Optimization of short hairpin RNA for lentiviral-mediated RNAi against *WAS*

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Abstract

The expression of short hairpin RNAs (shRNAs) with lentiviral vectors is useful to induce stable RNA interference, particularly in hematopoietic cells. Since primary cells integrate few copies of vector, we tested if several shRNA cassette modifications could improve knock-down efficacy. Using two shRNA sequences previously shown to inhibit the human *WAS* gene expression, we found that neither increasing the shRNA stem length from 19-nt to 29-nt, nor modifying the loop with 4-nt, 9-nt artificial loops or with the mir30 loop improved vector-induced shRNA efficacies. This cautions against extrapolating results obtained with synthetic molecules to shRNAs that are stably expressed from viral vectors. On the other hand, the duplication of the shRNA expression cassette resulted in twice as much knock-down per copy of integrated vector. This strategy allowed a strong suppression of WASp in $CD34^+$ cells and will facilitate future studies on the role of WASp in human cells.

Keywords: RNAi, shRNA, lentiviral vectors, Wiskott-Aldrich Syndrome.

Introduction

RNA interference (RNAi) has become a widely-used technique to silence gene expression. Classically, RNAi leads to the degradation of particular mRNAs through sequence-specific recognition and cleavage mediated by a nucleo-proteic complex, the RNA-induced silencing complex (RISC) (reviewed by [1]). Experimentally, RNAi can be induced in cells by synthetic double-stranded RNAs or by DNA-encoding small hairpin RNAs (shRNAs) [2]. When expressed from transfected plasmids or viral vectors, shRNAs are produced in the nucleus and are processed similarly to pre-micro RNAs (pre-miRNAs) [3]. Both pre-miRNA and shRNA utilize exportin 5 for nuclear export and in the cytoplasm, both are processed by Dicer to enter the effector RISC [4, 5]. The possibility to induce RNAi with DNA vector-encoded shRNAs has increased the range of application of this gene silencing technique. Several types of vectors encoding shRNAs have been developed and used effectively in a wide variety of cells. This includes retroviral vectors which are capable of stably integrating the shRNA cassette into the host cell genome to induce long-lasting silencing in various *in vitro* and *in vivo* model systems [1]. Lentiviral vectors are particularly useful to induce RNAi in hematopoeitic cells which are not easily transfected with oligonucleotides or plasmids. We have characterized several shRNAs delivered by HIV-1-derived lentiviral vectors that inhibit the expression of the Wiskott-Aldrich syndrome protein (WASp), a cytoskeletal regulator expressed exclusively in hematopoietic cells [6]. With the most efficient shRNA-encoding lentiviral vector, we observed 90% inhibition of WASp when at least 3 copies of the viral vector genome were integrated per cell in a Jurkat cell line model. A less efficient vector provided 90% inhibition with 10 copies integrated per cell. Because such a high level of

transduction cannot easily be reached in primary cells, we sought to improve the efficacy of these vectors through optimization of the shRNA cassette. Recent studies have shown that the cleavage efficiency of RISC is higher when this complex is loaded with a miRNA precursor rather than with the final product of Dicer processing, which is a 21-22 nucleotide-long siRNA duplex [7]. This led to the hypothesis that synthetic 21 nucleotide-long siRNA duplexes or shRNAs with 19 nucleotide-long stems may not be optimal to induce RNAi, as these molecules are not cleaved by Dicer. Indeed, lengthening the sequences to produce synthetic 27-mer siRNAs or 29-mer synthetic shRNAs has been reported to trigger more potent silencing in mammalian cell lines than the shorter 19-mer counterparts [8, 9]. However, with few target genes tested and no validation in the context of vector-encoded shRNA, we wondered if we could apply these observations to improve the silencing efficiency of our stably expressed 19-mer shRNAs directed against WASp. To our knowledge, there is no information available on the optimization of vector-encoded RNAi effectors according to their stem size. Moreover, we tested different shRNA loops, including the endogenous miR30 loop. This loop was already proposed to improve silencing efficiency compared to an artificial one [10]. Finally, we also duplicated the cassette. Only the latter strategy was found to improve knock-down with a low number of integrated vectors per cell.

Materials & Methods

Constructs

The construction of the pRRL-H1shRNA-PGKGFP vectors (W7-19/9, W8-19/9) was described previously [6]. A general schema of the vector is shown in figure 1-A. The corresponding 29-mer shRNA cassettes (shRNAs W7-29 and W8-29) are depicted in figure 1-B. The H1-shRNA cassette was duplicated in the constructs W7-19/9/2x and W8-miR/2x. All constructs were verified by restriction and sequence analysis. In the study, all H1-shRNA cassettes were cloned in the same orientation compared to the PGK-GFP cassette. The following sense oligonucleotides were used for cloning the shRNA downstream of the H1 promoter:

W7-29/4 (5'-GATCCCCTTGAGATGCTTGGACGAAAATGCTTGACGTTGGCGTCAAGCATTTTCGTCCAAGCATCTCAATTTTTGGAAA-3'), W8-29/4 (5'-GATCCCCTTCTCAGTTCTCTTCACTCAAGGATTGTTTTGGAACAATCCTTGAGTGAAGAGAACTGAGAATTTTTGGAAA-3'), W8-29/9 (5'-GATCCCCTTCTCAGTTCTCTTCACTCAAGGATTGTTTTCAAGAGAAACAATCCTTGAGTGAAGAGAACTGAGAATTTTTGGAAA-3'), W8-MIR (5'-GATCCCCGCGATCTCAGTTCTCTTCACTCACTGTGAAGCCACAGATGGGTGAGTGAAGAGAACTGAGACTGCTTTTTGGAAA-3'), CTRL-29/4 5'-GATCCCCCTATTGGTATTCCGCTCTTGTCATATTACTTGGGTAATATGACAAGAGCGGAATACCAATAGTTTTTGGAAA-3').

Generation and titration of lentiviruses

VSV-G-pseudotyped shRNA vectors (W7-19/9, W8-19/9, W7-29/4, W7-29/9, W8-29/4, W8-29/9, W8-miR, W7-19/9/2x and W8-miR/2x) were produced by transient quadritransfection in 293T cells, concentrated by ultracentrifugation. Transducing

units/ml (TU/ml) were determined by titration of the vector on HCT116 cells following quantification of GFP positive cells by flow cytometry, as in prior studies [6].

Cell culture, transient transfection and viral transduction

The human cell lines HT1080, HCT116 and 293T cells were cultured in Dulbecco's MEM medium supplemented with antibiotics, L-glutamine (Gibco BRL, Paisley, Scotland) and 10% fetal calf serum (FCS) (Hyclone, Logan, UT) in humidifed atmosphere at 37°C, 5% CO_2. Jurkat cells were cultured in RPMI 1640 medium supplemented with antibiotics, L-glutamine (GibcoBRL) and 10% FCS (Hyclone). For transduction, Jurkat cells were incubated at the concentration of 5 x 10^5 cells per ml with various concentrations of lentiviral vectors (from 5 x 10^5 and 10^7 TU/mL) in culture medium containing polybrene (4µg / ml, Sigma-Aldrich, Saint-Louis, MO). Partially transduced populations of cells were enriched in GFP^+ cells by flow cytometry cell sorting (MoFlo, Dako, Glostrup, Denmark).

Umbilical cord blood (UCB) samples were obtained from the Louise Michel Hospital (Courcouronnes, France) in accordance with national guidelines. UCB-derived $CD34^+$ hematopoietic progenitor cells were prepared using magnetic-activated cell sorting according to the manufacturer's recommendations (Miltenyi Biotec, Bergisch-Gladbach, Germany). Prior to transduction, $CD34^+$ cells were pre-activated with cytokines (flt-3 ligand 50ng/mL, c-kit ligand 25ng/mL, thrombopoietin 25ng/mL and interleukin-3 10ng/mL) (Peprotec, Rocky Hill, NJ) in XVIVO 20 medium (Biowhittaker, Walkersville, MD) and transduced the next day by adding 5 x 10^7 TU/mL of lentiviral vector twice, at 6 hours interval. The following day, cells were washed and cultured in the presence of the same cytokines for at least one week before analysis.

Western Blot

Protein extracts and electropheresis were performed as already described [6]. Briefly, 10 µg of protein per lane were separated by SDS-PAGE, transferred to nitrocellulose membranes which were blotted with a combination of the mouse monoclonal antibody D1 specific for human WASp (Santa Cruz Biotechnologies, Santa Cruz, CA) with the AC-15 antibody specific for human beta-actin (Sigma-Aldrich, Saint Louis, MO). These antibodies were detected with a goat anti-mouse IgG secondary antibody which was either conjugated to peroxidase (Jackson ImmunoResearch, West Grove, PA) and revealed by chemiluminescence (ECL Western blotting detection kit, Amersham, Piscataway, NJ) and Kodak Biomax film (Sigma Aldrich) or which was conjugated to Alexa 680 (LI-Cor Biosystems, Homburg, Germany) and revealed with the Odyssey Infrared Imaging System (set at 700 nm with 169 µm resolution, medium quality and intensity setting varying from 2 to 5; LI-Cor Biosystems). The quantification of WASp to beta-actin levels was done either by densitometry analysis of Kodak film scans using ImageJ software (NIH, Bethesda, MD) or using the Odyssey software (LI-Cor Biosystems).

Q-PCR

The number of vector copies per cell was determined by Q-PCR, amplifying from the genomic DNA the woodchuck post-transcriptional regulatory element (WPRE) sequences of the lentiviral vector in comparison with the human albumin gene as previously described [6].

Expression of the stress-induced gene *OAS-1* was measured by quantitative RT-PCR normalized to expression of the transcription factor *hTFIID*. Total RNA was extracted with the SV total RNA isolation system (Promega, Madison, WI) and 1 µg

was reverse transcribed into cDNA with the Super-Script II RT (Gibco BRL, Paisley, UK) using random hexamer primers. Amplification reactions (17 µl) contained 8 µl of sample cDNA and 9 µl of TaqMan buffer (TaqMan Universal PCR Master Mix, No AmpErase UNG, Applied Biosystems, Foster City, CA, USA), 0.2 µM primers (OAS-1 forward 5'- CGTGTTTCCGCATGCAAAT-3' and OAS-1 reverse 5'-ACCTCGGAAGCACCTTTCCT-3'), 0.1 µM OAS-1 TaqMan probe (5'-TGCCATTGACATCATCTGTGGGTTCCT-3') and consisted of 40 cycles at 95°C (15 s) then 60°C (1 min) on an ABI PRISM 7700 sequence detector (Perkin-Elmer, Applied Biosystems). Standard amplification curves were obtained by serial dilutions of a known cDNA sample. All PCR measures were performed at least in duplicate. Data were edited using the Primer Express software. Results were expressed as the ratio of *WAS* mRNA to *TFIID* (*TFIID* probe 5'-TGTGCACAGGAGCCAAGAGTGAAGA-3', *TFIID* forward primer 5'-GAGAGCCACGAACCACGG-3', *TFIID* reverse primer 5'-ACATCACAGCTCCCCACCAT-3').

Results

We have previously characterized the shRNA W7-19 and W8-19 targeting WASp and creating cellular models of the Wiskott-Aldrich syndrome [6]. To improve the utility of this approach in primary cells, we sought to obtain the highest RNAi effect with the lowest number of integrated copies by testing the effect of several modifications in stem length or loop. The shRNA W7-19/9 and W8-19/9 were designed according the rules first established in the field [11], e.g. with a 9-mer loop and a 19-mer stem. The stems of the shRNA W7-19/9 and W8-19/9 were extended to 29-mer according to Siolas *et al.* [9] so as to conserve the same 19-nt targeting sequence, adding sequences that were homologous to the targeted mRNA and as represented in figure 1-B. We constructed two versions of the W8-29 shRNA with two different loops. The loops were modified to compare the 9-nt loop described by Brummelkamp *et al.* [11] and the 4-nt loop of the 29-mer shRNAs reported in Siolas *et al.* [9]. We also introduced the endogenous miR30 loop in the 19-mer construct according to Boden *et al.* [10].

The various shRNAs were tested in Jurkat cells that naturally express the WASp target. To compare the various vectors, we correlated the efficacy of knock-down with the number of copies integrated per cell. For that, we tested stable and homogeneous populations of cells obtained by transducing with different concentrations of vector, followed by cell sorting (if necessary) and culture for at least two weeks. The expression of the vector reporter gene as expressed by the mean fluorescence intensity (mfi) of GFP was a reliable measure and directly proportional to the number of integrated vectors in cells (figure 2-A). Results of protein inhibition were therefore correlated to the mfi of GFP to evaluate the amount of integrated

vector per cell. Several independent experiments comparing the W8-19/9 shRNA to W8-29/4, W8-29/9 and W8-miR demonstrate that all shRNAs vectors were functional and equally efficient in Jurkat cells (figure 2-B). The various changes in stem or loop did not improve the efficacy of the W8-19/9 vector. To extend this investigation, we also tested two versions of the W7 shRNA: W7-19/9 and W7-29/4, in two different cell types. Lengthening the stem from 19 to 29-mer did not bring any benefit to the silencing effect when using lentiviral vectors carrying these constructs in Jurkat cells. In this case, the 29-mer vector was even less efficient than its 19-mer counterpart (figure 2-C). These results were confirmed by direct measurement of vector copy number in the cells by PCR. Finally, we transfected the shuttle plasmids carrying the 19-mer or the 29-mer shRNAs into 293T-WASP cells, which stably express the human WAS cDNA, and results showed that lengthening the shRNA stem did not improve silencing (data not shown). It is known that some shRNA sequences induce stress response genes in human cells [12] [13]. To assess if an eventual cytotoxicity could explain, at least in part, why the 29-mer shRNA WASp vectors were not more efficient that the 19-mer constructs, we tested their effects on HT1080 cells which are particularly prone to interferon response induced by double-stranded RNA [14]. Following transduction with the lentiviral vectors W8-19/9, W8-29/9, with control vectors ctrl-19/9 and ctrl-29/4 and with the W8-miR vector, the interferon response was monitored by the quantification of *OAS-1* mRNA, a classic interferon target gene [13]. Contrary to the introduction of a long double-stranded RNA in the cells, none of ten shRNA vectors induced significant *OAS-1* expression (figure 2-D).

As shown here and in our prior study [6], the extent of the lentiviral-induced knock-down is correlated to the number of proviral DNA copies integrated per cell.

Therefore, we constructed a double copy version of shRNA W7 and W8-miR to obtain W7-19/9/2x and W8-miR/2x, respectively. Comparison experiments showed that these double-copy vectors were twice as active as their single-copy counterpart, matching the theoretical curves of predicted efficacy (figure 3). So it is possible in this manner to improve RNAi with very potent shRNAs. Now, with the best construct, W7-19/9/2x, we can inhibit more than 90% of the WASp with only one copy of vector per cell. To verify that this was not limited to the Jurkat cell line model, we transduced human umbilical cord blood $CD34^+$ cells with this vector and obtained reproducible inhibitions of 80% of WASp with an average of 1.5 copies of integrated vector per cell (figure 4). Thus, duplicating the shRNA cassette in RNAi lentiviral vector can generate powerful tools for application of RNAi in primary hematopoietic cells.

Discussion

It has been postulated that changes in the shRNA structure may provide additional possibilities to improve RNAi efficacy [8-10]. Our study is the first to systematically test several modifications of shRNA cassettes for lentiviral vector-induced knock down. Using a model targeting the human *WAS* gene, we found that extending the stem of two different shRNAs from 19-mer to 29-mer did not generate more efficient constructs based on measurements of protein inhibition in correlation with the amount of vector integrated per cell. These results seem to be in contradiction with studies with synthetic shRNAs or siRNAs which have shown consistent improvement in the cases reported [9] [8]. Our results highlight the fact that expressed shRNA do not necessarily follow the same design rules than their synthetic counterparts. The discrepancy in these results could be explained by differences in the pathways for the processing of the different species of shRNAs in the cells. In the case of our lentiviral-encoded shRNA, once proviral integration has occurred, the cells produce a short shRNA transcript initiated from the H1 promoter and stopped at the 5T termination signal. Through various ribonucleic complexes, this transcript is processed in the nucleus, shuttled outside into the cytoplasm where it is brought into RISC in a manner similar to that of pre-miRNAs. So, in contrast to transfected RNAs, a DNA-encoded, endogenously-processed shRNA may not be as dependent upon Dicer cleavage for optimal incorporation into RISC. Alternatively, it can be argued that lengthening the shRNA stem may represent an improvement only when the initial sequences are suboptimal, which was the case in the prior studies where stem lengthening improved efficacy [9] [8]. Thus, changes in the shRNA structure may not improve shRNAs that are already effective such as W7 and W8.

In the present study, we also compared the effect of three different shRNA loops but none influenced shRNA efficiency. A prior study suggested that the loop of a natural miRNA could function more effectively than the 9-nt loop but a systematic comparison of the constructs in dose experiments was not performed, limiting the interpretation of these results [10]. Our data are consistent with the fact that many types of sequences and lengths of loops have been employed in effective shRNAs [15-17], suggesting that this element is not critically-determinant in shRNA activity.

Finally, vectors carrying two copies of the shRNA expression cassette were found to be twice as efficient based on the interference activity reported to the number of vector genomes integrated per cell, which was expected considering the dose-response relationship of endogenous shRNA. The multimerization strategy has already been used to amplify the efficacy of poorly-active shRNA in the case of limited choice of sequences [18]. We show here that it is also an efficient approach for shRNAs that are already efficient in a single-copy configuration.

Consistent with the notion that subcellular processing pathways may influence shRNA efficacy, there are several recent publications that propose microRNA backbones as the system of choice for RNAi [19-21]. While this may be the case, we nevertheless show here that the use of traditional 19-mer shRNA with a duplicated expression cassette can be an efficient mean to silence gene expression in human primary cells such as $CD34^+$ hematopoietic progenitor cells. With the advantages of lentiviral vectors in terms of stem cell transduction, the ability to severely knock-down an endogenous protein provides the opportunity to test the consequences on cellular

differentiation and proliferation in near-physiological conditions. Studies on human hematopoietic cell development, similar to those reported earlier in humanized mice with “traditional” shRNA cassette vectors [22], would be facilitated.

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Figure legends

Figure 1: A. Structure of the pRRL-H1-shRNA-PGK-eGFP-WPRE lentiviral transfer vector. H1-shRNA expression cassettes were duplicated from vectors W7-19/9 and W8-miR. B. Structure of the 19-mer, 29-mer and miR shRNA. The boxes contain the sequences common to the 19-mer and 29-mer constructs and that target WASp mRNA.

Figure 2: Effect of shRNA expression in Jurkat cells. Various concentrations of shRNA lentiviral vectors were used to transduce Jurkat cells. At low m.o.i. (<10) GFP^+ cells were sorted to correct for transduction efficiency. At higher vector concentrations this was not necessary as cells were 100% GFP^+. A. Vector integration was measured by quantitative PCR on genomic DNA and correlated to the GFP mfi; each dot represents an independent transduction. B. Jurkat cells were transduced with vectors shRNA W8-19/9, W8-29/9, W8-29/4 and W8-miR. WASp protein expression was measured by Western blot in GFP^+ cells. WASp protein amounts were calculated by densitometry from the western blot results and correlated to GFP mfi (representative of 7 experiments). C. Jurkat cells were transduced with different concentrations of vector W7-19/9 and W7-29/4. WASp protein expression was detected by Western blot in GFP^+ cells. D. Effect of shRNA expression on OAS-1 induction. HT1080 cells were either tranduced with lentiviral vectors coding for shRNA or transfected with dsRNA. OAS-1 mRNA was quantified by qRT-PCR, realized on total mRNA prepared 4 days post-transduction, and levels were normalized by levels of TFIID mRNA (representative of 2 experiments).

Figure 3: Effect of vectors carrying duplicated shRNA expression cassettes. Various concentrations of shRNA lentiviral vectors were used to transduce Jurkat cells. At low m.o.i. (<10) GFP^+ cells were sorted to correct for transduction efficiency. At higher vector concentrations this was not necessary as cells were 100% GFP^+. Theoretical curves were obtained by dividing per 2 the mfi values for a given WASp level. A double copy vector should have the same efficiency for twice less integrated vectors per cell (representative of two experiments).

Figure 4: Effect of W7-19/9/2x vector in human primary cells. $CD34^+$ cells were purified from three independent cord blood samples. Cells were transduced twice with vectors ctrl-19/9 or W7-19/9/2x. Protein extracts and genomic DNA were prepared two weeks after transduction. A. Western Blots of the three samples. B. WASp and actin levels were quantified by densitometry and vector copy numbers were evaluated by Q-PCR.

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