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Transcriptional Silencing by Single-Stranded RNAs Targeting a Noncoding RNA That Overlaps a Gene Promoter

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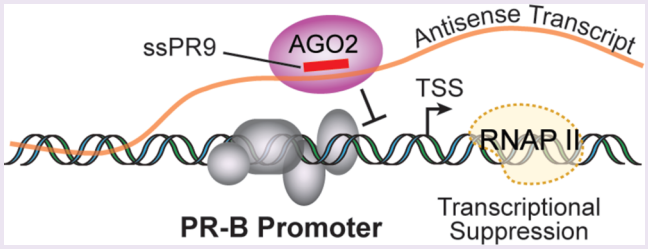
Supporting Information

ABSTRACT: RNAi using single-strand RNA would provide new options for therapeutic development and for investigating critical questions of mechanism. Using chemically modified single-strands, we test the hypothesis that single-stranded RNAs can engage the RNAi pathway and silence gene transcription. We find that a chemically modified single-stranded silencing RNA (ss-siRNA) designed to be complementary to a long noncoding RNA (lncRNA) requires argonaute protein, functions through the RNAi pathway, and inhibits gene transcription. These data expand the use of single-stranded RNA to cell nuclei.



Modulation of gene expression by double-stranded RNA (dsRNA) has the potential to be an important strategy for drug development.[1] dsRNA is routinely used as a powerful agent for silencing gene expression in cell culture, but progress in the clinic has been slowed by the need to develop lipid and other nanoparticle formulations to achieve useful levels of compound distribution to target tissues.

Antisense oligonucleotides (ASOs), by contrast, are usually observed to be a less potent and less robust silencing technology in cell culture but do not require complex formulations to achieve activity *in vivo*. ASOs are making good progress in several clinical trials using systemic delivery.[1] ASOs also contain only one strand, avoiding the need to assemble the duplex and reducing cost. An ideal gene silencing strategy would combine the simplicity and *in vivo* distribution of antisense oligonucleotides with the demonstrated ability of RNAi to efficiently silence gene expression.

Several studies have reported that single-stranded RNA can be active inside cells to block gene expression,[2−7] but potencies were low and follow-up investigations lacking. Recently, iterative design and chemical optimization of single-stranded RNA yielded single stranded silencing RNAs (ss-siRNAs) that were stable inside cells, engaged the RNAi induced silencing complex (RISC) protein machinery, silenced gene expression, and were active inside culture cells and animals.[8,9] ss-siRNAs targeting mRNA silenced PTEN expression[8] and allele-selectively blocked expression of mutant huntingtin protein.[9]

These recent studies have demonstrated that ss-siRNAs can successfully silence gene expression in cells and animals. ss-siRNAs, however, have been extensively chemically modified (Figure 1a). Most of the internucleotide linkages have phosphorothioate (PS) linkages, and every base is modified at the 2′ position. It is not clear, therefore, how broadly ss-siRNAs can be used given the diverse applications for duplex RNAs and their well-established sensitivity to the precise sequence of their target sites. Here, we challenge ss-siRNAs with a much different application: transcriptional silencing of gene expression in the nucleus.

Long noncoding RNAs (lncRNAs) are expressed throughout the genome within intergenic regions and overlapping protein-encoding mRNAs.[10] The overall importance of lncRNAs for regulating cellular processes is under intense debate.[11] It has been shown, however, that duplex RNAs complementary to gene promoters can affect transcription by associating with lncRNAs[12,13] and that promoter-targeted endogenous miRNAs are a novel class of regulatory nucleic acids.[14] The action of duplex RNAs is often assumed to occur in the cytoplasm, but in yeast, plants, and animals, it can occur in the nucleus[15] and lead to changes in transcription[16−18] or splicing.[19] In this study, we tested the hypothesis that ss-siRNAs can overcome thorough chemical modification and be used to recognize lncRNAs and regulate gene transcription.

ss-siRNAs comprise chemically modified nucleotides designed to stabilize the RNA strand against degradation by nucleases while maintaining the potential for recognition by RISC and gene silencing (Figure 1a). The modified RNA strand contains alternating 2′-fluoro (2′-*F*) and 2′-*O*-methyl (2′-*O*-Me) nucleotides with 2′-*O*-(2-methoxyethyl) (2′-*O*-MOE) modification at 5′ and 3′-termini. PS linkages are distributed throughout the strand to increase metabolic stability.

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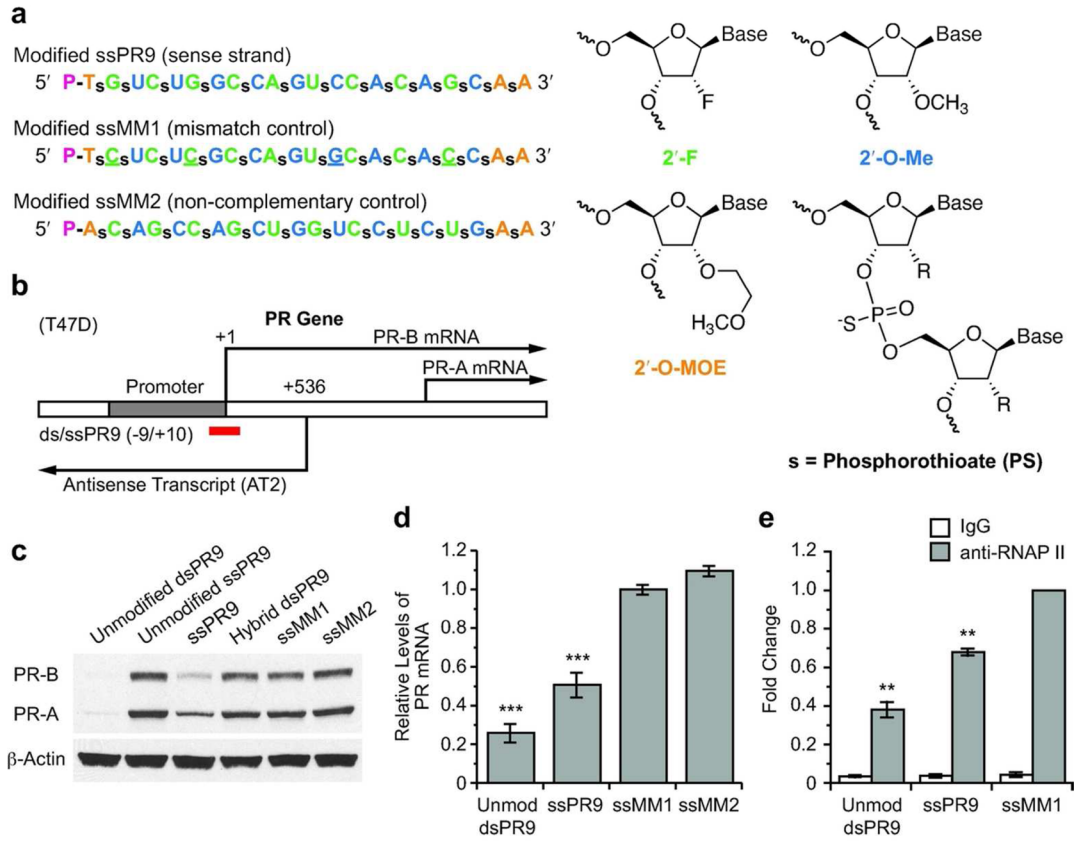

Figure 1. Inhibition of PR gene expression by ss-siRNAs targeting the PR gene promoter. (a) Sequences of chemically modified single-stranded RNAs (ss-siRNAs) used in this study (left) and chemical structures of modified nucleotides (right). Oligonucleotides are phosphorylated at the 5′ ends. Bases mismatched relative to ssPR9 are underlined. (b) Scheme of the PR gene promoter showing the target site for duplex or single-stranded PR9 within the antisense transcript (AT2). (c) Western blot analysis of PR expression after transfection with unmodified or chemically modified duplex or single-stranded RNAs (50 nM). Hybrid dsPR9 consists of ssPR9 (sense strand) and a complementary unmodified RNA antisense strand. The Western blot is representative of three independent experiments. (d) qPCR analysis after transfection of unmodified dsPR9, modified ssPR9, or modified control oligomers (ssMM1 and ssMM2) (50 nM), $n = 3$. (e) Chromatin immunoprecipitation (ChIP) for RNA polymerase II after treatment with unmodified dsPR9, modified ssPR9, or modified ssMM1, $n = 3$. Error shown is SD: **, $P < 0.01$; ***, $P < 0.001$ (t test).

Duplex RNAs complementary to gene promoters can inhibit gene transcription.[12,17] Silencing transcription in the nucleus by targeting a promoter is fundamentally different from silencing translation in the cytoplasm by targeting mRNA and offers a stringent test for the general application of ss-siRNA. We have extensively studied inhibition of progesterone receptor (PR) expression by promoter-targeted RNAs.[13,17,20] We designed an ss-siRNA (ssPR9) to mimic duplex RNA PR9 (dsPR9), a benchmark promoter-targeted RNA for modulating PR transcription. PR9 spans the region −9 to +10 relative to the most upstream transcription start site for PR, but there is no evidence that dsPR9 binds directly to the PR promoter. Instead, RNA immunoprecipitation has demonstrated that dsPR9 binds to an antisense transcript that overlaps the PR promoter and is synthesized in an opposite orientation (Figure 1b).[13]

We transfected ssPR9 into T47D breast cancer cells using cationic lipid. After four days, we harvested cells and discovered that ssPR9 inhibited PR expression with an efficacy similar to that produced by the parent duplex PR9 (Figure 1c). A hybrid duplex consisting of ssPR9 and a complementary unmodified RNA strand was inactive, suggesting that the chemical modifications needed for activity of ssPR9 block successful processing of the duplex. This result suggests that the rules governing the activity of duplex RNAs will not always govern the action of ssRNAs and that experimenters should be flexible in their design and use.

An unmodified single-stranded RNA analogous in sequence to ssPR9 that lacked chemical modifications was not an inhibitor, emphasizing the importance of chemical modifications for activity. ss-siRNAs with the same pattern of chemically modified bases but that were noncomplementary (ssMM2) or contained mismatches relative to ssPR9 (ssMM1) did not inhibit PR gene expression, consistent with a requirement for complementarity to the PR promoter.

We next examined whether inhibition occurred before or after RNA synthesis. Using quantitative PCR, we observed reduced levels of PR mRNA upon treatment with ssPR9 (Figure 1d). Chromatin immunoprecipitation with an anti-RNA polymerase II (RNAP II) antibody revealed reduced levels of RNAP II at the PR promoter (Figure 1e), consistent with inhibition of transcription. These results suggest that a passenger strand is not necessary for transcriptional silencing by ss-siRNAs in cell nuclei.

We next examined the potency and duration of promoter-targeted ss-siRNA and dsRNA action. PR has two isoforms, PR-B and PR-A, that are translated from mRNAs that initiate at different transcription start sites.[21] The PR-B transcription start site is upstream from PR-A, and we have previously shown that inhibition of PR-B expression results in silencing PR-A

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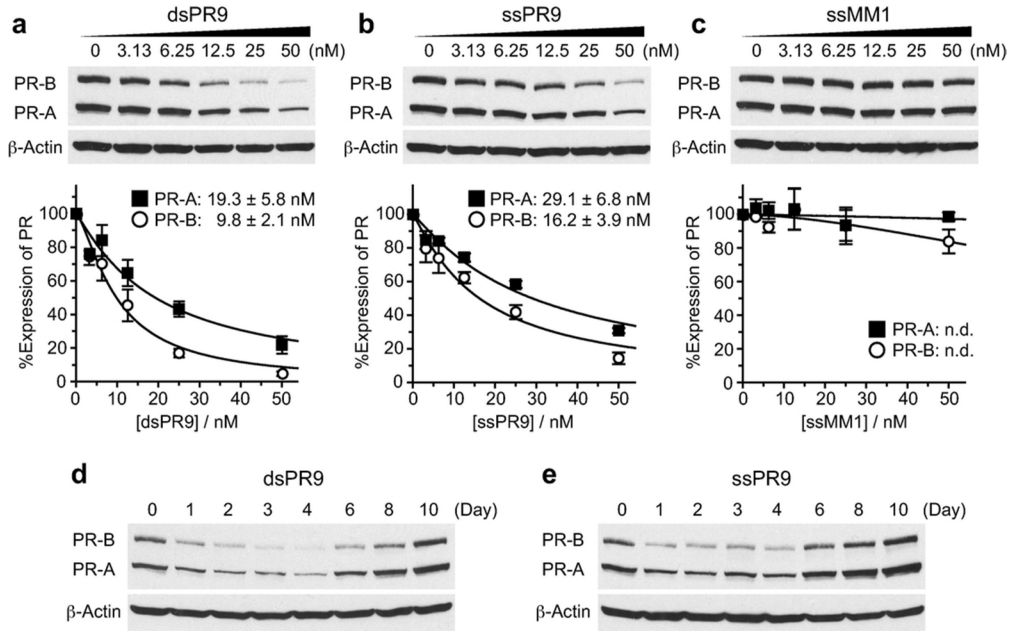

Figure 2. Potency and duration of effect for dsPR9 and ssPR9. (a–c) Western blots and their quantitation showing dose response profiles for unmodified dsPR9 (a; $n = 4$), modified ssPR9 (b; $n = 4$), and modified mismatch control ssMM1 (c; $n = 3$). Dose response data were fit to the following model equation: $y = 100(1 - x^m/(n^m + x^m))$, where y is percent expression of protein and x is concentration of oligomers. m and n are fitting parameters, where n is taken as the IC_{50} value. n.d. = not determined. (d,e) Western blots showing time-course profiles for unmodified dsPR9 (d; $n = 2$) and modified ssPR9 (e; $n = 2$). The Western blots are representative of independent replicates. Each oligomer was transfected into T47D cells at 50 nM using Lipofectamine RNAiMAX. Error shown is SD.

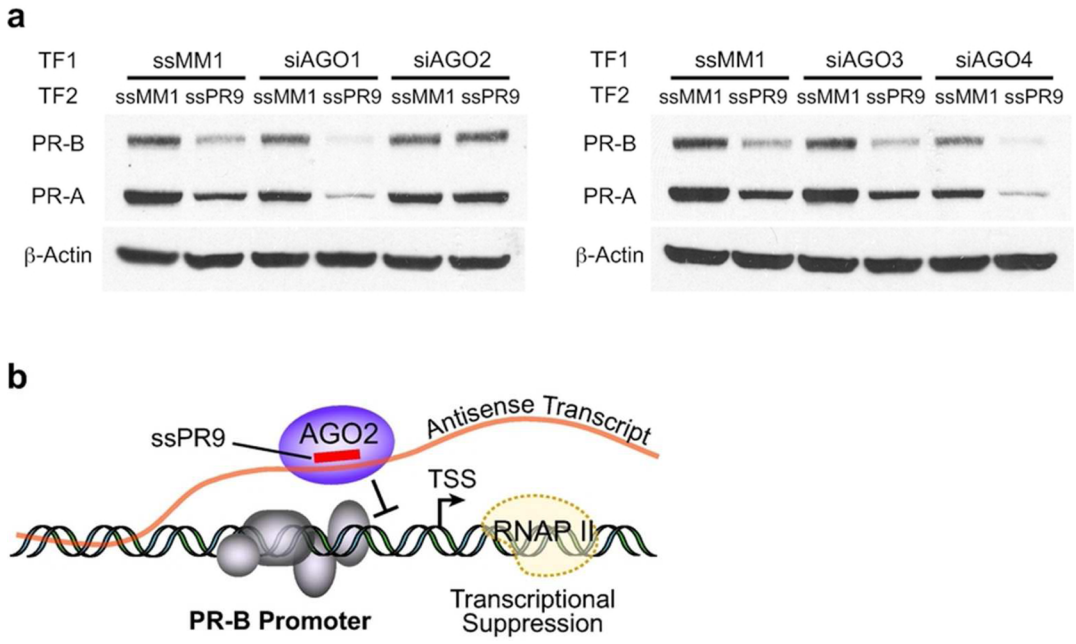

Figure 3. Requirement for AGO proteins during inhibition of PR expression by ssPR9. (a) ssMM1, siAGO1, siAGO2, siAGO3, or siAGO4 was transfected into T47D cells at 25 nM on day 0 (TF1). Two days later, second transfection (TF2) was performed using 50 nM mismatch ssMM1 or ssPR9. Cells were harvested for Western blot on day 6. The Western blots are representative of three independent experiments. Potency and selectivity of siAGOs were confirmed before this experiment (Supplementary Figure 1). (b) Schematic illustration of AGO2-mediated transcriptional silencing of PR by ssPR9.

regardless of the silencing method targets the gene promoter or PR mRNA.[17] PR-B contains a TATA-less promoter, and several different transcription start sites are spread out over a region covering approximately 50 bases.

We transfected RNAs into cells and measured the effect of RNA concentration on inhibition of PR expression. We found that ssPR9 silenced PR-B expression with an IC_{50} value of 16 nM and PR-A with an IC_{50} value of 29 nM (Figure 2b). These potencies are within 1.5–2-fold of those produced by analogous dsPR9 (Figure 2a). The mismatch-containing ss-siRNA caused little or no reduction in PR expression (Figure 2c).

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To establish the time-dependence of inhibition, we examined PR expression at up to 10 days after introducing RNA into cells. We found that inhibition of PR expression by ssPR9 was observed one day after transfection into T47D cells and persisted until day 6, similar to the timecourse for inhibition by dsPR9 (Figures 2d and 2e). Our data on the effect of varying RNA concentration and incubation time reinforce the conclusion that transcriptional silencing by ssPR9 is similar to unmodified duplex RNA PR9. This similarity is achieved in spite of heavy chemical modification and the absence of a passenger strand.

Previous studies had shown that chemically modified ss-siRNAs could function through the RNAi pathway to silence expression of mRNA. We examined whether the RNAi mechanism was also the foundation for transcriptional silencing by ss-siRNAs. To do this, we tested involvement of argonaute (AGO) protein. AGO is a central protein factor responsible for RNAi. There are four AGO variants in human cells, AGO1−4. AGO2 is the catalytic engine driving RNAi in the cytoplasm and post-transcriptional silencing of mRNA.[22,23] AGO proteins also exist in cell nuclei. In the nucleus, both AGO1 and AGO2 have been reported to be responsible for transcriptional gene silencing[20,24] with AGO2 identified as necessary for silencing PR expression by dsPR9.[20]

In an initial transfection, we used duplex RNAs targeting mRNAs encoding AGO1−4 to reduce AGO expression in T47D cells (Supplementary Figure 1). In a second transfection, we introduced ss-siRNA and tested whether reducing levels of AGO proteins would affect the efficiency of ss-siRNA-mediated inhibition of gene expression. We observed that reducing cellular levels of AGO1, AGO3, or AGO4 protein inside cells had no effect on subsequent gene silencing by ssPR9 (Figure 3a). By contrast, reducing levels of AGO2 blocked gene silencing by ssPR9. This data suggests that, like its double-stranded counterpart PR9, ssPR9 requires AGO2 protein and that silencing proceeds through a mechanism related to RNA interference (Figure 3b).

ssPR9 is chemically modified on almost every base and internucleotide linkage. It was previously shown that this heavy modification would not affect its ability to enter the RISC complex, target mRNA, and promote post-transcriptional gene silencing in the cytoplasm. Our laboratory targeted a trinucleotide repeat region and showed that inhibition could be achieved by a miRNA-like mechanism.[9] Lima and co-workers targeted a unique sequence within mRNA with a fully complementary ss-siRNA and reported gene silencing through an siRNA-like mechanism involving cleavage of the target transcript.[8]

Our data here show that extensive chemical modification is also compatible with entry into the nucleus and transcriptional gene silencing. ss-siRNAs, therefore, appear to be able to substitute for duplex RNAs in a wide variety of gene silencing applications. The activity of ssRNA in this application, however, is less potent (by 1.5−2-fold) than analogous duplex RNA, and the robustness of ssRNA will need to be evaluated on a case by case basis. More work will be needed to understand whether ss-siRNA function can always be predicted from the action of double stranded RNAs and to improve the potency of ss-siRNA action.

Successful silencing of genes in the clinic will require agents that maximize potency, biodistribution, and synthetic simplicity. We have shown that ss-siRNA can function through the RNAi pathway and inhibit gene transcription with a potency similar to that produced by duplex RNA. ss-siRNAs require extensive chemical modification to be active inside cells. Our data show that, in spite of this modification, they can achieve potent gene silencing not only by blocking translation in the cytoplasm but also by inhibiting gene transcription in the nucleus. The diverse activities of ss-siRNA suggest the potential to be widely applied agents for controlling gene expression and a novel silencing strategy for developing therapeutics.

METHODS

Cellular Delivery of Duplex/Single-Stranded RNAs and Gene Expression Assays. T47D cells (ATCC) were cultured at 5% CO_2 in RPMI-1640 (Sigma) supplemented with 10% (v/v) FBS (Sigma), 10 mM HEPES (Sigma), 0.5% (v/v) NEAA (Sigma), 10 μg mL^{-1} insulin (Sigma), and 1 mM sodium pyruvate (Sigma). Cells were plated in 6-well plates at 100,000 cells/well in RPMI-1640 2 days before transfection. Lipofectamine RNAiMAX (invitrogen) was used to deliver duplex RNAs or chemically modified single-stranded RNAs into T47D cells. Cells were harvested 3 days after transfection for qPCR and 4 days after transfection for Western blot. For double transfection experiments, siAGO1−4 or mismatch oligomer ssMM1 was transfected using reverse transfection protocol. Two days later, ssPR9 or ssMM1 was transfected using forward transfection protocol. Cells were harvested 4 days after the second transfection. Sequences of duplex and single-stranded RNAs are listed in Figure 1a or Supplementary Table 1.

Quantitative PCR (qPCR). Total RNA from T47D cells were isolated using TRI Reagent (Sigma). Two micrograms of total RNAs were treated with DNase I (Worthington) to remove genomic DNA. Treated RNAs were reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (ABI). qPCR was performed using TaqMan Gene Expression Assay (ABI) with 50 ng of cDNA as template.

Western Blot. Cells were lysed and protein concentrations were quantified by BCA assay (Thermo Scientific). Western blots were performed with 30 μg of protein per well. Primary antibodies used for immunoblotting were antiprogesterone receptor (6A1) mouse mAb (#3172, Cell Signaling) and anti-β-actin (A5441, Sigma). Protein was visualized using horseradish peroxidase-conjugated antimouse antibody (#715-035-150, Jackson Immunolabs) and supersignal developing solution (Thermo Scientific).

Chromatin Immunoprecipitation (ChIP). T47D cells were seeded at 1,500,000 cells in 15 cm dishes. Two days later, cells were transfected with duplex or single-stranded RNAs at 50 nM using Lipofectamine RNAiMAX. Three days after transfection, cells were cross-linked with 0.1% formaldehyde for 10 min and harvested. Cell nuclei were isolated using hypotonic lysis buffer (4 mL; 10 mM Tris-HCl [pH 7.5], 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% (v/v) NP-40). Nuclei were lysed in lysis buffer (1 mL; 1% (w/v) SDS, 10 mM EDTA, 50 mM Tris-HCl [pH 8.1], and 1× Roche protease inhibitors cocktail) and sonicated (2 pulses, 20% power, 20 s).

The cell lysate (40 μL) was incubated overnight with 2 μg of anti-RNA polymerase II antibody (05-623, Millipore) or normal mouse IgG (12-371, Millipore) in immunoprecipitation buffer (1 mL; 0.01% (w/v) SDS, 1.1% (v/v) Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl [pH 8.1], 167 mM NaCl, and 1× Roche protease inhibitors cocktail). After the antibodies were recovered with 40 μL of Protein G Plus/Protein A Agarose Beads (Calbiochem), the beads were washed with 1 mL of low salt (0.1% (w/v) SDS, 1% (v/v) Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.1], and 150 mM NaCl), high salt (see low salt but with 500 mM NaCl), LiCl solution (0.25 M LiCl, 1% (v/v) NP-40, 1% (w/v) deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl [pH 8.1]), and TE buffer (pH 8.0). Protein was eluted twice with 250 μL of elution buffer (1% (w/v) SDS and 0.1 M $NaHCO_3$) for 10 min at RT. Cross-linking was reversed by adding NaCl to a concentration of 200 mM and heating at 65 °C for at least 2 h. Protein was digested by incubating with Proteinase K (20 μg; invitrogen) at 42 °C for 50 min, followed by phenol extraction using an equal volume of phenol/chloroform/isoamyl alcohol. DNA in the

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aqueous layer was precipitated using 1/10 volume 3 M sodium acetate (pH 5.5), 2.2 volumes of ethanol, and glycogen (30 μg; ambion). The pellet was resuspended in 150 μL of nuclease-free water. qPCR was performed using iTaq SYBR Supermix (Biorad) and primers specific for the PR gene promoter (−37/+66; 5′-CCTAGAGGAG-GAGGCGTTGT-3′; 5′-ATTGAGAATGCCACCCACA-3′).

■ ASSOCIATED CONTENT

Supporting Information

Confirmation of potency and selectivity for siRNAs targeting AGO1-4 mRNA; sequences of RNAs used in this study. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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