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Chapter 6

Titers of lentiviral vectors encoding shRNAs and miRNAs are reduced by different mechanisms that require distinct repair strategies

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Abstract

RNAi-based gene therapy is a powerful approach to treat viral infections because of its high efficiency and sequence-specificity. The HIV-1 based lentiviral vector system is suitable for the delivery of RNAi inducers to HIV-1 susceptible cells due to its potency to transduce non-dividing cells, including hematopoietic stem cells, and its ability for stable transgene delivery into the host cell genome. The presence of antiviral shRNA and miRNA cassettes can negatively affect the lentiviral vector titers, which was tested for anti-HIV-1 shRNAs and miRNAs. We show that shRNAs that target the vector genomic RNA strongly reduced lentiviral vector titers, but inhibition of the RNAi pathway via saturation could rescue vector production. The presence of miRNAs in the vector RNA genome (sense orientation) results in a minor titer reduction due to Drosha processing. Vectors with the miRNAs in the antisense orientation have significant reduced titers due to self-targeting of the fully complementary vector target sequence by the mature miRNA. Another major cause for titer reduction of miRNA-vectors is likely due to promoter interference. The use of inducible promoters for expression of miRNA is therefore highly recommended. These results are important for further development and clinical application of lentiviral vectors encoding RNAi inducers.

Introduction

RNAi-mediated gene silencing is a powerful therapeutic approach to target disease-associated mRNAs and transcripts encoded by pathogenic viruses because of its high efficiency and sequence-specificity (1-3). In mammalian cells, stable RNAi can be obtained by intracellular expression of short hairpin RNAs (shRNAs) (4,5). These transcripts are transported to the cytoplasm by Exportin-5 and processed by Dicer into small interfering RNAs (siRNAs) of ~21 bp with 2-nt 3′ overhangs. The siRNA duplex is incorporated into the RNA induced silencing complex (RISC). The passenger strand of the siRNA is degraded and the guide strand of the siRNA programs RISC to cleave the perfectly complementary mRNA. Another vector based RNAi approach is the use of artificial miRNAs that closely resemble cellular miRNAs. These inhibitors are expressed as primary miRNAs (pri-miRNAs) that are cleaved by the RNase III-like endonuclease Drosha and its cofactor DGCR8 into precursor miRNAs (pre-miRNAs) (6). Pre-miRNAs are hairpin RNAs of ~70 nucleotides

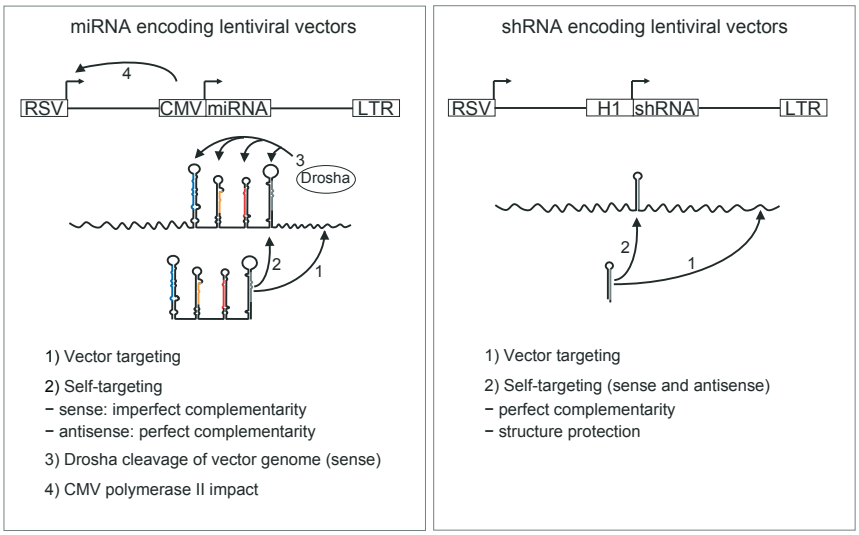

Figure 1. Possible causes for reduced titers of lentiviral vectors encoding miRNAs and shRNAs. The different mechanisms that can account for titer reduction in miRNA vectors (**left panel**) and shRNA vectors (**right panel**) are illustrated. First, anti-HIV-1 miRNA sequences can target similar sequences in the HIV-1 based lentiviral vector (mechanism 1, vector targeting). Second, mature miRNAs can target its own sequence as part of the lentiviral vector genome (mechanism 2, self-targeting). Third, miRNAs in the vector RNA genome can be processed by Drosha and thus leads to destruction of the vector genome (mechanism 3). Fourth, CMV-driven polymerase II transcription of the miRNA may interfere with RSV-driven polymerase II transcription of the vector RNA genome (mechanism 4). The situation for anti-HIV-1 shRNA expressing vectors seems less complex. The shRNAs can target HIV-1 sequences in the lentiviral vector (mechanism 1, vector targeting). Also, self-targeting of the shRNA sequence as part of the lentiviral vector genome can cause titer reduction (mechanism 2).

(nt) in size that are transported to the cytoplasm by Exportin-5 and further processed by Dicer into an imperfect ~22 nt miRNA duplex (7,8). The single stranded mature miRNA directs RISC to complementary mRNA sequences to cause mRNA cleavage or translational repression, depending on the complementarity between the miRNA and the mRNA target (9,10).

We and others previously demonstrated that potent HIV-1 inhibition can be obtained using antiviral shRNAs and designed miRNAs (11-16). However, HIV-1 can escape through the selection of a single nucleotide substitution within the target sequence (13,17). For a durable RNAi-based gene therapy against HIV-1, a combinatorial attack is required in which multiple viral sequences are targeted simultaneously (18). Therefore, we previously generated extended shRNA constructs that encode 2 or 3 active siRNAs (e2 or e3-shRNA) and an antiviral miRNA polycistron that encodes 4 active miRNAs (16,19,20).

For the delivery of RNAi-based antivirals to HIV-1 susceptible cells, the lentiviral vector system is very attractive because it is highly efficient in transducing non-dividing cells, including hematopoietic stem cells (21-24). In a durable HIV-1 treatment one can transduce CD34+ hematopoietic stem cells *ex vivo* with a lentiviral vector encoding HIV-1 specific shRNAs or miRNAs. The transduced cells should stably express the inhibitors and thus become resistant to HIV-1. Subsequently, the transduced stem cells can be engrafted back into the patient, where they will give rise to an HIV-1 resistant myeloid and lymphoid cell population. However, the presence of shRNA and miRNA cassettes can negatively affect the lentiviral vector titers, which may hamper clinical applications (25,26). It is therefore important to study the mechanisms by which RNAi inducers affect the vector titer in order to propose methods that could restore titers.

There are several possible causes for reduction in titers. These inhibitory possibilities on lentiviral vector titers are illustrated in figure 1, for miRNA cassettes on the left and shRNA cassettes on the right. An obvious problem for anti-HIV shRNAs and miRNAs can be that the target sequence is also present in the HIV-based vector genome. This vector targeting problem is marked as mechanism 1 in figure 1 and can simply be avoided by selecting antivirals that do not target the lentiviral vector. The shRNA or miRNA can possibly target its own sequence as part of the lentiviral vector genome (self-targeting) (Fig. 1, mechanism 2). Vector RNA genomes with a miRNA cassette face the additional problem that Drosha cleavage in the nucleus may inactivate the vector genome, either in the producer or transduced cell (Fig. 1, mechanism 3). It is also possible that transcription of the miRNA unit by polymerase II could interfere with transcription of the lentiviral vector (Fig. 1, mechanism 4). In general, expression of shRNAs or miRNAs may cause a titer reduction due to aspecific toxicity e.g. by an unwanted off-target effect. In addition, stable RNA hairpin structures introduced by the RNAi cassettes may affect vector titers by affecting RNA nuclear export, genomic RNA packaging, or reverse transcription in the target cells (27,28).

Here, we studied the effect of different RNAi inducers: single shRNA or multiplex e3-shRNA cassettes and single miRNA or multiplex miRNA expression cassettes on the titer of the lentiviral vector. We demonstrate that the titers were dramatically reduced by up to 1000-fold for some vectors compared to the control vector. The titer reduction of the shRNAs and miRNAs vectors is caused by different mechanisms. Based on this insight, we tested specific countermeasures that resulted in significantly improved titers. These insights are important for the clinical development of lentiviral vectors that induce RNAi to treat disease.

Materials and methods

DNA constructs

Lentiviral vector plasmids are derived from the construct (pRRLcpptpgkgfppreSsin) (29), which we renamed JS1. The JS1-based plasmids H1-shNef, H1-shLdr and H1-e3-shRNA were obtained by cloning of the H1-shRNA cassette from the original pSUPER construct (digestion with XhoI and PstI) into the corresponding sites of JS1. The JS1 plasmids CMV-miRNA S and CMV-4-miRNA S were obtained by digestion of the original pcDNA6.2 construct (16) with NruI and XhoI. The fragments were treated with Klenow enzyme according to the manufacturer's protocol (Roche, Mannheim, Germany) and inserted into the EcoRV site of JS1. The JS1 plasmids 4-miRNA S and 4-miRNA AS were obtained by PCR amplification of the miRNA polycistron from the original pcDNA6.2 construct with the primers XhoF:CGCTCGAGGAGGTGTTAATTCTAATTATCTATTTCA and PstR:CATCTGCAGGCATTGCAACCGATCCCAACCTGTGTA;PstF:CATCTGCAGGAGGTGTTAATTCTAATTATCTATT and XhoR:CGCTCGAGGCATTGCAACCGATCCCAACCTGTGT. The PCR fragments were digested with PstI and XhoI and inserted into the corresponding sites of JS1.

Luciferase reporters Luc-Pol, Luc-Gag, Luc-R/T, Luc-Nef and Luc-Ldr were described previously (18,30,31). The packaging plasmids pSYNGP (32), pRSV-rev (33), pVSVg (34) and the construct expressing 5xshRNA (Gag5, Pol1, Pol6, Pol9 and Pol47) from repeated H1 promoters were constructed as described previously (35). The Luc-ACDE reporter with four target sequences of the antiviral 4-miRNA polycistron will be described elsewhere (manuscript submitted). This reporter was also used to produce an RNAi decoy for CMV-miRNA S because it also encodes a target for this miRNA. The plasmid expressing the Adenovirus VA RNAs (pVA RNAs)(36), the Ebola VP35 protein (37,38), the CRM1 co-factor (39), the siRNA against Dicer and the shRNA against Luciferase (4) have been described elsewhere.

Cell culture and transfections

Human embryonic kidney (HEK) 293T adherent cells were grown in DMEM (Gibco BRL) supplemented with 10% fetal calf serum (FCS) (Hybond), minimal essential medium nonessential amino acids, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C and 5% CO_2. SupT1 suspension T cells were grown in advanced RPMI supplemented with 1% FCS, penicillin (30 U/ml) and streptomycin (30 μg/ml) and L-glutamine at 37°C and 5% CO_2.

To knock down Drosha in the producer cells, 3.0 x 10^5 HEK 293T cells were seeded in 6-wells plates. The next two consecutive days, the cells were transfected with 250 ng of shLuc or shDrosha using Lipofectamin 2000 reagent according to the manufac-

turer's protocol (Invitrogen, Carlsbad, CA). The third day, lentiviral vector production was initiated by co-transfection with the lentiviral vector and packaging constructs as described below.

Lentiviral vector production and transduction

For production of the lentiviral vector particles, 6.0 x 10^5 HEK 293T cells were seeded per well in 6-well plates in 2 ml of DMEM/10% FCS without antibiotics. The next day, medium was replaced with 0.4 ml DMEM/10% FCS without antibiotics. Subsequently, the control JS1 vector (0.95 μg), JS1-shNef, JS1-shLdr, JS1-e3-shRNA, JS1-CMV-miRNA S, JS1-CMV-4 miRNA and JS1-4-miRNA in sense or antisense orientation were co-transfected with the packaging plasmids pSYNGP (0.6 μg)(32), pRSV-Rev (0.25 μg)(33) and pVSVg (0.33 μg)(34) with Lipofectamine 2000 reagent as suggested by the manufacturer (Invitrogen, Carlsbad, CA). When indicated, competitor or suppressor plasmids (2.9 μg) were added to the co-transfection. Medium was replaced with 2 ml optimem on the second day. On the third day, medium containing lentiviral vector was harvested. Cellular debris was removed by centrifugation for 5 min at 1200 rpm. Production of lentiviral vector particles was determined by CA-p24 ELISA as previously described (40). Capsid values were corrected for between-session variation (41).

SupT1 cells were transduced with a dilution series of the lentiviral vector stocks to determine the titer. Three days post-transduction, cells were analyzed with FACS to detect eGFP positive cells. The titer is expressed as transducing units/ml vector stock, and values were corrected for between-session variation (41).

Luciferase assay

HEK 293T cells were plated one day before transfection in 24-well plates at a density of 1.3 x 10^5 cells per well in 500 μl DMEM/10% FCS without antibiotics and transfected using Lipofectamine 2000 reagents according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Cells were co-transfected with 100 ng of the firefly luciferase expression plasmid, 1 ng of renilla luciferase expression plasmid (pRL-CMV) and 100 ng of the JS1 constructs. Two days post-transfection, firefly and renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Relative luciferase activities were calculated from the ratio between firefly and renilla luciferase activities.

Results

Reduced titer of lentiviral vectors encoding RNAi inducers against HIV-1

We previously constructed several RNAi inducers against HIV-1, including shRNA and miRNA molecules (11-14). These inhibitors and the corresponding target sites in the HIV-1 RNA genome are indicated in Fig. 2a. For combinatorial RNAi approaches, we also generated constructs that target multiple sites in the HIV-1 RNA genome: the e3-shRNA and the antiviral 4-miRNA polycistron (16,20). The e3-shRNA encodes three siRNAs that target the nef19, pol1 and rev/tat (r/t) region (Fig. 2a). The antiviral 4-miRNA polycistron encodes four miRNAs that target pol47, gag, r/t and leader (ldr) sequences. The shRNA and e3-shRNA expression cassettes were cloned in the lentiviral vector JS1, which encodes the eGFP marker gene from an independent transcription unit

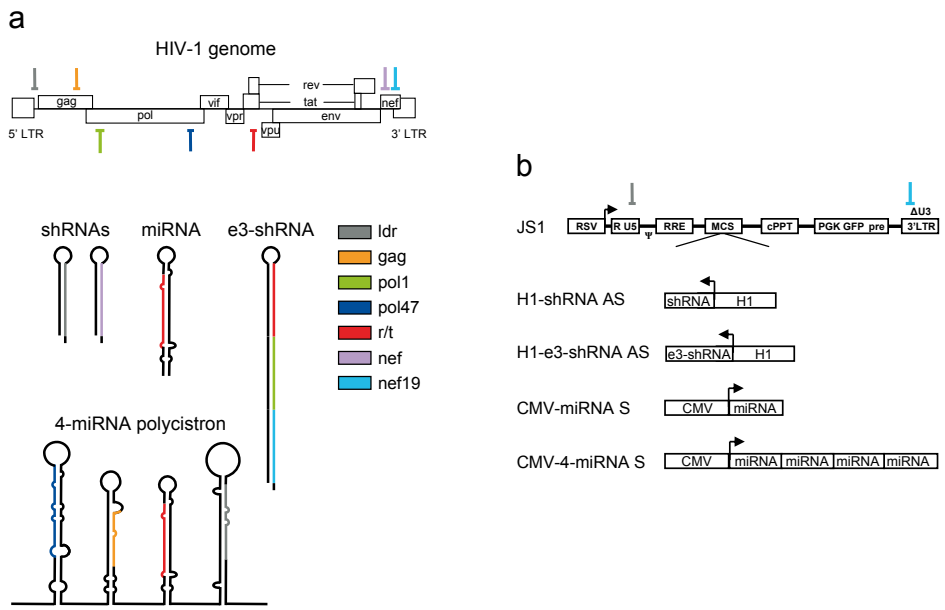

Figure 2. The RNAi inducers used in this study. (**a**) Structure of the shRNA, miRNA, e3-shRNA and the antiviral 4-miRNA polycistron and the target positions within the HIV-1 genome are indicated by color coding. (**b**) The JS1 lentiviral vector is shown at the top with the RNAi-inducing gene cassettes that were introduced into the multiple cloning site (MCS). The shRNA and e3-shRNA are driven by the polymerase III H1 promoter and were cloned in antisense (AS) orientation. The miRNAs are expressed from the polymerase II CMV promoter and were cloned in the sense (S) orientation.

driven by the PGK promoter (Fig. 2b). We used the H1 RNA polymerase III promoter to drive shRNA/e3-shRNA expression and these cassettes are cloned in the antisense orientation. The orientation of the shRNA expression cassette within the vector genome does not influence the titer (26). The expression of the miRNA units are controlled by the constitutive immediate early promoter of cytomegalovirus (CMV). These expression units were inserted into JS1 in the sense orientation to avoid interference with transcription of the lentiviral RNA genome.

To test whether shRNAs and miRNAs against HIV-1 affect the lentiviral vector in terms of production of viral particles and titers, we produced vesicular stomatitis virus (VSV) G-pseudotyped vector particles by transient co-transfection of HEK 293T cells. We studied whether the production of the lentiviral vector particles was affected by determining the capsid (CA-p24) level in the culture supernatant after 2 days. As a negative control, we included the JS1 lentiviral vector. No gross differences in capsid level were measured for the different lentiviral vectors (Fig. 3a). To determine the titer, we subsequently transduced SupT1 T cells with a dilution series of the produced vectors. The percentage of

eGFP positive cells was measured by FACS to determine the transduction units at three days post-transduction. Similar titers were measured for the control JS1 vector and the shNef vector (Fig. 3b). For the shLdr vector we measured a dramatic 188-fold reduction of titer. This reduction is caused by targeting of HIV-1 Ldr sequences that are also present in the vector backbone (Fig. 2b) (26). Thus, shLdr serves as a positive control for direct RNAi-attack on the lentiviral vector. The same mechanism may explain the drop in titer for e3-shRNA, which encodes the siNef19 inhibitor that attacks the lentiviral genome in the 3′ untranslated region (Fig. 2b). A profound titer reduction of 56- and 70-fold was observed for the miRNA and 4-miRNA vectors (Fig. 3b).

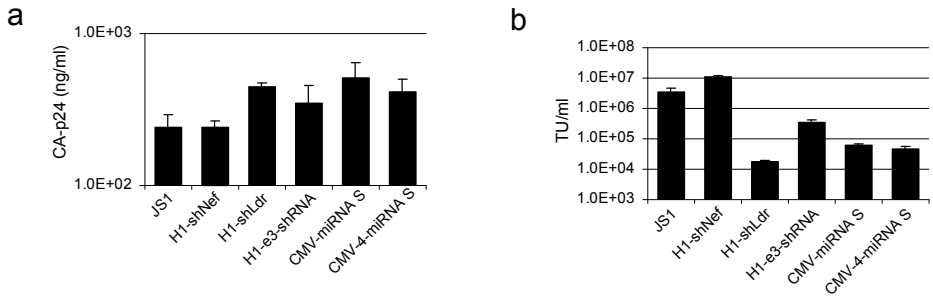

Figure 3. Lentiviral vector particle production and titers of the vectors encoding the RNAi inducers. (**a**) Lentiviral vector particle production was determined by measuring the CA-p24 levels of the lentiviral vector stocks. (**b**) Lentiviral vector titers (transducing units/ml, TU/ml) were determined by measuring the percentage of eGFP+ cells at 3 days post transduction. The mean values and standard deviations were shown from four independent transfections that were performed in duplo.

Inhibition of the RNAi pathway to increase the lentiviral vector titer

The shLdr and e3-shRNA constructs encode siRNAs that directly target vector RNA sequences (Fig. 2b). We therefore expected that inhibition of the RNAi pathway during vector production should lead to a (partial) restorement of the titers. To test this, we used different approaches to abort the RNAi mechanism by co-transfection of: an excess luciferase reporter with the corresponding shRNA target sequence (Luc) as RNAi target decoy, an excess shRNAs (a 5xshRNA plasmid encoding 5 shRNAs) to saturate the RNAi machinery, a plasmid encoding VA RNA of Adenovirus as Dicer inhibitor (36), a plasmid encoding the RNAi suppressor protein VP35 of Ebola virus (37,38), siRNAs against Dicer (4), or an shRNA against Drosha. Furthermore, we tested whether overexpression of the cellular transport protein CRM1 (39), which is involved in the nuclear export of unspliced and partially spliced viral RNAs, could improve production of viral particles and thereby the titer (42,43). We co-transfected the different RNAi inhibitors with the lentiviral vector and the standard set of packaging plasmids and subsequently determined the titer.

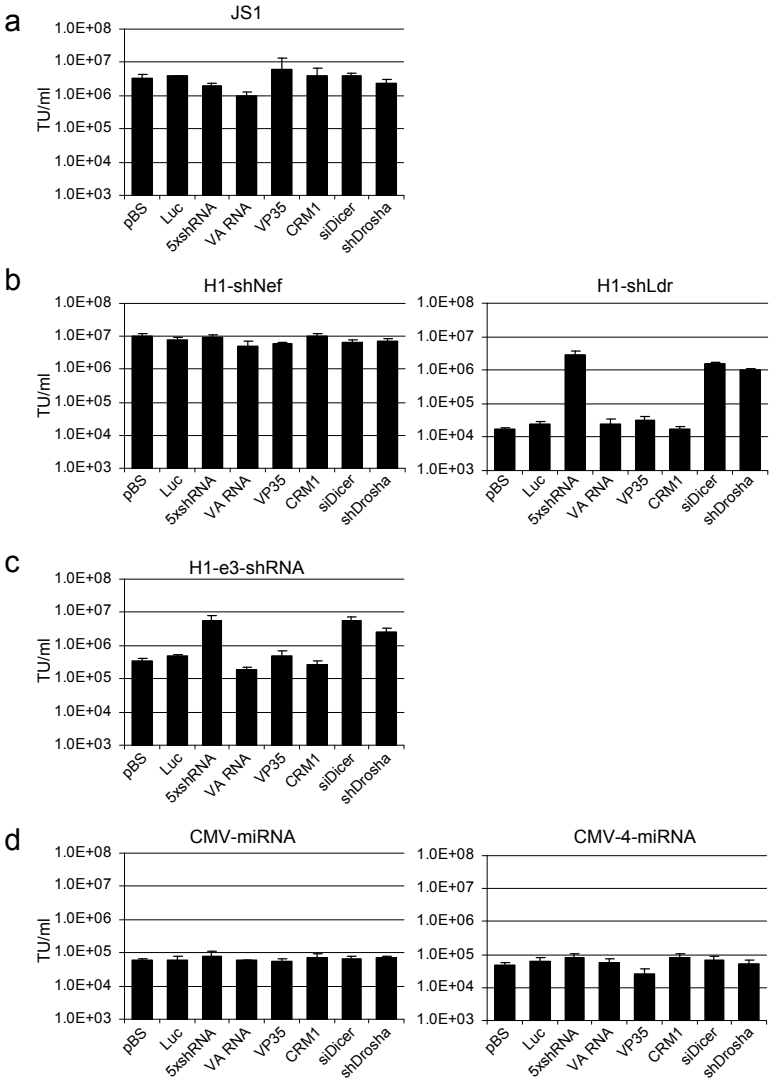

Figure 4. Inhibition of the RNAi pathway to improve the lentiviral vector titers. (**a**) Titers of the control JS1 lentiviral vector and the effect of saturation of the RNAi pathway. The plasmid encoding Bluescript (pBS) was used as a control. Decoy RNAi targets encoded in luciferase reporters, excess shRNA (p5xshRNA), VA RNA (pVA RNA), VP35 (pVP35), siRNA against Dicer (siDicer) and shRNA against Drosha (pshDrosha) were used to saturate the RNAi pathway. CRM1 (pCRM1) was used to increase nuclear export of the vector RNA genome export. The titers were determined by measuring the percentage of eGFP+ cells at 3 days post transduction. The same variations were tested for JS1 vectors with the following RNAi cassettes: (**b**) H1-shNef and H1-shLdr, (**c**) H1-e3-shRNA, (**d**) CMV-miRNA and CMV-4-miRNA. The mean values and standard deviations were shown that were based on four independent experiments.

The JS1 lentiviral vector was included as control and its titer is not significantly affected by the different effector molecules (Fig. 4a). Co-transfection of the RNAi inhibitors did not affect the titer of the shNef vector either (Fig. 4b, left). The titer of the shLdr vector is extremely low due to direct vector targeting, but was significantly improved by an excess shRNAs, siRNA against Dicer and the shRNA against Drosha (Fig. 4b, right). The titer of the shLdr vector can be improved to the level that is only 2-fold lower than that of the shNef vector. Most likely, this improvement is caused by saturation of the RNAi pathway, such that less shLdr-derived siRNA against vector sequences is incorporated into RISC. A very similar pattern of improvement was apparent for the e3-shRNA vector, although the initial defect is due to vector targeting, the magnitude of repair is more modest than observed for the shLdr vector (Fig. 4c). However, inhibition of the RNAi pathway did not significantly improve the titer of the two miRNA vectors (Fig. 4d). These results suggest a different cause of the titer reduction in the miRNA vectors.

Titer reduction of miRNA vectors is partially Drosha-dependent

The low titer observed for the miRNA vectors is likely caused by recognition and processing of the miRNAs as part of the vector RNA genome by the endonuclease Drosha (Fig. 1, mechanism 3). However, knocking down Drosha during vector production did not affect the titer of the miRNA vectors (Fig. 4d). This may be due to a long intracellular half-life of the Drosha enzyme. To intensify Drosha knockdown in the producer cells, we performed a new experiment in which the shDrosha plasmid was transfected two days and one day prior vector production. Drosha knockdown in the producer cells was confirmed by Western blot analysis (results not shown). Similar viral particle

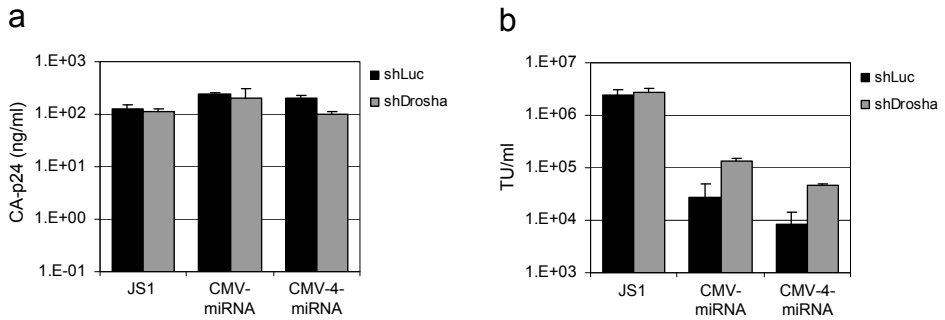

Figure 5. Titer decrease of miRNA vectors is partly Drosha-dependent. (**a**) miRNA expressing vectors were produced in cells where Drosha was knocked down using intensified shRNA treatment. As a negative control shRNAs against Luc (shLuc) was used. Capsid p24 levels were measured by ELISA for the JS1, CMV-miRNA, CMV-4-miRNA vectors. (**b**) Titers of these vectors that were produced in cells with or without Drosha knockdown. Averages and standard deviations were derived from three independent experiments.

production was measured for the JS1 control and the two miRNA vectors in the presence of shDrosha or the shLuc construct (Fig. 5a). The miRNA vectors showed the expected drop in titer. Interestingly, titers of both CMV-miRNA and CMV-4-miRNA showed a 10-fold improvement upon intensified Drosha knockdown (Fig. 5b). This up-regulation was not observed for the JS1 control, indicating that the titer reduction is at least partially Drosha-dependent.

Removal of the CMV promoter from miRNA vectors restores the titers

To study the titer problem of the CMV-4-miRNA vector in more detail, we made a new construct in which the miRNA unit is placed in the antisense orientation (CMV-4-miRNA AS, Fig. 6a). This means that the complement of the miRNA sequence will end up in the vector RNA genome, thus avoiding Drosha recognition. Consistent with the Drosha knockdown results, we measured a modest titer improvement compared to the sense vector (Fig. 6b). However, other explanations are required to explain the low titer of the CMV-4-miRNA AS vector. To test whether miRNA expression is the direct cause of the titer problem, we modified both the sense and antisense constructs by deletion of the CMV promoter (4-miRNA S and AS, Fig. 6a). By removal of the CMV promoter, the titer of both miRNA vectors was improved ~400 fold (Fig. 6b). These results indicate that either miRNA expression or the CMV promoter (its sequence or activity) caused the titer reduction. Notably, the titer of the promoterless miRNA vector in antisense orientation was restored to the level obtained with the control JS1 lentiviral vector. The titer of the miRNA vector in sense orientation still showed a minor reduction, which is in fact expected due to Drosha processing of the miRNA segment as part of the vector RNA genome (Fig. 1, mechanism 3).

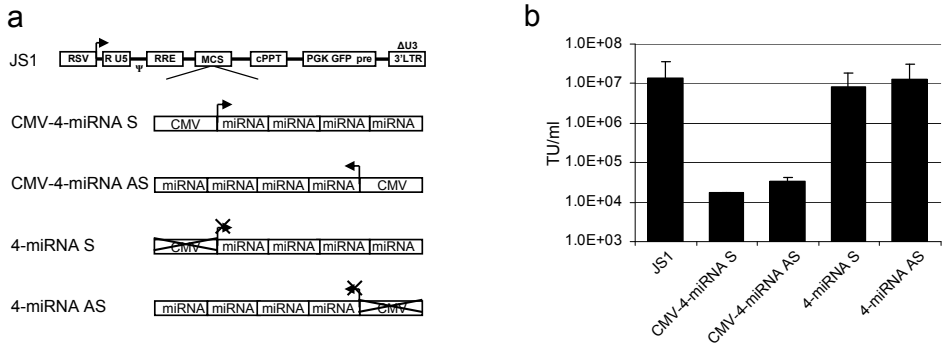

Figure 6. Absence of miRNA expression during vector production restores titers. (**a**) Schematic of the JS1 vector and the miRNA gene cassettes that were introduced into this vector. The antiviral 4-miRNA polycistron under the control of the CMV promoter was cloned in the sense or antisense orientation (CMV-4-miRNAs S and AS) into the JS1 vector. 4-miRNA S and 4-miRNA AS were generated by removal of the CMV promoter. (**b**) Titers of the vectors described in panel a were determined. Averages and standard deviations were derived from three independent experiments.

These results suggest that there are at least two causes for the titer reduction in miRNA vectors. Expression of the miRNA seems to have a major impact on the titer, and the sense constructs additionally encounter the problem of Drosha-mediated processing of the miRNA-containing vector genome. In addition, CMV-driven transcription of the miRNA unit may have a negative impact on the titer. We therefore decided to measure the level of mature miRNA expression from the lentiviral vectors. For this, we used four luciferase reporters with the appropriate HIV-1 targets. We observed very similar results for all reporters (Fig. 7). Potent inhibition of luciferase expression was measured for the miRNA constructs in the sense orientation, but to our surprise not for those in the antisense orientation (Fig. 7). These results indicate that both sense constructs express the miRNA. For the CMV-4-miRNA construct, this result is expected, but for the CMV-less construct this may be strange due to the lack of a promoter. In this case the miRNA is in fact processed from the vector genomic transcript during vector production. This means that these miRNAs will not be

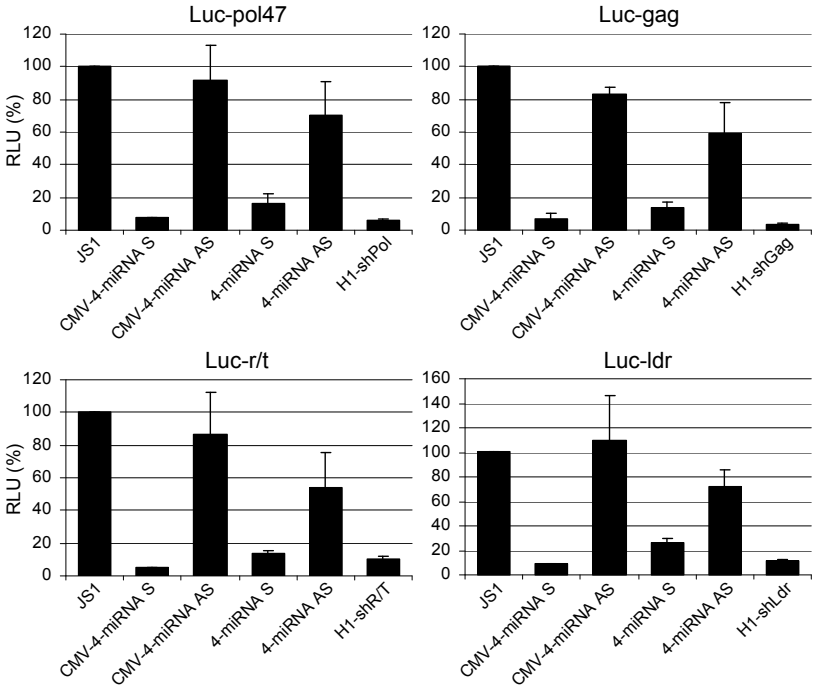

Figure 7. Luciferase reporter knockdown by the lentiviral constructs encoding the 4-miRNA polycistron. To test for miRNA expression and activity, we co-transfected the miRNA constructs with corresponding luciferase reporter constructs and a plasmid encoding Renilla luciferase. The normalized luciferase expression in the presence of the JS1 vector was set at 100% for each luciferase reporter. An shRNA construct against the specific target was used as positive control. The mean values and standard deviations were shown from two independent transfections that were performed in duplo.

produced in the transduced cells because we used a self-inactivating lentiviral vector that removes the upstream promoter during transduction. In contrast, the antisense CMV-4-miRNA construct did not express active miRNAs, which is likely due to the absence of a polyadenylation signal for the primary miRNA transcript, which may reduce the transcript stability. The sense constructs will produce pri-miRNA transcripts that use the polyadenylation signal within the 3′ LTR of the vector genome. These results indicate that it is not the expressed miRNAs that cause the severe titer reduction. This suggests that the CMV promoter (its sequence or activity), either in sense or antisense orientation, is causing the titer reduction.

Excluding miRNA-mediated titer reduction

Our results suggest that expression of antiviral miRNAs in the producer cells is not the cause of the poor vector titers. To confirm this, we co-transfected a miRNA expression plasmid with the CMV-4-miRNA cassette (16) during lentiviral vector production. As a negative control the plasmid encoding Bluescript (pBS) was used. The titer of the control JS1 vector was not affected by the expression of the antiviral 4-miRNA polycistron *in trans*, which confirms the observation that expression of the miRNAs does not cause the decrease in vector titer (Fig. 8). Likewise, the titer of the sense miRNA vectors was not significantly affected. These results suggest that the expressed mature miRNAs are not targeting the partially complementary sequences that are part of the lentiviral vector genome (Fig. 1 mechanism 2). However, when the miRNA cassette is present in the antisense orientation, a perfect complementary target will reside in the lentiviral vector genome for the miRNAs. Indeed, we observed a ~2 log titer reduction for the antisense miRNA vectors when the 4-miRNA construct was provided *in trans* during vector production. Very similar results were obtained in co-transfections with another 4-miRNA construct that expresses the same set of siRNAs, except the vector-targeting siLdr (results

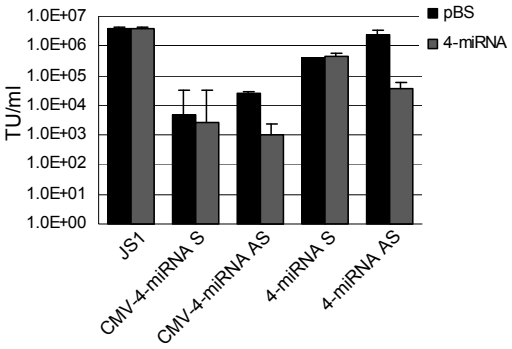

Figure 8. Titer reduction is not caused by miRNA expression. Titers of the set of the 4-miRNAs vectors that were produced in cells with or without 4-miRNA overexpression *in trans*. pBS was used as a negative control.

not shown). These combined results suggest that the titer reduction of miRNA-containing lentiviral vectors is due to a multiplicity of causes, including Drosha cleavage (sense only), the CMV promoter (sense and antisense) and miRNA-mediated self-targeting (antisense only).

Discussion

Lentiviral vectors encoding shRNAs or miRNAs are useful tools to specifically knock down disease-associated mRNAs and some are now being considered for clinical applications (44). Furthermore, these vectors are also powerful laboratory tools to study gene functions. Although relatively low vector titers are sufficient for many *in vitro* applications, high vector titers are important for clinical applications. In this study, we set out to test the negative impact of anti-HIV RNAi reagents, either shRNAs or miRNAs, on the lentiviral vector system that is well-suited for gene therapy applications in AIDS patients. We found that the insertion of a single shRNA expression cassette did not reduce the titer of the lentiviral vector. However, the lentiviral vector titer is severely reduced when the antiviral shRNA targets sequences of the vector system (Fig. 1, mechanism 1). We could repair the vector titer by inhibition of the RNAi pathway using excess shRNAs, siRNA against Dicer or shRNA against Drosha. The impact of the latter two reagents can in part be attributed to knockdown of Dicer and Drosha, but it is likely that also in these cases RNAi is predominantly inhibited via saturation.

A more complex situation was apparent for miRNA-expressing lentiviral vectors, which showed dramatically reduced titers. Inhibition of the RNAi pathway by saturation or the use of RNAi suppressors did not restore the titer. The reduction of the titer could be caused by Drosha recognition and processing of the pri-miRNA as part of the vector RNA genome (Fig. 1, mechanism 3) (sense orientation of miRNA unit). Indeed, intensified shRNA-mediated knockdown of Drosha resulted in a 10-fold improvement of the vector titer. Consistent with this idea is the observation that the titer is slightly improved when the miRNA cassette is placed in the antisense orientation to avoid Drosha processing of the vector RNA genome.

However, vectors with antisense miRNA cassettes face another problem because the mature miRNA may attack the fully complementary target sequence in the vector RNA genome in the producer cell, thus causing a drop in titer (Fig. 1, mechanism 2). In this study we showed that this mechanism causes a 2-log reduction in vector titers. This reduction could be mimicked by co-transfection of the miRNA cassette *in trans*. In this respect, miRNA vectors are different from shRNA expressing vectors that are not subjected to self-targeting because the target sequence is occluded in a stable RNA structure that is not a good RNAi target (26,45,46) (Fig, 1, right panel, mechanism 2). Similarly, Zhou et al. showed that shRNA expressing lentiviral vector do not have reduced titers caused by self-targeting (47). However, Poluri et al. showed

that lentiviral vectors encoding shRNAs have reduced titer because the vector RNA serves as a target for the expressed shRNA (25). The differences in results likely originate from the fact that Poluri et al. used a different lentiviral vector system. Consistent with our results, Poluri et al. showed that the titer reduction can be alleviated by inhibition of the RNAi pathway.

In addition to these orientation-specific vector problems, we also observed a strong negative effect by the presence of the CMV promoter of the miRNA cassette, and this effect was apparent in both orientations. These results indicate that either the sequence or the activity of the CMV promoter affects the titer. This titer reduction is only observed when the CMV promoter is present *in cis* in the lentiviral vector and not when provided on another plasmid *in trans*; thus excluding squelching effects. This *cis*-effect could be due to competition or interference of the internal CMV promoter with the RSV promoter that drives vector expression. It has previously been reported that the human CMV promoter is more active than the RSV promoter (48,49) and transcription of the RNAi cassette may be favored over expression of full length vector RNA during vector production. It has recently been reported that bidirectional promoter interference can occur between two promoters in lentiviral vector constructs (50). This promoter interference caused a marked reduction of transgene expression from the adjacent transcription unit. However, further studies are needed to elaborate on this hypothesis. To avoid any form of transcriptional interference, expression of the miRNA cassette during vector production should be prevented. This condition could be met by the use of a conditionally regulated promoter, which should be inactive during lentiviral vector production. Lentiviral vectors encoding shRNAs do not have reduced titers due to transcriptional interference. This is likely because transcription of the shRNA by RNA polymerase III does not interfere with transcription of the vector genome by RNA polymerase II.

Taken together, we showed that the titer reduction of miRNA-expressing lentiviral vectors is due to a multiplicity of causes. First, Drosha processing of the vector RNA genome causes a minor reduction in vector titer when the miRNA expression cassette is cloned in the sense orientation (Fig. 1, mechanism 3). Second, miRNA-mediated self-targeting causes a significant reduction in vector titer when the miRNA expression cassette is placed in the antisense orientation (Fig. 1, mechanism 2). Most importantly, the presence of the CMV promoter, in both orientations, seems detrimental for the vector titers (Fig. 1, mechanism 4). These insights allow us to present new design rules for miRNA-encoding lentiviral vectors. To avoid the Drosha effect, the miRNA cassette should be cloned in antisense orientation. However, this orientation causes the severe problem of self-targeting. This can be solved by the use of an inducible or tissue-specific promoter, which should be turned off during production of the lentiviral vector. Another advantage of such promoters is that miRNA expression can be strictly regulated to minimize off target effects. These findings have important implications for large-scale production of lentiviral vectors encoding RNAi inducers for clinical applications.

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