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Reduced levels of Ago2 expression result in increased siRNA competition in mammalian cells

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ABSTRACT

Administration of small interfering RNAs (siRNAs) leads to degradation of specific mRNAs utilizing the cellular RNA interference (RNAi) machinery. It has been demonstrated that co-administration of siRNAs may lead to attenuation of activity of one of the siRNAs. Utilizing antisense and siRNA-mediated RNA-induced silencing complex (RISC) gene reduction we show that siRNA competition is correlated with differences in the cellular expression levels of Ago2, while levels of other RISC proteins have no effect on competition. We also show that under certain conditions siRNA competition rather than reduction of cellular RISC levels may be responsible for apparent reduction in siRNA activity. Furthermore, exploiting siRNA competition, we show that the RISC pathway loads and results in detectable cleavage of the target RNA in ~2 h after transfection. The RISC pathway is also capable of being reloaded even in the absence of new protein synthesis. RISC reloading and subsequent induction of detectable cleavage of a new target RNA, requires about 9–12 h following the initial transfection.

INTRODUCTION

RNA interference (RNAi) is a mechanism by which double-stranded RNA triggers the inhibition of target gene expression by inducing sequence-specific target mRNA degradation (1). Short interfering RNAs (siRNAs), RNA duplexes of 21–23 nucleotides, are important intermediates in the RNAi pathway that lead to degradation of specific mRNAs through the RNA-induced silencing complex (RISC) (2). After cellular delivery of synthetic siRNAs, the double-stranded molecules are incorporated into the RISC-Loading Complex (RLC), which consists of the RNase, Dicer and TRBP (the HIV trans-activating response RNA-binding protein) (3). During assembly of RISC, one of the two siRNA strands, referred to as the passenger strand, is released, whereas the other strand (guide strand) binds to the Argonaut protein Ago2 (4). This strand then guides RISC to its complementary target RNA, which is finally cleaved by the RNase activity located in the Ago2 protein, triggering its destruction (5–9).

It has been shown that inactive siRNAs can compete with active siRNAs (10,11). Further, Koller *et al*. have also reported that two 'active' siRNAs can compete with each other; i.e. highly potent siRNAs are able to compete with less potent siRNAs, limiting the ability of siRNAs with lower potency to mediate mRNA degradation (12). This observation may be particularly important as it suggests that some component of the siRNA pathway is present in cells in limiting quantities and some properties of siRNA molecules must support discrimination between potent and less potent siRNA duplexes.

The purpose of the current investigation is to begin to dissect the molecular mechanisms by which siRNAs are differentiated and the mechanisms supporting competition. We show that under certain conditions, siRNA competition rather than reduction of cellular RISC levels may be responsible for apparent reduction in siRNA activity, suggesting that experiments in which siRNAs are co-administered should be interpreted with caution in order to distinguish the effects of target reduction from siRNA competition. We also show that siRNA competition, which varies significantly between cell lines, is correlated with differences in the expression levels of Ago2. While cellular Ago2 levels affect both siRNA competition and potency, levels of other RISC-associated proteins do not. Utilizing ASO and siRNA-mediated reduction of RISC proteins we further demonstrate that Ago2 appears to be a limiting component responsible, at least in part, for siRNA competition. Finally, we exploit the competition between siRNAs to better understand the kinetic characteristics of RISC loading and reloading. These results identify rate-limiting components for siRNA activity in cultured cells.

MATERIALS AND METHODS

Preparation of antisense oligonucleotides and siRNAs

Synthesis and purification of phosphorothioate/2′-MOE oligonucleotides was performed using an Applied

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Biosystems 380B automated DNA synthesizer as described previously (13). All ASOs were full phosphorothioate with 2′-*O*-methoxyethyl substitutions at positions 1–5 and 16–20 (boldface type). Residues 5–15 are unmodified 2′-oligodeoxynucleotides, so they can serve as substrates for RNase H. ASOs targeted to RISC genes were identified following screening of 78 ASOs as previously described (14). The sequences of the RNAse H ASOs are as follows. Ago2 (NM_012154, RefSeq ID: EIF2C2): **CTGC TGGAATGTTTC*CACTT*** (ISIS 136764); Dicer (NM_030621, RefSeq ID: DICER1): **GCTGA**CCTTTTTGCT **TCTCA (**ISIS 138648); TRBP (NM_004178, RefSeq ID: TARBP2): **TGCGG**TGGGCTGGCC**CAGAC** (ISIS 237288); Exportin5 (NM_020750, RefSeq ID: XPO5): **GTTAC**CATTCTGTAC**AGGTA** (ISIS 350560). *Silencer* Pre-designed siRNAs for knockdown of RISC proteins were obtained from Ambion (Austin, TX, USA). Ago2: siRNA ID# 133832; Dicer: siRNA ID# 137011; Exp5: siRNA ID# 109276; TRBP: siRNA ID# 139948. PTEN (NM_000314, RefSeq ID: PTEN) and Eg5 (NM_004523,RefSeq ID:KIF11) synthetic unmodified siRNAs were purchased from Dharmacon Research, Inc. (Boulder, CO, USA). The sequence of the Eg5 siRNA is CAACAAGGAUGAAGUCUAU (sense) and AUAGAC UUCAUCCUUGUUG (antisense). The sequence of the PTEN siRNA is AAGUAAGGACCAGAGACAA (sense) and UUGUCUCUGGUCCUUACUU (antisense). The sequence of the PTEN 40-mer 'Dicer-substrate' siRNA is **AAGUAAGGACCAGAGACAA**AAA GGGAGUAACU AUUCCCAG (sense) and CUGGGAAUAGUUACT CCCUUU**UUGUCUCUGGUCCUUACUU**CC (antisense). The sequence of the 19-mer is highlighted within the 40-mer. A two-base overhang was added to the antisense strand to ensure directionality of Dicer cleavage.

siRNA/ASO treatment

Tissue culture medium, trypsin and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA, USA). HeLa, T47D and U87-MG cells were obtained from the American Type Tissue Collection (Manassas, VA, USA) and cultured in DMEM supplemented with 10% fetal calf serum, streptomycin (0.1 μg/ml), and penicillin (100 units/ml). Treatment of cells with siRNA and RNAse H ASOs was performed using Opti-MEM media (Invitrogen) containing 2–5 μg/ml Lipofectamine 2000 and the indicated amount of siRNA/ASO for 3–5 h at 37°C, as described previously (14). For the generation of IC_{50} curves, cells were seeded in 96-well plates at 4000–6000 cells/well then treated at doses ranging from 2 pM to 60 nM in half-log serial dilutions ± 2–20 nM siRNA competitor (N = 4–8/dose). Treated cells were incubated overnight. The next day total RNA was purified from 96-well plates using an RNeasy 3000 BioRobot (Qiagen, Valencia, CA, USA).

Reduction of target mRNA expression was determined by qRT-PCR performed essentially as described elsewhere (15). Briefly, 200 ng of total RNA was analyzed in a final volume of 50 μl containing 200 nM gene-specific PCR primers, 0.2 mM of each dNTP, 75 nM fluorescently labeled oligonucleotide probe, 5 μl RT-PCR buffer, 5 mM $MgCl_2$, 2 U of Platinum *Taq* DNA Polymerase (Invitrogen), and 8 U of RNase inhibitor. Reverse transcription was performed for 30 min at 48°C followed by PCR: 40 thermal cycles of 30 s at 94°C and 1 min at 60°C using an ABI Prism 7700 Sequence Detector (Applied Biosystems). To avoid artifacts based upon well to well variation in cell number, mRNA levels were normalized to the total amount of RNA present in each reaction as determined by Ribogreen assay (16) (Invitrogen). IC_{50} curves and P-values were generated using Prism 4 software (GraphPad). Sigmoidal dose response was calculated according to Y = Bottom + (Top−Bottom)/(1 + 10)^((LogEC50-X))); where X is the logarithm of concentration and Y is the response. The sequence for the human Eg5 primer/probe set used in the RT-PCR reaction is GCC CCAAATGTGAAAGCATT for the forward primer, CTAAAGTGGGCTTTTTGTGAACTCT for the reverse primer and CCTTTAAGAGGCCTAACTC for the probe. The sequence for the human PTEN primer/probe set is AATGGCTAAGTGAAGATGACAAT for the forward primer, TGCACATATCATTACACCAG-TTCGT for the reverse primer and AGATGCCGTGTTTGATGGCTCC AGC for the probe.

Primer probe sets used to analyze the levels of targeted RISC genes are as follows. Human Ago2: CCAGCTACA CTCAGACCAACAGA (forward primer), GAAAACGG AGAATCTAATAAAATCAATGAC (reverse primer) and CGTGACAGCCAGCATCGAACATGAGA (probe). Human Dicer: ATT AACCTTTTGGTGTTTGATGAG TGT (forward primer), GCGAGGACATGATGGAC AAATT (reverse primer) and ATCTTGCAATC CTAGA CCACCCCTATCGAGAA (probe). Human TRBP: CAG CCCACCGCAAAGAAT (forward primer), TGCCACTC CCAATCTCAATG (reverse primer), and CACCATGAC CTGTCGAGTGGAGCGT (probe). Human Exp5: GCTG TGAATATTCTCGGTTTGATTT (forward primer), GGAAGCTAGTTTTGGGATCCAA (reverse primer) and TCCTCCCGAGCACAACAAGG AGAGG (probe).

Western blotting

Whole cell extracts were prepared by lysing cells in RIPA buffer containing complete protease inhibitor mix (Boehringer Mannheim). Protein concentration of the cell extracts was measured by Bradford assay. Equal amounts of protein (10–20 μg) were resolved on a NuPAGE Novex 8–16% Tris–glycine gel in Tris–Gly SDS running buffer (Invitrogen Life Technologies) and transferred to PVDF membranes (Invitrogen). The membranes were blocked for 1 h in PBS containing 0.05% Tween 20 (PBST) and 5% milk powder. After overnight incubation at 4°C with a 1/1000 dilution of a mouse monoclonal antibody to Ago2, Exportin 5 (Abnova), Dicer [13D6] (Abcam), or a 1/3000 dilution of a rabbit polyclonal antibody to TRBP (gift from J. Wu), the membranes were washed in PBST and incubated with a 1/5000 dilution of goat anti-rabbit or goat anti-mouse HRP-conjugated antibody in blocking buffer. Membranes were washed and developed using ECL detection system (Amersham Biosciences). Subsequently, membranes were

blocked for 2 h at room temperature in PBST plus 5% milk powder. After incubation at room temperature with a 1/5000 dilution of a mouse monoclonal tubulin antibody (no. T-5168; Sigma-Aldrich), the membranes were washed in PBST and incubated with a 1/5000 dilution of goat anti-mouse HRP-conjugated antibody in blocking buffer and developed as detailed above and exposed to film (Kodak). Bands were quantitated by densitometry and normalized to the tubulin signal intensity.

Extracts of HeLa cells transfected with plasmids expressing each of the analyzed proteins were also evaluated by western analysis to prove the specificity of each antibody and the molecular weight of the hybridized product (Supplementary Figure 1). The plasmids expressing Dicer and Exp5 under the control of the CMV promoter were obtained from OriGene Technologies, Inc. (Rockville, MD, USA). cDNAs for Ago2 and TRBP were isolated from HeLa cell cDNA by PCR and cloned into pcDNA3.1 (Invitrogen). For RISC gene overexpression assays, 10 μg of plasmid was introduced into cells at 50% confluence in 10 cm dishes using SuperFect Reagent (Qiagen). Following a 3 h treatment, plasmid was removed and fresh DMEM added to the cells, then incubated an additional 24 h prior to harvest and preparation of RIPA extracts.

Apoptosis detection

HeLa cells were seeded in 60 mM dishes at 50% confluency. The next day cells were transfected with ISIS 138648 (anti-Dicer) at 30 nM or a control ASO. Following the transfection, cells were split and seeded in 96-well plates at 5500 cells/well. Twenty-four hours later cells were treated with 50 nM PTEN 19- or 40-mer siRNA in the presence of Lipofectamine 2000 as detailed above. One to two days following the transfection, Caspase 3/7 levels were determined using an Apo-ONE Homogeneous Caspase-3/7 Assay kit (Promega) according to the manufacturer's protocol.

RISC protein reduction and overexpression

For RISC reduction, HeLa cells were seeded in 10 cm dishes at 650 000 cells per plate. The following day, RISC-specific siRNA/ASO (above) was added at 50 nM in Opti-MEM media in the presence of 4 μg/ml Lipofectamine 2000. Following a 4-h incubation, the transfection mixture was aspirated and DMEM added to the cells. Following an overnight incubation, cells were trypsinized, then seeded in 96-well plates at 4000–6000 cells per well or harvested for analysis of target RNA/protein reduction. Cells in 96-well plates were allowed to adhere for 4 h, then siRNAs were added in the presence of Lipofectamine 2000 reagent as detailed above. For siRNA competition experiments both siRNAs were pre-mixed then added simultaneously to the cells ($N = 4$/dose).

For RISC gene overexpression assays, 10 μg of plasmid was introduced into cells at 50% confluence in 10-cm dishes using SuperFect Reagent (Qiagen). Following a 3 h treatment, plasmid was removed and fresh DMEM added to the cells. Following an overnight incubation, cells were trypsinized then seeded in 96-well plates at 4000–6000 cells per well for use in competition experiments or harvested for analysis of target RNA/protein reduction.

Recombinant human Ago2 activity assay

One nanogram recombinant Ago2 (gift from J. Wu) was incubated at 37°C with 10 nM of either PTEN 19-mer or 40-mer siRNA guide strand in cleavage buffer (10 mM Tris, pH 7.5, 100 mM KCl, 2 mM $MgCl_2$, Protease Inhibitor and 0.5 mM DTT). After 2 h, 0.1 nM ^{32}P labeled target RNA was added. After 30 min cleavage reactions were quenched in gel loading buffer (Ambion, TX, USA). Cleavage products were resolved by denaturing PAGE and quantitated with Storm 850 Phosphorimager (Molecular Dynamics).

Kinetic analysis of siRNA competition

To analyze kinetics of RISC loading, HeLa cells were seeded in 96-well plates at 4500/well. The following day, cells were transfected with 300 pM Eg5 siRNA using Opti-MEM media containing 4 μg/ml Lipofectamine 2000. The Eg5/Lipofectamine 2000 complex was removed at timepoints between 0 and 240 min from the initiation of the transfection and replaced with a mixture of 300 pM Eg5 siRNA and 10 nM PTEN siRNA ($N = 4$/timepoint). Cells were incubated 4 h, then siRNA removed and fresh DMEM added. Cells were incubated overnight then total RNA was purified and Eg5 mRNA expression evaluated by qRT/PCR.

For RISC reloading kinetics, HeLa cells were seeded in 96-well plates at a density of 4000 cells/well. The following day cells were transfected with 10 nM PTEN competitor siRNA in Opti-MEM media containing 3 μg/ml Lipofectamine 2000. After 3 h the PTEN siRNA/Lipofectamine 2000 complex was removed, the cells washed with PBS, and fresh DMEM + 5% FCS added. A 20 nM Eg5 siRNA complexed with 4 μg/ml Lipofectamine 2000, then 1/20 volume added directly to the pre-treated cells (final concentration = 1 nM). Immediately following the PTEN siRNA pre-treatment and at intervals from 2–18 h, Eg5 siRNA was transfected at 300 pM as detailed above ($N = 8$/timepoint). For each timepoint the siRNA/lipid complex was removed after 3 h, cells washed with PBS, and fed with DMEM + 10% FCS. Cells were incubated overnight, then total RNA was purified and Eg5 mRNA expression evaluated by qRT/PCR. Where indicated the experiment was performed in the presence of 25 μg/ml cycloheximide (Sigma). Inhibition of protein synthesis was confirmed by measurement of cellular incorporation ^{35}S-Translabel (MP Biomedicals).

For kinetic analysis of siRNA activity HeLa cells were seeded in 96-well plates then treated with 10 nM Eg5 siRNA as detailed above. Cells were harvested and total RNA isolated beginning at 15 min from the initiation of transfection. The transfection mixture was removed from cells at 4 h for the 7 and 18 h timepoints and complete media added. Eg5 and PTEN mRNA expression was assessed by qRT/PCR and normalized to total RNA as measured by Ribogreen assay.

RESULTS

Levels of RISC components and magnitude of siRNA competition vary between cell lines

Previous work has suggested that RISC components may be rate limiting as co-transfection of two siRNAs resulted in loss of activity (11,12). In an attempt to correlate RISC levels with observed differences in siRNA competition and activity, the expression levels of Ago2, Dicer, TRBP, and Exportin-5 (Exp5) were assessed by western blot analysis in HeLa, T47D, U87-MG cells (Figure 1). The figures below each set of duplicate lanes represent the average normalized protein expression relative to HeLa cells. While HeLa and U87-MG cells were found to have similar levels of Dicer, levels were 3–5-fold higher in T47D cells. The levels of Ago2 and Exp5 were found to be lower in U87-MG cells than in HeLa and T47D cells which had roughly equivalent amounts of these proteins. TRBP levels varied somewhat less in the cell lines tested, differing by less than 2-fold.

To determine if siRNA competition correlated with differences in expression of the various RISC proteins, the activity of Eg5 siRNA was evaluated in the absence or presence of increasing amounts of PTEN siRNA in HeLa, T47D and U87-MG cells (Figure 2). Cells were transfected with Eg5 siRNA at concentrations ranging between 20 pM and 20 nM, alone or co-transfected with the PTEN competitor siRNA at concentrations of 2, 6 or 20 nM for 4 h as detailed in 'Materials and Methods' section. Following transfection, cells were incubated overnight then harvested and total RNA isolated. To detect small changes in siRNA potency, quantitative RT/PCR was performed to assess the reduction of Eg5 mRNA. In the absence of competitor siRNA, the IC_{50} for siRNA-mediated reduction of Eg5 was similar in HeLa and T47D cells (92 ± 18 and 76 ± 15 pM, respectively) and approximately 2-fold higher in U87-MG cells (198 ± 45 pM). In all cell lines, the IC_{50}s for Eg5 inhibition increased when co-transfected with increasing amounts of PTEN competitor siRNA. When nonspecific oligodeoxynucleotide carrier was added to the transfections to equalize the total amount of nucleic acid transfected, the results were identical eliminating the possibility that the observed levels of competition might be due to differences in transfection efficiency (Supplementary Figure 2). In HeLa cells, competition with PTEN siRNA at the highest concentration of 20 nM (open triangles) resulted in an increase in the IC_{50} for Eg5 siRNA to 772 ± 227 pM, approximately 8-fold higher than observed in the absence of competitor (Figure 2A). Similarly, in T47Ds, competition with 20 nM PTEN siRNA increased the IC_{50} nearly 7-fold (Figure 2B). In contrast, a similar magnitude of competition was achieved in U87 cells using only 6 nM PTEN siRNA competitor (circles; $IC_{50} = 1.2 \pm 0.3$ nM) and an over 19-fold increase in IC_{50} was observed in the presence of 20 nM competitor ($IC_{50} = 3.8 \pm 1.5$ nM, Figure 2C).

The competitive ability of three other siRNAs targeted to PTEN was also compared in HeLa and U87 cells (Supplementary Figure 3). In all cases the amount of competition observed was higher in U87 than HeLa cells, confirming that these relationships are not limited to a single siRNA.

Ago2 levels, but not Exp5, Dicer or TRBP limit siRNA activity and the magnitude of siRNA competition

The comparatively low levels of Ago2 and Exp5 in U87 cells relative to HeLa and T47D, together with the higher levels of competition in U87 cells suggested that differences in the magnitude of siRNA competition between cell lines may be inversely correlated with Ago2 and/or Exp5 levels. To explore this hypothesis and determine if reduction in innate levels of RISC components resulted in changes in siRNA competition and potency, siRNA or RNaseH-dependent antisense oligonucleotide (ASO) inhibitors of RISC components were employed. The activity of each agent used to reduce the levels of RISC was first determined in HeLa cells. Cells were transfected with siRNAs or ASOs at concentrations ranging between 2 pM and 60 nM. Following transfection, cells were incubated overnight, then harvested and total RNA isolated. Quantitative RT/PCR was performed to assess the reduction of each targeted mRNA. siRNAs targeting TRBP and Exp5 were the most potent with IC_{50}s for target mRNA reduction of 25 ± 5 and 38 ± 9 pM,

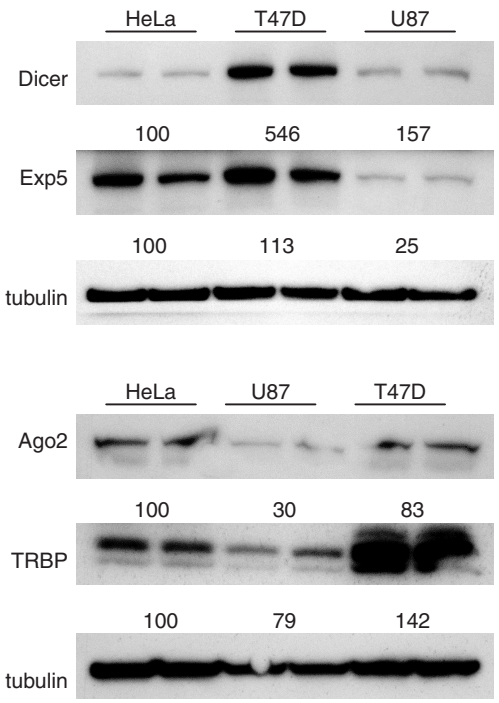
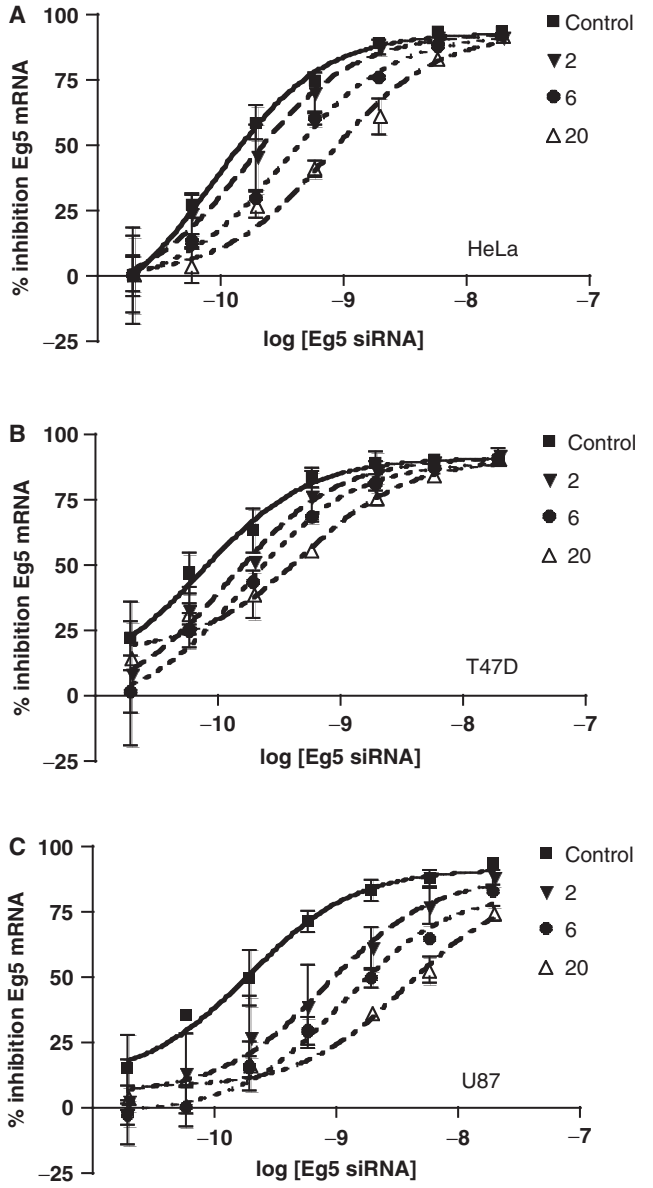


Figure 1. Protein levels of RISC components vary between cell lines. Western blots were prepared from RIPA lysates of HeLa, T47D or U87-MG cells as detailed in 'Materials and Methods' section. RISC proteins were detected using antibodies specific for Ago2, Dicer, Exp5 and TRBP proteins. An antibody to tubulin was used as a loading control and to normalize signals. Data are presented as the average percent expression for duplicate lanes relative to the levels of each protein in HeLa cells. Extracts of HeLa cells transfected with plasmids expressing each of the analyzed proteins were also evaluated by western analysis to prove the specificity of each antibody and the molecular weight of the protein (Supplementary Figure 1).

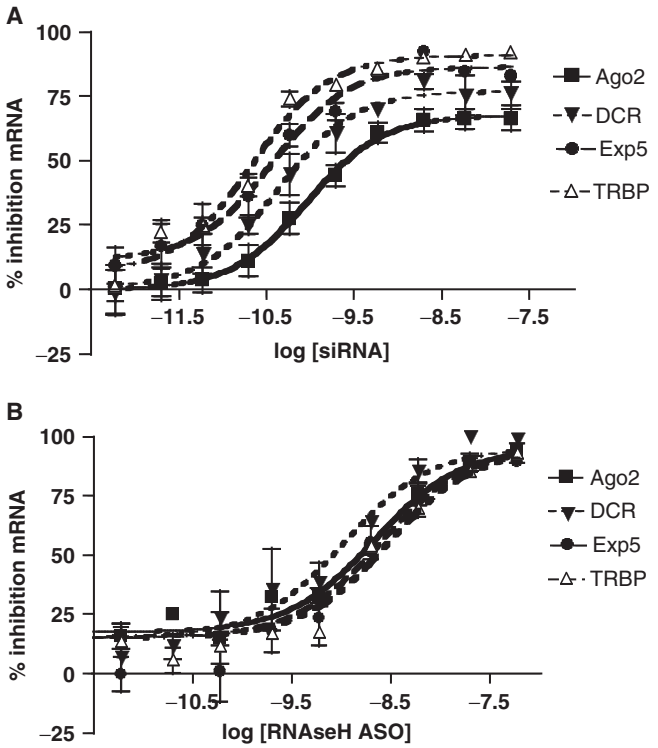


[PTEN siRNA]	0 nM	2 nM	6 nM	20 nM
	Eg5 siRNA 50% Inhibitory Concentration (pM)			
HeLa	92 ± 18	163 ± 27*	343 ± 53*	772 ± 227*
T47D	76 ± 15*	128 ± 37*	171 ± 35*	495 ± 114*
U87-MG	198 ± 45*	841 ± 292*	1160 ± 293*	3800 ± 1533*

Figure 2. The magnitude of PTEN and Eg5 siRNA competition varies between cell lines. Cells were treated at doses from 0.02 to 20 nM Eg5 siRNA in the absence or presence of PTEN competitor siRNA at a dose of 2, 6 or 20 nM for 4 h. The following day qRT/PCR was performed to assess the reduction of Eg5 mRNA. Inhibition versus Concentration curves are shown for Eg5 siRNA-mediated mRNA reduction in the presence of 0 (filled square), 2 (filled triangle), 6 (filled circle) or 20 (open triangle) nM PTEN siRNA. (**A**) HeLa cells. (**B**) T47D cells. (**C**) U87-MG cells. Calculated IC_{50}s with standard errors of the mean for Eg5 mRNA inhibition are shown at the bottom of the figure. *P*-values for IC_{50}s were also computed. Significant differences ($P \leq 0.01$) are indicated by ∗.

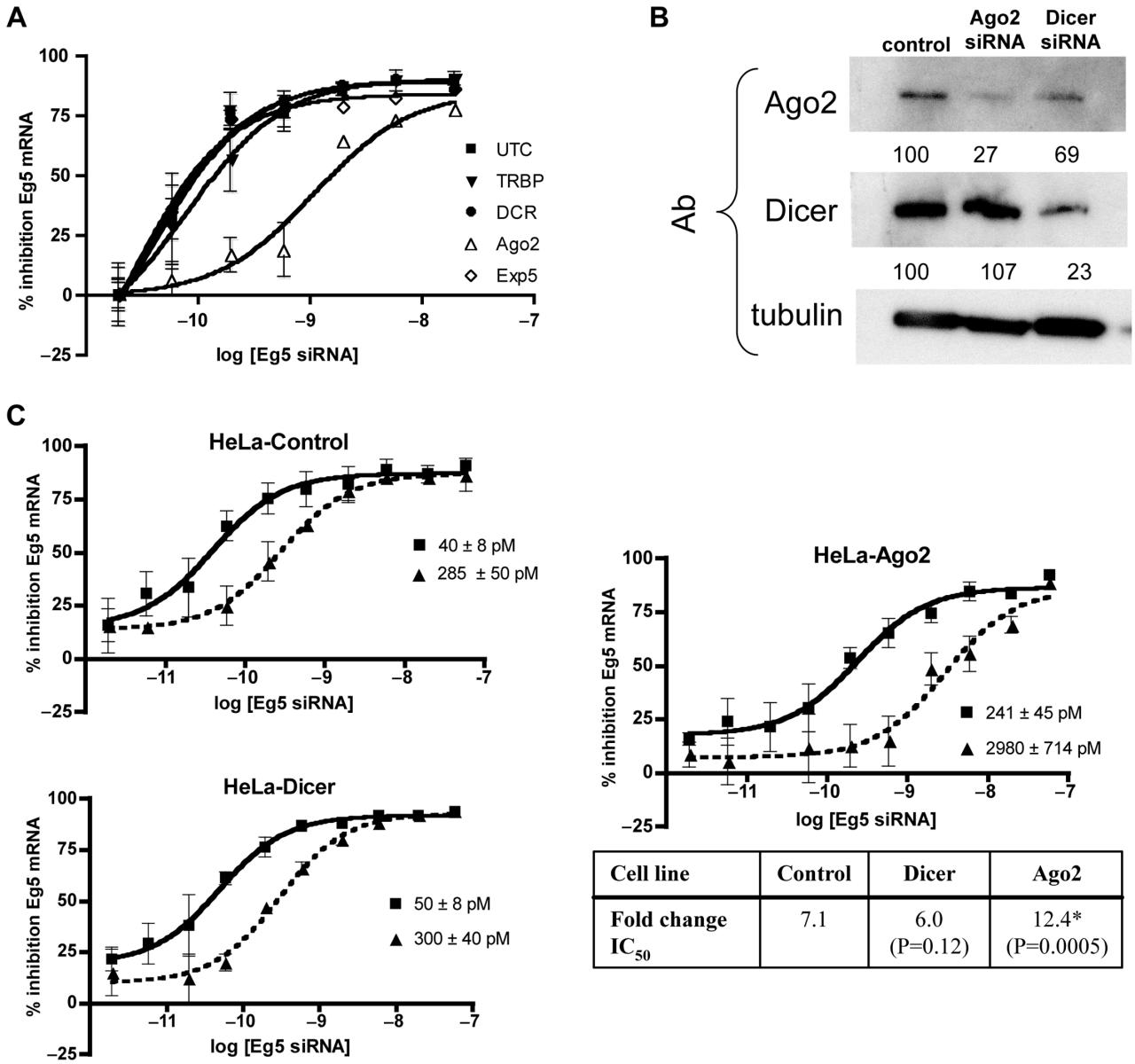
respectively (Figure 3A). The siRNA targeting Dicer was approximately 2-fold less potent with an IC_{50} of 67 ± 15 pM, while the siRNA targeting Ago2 was 4–6-fold less potent. This is not surprising as reduction of Ago2 has previously been shown to attenuate siRNA activity (17,18). ASO agents targeting RISC components had IC_{50}s in the 1–3 nM range (Figure 3B). Since these ASOs work via an RNAseH mechanism (19), reduction of RISC components would not be expected to affect their activity and, in fact, the ASO targeting Ago2 was approximately equipotent to those targeting TRBP and Exp5.

Initially, the effects of RISC protein reduction on siRNA potency were evaluated. HeLa cells were treated with ASOs targeted to TRBP, Ago2, Dicer and Exp5 at a concentration of 50 nM. Cells were split 24 h after the start of ASO treatment and seeded in 96-well plates. Cells were allowed to adhere to the plates for 4 h, then treated with siRNA targeting Eg5 at concentrations



Target	siRNA	RNAseH
Ago2	151 ± 38	2144 ± 328
Dicer	67 ± 15	1083 ± 291
TRBP	25 ± 5	2940 ± 636
Exp5	38 ± 9	2511 ± 889

Figure 3. RISC reduction. (**A**) RISC mRNA reduction by siRNAs. HeLa cells were treated with siRNAs targeted to Dicer, Ago2, TRBP or Exp5 at concentrations between 1 pM and 60 nM. After 24 h, cells were harvested and expression of the targeted RNA determined by qRT/PCR. (**B**) RISC mRNA reduction by RNAseH-dependent antisense oligonucleotides as above. All data are normalized to total RNA present as determined by Ribogreen assay. IC_{50}s (pM) are shown for each target at the bottom of the figure.



Cell line	Control	Dicer	Ago2
Fold change IC_{50}	7.1	6.0 (P=0.12)	12.4* (P=0.0005)

Figure 4. Ago2 reduction affects both potency and the magnitude of siRNA competition. (**A**) Hela cells were seeded in 10 cm dishes, then treated with ASO's targeted to TRBP, Ago2, Dicer, and Exp5 in the presence of lipid as detailed in Materials and Methods. Cells were split 24 hours after the start of ASO treatment and seeded in 96-well plates. Cells were allowed to adhere to the plates for 4 hours then treated with Eg5 siRNA at concentrations from 0.02 to 20 nM. Cells were harvested the following day and Eg5 mRNA levels assessed by qRT/PCR and normalized to total RNA as measured by Ribogreen assay to generate IC50 curves. (**B**) Levels of Ago2 and Dicer reduction in siRNA treated cells were assessed by Western blot 24 hours after initiation of treatment. The tubulin-normalized percent Ago2 and Dicer protein levels relative to control are shown below each lane. (**C**) HeLa cells were treated with siRNAs targeting Ago2 or Dicer. After 24 hours cells were seeded in 96 well plates then treated 4 hours later at Eg5 siRNA doses from 2 pM to 60 nM in the presence or absence of 10 nM PTEN competitor siRNA for 4 hours. The following day inhibition vs. concentration curves for Eg5 mRNA reduction were generated by qRT/PCR. Eg5 inhibition vs. concentration curves for HeLa cells in the presence (solid line) or absence (dotted line) of PTEN competitor siRNA. IC50's (pM) in the presence (▲) and absence (■) of competitor siRNA are shown.

from 0.02 to 20 nM. Cells were harvested the following day, RNA isolated, and Eg5 mRNA levels assessed by qRT/PCR. Reduction of Dicer, TRBP and Exp5 had no significant effect on Eg5 siRNA potency (Figure 4A) despite target RNA reduction of greater than 85% (data not shown). In contrast, Ago2 reduction resulted in an approximately 30-fold decrease in potency of the Eg5 siRNA. Effects of RISC protein reduction on siRNA competition were next evaluated. HeLa cells were treated with siRNAs targeted to Ago2 or Dicer at a dose of 50 nM to ensure maximum target reduction. Twenty-four hours later, cells were seeded in 96-well plates and IC_{50}s for Eg5 siRNA were assessed in the presence or absence of 10 nM PTEN siRNA competitor. Western blot analysis performed using the remaining cells at the time of Eg5 siRNA transfection confirmed that both Ago2 and Dicer protein levels were reduced by greater than 70% with siRNA treatment (Figure 4B). In control cells (Figure 4C,

Control), the IC_{50} for Eg5 siRNA in the absence of PTEN competitor was 40 ± 8 pM, while in the presence of competitor the IC_{50} was determined to be 285 ± 50 pM. Reduction of Dicer (-Dicer) had little effect on the siRNA activity or competition in HeLa cells with IC_{50}s in the absence and presence of PTEN competitor of 50 ± 8 and 300 ± 40 pM, respectively. In contrast, in cells in which Ago2 was reduced (-Ago2), there was a significant change in both siRNA potency and competition. The IC_{50} in the absence of PTEN competitor was determined to be 241 ± 45 pM, while competition with PTEN siRNA in the same cells resulted in an IC_{50} of 2.98 ± 0.7 nM. Comparing the Eg5 IC_{50}s in the absence of PTEN competition, there was a decrease in potency of approximately 6-fold in the cells treated with Ago2 ASO. In addition, the magnitude of PTEN competition was increased in these cells as the IC_{50} in the presence of competitor was approximately 12-fold greater than in the absence of competitor, compared to approximately 7-fold in HeLa cells with normal Ago2 levels. Therefore, the magnitude of competition was almost 2-fold greater in HeLa cells in which Ago2 levels were reduced. It is also interesting to note that Ago2 reduction in HeLa cells resulted in a change in siRNA potency and competition comparable to that observed in U87 cells (compare with Figure 2C). After reduction of Ago2 with siRNAs or ASOs, the level of Ago2 protein in HeLa cells appears to be roughly comparable to the level in untreated U87 cells (compare Figure 1 and Figure 4B).

In other experiments, Exp5 and TRBP were targeted, and reduction of these proteins had no effect on siRNA competition or potency (Supplementary Figure 4). These experimental results were also confirmed with ASO inhibitors of RISC to avoid potential artifacts of using two siRNAs. Ago2 reduction repeatedly and consistently resulted in decreases in siRNA potency and increases in competition using either ASOs or siRNAs to effect reduction of Ago2, while reduction of Dicer, TRBP and Exp5 had no effect on either parameter.

To further confirm the role of Ago2 levels with siRNA competition, the effect of Ago2 overexpression on siRNA competition was evaluated. U87-MG cells were transfected with an Ago2 expression plasmid as detailed in 'Materials and Methods' section. The following day, control and Ago2 overexpressing cells were transfected with Eg5 siRNA at doses from 2 pM to 60 nM in the presence or absence of 5 nM PTEN siRNA competitor. Increased levels of Ago2 expression in plasmid transfected cells were also confirmed by qRT/PCR. Total RNA was isolated 24 h later and IC_{50}s for Eg5 mRNA reduction determined by qRT/PCR. In control U87-MG cells, the IC_{50} was determined to be 291 ± 79 pM while in cells overexpressing of Ago2, the IC_{50} was determined to be 65 ± 18 pM; a 4–5-fold increase in siRNA potency (Table 1). The IC_{50} of the Eg5 siRNA in the presence of PTEN siRNA increased 5.6-fold in untreated cells and only 2.1-fold in cells previously transfected with Ago2 plasmid. In other experiments overexpression of Dicer, Exp5 or TRBP had no effect on the potency of Eg5 siRNA or competition due to PTEN siRNA (data not shown).

Table 1. Overexpression of Ago2 in U87-MG cells affects siRNA potency and competition

Cell line	Control		Ago2+	
10 nM PTEN siRNA	–	+	–	+
IC_{50} (pM)	291 ± 79	1640 ± 499	65 ± 18	138 ± 40
Fold change IC_{50}	5.6		2.1	

U87-MG cells were transfected with pCMV-Ago2. After 24 h Ago2 overexpression was confirmed by western blot and cells were seeded in 96-well plates then treated with Eg5 siRNA at doses from 2 pM to 60 nM ($N = 4$/dose) in the presence or absence of 10 nM PTEN competitor siRNA for 4 h. The following day inhibition versus concentration curves for Eg5 mRNA reduction were generated by qRT/PCR. Calculated IC_{50}s with standard error for control and Ago2 overexpressing cells, along with the fold change in IC_{50} in the presence of competitor are shown.

In our system, reduction of Dicer did not affect siRNA potency. Two reports in which siRNAs were used to reduce Dicer suggested that it was required for siRNA activity (17,20), but studies in which the Dicer gene was knocked out suggested that Dicer was required for miRNA processing and activity, but not required for siRNA activity (21,22). To address the possibility that the levels of Dicer protein reduction in our system were not sufficient to observe an effect on siRNA activity, the effect of Dicer reduction on the potency of the 19-mer PTEN siRNA was compared with a 40-mer RNA duplex targeting the same site. It has been shown RNA duplexes 27 base pairs and greater in length require the presence of Dicer for cleavage into 21-22 base duplexes capable of activating RISC (23). HeLa cells were treated with 50 nM ASO targeting either Ago2 or Dicer. Twenty-four hours later, the cells were seeded in 96-well plates and IC_{50}s for PTEN siRNA were assessed for the 19-mer or 40-mer PTEN siRNAs. Reduction of Ago2 and Dicer was also evaluated by qRT/PCR and found to be greater than 80% for both genes. While Ago2 reduction resulted in decreased potency of the 19-mer siRNA, reduction of Dicer had no effect (Figure 5A). Conversely, both Ago2 and Dicer reduction resulted in a similar decrease in the potency of the 40-mer siRNA (Figure 5B). To ensure that these results were not complicated by the activation of the dsRNA-dependent interferon response (24), treated cells were also monitored for apoptosis. HeLa cells were either mock transfected or transfected with 30 nM ISIS 138648 to reduce Dicer expression. The following day cells were treated with either the 19-mer or 40-mer PTEN siRNA at a concentration of 50 nM. Caspase 3/7 levels were then determined at 18 or 42 h posttransfection as detailed in 'Materials and Methods' section. Treatment with either the 19-mer or 40-mer resulted in no increased caspase levels when compared with untreated cells at any time-point (data not shown).

To confirm that processing of the 40-mer siRNA substrate was required for Ago2-mediated activity, *in vitro* cleavage reactions were performed with purified Ago2. Either PTEN 19-mer or 40-mer siRNA guide strand was incubated with ^{32}P labeled target RNA in the presence of recombinant Ago2 as detailed in 'Materials and Methods' section. Cleavage products were resolved by

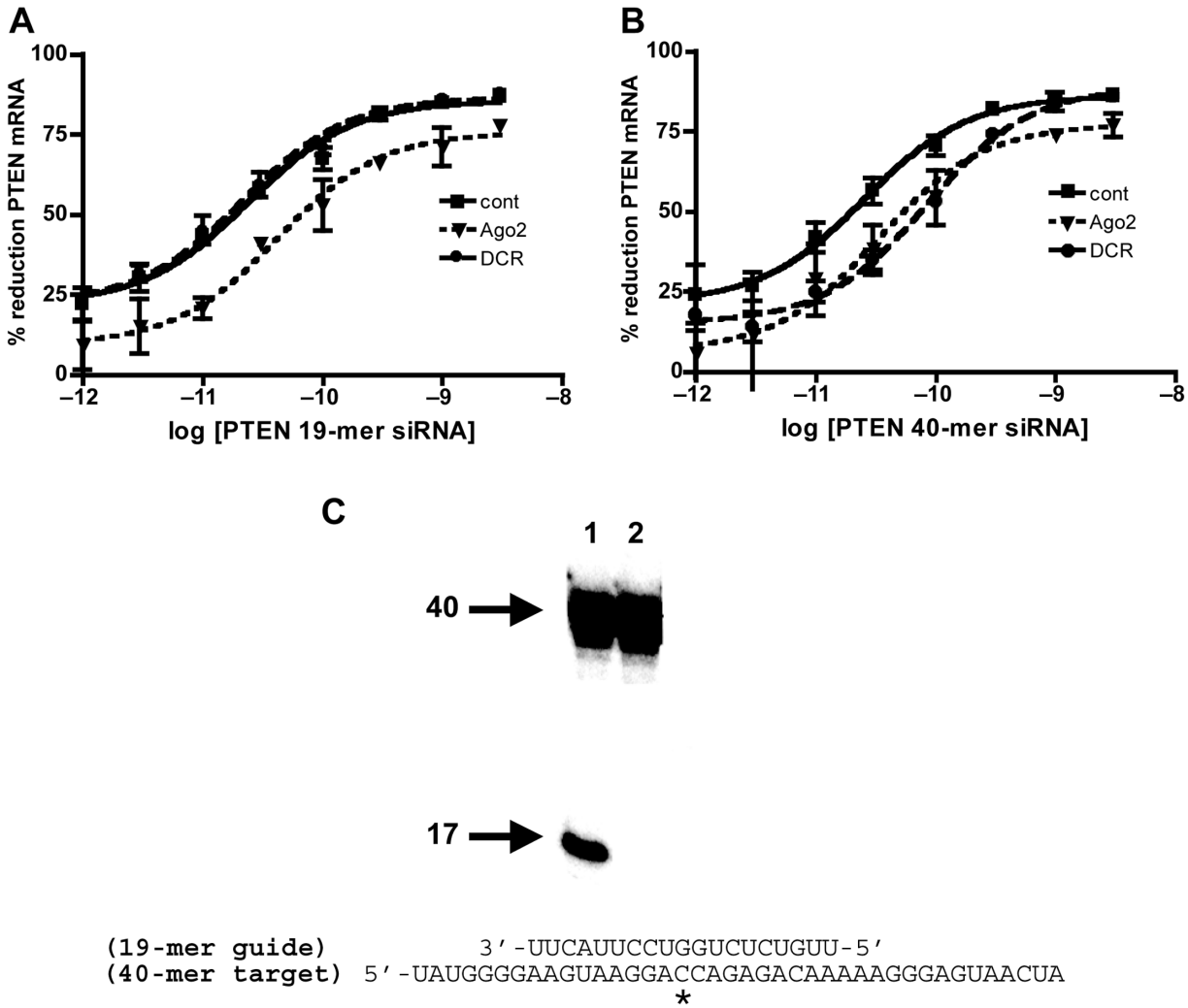
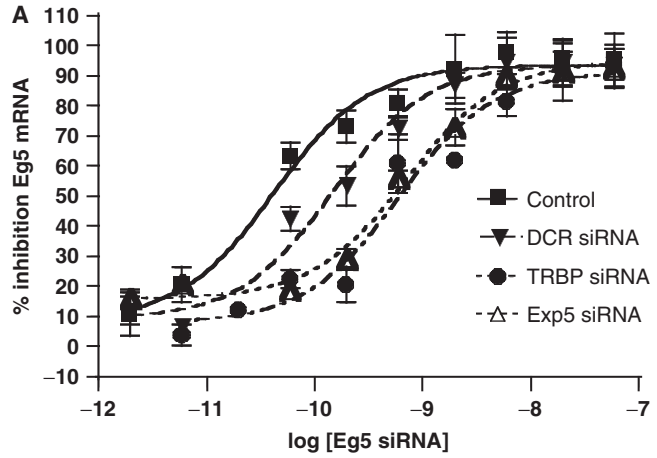


Figure 5. Dicer reduction affects 40-mer, but not 19-mer siRNA activity. (**A**) 19-mer PTEN siRNA activity was evaluated in control HeLa cells (filled square) or in HeLa cells that had been treated 24 h previously with ASOs targeting Ago2 (filled triangle) and Dicer (filled circle). (**B**) 40-mer PTEN siRNA activity in Ago2/Dicer-reduced HeLa cells. (**C**) Recombinant human Ago2 activity assay. Recombinant Ago2 was incubated with either PTEN 19-mer or 40-mer siRNA guide strand in cleavage buffer and ^{32}P labeled target RNA as detailed in 'Materials and Methods' section. Cleavage products were resolved by denaturing PAGE. Lane 1: 19-mer siRNA guide strand; Lane 2: 40-mer siRNA guide strand.

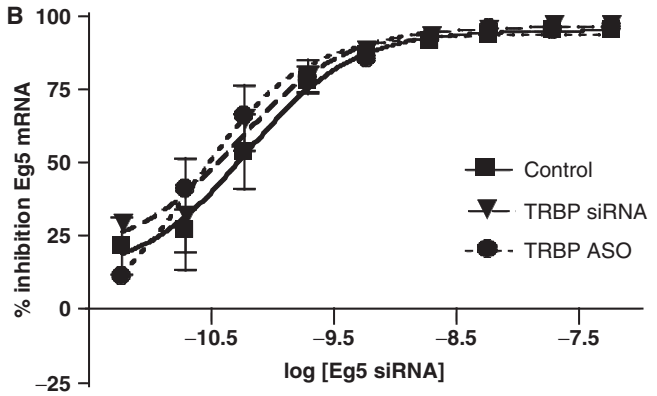
denaturing PAGE and quantitated. While the 19-mer produced a cleavage product of the expected size, no activity was observed with the 40-mer guide strand (Figure 5C). Therefore, activity observed for the 40-mer when transfected into cells must be dependent upon cleavage by Dicer to a length capable of loading Ago2 and directing target cleavage. Taken together these data suggest that while reduction of Dicer is sufficient to affect processing of a 40-base pair substrate, only Ago2 reduction affects the potency of the 19-mer siRNA.

Others have reported that, like Dicer, reduction of TRBP results in a decrease in siRNA activity (20). Since in these experiments siRNAs were co-transfected, it is possible that siRNA competition rather than reduction of cellular levels of Dicer or TRBP may be responsible for the reported reduction in siRNA activity. To address this possibility, IC_{50}s for Eg5 siRNA were determined following co-transfection with siRNAs targeting RISC genes at a concentration of 10 nM, rather than waiting 24 h between RISC siRNA and Eg5 siRNA treatment. Under these conditions Eg5 siRNA potency decreased by 3.5-fold in the presence of Dicer siRNA and over 15-fold in the presence of TRBP or Exp5 siRNA (Figure 6A).

To confirm these results, HeLa cells were transfected with either the TRBP siRNA or an RNAseH-dependent ASO complementary to the same target. Twenty-four hours later, the cells were transfected with Eg5 siRNA at doses ranging from 2 pM to 60 nM. Cells were harvested the following day and the IC_{50}s for Eg5 mRNA reduction determined. The IC_{50}s in cells treated with either the TRBP ASO or siRNA were not significantly different than in cells with no TRBP reduction (Figure 6B). Eg5 siRNA IC_{50}s were also determined in cells co-transfected with 15 nM TRBP ASO or siRNA. Under these conditions, while the ASO did not have a significant effect on the potency of the Eg5 siRNA, the potency in the presence of



siRNA	None	Dicer	TRBP	Exp5
IC_{50} Eg5 siRNA (pM)	39 ± 8	137 ± 32	639 ± 161	600 ± 138
Fold change Eg5 IC_{50}	---	3.5	16.4	15.4



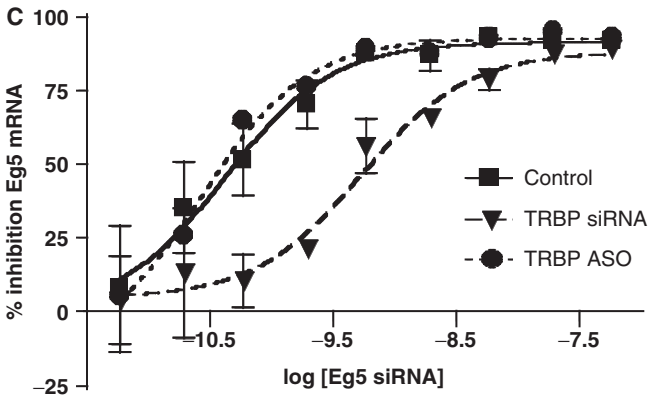


Figure 6. RISC siRNAs can compete for siRNA activity, but competition does not occur if transfection of siRNAs are separated by 24 h. (**A**) HeLa cells were treated at doses from 2 pM to 60 nM Eg5 siRNA in the absence or presence of Dicer, TRBP or Exp5 siRNA at a dose of 10 nM for 4 h. The following day qRT/PCR was performed to assess the reduction of Eg5 mRNA. Inhibition versus concentration curves are shown for Eg5 siRNA-mediated mRNA reduction with no competitor (filled square), Dicer siRNA (filled triangle), TRBP siRNA (filled circle) or Exp5 siRNA (open triangle). (**B**) HeLa cells were transfected with 15 nM siRNA or ASO targeting TRBP. After 24 h cells were transfected again with Eg5 siRNA at doses from 2 pM to 60 nM for 4 h. The following day IC_{50}s for Eg5 mRNA reduction were generated by qRT/PCR. (**C**) HeLa cells were co-transfected with 15 nM siRNA or ASO targeting TRBP and with Eg5 siRNA at doses from 2 pM to 60 nM for 4 h. The following day IC_{50}s for Eg5 mRNA reduction were generated by qRT/PCR. Eg5 siRNA only (filled square), +TRBP siRNA (filled triangle), +TRBP ASO (filled circle).

the TRBP siRNA decreased appreciably (Figure 6C). Results of experiments using Dicer siRNA were similar (data not shown). These findings reveal that siRNAs directed to RISC components can themselves compete to different extents when co-transfected with another siRNA (reporter), however, competition does not occur if there is a period of recovery of 24 h or less between transfection of the two siRNAs. When transfected 24 h apart, rather than co-transfected, only Ago2 siRNA had an effect on the potency of 19–21-mer siRNAs, suggesting that only reduction of Ago2, but not other RISC proteins, affects siRNA activity. Furthermore, these data confirm that DNA-like ASOs work via a pathway that differs from the RISC pathway and can serve as effective controls for siRNA mechanistic studies.

Kinetics of RISC loading and reloading

Since our data suggested that new RISC must become available for siRNA activity in a period less than 24 h we sought to use siRNA competition to evaluate the kinetics of RISC loading and reloading. We define 'loading' as the process between transfection of an siRNA and the induction of detectable cleavage of the target mRNA. Obviously the precise time required to load RISC is not determined. We define 'reloading' as the steps required to release an siRNA from RISC, acquire a new siRNA, and cleave the new target mRNA. To evaluate RISC loading kinetics, Eg5 siRNA activity was measured in the presence of the PTEN competitor siRNA added simultaneously or at various times after the initiation of transfection of the Eg5 siRNA. In the absence of PTEN siRNA competition, 78.3 ± 11.2% reduction of Eg5 message was observed in cells treated with 300 pM Eg5 siRNA, while in the presence of 10 nM PTEN siRNA competitor added simultaneously only 40.6 ± 11.5% reduction was observed (Figure 7A). When the addition of PTEN competitor siRNA was delayed from 5 to 30 min no effect on competition was observed. When addition of PTEN siRNA was delayed by 60 min, the percent reduction of Eg5 siRNA increased to 65.8 ± 12.5% and by 120 min the percent reduction was comparable to that observed with Eg5 siRNA treatment alone. These results indicate that the Eg5 siRNA maximally loaded the RISC available in the cells in 1–2 h. Once RISC was loaded with the Eg5 siRNA, the more potent PTEN siRNA, the guide strand of which displays higher affinity for purified Ago2, was unable to compete, indicating that the association of siRNA with activated RISC is stable. To exclude the possibility that differences in competition are the result of a decreased ability of the cells to be double transfected with time, cells were pre-treated for 2 h with the transfection mixture minus siRNA. Cells were then treated with Eg5 siRNA. As a control, cells were

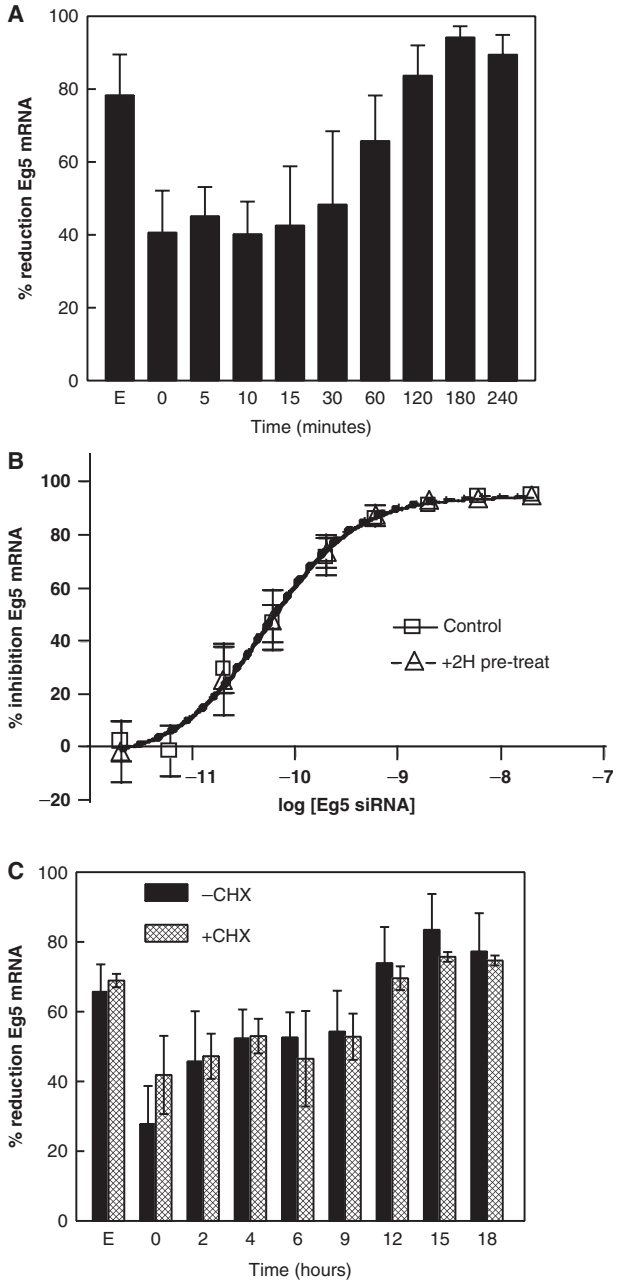


Figure 7. Kinetics of RISC loading and reloading. (**A**) RISC loading. HeLa cells were treated with Eg5 siRNA at 300 pM. 10 nM PTEN siRNA competitor was added between 0 and 240 min after the initiation of the Eg5 siRNA transfection. Transfections were carried out for a total of 5 h. Cells were harvested the following day and total RNA purified. The percent inhibition of Eg5 mRNA is shown at the various timepoints for the addition of PTEN competitor. E = Eg5 siRNA only, no competitor. (**B**) Cells were pre-treated for 2 h with the transfection mixture minus siRNA. Cells were then treated with 2 pM to 60 nM Eg5 siRNA. As a control, cells were transfected with the siRNA without lipid pre-treatment. IC_{50}s (pM) for Eg5 reduction determined the following day by qRT/PCR. Control cells (open square). Lipid pre-treated cells (open triangle). (**C**) RISC reloading. HeLa cells were treated for 3 h with PTEN siRNA. Cells were washed then treated with 300 pM Eg5 siRNA at 0–18 h following the removal of the PTEN siRNA. Transfections were carried out for 3 h. The following day cells were harvested and total RNA purified. The percent inhibition of Eg5 mRNA is shown at the various timepoints following removal of PTEN competitor siRNA. The experiment was performed in the presence (solid bars) or absence (striped bars) of 25 μg/ml cycloheximide (CHX).

transfected with the siRNA without lipid pre-treatment. IC_{50} curves for Eg5 reduction determined the following day were virtually super-imposable (Figure 7B) suggesting that the results of the RISC loading experiments are not simply a transfection artifact.

To evaluate the reloading of RISC, HeLa cells were pre-treated for 3 h with 15 nM PTEN siRNA. Cells were washed, then 300 pM Eg5 siRNA added at times following PTEN siRNA treatment. Eg5 siRNA transfections were carried out for 3 h, then the cells incubated overnight. The following day RNA was purified and the percent reduction of Eg5 mRNA determined by qRT/PCR. Cells that were not pre-treated with PTEN siRNA (E) showed greater than 65% reduction of Eg5 mRNA (Figure 7C, solid bars). In contrast, cells pre-treated with PTEN siRNA displayed approximately 28% reduction of Eg5 mRNA at the same dose when the Eg5 siRNA was transfected immediately after PTEN siRNA treatment ($T = 0$). After 2 h the percent reduction was increased from 28% approximately 46% and increased to 55% by 9 h. After 12 h, the PTEN siRNA failed to attenuate the effects of Eg5 siRNA. To determine if the loss of competition resulted from unloading of RISC or because of new synthesis of Ago2, protein synthesis was inhibited in HeLa cells in similar experiments. When cycloheximide was added to cells following the PTEN siRNA pre-treatment, Eg5 mRNA reduction followed the same pattern as in the absence of cycloheximide (Figure 7C, crosshatched bars). In these experiments, S^{35} methionine incorporation was determined to be inhibited by greater than 95% (data not shown). The similarity of recovery of Eg5 inhibition in the presence or absence of cycloheximide suggests that new protein synthesis is not required. Instead, RISC must in some manner be unloaded and recycled.

Together, these kinetic data indicate that loading of RISC after transfection is fairly rapid; within 2 h of the initiation of transfection, RISC is loaded and can result in detectable cleavage of target mRNA. RISC recycling is a slower process taking as long as 12 h to complete.

DISCUSSION

It has previously been observed that co-administration of two siRNAs can result in inhibition of siRNA activity (11). The objective of this study is to begin to understand the molecular mechanisms by which siRNA competition takes place and to use the competition between siRNAs to define basic characteristics of the RISC pathway.

In this study, we demonstrate that the levels of several protein components of the RISC pathway vary from cell line to cell line (Figure 1). The potencies of siRNAs also varied as a function of the cell lines (Figure 2). Moreover, the extent of competition between siRNAs varied in a similar fashion to potency. In the competition assay, both siRNAs were transfected simultaneously, so differences in the efficiency of transfection cannot account for these observations. Ago2 and Exp5 are significantly less abundant in U87 cells relative to HeLa and T47D cells. Only reduction of Ago2 by either a siRNA or by an RNase H-dependent ASO reduced the potency of Eg5 siRNA (and other siRNAs) and the extent of competition

(Figure 4). Reduction of Dicer, TRBP or Exp5 by more than 70% had no effect on either parameter. To confirm the unique importance of Ago2, we also overexpressed each of the proteins. Only Ago2 overexpression resulted in an increase in potency and an attenuation of competition (Table 1). These results confirm and extend previously reported observations of other investigators who demonstrated that Ago2 is an essential component for siRNA-directed RNA interference (6–8,18). Moreover, these observations appear to be generalizeable as we have studied multiple siRNAs and several cell lines and the results are consistent (Figures 2 and Supplementary Figure S3). The data from experiments in which we studied three siRNAs designed to bind to different gene targets (Figure 6A) demonstrate that these observations are not simply due to differences in the target RNAs.

Since Dicer was expressed at much higher levels in T47D than HeLa cells (Figure 1) and since the degree of competition was very similar in these cell lines (Figure 2), it is not surprising that reduction of Dicer had no effect on competition. However, in our system, reduction of Dicer did not affect siRNA potency and this differs from previous reports showing siRNA-mediated depletion of Dicer-diminished RISC-mediated reporter gene silencing (17). Another study also reported that depletion of Dicer and TRBP diminished RISC-mediated reporter gene silencing (20). In both of these studies the authors observed a decrease in reporter siRNA activity when Dicer and/or TRBP were targeted with siRNAs. Our experiments differed from the previously published experiments in which the siRNAs were co-transfected, so we hypothesized that the observed reduction in reporter activity was due to siRNA competition rather than reduction of cellular levels of Dicer or TRBP. To confirm this, we evaluated our siRNAs directed to Dicer, TRBP and Exp5 for competitive ability and found that all were efficient competitors of Eg5 when co-transfected into HeLa cells (Figure 6A and C). However, when transfection of the reporter siRNA (Eg5) was delayed by 24 h or when cells were co-transfected with an ASO inhibitor, we never observed an effect on siRNA activity (Figures 4B and 6B). It should also be noted that siRNAs, but not shRNAs, have been shown to be active in Dicer knockout ES cells (21) and that Dicer immunodepleted HeLa S100 extracts retain the ability for targeting siRNA-directed target RNA cleavage (22,25). Our data confirm siRNA activity independent of Dicer. However, the activity of a 40-mer 'Dicer-substrate' siRNA was found to be attenuated by Dicer reduction in HeLa cells (Figure 5) demonstrating Dicer protein reduction in our system was sufficient to observe an effect on siRNA activity and suggesting that a 19-21 base siRNA does not require Dicer to form active RISC.

The lack of an observed effect of Exp5 reduction on siRNA activity (Supplementary Figure 4) is in agreement with a previous study demonstrating that Exp5 overexpression enhances endogenous miRNA activity but not siRNA activity (26). More recent experiments suggest that in the presence of low siRNA concentrations, Exp5 can play a role in siRNA activity (27), however we did not see an effect of Exp5 reduction on siRNA activity or competition even at the lowest concentrations tested. Our data therefore support the current theory that Exp5 is principally involved in the nuclear export of miRNAs and shRNAs (28,29).

Using the competition assay, we have demonstrated that RISC 'loading' requires 1–2 h following the initiation of transfection (Figure 7A). Presumably during that time, the duplex interacts with components of the RISC pathway and the guide strand is ultimately translocated to Ago2 and induces cleavage of the target mRNA. For these experiments RISC loading is defined by the time when the fragment of the gene amplified by qRT/PCR is degraded. For Eg5 mRNA the siRNA target site is located towards the 5′ end of the transcript, while the qRT/PCR primer-probe set used is located in the 3′ UTR. Holen (30) has shown that some siRNAs leave cleavage fragments that may indicate a slowed RNAi cleavage complex. However, we have also employed a primer-probe set located 5′ of the siRNA cleavage site and found that the kinetics of Eg5 mRNA reduction were identical (data not shown), so it is likely that the mRNA target is rapidly degraded following siRNA cleavage.

Addition of the PTEN siRNA more than 2 h after the addition of Eg5 siRNA, resulted in no inhibition of Eg5 RNA cleavage, i.e. PTEN was no longer able to compete Eg5 siRNA from Ago2. We speculate that this is due to changes in the proteins to which the siRNA is bound and/or the nature of the binding interaction of the guide strand with Ago2. Perhaps the simplest explanation is that once the target mRNA is recruited to the guide strand loaded Ago2, the binding is essentially irreversible until the mRNA is degraded and Ago2 regenerated. Liu *et al.* (31) created mutations in the PAZ domain that abolished its ability to bind siRNAs, then followed the subcellular localization of Ago2. In contrast to wild-type Ago2, PAZ domain mutants failed to accumulate in P-bodies, suggesting that binding to RNA is critical for their localization there. It is possible that competition does not take place after mRNA binding due to differences in the cellular compartmentalization of free siRNAs and those associated with Ago2 and target mRNA in P-bodies. Obviously, other possibilities exist, and much more work is needed before we can move beyond speculation.

We also used the competition assay to determine the kinetics of RISC 'reloading'. We demonstrated that programmed RISC in HeLa cells reloaded in 9–12 h and that reloading did not require new protein synthesis, i.e. Ago2 can be recycled (Figure 7C). Why is the process so slow? RISC assembled in *D. melanogaster* extracts uses ATP hydrolysis to accelerate product release (32). However, it has been demonstrated that purified Ago2 can engage in only one catalytic round, that product cleavage is not ATP-dependent, and that product release is extremely slow or non-existent (9). These data strongly suggest that a protein other than Ago2 uses the energy of ATP hydrolysis to facilitate product release in the more complete RISC formed *in vivo*. This is similar to the behavior of Dicer (33). So the simplest explanation for the unloading kinetics is that product release, facilitated by other proteins, is extremely slow. Perhaps unloading of RISC requires degradation of the mRNA and perhaps this

can only happen when appropriate additional nucleases are recruited to the fully loaded RISC in P-bodies (34). It is also important to remember that, in fact, we are measuring reloading with Eg5 siRNA. Reloading of RISC with Eg5 siRNA can only happen when the PTEN siRNA has been degraded or diluted sufficiently that its concentration is insufficient to compete with Eg5 siRNA. Clearly, during the process we call reloading, PTEN siRNA is being degraded/diluted and the rate of that process will also affect the rate of perceived reloading. In our system, we typically see siRNA effects lasting approximately 48 h (35). We do not know why RISC reloading appears to be more rapid than the loss of siRNA effects, however the duration of effect of an siRNA is a product of the stability of the siRNA, the stability of the interaction of the siRNA with RISC, and the time required to replace the mRNA that has been lost. Perhaps by 12 h, the concentration of the just-transfected Eg5 siRNA is high enough to out-compete the PTEN siRNA still present in the cell for available RISC. However, in the absence of the newly transfected siRNA, the PTEN siRNA continues to reload and exert its effects. Again, this speculation can only be evaluated with further work.

It has previously been demonstrated that siRNA competition is related to potency; i.e. highly potent siRNAs are able to compete with less potent siRNAs, limiting the ability of siRNAs with lower potency to mediate mRNA degradation (12). We also observed that PTEN siRNA is competitively advantaged relative to Eg5 siRNA. When co-administered, the PTEN siRNA consistently led to the degradation of PTEN mRNA and reduced the degradation of Eg5 mRNA directed by the Eg5 siRNA. However Eg5 siRNA only weakly reduced PTEN siRNA activity when the siRNAs were co-transfected (Supplementary Figure 5). Since both siRNAs are blunt-ended 19-mer duplexes of unmodified RNA, transfected simultaneously into the same cells, the competitive advantage enjoyed by the PTEN siRNA must be due to its sequence relative to the Eg5 sequence. We asked whether the basis for differences between siRNAs lies in the affinity of the guide strand for Ago2 and found no correlation (Lima *et al*, submitted for publication). However, it is possible that in our system, which uses purified Ago2 protein, accessory factors are missing that are required to facilitate Ago2 binding and competition in the cell.

Clearly, this study has implications for both *in vitro* gene functionalization and *in vivo* co-administration of siRNAs. *In vitro* experiments in which siRNAs are co-administered should be interpreted with caution in order to distinguish the effects of target reduction from siRNA competition. It has been shown that i*n vivo* Ago2 levels vary between cell types (36). Therefore it is possible the potencies of siRNAs in various cells and tissues may vary widely, both because of differences in the distribution of siRNAs and the relative levels of Ago2. Further, there may be significant and unpredictable competition between siRNAs if co-administered *in vivo* depending on the relative potencies of the siRNAs used and the tissue targeted.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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