# DNA-modified siRNA-dependent gene silencing with reduced off-target effect is induced through a pathway parallel to that for siRNA-mediated RNA interference

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**Abstract:**
**RNA interference (RNAi) is mediated by 21 nucleotides of short interfering RNA (siRNA) through sequence-specific cleavage of the cognate transcript. The position-dependent function of ribonucleotide residues in siRNA was analyzed by systematic DNA substitution. The results indicated that eight nucleotides from the 5' end of the guide strand and its complementary sequence are replaceable with DNA counterparts without any substantial loss of gene silencing activity. However, the remaining duplex including the 3' end of the guide strand could not be replaced with DNA, probably because of binding of RNA-binding proteins such as Argonaute and TRBP2. In addition, due to the reduced stability of a DNA–RNA hybrid other than the RNA duplex, previous studies have reported that the guide strand of the DNA-modified siRNA was, in most cases, incapable of exerting unintended off-target gene silencing (Ui-Tei *et al.*, *Nucleic Acids Res.* 36, 2136-2151, 2008). Argonaute and TRBP2 were found to be necessary for inducing both nonmodified and DNA-modified siRNA-mediated gene silencing. Although the major target cleavage sites by nonmodified and DNA-modified siRNAs were identical to each other, transcript cleavage at minor sites was prevented by the presence of the DNA arm. Unlike the 5' end of the nonmodified-siRNA guide strand, the 5' end of the guide strand of DNA-modified siRNA appeared dispensable for seed positioning on the Argonaute surface.**

## 1. INTRODUCTION

RNA interference (RNAi) is an evolutionarily conserved process induced by double-stranded RNA (dsRNA) [1]. In mammals, short interfering RNA (siRNA), 19 bp in length dsRNA with 2-nucleotide 3' overhangs, is widely used for inducing RNAi [2]. In *Drosophila* cells, a heterodimer of Dicer2 and R2D2 is thought to sense the differential stability of siRNA duplex ends in determining the guide strand [3–7]. The Dicer2/R2D2 dimer in the RNA-induced silencing complex (RISC) loading complex (RLC) is replaced by Argonaute (Ago) while siRNA undergoes unwinding. The 5' and 3' ends of the guide strand may be anchored in pockets formed in PAZ and Mid domains of Ago, respectively [5, 8, 9]. The 5'-proximal "seed" nucleotides [10], which occupy positions 2–8 measured from the 5' end and are present on the surface of the Mid-PIWI-L1 lobe in a quasi-helical form, may serve as the nucleation site for mRNA [8]. In mammals, target RNA is cleaved by the RNase activity within Ago2 that forms the core of RISC [11,12]. siRNA with only seed sequence homology to the target is also capable of inducing gene silencing [13]. This seed-dependent gene silencing may be the major cause of the off-target effect [14–16].

Previously, we systematically examined the effects of deoxyribonucleotide substitutions in siRNA on gene silencing [17]. The 5' proximal eight nucleotides containing the seed sequence of the guide strand and the complementary nucleotides could be replaced with their DNA counterparts without substantial loss of gene silencing activity. Surprisingly, virtually no off-target effects were associated with these DNA-modified siRNAs, indicating that the DNA seed arm in RISC may exert very few off-target effects.

Here, we present additional experimental data essential to further understand the molecular mechanism of gene silencing due to DNA-modified siRNA. These results, along with those of previous experiments [17], suggest that DNA-modified siRNA-dependent gene silencing is induced in transfected cells through a series of molecular reactions very similar to that for authentic RNAi.

## 2. EXPERIMENTAL PROCEDURES

### 2.1 Synthesis of siRNA and DNA–RNA chimeras, construction of psiCHECK-sm plasmids and seed sequence activity assay

siRNAs and DNA–RNA chimeras were synthesized by and purchased from Promega. The construction of psiCHECK plasmids and the seed sequence assay have been described previously [17].

### 2.2 RT-PCR

The plasmids pGL3-Control (1 μg) and pRL-SV40 (10 ng) were introduced into HeLa cells using Lipofectamine 2000 (Invitrogen) with 5 nM siLuc-36 or 50 nM cognate DNA-modified siLuc-36 along with 50 nM siRNAs for human Dicer, Ago1, 2, 3, 4, TRBP2 and PACT. RNA was extracted 24 h after transfection using an RNeasy kit (QIAGEN). Possible changes in the relative fractions of each RNA were examined by RT-PCR using an RNA LA-PCR kit (Takara).

### 2.3 Target cleavage assay

The target sequences for firefly *luc* siRNAs, siLuc2-153 and siLuc-36, were inserted into the *Eco*RI/*Xho*I sites of pTREC [18] to generate pTREC-2-153 and pTREC-36, respectively. The constructs (0.5 μg) were introduced by transfection into

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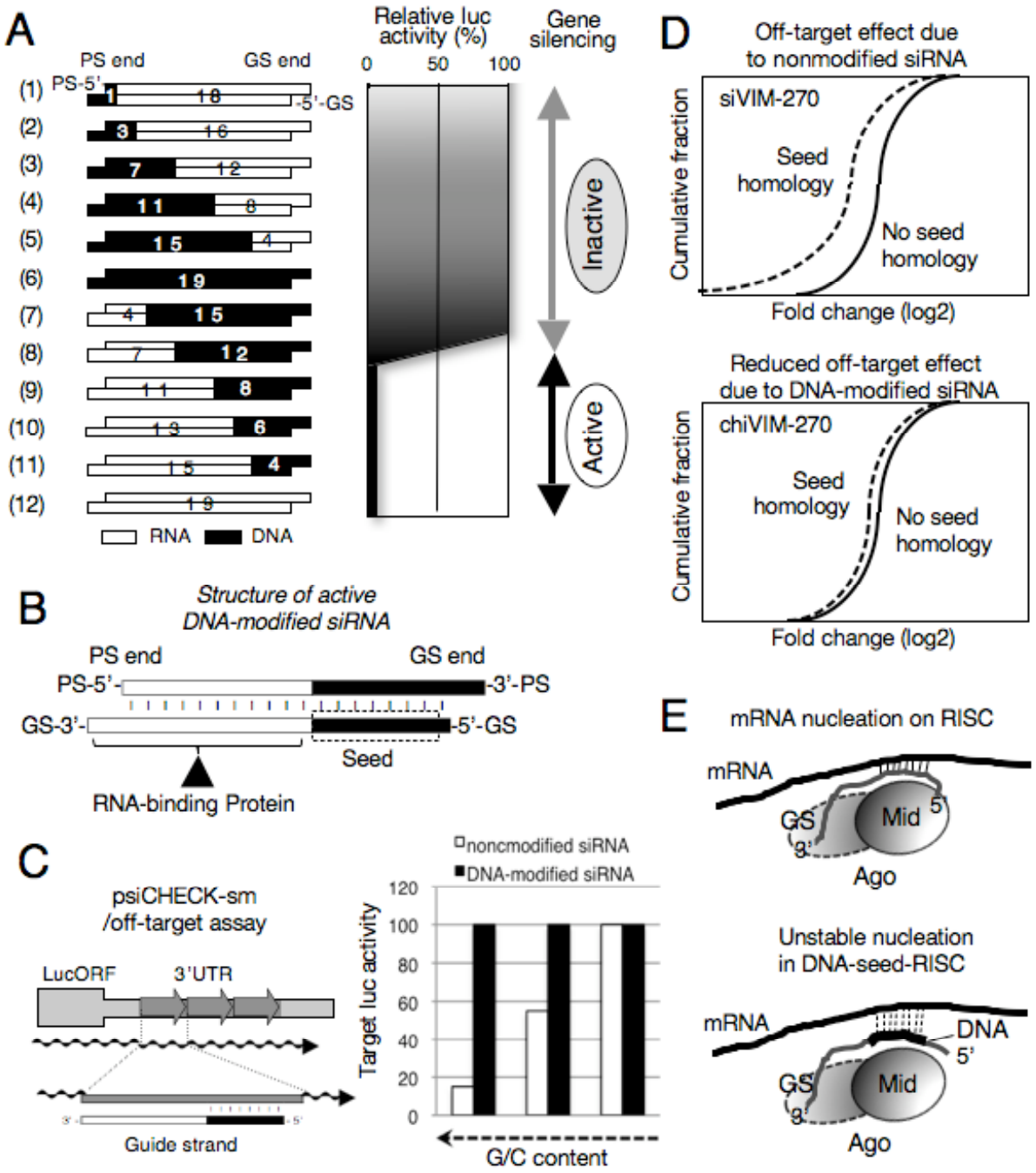


**Fig. 1** Basic framework of DNA-modified siRNA-dependent gene silencing. (**A**) Effects of DNA replacement on gene silencing activity. Passenger (PS) and guide (GS) strands are shown on the top and bottom, respectively. Open rectangles, RNA; Filled rectangles; DNA. GS end, the end of the siRNA containing the 5' end of the guide strand; PS end, siRNA end containing the 5' end of the passenger strand. The gene silencing activity of modified siRNA with less than 9 bp of DNA from the GS end is essentially identical to that of nonmodified siRNA. (**B**) Structure of active DNA-modified siRNA. (**C**) Reporter-dependent off-target assay. Note that virtually no off-target effect is associated with DNA-modified siRNA treatment. (**D**) Microarray profiling of seed-dependent off-target activity. In contrast to nonmodified siRNA treatment, DNA-modified siRNA treatment was associated with almost no unintended gene silencing. (**E**) Unstable mRNA/RISC nucleation complex formation by DNA-modified siRNA.

HeLa cells with or without cognate siRNA or DNA-modified siRNA, and cells were recovered 24 h after transfection. Total RNA was prepared using QuickPrep Micro mRNA Purification kit (Amersham). Primer extension was carried out using $^{32}$P-labelled primer (5'-CTCGAAGCATTAACCCTCACT-3') and a Primer Extension System-AMV Reverse Transcription kit (Promega). Reaction products were size-fractionated by

electrophoresis on 6% polyacrylamide gel containing 7 M urea in TBE buffer. Cleavage sites were determined by paralleled sequence gel electrophoresis. DNA sequence analysis was performed using a Thermo Sequenace Fluorescent Labelled Primer Cycle Sequencing kit (Amersham), although fluorescent-labelled dideoxyribonucleotides were replaced with $^{32}$P-labelled dideoxyribonucleotides.

**2.4 Electrophoresis gel mobility shift analysis**

A DNA fragment corresponding to the PAZ domain of human Ago1 (amino acid residues 222–376) was amplified from a cDNA mixture of total RNA extracted from HeLa cells by PCR using the following primers: 5'-CCTGGGATCCTTTTATAAGGCACAGCCAGTGATTG-3' and 5'-GGAGGAGGCGGCCGCAGGCAGCAGCCACCACCAC CACCACCACTGACTCCTCCTGTCTGTCTGGAGCGGA TCTAGC-3'. The amplified fragment was digested with *Bam*HI and *Not*I, and inserted into pGEX-6P2 (Amersham). The PAZ domain was expressed in the *Escherichia coli* BL21 codon plus (Stratagene) as a glutathione S-transferase (GST) fusion protein and purified with a glutathione Sepharose column (Amersham) according to the manufacturers' instructions. The region encoding the full length human TRBP2 was amplified by PCR using the following primers: 5'-TTTTTTTTTTCATATGCTGGCCGCCAACCCAGGCAA GA-3' and 5'-TTTTCCTTTTGCGGCCGCTCACTTGCTGCCTGCCATG ATCTTGAGGTA-3'. The amplified fragments were digested with *Nde*I and *Not*I and cloned into pET-28a (Novagen). TRBP2 protein was produced in Rosetta (DE3) pLysS as an amino-terminal hexa-histidine-fusion protein, and purified with NTA agarose. Binding to siRNA or its derivatives (5 fmol/µl) was carried out in 25 mM Tris-HCl (pH 8.0), 150 mM NaCl, 125 mM imidazole, 13 % glycerol, 50 ng/µl salmon sperm DNA, and 2 U/µl RNasin for 30min at room temperature. The mobility shift assay was conducted on a 5% polyacrylamide gel and analyzed quantitatively using an FLA-2000 image analyzer (Fujifilm).

## 3. RESULTS AND DISCUSSION

**3.1 Basic framework of DNA-modified siRNA-dependent gene silencing associated with little off-target effect**

In our previous study, we discovered and characterized the basic properties of DNA-modified siRNA-dependent gene silencing [17]. Here, we first present a summary of our previous work and describe its implications to clarify the basic framework of gene silencing due to DNA-modified siRNA.

To examine the effects of DNA replacement in siRNA on gene silencing in mammalian cells, various types of siRNAs with DNA substitution were constructed based on the sequences of highly functional class I anti-*luc* siRNAs [17, 19]. In DNA-modified siRNAs 9–11 in **Fig. 1A** (left panel), ribonucleotides were progressively replaced with deoxyribonucleotides from the GS end, which contains the 5' end of the guide strand, while in modified siRNAs 1–6, deoxyribonucleotide substitutions occurred progressively from the PS end with the 5' end of the passenger strand.

As shown schematically in **Fig. 1A** (right panel), nearly all *luc* gene activity was virtually completely abolished after transfection with modified siRNAs with DNA substitution in the region ≤ 8-bp from the GS end. Gene silencing activity of modified siRNA with 8-bp GS-DNA substitution was essentially identical to that of nonmodified cognate siRNA (**Fig. 1A**, 12), indicating that modified siRNA with 8-bp GS-DNA substitution can be used as a reagent for gene silencing with similar to that of nonmodified authentic siRNA. In contrast, all modified siRNAs with DNA replacement from the PS end showed little gene silencing effect (**Fig. 1A**, 1-6), indicating that RNA in the vicinity to the PS end is essential for gene silencing (**Fig. 1B**). Similar results were obtained when many other class I DNA-modified siRNAs were used for transfection in place of anti-*luc* siRNAs. Furthermore, our analysis indicated that the effect of DNA replacement is mainly due to changes in the guide strand [17]. Therefore, we considered that mammalian gene silencing due to siRNAs with 8-bp GS-DNA substitution is due primarily to interactions between the 5' proximal guide strand region and RNA-binding protein moieties of RISC and/or RLC (**Fig. 1B)**.

DNA positions 2–8 of the guide strand in active DNA-modified siRNA corresponds to the seed region of microRNA that has been implicated in gene regulation. In nonmodified siRNA-dependent gene silencing, the corresponding "seed" region of siRNA is considered to play an essential role in the entry or nucleation stage of mRNA recognition and to be responsible for gene silencing due to off-target effects (see **Fig. 1E**) [14–16].

A DNA–RNA hybrid is generally thought to be less stable thermodynamically than an RNA duplex [20-23]. Thus, the seed activity or off-target effect inducing activity due to the DNA seed arm in the putative RISC which contains the guide strand of modified siRNA with 8-bp GS-DNA substitution is expected to be considerably weaker than that of the nonmodified siRNA RISC. To clarify this point, the off-target effect due to siRNA with 8-bp GS-DNA substitution was examined using the psiCHECK-sm expression system and genome-wide microarray expression profiling.

In the psiCHECK-sm expression system, three consecutive copies of an identical seed matched (sm) target sequence, which possesses homology only to the seed sequence of nonmodified or DNA-modified siRNAs, were introduced into the 3' untranslated region of psiCHECK-1 *Renilla luc* (**Fig. 1C**, left panel). Many sm-targets for highly functional siRNAs were examined as inserts. A considerable reduction in relative *luc* activity was observed in sm-target expressing cells transfected especially with certain

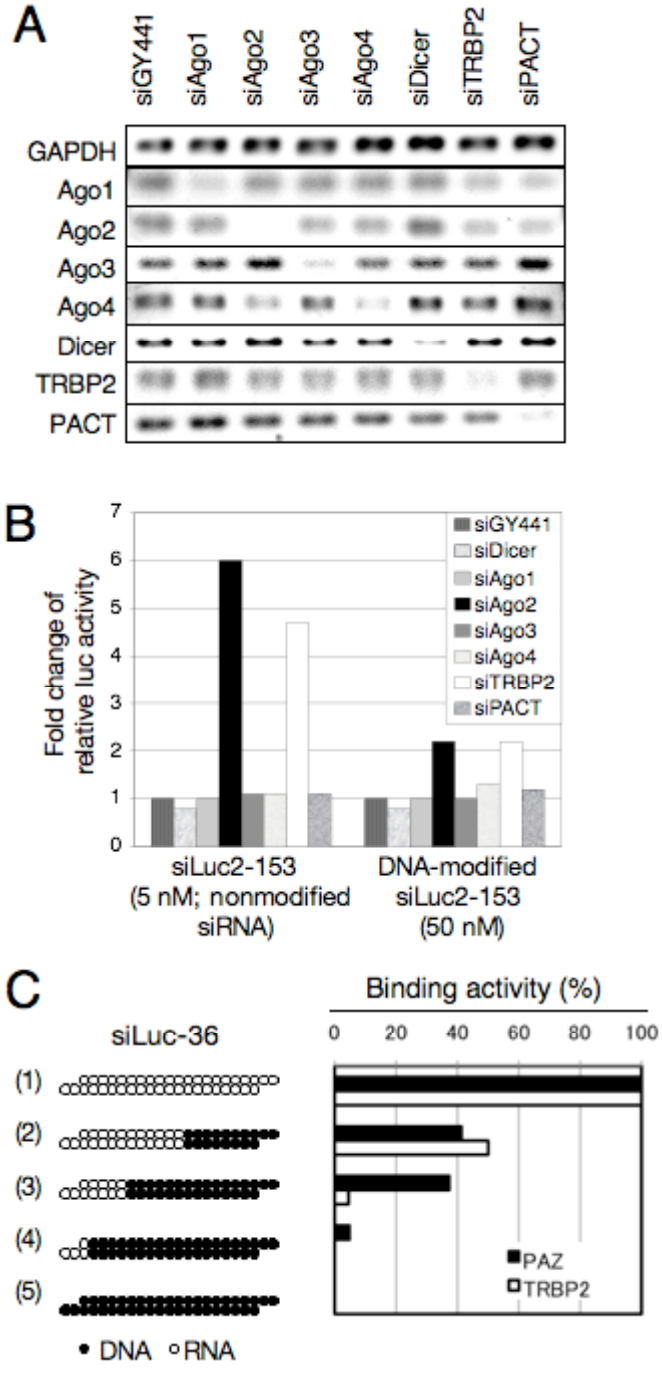


**Fig. 2** Requirement of Ago2 and TRBP2 for gene silencing due to nonmodified siRNA and DNA-modified siRNA in HeLa cells. (**A**) HeLa cells were transfected with corresponding siRNA (50 nM). RNA expression was detected 24 h after transfection by RT-PCR. The number of amplification cycles were 17, 26, 25, 27, 25, 26, 25 and 25 for GAPDH, Ago1–Ago4, Dicer, TRBP2 and PACT RNA, respectively. Guide strand and passenger strand sequences of siRNAs are as follow: siDicer (5'-GCUUGAGUUGAUUACAAGACU (guide strand) and 5'-UCUUGUAAUCAACUCAAGCAU (passenger strand)), siAgo1 (5'-GGCAGCGCUGUAUUAAAAAGC and 5'-UUUUUAAUACAGCGCUGCCCA), siAgo2 (5'-CGAUCGGCAAGAAGAGAUUAG and 5'-AAUCUCUUCUUGCCGAUCGGG), siAgo3 (5'-CCUACCUGCCACUAGAAGUCU and 5'-ACUUCUAGUGGCAGGUAGGUG), siAgo4 (5'-CGAAAGGCAUGUAUUAGCUUG and 5'-AGCUAAUACAUGCCUUUCGAA), siTRBP2 (5'-CGUCAGCUACCUGGAUAUUGA and 5'-AAUAUCCAGGUAGCUGACGUG) and siPACT (5'-GCCAAUGCAAGUAUUUGCUUU and 5'-AGCAAAUACUUGCAUUGGCUU). The following primers were used for RT-PCR: GAPDH (5'- CAAGGCTGAGAACGGGAAGCT and 5'- GACACGGAAGGCCATGCCAGT), Dicer (5'-CGCTCTGGAGAGGTTACCAT and 5'-CAGGTTTTTCAAGCCGAAGA), Ago1 (5'-GGCCAGGAACAAAAGCATAC and 5'-TCTGGAGCGGATCTAGCTGT), Ago2 (5'-ACATTGTGGCAGGACAAAGA and 5'-GAAACTTGCACTTCGCATCA), Ago3 (5'-ACCTTCCCTGTCTGCAAGTC and 5'-CAGATCTTGCTGTTGCCTTG), Ago4 (5'-TGTGGTTGGCAGTATGGATG and 5'-TGGAATAGAGACTGAGCGAGTG), TRBP2 (5'-AGGTGGAGCCTGATGATGAC and 5'-GCAGAGCCATGACACACAGT) and PACT (5'-CCTGCACAGGTGAAGGTACA and 5'-TGGAAGGGTCAGGCATTAAG). (**B**) HeLa cells were simultaneously transfected with pGL3-Control DNA, pRL-SV40 DNA and siRNA (50 nM) against Dicer, Ago1-4, TRBP2 or PACT. In addition, either nonmodified siLuc2-153 (5 nM) or its modified siRNA counterpart (50nM) was used for *luc* gene silencing. As an siRNA control, siGY441 (50nM), an siRNA for EGFP knockdown, was used. Gene silencing activity was measured 24 h after transfection and normalized with the activity of siGY441. (**C**) Binding of the PAZ domain of Ago1 and TRBP2 to siRNA (siLuc-36), modified siRNA and siDNA. The 5' ends of the guide strand and the passenger strand were phosphorylated with [$\gamma$-$^{32}$P] ATP and with cold ATP, respectively. A mobility shift assay was carried out using PAZ and TRBP2 proteins purified from *E. coli* cells. Relative binding activity was determined densitometrically.

nonmodified siRNAs with a higher G/C content in the seed region (U.-T. *et al.*, in preparation). However, virtually no sm target knockdown was observed by treatment with DNA-modified siRNAs irrespective of G/C content in the seed region (**Fig. 1C**, right panel). The seed activity of authentic siRNA increased with increasing copy number of the sm target, but no such effect was observed in the case of DNA-modified siRNA treatment [17], supporting the suggestion that most, if not all, functional siRNAs with 8-bp GS-DNA substitution induce virtually no seed sequence-mediated off-target effect.

The off-target effect due to DNA-modified-siRNA was also examined by microarray analysis using RNA extracted from cells transfected with 8-bp GS-DNA-modified and nonmodified anti-vimentin siRNAs (siVIM-270 and chiVIM-270, respectively), both recognizing the CDS of vimentin mRNA [17]. A total of 16,783 transcripts, 6 % of which showed similarity in the 3'UTR to the seed sequence of siVIM-270, were examined. As shown schematically in **Fig. 1D**, unintended gene silencing due to chiVIM-270 was significantly less prominent than that due to siVIM-270, whereas intended gene silencing due to chiVIM-270 was as effective as that due to siVIM-270.

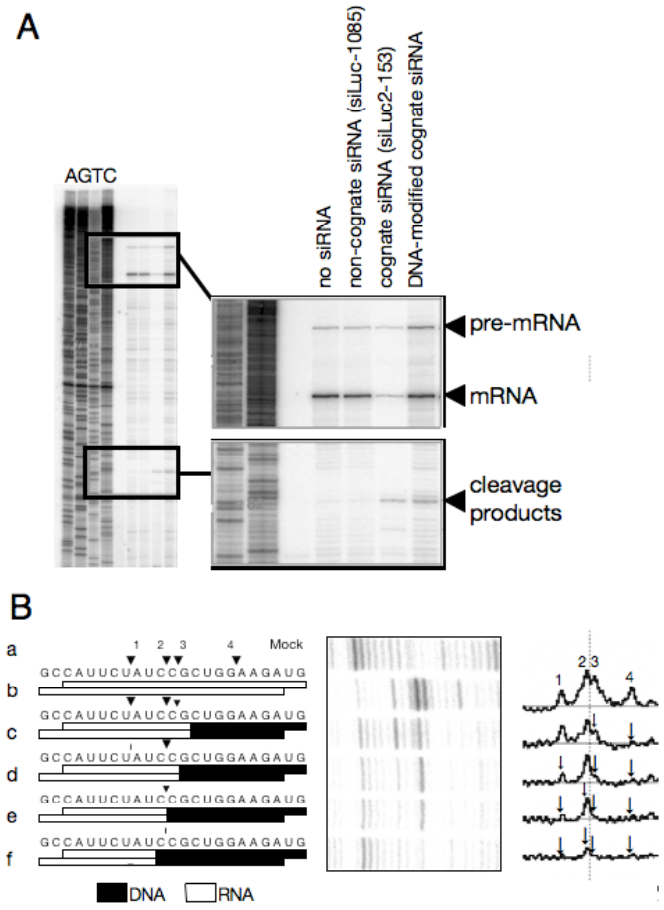


**Fig. 3** DNA-modified siRNA-mediated target mRNA cleavage. (**A**) HeLa cells were co-transfected with pTREC-2-153 and siLuc-1085 (negative control siRNA), siLuc2-153 (target-cleavable siRNA) or its DNA-modified cognate siRNA. RNA was extracted 24 h after transfection. Cleavage sites were determined by primer extension. Sequence ladder was prepared using the same pTREC construct. The panels on the right show enlarged photographs of the regions enclosed by rectangles in the left panel. Note that the position of the main cleavage site is identical between nonmodified siRNA-dependent RNAi and DNA-modified siRNA-dependent gene silencing. (**B**) HeLa cells were co-transfected with pTREC-36 and nonmodified or DNA-modified versions of siLuc36. Triangles 2 and 3, major cleavage sites; Triangle 1 and 4, minor cleavage sites.

Taken together, these results suggest that replacement of the seed arm of highly functional class I siRNAs with cognate DNA sequences leads to almost complete loss of off-target effects with no substantial effect on gene silencing activity in mammalian cells. Thus, it is quite likely that siRNA with DNA seed requires not only DNA seed/target mRNA interactions but non-seed RNA/target mRNA interactions for stable "mRNA-RISC complex" formation (**Fig. 1E**).

### 3.2 Involvement of interactions between Ago/TRBP2 and the PS terminal RNA half in gene silencing due to DNA-modified siRNA

Gene silencing through RNAi is carried out by RISC. The human Ago family has eight members, four of which (Ago1–4) are closely related in sequence and co-expressed in many cell types [24]. TRBP2 [25] and PACT [26] are putative mammalian counterparts of the *Drosophila* RLC protein, R2D2. The Dicer–R2D2 complex is considered to sense the differential stability of siRNA duplex ends to determine which strand will be loaded into RISC as the guide strand [3]. We examined whether these proteins are responsible for gene silencing due to transfection with nonmodified siRNA and modified siRNA with an 8-bp DNA seed arm in HeLa cells by reducing their activities using highly functional class I siRNAs (**Fig. 2A**). As shown previously [17] and in **Fig. 2B**, reduction of Ago2 and TRBP2 siRNAs clearly prevented *luc* gene silencing due to DNA-modified and nonmodified siRNAs (siLuc2-153). Ago2 and TRBP2 siRNA treatment increased relative *luc* activity by 5- and 2-fold when nonmodified and DNA-modified siRNAs were used for target gene silencing, respectively. No appreciable prevention of target gene silencing was observed with reduction of other factors. These results suggested that Ago2 and TRBP2 are involved in gene silencing not only due to nonmodified siRNA but also to DNA-modified siRNA. The absence of a gene-silencing prevention effect by siRNAs for factors other than Ago2 and TRBP2 may indicate that these factors are not involved in gene silencing or that they are functionally redundant in the cells examined. This remains to be clarified in future studies.

TRBP2 and PAZ domain of Ago contain dsRNA binding domains [24, 25] and, as anticipated, are capable of binding to dsRNA but not dsDNA **(Fig. 2C**). **Fig. 2C** also shows that both PAZ and TRBP2 are capable of binding to siRNA with 8-bp GS-DNA, indicating that the PS terminal RNA region of siRNA includes binding sites for TRBP2 and Ago. However, TRBP2 and Ago differ from each other in the manner of binding to the RNA portion of DNA-modified siRNA. TRBP2 requires binding sites situated in a more central region for effective binding to the chimera. Ago may also require some additional terminal sites for effective binding (**Fig. 2C**).

Thus, as with functional siRNA, modified siRNA with DNA seed appears to be capable of forming RLC with TRBP2 and RISC with Ago2 in mammalian cells and these complexes are required for gene silencing due to modified siRNA with an 8-bp GS-DNA substitution as in the case of that due to nonmodified siRNA.

### 3.3 Prevention of target mRNA cleavage at minor cleavage sites in DNA-modified siRNA-dependent gene silencing

In RNAi, the target mRNA is cleaved at a point corresponding to the center of the guide strand via RNase H-like activity of the PIWI domain of Ago2 [11, 12, 27, 28]. Previously [17], we determined the major cleavage site in the target *luc* mRNA, which was induced by transfection of DNA-modified siRNA (siLuc2-153) with 8-bp GS-DNA substitution. HeLa cells were transfected with a target-expression plasmid, pTREC-2-153 and either the DNA–

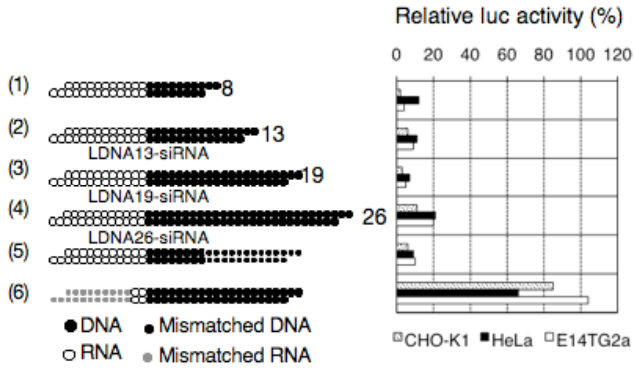


**Fig. 4** Seed positioning in DNA-modified siRNA-containing RISC is determined by the RNA terminal half of the guide strand. Cells were transfected with pGL3-Control DNA, pRL-SV40 and siLuc-36 derivatives (50 nM). LDNAn-siRNA is a version of DNA-modified siLuc-36 with (n-8)-nucleotide DNA extension. Relative *luc* activity was measured 24 h after transfection using CHO-K1, E14TG2a and HeLa cells. The guide and passenger strand sequences of DNA-modified siRNAs are as follows (upper case, RNA; lower case, DNA): (1) 5'-CAUUCUAUCCGctggaagatg-3' (guide strand) and 5'-tcttccagCGGAUAGAAUGGC-3' (passenger strand), (2) LDNA13-siRNA; 5'-CAUUCUAUCCGctggaagatggaacc-3' and 5'-ttccatcttccagCGGAUAGAAUGGC-3', (3) LDNA19-siRNA; 5'-CAUUCUAUCCGctggaagatggaaccgctgga-3' and 5'-cagcggttccatcttccagCGGAUAGAAUGGC-3', (4) LDNA26-siRNA; 5'-CAUUCUAUCCGctggaagatggaaccgctggagagcaac-3' and 5'-tgctctccagcggttccatcttccagCGGAUAGAAUGGC-3', (5) 5'-CAUUCUAUCCGctggaagagttccaatagttc-3' and 5'-actattggaactcttccagCGGAUAGAAUGGC-3', (6) 5'-ACGGAGCGACGctggaagatggaaccgctgga-3' and 5'-cagcggttccatcttccagCGUCGCUCCGUUA-3'. Mismatched sequences are underlined. Note that modified siRNA (5) but not (6) recognized the *luc* target correctly.

modified or nonmodified siRNA (siLuc2-153). Target mRNA cleavage was examined by primer extension. The position of the major cleavage site due to the DNA-modified siRNA was identical to that of RNAi induced by the authentic siRNA counterpart (**Fig. 3A**) [17]. In both cases, the main cleavage point was situated between positions 10 and 11, as measured from the 5' end of the guide strand.

As shown in **Fig. 3B**, densitometric analysis of primer extension products generated by nonmodified siRNA treatment indicated that target mRNA may be cleaved not only at the major cleavage sites (sites 2 and 3 in **Fig.3B, b**) but also at two minor sites (sites 1 and 4) separated by about ten nucleotides, or one turn of the Watson-Crick helix. Although neither an increase in siRNA concentration nor passenger-strand replacement with DNA had a marked effect on the cleavage pattern (data not shown), this pattern was quite sensitive to changes in the DNA arm length of DNA-modified siRNA (**Fig. 3B**). The cleavage of mRNA target at multiple sites therefore may not be a primer extension artifact but an intrinsic characteristic. The presence of the 8-bp DNA guide brought about a complete loss of cleavage at site 4 (**c**). Target mRNA cleavage at sites 1 and 4 was eliminated almost completely by the 9–10-bp DNA arm (**d, e**). The 11-bp DNA arm significantly suppressed target cleavage even at the major cleavage site (**f**).

Considering the conformation of target mRNA/guide strand with 5' proximal DNA, mRNA cleavage at the minor sites is unlikely to be attributable to the Ago RNase H activity. Double-stranded DNA surrounding the nucleosome core protein surface is cleaved at an interval of ten nucleotides by non-specific DNase [29]. Therefore, we consider that mRNA cleavage at minor sites is due to attack of a duplex consisting of mRNA and a guide strand with the DNA arm bound to the surface of Ago by non-specific cellular RNase and that this RNase activity is prevented by the presence of DNA in the vicinity of a cleavable site.

### 3.4 Seed positioning in DNA-modified siRNA containing RISC

In the authentic RNAi, the seed arm in RISC is believed to be correctly positioned via the interaction between the 5' phosphate of the guide strand and the Mid domain pocket [5, 8, 9]. Therefore, this was examined in DNA-modified siRNA-dependent gene silencing using siRNA with a longer DNA arm (LDNA-siRNA; **Fig. 4**, 2-4). For example, LDNA19-siRNA is an active modified siRNA with 8-bp GS-DNA associated with an 11-bp DNA extension (19-bp in total length). As shown in **Fig. 4** (1–4**)**, the extension of the DNA arm did not affect gene silencing activity. If the 5' end of the guide strand is a determinant of seed positioning of RISC, DNA extension should change the mRNA target sequence. To examine this possibility, an LDNA19-type siRNA was constructed containing DNA extension of different sequences. The results indicated that 8 bp of DNA immediately adjacent to the RNA is capable of acting as a seed for mRNA recognition, but no seed activity was found to be associated with the DNA extension, indicating that, in contrast to the case of nonmodified siRNA, the 3' but not 5' GS end may be responsible for positioning of the seed in DNA-modified siRNA-containing RISC. Consistent with this, changes in the RNA sequence in the 3' end of guide strand significantly affected target silencing (**Fig. 4**, 6).

## 4. CONCLUDING REMARKS

We found that class I siRNAs with DNA seed arm are capable of inducing highly effective gene silencing without any substantial off-target effect in mammalian cells. The presence of the DNA arm in the GS terminal half eliminates minor cleavage of mRNA possibly due to cellular non-specific RNase. The molecular mechanism of DNA-modified siRNA-dependent gene silencing is similar to that of nonmodified siRNA-dependent gene silencing. However, in DNA-modified siRNA-dependent gene silencing, the 5' end of the guide strand may not be involved in correct positioning of the seed sequence in RISC.

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