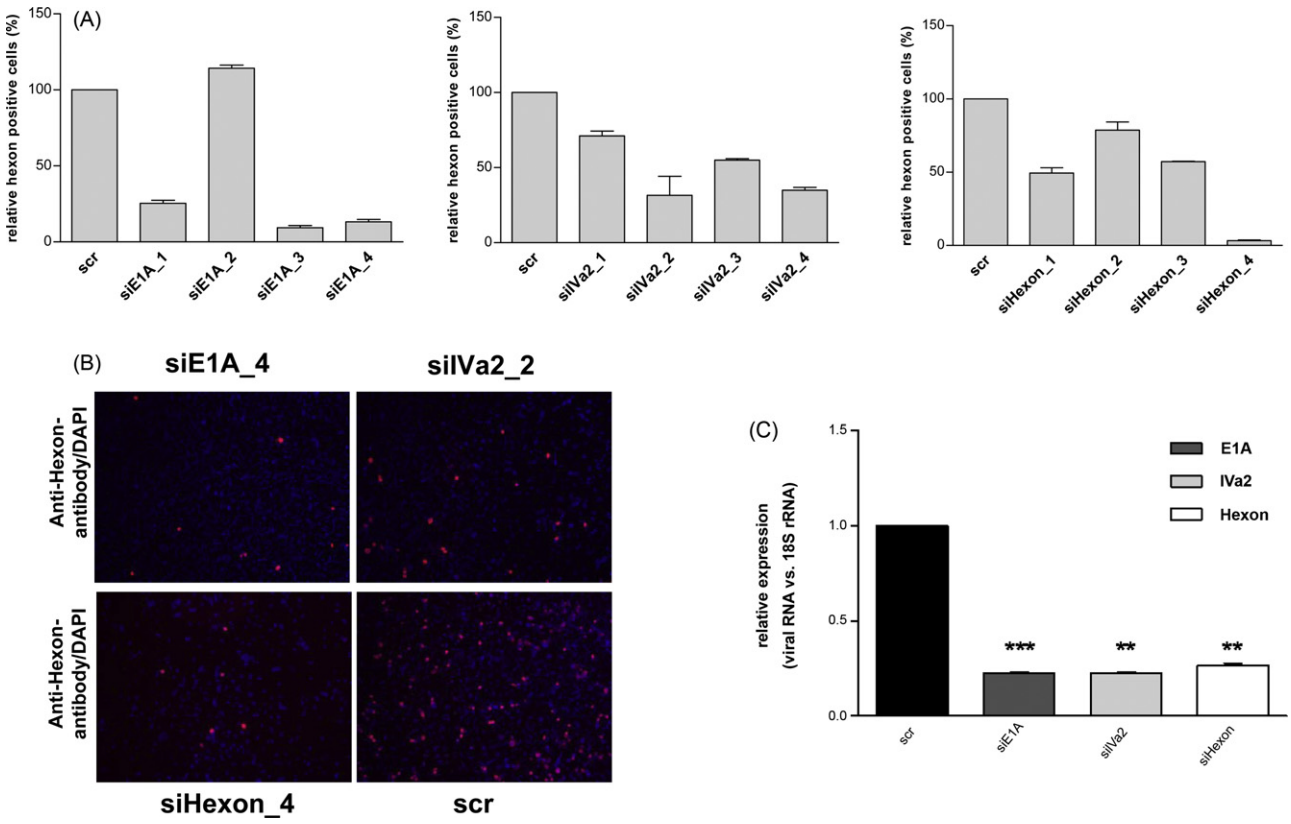

Fig. 1. Evaluation of siRNAs directed against adenoviral E1A, IVa2 and hexon. (A) Quantitative analysis of hexon protein expressing cells. HeLa cells seeded in 24 well plates were transfected with 100 nM of the indicated siRNAs and infected with Ad5TRE-E1A at an moi of 5. Cells were fixed 48 h later and hexon protein immunoreactivities (red) were detected with hexon specific antibodies. Cells were co-stained with DAPI (blue) to visualize the cell nuclei. Detection of hexon protein immunoreactivity was also used to assess antiviral efficiency of IVa2 siRNAs as there is no commercially available antibody for IVa2 and for E1A siRNAs. The number of cells positively stained for hexon protein was determined by counting each four areas per well and sample of two independent experiments. Amount of hexon protein positive cells treated with anti-adenovirus siRNAs is given as percentage of positive cells of scrambled (scr) siRNA control (=100%). Values are given as mean values ± S.E.M. (B) Hexon protein staining of most efficient siRNAs according to the experiment described under A. (C) Silencing of E1A, IVa2 and hexon mRNA expression by target gene specific siRNAs. HeLa cells were treated as described under A. Total RNA was isolated 48 h after virus infection and E1A, IVa2 and hexon mRNA expression determined by real-time RT-PCR. Values are given as mean values ± S.E.M. Significance: $^{**}p < 0.01$; $^{***}p < 0.001$.

determined with the Dual-Luciferase Reporter Assay (Promega). Luciferase activity was measured in a Lumat LB 9501 luminometer (Berthold Technologies, Bad Wildbad, Germany).

2.9. Plaque assay

For release of the virus from infected cells, cells were scraped into 1 ml DMEM and subjected to 4 freeze–thaw cycles. The cellular debris was removed by centrifugation and the supernatant was used for re-infection of HEK293 cells. Two hours post-infection, cells were washed and overlaid with 1.25% low melting point agarose dissolved in DMEM growth medium supplemented with 5% FCS. Plaques were counted 2 weeks later and plaque forming units were calculated.

2.10. Cell killing assay

HeLa cells were grown to 60–80% confluence and transfected with siRNAs at a final concentration of 75 nM as described earlier. Immediately after transfection, cells were infected with replication competent adenovirus Ad5TRE-E1A or the control adenoviral vector Ad5CMVluc at an moi of 10. After a 4 h incubation at 37 °C, the medium was replaced with fresh DMEM growth medium containing 10% FCS and 1% penicillin/streptomycin and cytopathic effect (CPE) was monitored by crystal violet staining 4 days later.

2.11. Statistical analysis

All data were expressed as mean ± standard error of mean (S.E.M.). To test for statistical significance, Student's *t*-test was applied. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Evaluation of siRNAs directed against the adenoviral E1A, IVa2 and hexon genes

For initial comparison of the antiviral efficiency of siRNAs directed against different adenoviral target genes, four siRNAs against each of the mRNAs encoding E1A, IVa2 or hexon (Table 1) were designed and analyzed. HeLa cells were transfected with 100 nM (final concentration) of the anti-adenoviral siRNAs or a scrambled control siRNA and infected with an moi of 5 of the replication competent adenovirus Ad5TRE-E1A for 4 h and analyzed 48 h later. Ad5TRE-E1A is an adenovirus 5 mutant, which selectively replicates in tumor cells. Its replication levels are slightly lower than those of wild type adenovirus (Fechner et al., 2003). As sufficient production of E1A and IVa2 is required to produce hexon protein during the adenoviral replication cycle we hypothesized that hexon protein expression should not only be useable to determine efficiency of hexon siRNAs but also to determine the efficiency of the E1A and IVa2 siRNAs. Therefore, all samples were

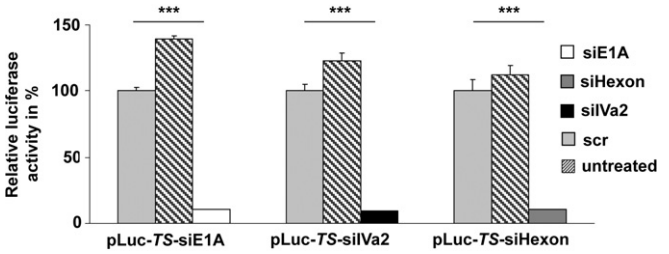

Fig. 2. Silencing efficiency of individual anti-adenoviral siRNAs. HeLa cells were co-transfected with one of the siE1A, siHexon and siIVa2 at a concentration of 10 nM and a firefly luciferase plasmid carrying the respective target sequence of each siRNA in its 3′ UTR. In addition, a renilla luciferase expressing plasmid was co-transfected as internal standard. Cells were lysed 48 h after transfection and activity of firefly and renilla luciferase was measured. The relative firefly luciferase activity defined as the ratio of firefly to renilla luciferase activity is shown. Values are given as mean values ± S.E.M. of two independent experiments each performed in triplicate. scr = scrambled siRNA. Significance: $^{***}p < 0.001$.

monitored for hexon protein expression using indirect immunofluorescence technique. Several of the siRNAs directed against E1A (especially siE1A_1, _3 and _4), hexon (siHexon_4) and IVa2 (especially siIVa2 _2 and _4) showed a distinct antiviral activity (Fig. 1A and B).

The most efficient siRNAs (siE1A_4, siIVa2_2, siHexon_4) for each of the different target genes was selected and the three siRNAs – hereafter referred to as siE1A, siIVa2 and siHexon – were analyzed in more detail to confirm the efficiency and specificity of the silencing approach. For this propose we first determined siE1A, siIVa2 and siHexon (100 nM) silencing activity by measurement of the respective E1A, IVa2 and hexon mRNA expression in Ad5TRE-E1A (5 moi) infected cells by using target gene specific quantitative RT-PCR. As shown in Fig. 1C all three siRNAs were able to downregulate their respective target gene by approximately 75%. To confirm that silencing indeed resulted from interaction of the siRNA with its respective target the target sequences of the selected siRNAs were inserted into the 3′ UTR of a luciferase reporter. Co-transfection of HeLa cells with 10 nM siRNAs and the respective reporter gene constructs revealed strong and sequence-specific inhibition of luciferase activity. Moreover, the silencing efficiency was comparable for the three siRNAs siE1A, siIVa2 and siHexon (Fig. 2). Similar results were obtained when the siRNAs were used at lower concentrations of 5 nM or 1 nM (data not shown). siE1A, siIVa2 and siHexon were unable to silence luciferase expression in these constructs which contain adenoviral target

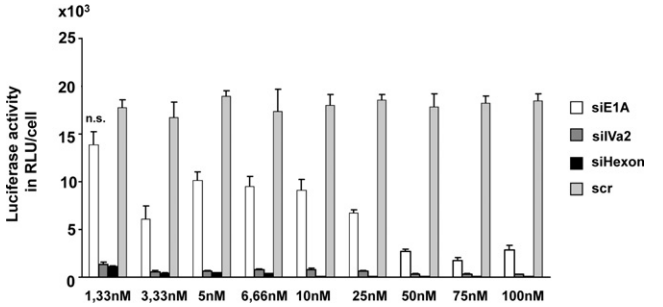

Fig. 3. Dose-dependent inhibition of adenoviral production by siE1A, siIVa2 and siHexon. HeLa cells were transfected with siE1A, siIVa2 and siHexon as indicated and infected with Ad5TRE-E1A at an moi of 1 and Ad5CMVluc at an moi of 2. Inhibition of virus production was determined by trans-complementation assay 36 h after transfection. siHexon and siIVa2 showed substantially stronger inhibition of virus production as compared to siE1A. Values are given as mean values ± S.E.M. scr = scrambled siRNA. Significance: siE1A treatment at a concentration of 1.33 nM failed to reduce adenoviral production compared to scrambled siRNA (scr) (n.s.: not significant), whereas all other anti-adenoviral siRNA treatment groups resulted in significant lower viral production compared to scr, $p < 0.05$.

sequences that did not match their specific siRNA target sequence (data not shown), respectively. Furthermore, long-term investigations demonstrated that treatment with anti-adenoviral siRNAs resulted in stable and efficient silencing over a 6 days investigation period (Figure I in the online-only Data Supplement). Finally, to exclude general non-specific off-target effects of anti-adenovirus siRNAs on virus replication HeLa cells were transfected with the siE1A, siIVa2 and siHexon and infected with low moi's of coxsackievirus B3 (CVB3), an RNA virus that can efficiently infect and lyse HeLa cells. siRNA treatment did not change replication or lytic activity of CVB3 in HeLa cells (data not shown).

In summary, these results confirm that the observed reduction of hexon protein and E1A, IVa2 and hexon mRNA expression following siE1A, siIVa2 and siHexon treatment was a result of specific siRNA-mediated knockdown of the respective adenoviral genes. Moreover, they indicate that the three selected adenoviral siRNAs had similar silencing efficiencies and do not induce general off-target effects.

3.2. siRNAs directed against IVa2 and hexon are more efficient in inhibiting adenoviral production than the siRNA directed against E1A

In further experiments we assessed the capacities of siE1A, siIVa2 and siHexon to inhibit adenovirus replication in a quantitative manner. To this end, trans-complementation assays with co-infection of HeLa cells with Ad5TRE-E1A at an moi of 1 and Ad5CMVluc, which expresses luciferase, at an moi of 2 were performed. In this system, luciferase expression levels can then be used as an indirect measure to quantitatively assay adenoviral production as demonstrated previously (Fechner et al., 2000). To inhibit adenovirus replication, the three siRNAs were added at concentrations ranging from 1.33 to 100 nM. While siE1A caused no significant reduction of virus replication at the lowest concentration used, siIVa2 and siHexon already brought about a more than 90% inhibition of adenoviral production at the same concentration (Fig. 3). The maximal level of inhibition of virus replication was approximately 99% for siIVa2 and siHexon at the highest concentration of siRNA used, whereas siE1A only led to a maximal inhibition of ~90% at an siRNA concentration of 75 nM.

The results of the trans-complementation assay were confirmed by direct measurement of viral production. HeLa cells were infected with Ad5TRE-E1A at an moi of 1 and transfected with 5 nM of the respective siRNAs. Virus titres were determined by plaque assay 48 h thereafter. In line with the results described above, siE1A showed the lowest inhibitory efficiency with about 60% reduction of virus replication, while antiviral efficiencies of the siRNAs targeting IVa2 and hexon were substantially higher with more than 90% inhibition at the same concentration (Fig. 4). At a higher siRNA dose of 75 nM all three siRNAs inhibit adenovirus replication to similar extents (not shown). These data confirm that siE1A has lower efficiency to inhibit adenoviral replication than the two siRNAs directed against the late adenoviral genes. The data also demonstrate that determination of hexon protein expression as carried out above (Fig. 1A and B) obviously does not result in a very accurate quantitative measurement of the inhibitory efficiency of the anti-adenoviral siRNAs

3.3. Combination of different anti-adenoviral siRNAs has similar effects on inhibition of adenoviral production as compared to the single siRNA approach

To monitor possible improved effects resulting from the simultaneous inhibition of different adenoviral genes, HeLa cells were transfected with different combinations of siE1A, siIVa2 and siHexon. The total concentration of siRNAs was kept constant

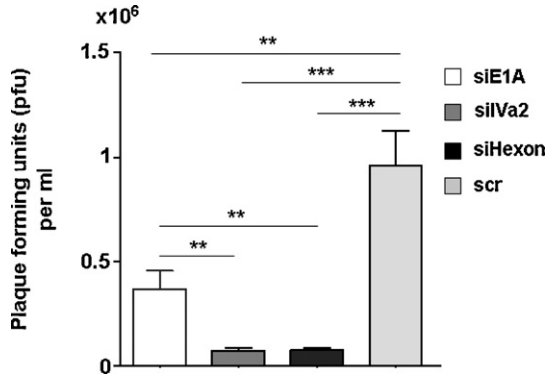

Fig. 4. Comparison of antiviral efficacy of siE1A, siIVa2 and siHexon. HeLa cells were infected with 1 moi of Ad5TRE-E1A and incubated for 2 h. Transfection was carried out with 5 nM of the indicated siRNAs and cells were incubated for another 48 h. Virus was released by four freeze/thaw cycles and plaque assays were performed. Values are given as mean values ± S.E.M. of three independent experiments. scr = scrambled siRNA. Significance: $^{***}p < 0.001$; $^{**}p < 0.01$.

at either 75, 1 or 0.5 nM, respectively and partitioned equally between the different siRNAs. After transfection of the siRNAs, cells were infected with Ad5TRE-E1A at an moi of 10 and Ad5CMVluc at an moi of 2 and virus replication was determined by trans-complementation assay. The treatment of cells with different combinations of siRNAs at total concentrations of 75 and 1 and 0.5 nM did not result in a stronger inhibition of adenovirus replication than the most efficient individual siRNAs used at the same total concentration (Fig. 5). siIVa2 and siHexon as single agents resulted in similar luciferase levels as the combined treatments, whereas siE1A displayed a lower efficiency, which is in line with the results shown above.

3.4. Combination of siE1A with siIVa2 or siHexon have synergistic inhibitory effects on adenovirus-induced cell killing activity

The physiologically most relevant parameter for treatment of adenovirus infections is a reduction of adenovirus-induced cytotoxicity. In final experiments addressing this question, HeLa cells infected with Ad5TRE-E1A at an moi of 10 and additionally transfected with siRNAs individually or in different combinations at a total concentration of 75 nM were scored for cell viability 4 days post-infection. As can be seen in Fig. 6, the application of any of the siRNAs as a single agent was insufficient to inhibit adenovirus-induced cell killing and virtually all cells were lysed after 4 days. In

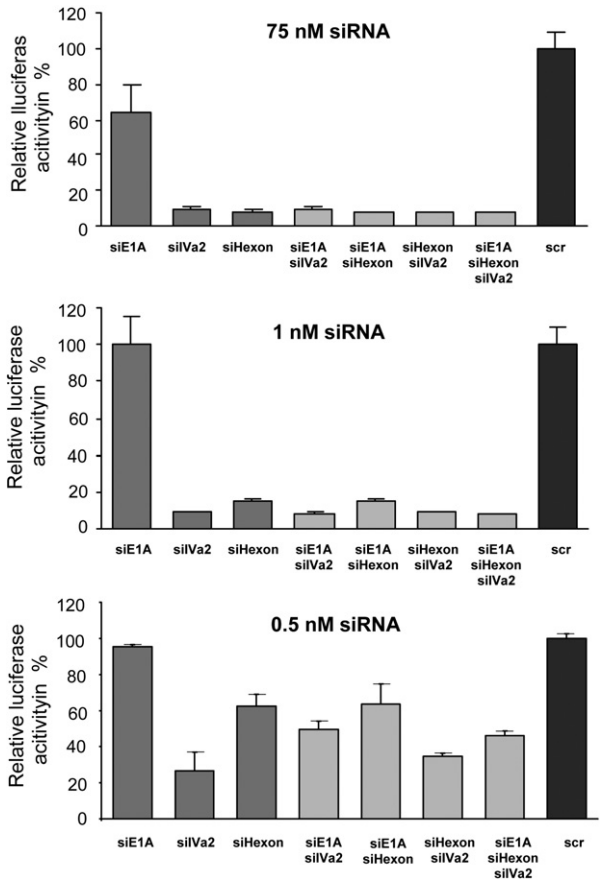

Fig. 5. Inhibition of adenovirus replication by combination of siRNAs targeting different adenoviral genes. HeLa cells were transfected with a total siRNA dose of 75, 1 and 0.5 nM per well, respectively. The siRNA dose was partitioned in equal amounts in cases in which more than one siRNA was transfected. After transfection, cells were infected with 10 moi of Ad5TRE-E1A and 2 moi of Ad5CMVluc for 4 h. Inhibition of adenoviral production was determined by trans-complementation assay 36 h after transfection. Values are given as mean values ± S.E.M. of two independent experiments each performed in triplicate. scr = scrambled siRNA.

sharp contrast, combinations of siRNAs, which included the siRNA directed against E1A, efficiently protected cells against adenovirus-induced cell lysis. These results demonstrate that combinations of an siRNA against the early adenoviral E1A and a late adenoviral gene are superior to isolated siRNAs or the siRNA combination directed

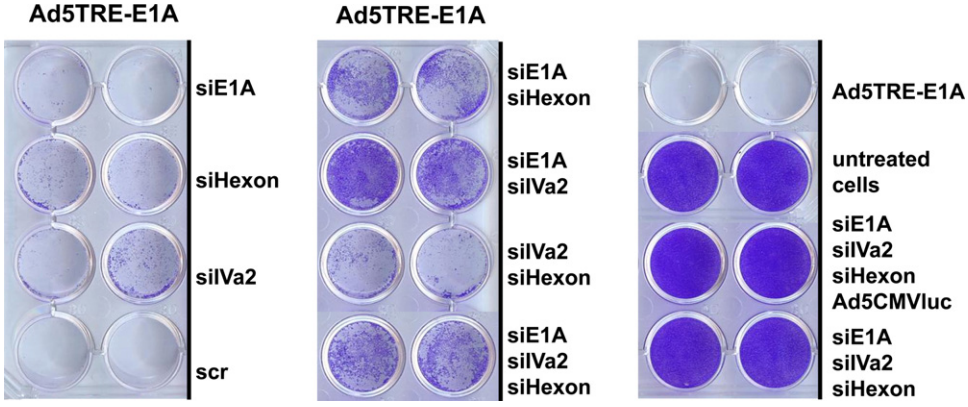

Fig. 6. Inhibition of adenovirus-mediated cytolysis by siRNAs targeting different adenoviral genes. HeLa cells were transfected with 75 nM of the indicated siRNAs. Immediately after transfection, cells were infected with 10 moi of Ad5TRE-E1A or 10 moi of the control adenovector Ad5CMVluc. After a 4 h incubation period medium was replaced by fresh medium. The siRNA concentration of 75 nM was partitioned in equal amounts in cases in which more than one siRNA was transfected. Cytopathic effects were monitored by staining viable cells with crystal violet 4 days after adenovirus infection. scr = scrambled siRNA. The figure shows one of two experiments, both of which gave similar results.

against the late adenoviral genes IVa2 and hexon in the prevention of adenovirus-mediated cell killing.

4. Discussion

Adenovirus infections typically result in mild illness and the immune system is usually capable of eliminating the virus within a few days. In some cases, however, particularly in immunosuppressed patients, adenovirus infection can lead to severe organ dysfunction and death (Hough et al., 2005; Abarca et al., 2008; Valdes et al., 2008). Currently, there is no specific anti-adenoviral drug therapy available and adenovirus infections can only be treated by palliative care. Recently, it was shown that adenovirus 11 replication can be inhibited by RNAi-mediated silencing targeting the adenoviral E1A gene (Chung et al., 2007). In our present study, we performed a further refinement of this approach by analyzing the susceptibility of various adenoviral genes to RNAi and the possibility of targeting multiple functions for enhanced antiviral efficiency. The inability of the adenovirus to generate infectious virus particles following gene deletions was the central criterion for selection of anti-adenoviral siRNAs. Several genes as the E3 genes that are dispensable for the replication of virus and the E1B genes whose deletion only reduces virus replication (Fechner et al., 2003) do not represent appropriate siRNA targets in order to achieve highly efficient inhibition of adenovirus infection. A similar assessment can be suggested for RNAi directed against the fiber protein. The fiber carries a ^{91}KKTK^{94} domain in its shaft domain for binding to heparan sulfate glycosaminoclycans (HSG) (Dechecchi et al., 2000) and on its knob a domain for binding of the domain I of the coxsackievirus adenovirus receptor (CAR) (Bewley et al., 1999). Both structures are important for adenovirus uptake into the target cells *in vitro*. However, mutation of the fiber in CAR and HSG binding sites does not affect adenovirus uptake into hepatocytes *in vivo* (Bayo-Puxan et al., 2006).

The target genes E1A, IVa2 and hexon used in this study are essential for adenovirus infection. Other genes such as the E2A (DBP) and E2B (pTP and Pol) genes which provide the machinery for replication of virus DNA and are essential for transcription of late genes (Hay et al., 1995), the pentone base which represent an important structural component of the virus capsid and mediates adenovirus internalization (Wickham et al., 1993) and pIX which is involved in capsid formation (Sargent et al., 2004) may also be regarded as potential targets for anti-adenoviral RNAi. The primary aim of this study, however, was to analyze whether combination of siRNAs would enhance anti-adenoviral efficiency in a proof-of-concept study. Therefore, only two additional candidate genes were selected in addition to E1A, which was already used successfully as target gene before (Chung et al., 2007). IVa2 was chosen since several studies demonstrated that it is essential for capsid assembly (Tribouley et al., 1994; Zhang and Imperiale, 2003) and encapsidation of the viral genome (Gustin et al., 1996; Zhang et al., 2001; Tyler et al., 2007). Hexon was included as it is essential for formation of the capsid and is important for adenovirus uptake into the liver *in vivo* (Waddington et al., 2008).

Four siRNAs were initially tested for each target gene, and several of them were able to efficiently inhibit adenoviral replication. With reporter gene constructs harbouring the corresponding target sequences, we confirmed that the inhibition was through siRNA-mediated sequence-specific silencing and not through indirect and unspecific side effects. More importantly, we were able to select siRNAs for the different target genes with potentially comparable silencing efficiencies. Surprisingly, however, when these siRNAs were directly compared for their ability to inhibit adenovirus replication, the maximal level of inhibition was distinctly stronger with anti-IVa2 and anti-Hexon siRNAs (about 99% inhibition) than with anti-E1A siRNA (only about 90% inhibition).

E1A is the first gene to be transcribed after transfer of the adenoviral genome into the nucleus. The E1A gene products, which are generated by differential splicing, transactivate both cellular and adenoviral early functions through interaction with components of the general transcription machinery and sequence-specific transcription factors. In addition, E1A modulates the cell cycle through interaction with proteins of the Rb family to create an optimal cellular environment for viral replication (Nevins et al., 1988; Russell, 2000). It is well known that the elimination of the E1A gene abolishes the ability of adenoviruses to replicate, a fact that was utilized for the generation of replication-deficient adenovirus vectors for gene therapy. However, we and others found that even a minimal expression of E1A may be sufficient to enable adenovirus replication *in vitro* (McCart et al., 2002; Fechner et al., 2003; Hurtado Picó et al., 2005; Ylosmaki et al., 2008). Even a 40-fold reduction of the E1A protein level did not significantly decrease the rate of replication of adenovirus 5 in HeLa cells (Hitt and Graham, 1990). This finding may explain why employment of a highly efficient anti-E1A siRNA, as found in this study (Figs. 1 and 2), only results in a moderate inhibition of adenoviral production.

Combination of siRNAs targeting different viral genes or different target sites of a gene has been shown to enhance the antiviral efficiency of RNAi-mediated inhibition. Furthermore, the combination of siRNAs is considered to be a suitable strategy to counteract the problem of viral escape as a consequence of the high mutation rate of many viruses (Schubert et al., 2005; ter Brake et al., 2006; Xin et al., 2008; Fulton et al., 2009). The mutation rate of adenoviruses, however, is comparatively low. Therefore, the attempt to silence multiple genes simultaneously does not primarily follow the aim of preventing viral escape, but rather to improve the antiviral activity by simultaneously targeting the complex adenovirus replication cycle at different critical points. According to the results obtained in the present study, the combination of anti-adenoviral siRNAs does not enhance inhibition of adenoviral production if applied at the same total concentration as single highly efficient siRNAs. This finding reveals failure of specific synergistic effects of the combination of siRNAs. The question, whether combinations of siRNAs can be expected to exert synergistic effects has not been clearly answered yet. While enhanced gene silencing effects were reported for combinations of siRNAs targeting an endogenously expressed gene (Ji et al., 2003) or HIV (ter Brake et al., 2006), no synergistic antiviral effects were observed for a combination of two shRNAs against CVB3 (Ahn et al., 2005). Likewise, we did also not find an increased inhibitory effect against echovirus 30, when combining an shRNA against the virus with a second shRNA targeting its cellular receptor (Rothe et al., 2010). Since the synergistic effect might only be pronounced at low siRNA concentrations, we tested combinations of siRNAs at high and low concentrations in the present studies, but we did not observe any synergistic effects of the siRNA combinations at any of the concentrations tested. The reason for the lack of the effects might be the competition of siRNAs for RISC, which was shown to reduce each other's activity (Koller et al., 2006). A second study confirmed that combinatorial delivery of siRNAs reduces RNAi efficacy by selective incorporation into RISC even under non-saturating conditions (Castanotto et al., 2007). The competition of siRNAs for RISC might thus explain the lack of synergistic effects after combination of siRNAs against adenovirus in the present study. However, the combination of the different anti-adenoviral siRNAs was as effective as the application of a single highly effective siRNA and therefore is not principally adverse in the potential therapeutic application. Moreover, with regard to the possible development of escape mutants in an *in vivo* setting, it may of course be of some advantage.

In contrast to the inhibition of adenovirus replication, the combinations of siRNAs targeting E1A and siRNAs directed against late adenoviral genes IVa2 and hexon were distinctly superior in pro-

tecting cells from adenovirus-induced cell lysis as compared to any of the individual siRNAs. These data not only show an enhanced anti-adenoviral efficiency following simultaneous silencing of different adenoviral genes as a result of synergistic anti-adenoviral siRNA activity, but also indicate that E1A silencing is crucial to prevent cell killing activity of the adenovirus. Furthermore, the data demonstrate that the extent of the inhibition of adenoviral production does not necessarily correlate with the degree of inhibition of adenovirus-induced cell lysis. The latter finding may be due to functions of E1A not directly involved in viral replication such as its well-documented pro-apoptotic properties (White, 2001; Levine, 2009). Regardless of the exact mechanism, by which co-silencing of E1A results in inhibition of adenovirus induced cytotoxicity, two important conclusions can be drawn from the present results. First, E1A-induced cytotoxicity seems to require higher E1A concentration than those required to support adenoviral replication and thus is more amenable to RNAi-mediated inhibition. Second, inhibition of E1A alone by RNAi is clearly not sufficient to efficiently inhibit cell lysis, which involves both E1A-dependent processes as well as functions mediated by the actual replication of the virus. According to our results, the latter can be efficiently inhibited by additional targeting of late adenoviral genes by RNAi.

5. Conclusion

In summary, we have shown for the first time that RNAi-mediated knockdown of genes expressed during the late phase of adenovirus replication is sufficient to inhibit adenovirus replication and that an enhanced anti-adenoviral efficiency can be achieved by combination of siRNA mediating silencing of early and late adenoviral genes. Further studies, however, are necessary to better understand the molecular mechanisms determining the specific function of E1A in this context.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2010.08.002.

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