Chapter 2

Design of Ligand-Responsive Small Interfering RNAs

ABSTRACT

Small interfering (si)RNAs are duplexed RNAs of 19 base pairs that induce potent and specific gene silencing in metazoans through the RNA interference pathway. We have designed siRNAs, which we call siSwitches, that activate gene silencing only in the presence or absence of a selected ligand. Rational integration of siRNAs and ligand-binding aptamers imparted tunable ligand control of siRNA formation and Dicer processing in vitro. Surprisingly, siSwitches produced by T7 transcription and not chemical synthesis induced potent silencing in mammalian cells even when one of the two siRNA strands were absent, suggesting the presence of spurious T7 transcription products that activate gene silencing. Plasmid-based expression of siRNAs with 25 base pairs from U6 promoters induced greater silencing than the traditional siRNA counterparts, supporting further research in the endogenous expression of siSwitches toward applications in advanced genetic screens and gene-specific therapeutics.

INTRODUCTION

RNA interference (RNAi) is a revolutionary genetic tool that enacts targeted post-transcriptional gene silencing in most organisms including humans. Applied double-stranded or hairpin RNAs are processed by the RNase III enzyme Dicer to 19-base pair duplexes termed small interfering (si)RNAs (Bernstein *et al*, 2001). One of the two siRNA strands termed the guide strand is retained by the RNA induced silencing complex (RISC) and serves as a template for the targeting of transcripts with complementary sequences. Researchers have implemented siRNAs with duplexes of 19 base pairs or 27 base pairs that respectively bypass or undergo Dicer processing to enact gene silencing (Elbashir *et al*, 2001; Kim *et al*, 2005). Simple base pairing interactions between the guide strand and transcript dictate target recognition, lending to the rational design of siRNAs that specifically target any single expressed gene (Tilesi *et al*, 2009). In turn, siRNAs have been used as the basis for high-throughput genetic screens (Echeverri and Perrimon, 2006) and emerging treatment strategies to quell viral infection and genetic diseases such as cancer (Aagaard and Rossi, 2007).

The regulatory capabilities of siRNAs represent only one of many unique qualities afforded to RNA. As a second example, RNAs termed aptamers can bind diverse molecules or ligands with high affinity and specificity. These molecules can be selected through a standard in vitro procedure (Ellington and Szostak, 1990; Tuerk and Gold, 1990), resulting in the identification of aptamer sequences against proteins, metals, metabolites, and small molecule drugs. Utilizing these versatile recognition properties, researchers have integrated aptamers into multifunctional RNAs that activate or repress gene expression in the presence or absence of the recognized ligand (Suess and Weigand,

2008). Researchers have exploited a diversity of regulatory mechanisms in bacteria, yeast, and mammalian cells in the design of these multifunctional RNAs, although none to-date have integrated aptamers and siRNAs for ligand control of RNAi.

In this report, we describe a strategy to engineer ligand control of RNAi through the rational combination of siRNAs and aptamers. The modular and tunable designs are based on a previous framework developed in our group for ligand control of eukaryotic gene expression through antisense inhibition (Bayer and Smolke, 2005). Under our design, ligand binding controls whether or not the siRNA can undergo further processing to activate RNAi. By coupling in vitro aptamer selection technologies and rational siRNA design, virtually any gene can be controlled by a diversity of molecules located in the intracellular environment.

RESULTS AND DISCUSSION

We devised two design schemes that rationally combine aptamers and siRNAs to introduce ligand control of siRNA processing and subsequent RNAi-mediated gene silencing (Figure 2.1). We call the resulting regulatory molecules small interfering switches or siSwitches. In both design schemes, hybridization of the guide strand and the passenger strand form the siRNA that undergoes Dicer processing. Ligand binding to the aptamer stabilizes one of two conformations that either expose or sequester the passenger strand to promote (OFF behavior) or inhibit (ON behavior) siRNA formation, respectively. In the scheme for ON behavior, the aptamer is flanked by the siRNA passenger strand and a competing strand complementary to the passenger strand (Figure 2.1A). The competing strand is intentionally shorter than the passenger strand to avoid

Dicer recognition and processing. In the absence of ligand, the guide strand preferentially displaces the competing strand and hybridizes to the passenger strand, thereby forming an siRNA. Ligand binding stabilizes the aptamer stem, which coincides with base pairing between the passenger strand and competing strand. Thus, the addition of ligand is anticipated to increase target gene levels by inhibiting siRNA formation and subsequent Dicer processing.

In the scheme for OFF behavior, the passenger strand and the aptamer are separated by an intervening sequence complementary to the passenger strand (Figure 2.1B). In the absence of ligand, the intervening sequence base pairs to the passenger strand, sequestering it from guide strand hybridization. Ligand binding to the aptamer displaces the passenger strand, allowing guide strand hybridization, siRNA formation, and subsequent gene silencing. Overall, the two schemes allow either activation or repression of target gene levels in the presence of the cognate ligand by controlling the activity of RNAi.

An important design consideration for both ON and OFF behavior is the number of base pairs in the formed siRNA. In mammals, double-stranded RNAs consisting of more than 30 base pairs efficiently activate the interferon response, which shuts down global protein synthesis. To maintain targeted gene silencing through RNAi, the formed siRNA should be less than the cutoff of 30 base pairs.

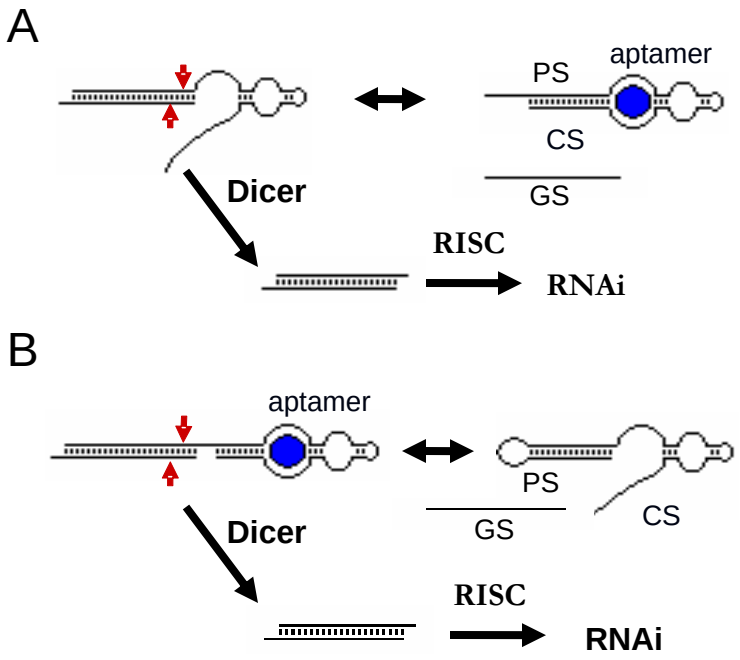


Figure 2.1 Design schemes for ligand-regulated siRNAs or siSwitches that rely upon the necessity of a 2-nt 3'overhange for efficient Dicer processing. Designs combine two strands of RNA. One encodes the guide strand (GS) and the other encodes the passenger strand (PS), a ligand-binding aptamer, and a competing strand (CS) complementary to the passenger strand. Ligand binding (blue circle) stabilizes an aptamer-formed conformation such that the guide strand can or cannot hybridize. Hybridization produces an siRNA with the necessary overhang that can undergo Dicer processing, subsequently activating gene silencing. Designs exhibiting ON behavior (**A**) or OFF behavior (**B**) as designated by the expression level of the target gene in the presence of the ligand. Red arrows designate Dicer cleavage sites.

We first evaluated the behavior of siSwitches in vitro (Figure 2.2). An initial siSwitch (S1) was constructed following the design scheme for ON behavior. S1 contains a passenger strand matching the fluorescent protein encoding gene *gfp*, an in vitro-selected aptamer against the small molecule theophylline (Jenison *et al*, 1994; Zimmermann *et al*, 2000), and a competing strand that forms 19 base pairs with the passenger strand (Figure 2.2A). To assess the impact of ligand binding on siRNA formation and Dicer processing in vitro, T7-transcribed and 5'-radiolabeled S1 was incubated with a 2-fold molar excess of an unlabeled 28-nt guide strand (aS1) and varying concentrations of theophylline in the presence or absence of Dicer. RNAs were then resolved by nondenaturing PAGE (Figure 2.2B). In the absence of Dicer, S1 predominantly hybridized with aS1 to form an siRNA and theophylline addition partially inhibited siRNA formation. The presence of Dicer removed all traces of full-length S1 even in the presence of theophylline. These results suggest that theophylline addition inhibited siRNA formation in line with the predicted behavior for ON-acting siSwitches, although irreversible Dicer processing drove the partitioning toward siRNA formation until the entire S1 pool had been cleaved.

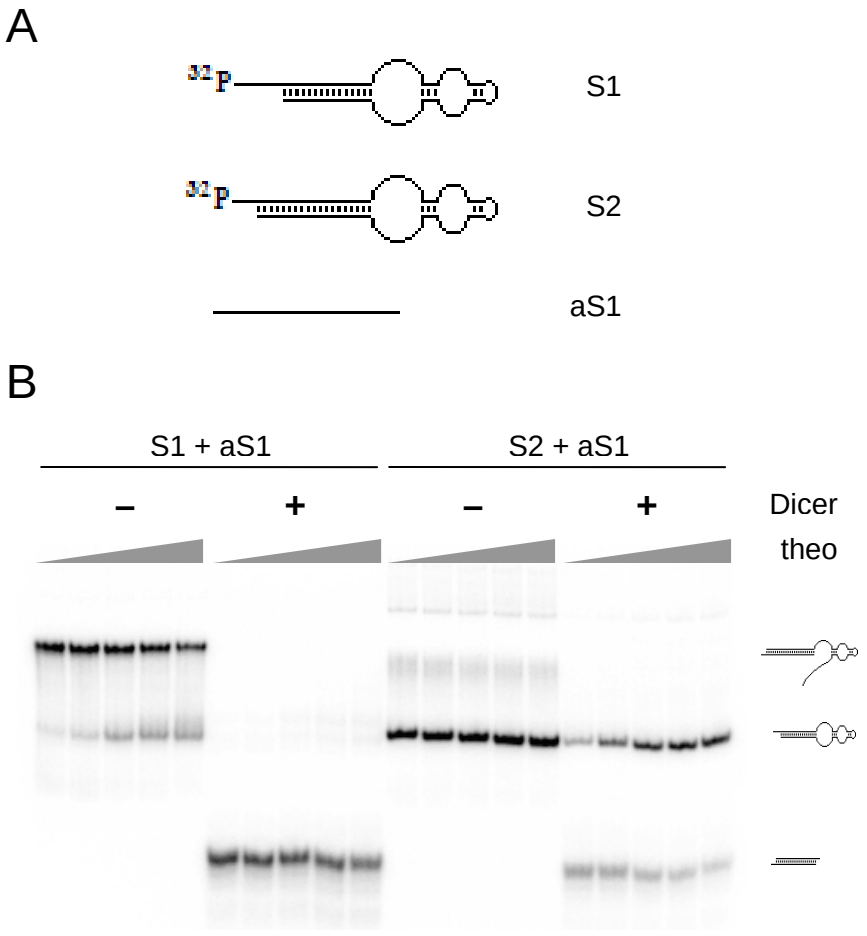


Figure 2.2 Length of the competing stem affects the extent of binding and Dicer processing in vitro. (**A**) Schematic of two ON-behaving long siRNAs with competing strand lengths of 19-nt (S1) and 23-nt (S2) with radiolabeled 5' phosphates. The antisense strand was unlabeled in these experiments. (**B**) Non-denaturing gel results of both sense strands incubated with a 200% molar excess of the antisense strand in the presence (+) or absence (–) of Dicer and varying concentrations of theophylline (theo). Specific theophylline concentrations used (mM): 0.01, 0.1, 1, 10, 25. Gel results are representative of two independent experiments.

The substantial processing even in the presence of theophylline highlights the irreversibility of the Dicer processing step, which may lead to extensive gene silencing in vivo for ON behavior even in the presence of ligand. Therefore, it would be beneficial to tune the extent of processing to manipulate the relationship between ligand concentration and target gene levels. One tuning strategy was suggested by two reports of synthetic RNA-based gene regulation, where the complementarity between hybridizing RNAs was modified to modulate the energetics of complex formation (Bayer and Smolke, 2005; Isaacs *et al*, 2004). We adopted a similar strategy by extending the competing strand of S1 to form 19 base pairs with the passenger strand (Figure 2.2A). The designed siSwitch (S2) was subjected to the in vitro processing assay to compare theophylline control of siRNA formation and Dicer processing to that of its counterpart S1. In vitro results showed that S2 was less prone to form an siRNA and Dicer processing was more sensitive to theophylline addition. By extending the competing strand, siRNA formation was less thermodynamically favorable, providing fewer formed siRNAs for Dicer processing. Modulating the competing strand length can be considered a general design strategy to tailor siSwitch behavior to in vivo silencing requirements.

We next explored the behavior of siSwitches in vivo through two modes of delivery: transfection and endogenous expression. Transfection was initially evaluated based on the predominance of this mode of siRNA delivery in both basic research and disease treatment applications. Transfected siRNAs can be generally produced through chemical synthesis or in vitro transcription, where the latter is more economically viable to mass produce RNAs greater than 50 nts. However, delivery of T7-transcribed RNAs has been shown to induce an innate immune response through PKR recognition of the 5’

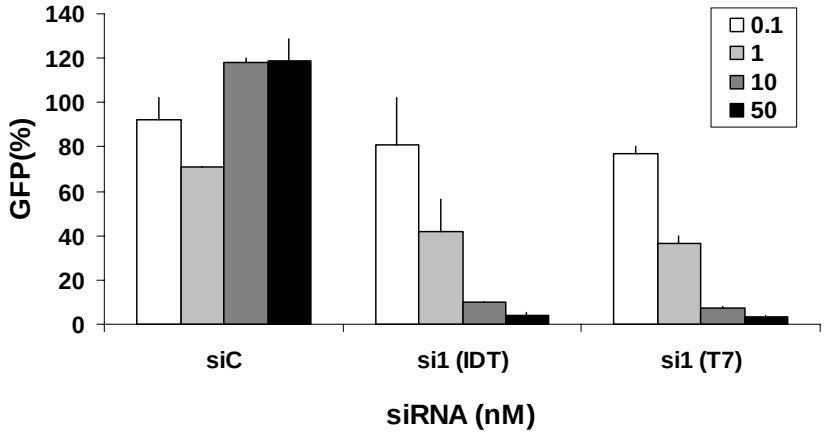
triphosphate (Kim *et al*, 2004). To initially test the silencing capacity of T7-transcribed RNAs through RNAi, we transfected cells with chemically-synthesized or T7-transcribed siRNAs designed to target GFP. 5' phosphates on T7-transcribed siRNAs were removed by phosphatase treatment. Both GFP-targeting siRNAs effectively reduced fluorescence levels, which were insensitive to a T7-transcribed siRNA with a scrambled guide strand (Figure 2.3A).

We anticipated that siSwitches should show the same targeting specificity as the siRNAs when both siRNA strands are present and designed to target GFP. Surprisingly, transfection of the T7-transcribed passenger strands or the guide strands for both ON- and OFF-behaving designs significantly silenced GFP levels (Figure 2.3B). Silencing was not observed for a scrambled guide strand (aSC), suggesting that silencing was sequence-specific. To evaluate whether silencing was linked to T7 transcription, we transfected cells with a T7-transcribed or chemically synthesized guide strand (aS1) targeting GFP and measured relative GFP levels. Only the T7-transcribed guide strand induced GFP silencing, eliminating the possibility of antisense inhibition. One interpretation is that T7 transcription for siSwitches produces spurious products that activate RNAi, either as separate species or extensions of the templated RNAs. These spurious products must be removed to allow ligand control of RNAi-mediated silencing by siSwitches produced through T7 transcription. Alternatively, chemical synthesis may become a viable option with further improvements in RNA synthesis technologies and corresponding cost reductions.

An alternative possibility is endogenously expressing siSwitches in cells to introduce ligand control of gene silencing. Previous work showed that plasmid-based

expression of individual siRNA strands from U6 promoters could significantly silence a target gene through RNAi (Miyagishi and Taira, 2002; Wu *et al*, 2005; Yu *et al*, 2002). Only siRNA duplexes with 19 base pairs were tested, where siRNAs of this length do not undergo Dicer processing. More recent work showed that delivery of longer siRNAs improved silencing efficiency (Kim *et al*, 2005). These longer siRNAs undergo Dicer processing, resulting in rapid loading of the guide strand into RISC (Gregory *et al*, 2005; Maniataki and Mourelatos, 2005). Since these longer siRNAs have not been tested under endogenous expression and form the basis of our siSwitch designs, we evaluated the improved silencing efficiency of longer siRNAs under endogenous expression. The strands of GFP-targeting siRNAs with 19 or 26 base pairs were encoded in plasmids downstream of U6 promoters. The resulting plasmids were cotransfected into HeLa cells with a GFP reporter plasmid. The relative fluorescence showed a much larger decrease in GFP for cells transfected with the longer siRNAs, supporting the improved silencing efficiency of longer siRNAs (Figure 2.4).

A


140
120
100
80
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GFP(%)
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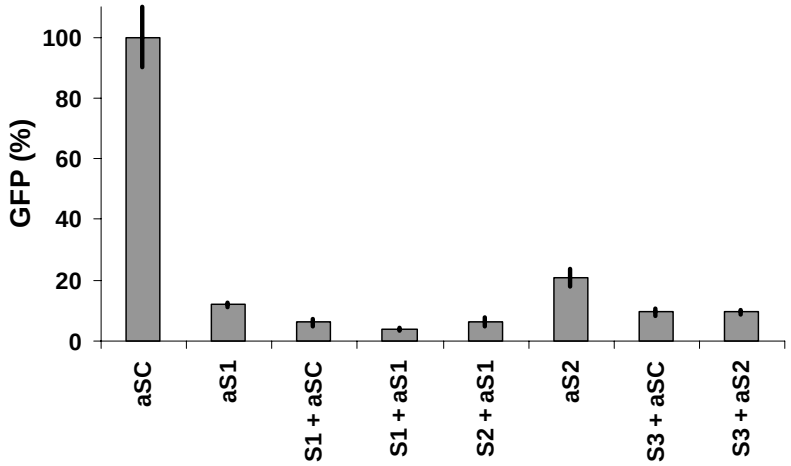
si1 (IDT)
si1 (T7)
siRNA (nM)

B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GFP (%)
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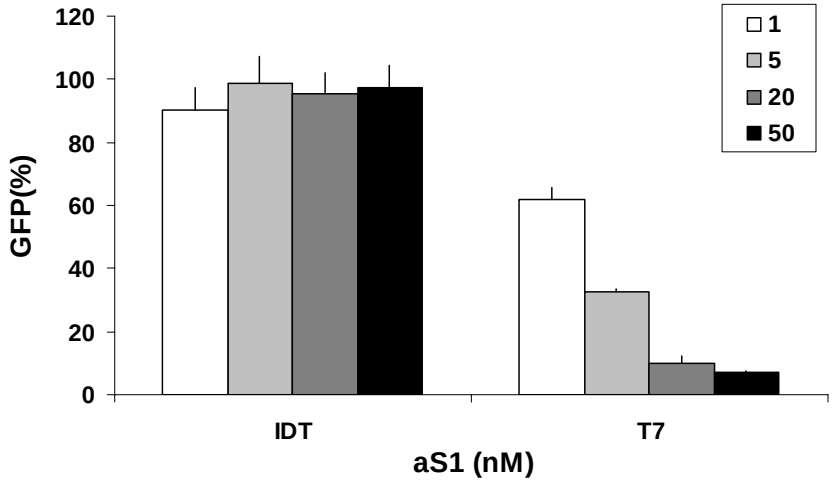
120
100
80
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GFP(%)
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5
20
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IDT
T7
aS1 (nM)

Figure 2.3 Relative EGFP knockdown by T7-transcribed siRNAs in HEK293T tTA-d2EGFP. (**A**) T7-transcribed siRNAs produce sequence-specific knockdown of EGFP. Compared to untransfected cells, a scrambled 25-bp siRNA (siC) produces negligible knockdown, whereas chemically-synthesized (IDT) and T7-transcribed (T7) 25-bp siRNAs (si1) produce dose-dependent knockdown of fluorescence. (**B**) Individual molecules of T7-transcribed ligand-regulated siRNAs produce sequence-specific knockdown of EGFP. Individual molecules were transfected at a concentration of 10 nM, where trials with two RNA strands had a total RNA concentration of 20 nM. Sc, Scrambled RNA strand; aS1, antisense strand for ON behavior; S1, siSwitch for ON behavior containing sense strand and theophylline aptamer; aS2, antisense strand for OFF behavior; S3, sense strand for OFF behavior. (**C**) T7-transcribed antisense RNAs produce sequence-specific knockdown. Chemically-synthesized antisense RNA (IDT) produced no knockdown compared to untransfected cells, while T7-transcribed antisense RNAs produced dose-dependent knockdown of EGFP. Error bars for all three graphs represent three independent measurements.

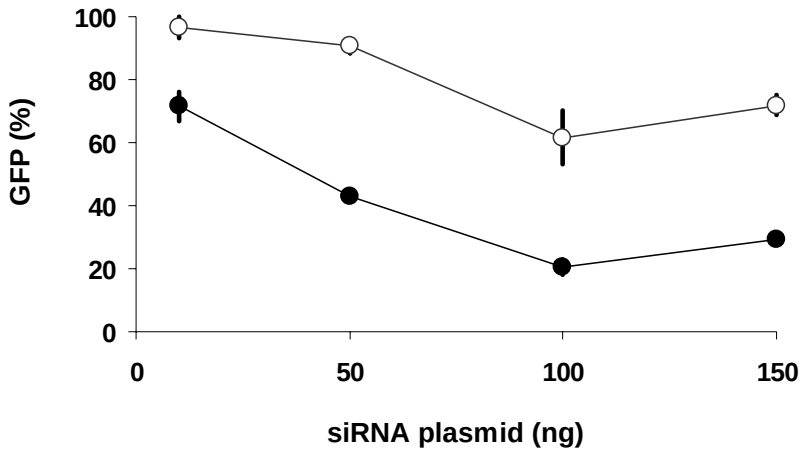

Figure 2.4 Endogenously-expressed long siRNAs induce greater EGFP knockdown than regular siRNAs. Both strands of 19 bp (white) or 25 bp siRNAs (black) targeting EGFP were placed downstream of a U6 promoter encoded in a single plasmid. Different amounts of the siRNA-encoding plasmids were cotransfected with 10 ng of an EGFP-expressing reporter plasmid into HeLa cells. Measured fluorescence was normalized to the fluorescence of cells transfected with the corresponding amount of an empty plasmid. Error bars represent the standard deviation of two independent transfections.

Future work should focus on testing both ON and OFF designs for siSwitches under endogenous expression. Plasmids encoding siSwitches could be transfected in a cell line stably expressing GFP to facilitate the evaluation of siSwitch activity. Once a base assay is developed, aptamer swapping and performance tuning should be evaluated to ascertain how well siSwitches can be tailored to meet any regulatory demands. siSwitch performance should also be compared to the other designs documented in Chapters 3 and 4 to assess which engineered mode of ligand control of RNAi is best for any desired application.

MATERIALS AND METHODS

T7 transcription. All reagents and solutions for T7 transcription were RNase-free. Shorter DNA templates for the T7 transcription reaction were synthesized (IDT) and annealed. For longer templates, the top and bottom strands were ordered truncated at the 3' end. Full-length templates were produced by annealing these strands, adding Klenow (NEB), the supplied buffer, and enough dNTPs for a final concentration of 0.33 mM and incubated at room temperature for 30 mins. Templates were then ethanol-precipitated prior to the T7 transcription reaction. The sequence of the final template contained the T7 promoter sequence (TTCTAATACGACTCACTATAG) at the 5' end. The final sequences of synthesized or transcribed RNAs are reported in Table 2.1. RNA was transcribed using the Ampliscribe T7 transcription kit (Epicentre) according to the manufacturer's instructions. To remove 5' phosphates, 1 U calf intestinal phosphate (NEB) was added to the transcription reaction, followed by incubation at 37 ºC for 1 hr. Unincorporated ribonucleotides were removed using NucAway spin columns (Ambion) and the transcribed RNA was further purified by PAGE. RNA concentration was estimated from the measured absorbance at 260 nm using a DU530 spectrophotometer (Beckman Coulter).

In vitro Dicer assay. Transcribed RNA was 5' labeled by incubating the RNA with γ-[^{32}P]-ATP and T4 polynucleotide kinase (NEB) for 30 min at 37 ºC and purified using the NucAway spin column. Approximately 1 nmol of the labeled RNA was incubated for 16 hrs in 1X Dicer reaction buffer (20 mM Tris-HCl, 150 mM NaCl, 2.5 mM $MgCl_2$, pH 8.0) with varying concentrations of theophylline in the presence or absence of 0.5 U of

Dicer (Stratagene) for a total volume of 10 µl. After the incubation, each sample was combined with 2 µl of 6X loading buffer (40% sucrose), resolved on a 12% non-denaturing polyacrylamide gel, and imaged on an FX phosphorimager (BioRad).

Plasmid construction. The endogenously-expressed siRNAs were encoded in pcDNA3.1(+) (Invitrogen), where the two siRNA strands were separately placed downstream of U6 promoters. The U6 promoters were cloned into BglII/MfeI and SpeI/XhoI of pcDNA3.1(+) after amplification from pSilencer 2.1 puro. Each siRNA strand was encoded in a 5' tail of the reverse oligo primer. sU6.fwd 5'-AATAAGATCTCCCCAGTGGAAAGACGCGCA-3', aU6.fwd 5'- AATAACTAGTCCCCAGTGGAAAGACGCGCA-3', 19nt-sU6.rev 5'-AATACAATTGAAAAAAGATGAACTTCAGGGTCAG**C**GGATCCCGCGTCCTTTCCACA-3', 19nt-aU6.rev 5'-AATACTCGAGAAAAAAGCTGACCCTGAAGTTCAT**C**GGATCCCGCGTCCTTTCCACA-3', 25nt-sU6.rev 5'-AATACAATTGAAAAAATGCAGATGAACTTCAGGGTCAG**C**GGATCCCGCGTCCTTTCCACA-3', 25nt-aU6.rev 5'-AATACTCGAGAAAAAAGCTGACCCTGAAGTTCATCTGCATTT**C**GGATCCCGCGTCCTTTCCACA-3'. Underlined text designates restriction sites while each bolded 'C' marks the start of transcription from the U6 promoter. All restriction enzymes and T4 DNA ligase were purchased from NEB. All constructs were sequence-verified (Laragen).

Cell culture and transfection. HEK293T and HeLa cells were maintained in minimal essential medium alpha media (Invitrogen) with 10% fetal bovine serum (Invitrogen) at 37°C in a 5% CO_2-humidified incubator. Cells were transfected 1 day after seeding with

either plasmid DNA, T7-transcribed RNA, or chemically-synthesized RNA (IDT) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. pcDNA3.1(+) encoding EGFP was transfected to evaluate siRNA potency. pSilencer 2.1 puro served as a negative control. One day post-transfection, the media was replaced.

Quantification of fluorescence. Three days post-transfection, the media was removed from each well and 100 µl (for 48-well plate) of M-PER Mammalian Protein Extraction Reagent (Pierce) was added to each well. After 5 min of agitation, each well was scraped with a pipette tip and the lysed cells were transferred to a 1.5 ml microcentrifuge tube. Following a 10 min spin at 4 ºC and 14K rpm, the supernatant was transferred to a fresh tube and assayed for total protein using the BCA kit (Sigma). The remaining supernatant was transferred to a 386-well plate and assayed for fluorescence using a Safire (Tecan) fluorescent plate reader set to the appropriate excitation (EGFP, 485 nm) and emission (EGFP, 515 nm) wavelengths. Sample fluorescence was normalized to total protein levels then normalized such that 100% represents relative GFP levels for cells transfected with the negative control plasmid.

One drawback to this procedure is that the measured fluorescence is a population average; hence confounding factors such as variable transfection efficiency bias the fluorescent data. To correct for this, flow cytometry should be used in the future, since the effects of transfection efficiency can be ascertained and removed from the data.

Table 2.1 Sequence of synthesized and T7-transcribed RNAs. Sequences are written 5' to 3'. The theophylline aptamer is indicated in blue text. The guide strand (GS) and passenger strand (PS) are shown for the siRNAs tested in Figure 2.3.

Name	Sequence
S1	GCUGACCCUGAAGUUCAUCUGCGGUGAUACCAGCAUCGUCUUGAUGCCCUUGGCAGCACCGCAGAUGAACUUCAG
S2	GCUGACCCUGAAGUUCAUCUGCGGUGAUACCAGCAUCGUCUUGAUGCCCUUGGCAGCACCGCAGAUGAACUUCAGGGUC
S3	GCUGACCCUGAAGUUCAUCUGCGAACUUCAGGGUCAGCGAUACCAGCAUCGUCUUGAUGCCCUUGGCAGCGCUGACCCUU
aS1	GCACCGCAGAUGAACUUCAGGGUCAGCU
aS2	GUUCGCAGAUGAACUUCAGGGUCAGCU
aSC	GUUGCACUGGCUCUACAACUAGACCGCA
si1 (GS)	GGUGCAGAUGAACUUCAGGGUCAGCUU
si1 (PS)	GCUGACCCUGAAGUUCAUCUGCACC
siC (GS)	GUUGCACUGGCUCUACAACUAGACCGC
siC (PS)	GGUCUAGUUGUAGAGCCAGUGCAAC

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