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Competition potency of siRNA is specified by the 5′-half sequence of the guide strand

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Abstract

Small-interfering RNAs (siRNAs) execute specific cellular gene silencing by exploiting the endogenous RNA interference (RNAi) pathway. Therefore, excess amounts of siRNAs can saturate cellular RNAi machineries. Indeed, some siRNAs saturate the RNA-induced silencing complex (RISC) and competitively inhibit silencing by other siRNAs. However, the molecular feature of siRNAs that specifies competition potency has been undetermined. While previous reports suggested a correlation between the competition potency and silencing efficiency of siRNAs, we found that the silencing efficiency was insufficient to explain the competition potency. Instead, we show that the nucleotide sequence of the 5′-half of the guide strand determines the competition potency of an siRNA. Our finding provides important information for understanding the mechanistic basis of competition in combinatorial RNAi treatment.

Keywords: RNA interference; siRNA; Competition; Guide strand

Small interfering RNAs (siRNAs) are short (usually 19–21 base pairs), double-stranded RNAs (dsRNAs) that can catalyze the destruction of cellular mRNAs in a sequence-specific manner [1]. The degradation of mRNA molecules by siRNAs is mediated by the endogenous RNA interference (RNAi) pathway, which is present in most cell types [2]. To overcome the transient nature of siRNA-mediated gene inhibition and achieve sustained RNAi, short hairpin RNAs (shRNAs) have been developed [3,4]. Like endogenous microRNAs (miRNAs), shRNAs can be stably expressed in the nucleus as hairpin precursors, exported to the cytoplasm by Exportin-5 (Exp5), and then processed by Dicer to enter the RNA-induced silencing complex (RISC), which participates in the targeted destruction of mRNAs [5].

Because of its high efficiency in gene silencing and its success rate, RNAi-mediated gene silencing has quickly become a method of choice for drug target validation and is currently being developed as a therapeutic strategy for combating a variety of diseases, including viral infections and cancers [6,7]. However, recent reports have documented some unexpected side-effects of RNAi-mediated gene silencing [8]. These include the suppression of non-target genes [9,10], non-specific induction of the innate immune response [11–17], and saturation of the RNAi machinery by excess amounts of siRNAs and shRNAs [18–20].

Because they exploit the endogenous RNA interference (RNAi) pathway, excess amounts of siRNAs can saturate the cellular RNAi machinery. One consequence of saturation of the RNAi machinery by exogenous siRNAs is that the simultaneous introduction of two distinct siRNAs often results in the loss of the gene-silencing activity of one of the siRNAs. This phenomenon, known as competitive inhibition, has been reported by several groups [21–27]. It has been suggested that some component of the RNAi pathway, presumably RISC, may be saturated by the exogenously introduced siRNAs [25]. However, the molecular

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features of siRNAs that specify competition potency have not yet been deciphered. In addition, there have been conflicting reports regarding the correlation between gene silencing efficiency and competition potency of siRNAs. One study reported that siRNAs which lacked the ability to silence target gene expression were able to effectively inhibit the activity of siRNAs that display high silencing efficiency [24]. A second study suggested that silencing efficiency correlated with competition potency [25]. Both these studies, however, tested only a limited number of siRNAs.

In this report, we sought to determine whether a correlation exists between the silencing efficiency and competition potency of siRNAs. In contrast to previous reports, we found no absolute correlation between these two activities. Instead, we found that competition potency was specified by the nucleotide sequence of the 5′-half of the antisense strand of an siRNA.

Materials and methods

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

Preparation of siRNA. 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 the siRNAs used were:

siCREB3 5′-GGAGUACACAGUCUGAGCC(dTdT)-3′ (Sense)
3′-(dTdT)CCUCAUGUGUCAGACUCGG-5′ (Antisense)
siTIG3 5′-CUGUCUCAGGCGUUCUCUA(dTdT)-3′ (Sense)
3′-(dTdT)GACAGAGUCCGCAAGAGAU-5′ (Antisense)
siDBP 5′-UCGAAGACAUCGCUUCUCA(dTdT)-3′ (Sense)
3′-(dTdT)AGCUUCUGUAGCGAAGAGU-5′ (Antisense)
siIntegrin 5′-UGAACUGCACUUCAGAUAU(UU)-3′ (Sense)
3′-(UU)ACUUGACGUGAAGUCUAUA-5′ (Antisense)
siXBP-1 5′-ACAGCAAGUGGUAGAUUUA(dTdT)-3′ (Sense)
3′-(dTdT)UGUCGUUCACCAUCUAAAU-5′ (Antisense)
siMyD88 5′-CUGGAACAGACAAACUAUC(dTdT)-3′ (Sense)
3′-(dTdT)GACCUUGUCUGUUUGAUAG-5′ (Antisense)
siBCL2 5′-GUGAAGUCAACAUGCCUGC(UU)-3′ (Sense)
3′-(UU)CACUUCAGUUGUACGGACG-5′ (Antisense)
siTRIF 5′-GACCAGACGCCACUCCAAC(dTdT)-3′ (Sense)
3′-(dTdT)CUGGUCUGCGGUGAGGUUG-5′ (Antisense)
siTI-P1 5′-ACAGCAAGUGCGUUCUCUA(dTdT)-3′ (Sense)
3′-(dTdT)UGUCGUUCACGCAAGAGAU-5′ (Antisense)
siXB-G3 5′-CUGUCUCAGGGUAGAUUUA(dTdT)-3′ (Sense)
3′-(dTdT)GACAGAGUCCCAUCUAAAU-5′ (Antisense)
siTI-D88 5′-CUGGAACAGGCGUUCUCUA(dTdT)-3′ (Sense)
3′-(dTdT)GACCUUGUCCGCAAGAGAU-5′ (Antisense)
siMy-G3 5′-CUGUCUCAGACAAACUAUC(dTdT)-3′ (Sense)
3′-(dTdT)GACAGAGUCUGUUUGAUAG-5′ (Antisense)
siDB-D88 5′-CUGGAACAGUCGCUUCUCA(dTdT)-3′ (Sense)
3′-(dTdT)GACCUUGUCAGCGAAGAGU-5′ (Antisense)
siMy-BP 5′-UCGAAGACAACAAACUAUC(dTdT)-3′ (Sense)
3′-(dTdT)AGCUUCUGUUGUUUGAUAG-5′ (Antisense)

siRNA transfection. For the siRNA competition assays shown in Fig. 1, one day before transfection, HeLa cells in complete medium without antibiotics were plated in 12-well plates and incubated for 24 h at 37 °C until they reached 50% confluency. Transfection was then carried out, according to the manufacturers' instructions, using Oligofectamine reagent (Invitrogen) and the target siRNA (siCREB3) at a final concentration of 10 nM with or without various competitor siRNAs at a final concentration of 50 nM. After 48 h of incubation at 37 °C, the transfected cells were harvested, RNA was isolated, and reverse transcription-polymerase chain reactions (RT-PCR) were performed.

Quantitative real-time PCR. Cells were harvested 48 h after transfection,, and total RNA was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. The purified total RNA (1 μg) was used for cDNA synthesis using the ImProm-II Reverse Transcription System (Promega) and an oligo-$(dT)_{15}$ primer. Aliquots (1/10) of the cDNA reaction mixture were analyzed by quantitative real-time PCR on a Rotor-Gene 3000 PCR machine (Corbett Research) according to the manufacturer's protocol. Primer sequences for the PCRs were: TIG3 forward, 5′-AGA TTT TCC GCC TTG GCT AT-3′ and reverse, 5′-TTT CAC CTC TGC ACT GTT GC-3′; DBP forward, 5′-CCT CGA AGA CAT CGC TTC TC-3′ and reverse, 5′-GCA CCG ATA TCT GGT TCT CC-3′; Integrin forward, 5′-CGT ATC TGC GGG ATG AAT CT-3′ and reverse, 5′-GGG TTG CAA GCC TGT TGT AT-3′; XBP-1 forward, 5′-GGA GTT AAG ACA GCG CTT GG-3′ and reverse, 5′-ACT GGG TCC AAG TTG TCC AG-3′; CREB3 forward, 5′-TTC TGA GGT ACC GAG CGA CT-3′ and reverse, 5′-GGA GGG AGT AGG TGT GGT CA-3′; MyD88 forward, 5′-TGC AGA GCA AGG AAT GTG AC-3′ and reverse, 5′-AGG ATG CTG GGG AAC TCT TT-3′; BCL2 forward, 5′-ATG TGT GTG GAG AGC GTC AA-3′ and reverse, 5′-ACA GTT CCA CAA AGG CAT CC-3′; TRIF forward, 5′-CCC TGT GGA CAG TGG AAG AT-3′ and reverse, 5′-CAA GAC CCT TCA CCC AGA AA-3′; GAPDH forward, 5′-GAG TCA ACG GAT TTG GTC GT-3′ and reverse, 5′-GAC AAG CTT CCC GTT CTC AG-3′.

Results and discussion

While working with siRNAs, we observed that co-transfection of two different siRNAs sometimes resulted in the inhibition of the silencing efficiency of one siRNA, a phenomenon known as competitive inhibition [21–27]. By testing various combinations of siRNAs with different targets and silencing efficiency (Fig. 1A), we found that the silencing efficiency of siCREB3 was reduced by several competitor siRNAs (Fig. 1B). However, the relative competition potency of these siRNAs was not identical. For example, siTIG-3 and siDBP efficiently reduced silencing by siCREB3, whereas siXBP-1 and siMyD88 had only marginal effects (Fig. 1B), indicating that competition potency was unique to each siRNA.

Because none of the competitor siRNAs alone had significant effects on the level of CREB3 mRNA (Supplementary Fig. 1), the effects on CREB3 mRNA level of competitor siRNAs in the presence of siCREB3 were most likely due to the competitive inhibition of silencing by siCREB3, and not due to off-target effects or alterations in secondary gene expression triggered by the competitor siRNAs.

It has been suggested that the competition potency of an siRNA is either positively [25] or negatively [24] correlated to the silencing efficiency. However, we did not observe an absolute correlation between these activities (Fig. 1). For example, siTIG3 and siDBP, which had the highest gene silencing efficiency, were also the most potent competitors, results consistent with a positive correlation between silencing efficiency and competition potency. In contrast, siBCL2

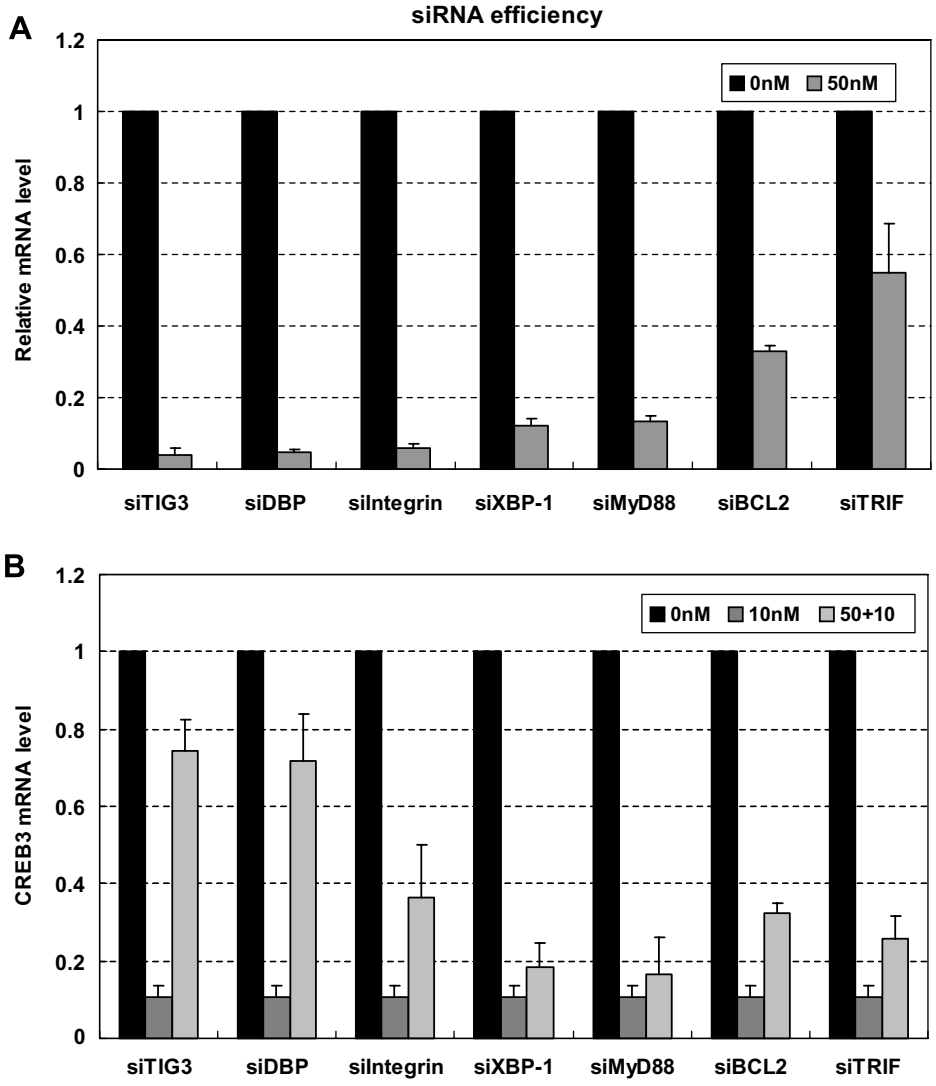

Fig. 1. Lack of correlation between siRNA silencing activity and competition potency. (A) Efficiency of target gene silencing by each competitor siRNA. The reductions in the amounts of target mRNAs in HeLa cells in which each siRNA was transfected at a concentration of 50 nM were measured by quantitative real-time RT-PCR. Values shown on the *y*-axis are mean + standard deviations (SD) of at least two experiments. 0 nM (dark bars), oligofectamine only; 50 nM (gray bars), 50 nM of each siRNA transfected. (B) siRNAs for the *CREB3* target mRNA (siCREB3) and competitor siRNAs designated in each panel were mixed at the indicated concentrations and co-transfected into HeLa cells. The reductions in the amounts of target mRNAs in the transfected cells were measured using real-time RT-PCR. Values shown on the *y*-axis are mean + SD of duplicate experiments. 0 nM (dark bars), oligofectamine only, 10 nM (dark gray bars), 10 nM of siCREB3 transfected; 50 + 10 (lightly shaded bars), 50 nM competitor siRNA with 10 nM siCREB3.

and siTRIF, which had silencing efficiencies less than those of siXBP1 and siMyD88, had better competition potencies than the latter, indicating that silencing efficiency alone was insufficient to explain the competition potency of siRNAs, and that this activity may be due to other features of siRNAs.

One drawback of this experiment is that because these siRNAs targeted different mRNAs, the structure and/or stability of the target mRNA, in addition to the efficiency of RISC assembly, may have affected the silencing efficiency of each siRNA. Thus, the lack of correlation between silencing efficiency and competition potency of these siRNAs may not be a true reflection of the mechanistic difference between the two phenomena. By using different chemical modifications of the same siRNA, we demonstrated that the two phenomena are truly separable and may involve different mechanisms (see below).

To identify the molecular feature of siRNAs that specifies their competition potencies, we tested the competitive abilities of several chemically modified siRNAs. By introducing a fluorescein moiety at the 5′-end of the antisense (AS) strand of the potent competitor siTIG3, designated siT5asF, the competition potency of this siRNA was reduced considerably (Fig. 2A). When a fluorescein moiety was introduced at the 3′-end of the AS strand (siT3asF), however, the competition potency of the resulting siRNA was similar to that of unmodified siTIG3 (Fig. 2A). Introduction of fluorescein at the 5′ end of the AS strand also

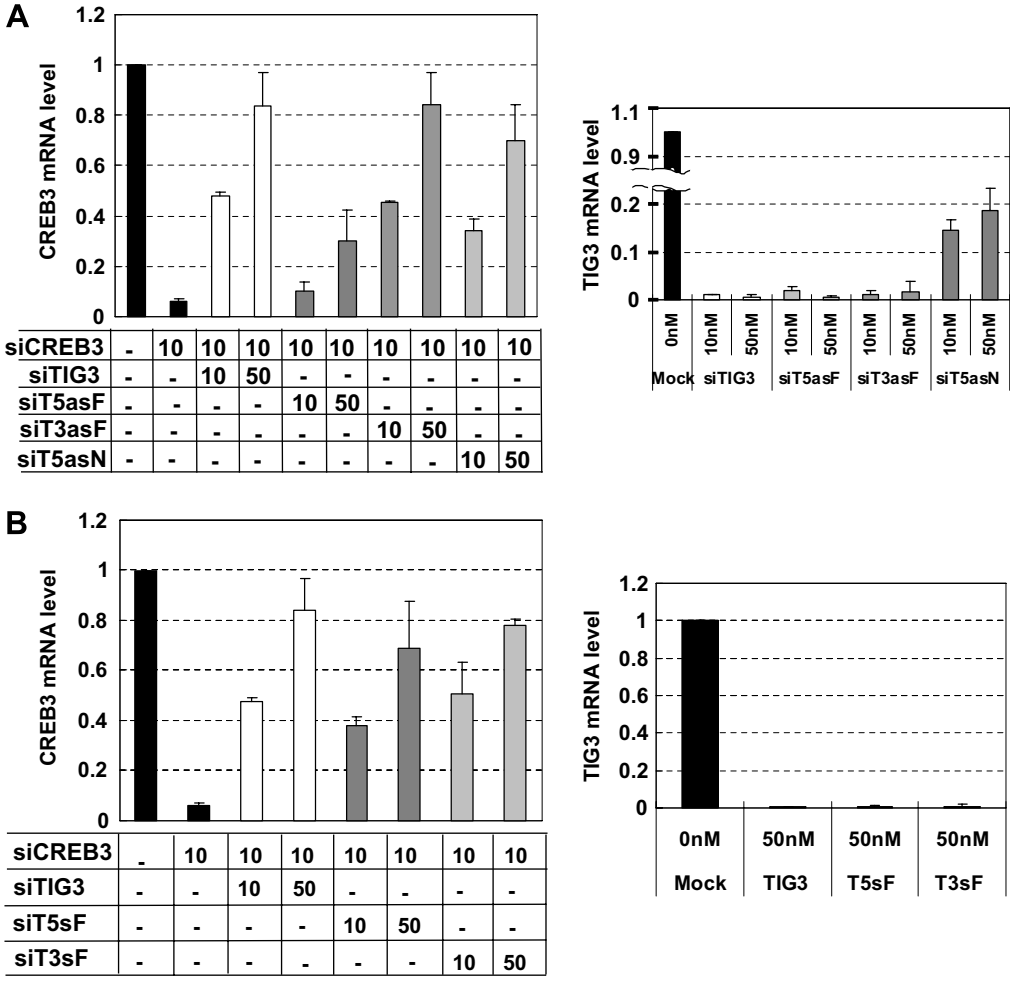

Fig. 2. Competition potency of chemically modified siRNAs. (A) Competition potencies and silencing efficiencies of chemically modified siRNAs. Chemically modified siTIG3 derivatives at a concentration of either 10 nM or 50 nM were mixed with 10 nM siCREB3 and co-transfected into HeLa cells. The amounts of *CREB3* and *TIG3* mRNA were measured by quantitative real-time RT-PCR, 48 h after transfection. The numbers shown in the table below the graph on the left are the concentrations of the transfected siRNAs in nM, and each column corresponds to the bar directly above it. Values are mean + SD of at least triplicate experiments. siT5asF, fluorescein modification at the 5′-end of the AS strand of siTIG3; siT3asF, fluorescein modification at the 3′-end of the AS strand of siTIG3. (B) Sense strand 5′- or 3′-fluorescein modified siTIG3 was mixed with siCREB3 and transfected into HeLa cells. After 48 h, the mRNA levels of *CREB3* and *TIG3* were measured by quantitative real-time PCR. Values are mean + SD of triplicate experiments. siT5sF, fluorescein modification at the 5′-end of the S strand of siTIG3; siT3sF, fluorescein modification at the 3′-end of the S strand of siTIG3.

reduced the competition potency of another competitor siRNA, siDBP (Supplementary Fig. 2), whereas fluorescein modification of either the 5′- or 3′-end of the sense (S) strand did not affect the competition potency of siTIG3 (Fig. 2B). These results indicate that the 5′-end of the AS strand is important for the competition potency of an siRNA.

Somewhat unexpectedly, and in contrast to fluorescein modification, amine (NH_2-) modification of the 5′-end of the AS strand of siTIG3, designated siT5asN, only slightly affected the competition potency of the siRNA (Fig. 2A). This difference may have been caused by the difference in bulkiness between the larger fluorescein moiety and the smaller amine moiety. Nonetheless, both chemical modifications reduced the silencing efficiency of siRNA (Fig. 2A). Interestingly, 5′-AS amine modification had more negative effects on the silencing efficiency of the competitor siRNA than did 5′-AS fluorescein modification, albeit the former maintained stronger competition potency than the latter (Fig. 2A). These findings further indicated that silencing efficiency and competition potency are separate phenomena, and likely involve different mechanisms.

Because the only difference between siRNAs is their nucleotide sequences, we hypothesized that the sequence near the 5′-end of the AS strand might be important in specifying the competition potency of siRNAs. To test this idea, we generated chimeric siRNAs by fusing the 5′-AS half of a strong (or weak) competitor siRNA with the 3′-AS half of a weak (or strong) competitor siRNA. For example, we fused the 5′-AS half of siTIG3, a strong competitor, to the 3′-AS half of siXBP-1, a poor competitor, to generate siTI-P1 (Fig. 3A). Using this strategy, six different chimeric siRNA duplexes were made (Fig. 3A). We then tested the ability of these chimeric siRNAs to compete with

A

siTI-P1

```
AS 5′-        UAGAAAGCGC ACUUGCUGU(dTdT)-3′
SS 3′- (dTdT)AUCUCUUGCG UGAACGACA       -5′
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siXB-G3

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AS 5′-       UAAAUCUACC CUGAGACAG(dTdT)-3′
SS 3′-(dTdT)AUUUAGAUGG GACUCUGUC       -5′
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siTI-D88

```
AS 5′-       UAGAGAACGC CUGUUCCAG(dTdT)-3′
SS 3′-(dTdT)AUCUCUUGCG GACAAGGUC       -5′
```

siMy-G3

```
AS 5′-       GAUAGUUUGU CUGAGACAG(dTdT)-3′
SS 3′-(dTdT)CUAUCAAACA GACUCUGUC       -5′
```

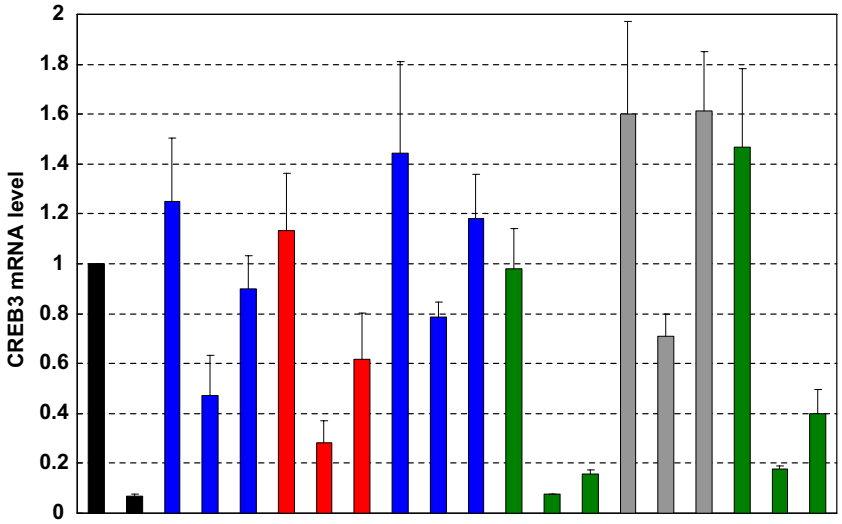
siDB-D88

```
AS 5′-       UGAGAAGCGA CUGUUCCAG(dTdT)-3′
SS 3′-(dTdT)ACUCUUCGCU GACAAGGUC       -5′
```

siMy-BP

```
AS 5′-       GAUAGUUUGU UGUCUUCGA(dTdT)-3′
SS 3′-(dTdT)CUAUCAAACA ACAGAAGCU       -5′
```

B



siCREB3	-	10	-	10	10	-	10	10	-	10	10	-	10	10	-	10	10	-	10	10
siTI-P1	-	-	50	10	50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
siXB-G3	-	-	-	-	-	50	10	50	-	-	-	-	-	-	-	-	-	-	-	-
siTI-D88	-	-	-	-	-	-	-	-	50	10	50	-	-	-	-	-	-	-	-	-
siMy-G3	-	-	-	-	-	-	-	-	-	-	-	50	10	50	-	-	-	-	-	-
siDB-D88	-	-	-	-	-	-	-	-	-	-	-	-	-	-	50	10	50	-	-	-
siMy-BP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	50	10	50

Fig. 3. Competition potency of chimeric siRNAs. (A) Schematic drawing and sequences of the chimeric siRNAs. (B) Chimeric siRNA was co-transfected into HeLa cells with siCREB3, and *CREB3* mRNA concentrations were measured by quantitative real-time PCR. The numbers shown in the table below the graph on the left are the concentrations of the transfected siRNAs in nM, and each column corresponds to the bar directly above it. Values are mean + SD of triplicate experiments.

siCREB3 by measuring their effect on siCREB3-mediated silencing of the *CREB3* gene in co-transfected HeLa cells. We observed that, siTI-P1 and siTI-D88, which contained the 5′-AS half of the siTIG3 duplex, a strong competitor siRNA, showed strong competition potency at both concentrations tested (10 and 50 nM) (Fig. 3B). siDB-D88, which contains the 5′-AS half of siDBP, another strong competitor siRNA, also showed strong competition potency (Fig. 3B). On the other hand, siXB-G3, siMy-G3, and siMy-BP, which carried the 5′-AS half of weak competitors (siXBP1, siMyD88, and siMyD88, respectively), displayed reduced competition potency at both concentrations tested (Fig. 3B). In summary, all three chimeric siRNAs which contained 5′-AS half of strong competitor siRNAs were strong competitors, whereas all three chimeric siRNAs which contained 5′-AS half of weak competitor siRNAs were weak competitors. These results were in complete agreement with our hypothesis that the sequence near the 5′-end of the guide strand of an siRNA specified its competition potency.

A recent study [27] showed that cellular downregulation of TRBP resulted in a loss of differential competition by siRNAs. Since TRBP identifies the guide strand prior to passage onto Ago-2 [28], our finding that the 5′-end of the guide strand sequence specified the competition potency was consistent with this observation. While our study clearly demonstrated the importance of siRNA sequence for competition potency, the limited number of siRNAs tested here did not permit identification of the exact sequence features, such as sequence motifs or composition, responsible for competition. Future study should focus on the identification of such sequence features responsible for the competition potency of siRNAs, which will require large scale competition experiments using a number of different siRNAs. Identification of the sequence features which govern the competition potency will facilitate mechanistic studies of the saturation of the RNAi machinery by siRNAs. In addition, chemical and/or structural modification of siRNAs, which could maintain the silencing efficiency but reduce competition potency, should be investigated. These studies will help researchers to develop an efficient combinatorial RNAi strategy [29].

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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.2007.12.099.

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