Functional shRNA expression system with reduced off-target effects

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Abstract:
The capability to induce off-target effects by small interfering RNA (siRNA) is highly correlated with the calculated melting temperature (T_m) of the protein-free seed-target duplex, indicating that its thermodynamic stability is a major factor in determining the degree of the off-target effect. In this study, we analyzed changes in microarray expression profiles following transfection of short hairpin RNAs (shRNAs). In a similar manner to siRNA, no or little off-target effects were observed by shRNA with a low T_m value in the seed-target duplex, whereas a high degree of off-target effect was induced by shRNA with a high T_m. Furthermore, shRNAs were often cleaved by Drosha and/or Dicer at the shifted sites in their primary transcripts, resulting in unexpected siRNA products with shifted sequences. Based on these results, the following conditions should be satisfied to ensure a functional shRNA with reduced off-target effects: the presence of A/U residues at nucleotide positions 1 and -1 from the 5′ end of the guide strand, three to six A/Us in nucleotide positions 2–7, G/C at positions 19 and 18, and no GC stretch of more than nine nucleotides. In addition, the T_m values of the seed regions in both strands should be low.

1. INTRODUCTION

RNA interference (RNAi) is an evolutionarily conserved, sequence-specific gene-silencing process triggered by the presence of double-stranded RNA (dsRNA); it is involved in the innate defense against viral infection and transposable elements [1]. RNAi has also been used as a research tool to control the expression of specific genes in numerous experimental organisms and has potential as a therapeutic strategy for several human diseases [2]. RNAi silencing is triggered by small interfering RNA (siRNA) helices, which comprise 21 nucleotides (nts) containing 2-nt 3′ overhangs in mammalian cells. siRNA incorporated into the cells is transferred to the RNAi effector complex, resulting in an RNA-induced silencing complex (RISC) [3]. However, the two strands of siRNA are handled differently. The strand with the less stable duplex at its 5′ end is incorporated preferentially into RISC [4–8]. The activated RISC is a ribonucleoprotein complex consisting of at least an Argonaute protein and a single-stranded siRNA [9–11]. In human and mouse cells, four Argonaute family proteins (human and mouse Ago1–4) have been found [12]. Among them, only Ago2 is endonucleolytically active [10, 11, 13]. The RISC containing the siRNA guide strand and Ago2 binds to the target RNA and silences gene expression by cleaving target mRNA by an RNase H-like active site in Ago2 [10,14].

Vector-based short hairpin RNA (shRNA) expression systems are used to induce sustained RNAi in cells with the help of plasmid constructs. Early designs of shRNAs had stems of 19–29 nucleotides with minimal flanking RNA sequences and were expressed using strong RNA polymerase III (pol III) promoters, such as those that drive the expression of endogenous U6 small nuclear RNA (snRNA) or H1 RNA [15–17]. Later, an expression system that mimics natural microRNA (miRNA) was constructed [18,19]. Natural miRNAs are a class of endogenous dsRNAs that are transcribed by pol II as long primary 5′-capped and polyadenylated miRNAs (pri-miRNAs) [20,21]. The pri-miRNA is cropped first to release 70–90 nts of a hairpin-shaped precursor (pre-miRNA) by a member of the ribonuclease III family (RNase III), Drosha, and its cofactor, DGCR8, in the context of the Micro-processor complex [22–24]. The pre-miRNA remains with a 2-nt 3′ overhang at one end, which is critical for miRNA biogenesis because it defines the position at one end of the molecule. This distinctive structure is recognized by the Exportin-5-Ran-GTP heterodimer, and the pre-miRNA is shuttled to the cytoplasm [25,26]. In the cytoplasm, the Dicer, an RNase III type protein, cuts the transported pre-miRNA to generate ~22 nt miRNA duplex [27–29]. One strand of the Dicer product remains as a mature miRNA and is assembled into an effector complex known as the miRNA-induced silencing complex (miRISC) [4,5], which exerts a gene-silencing effect. Thus, the pol II-driven shRNA expression system is constructed by replacing the miRNA region with siRNA.

Recent microarray profiling studies have revealed that "off-target" or unintended gene silencing is frequently brought about by sequence complementarity between target mRNA and the "seed" region of the siRNA (nucleotides 2–8 from the 5′ end of the siRNA guide strand) [30–35] through a mechanism similar to that of target silencing by miRNAs [36–38]. Furthermore, we have shown that the capability of siRNA to induce off-target effects is highly correlated with the thermodynamic stability or calculated melting temperature (T_m) for the formation of the protein-free seed-target duplex [35]. siRNAs with lower seed-target duplex stabilities (benchmark T_m value < 21.5 ºC) should minimize off-target effects.

In this study, we analyzed changes in microarray expression profiles by transfecting HeLa cells with shRNA expression constructs. Seed-dependent off-target gene silencing was induced by shRNA in a similar manner as that by siRNA; no or little off-target effect was observed

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after introducing shRNA with a low T_m in the seed-target duplex, whereas a high degree off-target effect was induced by shRNA with a high T_m. Furthermore, it was also evident that primary shRNA transcripts are often cleaved at the shifted sites. In view of these results, we proposed feasible rules for constructing shRNA with a low off-target effect.

2. EXPERIMENTAL PROCEDURE

2.1. Cell culture

Human HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco-BRL) at 37°C. Media was supplemented with 10% heat-inactivated fetal bovine serum (FBS; Mitsubishi Kagaku) and antibiotics (10 units/ml penicillin [Meiji] and 50 μg/ml streptomycin [Meiji]).

2.2. Preparation of siRNA and construction of the shRNA expression plasmid

RNA oligonucleotides were purchased from Proligo (Sigma-Aldrich) and siRNA was prepared as described previously (**Fig. 1A**) [8]. The siRNA sequences were as follows:
siVIM269, 5′-GCCAUCAACACCGAGUUCAAG-3′ and 5′-UGAACUCGGUGUUGAUGGCGU-3′;
siVIM270, 5′-CCAUCAACACCGAGUUCAAGA-3′ and 5′-UUGAACUCGGUGUUGAUGGCG-3′;
and siVIM812, 5′-GUACGUCAGCAAUAUGAAAGU-3′ and 5′-UUUCAUAUUGCUGACGUACGU-3′.

The pol III-driven shRNA expression vectors were constructed by inserting annealed synthetic oligonucleotides consisting of two strands composed of single-stranded

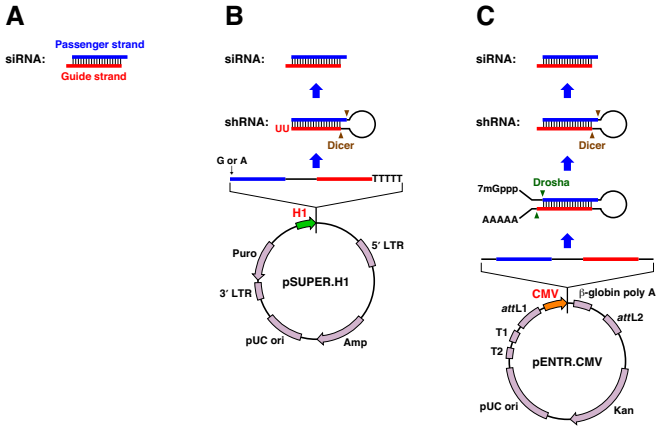

Fig. 1 The structures of siRNA and shRNA expression constructs. (**A**) siRNA. Blue line: passenger strand. Red line: guide strand. (**B**) pSUPER.H1 vector. A primary transcript driven by the H1 promoter preferentially starts at G or A and terminates at the poly(T) tract. shRNA is cleaved by Dicer (brown triangle) to generate mature siRNA. (**C**) pENTR.CMV vector. The 5'-capped and polyadenylated transcript is driven by the CMV promoter. The transcript is first cleaved by Drosha (green triangle) and subsequently by Dicer (brown triangle) to generate mature siRNA.

67-mer oligonucleotides into BamHI/HindIII sites in the pSUPER.retro.Puro vector (OligoEngine) containing an H1 promoter; this construct was named pSUPER.H1 (**Fig. 1B**). From the inserted oligonucleotides, the transcript was encoded in the following order: (i) a 21-nt siRNA guide strand; (ii) a human miR-23a loop, and (iii) a 19-nt passenger strand of the identical siRNA lacking a 3′ overhang. The inserted synthetic oligonucleotide sequences were as follows:
pSUPER.H1-VIM269, 5′-GATCCCGCCATCAACACCG AGTTCAAGGCTTCCTGTCACCTTGAACTCGGTGTT GATGGCTTTTTTA-3′ and 5′-AGCTTAAAAAAGCCAT CAACACCGAGTTCAAGGTGACAGGAAGCCTTGAA CTCGGTGTTGATGGCGG-3′;
and pSUPER.H1-VIM812, 5′-GATCCCGTACGTCAGCA ATATGAAAGTGCTTCCTGTCACACTTTCATATTGCT GACGTACTTTTTTA-3′ and 5′-AGCTTAAAAAAGTAC GTCAGCAATATGAAAGTGTGACAGGAAGCACTTT CATATTGCTGACGTACGG-3′.

The pol II-driven shRNA expression vector was a derivative of pENTR.U6 (Invitrogen), and was named pENTR.CMV (**Fig. 1C**). It contains the cytomegalovirus (CMV) immediate-early enhancer/promoter and the genome region from which human miR-187 is transcribed. The CMV promoter and the miR-187 expression region were PCR-amplified from pSH-CMV using primers (5′-CC GCTCGAGTCAATATTGGCCATTAGC-3′ and 5′-CTA GCTAGCTAGTTCATTAATGCAGTCGAGTTC-3′) and digested with XhoI/NheI. The digested product was ligated into pENTR.U6 digested with SalI/XbaI. pSH-CMV was a derivative of pTRE2 (Clontech). The Tet response element (TRE) in pTRE2 was replaced with the CMV promoter of pCI-neo (Promega) and the genomic sequence with the miR-187 expression site was inserted into pTRE2. The CMV promoter was PCR-amplified with primers (5′-TTAA TTGGCGCGCCTCAATATTGGCCATTAGC-3′ and 5′-AACTGCAGAACCAATGCATGATCTGACGGTTCA CTAAACG-3′) and digested with AscI/NsiI. The product was inserted into pTRE2 previously treated with the same restriction enzymes. The human miR-187 expression region (271 bp) was PCR-amplified from HeLa genomic DNA using primers (5′-CGCGGATCCATCGGGATGCACAGC AAGT-3′ and 5′-GCTCTAGACCCACCAGAGCCTGGAC TTTC-3′) and digested with BamH1/XbaI and ligated into pTRE2 previously digested with the same restriction enzymes. From the annealed oligonucleotides inserted into DraIII/PshAI sites in the miR-187 expression region, the transcript was encoded in the following order: (i) the 5′-flanking region of miR-187, (ii) a 21-nt siRNA guide strand, (iii) a human miR-30 loop, (iv) a 19-nt passenger strand of the identical siRNA lacking a 3′ overhang, and (v) a 3′-flanking region of miR-187. The inserted oligonucleotide sequences were as follows:
pENTR.CMV-VIM269, 5′-GTGACCCTCCAGCCAUCA ACACCGAGUUCAAGTGTGAAGCCACAGATGGCTT GAACTCGGTGTTGATGGCTTGAGGGACGC-3′ and

5′-GCGTCCCTCAAGCCAUCAACACCGAGUUCAAGC CATCTGTGGCTTCACACTTGAACTCGGTGTTGATG GCTGGAGGGTCACACT-3′;
and pENTR.CMV-VIM812, 5′-GTGACCCTCCAGUACG UCAGCAAUAUGAAAGUTGTGAAGCCACAGATGGA CTTTCATATTGCTGACGTACTTGAGGGACGC-3′ and 5′-GCGTCCCTCAAGUACGUCAGCAAUAUGAAAGU CCATCTGTGGCTTCACAACTTTCATATTGCTGACG TACTGGAGGGTCACACT-3′.

2.3. Microarray analysis

HeLa cells (1×10^5 cells/ml) were inoculated 24 h prior to transfection into individual wells of a culture plate. Cells were transfected with 50 nM siRNA, 500 ng pSUPER.H1, or 500 ng pENTR.CMV. Total RNA (3 μg) was purified using the RNeasy Kit (Qiagen) 24 h after transfection, and hybridized to the Human Genome U133 Plus 2.0 GeneChip (Affymetrix) containing about 47,400 human transcripts according to the manufacturer's protocol. RNA from mock-transfected cells treated with transfection reagent in the absence of siRNA were used as the siRNA transfection control. The plasmids pSUPER.H1 and pENTR.CMV, both without the shRNA transcription unit, were used as shRNA controls. The transcript expression value was calculated using Microarray Suite 5.0 (MAS5) [39] with quantile normalization [40]. Transcripts with hybridization signals strong enough to be called present (P) were used in this study. To identify downregulated transcripts on the array, the cumulative distribution and mean fold changes of each transcript groups with the site versus those with no canonical site were compared.

2.4. Motif analysis of 3′ UTR and CDS

The probe sequences, which were taken from the annotation table provided on the Affymetrix website (http://www.affymetrix.com/), were mapped to the RefSeq human mRNA sequences (release 24) to identify the target transcripts. We found 67,220 annotations for human transcripts that corresponded to 54,675 of the probe sets. In microarray analyses using siVIM269, siVIM270, and siVIM812, the number of transcripts identified as P by

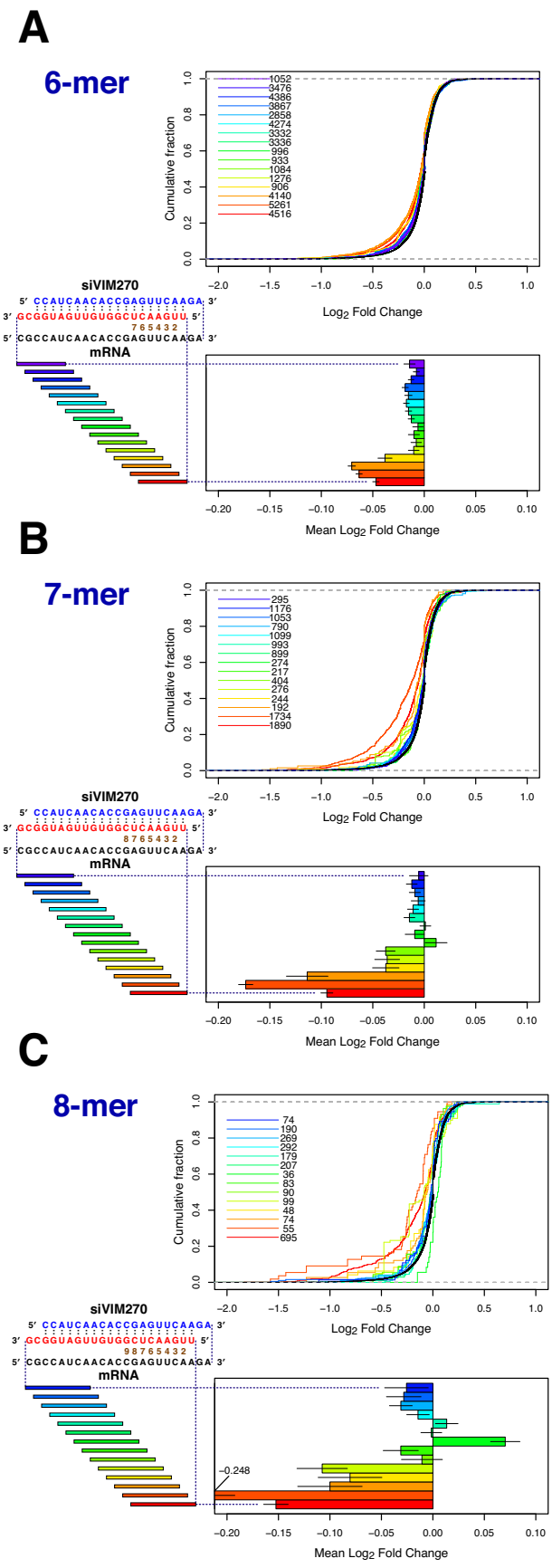

Fig. 2 Microarray transcript profiles following transfection of siVIM270. Transcripts possessing the complementarity to a 6-mer (**A**), 7-mer (**B**), and 8-mer (**C**) guide strand sequences of siVIM270 were divided into 16, 15, and 14 groups, respectively, based on their positions in the siRNA. (upper panel) Change in gene expression is shown by log2-fold change ratio to mock transfection. The cumulative distribution of transcript groups is colored as shown in each figure. The number of transcripts belonging to each group is presented at the side of each colored line. (lower panel) Blue character: passenger strand sequence. Red character: guide strand sequence. Black character: target mRNA sequence. Histograms of mean fold changes (log2) of each transcript groups are represented on the right.

MAS5 was estimated at 19,856. Of these, 16,783, which represented RefSeq entries (25%) with the 3′ UTR, were considered here. In the experiments using pSUPER.H1-VIM269 and pSUPER.H1-VIM812, 17,858 of 21,543 transcripts defined as P were analyzed. In the experiments using pENTR.CMV-VIM269 and pENTR.CMV-VIM812, 17,435 of 20,957 transcripts were analyzed. Seven nucleotide sequences matching the seed region (nucleotides 2–8) of both strands of siRNA were assigned as extracted 3′ UTR sequences.

3. RESULTS AND DISCUSSION

3.1 Thermodynamic stability of the seed-target duplex determines shRNA off-target effects

To confirm the notion that off-target gene silencing induced by shRNA is also determined primarily by seed-target duplex stability as it is by siRNA, genome-wide expression profiling was performed using three siRNAs and four shRNA expression plasmids, all of which target the human vimentin gene. Two siRNAs, siVIM269 and siVIM270, which have seed sequences with T_m values in 100 mM NaCl of 27.4°C and 26.5°C, respectively, were predicted as good off-target inducers. The seed duplex generated by siVIM812 had a T_m value of 8.8°C and was considered a poor off-target inducer. HeLa cells were transfected with each of 50 nM siRNA and their corresponding shRNA expression constructs, and the changes in the expression level of transcripts were analyzed after 24 h. siVIM269 and siVIM270 decreased the expression level of target vimentin gene to 26.8 and 15.8 %, respectively, and siVIM812 decreased vimentin expression to 15.2 %. The expression levels of the transcripts with seed complementary sequences (positions 2–8 of the siRNA guide strand) in 3′ UTRs were most significantly reduced by siVIM269 (**Fig. 3A**) and siVIM270 (**Fig. 2A**). To evaluate the off-target effects of every 6-, 7-, and 8-mer sequence in the siRNA guide strand, we analyzed differences in transcriptional expression levels with complementarities with siVIM270 sequence (**Fig. 2**). Although the transcripts with 6-mer complementarities at positions 2–7 and 3–8 were similarly reduced (**Fig. 2A**), those with 7-mer complementarities at positions 2–8 were prominently reduced (**Fig. 2B**). Highly reduced transcripts contained complementarities at positions 2–9 in 8-mer complementary transcripts whereas the neighboring 8-mers also reduced considerably (**Fig. 2C**). These results confirm that seed-dependent off-target effects are highly correlated with the 7-mer sequence positioned at 2–8 in the seed region. The transfection with siVIM812 exhibited no or little off-target effect (**Fig. 3D**). To evaluate the off-target effects induced by shRNA expression plasmids, we used two siRNA (siVIM269 and siVIM812) sequences inserted into two types of shRNA expression vectors, pol III-driven pSUPER.H1 and pol II-driven pENTR.CMV, respectively. The shRNA expression plasmids were transfected into HeLa cells at 0.5 μg/well and analyzed after 24 h. Both pSUPER.H1-VIM269 and pENTR.CMV-VIM269 plasmids induced high levels of off-target effects (**Fig. 3B,C**), and decreased the expression level of vimentin to 46.0 and 32.7 %. However, trace off-target effects were observed with pSUPER.H1-VIM812 and pENTR.CMV-VIM812 (**Fig. 3E,F**), although vimentin expression was clearly reduced to 54.0 and 47.7 % by these constructs, respectively. These results indicate that seed-dependent off-target effects are induced in a similar manner as that induced by siRNA; the degree of off-target effect is primarily governed by the thermodynamic stability or T_m value of the seed-target duplex.

3.2 Shift in cleavage site by Dicer and Drosha

The primary transcript of pol III-driven shRNA is cleaved by Dicer to generate an siRNA duplex (**Fig. 1B**), and pol II-driven shRNA is processed by Drosha before Dicer cleavage (**Fig. 1C**). We investigated the displacement of the cleavage site by Dicer or Drosha. Dicer cleaves the site close to the loop as shown in **Figures 1B and C**. Because both of our pol III- and pol II-driven shRNA expression plasmids had only 1 nt of clearance to the root of the loop region from the 3′ end of the passenger strand, the cleaved site could be shifted only up to 1 nt. siVIM269 significantly reduced the expression of transcripts with seed complementarities between the siRNA sequence at positions 2–8 from the 5′ end of the guide strand and 3′ UTRs (**Fig. 3A**). However, the transcripts with complementarities in the 7-mer at positions 1–7 were mostly reduced by the transfection of pSUPER.H1-VIM269 and pENTR.CMV-VIM269 (**Fig. 3B,C**). This indicates that, as anticipated, the processed site by Dicer was shifted 1 nt towards the loop. Drosha cleaves out the flanking region of the primary transcript of shRNA driven by the pol II promoter (**Fig. 1C**). The passenger strand of siVIM812 showed a seed-dependent off-target effect (**Fig. 3G**). The transcripts with complementarity in positions 2–8 were also significantly reduced by the addition of siVIM812. The passenger strand of shVIM812 derived from pENTR.CMV-VIM812 decreased transcripts with complementarities in positions 3–9 as well as 2–8 (**Fig. 3I**), indicating that a part of the sites processed by Drosha in shVIM812 was shifted 1 nt away from the flanking region. These results clearly demonstrate that the sites processed by Dicer and Drosha fluctuate, although the shift in cleavage site may differ according to the inserted shRNA sequence.

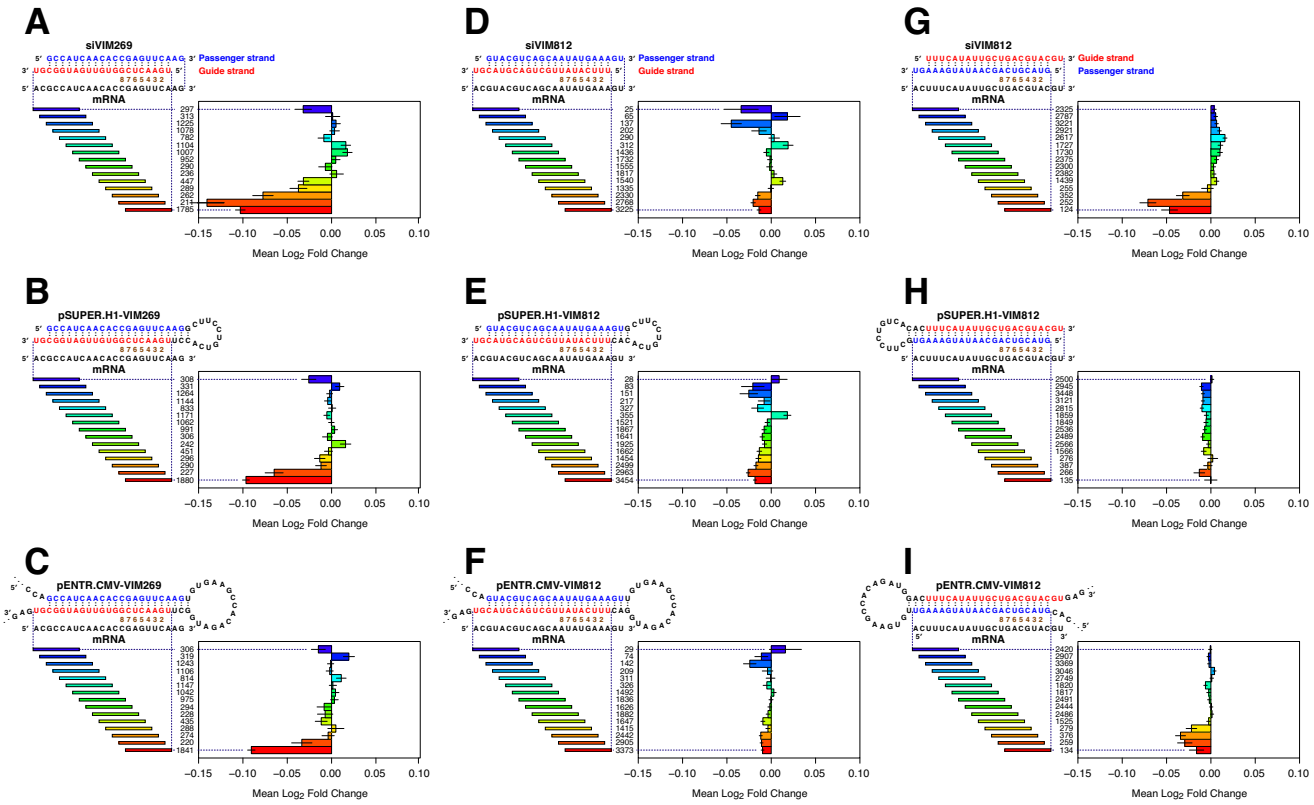

Fig. 3 Seed-dependent off-target effects after transfection of siRNA and shRNA. Changes in the expression levels of the transcripts possessing the 7 nt complementarity with the siRNA guide strand are shown as mean log2-fold change. siRNA and shRNA structures are shown in the upper left. Blue character: passenger strand sequence. Red character: guide strand sequence. Black character: target mRNA sequence. The number of transcripts belonging to each group is presented at the side of each colored line. Histograms of the mean fold changes (log2) of each transcript group are represented on the right. The off-target effect of the passenger strand is also analyzed in **G–I**.

Furthermore, no or little off-target effect was observed in the transcripts with seed complementarity with the passenger strand of shVIM812 derived from pSUPER.H1-VIM812 (**Fig. 3H**). The 5′ end of the primary transcript from pSUPER.H1-VIM812 corresponded to the transcription start site and the 3′ end corresponded to the termination site. Therefore, the results suggest that the start and/or termination site are not rigid and ambiguous.

3.3 Design of shRNA expression system with reduced off-target effects

We reported previously that highly functional siRNA in mammalian RNAi simultaneously satisfies the following four sequence conditions (**Fig. 4A**): A or U residues at position 1 from the 5′ end of the guide strand, three to six A/Us in positions 2–7, G/C at position 19; and no GC stretch of more than nine nucleotides present. Therefore, oligonucleotides inserted into shRNA expression vectors to transcribe shRNAs must at least satisfy these sequence conditions (**Fig. 4B and C**).

In this study, we found that the transcription start site might not be strictly defined, and that the Dicer cleavage site could be shifted at the position between -1 and -2 of the guide strand and the corresponding sites in the passenger strand (**Fig. 4D**). In the pol II system, the cleavage sites of both Drosha and Dicer were often shifted 1 nt (**Fig. 4E**). Furthermore, shRNA as well as siRNA (**Fig. 4F**) with low T_m (< 21.5°C) at seed regions are preferred to reduce off-target effect.

Considering the sequence rules for the design of functional siRNA, the thermodynamic stability of the seed-target duplex, and the shift in Dicer and Drosha cleavage sites, a functional shRNA with reduced off-target effects should satisfy the following conditions: the presence of A/U residues at nucleotide positions 1 and -1 from the 5′ end of the guide strand, three to six A/Us in nucleotide positions 2–7, G/C at positions 19 and 18, and no stretch of more than nine GC base pairs. In addition, the T_m values of 7 nt at positions 2–8 of the guide strand and 11–17 of the passenger strand should be low (**Fig. 4G,H**). Furthermore, particularly in the pSUPER.H1 system, a G residue should be the first nucleotide because the RNA pol III promoter prefers to initiate transcription with a purine. Stretches of ≥ 4 T's or A's in the siRNA sequence should also be avoided due to the addition of a poly(T) tract as a termination signal for RNA pol III (**Fig. 4G**).

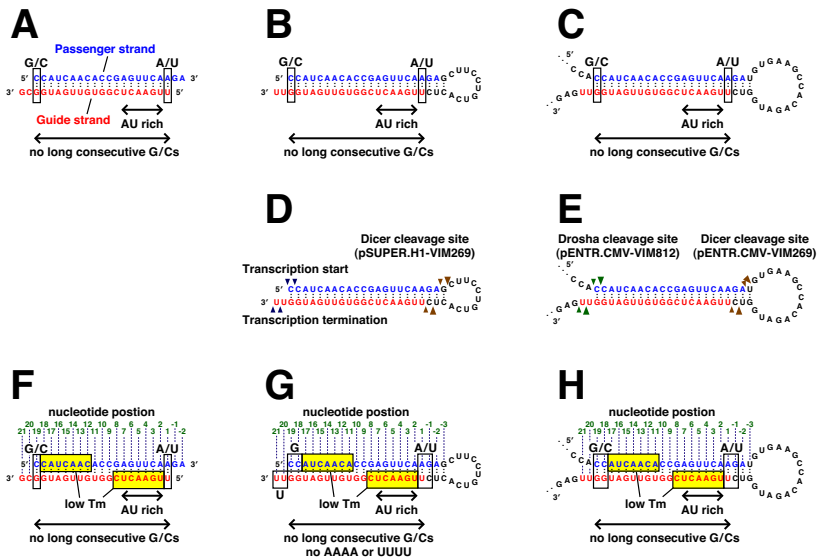

Fig. 4 The functional and reduced off-target effect of siRNA and shRNA sequences. Functional siRNA (**A**) and shRNAs derived from pSUPER.H1 (**B**) and pENTR.CMV (**C**). The potential cleavage sites by Drosha and/or Dicer in shRNAs derived from pSUPER.H1 (**D**) and pENTR.CMV (**E**). The functional and reduced off-target effect of siRNA (**F**), shRNAs derived from pSUPER.H1 (**G**), and pENTR.CMV (**H**). Blue character: passenger strand sequence. Red character: guide strand sequence. Green triangles indicate Drosha cleavage sites (small triangle: original cleavage site; large triangle: shifted cleavage site). Brown triangles indicate Dicer cleavage sites (small triangle: original cleavage site; large triangle: shifted cleavage site). Blue triangles indicate transcription start or termination sites. Yellow box indicates the region with low thermodynamic stability (T_m). Nucleotide positions are shown in the upper regions in **F, G,** and **H**. Position 1 indicates the 5′ end of the guide strand.

4. CONCLUSION

In summary, we found that shRNA induces off-target effects depending on the thermodynamic stability or T_m of the seed-target duplex, in a similar manner as does siRNA. However, the processing of shRNA by Drosha or Dicer was not rigid but rather fluctuated slightly. Based on these results, a functional shRNA with reduced off-target effects should satisfy the following conditions: the presence of A/U residues at nucleotide positions 1 and -1 from the 5′ end of the guide strand, three to six A/Us in nucleotide positions 2–7, G/C at positions 19 and 18, and no GC stretch of more than nine nucleotides. In addition, the T_m values of the seed region in both strands should be low.

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