Thermodynamic control of small RNA-mediated gene silencing

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INTRODUCTION

Small RNA molecules, including small interfering RNA (siRNA) and microRNA (miRNA), are key regulators of posttranscriptional gene silencing referred to as RNA interference (RNAi) or RNA silencing. In RNAi, siRNA loaded onto the RNA-induced silencing complex (RISC) downregulates target gene expression by cleaving mRNA whose sequence is perfectly complementary to the siRNA guide strand. We previously showed that highly functional siRNAs possessed the following characteristics: A or U residues at nucleotide position 1 measured from the 5′ terminal, four to seven A/Us in positions 1–7, and G or C residues at position 19. This finding indicated that an RNA strand with a thermodynamically unstable 5′ terminal is easily retained in the RISC and functions as a guide strand. In addition, it is clear that unintended genes with complementarities only in the seed region (positions 2–8) are also downregulated by off-target effects. siRNA efficiency is mainly determined by the Watson–Crick base-pairing stability formed between the siRNA seed region and target mRNA. siRNAs with a low seed-target duplex melting temperature (Tm) have little or no seed-dependent off-target activity. Thus, important parts of the RNA silencing machinery may be regulated by nucleotide base-pairing thermodynamic stability. A mechanistic understanding of thermodynamic control may enable an efficient target gene-specific RNAi for functional genomics and safe therapeutic applications.

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FIGURE 1 | The mechanism of RNAi and its off-target effect in mammalian cells. An RNA strand with A or U at position 1, four to seven AUUs in positions 1–7 and G/C at position 19 (measured from the guide strand 5' end) are easily unwound from the 5' end and retained in the RISC. The passenger strand is dissociated from the Ago1-4-containing RISC following unwinding, but cleaved in the Ago2-containing RISC. The guide strand recognizes target and off-target transcripts with complementary sequences to seed region positions 2–8. The target transcript, which has complete complementarity in positions 9–21, in addition to positions 2–8, is knocked down by RNAi. Conversely, off-target transcripts are downregulated according to the thermodynamic stability in the duplex formed between the siRNA seed region and target mRNA.
guide strand (Figure 1). In addition, a GC stretch of no more than nine nucleotides occurs in class I siRNA sequences. Class III siRNAs have opposite features with respect to the first three conditions and cause the least RNAi-silencing effects. The remaining siRNAs belong to class II and are a mixture of functional and non-functional siRNAs.

We and others demonstrated that functional siRNA with an unstable RNA strand 5’ terminal in the siRNA duplex is functional as a guide strand (Amarzguioui and Prydz, 2004; Reynolds et al., 2004; Ui-Tei et al., 2004); A or U residues at the 5’ end of the guide strand are especially important. In RNAi, thermodynamic asymmetry is not essential for target gene silencing because the passenger strand of most double-stranded siRNAs loaded onto RISC are cleaved by catalytic activity of the Ago2 protein and degraded (Figure 1; Kawamata et al., 2009; Yoda et al., 2010). Thus, in this case, A/U nucleotide itself at 5’ terminal might be strongly contributed to the RNAi activity, as nucleotide monophosphates, AMP, and UMP, bind to Ago2 with up to 30-fold higher affinity than either CMP or GMP (Frank et al., 2010). However, when the siRNA duplex is loaded into other Ago proteins without slicer activity, siRNAs might be unwound into a single-strand from the thermodynamically unstable 5’ terminal as shown in miRNA-mediated gene silencing (Figure 1; Matranga et al., 2005; Miyoshi et al., 2005; Leuschner et al., 2006; Kawamata et al., 2009; Yoda et al., 2010). As off-target gene silencing is performed using both mechanisms for eliminating the passenger strand, siRNA thermodynamic asymmetry in addition to A/U nucleotide itself at the 5’ terminal might be involved in seed-dependent off-target effects.

**SEED-DEPENDENT OFF-TARGET EFFECT EFFICIENCY VARIES DEPENDING ON SEED SEQUENCE**

Accumulated evidence from large-scale knockdown experiments (Jackson et al., 2003, 2006a; Scacheri et al., 2004; Lin et al., 2005; Birmingham et al., 2006) suggests that siRNA can generate off-target effects through a mechanism similar to that of miRNA target silencing (Lewis et al., 2005; Lim et al., 2005; Grimson et al., 2007). The 3’UTRs of off-target transcripts or miRNA targets are complementary to the guide strand seed region (i.e., nucleotide positions 2–8; Figure 2; Lim et al., 2005; Lin et al., 2005; Birmingham et al., 2006; Jackson et al., 2006a). We determined the relationship between class I siRNA seed sequences and off-target effect using the expression reporter plasmid, psiCHECK, which encodes the Renilla luciferase gene. Three tandem repeats of seed-matched target sequences (Figure 3C) complementary to the entire seed-containing region (positions 1–8), but not to the remaining non-seed region (positions 9–21), were introduced into the region corresponding to the 3’UTR of the luciferase mRNA to generate psiCHECK-sm and used to determine the efficiency of the seed-dependent unintended off-target effect (see Figure 4A; Ui-Tei et al., 2008a). Although all siRNAs examined exhibited high activity for intended gene silencing at 50 nM, the off-target gene silencing calculated using psiCHECK-sm was much less effective and more susceptible to changes in siRNA concentration (Ui-Tei et al., 2008a). These findings indicated that variations in the efficiency of unintended off-target gene silencing were due to a difference in the interactions between the guide strand RNA entrapped in the RISC and mRNA.

**SEED-DEPENDENT OFF-TARGET EFFECT EFFICIENCY VARIES DEPENDING ON SEED REGION GC CONTENT**

Class I siRNA seed region GC content used in our previous study (Ui-Tei et al., 2008a) ranged from 0 to 57%. To further determine the relationship between seed region GC content exceeding 57% and off-targeting efficiency of the corresponding siRNA, six functional class II siRNAs with high GC content in the seed region were arbitrarily selected (Figure 3A), and their capability to exert off-target effects was examined using luciferase reporter assays (Figure 4; Ui-Tei et al., 2009). Note that two of the six class II siRNAs (siLuc-1063 and siLuc-1430) possessed a 100% GC content
in the seed region (Figure 3A). In contrast to class I siRNAs, which have little or no off-target effects, class II siRNAs were frequently associated with a considerable level of off-target gene silencing on the seed-matched targets (Figure 4). This apparent difference in the off-target effect may be due to differences in the GC content in the seed region between functional class I and II siRNAs.

SEED-DEPENDENT OFF-TARGET EFFECT IS DETERMINED BY THERMODYNAMIC STABILITY IN THE DUPLEX FORMED BETWEEN THE siRNA GUIDE STRAND SEED REGION AND TARGET mRNA

The results shown above indicated that siRNAs with high GC content in the seed sequence have strong seed-dependent off-target
FIGURE 4 | Concentration-dependent gene silencing effects of authentic siRNA of seed-matched targets. Both class I siRNAs and functional class II siRNAs were included. The gene silencing effects were examined using HeLa cells transfected with psiCHECK-sm plasmids containing various seed-matched targets. The relative luciferase (luc) activity in transfected HeLa cells was determined using a dual luciferase assay. (A) Authentic, non-modified siRNA psiCHECK-sm plasmid structures and gene silencing mechanism. Three tandem repeats of seed-matched target sequences were introduced into the region corresponding to the 3′UTR of the luciferase mRNA. In (B–R), the effects of non-modified siRNA transfection on seed-matched targets are shown. (B–L) class I siRNAs, (M–R) class II siRNAs. (B) siLuc-309, (C) siVIM-812, (D) siGRK4-934, (E) siOct-421, (F) siLuc-774, (G) siVIM-1128, (H) siLuc2-153, (I) siVIM-596, (J) siOct-797, (K) siVIM-1128, (L) siLuc-270, (M) siGRK4-189, (N) siLuc-1048, (O) siLuc-49, (P) siLuc-1048, (Q) siLuc-1063, (R) siLuc-1430. siRNA sequences and structures are shown in Figure 3A.
FIGURE 5 | The close relationship between the efficiency of seed-dependent off-target gene silencing and seed-target duplex thermodynamic stability. Both class I siRNAs and functional class II siRNAs (A–C) and class I chiRNAs and functional class II chiRNAs (D–F) were analyzed. Solid red circles and open red circles represent the class I and II siRNA data, respectively. Solid blue circles and open blue circles represent the class I and II chiRNA data, respectively. (A,D) The calculated $T_m$ of the seed-target duplex decreased with increasing standard free energy ($\Delta G$) for seed-target duplex formation (correlation coefficient: −0.98 and −0.91, respectively). (B,E) Luciferase activity (seed-dependent off-target gene silencing at a 50 nM siRNA concentration) was positively correlated with $\Delta G$ (correlation coefficient: 0.72 and 0.71, respectively). (C,F) The correlation between the seed-dependent gene silencing activity (luciferase activity) and the calculated $T_m$ of the seed-target duplex. Luciferase activity based on seed-dependent gene silencing with 50 nM siRNA was obtained from Figures 4 and 7, respectively. Seed-target duplex $\Delta G$ and $T_m$ were calculated using the nearest neighbor method. The relative luciferase activity and calculated $T_m$ were correlated with each other and had a coefficient of −0.76 and −0.79, respectively.
### Table 1 | Relative luciferase activities and \( T_m \), \( \Delta G \), and \( K_d \)s at seed regions of class I and II siRNAs.

|               | Luciferase activity (% at 50 nM) | Seed region GC number | \( T_m \) 2–8 (°C) | \( \Delta G \) 2–8 (kcal/mol) | \( K_d \) (M) |
|---------------|----------------------------------|-----------------------|---------------------|-------------------------------|--------------|
| **CLASS I**   |                                  |                       |                     |                               |              |
| siLuc-309     | 76                               | 0                     | −8.1                | −72                           | 5.3 \times 10^{-6} |
| siVIM-812     | 95                               | 1                     | 8.8                 | −9.3                          | 1.5 \times 10^{-7} |
| siGRK4-934    | 102                              | 1                     | 6.7                 | −9.7                          | 9.2 \times 10^{-8} |
| siOct-821     | 64                               | 2                     | 12.2                | −10                           | 2.8 \times 10^{-8} |
| siLuc-774     | 75                               | 2                     | 14.6                | −10                           | 4.7 \times 10^{-8} |
| siVIM-1128    | 79                               | 3                     | 21.2                | −12.8                         | 4.1 \times 10^{-10} |
| siLuc2-153    | 52                               | 3                     | 21.0                | −11.7                         | 2.6 \times 10^{-9} |
| siVIM-596     | 49                               | 3                     | 26.4                | −11.6                         | 3.1 \times 10^{-8} |
| siOct-797     | 29                               | 3                     | 25.7                | −12.4                         | 8.1 \times 10^{-10} |
| siVIM-270     | 25                               | 3                     | 26.2                | −12.2                         | 1.1 \times 10^{-9} |
| siLuc-36      | 49                               | 4                     | 28.4                | −13                           | 3.0 \times 10^{-10} |
| **CLASS II**  |                                  |                       |                     |                               |              |
| siGRK4-189    | 9                                | 4                     | 40.1                | −15.5                         | 4.3 \times 10^{-12} |
| siLuc-1120    | 44                               | 5                     | 42.3                | −16.7                         | 5.7 \times 10^{-13} |
| siLuc-49      | 6                                | 6                     | 46.3                | −17.6                         | 1.3 \times 10^{-13} |
| siLuc-1048    | 30                               | 6                     | 49.7                | −17.8                         | 8.9 \times 10^{-14} |
| siLuc-1063    | 18                               | 7                     | 54.5                | −18.6                         | 2.3 \times 10^{-14} |
| siLuc-1430    | 20                               | 7                     | 54.5                | −18.6                         | 2.3 \times 10^{-14} |

**FIGURE 6** Correlation between seed-dependent gene silencing activity of siRNA and chRNA and calculated \( T_m \) of the protein-free seed duplex. Gene silencing activity was measured using relative luciferase activity in HeLa cells transfected with psiCHECK-sm and cognate siRNAs or chRNAs at a 50 nM concentration, as shown in Figures 4 and 7. \( T_m \) of the protein-free seed region (positions 2–8) was determined using the nearest neighbor method. All possible 7-nt seed sequences (\( 4^7 = 16,384 \)) were ordered as a function of GC content and \( T_m \) values of their double-stranded counterparts with RNA (A) and DNA (B). Note that because of its definition, class I siRNA or chRNA cannot possess more than four GCs in the seed region. Open red (A) or blue (B) circles represent combinations of target and siRNA resulting in less than 50% relative luciferase activity. Solid red (A) or blue (B) circles represent combinations of target and siRNA with little or no off-target effect (luciferase activity >50%). The horizontal line at 21–25°C (A) or 28–41°C (B) may correspond to 50% luciferase activity reduction.

Effects. Thus, one possible efficiency regulator of the seed-dependent off-target effect might be the thermodynamic stability of the nucleotide duplex. The melting temperature (\( T_m \)) and standard free energy change (\( \Delta G \)) of the seed–target duplex formation are good measures of the thermodynamic stability of the protein-free seed–target duplex. In a previous experiment using class I siRNAs (Ui-Tei et al., 2008a), we verified a close linear relationship between \( \Delta G \) and \( T_m \) in seed region positions 2–8; a strong positive correlation between luciferase activity and \( \Delta G (r = 0.69) \), and a strong negative correlation between \( T_m \) and luciferase activity (\( r = −0.72 \)) was found. By replacing class I siRNAs with a mixture of class I and II siRNAs, the \( \Delta G \) range expanded from −13 and
Table 2 | Thermodynamic siRNA modification.

| Chemical modification | Nucleotide position | Modified base-pairing stability | Functional modification | Reference |
|-----------------------|--------------------|---------------------------------|-------------------------|-----------|
| LNA                   | The 5’ end of the passenger strand | Increase the stability at 5’ end of the passenger strand | Enhancement of selective RISC loading of the guide strand | Elmén et al. (2005) |
| 4'-Thionobonucleoside | Four residues on both ends of the passenger strand and 3’ end of the guide strand | Increase the stability at 3’ end of the guide strand | Reduction of the seed-dependent off-target effect | Jackson et al. (2006b) |
| 2-Thiouracil          | The 3’ end of the guide strand | Increase the stability at 3’ end of the guide strand | Increase the stability at 5’ end of the passenger strand | Sipa et al. (2007) |
| Dihydrouracil         | The 3’ end of the guide strand | Decrease the stability at 3’ end of the guide strand | The conformational alteration of RISC by the guide strand modification reduces the rate of RISC formation to dissociate off-target transcripts with weaker binding to the guide strands | Jackson et al. (2008b) |
| 2'-O-methyl           | Position 2 of the guide strand and positions 1 + 2 of the passenger strand | The conformational alteration of RISC by the guide strand modification reduces the rate of RISC formation to dissociate off-target transcripts with weaker binding to the guide strands | The conformational alteration of RISC by the guide strand modification reduces the rate of RISC formation to dissociate off-target transcripts with weaker binding to the guide strands | Sipa et al. (2007) |
| 2'-Deoxy (DNA)        | Positions 1–8 of the guide strand and positions 12–21 of the passenger strand | Decrease the stability in the seed region of the guide strand | Increase the stability at 3’ end of the passenger strand | Jackson et al. (2008b) |

Table 3 | Relative luciferase activities and Tm,s, ΔGs, and Kds at seed regions of class I and II chiRNAs.

| CLASS I | Luciferase activity (% at 50 nM) | Seed region GC number | Tm 2–8 (˚C) | ΔG 2–8 (kcal/mol) | Kd (M) |
|---------|----------------------------------|-----------------------|-------------|-------------------|-------|
| chiLuc-309 | 103          | 0                  | −12.2       | −4.9              | 2.6 × 10⁻⁴ |
| chiVIM-812   | 94           | 1                  | −0.5        | −5.5              | 9.3 × 10⁻⁵ |
| chiGRK4-934  | 95           | 1                  | 0.2         | −5.6              | 7.3 × 10⁻⁵ |
| chiOct-821   | 95           | 2                  | 4.7         | −7.1              | 6.2 × 10⁻⁶ |
| chiLuc-774   | 65           | 2                  | −4.8        | −6.8              | 1.0 × 10⁻⁶ |
| chiVIM-1128  | 79           | 3                  | 8.8         | −7.9              | 1.6 × 10⁻⁵ |
| chiLuc-2153  | 91           | 3                  | 5.4         | −7.8              | 1.9 × 10⁻⁵ |
| chiVIM-596   | 63           | 3                  | 8.8         | −7.8              | 1.9 × 10⁻⁵ |
| chiOct-797   | 84           | 3                  | 1.3         | −7.8              | 1.9 × 10⁻⁵ |
| chiVIM-270   | 78           | 3                  | 3.1         | −7.6              | 2.7 × 10⁻⁵ |
| chiLuc-36    | 68           | 4                  | 19          | −8.0              | 1.4 × 10⁻⁵ |

CLASS II

| CLASS II | Luciferase activity (% at 50 nM) | Seed region GC number | Tm 2–8 (˚C) | ΔG 2–8 (kcal/mol) | Kd (M) |
|----------|----------------------------------|-----------------------|-------------|-------------------|-------|
| chiGRK4-189 | 12           | 4                  | 28.1        | −9.6              | 9.2 × 10⁻⁸ |
| chiLuc-1120  | 69           | 5                  | 31.1        | −9.8              | 6.5 × 10⁻⁸ |
| chiLuc-49    | 31           | 6                  | 30.4        | −11.0             | 8.6 × 10⁻⁹ |
| chiLuc-1048  | 16           | 6                  | 45.2        | −9.7              | 7.7 × 10⁻⁸ |
| chiLuc-1063  | 58           | 7                  | 41.4        | −12.0             | 1.6 × 10⁻⁹ |
| chiLuc-1430  | 53           | 7                  | 35          | −12.0             | 1.6 × 10⁻⁹ |

−7 to between −19 and −7 kcal/mol (Figures 5A,B), while the Tm range expanded from −8 and 28˚C to −8 and 55˚C (Figures 5A,C). Correlation coefficients between luciferase activity and ΔG or Tm were 0.72 or −0.76, respectively, indicating a close relationship between the seed-dependent off-target effect and the seed duplex ΔG and Tm. The linear relationships among these parameters were almost invariant (Figure 5A). The dissociation constant (Kd) of the 17 siRNAs calculated using the formula ΔG = −RTln(Kd) indicated that the highest Kd was more than 10⁸ times greater than the lowest one (Table 1). 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 formed between the seed region of the siRNA guide strand and its mRNA counterpart. In Figure 6A, all possible 7-nt seed sequences (4⁷ = 16,384) were ordered as a function of GC content and Tm values of their double-stranded counterparts, and the siRNAs were plotted against the absence or presence of off-target effects. The data suggest that Tm values between 21 and 25˚C serve as a Tm boundary, which may discriminate off-target-free seed sequences from off-target-positive ones. Approximately 22% of 7-nt sequences had Tm values under 21˚C, indicating that limited seed sequences
FIGURE 7 | Concentration-dependent gene silencing effects of DNA-seed-containing chiRNA for seed-matched targets. Both class I chiRNAs and functional class II chiRNAs were included. The gene silencing effects were examined using HeLa cells transfected with psiCHECK-sm plasmids containing various seed-matched targets. The relative luciferase (luc) activity in transfected HeLa cells was determined using a dual luciferase assay. (A) chiRNA psiCHECK-sm plasmid structures and gene silencing mechanism. Three tandem repeats of seed-matched target sequences were introduced into the region corresponding to the 3’ UTR of the luciferase mRNA. In (B–R), the effects of chiRNAs transfection on seed-matched targets are shown. (B–L) class I chiRNAs, (M–R) class II chiRNAs. (B) chiLuc-309, (C) chiVIM-812, (D) chiGRK4-934, (E) chiOct-821, (F) chiLuc-774, (G) chiVIM-1128, (H) chiLuc-791, (I) chiVIM-1128, (J) chiLuc-2153, (K) chiVIM-596, (L) chiLuc-36, (M) chiGRK4-189, (N) chiLuc-1120, (O) chiLuc-1120, (P) chiLuc-49, (Q) chiLuc-1048, (R) chiLuc-1063. chiRNA sequences and structures are shown in Figure 3B.
are available for selecting siRNAs with reduced off-target effects.

**THERMODYNAMIC CONTROL OF RNA STRAND INCORPORATION INTO THE RISC BY CHEMICAL MODIFICATIONS**

RNA strand incorporation into the RISC is determined by siRNA duplex thermodynamics. The RNA strand with lowest binding stability in the 5′ end of the guide strand is preferentially incorporated into the RISC. Thus, rational chemical modifications can be used to improve selective guide strand loading into the RISC (Table 2).

High-affinity modifications [e.g., locked nucleic acid (LNA)] at the 5′ end of the passenger strand increase selective loading of the guide strand (Elmén et al., 2005). In addition, base modifications of a high-affinity 2-thiouracil base at the 3′ end of the guide strand and a low-affinity dihydouracil base at the 3′ end of the passenger strand can be used to the same effect (Sipa et al., 2007). Furthermore, a moderately active siRNA duplex is significantly improved by modifying the high-affinity 4′-thioribonucleoside (Hoshika et al., 2007). Similarly, other modifications, such as high-affinity 5-methyluracil and 5-methylcytosine modifications (Terrazas and Kool, 2009), or low-affinity 2,4-difluorotoluene and 5-nitroindole modifications (Addepalli et al., 2010), may also control the efficiency of RISC loading.

**ELIMINATION OF SEED-DEPENDENT OFF-TARGET EFFECT BY CHEMICAL MODIFICATIONS**

The seed-dependent off-target effect is also eliminated by chemical modifications (Table 2). 2′-O-methyl modification of the guide strand seed region alters the RISC conformation and reduces seed-dependent off-target effects by dissociating off-target transcripts with weak binding to the guide strand (Jackson et al., 2006b). Low-affinity dihydouracil base, 2,4-difluorotoluene, or 5-nitroindole modifications in the seed region may also reduce the seed-dependent off-target effects. Furthermore, we revealed that 2′-deoxy modification (DNA replacement) of nucleotides 1–8 in the guide strand and 12–21 in the passenger strand (DNA:RNA chimeric siRNA, chiRNA; Figure 3B) reduces thermodynamic stability in the seed-target duplex, and almost completely eliminates off-target effects with little or no loss of target gene silencing activity (Ui-Tei et al., 2008b). 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 of the passenger strand. As shown in Figure 7, most functional class II siRNAs could not effectively eliminate the off-target effects by DNA replacement in the seed region (Ui-Tei et al., 2009). We examined the relationship between the relative luciferase activity and the ΔG or T_m of the DNA:RNA seed duplex in 11 class I and six class II chiRNAs (Figure 5D–F). We verified a close linear relationship between ΔG and T_m in the seed region (Figure 5D), a strong positive correlation between luciferase activity and ΔG (r = 0.71) and a strong negative correlation between T_m and luciferase activity (r = −0.79), irrespective of the presence or absence of DNA replacement in the seed region (Figure 5E,F). However, DNA replacement increased ΔG and reduced both the seed-target duplex T_m and luciferase activity considerably. T_m was reduced to less than −20°C in all the class I chiRNAs, while the relative luciferase activity at 50 nM exceeded 60%, the minimum relative luciferase activity necessary for a practical off-target effect. In contrast, the relative luciferase activity at 50 nM was 30% or less in three of six cases treated with functional class II chiRNAs, even though the seed-target T_m was reduced; this demonstrates a strong negative correlation with ΔG for seed-target duplex formation (Figure 5D). Therefore, it appears that the reduced off-target effect in chiRNA-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 Tables 1 and 3, DNA replacement throughout the guide
strand seed region is roughly equivalent to a 13°C reduction in $T_m$, a 5 kcal/mol increment in $\Delta G$, and about two to three G/C → A/U changes in the seed duplex. In Figure 6B, $T_m$ values of all possible 7-nt DNA:RNA hybrids are ordered and plotted against siRNAs with or without off-target silencing activity. For DNA:RNA hybrids, 28–41°C might be a boundary line that discriminates off-target-free from off-target-positive seed sequences. However, this boundary had higher $T_m$ values, as compared to those of RNA duplexes shown in Figure 6A. This might be partially due to different parameters used in calculating $T_m$ values of RNA duplexes and DNA:RNA hybrids. The proportion of 7-nt DNA:RNA hybrids with $T_m$ values under 28°C was about 88%, indicating that most 7-nt sequences are available for off-target effect-reduced RNA silencing by replacing RNA with DNA in the seed region.

**GENOME-WIDE ANALYSIS REVEALED THAT siRNA WITH LOW STABILITY IN THE SEED-TARGET DUPLEX IS CAPABLE OF INDUCING TARGET GENE-SPECIFIC SILENCING**

The hypothesis that off-target gene silencing is determined primarily by seed-target duplex stability was apparent in genome-wide expression profiling using class I siRNA with high or low seed $T_m$ value (Figure 8). The reporter assay described above predicted that siRNA with high seed $T_m$ value would be good inducer, while that with low seed $T_m$ value would be poor inducer of the off-target effect.

As anticipated, both siRNAs effectively reduced the amount of intended RNAi (red arrows in Figure 8). In contrast, a high level of off-target effects was evident in the case of transfection with siRNA with high seed $T_m$ value. Conversely, transfection with siRNA with low seed $T_m$ value exhibited little off-target effect. Thus, it was concluded that the level of off-target gene silencing is determined by the thermodynamic stability of the seed duplex formed between the siRNA guide strand and the target mRNA.

**CONCLUSION**

In this review, we demonstrated that siRNA seed-dependent off-target effect efficiency is controlled by thermodynamic properties of the nucleotide duplex. This conclusion was drawn from the following: (1) The functional siRNA duplex is asymmetric in its terminal nucleotide base-pairing. An RNA strand with an unstable 5’ terminal is effective as a guide strand, probably because it is easily retained in the RISC. (2) The siRNA off-target effect efficacy can be determined by seed region nucleotide duplex thermodynamic properties. The seed-dependent off-target effect efficiency is positively and negatively correlated with $\Delta G$ and $T_m$ in seed region positions 2–8. Thus, small RNA-mediated gene silencing is partly regulated by nucleotide base-pairing thermodynamic stability.

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