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Validating siRNA using a reporter made from synthetic DNA oligonucleotides

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Abstract

Only a small fraction of all siRNAs are effective in silencing their target genes, and siRNA efficacy can only be determined experimentally. Previously described reporter-based siRNA validation methods all rely on the availability of physical cDNA clones, and this limits the high throughput applicability of the method. In the current report, we used short synthetic DNA fragment containing a siRNA targeting site, instead of cDNA, to fuse with a reporter gene. When targeting such transcripts with different siRNAs, we found that such constructs can faithfully report the efficacy of the corresponding siRNAs in a sequence specific manner, even when the inserted DNA fragment is essentially only long enough to cover the targeting site. The efficacy of both vector-based siRNA and synthetic siRNA can be evaluated using this system. Since only readily available short synthetic DNA fragments are needed for forming the evaluation vector, this method provides an appealing way of validating siRNAs in high throughput.

Keywords: siRNA; Target site; Dual-luciferase assay; Efficacy validation; Reporter; Oligonucleotides

RNA interference (RNAi or posttranscriptional gene silencing) is a process in which a double-stranded RNA triggers the degradation of homologous mRNAs, and it has become widely adapted for deciphering gene functions. Successful implementation of this method (in the form of siRNA produced chemically or from vectors) for gene silencing in mammalian cells represents an important step in approaching the daunting task of functional annotation of the human genomes, and it also opened new prospects for gene therapy [1–3].

While perfectly suited for large-scale functional genomics, it is also true that only a small fraction of all siRNAs designed so far are efficacious. People have started to uncover some mechanisms that might have impact on the efficacy of siRNA [4], but until such mechanisms are more thoroughly investigated, researchers have to rely on experimental tools to assess the efficacy of each siRNA. Due to the excessive labor and cost for carrying out large-scale siRNA validation using existing methods, the most feasible alternative is using redundant siRNAs without validating their silencing efficacies (“silencing efficacy” is proposed to be referred as “efficacy,” and efficacy is used in this paper) [5]. This would be translated into huge waste of time and resources.

Although several studies suggested that siRNA might have sequence preferences [1,4,6,7], and this has resulted in different algorithms that can help researchers to filter out siRNAs that might be active statistically, such software does not provide definite indication about the efficacy of any individual siRNA, not to mention the silencing strength of the reagent which is of critical importance for interpretation of knockdown experiments. In practice, three or more siRNAs are normally chosen (or designed by computer program) for each gene to be studied, and each siRNA is experimentally

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tested against its natural target (or fusion gene including full-length cDNA) in order to identify a good siRNA. The suppression activities of siRNA are usually evaluated by methods including quantitative PCR, Northern blot and Western blot methods, which are still very laborious. To validate siRNAs for each gene, the experimental process and conditions have to be optimized individually. This imposes a serious restraint on the throughput of any large-scale siRNA validation effort.

A new type of siRNA validation tool has been created to circumvent the difficulties of siRNA efficacy evaluation. In this approach, long target cDNA fragments were fused into different reporter genes [8,9]. It was found that siRNA attacking on the target could result in efficient silencing of the reporter gene by disrupting the open reading frame. These elegant methods, however, are all subjected to limitations by the availability of physical clones of full-length (or even partial) cDNA. The high level of error rate of IMAGE clones calls for the sequencing of any clone obtained, which is time-consuming and costly. Sequence-verified clones still remain to be prohibitively expensive for individual users, although the situation has been improved for mouse cDNAs. Additionally, such methods were configured to work only with siRNA targeting the coding region of mRNA.

In order to carry out siRNA validation more robustly and cost-effectively, we developed a reporter-based siRNA validation system that eliminated the need for cDNA clones. In this system, short oligonucleotides corresponding to the target sites of tested siRNA were inserted into a reporter plasmid referred to as siQuant construct, and the fusion construct was used directly for validating the siRNA efficacy. We demonstrated that siRNA silencing efficacy on the fusion constructs reflects the efficacy on its endogenous target genes. This paves the way for genome-wide siRNA validation in a high throughput manner in any molecular biology laboratory.

Results and discussion

Rationale of the efficacy assay

It has been well elucidated that RNAi is a sequence-dependent expression-silencing phenomenon. In theory, two types of factors could contribute to siRNA activity: (a) sequence properties of the siRNA (the intrinsic gene silencing properties of the siRNA) and (b) the environmental factors. Although several reports have suggested that the secondary structures of mRNA and mRNA binding proteins might interfere with the target site accessibility for RISC complex [10], it is generally believed that mRNAs could adopt more flexible secondary structures than their rRNA and tRNA counterparts, whose functions are more structure-based. Data from our recent experiments and other studies, however, showed that the efficacy of siRNA is to a large extent secondary structure-independent [4,11,12], suggesting that the sequence properties play the major role in determining siRNA efficacy. If this hypothesis were true, then it could be possible to assess the efficacy of a particular siRNA in a sequence context that is unlikely to recruit any of the authentic environmental factors. With this thought in mind, we started to explore whether the siRNA efficacy can be analyzed out of its original sequence (secondary structure) context, and how faithful are such data in reflecting the silencing efficacy of the siRNA on the endogenous target genes.

A modified *firefly* luciferase expression vector [8] was used to incorporate a short synthetic double-stranded DNA fragment immediately after the translation start codon (ATG) of the luciferase gene (Fig. 1). The short DNA fragment was designed to harbor the siRNA target sites and was chosen to be 38 and 19 bp, respectively, a length that could be dealt with easily by chemical synthesis (Table 1). They were designed mostly to be incorporated in-frame with the luciferase open reading frame. Out-of-ORF fusions were also tested in order to understand whether sequence containing unavoidable stop-codons could be analyzed using this system. After siRNA target sites were cloned into this expression vector, they were co-transfected into HEK293 cells, together with respective cognate siRNA and *Renilla* luciferase expression vector (pRL-TK) as the control. The activity of tested siRNAs was measured by dual-luciferase assay.

Effective siRNA can silence the expression of luciferase gene fused with a short siRNA target site

The first question we tried to answer was whether a proven efficacious siRNA could successfully silence its target site if such a site was grafted out of its authentic mRNA (sequence and/or structural) context. Towards

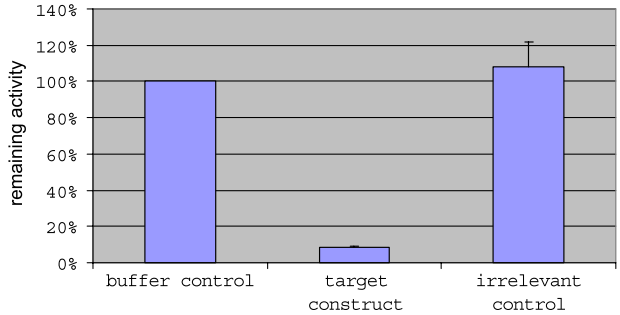

Fig. 1. Silencing of the artificial target gene is sequence-dependent. Efficient silencing of recombinant luciferase gene containing an inserted target site was achieved by a cognate siRNA whereas no inhibition was observed for the same siRNA on a recombinant gene containing an irrelevant sequence. Normalized expression ratios between the *firefly* luciferase activities and the *Renilla* luciferase activities are shown. The plotted data are averages from triplicates.

Table 1
Sequences of synthetic DNA oligonucleotides used in this study to construct different siQuant plasmids for carrying out the efficacy assay

Names of recombinant siQuant plasmid	Synthetic short DNA fragments inserted in each siQuant plasmid
CD46-siteA-38mer	GATCTATTTTTCACTTATTGGAGAGAGCACGATTTATTGTGGTGGGCC ATAAAAAGTGAATAACCTCTCTCGTGCTAAATAACACCAC
CD46-siteA-19mer	GATCTCACTTATTGGAGAGAGCACGATTGGGCC AGTGAATAACCTCTCTCGTGCTAAC
Out-of-frame construct	GATCTGACTTATTGGAGAGAGCACGATTGGGCC ACTGAATAACCTCTCTCGTGCTAAC
Frame-control construct	GATCTTACTTATTGGAGAGAGCACGATTGGGCC AATGAATAACCTCTCTCGTGCTAAC
CD46-siteB-38mer	GATCTCTACTTACAAGCCTCCAGTCTCAAATTATCCAGGATATGGGCC AGATGAATGTTCGGAGGTCAGAGTTTAATAGGTCCTATAC
CD46-siteB-19mer	GATCTACAAGCCTCCAGTCTCAAATTATGGGCC ATGTTCGGAGGTCAGAGTTTAATAC
Fas297	GATCTCAGGGAAGGAGTACATGGACACCGGGCC AGTCCCTTCCTCATGTACCTGTGGC
Fas297m	GATCTCAGGGAAGGAATACATGGACACCGGGCC AGTCCCTTCCTTATGTACCTGTGGC
Fas679	GATCTCAACCATACCAATGAATGCCTCCGGGCC AGTTGGTATGGTTACTTACGGAGGC
Fas873	GATCTCATCTCATGGGAAGAGTGATGCCGGGCC AGTAGAGTACCCTTCTCACTACGGC
Cyclin G1	GATCTCAGATCTACTTAGTCTAACTCCCGGGCC AGTCTAGATGAATCAGATTGAGGGC
Dusp6	GATCTCAGAGTTTGGCATCAAGTACACCGGGCC AGTCTCAAACCGTAGTTCATGTGGC
NPY	GATCTCAATGAGAGAAAGCACAGAAAACGGGCC AGTTACTCTCTTTCGTGTCTTTTGC

For making each siQuant plasmid two oligos were synthesized to form the short duplex as shown in the right panel. Note that all such duplexes have universal left and right over-hangs.

this end, an effective siRNA targeting an endogenous gene (CD46) was chosen from our earlier study [13], and an expression vector containing its 19mer target site was constructed as described in Materials and methods. Another 19mer fragment from the same gene was used as irrelevant sequence control. The effective siRNA (CD46-604) was then tested against both the artificial target and the irrelevant control. As shown in Fig. 1, HEK293 cells transfected with the target construct exhibited a significant reduction (92% knockdown) of luciferase gene expression compared to cells transfected with the irrelevant control construct. No difference of *Renilla* luciferase expression, which was used as an internal control for the transfection experiment, was observed. No luciferase silencing was observed when the siRNA was used to treat the *firefly* luciferase without the fused target site, suggesting that the silencing effect of the CD46 siRNA was achieved by attacking the inserted target site in a sequence specific manner.

The same experiments were performed with several effective siRNAs from other genes including: Cyclin G1, Dsup6, and NPY. Oligos as outlined in Table 1 were inserted into the same cloning site, then the recombinant plasmids were analyzed for fusion gene expression and the impact of cognate siRNAs. All effective siRNAs could knock down the expression of respective fusion luciferase gene through attacking the inserted cognate target sites. These data showed that an efficacious siRNA does have the capacity to find and cleave its target site, even if such a targeting is grafted out of its authentic sequence context and inserted into a reporter gene such as luciferase.

Optimization of siRNA target site

The next question we explored was the optimal length of the siRNA target site for this system. Desirably the shorter the region, the better if such a length could work as expected. To address this issue, four plasmids containing respective 19mer and 38mer fragments covering the target regions of two siRNAs were constructed and assessed for their capabilities to mediate siRNA-induced knockdown of the fusion target genes (Table 1). As shown in Fig. 2, no significant difference of inhibition has been observed among the plasmids containing 19mer or 38mer target sites for each siRNA, implying that the cognate sequence of siRNA, instead of the flanking sequence, was enough to serve as target site for the siRNA to accomplish the suppressive mission.

Since the above results suggested that the 19mer target site might contain most (if not all) necessary information for determining whether a siRNA worked or not, all subsequent experiments were carried out with the siQuant constructs containing 19mer siRNA target sites.

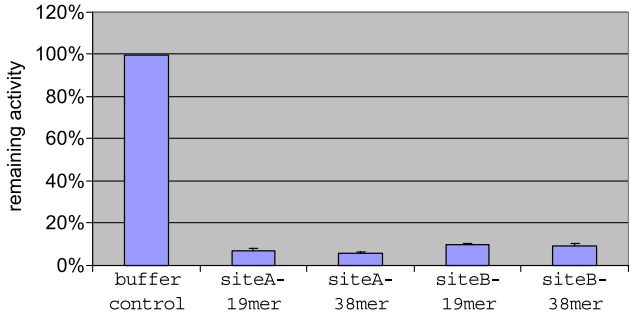

Fig. 2. Target site optimization. Four plasmids containing 19mer and 38mer oligonucleotides, respectively, covering the target siteA and siteB of CD46 were tested for their abilities to elicit the knock down of coupled luciferase gene. No significant difference of inhibition was observed among the plasmids containing 19mer and 38mer target site for each siRNA. Normalized expression ratios between the *firefly* luciferase activities and the *Renilla* luciferase activities are shown. The plotted data are averages from triplicates.

The reporter-based assay showed similar efficacy as demonstrated by targeting endogenous transcripts

Another critical issue was whether the efficacy observed using a siRNA on a reporter gene fused with its target sites could be used to predict the efficacy of the siRNA on its endogenous target gene. We designed three siRNAs against Fas mRNA (Table 2) and conducted a double-blinded test where the siRNA silencing activities were evaluated by one researcher using the fusion luciferase system described above, and the same siRNAs were used to target Fas mRNA by another researcher.

Efficacy assay showed that siRNA Fas679 and Fas297 could suppress the reporter gene expression by targeting their fusion sites, and the luciferase activities were knocked down to 12% and 14% of untreated controls, respectively, whereas Fas873 siRNA did not affect the expression of fusion gene (Fig. 3A). As internal control, the activities of *Renilla* luciferase were not altered as judged from the raw data. In parallel, Northern blot analysis was performed in AML12 cell line using the same siRNAs to silence endogenous Fas gene. The suppression profiles observed for the authentic Fas gene expression closely matched the corresponding profiles obtained from efficacy assays where the fusion reporter genes were used (Fig. 3B). The expression of β-actin gene, which was used as internal control for Northern blot assay, was not affected by these siRNAs.

Table 2
The siRNAs used in this study

Names of siRNA	Sequences of the siRNA
CD46-siteA	CUUAUUGGAGAGAGCACGATT
	TTGAAUAACCUCUCUCGUGCU
CD46-siteB	GCCUCCAGUCUCAAAUUAUTT
	TTCGGAGGUCAGAGUUUAAUA
Fas297	GGGAAGGAGTACATGGACATT
	TTCCCUUCCUCAUGUACCUGU
Fas679	CCATACCAATGAATGCCTCTT
	TTGGUAUGGUUACUUACGGAG
Fas873	TCTCATGGGAAGAGTGATGTT
	TTAGAGUACCCUUCUCACUAC
Cyclin G1	GATCTACTTAGTCTAACTCTT
	TTCUAGAUGAAUCAGAUUGAG
Dusp6	GAGTTTGGCATCAAGTACATT
	TTCUCAAACCGUAGUUCAUGU
NPY	TGAGAGAAAGCACAGAAAATT
	TTACUCUCUUUCGUGUCUUUU

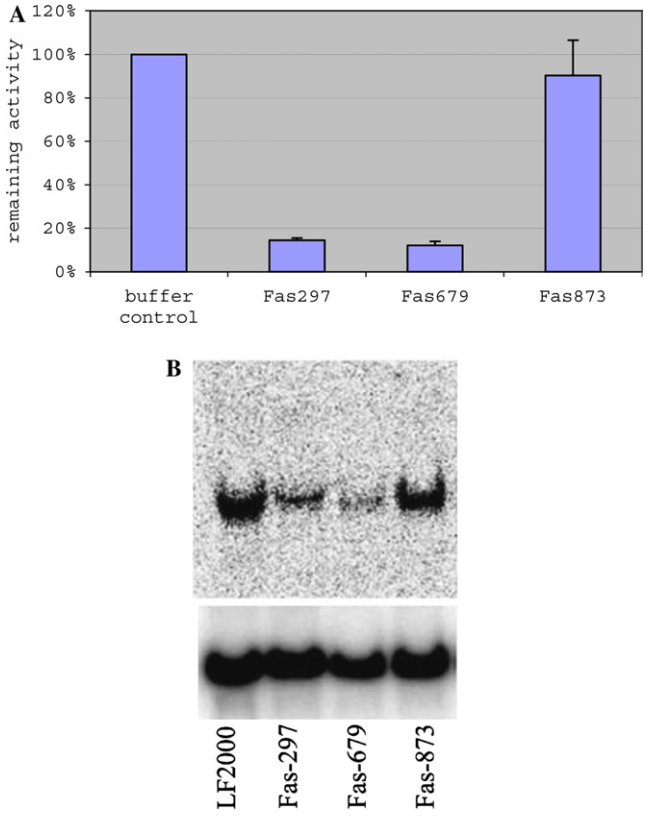

Fig. 3. Expression inhibition of recombinant gene and endogenous mRNA by Fas siRNAs. (A) Three Fas siRNAs (Fas297, Fas679, and Fas873) were used to knock down the fusion target genes containing respective cognate sequences. Normalized expression ratios between the *firefly* luciferase activities and the *Renilla* luciferase activities are shown. The plotted data are averages from triplicates. (B) Northern blot assay for the inhibition of endogenous Fas mRNA in mouse AML12 cells by the same siRNAs. β-Actin cDNA probe was used as hybridization control to ensure that the same amount of total RNA was loaded in each lane. Transfection control with only Lipofectamine 2000 was shown at the left lane.

The specificity of the efficacy assay was further demonstrated by a mismatch test

Several studies have shown that mismatches between the siRNA and its target site could affect the suppression activities of various siRNAs [12]. Therefore, we went further to test whether the efficacy assay system we set up had similar sensitivities to mismatches. We modified

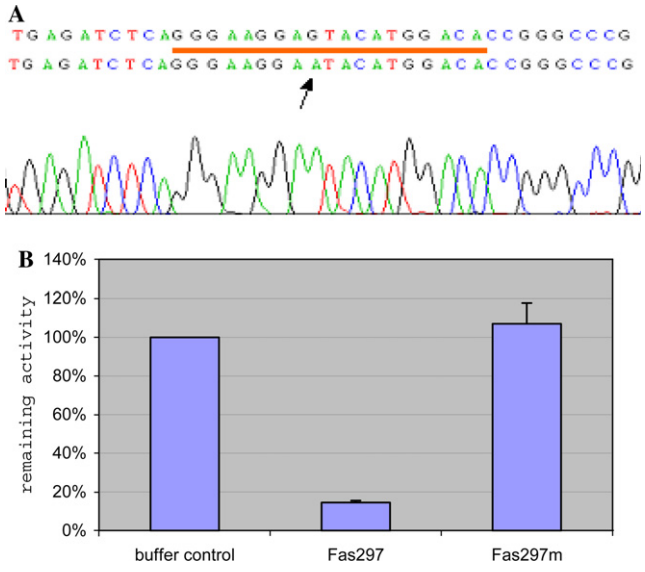

Fig. 4. Single point mutation in the target sequence eliminates the inhibition of an active siRNA. (A) Sequence comparison of Fas297 and Fas297m. The arrow indicated the G → A mutation introduced into Fas297m recombinant gene. (B) Expression knockdown of Fas297 and Fas297m on recombinant genes. Normalized expression ratios between the *firefly* luciferase activities and the *Renilla* luciferase activities are shown. The plotted data are averages from triplicates.

the target site of Fas297 siRNA and tested whether the introduced mismatch could alter the suppression activity of this otherwise proven active siRNA in cultured cells. A single nucleotide mutation (G → C) was thus constructed at the position 9 of the sense target strand in fusion luciferase gene (Fig. 4A). Both the original and modified constructs were tested using the same siRNA. The result showed that this single mismatch in the target site rendered the effective siRNA inactive for its mutated target (Fig. 4B). Since we knew that the siRNA can be readily integrated into the RNAi pathway, this experiment suggested that the change of the silencing effect for the reporter gene was caused by the point mutation. This result thus further demonstrated that our efficacy assay was dependent on the siRNA target sites in a highly sequence specific manner.

In-frame vs. out-of-ORF fusion of the siRNA target site

After demonstrating that in-frame fusion of the siRNA target site appeared to be well suited for efficacy assay, we went further to investigate whether siRNA target sites harboring stop codons could be used in such an assay as well. Normally one can avoid such complications by shifting frames slightly, but we thought that the out-of-ORF cases would not only be useful for assessing the sites with unavoidable stop codons, but should also be very helpful for target selection within or out of the open reading frames.

In this experiment, we used the same siRNA and target sites as we used for the in-frame studies, except that an artificial in-frame stop codon was introduced to exclude the target site from the ORF (Fig. 5). A strong luciferase activity could still be observed from such a fusion construct, suggesting that the ribosomes might have used the ATG immediately after the insertion site to synthesize a fully functional enzyme. After titrating plasmid amount to get comparable luciferase activities, we compared the silencing effect of the same siRNA on sites that was in the ORF and that was excluded from the ORF by a stop codon. It was observed that the siRNA could also induce efficient silencing of the reporter gene for the out-of-frame construct although the silencing efficiency seemed to be slightly reduced in comparison to inhibition of the in-frame fusion construct. The difference of silencing efficacy in correlation to the frame situation could be due to two reasons: (a) in an out-of-ORF targeting event, the cleaved target mRNA has to be further degraded to remove the downstream ATG site. (b) The insertion of the stop codon might have impacts on ribosome entry or the recruitments of other protein factors that could result in the change of accessibility in an otherwise accessible site. This phenomenon will be further investigated.

In summary, we established a siRNA efficacy validation system where the efficacy of a siRNA could be read out by measuring the activity of a reporter enzyme, whose mRNA was tagged by a short sequence representing the target site of the tested siRNA. Our data demonstrated that siRNAs could sequence-dependently knock down their target-site-coupled luciferase gene expressions and the suppression profiles against these artificial targets could be verified by targeting their natural target genes. In comparison to similar systems where the tagging sequences have to be large cDNA fragments, the current method presents a significant potential for high throughput applications due to the fact that the tagging sequences can be readily available through high throughput oligonucleotide synthesis, thus avoiding the restraint imposed by the availability of correct cDNA. Other advantages of the current efficacy assay system include: (a) the universal cloning scheme makes it much simpler than the individual frame adjustment for cDNA; (b) sequences with or without stop codon can be assessed using the same scheme; and (c) this system is internally controlled. In addition to making the results more consistent and reliable, internal control eliminates variability that may arise as a consequence of indirect normalization, differences of mRNA abundance, and difference of translation efficacies. The read-out from the efficacy assay would provide a relative guide on the efficacies of different siRNAs targeting the same gene. We have attempted on-chip synthesis of the tagging sequences for large-scale siRNA validation with promising initial results. This makes it highly feasible to validate siRNAs against all human and mouse genes in a short period of time.

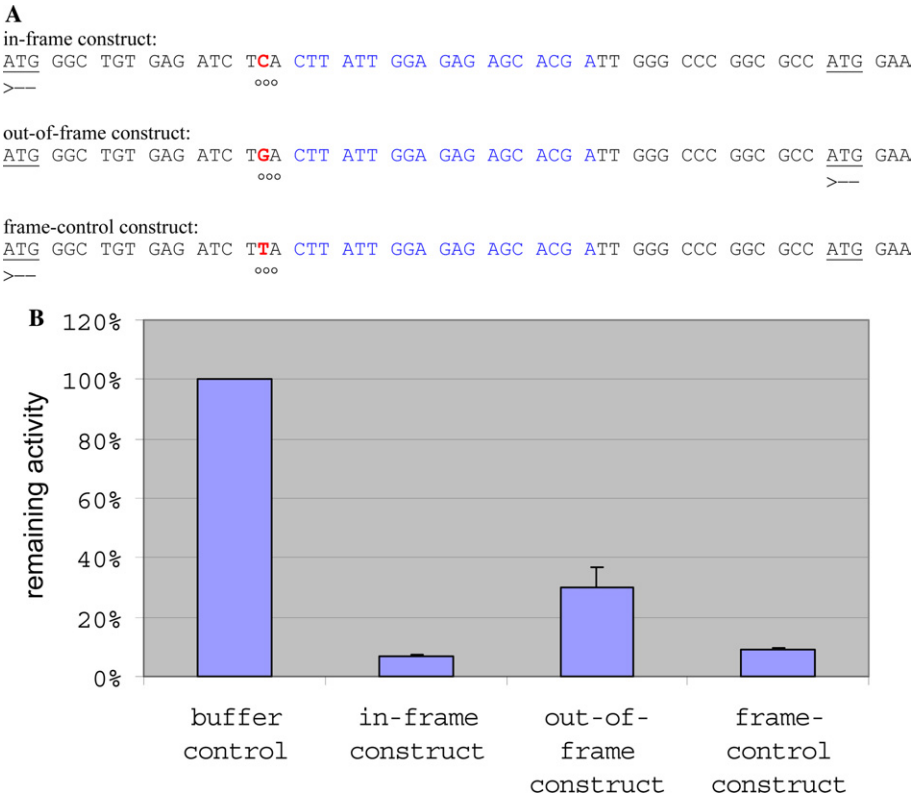

Fig. 5. Comparison of in-frame and out-of-frame fusion of the siRNA target. (A) Three versions of constructs were examined in this experiment. For the in-frame construct, the first ATG codon (labeled by >—) was used for *firefly* luciferase gene translation. For the out-of-frame construct, a point mutation (C → G) (red letters) was introduced to force the *firefly* luciferase translation start from the second ATG codon (labeled by >—) and leave the siRNA target site out of the ORF. For the frame-control construct, a point mutation was introduced at the same position as in the out-of-frame construct without stopping the translation. (B) Expression knockdown of three constructs. Normalized expression ratios between the *firefly* luciferase activities and the *Renilla* luciferase activities are shown. The plotted data are averages from triplicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

Materials and methods

Oligonucleotides. All DNA oligonucleotides used for target sites cloning (Table 1) were obtained from MWG Biotech AG (Ebersberg, Germany). RNA oligonucleotides were obtained from Dharmacon Research (Lafayette, CO, USA) and GENSET SA (Paris, France). SiRNA duplex was prepared by mixing complementary sense-strand RNA and antisense-strand RNA at equal concentration at 50 μM in MQ water. The mixture was incubated in boiling water for 1 min and cooled down overnight to allow the efficient formation of siRNA duplex. The quality of the RNA duplexes was assessed on a 15% PAGE gel.

Plasmid construction and cloning of siRNA target site. The luciferase expression plasmid (pTRE_PSKH1_Luc) was kindly provided by Dr. Hans Prydz [14]. A cloning site was introduced immediately after the start codon of *firefly* luciferase gene by polymerase chain reaction (PCR) using the site forward primers 5′-TGCTAGATCTCACA GCCCATGGTGCGGAT-3′ and site reverse primer 5′-CATGGG CCCGGCGCCATGGAAGACGCCA-3′. The design of the primer pair incorporated a *Bgl*II and an *Apa*I restriction sites to facilitate cloning. The list of oligonucleotides used for target sites cloning is summarized in Table 1. Oligo sets, designed with the appropriate restriction sites, were annealed and subcloned into the *Bgl*II–*Apa*I sites of constructed plasmid. Sequencing was performed to verify the inserted sequences using the Big-DYE terminator Method (Perkin–Elmer). The tested siRNAs are listed in Table 2.

Transfection and dual-luciferase assay. To investigate the suppression effect of these siRNA on their respective constructs, we carried out the dual luciferase reporter assay. Human embryonic kidney (HEK293) cells were maintained in DMEM (Life Technologies, Gibco) and seeded in 24-well multi-dishes (0.5 ml medium/well) to reach about 50% confluence at transfection. The cells were grown for 24 h and the culture medium was changed to OPTIMEM (Gibco), 0.5 ml/well, before transfection. The cells were co-transfected with plasmids and siRNA duplex in the presence of Lipofectamine 2000 (Invitrogen, USA) at a final concentration of 0.17% (total transfection volume, 0.6 ml). For each well, 0.17 g of recombination plasmid and 0.017 g pRL-TK were used. The final concentration of siRNA is 13 nM. The transfection medium was changed to culture medium (1 ml) after 4 h. All experiments were performed in triplicates and repeated at least twice. Cells were harvested in 24 h by passive cell lysis and the dual-luciferase assay was conducted. Luciferase activities were determined with 10 μl cell lysate using the Dual-Luciferase Assay System (Promega) by NOVOStar (BMG Labtechnologies GmbH, Germany). The *firefly*/*Renilla* activity ratio was generated for each well, and the inhibition efficiency of each siRNA was calculated by normalizing to respective buffer control.

Transfection and northern blot assay. The mouse AML12 cell line (ATCC; CRL-254) was cultured in DMEM/F12 (Life Technologies, Gibco) supplemented with 40 ng/ml dexamethasone (Sigma), 10% fetal bovine serum (Life Technologies, Gibco), 1× Insulin–Transferrin–Selenium-X (Life Technologies, Gibco), and 1× Penicillin–Streptomycin–Glutamine (Life Technologies, Gibco). For transfection, the medium described above was used without antibiotics. Transfection was done in accordance with the manufacturer's manual with 0.32% v/v of LipofectAmine2000, on cells between passages 5 and 18. Briefly, cells were counted and seeded at around 1,000,000 cells/25 cm^2 flask. Typically after 24 h, when the cell cultures were around 90% confluent,

liposome-plasmids/siRNA complexes formed in OptiMem were added to the cultures.

Twenty-four hours after transfection, the cells were harvested in 1 ml RNAwiz solution (Ambion) according to the manufacturer's instruction. Total RNA, 10–12 μg, was separated by electrophoresis in an ethidium bromide-containing agarose–formaldehyde gel [15]. The intensity of the 18S and 28S rRNA bands under UV light was checked to verify that all samples were equally loaded and that no RNA degradation had occurred. The β-actin cDNA probe used was a fragment described earlier [16] and the Fas cDNA was an *Eco*RI digest of plasmid pMFI [17]. The probes were labeled with [γ-^{32}P]dCTP with Ready-To-Go DNA labeling beads (Amersham–Pharmacia Biotech). Hybridization and stringency wash was performed as previously described [15], and the signal was detected by a BAS 1500 scanner (Fuji).

Acknowledgments

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