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Identification of sequence features that predict competition potency of siRNAs

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ABSTRACT

Small interfering RNAs (siRNAs) specifically knock-down target mRNAs via RNA interference (RNAi) mechanism. During this process, introduction of excess amount of exogenous siRNAs could lead to the saturation of cellular RNAi machinery. One consequence of RNAi machinery saturation is the competition between two simultaneously introduced siRNAs, during which one siRNA loses gene silencing activity. Although competition phenomena have been well characterized, the molecular and sequence features of siRNAs that specify the competition potency remain poorly understood. Here, for the first time, we performed a large-scale siRNA competition potency analysis by measuring the competition potency of 56 different siRNAs and ranking them based on their competition potency. We have also established an algorithm to predict the competition potency of siRNAs based upon the conserved sequence features of strong and weak competitor siRNAs. The present study supports our hypothesis that the competition potency of siRNAs is specified by the 5′-half antisense sequence and provides a useful guideline to design siRNAs with minimal RNAi machinery saturation.

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1. Introduction

Small interfering RNAs (siRNAs) are short (19–21 bp), double-stranded RNAs (dsRNAs) which trigger sequence-specific mRNA degradation via the endogenous RNA interference (RNAi) pathway [1,2]. Because of the high gene silencing activity and simple strategy, siRNA-mediated gene silencing has become a method of choice for validating target genes in functional genomics field, and is currently being developed as therapeutics toward several diseases including viral infection and cancer [3,4].

However, several studies have demonstrated unexpected non-specific effects triggered by siRNAs such as off-target gene silencing [5,6], activation of innate immune responses [7–12], and saturation of RNAi machinery by excess amounts of exogenously introduced siRNAs [13,14] or short hairpin RNAs (shRNAs) [15–17]. To develop siRNAs as a specific gene knock-down tool and a therapeutic agent without unwanted side effects, it is imperative to understand the mechanism of non-specific effects triggered by siRNAs and devise ways to circumvent them.

One phenomenon observed due to the saturation of endogenous RNAi machinery by exogenously introduced siRNAs is the competition between different siRNAs. Several studies reported that when the mixture of two siRNAs is co-transfected, gene silencing activity of one siRNA is often reduced, whereas the other siRNA functions normally [13,14,18–26]. Understanding the competition potency of siRNA is important to achieve successful multiple siRNA treatments for pathway analysis or antiviral therapeutic strategy. More recently, bioinformatic analysis revealed that exogenously introduced siRNAs could perturb cellular microRNA (miRNA) function, resulting in non-specific gene expression pattern [27]. This report further underscores the importance of using siRNAs with minimal saturation of the RNAi machinery.

Different siRNAs show different levels of competition potency, i.e., the ability to saturate the RNAi machinery. However, the exact mechanism or the sequence feature of siRNA affecting competition potency remains poorly understood. While the protein component of RNAi machinery saturation was suggested to be Argonaute-2 (Ago2) [22], it is unclear which features of siRNAs dictate strong vs. weak competition potency. There have been conflicting reports regarding correlation between gene silencing efficiency and competition potency of siRNAs [21,22]. In our previous study, we have shown the lack of correlation between gene silencing efficiency and competition potency of siRNAs [25]. We also found that the competition potency of siRNA was specified by the 5′-half sequence of the guide strand [25]. A recent study suggested the RISC loading efficiency of siRNAs as a major determinant of competition potency [26]. All these studies tested only a few siRNAs, so that the generalization of the claims is rather limited.

In this study, we present, for the first time, a large-scale analysis of siRNA competition potency. A total of 56 siRNAs was tested and

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ranked based upon their competition potency, which resulted in successful separation of siRNAs with strong or weak competition potency. Based on these results, we were able to identify the sequence features of both strong and weak competitor siRNAs. We also show that the RISC loading efficiency, predicted by the thermodynamic stability of the siRNA ends, does not correlate with the competition potency.

2. Materials and methods

2.1. Cell culture

A HeLa cell line was purchased from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% fetal bovine serum and penicillin/streptomycin (100 U/ml penicillin and 100 μg/ml streptomycin), and cultured at 37 °C with 5% CO_2. Cells were regularly subcultured to maintain exponential growth.

2.2. Preparation of siRNA or antisense oligonucleotide (ASO)

siRNA oligonucleotides were synthesized by Bioneer (Daejeon, Korea). Sense and antisense RNA oligonucleotides were mixed at a final concentration of 100 μM, denatured at 90 °C for 2 min, and then annealed at 37 °C for 1 h. Sequences of siRNA used were described in Table S2. Ago2-specific ASO was synthesized as previously described [24]. The ASO sequence was **CTGCT**GGAATGTTTC **CACTT**, with fully phosphorothioated backbones. 2′-*O*-Methyl modified nucleotides were marked in bold characters.

2.3. Transfection of siRNA or ASO

One day before transfection, HeLa cells were plated in 12-well plates with complete medium without antibiotics and were incubated for 24 h at 37 °C until they reached 50% confluency. For siRNA competition assay, cells were transfected with siCREB3 either alone or in combination with competitor siRNAs using lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. For the co-transfection of ASO with siRNAs, siRNAs with indicated concentrations were mixed with final 25 nM of ASO which were subsequently reverse-transfected into the HeLa cells using G-fectin as manufacturer's instruction (Genolution Pharmaceuticals). After 48 h incubation at 37 °C, the transfected cells were harvested, RNA was isolated, and reverse transcription-polymerase chain reaction (RT-PCR) was performed. For Western blot, a final concentration of 25 nM Ago2-targeting ASO was reverse-transfected to 10^6 HeLa cells using G-fectin. After 48 h incubation, cells were washed, lysed, and then Western blot was performed.

2.4. Quantitative real-time PCR

Cells were harvested 48 h after transfection, and total RNA was extracted using Trizol (Ambion) according to the manufacturer's instructions. Five hundred nanograms of total RNA was used for cDNA synthesis using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and an oligo-(dT) 15 primer. Aliquots (1/10) of the cDNA reaction mixture were analyzed by quantitative real-time PCR on a StepOne™ Real-Time PCR Systems (Applied Biosystems) according to the manufacturer's protocol. Primer sequences for the PCRs were: CREB3 forward, 5′-TTC TGA GGT ACC GAG CGA CT-3′ and reverse, 5′-GGA GGG AGT AGG TGT GGT CA-3′; MAVS forward, 5′-GGG TAA ACA GGG TGC AGA GA-3′ and reverse, 5′-ACA GGC ATC AAG GTG GTA GG-3′; GAPDH forward, 5′-GAG TCA ACG GAT TTG GTC GT-3′ and reverse, 5′-GAC AAG CTT CCC GTT CTC AG-3′.

2.5. Measurement of siRNA base frequency

siRNA base frequency was calculated by web-based logo program (WebLogo3 ver. 2.8.2) [28]. Sequences of total, strong, or weak competitors were uploaded in FASTA format and frequency plot was presented (Fig. 2).

2.6. Measurement of thermodynamic asymmetry of siRNAs

The first 4 nts from the 5′-end of both antisense and sense strand of siRNAs were used for the calculation of thermodynamic asymmetry of siRNAs to determine the strand loading bias [26]. The 5′-end thermodynamic stability difference between antisense and sense strands were indicated as positive or negative scores.

2.7. Western blot

Forty-eight hours after transfection, cells were washed twice with 1× PBS, lysed with RIPA buffer (50 mM Tris–Cl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 2 mM EDTA). Protein concentration was measured by BCA method. Thirty micrograms of total lysate was mixed with 5× sample buffer and loaded to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Samples were electrophoretically transferred onto PVDF membrane (Immune-Blot™ PVDF membrane, Bio-Rad). Proteins of interest were detected with specific antibodies as indicated (1:2000 dilution), and then visualized with an enhanced chemiluminescence (ECL plus) reagent (Amersham Bioscience). Mouse monoclonal anti-Ago2 antibody (ab57113) was purchased from Abcam.

3. Results and discussion

3.1. The position of antisense 5′-half sequence is essential for the competition potency

Competition phenomenon is dependent upon the amount of siRNA taken up by the cells. To identify siRNA concentration optimal for competition study, we first performed competition experiments using siCREB3, a CREB3-targeting siRNA which was identified as a weak competitor from our previous study, and siMAVS, a strong competitor siRNA, with varying siRNA concentrations (1, 3, and 10 nM). We found that the maximal competition between siRNAs was observed at 10 nM siRNA concentration, and reducing the siRNA concentration to 3 and 1 nM reduced the extent of competition (Fig. S1). Therefore, for our purpose of identifying strong and weak competitors, we decided to use 10 nM siRNA concentration to maximize the extent of competition. Another important point to consider is that, at this siRNA concentration, the competitor siRNA should not interfere with normal CREB3 mRNA level when transfected alone. As shown in Fig. S1, at all tested concentration range, the siMAVS treatment did not significantly affect CREB3 mRNA level. Therefore, we conclude that the competition phenomenon we observe is a specific effect of RNAi machinery saturation.

In our previous study, we demonstrated that the competition potency of siRNA was dependent on antisense 5′-half sequence of siRNAs, by testing the competition potency of chimeric siRNAs with antisense 5′-half sequences from the strong (or weak) competitors and antisense 3′-half sequences from the weak (or strong) competitors [25]. Further with these observations, we wondered whether the position of antisense 5′-half sequence of siRNAs matters to dictate the competition potency. To test this hypothesis, we generated another chimeric siRNAs, such as siMyDB and siMyTI, in which the antisense 5′-half sequences of strong competitors, such

as siTIG3 and siDBP, were directly attached to the 3′-end of the antisense 5′-half sequences of a weak competitor, siMyD88. These chimeric siRNAs were co-transfected into the cells with the siCREB3 (Fig. S2A). The gene silencing efficiency of siCREB3 was only slightly affected by siMyD88, siMyDB, and siMyTI, whereas siDBP and siTIG3 significantly inhibited siCREB3 gene silencing efficiency (Fig. S2B). The data further support our previous observation that the position of antisense 5′-half sequence is important for dictating the competition potency of siRNAs.

3.2. Identification of the consensus sequence which specifies competition potency of siRNAs

Previous studies failed to identify the general feature of siRNAs which dictates the competition potency of siRNAs, because they tested only a few siRNAs in their studies. Based on our finding that the position and the sequence of the 5′-half antisense are important for siRNA competition potency, here we conducted, for the first time, a large-scale analysis of siRNA competition potency. For this, we randomly chose 56 different siRNAs targeting various target mRNAs. These siRNAs (10 or 50 nM, respectively) were co-transfected into cells with siCREB3 (10 nM), and the competition potency of each siRNA was measured (Fig. 1A and B). A wide range of competition potency was observed for different siRNA competitors. The inhibition of siCREB3 gene silencing efficiency by competitor siRNAs ranged minimum 1.06-fold to maximum 5.58-fold in the case of 10 nM competitor siRNAs, or minimum 1.83-fold to maximum 7.35-fold in the case of 50 nM competitor siRNAs, compared with control transfected with siCREB3 only (Fig. 1A and B). We then calculated the median value of competition potency and used this value to separate strong and weak competitors. Median values of 2.26 (10 nM competitor siRNAs) and 3.93 (50 nM competitor siRNAs) were obtained compared with siCREB3 only transfected control, respectively. By comparing the competition potency of each individual siRNA with the median values, we selected 14 strong competitor siRNAs and 20 weak competitor siRNAs (Table S1).

We then aligned and graphically represented the sequence frequencies on each position by using total siRNAs, strong competitor siRNAs, and weak competitor siRNAs, respectively [28] (Fig. 2). From this analysis, we found an over-represented sequence feature specific to the strong competitor siRNAs, (U/A)ACAGANGN(A/C)NNNNNNCNNNN. Interestingly, the alignment of antisense 5′-half of siRNA showed a rather prominent consensus sequence compared with the antisense 3′-half sequence, which well correlates with our previous results that the antisense 5′-half sequence dictates competition potency of siRNAs [25].

To confirm whether the consensus sequence derived from the strong competitor siRNAs is indeed relevant to the strong compe-

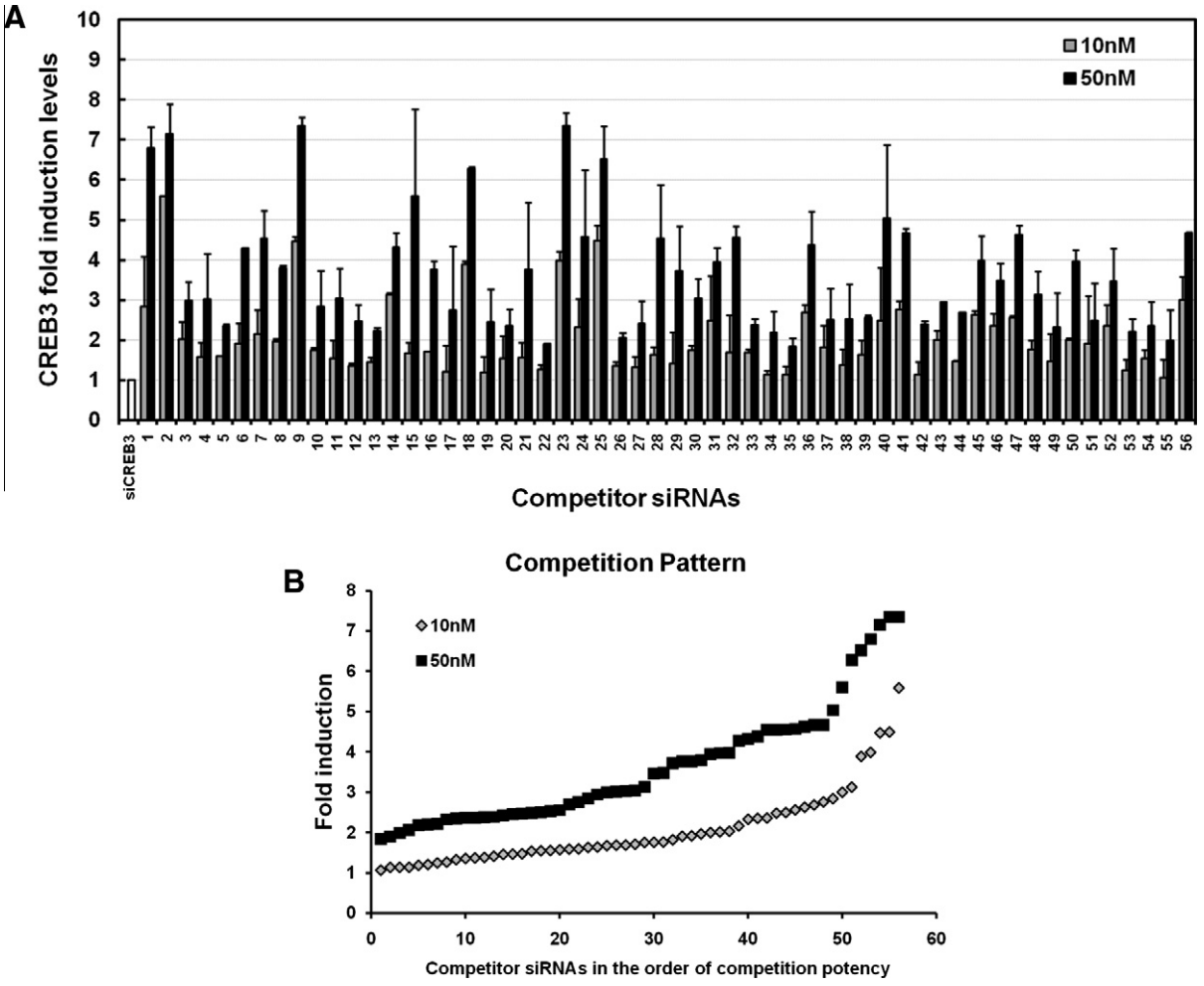

Fig. 1. Measurements of competition potency of various siRNAs. (A) Endogenous CREB3 mRNA levels that were regulated by either siCREB3 only control or siCREB3 with a variety of competitor siRNAs were measured by quantitative RT-PCR. Ten nanomolars (gray bar) or 50 nM (black bar) of competitor siRNAs was mixed with 10 nM of the weak competitor siRNA (siCREB3) and transfected into the HeLa cells. Values shown on the *y*-axis are means + standard deviations (SD) from at least two experiments. (B) Each 10 nM (gray spot) and 50 nM (black spot) of competitors was arranged in regular sequence.

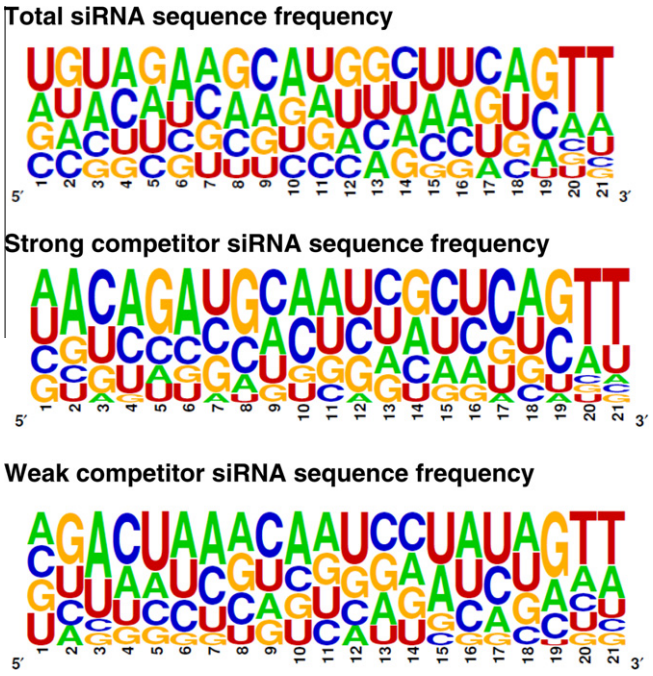

Fig. 2. Base frequencies of tested total, strong, and weak competitor siRNAs. The guide sequences of total, strong, or weak competitor siRNAs were represented by WebLogo, the web-based sequence alignment program http://weblogo.berkeley.edu/. The *x*-axis represents the identities of antisense 5′–3′ sequences of siRNAs while the values on the *y*-axis are the frequencies of nucleotide appearance.

tition potency, we synthesized a siRNA sequence based upon the most over-represented sequence from the sequence alignment. As expected, the newly designed siRNA based upon the consensus sequence from strong competitor siRNAs strongly inhibited the gene silencing efficiency of siCREB3 (Fig. 3).

Next, we generated a scoring matrix for antisense 5′-half sequences for strong competitor siRNAs and weak competitor siRNAs, respectively (Table 1A). For strong competitor siRNAs, the base frequencies of each position within the 11 nts from the 5′-antisense strand of 14 strong competitor siRNAs were calculated and used to generate the scoring matrix (Table 1A). The scoring matrix for weak competitor siRNAs was generated similarly, using the sequences of 20 weak competitor siRNAs (Table 1A). To verify this scoring method, we analyzed the scores of selected strong and weak competitor siRNAs using Table 1A. Sequences and scores of the selected siRNAs are shown in Table 1B. We found that 86% of strong competitor siRNAs (12/14) scored higher using strong competitor scoring matrix than weak competitor scoring matrix, whereas 85% of weak competitor siRNAs (17/20) scored higher using weak competitor scoring matrix than strong competitor scoring matrix. This result further supports the idea that specific nucleotide sequences within the antisense 5′-half of siRNAs determines competition potency. In addition, the scoring matrix shown in Table 1A could be used as a useful guide to design siRNAs with minimal RNAi machinery saturation.

Within the consensus sequence derived from the antisense 5′-half sequences of strong competitor siRNAs, the emergence of A/U and A as the over-represented sequence of the 1st and 2nd nucleotide of the strong competitor siRNAs may support the recent suggestion that the thermodynamic stability of the antisense 5′-end determines the RISC loading efficacy of siRNAs, which in turn specifies the competition potency [26,29]. To determine the correlation of competition potency with the 5′-end thermodynamic asymmetry of siRNAs, we calculated the asymmetry score of all the siRNAs tested by comparing the thermodynamic stability of the first 4 nts of two ends of siRNA duplex as described [26]. As shown in Fig. S3, there was no correlation between the competition potency and thermodynamic asymmetry of siRNAs (R^2 = 0.0281). These results suggest that, contrary to the previous report, the thermodynamic asymmetry may not be used as a major guideline to accurately predict the siRNA competition potency.

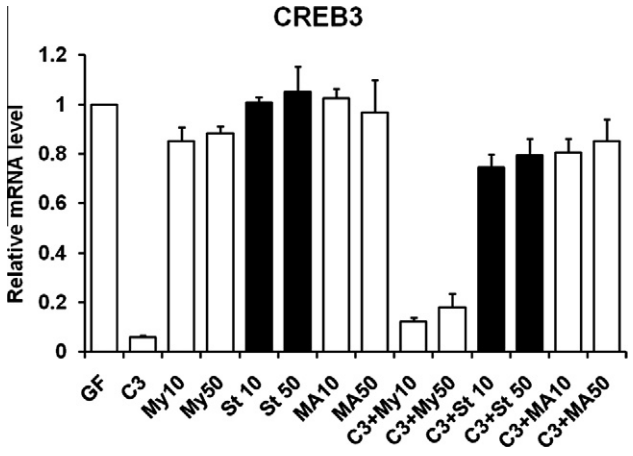

Fig. 3. Competition potency of an artificial strong competitor siRNA. Competition between siCREB3 with siMyD88 (a validated weak competitor), siSt (a predicted strong competitor sequence from the sequence alignment result), or siMAVS (a validated strong competitor), was measured by quantitative RT-PCR with indicated concentration. Values shown on the *y*-axis are means + standard deviations (SD) of at least three independent experiments. GF; transfection reagent only, C3; siCREB3, My10; siMyD88 10 nM, My50; siMyD88 50 nM, St10; siSt 10 nM, St50; siSt 50 nM, MA10; siMAVS 10 nM, MA50; siMAVS 50 nM, C3 + My10; siCREB3 + siMyD88 10 nM, C3 + My50; siCREB3 + siMyD88 50 nM, C3 + St10; siCREB3 + siSt 10 nM, C3 + St50; siCREB3 + siSt 50 nM, C3 + MA10; siCREB3 + siMAVS 10 nM, C3 + MA50; siCREB3 + siMAVS 50 nM.

3.3. Competition potency of siRNAs correlates with Ago2 dependency

A previous study suggested that the RISC component which is the target of the siRNA competition phenomenon might be Ago2, as ASO-mediated reduction of the Ago2 level resulted in the enhanced competition [24]. We hypothesized that, if Ago2 is indeed the machinery saturated by siRNAs, the gene silencing efficiency of weak competitor might be sensitive to the cellular Ago2 protein level, whereas that of strong competitor might be relatively insensitive. To test this hypothesis, we co-transfected an Ago2-targeting ASO along with a weak competitor siRNA, siCREB3, or a strong competitor siRNA, siMAVS, respectively. The Ago2 ASO treatment resulted in ~50% reduction of both Ago2 mRNA and protein levels (Fig. S4B). Forty-eight hours after transfection, endogenous CREB3 or MAVS mRNA levels were observed. As expected, we found that the gene silencing efficiency of a weak competitor, siCREB3, was

Table 1A
Scoring matrix for calculating nucleotide frequency of competitor siRNAs.

	Antisense strand 5′-nucleotide position										
	1	2	3	4	5	6	7	8	9	10	11
Strong competitor scoring											
A	0.29	0.50	0.07	0.43	0.14	0.50	0.07	0.14	0.29	0.36	0.36
C	0.21	0.14	0.43	0.29	0.21	0.21	0.29	0.29	0.36	0.36	0.14
G	0.21	0.21	0.21	0.07	0.50	0.14	0.29	0.50	0.14	0.14	0.21
U	0.29	0.14	0.29	0.21	0.14	0.14	0.36	0.07	0.21	0.14	0.29
Weak competitor scoring											
A	0.25	0.15	0.5	0.25	0.2	0.35	0.4	0.3	0.2	0.4	0.3
C	0.25	0.2	0.1	0.4	0.2	0.25	0.3	0.25	0.35	0.2	0.2
G	0.25	0.4	0.1	0.15	0.15	0.1	0.1	0.3	0.2	0.2	0.25
U	0.25	0.25	0.3	0.2	0.45	0.3	0.2	0.15	0.25	0.2	0.25

Table 1B
Sequences and scores of sorted competitors.

Strong competitor score[b]				Weak competitor score[b]			
siRNA No.	Sequence (As)[a]	Strong	Weak	siRNA No.	Sequence (As)	Strong	Weak
1	5′-**CAG CAU GGC CG**C CGC CAU CTT-3′	3.21	2.6	3	5′-**AUG GAU AGU GA**U GGA AUC GTT-3′	2.50	2.70
2	5′-**UUC ACA GGC AU**C AAG GUG GTT-3′	3.8	2.8	4	5′-**UCU CUA ACU AG**A AGC UUG UTT-3′	2.79	3.50
7	5′-**AAG UCA CGA AG**C CUA CUC GTT-3′	3.57	2.7	5	5′-**AGU CCA GAG UA**U CCA AGA CTT-3′	2.86	2.65
9	5′-**AAC AGA CCC UA**G ACA UCU GTT-3′	4.09	2.65	10	5′-**AGA AAU GGA GC**C ACU ACU GTT-3′	2.64	2.90
14	5′-**UCU AGA GGA AA**U GGC UCU GTT-3′	3.95	2.8	11	5′-**AUU AUU CUU GU**G UGA AAA G UC-3′	2.43	2.95
18	5′-**AGU CAC UAU AG**G UCU CCU UTT-3′	2.71	3.2	13	5′-**GGA CAC CAG UU**C AGA AAA G GA-3′	2.14	3.25
24	5′-**CAU UCC UCC GA**U CGC ACA C AU-3′	3.14	2.65	17	5′-**GAU AGU UUG UC**U GUU CCA GTT-3′	2.93	2.35
25	5′-**GCU GGU CGU GU**G UCC ACC U CA-3′	2.78	2.65	20	5′-**CGA GGU AGU AG**U CUA AGU ATT-3′	2.57	3.35
31	5′-**UAG AGA ACG CC**U GAG ACA GTT-3′	3.43	2.5	26	5′-**UCU UUA AGC GA**U UAC UCA GTT-3′	3.00	3.30
40	5′-**GGC AGG UCC CU**C UGU UGA C GC-3′	3.58	2.5	30	5′-**UGA UUA UCA CU**U GCU CUG AUU TT-3′	3.00	3.25
41	5′-**UUC CUA UGA CU**U CAU UGA A AU-3′	3.59	2.95	33	5′-**UGA AGC UGC CA**U CAU CCU GTT-3′	3.64	3.15
45	5′-**GGA CGG UUG AA**G AAA UUG C UU-3′	2.71	3.05	34	5′-**CAA GCA ACC AU**G GCU UUC GUCTT-3′	2.93	3.25
47	5′-**AAC AUC CAA UC**U GUC CCG GTT-3′	3	2.65	37	5′-**UGC UCC ACA AU**C ACU UCU GGA-3′	2.86	2.90
56	5′-**CAC UGA GGU CA**A UGU GGA C AG-3′	4.07	2.35	43	5′-**GUG AUA ACC AG**U GUG UAG C CA-3′	2.93	3.30
				48	5′-**CAA CCG CAA AG**A AGG GUU GCG UUU A-3′	2.71	3.05
				49	5′-**CUU UUU CAG CC**A CAG GAA A AA-3′	2.21	2.95
				51	5′-**AGA CAA CAC CA**G CAA AUC A AU-3′	3.00	3.55
				53	5′-**GCC CUC CAC UC**G GCU CCG C AU-3′	2.50	3.00
				54	5’-**GUA CUC AGC AA**C CUU CUG A AG-3’	2.71	3.85
				55	5’-**CCA CUG UUU AG**G UAA UUU C CA-3’	2.21	3.15

[a] 11 nt of 5′-antisense strand of siRNAs are marked in bold characters.
[b] Calculated scores from Table 1A are indicated.

significantly reduced by the Ago2 ASO treatment, whereas that of siMAVS, a strong competitor, was not affected (Fig. S4A). The data suggest that the siRNA competition potency correlates with the Ago2 dependency, and supports the previous proposal that the interaction with Ago2 is a major mechanistic point in RNAi machinery saturation by siRNAs.

Although a number of studies have reported the competition phenomenon, which is a consequence of endogenous RNAi machinery saturation, the molecular feature of siRNAs which specifies the competition potency is still poorly understood. Previously, using a limited set of siRNAs, we found that the 5′-half sequence of antisense strand is critical for determining competition potency of siRNAs [25]. In this study, we performed the first large-scale analysis of siRNA competition potency to verify our previous conclusion, as well as to identify sequence features that specify competition potency of siRNAs. Contrary to a recent report, there was no correlation between the thermodynamic asymmetry of siRNA ends and competition potency. We also found that the competition potency correlated with the Ago2 dependency of siRNAs, supporting the previous report that Ago2 is the main target molecule for RNAi machinery saturation [14,22,24–26].

Taking these findings together, we propose a model for siRNA competition, i.e., RNAi machinery saturation by siRNAs. We suggest that the key feature of siRNA competition phenomenon is the interaction between antisense 5′-end sequence of siRNAs and Ago2, the catalytic component of RISC machinery. In this model, a strong competitor sequence might have higher binding affinity to Ago2, which would result in an increased occupancy of RISC complex. Further biochemical and structural analysis will clearly reveal the molecular mechanism of RNAi machinery saturation by siRNAs, which will help researchers to design siRNAs not only suitable for gene silencing with minimal non-specific effects but also optimal for combinatorial RNAi strategies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.06.041.

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