Reduced base–base interactions between the DNA seed and RNA target are the major determinants of a significant reduction in the off-target effect due to DNA-seed-containing siRNA

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Abstract:
Previously, we showed that DNA replacement of the seed-containing region of class I siRNAs significantly reduced the off-target effect without substantial loss of target gene silencing activity (Ui-Tei et al, Nucleic Acids Res., 36, 2136-2151, 2008). Separately, we also showed that the level of the off-target effect due to non-modified class I siRNA is determined primarily by the calculated melting temperature (T_m) of the seed-target duplex, indicating that, at least in class I-siRNA-mediated gene silencing, T_m or thermodynamic stability of the complex between the siRNA seed and target mRNA is the most important factor determining the degree of off-target effects (Ui-Tei et al., Nucleic Acids Res., 36, 7100-7109, 2008). Here, using non-modified siRNAs other than class I siRNAs and DNA-seed-containing siRNAs, we examined the relationship between the thermodynamic stability of the seed–target duplex and the degree of the off-target effect. We found that the degree of the off-target effects is generally determined primarily by the T_m of the corresponding seed-target duplex, indicating that its thermodynamic stability is the most important general determinant of the degree of the off-target effect in gene silencing.

1. INTRODUCTION

RNA interference (RNAi) has been used to study gene function in a variety of organisms and it holds great promise for therapeutic applications. However, a growing body of evidence from microarray profiling and large-scale knockdown experiments shows that gene-silencing due to authentic siRNA is associated with activity that knocks down many unintended nontarget genes (*i.e.* the off-target effect) [1–5]. Most, if not all, transcripts of these false-positive genes possess virtually complete complementarity only to the siRNA "seed" region (nucleotide positions 2–8 from the 5'-end of the guide strand) [3–7]. Consistent with this, the capability of class I siRNAs [8] to induce the off-target effect was found to be highly correlated with the thermodynamic stability or calculated melting temperature (T_m) of the duplex formed between the guide-stand seed arm and target mRNA (seed-target, or seed duplex) [7].

Previously, we examined the effects of systematic deoxyribonucleotide substitutions of highly functional siRNAs on gene silencing, and found that the 5'-proximal seed arm of the guide strand could be replaced with cognate deoxyribonucleotides with little or no loss of gene silencing activity [9]. In contrast, replacing the 3'-proximal RNA sequence of the guide strand with its DNA counterpart resulted in almost complete loss of gene silencing activity. Unlike non-modified siRNAs, DNA-modified siRNAs, in which the seed region consists of DNA and the remainder is RNA, exerted a limited, if any, off-target effect. These findings imply that most mammalian genes can be knocked down effectively without a substantial off-target effect by treating cells with a special class of DNA-seed-containing siRNAs.

In this study, we further confirmed the close relationship between thermodynamic stability in seed-target base-pairing and the level of the off-target effect by analyzing gene silencing in mammalian cells transfected with DNA-seed-containing siRNAs or non-modified siRNAs other than those belonging to class I.

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 for both cell lines were supplemented with 10 % heat-inactivated fetal bovine serum (FBS; Mitsubishi Kagaku) and antibiotics (10 units/ml of penicillin (Meiji) and 50 μg/ml of streptomycin (Meiji)).

2.2. siRNA and DNA-seed-containing siRNA preparation

Passenger- and guide-strand RNA or DNA-substituted RNA oligonucleotides (**Table 1**) were chemically synthesized (Proligo), mixed in a 1:1 fashion in 10 mM NaCl and 20 mM Tris–HCl (pH 7.5), and annealed by incubating at 95ºC for 15 min, 37ºC for 30 min, and 25ºC for 30 min. Annealed products were examined using 3 % agarose gel electrophoresis in TBE buffer, which can separate 21-bp long double-stranded siRNA from 21-nt long single-stranded RNA. Almost all RNA was recovered as dsRNA.

2.3. Construction of psiCHECK derivatives and the assay of seed-dependent off-target activity

The plasmids psiCHECK-cm included 3 copies of completely matched (cm) target sequences while

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psiCHECK-sm included 3 copies of seed-matched (sm) target sequences (see **Table 2** and **Fig. 2A**). Chemically synthesized oligodeoxynucleotides (75 bp in length) including three copies of the same 23bp sm or cm target sequences with cohesive XhoI/EcoRI ends were inserted into the psiCHECK-1 (Promega) XhoI/EcoRI site, which is situated in the region encoding the 3' UTR of *Renilla luc* mRNA. The cm sequence is completely matched that of the guide strand of non-modified or DNA-modified siRNAs, and is expressed as a part of the target mRNA in transfected cells. In contrast, the sm sequence in the target mRNA consists of two parts. Its 3'-terminal third is complementary in sequence to the 8bp-long 5' proximal region of the guide strand of siRNA or DNA-seed-containing siRNA, while the remaining two-thirds, totally non-homologous. One milliliter of HeLa cell suspension (1 x 10^5 cells/ml) was inoculated in a 1.5-cm well 24 hours prior to transfection. Cells were transfected simultaneously with one of psiCHECK derivatives (10 ng each), pGL3-Control (Promega, 0.5 μg) or phLuc-Control (0.5 μg) [7], and siRNA or DNA-seed-containing siRNA. Lipofectamine 2000 reagent (Invitrogen) was used for transfection. Cells were harvested and luc activity was measured using the Dual-Luciferase Reporter Assay System (Promega). The reduction of *Renilla* luc activity by the addition of siRNA against the firefly *luc* gene is normalized with firefly luc activity (internal control). pGL3-Control, encoding the firefly *luc,* served as a control for *Renilla luc* silencing assay for the siRNAs (siVIM-270, -596, -812, and -1128, siOct-797, and -821, siGRK4-189, and -934) along with cognate DNA-seed-containing siRNAs. The gene silencing assay for siRNAs (siLuc-36, -49, -309, -774, -1048, -1063, -1120, -1430 and 2-153) and cognate DNA-seed-containing siRNAs was carried out using as a control phLuc-Control coding for a modified firefly *luc* gene. The siGY441 is an siRNA for knock-down of GFP and serves as an siRNA control.

2.4. Calculation of melting temperature

Melting temperature (Tm) was calculated based on the nearest-neighbor model [10] and the thermodynamic values for RNA-RNA [11] and RNA-DNA [12]. The formula for calculating Tm is as follows. Tm ={(1000 x ΔH)/(A + ΔS + R ln (Ct/4))}-273.15 + 16.6log[Na^+]. ΔH (Kcal/mol), sum of nearest neighbor enthalpy change. A, helix initiation constant (-10.8). ΔS, sum of nearest neighbor entropy change. R, gas constant (1.987 cal/deg/mol). Ct, total molecular concentration of strand (100 μM). [Na^+] was fixed at 100 mM. Standard Gibbs free energy change (ΔG) and dissociation constant (Kd) were calculated as described previously [7].

3. RESULTS AND DISCUSSION

3.1 Variation in the efficiency of off-target gene silencing due to functional class II siRNAs with a seed-GC content exceeding 57 %

Three classes of siRNAs exist based on their RNAi gene-silencing activity [8]. Class I siRNAs, which are highly functional in mammalian RNAi, have A or U residues at nucleotide position 1, three to six A/Us in nucleotide positions 2–7 and G/C at position 19, with the nucleotide position measured from the 5'-end of the guide strand, as shown in **Fig. 1A**. In addition, no GC stretch of more than nine nucleotides occurs in class I siRNA sequences. Class III siRNAs have the opposite features with respect to the first three conditions and cause little or no mammalian gene silencing. The remaining siRNAs belong to class II and are a mixture of highly or intermediately functional siRNAs and nonfunctional siRNAs. Previously [7], we determined that thermodynamic stability and Watson–Crick base-pairing in the seed–target duplex [the duplex formed between the guide-strand seed (positions 2–8) and target mRNA] are the major determinants of the efficiency of the off-target effects due to class I siRNAs, for which the seed-region GC content ranges from 0% to 57%.

To determine the relationship between the thermodynamic stability of seed–target duplexes with GC content exceeding 57% and the off-targeting efficiency of the corresponding siRNA, six functional class II siRNAs with high seed-GC contents were chosen arbitrarily, and their capability to exert off-target effects was examined (**Fig. 2M–R**). Note that two of the six class II siRNAs examined here (siLuc-1430 and siLuc-1063) possess a 100%-GC seed region (**Table 1**). To compare the degrees of the off-target effect and target gene silencing, 17 pairs of completely matched (cm) and seed-matched (sm) target sequences corresponding to the above-mentioned six class

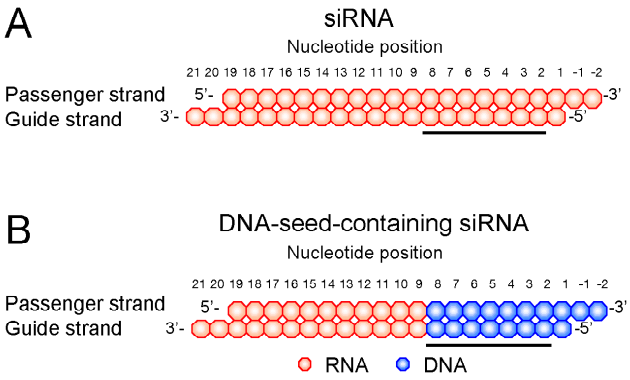
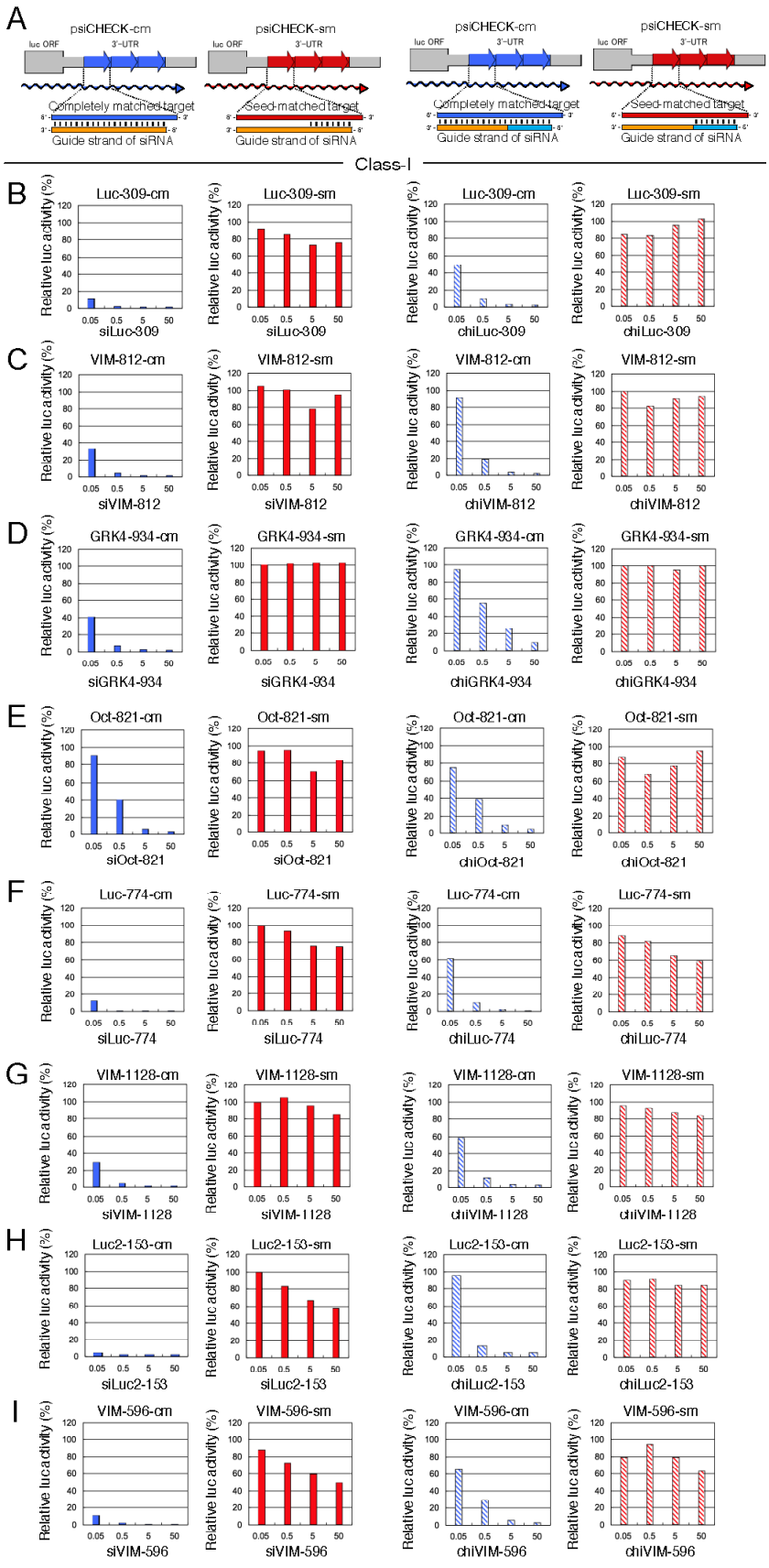
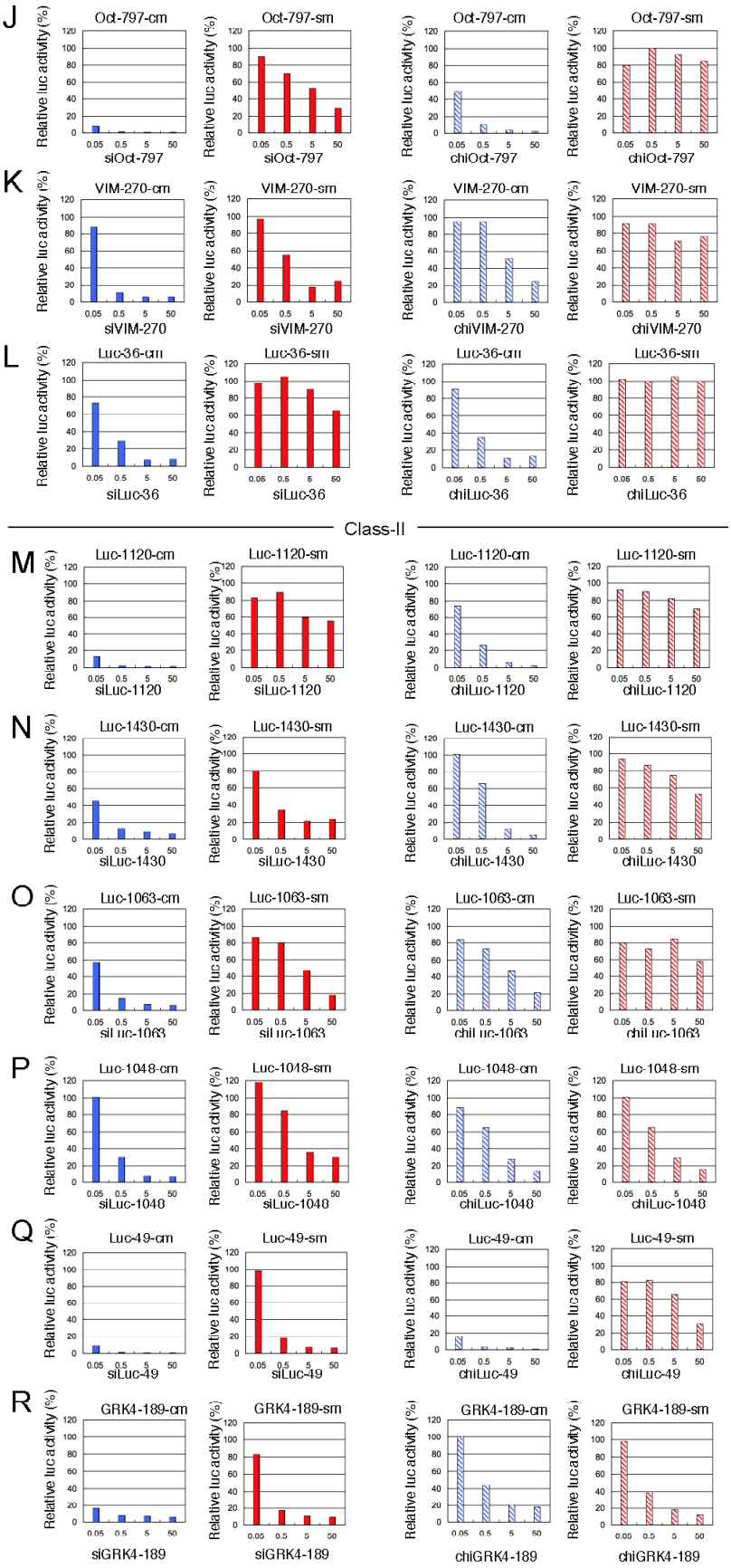


Fig. 1 Structures of siRNA (**A**) and DNA-seed-containing siRNA (**B**). The nucleotide position is counted from the 5'-end of the guide strand. The seed region (positions 2–8) is underlined. In **B**, the DNA and RNA nucleotides are blue and red, respectively. Note that the 5' one-third of the guide strand containing the seed region and its complementary region in the passenger strand with the 3'-overhang are replaced with DNA.



II siRNAs along with 11 arbitrarily chosen class I siRNAs (**Table 1**) were introduced into an expression reporter plasmid, psiCHECK (**Fig. 2A**), and the change in luc activity in transfected HeLa cells was examined as a function of the siRNA concentration (**Fig. 2B-R**). psiCHECK encodes the *Renilla luc* gene. Three tandem repeats of cm or sm target sequences were introduced into the region corresponding to the 3'-UTR of the *luc* mRNA to generate psiCHECK-cm or psiCHECK-sm, respectively (**Fig. 2A**). The 21-nuleotide-long cm targets were completely complementary to the siRNA guide strands (**Table 2**) and were used to determine the efficiency of intended gene silencing, basically RNAi (**Fig. 2**). The sm sequence was completely comlementary to the entire seed-containing region (positions 1–8), but not to the remaining non-seed region, positions 9–21 (**Table 2**), and was used to determine the efficiency of the seed-dependent unintended off-target effect (**Fig. 2**). Under our experimental conditions, the amount of *luc* mRNA produced within cells was estimated to be about 300 copies per ng of total RNA [7], a value

Fig. 2 Dose-dependent gene silencing effects of authentic siRNA and DNA-seed-containing siRNA for completely matched (cm) and seed-matched (sm) targets. Both class I siRNAs and functional class II siRNAs are included. The gene-silencing effects were examined using HeLa cells transfected with psiCHECK-cm or -sm plasmids containing various cm and sm targets. The relative luc activity in transfected HeLa cells was determined using the dual luciferase assay. (**A**) The structures of the psiCHECK-cm and -sm plasmids and the mechanism of gene silencing with authentic non-modified siRNAs (left) and DNA-seed-containing siRNAs (right). Three tandem repeats of cm or sm target sequences were introduced into the region corresponding to the 3'-UTR of the *luc* mRNA. In **B–R**, the effects of transfection of non-modified siRNAs (left pair) and DNA-seed-containing siRNAs (right pair) on cm and sm-targets are shown. (**B–L**) class I siRNAs, (**M–R**) class II siRNAs, (**B**) siLuc-309, (**C**) VIM-812, (**D**) GRK4-934, (**E**) Oct-821, (**F**) Luc-774, (**G**) VIM-1128, (**H**) Luc2-153, (**I**) VIM-596, (**J**) Oct-797, (**K**) VIM-270, (**L**) Luc-36, (**M**) Luc-1120, (**N**) Luc-1430, (**O**) Luc-1063, (P) Luc-1048, (**Q**) Luc-49, (**R**) GRK4-189. The sequences of the siRNA and DNA-seed-containing siRNAs, and sm and cm target sequences are shown in **Tables 1** and **2**.

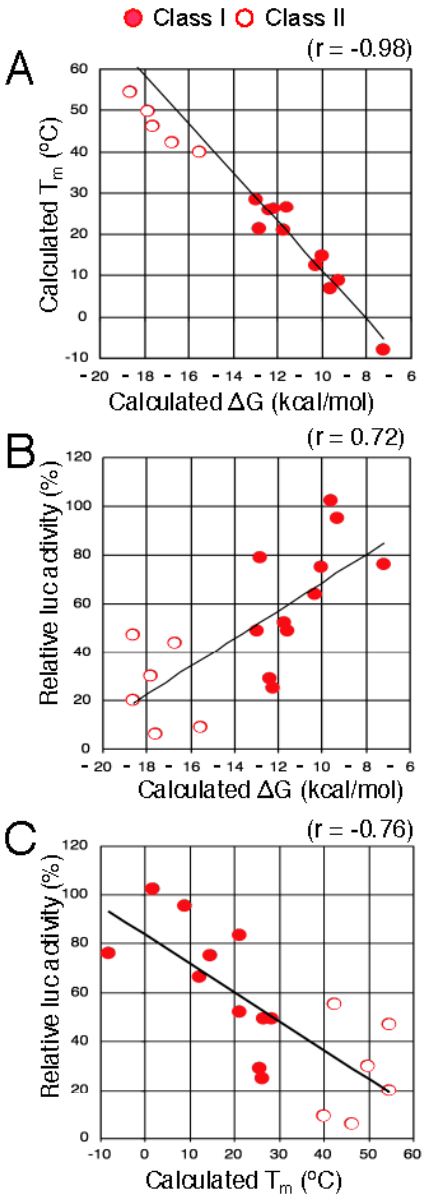


one-hundredth that of β-actin mRNA. The luc activities measured using different psiCHECK-sm and -cm constructs targeted by the corresponding siRNAs were roughly proportional to the levels of mRNA [7], indicating that at least under these conditions most, if not all, of the luc activity reduction due not only to RNAi, but also to the off-target effect, is attributable to siRNA-dependent *luc* mRNA degradation.

As with the 11 class I siRNAs with 0–57 % GC content in the seed region, all six functional class II siRNAs examined here, for which the seed-region GC content ranged from 57% to 100%, showed high activity of the intended gene silencing at 50 nM (**Fig. 2M–R**). Even at 0.5 nM, most, if not all, of the class II siRNAs examined here reduced the activity of the *luc* gene with the cm target to less than 20 %. In contrast to class I siRNAs, which have little or no off-target effect, the class II siRNAs were frequently associated with a considerable level of off-target gene silencing (**Fig. 2**).

The T_m and standard free energy change (ΔG) for the formation of the seed–target duplex are good measures of the thermodynamic stability of the protein-free seed–target duplex. In a previous experiment [7], using class I siRNAs, we verified the close linear relationship between ΔG and Tm, a positive strong correlation between sm-luc activity and ΔG, and a negative strong correlation between Tm and sm-luc activity. Note that the relative sm-luc activity is compromised by the off-target effect. By replacing class I siRNAs with a mixture of class I and II siRNAs, the range of ΔG expanded from between –13 and –7 to between –19 and –7 kcal/mol (**Fig. 3A,B,D**), while the range of T_m expanded from between –8 and 28ºC to –8 and 55ºC (**Fig. 3A,C,D**). However, as shown in **Fig. 3A–C**, the linear relationships among these parameters were almost invariant.

The value of ΔG may be converted into the dissociation constant (Kd) using the formula $\Delta G = -RT\ln(1/K_d)$. The K_d values of the 17 siRNAs were calculated and are listed in **Fig. 3D**. The highest Kd was more than 10^8 times greater than the lowest one. Therefore, it may follow that in both functional class I and II siRNA-mediated gene silencing, the degree of off-target effects is governed primarily by the thermodynamic stability of the seed-target duplex, which is 7 base pairs long.



D

	Luc act (%)	Tm 2-8 (℃)	ΔG 2-8 (kcal/mol)	Kd (nM)
Class I				
smLuc-309	76	-8.1	-7.2	5.3E-06
smGRK4-934	102	6.7	-9.6	9.2E-08
smVIM-812	95	8.8	-9.3	1.5E-07
smOct-821	64	12.2	-10.3	2.8E-08
smLuc-774	75	14.6	-10	4.7E-08
smLuc2-153	52	21	-11.7	2.6E-09
smOct-797	29	25.7	-12.4	8.1E-10
smVIM-270	25	26.2	-12.2	1.1E-09
smVIM-596	49	26.4	-11.6	3.1E-09
smLuc-36	49	28.4	-13	3.0E-10
smVIM-1128	79	21.2	-12.8	4.1E-10
Class II				
smGRK4-189	9	40.1	-15.5	4.3E-12
smLuc-1120	44	42.3	-16.7	5.7E-13
suLuc-49	6.1	46.3	-17.6	1.3E-13
smLuc-1048	30	49.7	-17.8	8.9E-14
smLuc-1063	47	54.5	-18.6	2.3E-14
smLuc-1430	20	54.5	-18.6	2.3E-14

Table 1 Sequences of the guide strand and passenger strand of siRNAs and the corresponding DNA-seed-containing siRNAs.

Authentic siRNA	Passenger strand (5'→3')	Guide strand (5'→3')
Class I		
siLuc-309	CCGCGAACGACAUUUAUAAUG	UUAUAAAUGUCGUUCGCGGGC
siVIM-812	GUACGUCAGCAAUAUGAAAGU	UUUCAUAUUGCUGACGUACGU
siGRK4-934	CUUGAAGCCUGAGAAUAUUCU	AAUAUUCUCAGGCUUCAAGUC
siOct-821	CGCCAGAAGGGCAAAAGAUCA	AUCUUUUGCCCUUCUGGCGCC
siLuc-774	GAUUUCGAGUCGUCUUAAUGU	AUUAAGACGACUCGAAAUCCA
siVIM-1128	CUCGUCACCUUCGUGAAUACC	UAUUCACGAAGGUGACGAGCC
siLuc2-153	CGUACGCGGAAUACUUCGAUU	UCGAAGUAUUCCGCGUACGUG
siVIM-596	GAAAACACCCUGCAAUCUUUC	AAGAUUGCAGGGUGUUUUCGG
siOct-797	GUUCGAGUAUGGUUCUGUAAC	UACAGAACCAUACUCGAACCA
siVIM-270	CCAUCAACACCGAGUUCAAGA	UUGAACUCGGUGUUGAUGGCG
siLuc-36	CAUUCUAUCCGCUGGAAGAUG	UCUUCCAGCGGAUAGAAUGGC
Class II		
siLuc-1120	GGAUCUGGAUACCGGGAAAAC	UUUCCCGGUAUCCAGAUCCAC
siLuc-1430	GGUGAACUUCCCGCCGCCGUU	CGGCGGCGGGAAGUUCACCGG
siLuc-1063	GGAUGAUAAACCGGGCGCGGU	CGCGCCCGGUUUAUCAUCCCC
siLuc-1048	GAUUACACCCGAGGGGGAUGA	AUCCCCCUCGGGUGUAAUCAG
siLuc-49	GGAAGAUGGAACCGCUGGAGA	UCCAGCGGUUCCAUCUUCCAG
siGRK4-189	GACGUCUCUUCAGGCAGUUCU	AACUGCCUGAAGAGACGUCUU
Control		
siGY441	GCCACAACGUCUAUAUCAUGG	AUGAUAUAGACGUUGUGGCUG

DNA-seed-containing siRNA	Passenger strand (5'→3')	Guide strand (5'→3')
Class I		
chiLuc-309	CCGCGAACGACatttataatg	ttataaatGUCGUUCGCGGGC
chiVIM-812	GUACGUCAGCAatatgaaagt	tttcatatUGCUGACGUACGU
chiGRK4-934	CUUGAAGCCUGagaatattct	aatattctCAGGCUUCAAGUC
chiOct-821	CGCCAGAAGGGcaaaagatca	atcttttgCCCUUCUGGCGCC
chiLuc-774	GAUUUCGAGUCgtcttaatgt	attaagacGACUCGAAAUCCA
chiVIM-1128	CUCGUCACCUUcgtgaatacc	tattcacgAAGGUGACGAGCC
chiLuc2-153	CGUACGCGGAAtacttcgatt	tcgaagtaUUCCGCGUACGUG
chiVIM-596	GAAAACACCCUgcaatctttc	aagattgcAGGGUGUUUUCGG
chiOct-797	GUUCGAGUAUGgttctgtaac	tacagaacCAUACUCGAACCA
chiVIM-270	CCAUCAACACCgagttcaaga	ttgaactcGGUGUUGAUGGCG
chiLuc-36	CAUUCUAUCCGctggaagatg	tcttccagCGGAUAGAAUGGC
Class II		
chiLuc-1120	GGAUCUGGAUAccgggaaaac	tttcccggUAUCCAGAUCCAC
chiLuc-1430	GGUGAACUUCCcgccgccgtt	cggcggcgGGAAGUUCACCGG
chiLuc-1063	GGAUGAUAAACcgggcgcggt	cgcgcccgGUUUAUCAUCCCC
chiLuc-1048	GAUUACACCCGaggggggatga	atcccccgCGGGUGUAAUCAG
chiLuc-49	GGAAGAUGGAAccgctggaga	tccagcggUUCCAUCUUCCAG
chiGRK4-189	GACGUCUCUUCaggcagttct	aactgcctGAAGAGACGUCUU
Control		
chiGRK4-189	GACGUCUCUUCaggcagttct	aactgcctGAAGAGACGUCUU

Lower case, deoxyribonucleotide. Upper case, ribonucleotide. Red and blue indicate the A or U (T) residue and the G or C residue in the seed region, respectively.

Table 2 Sequences of the cm- and sm-targets.

sm-Target	Target sequence (5'→3')
Luc-309-sm	ACUAACCCCUAAUAUUUAUAAUG
VIM-812-sm	CUAGGUGAAACUUAUAUGAAAGU
GRK4-934-sm	AGGCAAGCGGCGAAGAAUAUUCU
Oct-821-sm	CACUGAGCGGUUUCAAAAGAUCA
Luc-774-sm	UAGGAUGCCAUCGGUCUUAAUGU
VIM-1128-sm	GAGGCAAAUGACGCGUGAAUACC
Luc2-153-sm	AAGCGGUGCCAAGUACUUCGAAA
VIM-596-sm	CGUUACUCUCGGAGCAAUCUUUC
Oct-797-sm	GCUGAAGCUAGUAGUUCUGUAAC
VIM-270-sm	AAGGAAGGGAGAGGAGUUCAAGA
Luc-36-sm	CUUUGGAUGAAACCUGGAAGAUG
Luc-1120-sm	CCUACAGCGGCUGCCGGGAAAAC
Luc-1430-sm	CACAGAGGACUAACGCCGCCGUU
Luc-1063-sm	CUCUGCGAGGUCACGGGCGCGGU
Luc-1048-sm	UGUGGGAGUGCAAAGGGGGAUGA
Luc-49-sm	ACACACUAGGACCCCGCUGGAGA
GRK4-189-sm	AAUUUUUGUAAGAAGGCAGUAA

cm-Target	Target sequence (5'→3')
Luc-309-cm	GCCCGCGAACGACAUUUAUAAUG
VIM-812-cm	ACGUACGUCAGCAAUAUGAAAGU
GRK4-934-cm	GACUUGAAGCCUGAGAAUAUUCU
Oct-821-cm	GGCGCCAGAAGGGCAAAAGAUCA
Luc-774-cm	UGGAUUUCGAGUCGUCUUAAUGU
VIM-1128-cm	GGCUCGUCACCUUCGUGAAUACC
Luc2-153-cm	CACGUACGCGGAAUACUUCGAAA
VIM-596-cm	CCGAAAACACCCUGCAAUCUUUC
Oct-797-cm	UGGUUCGAGUAUGGUUCUGUAAC
VIM-270-cm	CGCCAUCAACACCGAGUUCAAGA
Luc-36-cm	GCCAUUCUAUCCGCUGGAAGAUG
Luc-1120-cm	GUGGAUCUGGAUACCGGGAAAAC
Luc-1430-cm	CCGGUGAACUUCCCGCCGCCGUU
Luc-1063-cm	GGGGAUGAUAAACCGGGCGCGGU
Luc-1048-cm	CUGAUUACACCCGAGGGGGAUGA
Luc-49-cm	CUGGAAGAUGGAACCGCUGGAGA
GRK4-189-cm	AAGACGUCUCUUCAGGCAGUUCU

3.2 Effects of DNA replacement in the siRNA seed region on the seed-dependent off-target effect

Our previous experiment showed that DNA replacement of the seed-containing sequence (DNA-seed-containing siRNA; **Fig. 1B**) in class I siRNAs almost completely eliminates off-target effects [7] (also see **Fig. 2B–L**). In contrast, as shown in **Fig. 2M–R**, functional class II siRNAs frequently could not effectively eliminate the off-target effects by DNA replacement in the seed region. This apparent difference in the off-target effect may be due to the great difference in the seed-GC content between functional class I and II siRNAs.

To clarify this point, we

Fig. 3 The close relationship between the efficiency of seed-dependent off-target gene silencing and the thermodynamic stability of the seed-target duplex. Both class I siRNAs and functional class II siRNAs were used for the analysis. Solid red circles and open red circles show the class I and II siRNA data, respectively. (**A**) The calculated T_m of the seed-target duplex decreases with increases in the standard free-energy (ΔG) for seed duplex formation (correlation coefficient; –0.98). (**B**) The sm-luc activity, which is the seed-dependent off-target gene silencing at a 50nM siRNA concentration, was positively correlated with ΔG (correlation coefficient; 0.72). (**C**) The correlation between the seed-dependent gene silencing activity (sm-luc activity) and the calculated Tm of the seed-target duplex. A set of sm-luc activities based on the seed-dependent gene silencing at a 50nM siRNA concentration was obtained from **Fig. 2**. The T_m of the seed-target duplex was calculated using the nearest neighbor method. The relative sm-luc activity and calculated Tm were correlated with each other and had a coefficient of –0.76. (**D**) List of the seed-target duplex dissociation constants.

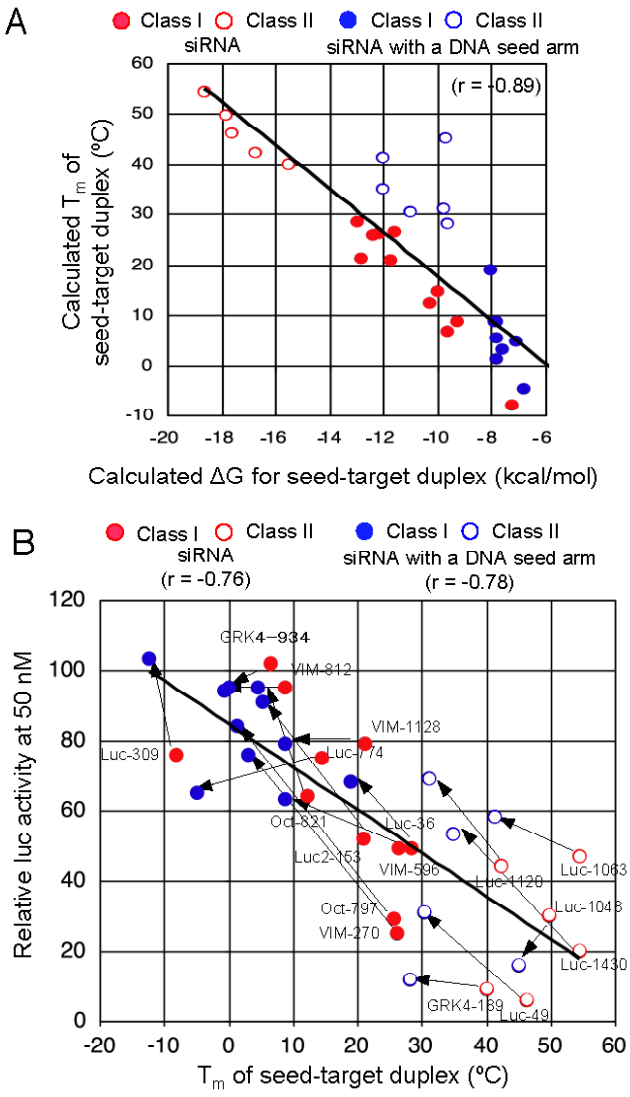


Fig. 4 The close relationship between the efficiency of DNA-seed-dependent off-target gene silencing and the thermodynamic stability of the seed-target duplex. Solid and open red circles show the data obatined by transfecting class I and II nonmodified siRNAs, respectively. Solid and open blue circles show the data obtained by transfecting class I and II DNA-seed-containing siRNAs, respectively. (**A**) A strong negative correlation was observed between the calculated T_m and ΔG with a correlation coefficient of –0.89. (**B**) The correlation between the sm-luc activity and T_m. The abscissa indicates the calculated T_m for the seed-target duplex. The ordinate is the relative sm-luc activity at a 50 nM siRNA concentration. Arrows indicate shifts induced on DNA replacement of the siRNA-seed sequence. The correlation coefficient between the luc activity and T_m of the RNA-seed and RNA target duplex was –0.76, while that between the sm-luc activity and T_m of the duplex between DNA-seed and target mRNA was –0.78.

Table 3 DNA-replacement-dependent changes in the Tm, Δ G and dissosiation constants.

siRNA	GC content	Δ Tm (℃)	ΔΔG (kcal/mol)	Kd-hybrid / Kd-RNA
Class I				
siLuc-309	0	-4.1	2.3	2.0E-02
siGRK4-934	1	-6.5	4.0	1.2E-03
siVIM-812	1	-9.3	3.8	1.6E-03
siOct-821	2	-7.5	3.2	4.5E-03
siLuc-774	2	-19.4	3.2	4.7E-03
siLuc2-153	3	-15.6	3.9	1.4E-03
siOct-797	3	-24.4	4.6	4.3E-04
siVIM-270	3	-23.1	4.6	4.1E-04
siVIM-596	3	-17.6	3.8	1.6E-03
siLuc-36	3	-9.4	5.0	2.1E-04
siVIM-1128	4	-12.4	4.9	2.6E-04
Class II				
siGRK4-189	5	-12.0	5.9	4.7E-05
siLuc-1120	7	-11.2	6.9	8.8E-06
siLuc-49	7	-15.9	6.6	1.5E-05
siLuc-1048	5	-4.5	8.1	1.2E-06
siLuc-1063	6	-13.1	6.6	1.4E-05
siLuc-1430	4	-19.5	6.6	1.4E-05
Mean		-13	5	2.E-03

"GC conent" indicates the number of G and C in the seed region. Δ Tm = [Tm value between the seed sequence of the DNA-seed-containing siRNA and target mRNA (DNA-RNA hybrid)] - [Tm value between the siRNA seed sequence and target mRNA (RNA duplex)]. ΔΔG = (ΔG for the DNA-RNA hybrid) - (ΔG of the RNA duplex). Kd, dissociation constant. Kd-hybrid/Kd-RNA = (Kd for DNA-RNA hybrid)/(Kd of the RNA duplex).

examined the relationship between the relative sm-luc activity, which is compromised by the off-target effect, and the T_m of the DNA-RNA seed duplex in 11 class I and six class II siRNAs (**Fig. 4**). We found that the linear relationship between T_m and the relative sm-luc activity (off-target effect) is essentially invariant irrespective of the presence or absence of DNA replacement in the seed-containing region. However, DNA replacement reduced both the seed-duplex T_m and sm-luc activity considerably. In all the class I DNA-seed-containing siRNAs, the T_m was reduced to less than 20ºC, while the relative sm-luc activity at 50 nM exceeded 60%, the minimum relative luc activity necessary for a practical off-target effect. In contrast, in three of six cases treated with functional class II siRNA, the relative sm-luc activity at 50 nM was 30 % or less, although the seed-T_m was reduced, which shows a strong negative correlation to with the Δ G for seed-target duplex formation (**Fig. 4A**). Therefore, it appears that the reduced off-target effect in DNA-seed-containing siRNA–dependent gene silencing is generally attributable to a reduction in the thermodynamic stability of the DNA–RNA hybrid in the seed–target duplex. According to **Fig. 4A and B** and **Table 3**, DNA replacement throughout the guide-strand seed region is

roughly equivalent to a 13ºC reduction in T_m, a 5 kcal/mol increment in ΔG, and about two to three G/C→A/U changes in the seed duplex (**Table 3**).

4. CONCLUSION

In summary, DNA substitution in the siRNA-seed-containing sequence significantly reduced the seed-dependent off-target effect without changing the T_m /off-target strength linear relationship. These results suggest that as with authentic siRNA-mediated gene silencing, the thermodynamic stability or Tm of the seed-target duplex is the most important determinant of the off-target effect in gene silencing due to DNA-seed-containing siRNA.

ACKNOWLEGEMENTS

This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan to K.U-T.

REFERENCES

[1] Jackson,A.L., Bartz,S.R., Schelter,J., Kobayashi,S.V., Burchard,J., Mao,M., Li,B., Cavet,G. and Linsley,P.S. (2003) Expression profiling reveals off-target gene regulation by RNAi. *Nat. Biotech*., **21**, 635-637.

[2] Scacheri, P.C., Rozenblatt-Rosen,O., Caplen,N.J., Wolfsberg,T.G., Umayam,L., Lee,J.C., Hughes,C.V., Shanmugam,K.S., Bhattacharjee,A., Meyerson,M. and Collins,F.S. (2004) Short interfering RNAs can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells. *Proc. Natl. Acad. Sci., USA*., **101**, 1892-1897.

[3] Lin,X., Ruan,X., Anderson,M.G, McDowell,J.A., Kroeger,P.E., Resik,S.W. and Shen,Y. (2005) siRNA-mediated off-target gene silencing triggered by a 7 nt complementation. *Nucleic Acids Res*., **33**, 4527-4525.

[4] Jackson,A.L., Burchard,J., Schelter,J., Chau,B.N., Cleary,M., Lim,L. and Linsley,P.S. (2006) Widespread siRNA "off-target" transcript silencing mediated by seed region sequence complementarity. *RNA,* **12,** 1179-1187.

[5] Birmingham,A., Anderson,E.M., Reynolds,A., Ilsley-Tyree,D., Leake,D., Fedorov,Y., Baskerville,S., Maksimova,E., Robinson,K., Karpilow,J., Marshall,W.S. and Khvorova,A. (2006) 3′ UTR seed matches, but not overall identity, are associated with RNAi off-targets. *Nat. Methods,* **3,** 199-204.

[6] Grimson, A., Farh,K.K., Johnston,W.K., Garrett-Engele,P., Lim,L.P. and Bartel,D.P. (2007) MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol. Cell,* **27,** 91-105.

[7] Ui-Tei,K., Naito,Y., Nishi,K., Juni,A. and Saigo,K. (2008) Thermodynamic stability and Watson-Crick base pairing in the seed duplex are major determinants of the efficiency of the siRNA-based off-target effect. *Nucleic Acids Res.,* **36,** 7100-7109.

[8] Ui-Tei,K., Naito,Y., Takahashi,F., Haraguchi,T., Ohki-Hamazaki,H., Juni, A., Ueda,R. and Saigo,K. (2004) Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference. *Nucleic Acids Res.,* **32,** 936-948.

[9] Ui-Tei,K., Naito,Y., Zenno,S., Nishi,K., Yamato,K., Takahashi,F., Juni, A. and Saigo,K. (2008) Functional dissection of siRNA sequence by systematic DNA sustitution: modified siRNA with a DNA seed arm is a powerful tool for mammalian gene silencing with significantly reduced off-target effect. *Nucleic Acids Res.,* **36,** 2136-2151.

[10] Panjkovich, A. and Melo, F. (2005) Comparison of different melting temperature calculation methods for short DNA sequences. *Bioinformatics*, **21,** 711-722.

[11] Freier, A. M., Keirzek, R., Jaeger, J. A., Sugimoto, N., Caruthers, M. H., Neilson, T. and Turner, D. H. (1986) Improved free-energy parameters for predictions of RNA duplex stability. *Proc. Natl. Acad. Sci. USA*, **83,** 9373-9377.

[12] Sugimoto, N., Nakano, S., Katoh, M., Matsumura, A., Nakamuta, H., Ohmichi, T., Yoneyama, M. and Sasaki, M. (1995) Thermodynamic parameters to predict stability of RNA/DNA hybrid duplexes. *Biochemistry*, **34,** 11211-11216.

[13] Lim, L.P., Lau,N.C., Garrett-Engele,P., Grimson,A., Schelter,J.M., Castle,J., Bartel,D.P., Linsley,P.S. and Johnson,J.M. (2005) Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature,* **433,** 769-773.

[14] Lewis,B.P., Burge,C.B. and Bartel,D.P. (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell,* **120**, 15-20.

[15] Borer, P.M., Dengler, B., Tinoco, I.Jr. & Uhlenbeck, O.C. Stability of ribonucleic acid double-stranded helices. *J. Mol. Biol*. **86,** 843-853 (1974).

[16] Breslauer, K.J., Frank, R., Blocker, H. & Marky, L.A. Predicting DNA duplex stability from the base sequence. *Proc. Natl. Acad. Sci. USA* **83,** 3746-3750 (1986).