Enhancement of RNAi activity by improved siRNA duplexes

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Abstract RNA interference (RNAi) is a powerful tool for suppressing the expression of a gene of interest, in which 21–25 nucleotide short interfering RNA (siRNA) duplexes homologous to the silenced gene function as sequence-specific RNAi mediators. The present study shows that newly designed siRNA duplexes, 'fork-siRNA duplexes', whose sense-stranded siRNA elements carry one to four nucleotide mismatches at the 3′-ends against the antisense-stranded siRNA elements, can enhance RNAi activity over conventional siRNA duplexes in cultured mammalian cells.

Key words: RNA interference (RNAi); Fork-siRNA; Mismatch; Human cell

1. Introduction

RNA interference (RNAi) is the process of sequence-specific posttranscriptional gene silencing triggered by double-stranded RNAs (dsRNAs) homologous to the silenced genes. This intriguing gene silencing has been found in various kinds of species including flies, worms, protozoa, vertebrates and higher plants (reviewed in [1–4]). DsRNAs introduced or generated in cells are subjected to digestion with an RNase III-like enzyme, Dicer, into 21–25 nucleotide (nt) RNA duplexes [5–8], and the resultant duplexes, referred to as short interfering RNA (siRNA) duplexes, function as essential sequence-specific mediators of RNAi in the RNA-induced silencing complexes (RISCs) [7,8]. In mammalian cells except for some undifferentiated cells [9–12], long dsRNAs (>30 bp) trigger a rapid and non-specific RNA degradation involving the sequence-non-specific RNase, RNase L [13], and a rapid translation inhibition involving the interferon-inducible, dsRNA-activated protein kinase, PKR [14]; thereby the sequence-specific RNAi activity induced by long dsRNAs appears to be masked. Elbashir et al. [15] have shown that synthetic 21-nt siRNA duplexes can induce the sequence-specific RNAi activity in cultured mammalian cells without triggering the rapid and non-specific RNA degradation and translation inhibition. RNAi induction by synthetic siRNA duplexes appears to have paved the way for studying the molecular mechanism of mammalian RNAi, and also provided us with a powerful reverse genetic tool for suppressing the expression of a gene of interest in mammalian cells [16].

While direct introduction of synthetic siRNA duplexes into cells is often used for induction of mammalian RNAi nowadays, it has been known that different siRNAs induce different levels of RNAi activity [17,18]. Therefore, in order to realize efficient RNAi induction by synthetic siRNAs in mammalian cells, it is important to understand the properties of the siRNA duplexes conferring a strong RNAi activity. In the present study, the effect of various types of synthetic siRNAs on the induction of RNAi in mammalian cells was investigated and an improvement of the siRNA duplexes for enhancing RNAi activity was found.

2. Materials and methods

2.1. Preparation of oligonucleotides

RNA and DNA synthetic oligonucleotides were obtained from PROLIGO and SIGMAGENOSIS, respectively. For preparation of RNA duplexes, sense- and antisense-stranded RNAs (ssRNA and asRNA) (20 μM each) were mixed in an annealing buffer (30 μM HEPES pH 7.4, 100 μM potassium acetate, 2 μM magnesium acetate), heat-denatured at 90°C for 3 min, and annealed at 37°C overnight. Non-silencing siRNA duplex (Qiagen) was used as a negative control.

2.2. Cell culture, transfection, and luciferase assay

HeLa cells were grown as described previously [17]. The day before transfection, cells were trypsinized, diluted with fresh medium without antibiotics, and seeded into 24-well culture plates (approximately 0.5×10^{-5} cells/well). Cotransfection of synthetic siRNA duplexes with reporter plasmids was carried out using lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions, and to each well, 0.24 μg (40 nM) of siRNA duplexes against *Photinus luciferase*, 0.2 μg of pGL3-control plasmid (Promega) carrying *P. luciferase* and 0.05 μg of phRL-TK plasmid (Promega) carrying *Renilla luciferase* as a control were applied. 24 h after transfection, cell lysate was prepared and the expression levels of luciferase were examined by a dual luciferase reporter assay system (Promega) according to the directions provided by the manufacturer. For silencing the expression of the endogenous *LAMIN A/C* and *DNMT1* genes, 0.6 μg (100 nM) of each synthetic siRNA duplex against either of the genes was transfected into cells using jetSI transfection reagent (Polyplus transfection) according to the manufacturer's instructions.

2.3. Real-time polymerase chain reaction (PCR)

48 h after transfection, total RNA was extracted with Trizol reagent (Invitrogen) and subjected to cDNA synthesis using oligo(dT) primers and a Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The resultant cDNAs were examined by real-time PCR using the ABI Prism 7000 sequence detection system (Applied Biosystems) with a SYBER Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. The PCR primers used in the real-time PCR were as follows: for detection of the *LAMIN A/C* transcript, Hs.Lamin-F: 5′-GAGGCCAAGAAGCAACTTCA-3′; Hs.Lamin-R: 5′-AGCTCCTCACTGTAGATGTTCTT-3′; for detection of the *DNMT1* transcript, Hs.Dnmt1-F: 5′-GAAGGAGAAATTGAATCTCTTGCAC-3′; Hs.Dnmt1-R: 5′-GGGATTTGACTTTAGCCAGGTAG-3′; for detection of the *G3PDH* transcript as a control, Hs.G3PDH-F: 5′-TGCCAAATAT-

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GATGACATCAAGAAG-3′; Hs.G3PDH-R: 5′-TGTCGCTGTT-GAAGTCAGAG-3′.

3. Results and discussion

3.1. RNAi induction by synthetic siRNA duplexes possessing mismatched sequences at their termini

Various siRNA duplexes were constructed against the *P. luciferase* gene, and the effect of the duplexes on the suppression of the expression of *P. luciferase* was examined by co-transfection of the duplexes with a pGL3-control plasmid carrying the *P. luciferase* gene and a phRL-TK plasmid carrying the *R. luciferase* gene as a control into HeLa cells. As the target sites of the siRNAs, two sites conferring different levels of RNAi activity [17] were chosen: one is the La2 siRNA, conferring a strong RNAi activity (~98% suppression), and the other is the La21 siRNA, conferring a moderate RNAi activity (~50% suppression) (Fig. 1a). Table 1 shows the siRNA duplexes synthesized in this study: the newly designed duplexes possess mismatched sequences at their termini due to introduction of base substitutions into the sense-stranded siRNA (ss-siRNA) elements (note that the antisense-stranded siRNA (as-siRNA) elements remain intact).

Fig. 1 shows the results of the dual luciferase assay. When the La2 siRNA duplexes composed of the 3′-end mismatched ss-siRNA and intact as-siRNA elements, the La2-3′m1~4 duplexes, were used as RNAi mediators, they, like the conventional La2 siRNA duplex, the La2-conv. duplex, could trigger strong RNAi activities. Interestingly, the levels of the RNAi activities induced by the La2-3′m1~4 duplexes, i.e. the levels of silencing of the *P. luciferase* gene expression, appeared to be increased as compared with those of the La2-conv. duplex. In addition, the La2-3′BL duplex possessing no overhangs at the 3′-end of the ss-siRNA element could also induce a strong RNAi activity, and the level of its activity appeared to be higher than that of the La2-conv. duplex, which agrees with the previous report [19]. Of the La2-3′m1~4 and La2-3′BL duplexes used, the La2-3′m2 duplex, carrying 2-nt mismatches at the 3′-end of the ss-siRNA element, appears to confer the strongest RNAi activity (Fig. 1b). In contrast, when base substitutions were introduced into the 5′-end of the ss-siRNA element, the resultant siRNA duplex, the La2-5′m2 duplex, decreased the level of suppression of the expression of *P. luciferase* as compared with that of the La2-conv. duplex (Fig. 1b). Therefore, these observations suggest that the introduction of mismatches at the 3′-ends, but not at the 5′-ends, of the ss-siRNA elements in siRNA duplexes does not prevent the induction and activation of RNAi, and also that such siRNA duplexes carrying mismatches at the 3′-ends of the ss-siRNA elements likely possess potential for increasing RNAi activity.

Similar results were also obtained when the La21-3′m1~4, La21-3′BL and La21-5′m2 duplexes were used as RNAi mediators (Fig. 1c). The important point to note is that the levels of RNAi activities induced by the La21-3′m1~4 and La21-3′BL duplexes were significantly increased over those of the La21-conv. duplex. In addition, the highest level of the RNAi activity with these duplexes was detected by using the La21-3′m2 duplex carrying the 2-nt mismatches at the 3′-end of the ss-siRNA element, which agrees with the results of the La2-3′m2 duplex as shown in Fig. 1b. When the La21-5′m2 duplex was used as an RNAi mediator, a significant loss of the RNAi activity was observed, which also agrees with the results of the La2-5′m2 duplex as shown in Fig. 1b. Therefore, these results strongly suggest that siRNA duplexes carrying mismatches at the 3′-ends of the ss-siRNA elements can induce a more efficient RNAi activity than conventional siRNA duplexes, and siRNA duplexes carrying mismatches at the 5′-ends of the ss-

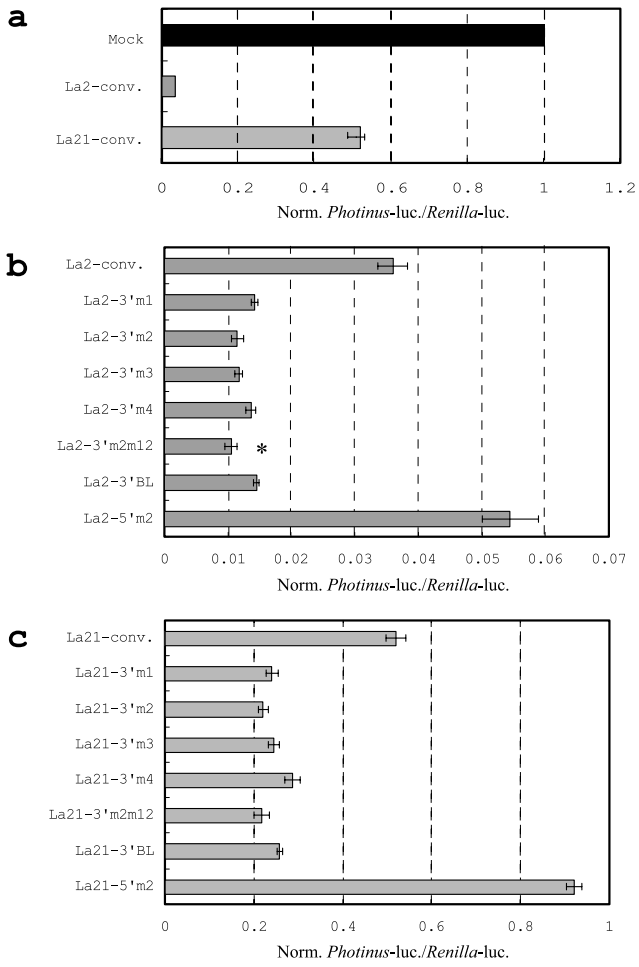

Fig. 1. Silencing of the expression of exogenous reporter gene with various types of synthetic siRNA duplexes. a: Gene silencing of *P. luciferase* with conventional 21-nt siRNA duplexes. The La2-conv. or La21-conv. siRNA duplex against the *P. luciferase* gene together with pGL3-control and phRL-TK plasmids carrying *P. luciferase* and *R. luciferase* reporter genes, respectively, were cotransfected into HeLa cells using lipofectamine 2000 transfection reagent (Invitrogen). 24 h after transfection, cell lysate was prepared and dual luciferase assay was carried out using a dual luciferase reporter assay system (Promega). Ratios of normalized target (*Photinus*) luciferase activity to control (*Renilla*) luciferase activity are shown: the ratios of luciferase activity determined in the presence of either La2 or La21 siRNA duplex are normalized to the ratio obtained for a control in the presence of a non-silencing siRNA duplex (Qiagen) (mock). Data are averages of at least four independent experiments. Error bars represent standard errors. b, c: Gene silencing of *P. luciferase* with various types of La2 (b) and La21 (c) siRNA duplexes. Various types of synthetic La2 or La21 siRNA duplexes (Table 1) together with pGL3-control and phRL-TK reporter plasmids were cotransfected into HeLa cells, and the expression levels of luciferase were examined as in a. Ratios of normalized target (*Photinus*) luciferase activity to control (*Renilla*) luciferase activity are shown as in a. Data are averages of at least four independent experiments. Error bars indicate standard errors. Statistical analyses were carried out by using Student's *t*-test. Asterisk indicates statistically significant difference ($P < 0.01$) against the data of the La2-3′m2.

Table 1
Synthetic siRNAs used in this study

name	Sequence
La2-conv.	5' GGAAGACGCCAAAAACAUAUU 3' 3' UUCCUUCUGCGGUUUUUGUAU 5'
La2-3'm1	5' GGAAGACGCCAAAAACAUU 3' 3' UUCCUUCUGCGGUUUUUGUAU 5'
La2-3'm2	5' GGAAGACGCCAAAAACAAU 3' 3' UUCCUUCUGCGGUUUUUGUAU 5'
La2-3'm3	5' GGAAGACGCCAAAAACUAU 3' 3' UUCCUUCUGCGGUUUUUGUAU 5'
La2-3'm4	5' GGAAGACGCCAAAAAUUAU 3' 3' UUCCUUCUGCGGUUUUUGUAU 5'
La2-5'm2	5' UUAAGACGCCAAAAACAUAUU 3' 3' UUCCUUCUGCGGUUUUUGUAU 5'
La2-3'm2m12	5' GGAAGACUCCAAAAACAAU 3' 3' UUCCUUCUGCGGUUUUUGUAU 5'
La2-3'BL	5' GGAAGACGCCAAAAACAUA 3' 3' UUCCUUCUGCGGUUUUUGUAU 5'
La21-conv.	5' ACCGCUGGAGAGCAACUGCUU 3' 3' UUUGGCGACCUCUCGUUGACG 5'
La21-3'm1	5' ACCGCUGGAGAGCAACUGU 3' 3' UUUGGCGACCUCUCGUUGACG 5'
La21-3'm2	5' ACCGCUGGAGAGCAACUUU 3' 3' UUUGGCGACCUCUCGUUGACG 5'
La21-3'm3	5' ACCGCUGGAGAGCAACAUU 3' 3' UUUGGCGACCUCUCGUUGACG 5'
La21-3'm4	5' ACCGCUGGAGAGCAAUAUU 3' 3' UUUGGCGACCUCUCGUUGACG 5'
La21-5'm2	5' UACGCUGGAGAGCAACUGCUU 3' 3' UUUGGCGACCUCUCGUUGACG 5'
La21-3'm2m12	5' ACCGCUGUAGAGCAACUUU 3' 3' UUUGGCGACCUCUCGUUGACG 5'
La21-3'BL	5' ACCGCUGGAGAGCAACUGC 3' 3' UUUGGCGACCUCUCGUUGACG 5'
Lam-conv.	5' UGCUGAGAGGAACAGCAACCU 3' 3' AGACGACUCUCCUUGUCGUUG 5'
Lam-3'm2m12	5' UGCUGAGUGGAACAGCAUU 3' 3' AGACGACUCUCCUUGUCGUUG 5'
Nat.Lam-conv.	5' CUGGACUUCCCAGAAGAACAUU 3' 3' UUGACCCUGAAGGUCUUCUUGU 5'
Nat.Lam-3'm2m12	5' CUGGACUACCCAGAAGAAUU 3' 3' UUGACCCUGAAGGUCUUCUUGU 5'
Dn1(#1)-conv.	5' GUCCGCAGGCGGCUCAAAGAUU 3' 3' UUCAGGCGUCCCGCCGAGUUUCU 5'
Dn1(#1)-3'm2m12	5' GUCCGCAGUCGGCUCAAAUU 3' 3' UUCAGGCGUCCCGCCGAGUUUCU 5'
Dn1(#2)-conv.	5' GUGACUUGGAAACCAAAUUUU 3' 3' UUCACUGAACCUUUGGUUUAA 5'
Dn1(#2)-3'm2m12	5' GUGACUUUGAAACCAAAAA 3' 3' UUCACUGAACCUUUGGUUUAA 5'

a

	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
La2	G	G	A	A	G	A	C	G	C	C	A	A	A	A	A	C	A	U	A
GL3UU	C	U	U	A	C	G	C	U	G	A	G	U	A	C	U	U	C	G	A
hTF167i	G	C	G	C	U	U	C	A	G	G	C	A	C	U	A	C	A	A	A
hTF173i	G	A	G	G	C	A	C	U	A	C	A	A	A	U	A	C	U	G	U
hTF372i	G	A	A	G	C	A	G	A	C	G	U	A	C	U	U	G	G	C	A
hTF256i	C	C	C	G	U	C	A	A	U	C	A	A	G	U	C	U	A	C	A
hTF164i	C	C	G	G	C	G	C	U	U	C	A	G	G	C	A	C	U	A	C

b

	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
La100	G	C	A	G	U	U	G	C	G	C	C	C	G	C	G	A	A	C	G
La21	A	C	C	G	C	U	G	G	A	G	A	G	C	A	A	C	U	C	G
hTF562i	C	G	G	A	C	U	U	U	A	G	U	C	A	G	A	A	G	G	A
hTF459i	C	U	C	C	C	C	A	G	A	G	U	T	C	A	C	A	C	C	U
hTF478i	U	A	C	C	U	G	G	A	G	A	C	A	A	A	C	C	U	C	G
hTF929i	G	C	U	G	G	A	A	G	G	A	G	A	A	C	U	C	C	C	C
hTF77i	U	G	G	A	G	A	C	C	C	C	U	G	C	C	U	G	G	C	C

Fig. 2. Target nucleotide sequences of synthetic siRNA duplexes conferring strong (a) and moderate (b) RNAi activities. Sequence data are derived from previous studies [17,18], and aligned such that the 3′-ends of the sequences can be matched. The names of siRNAs examined and nucleotide positions from the 3′-end of the sequence are shown.

siRNA elements can reduce RNAi activity. I have named the siRNA duplexes carrying mismatches at the 3′-ends of the ss-siRNA elements 'fork-siRNA duplexes'.

3.2. *Target sequences of siRNAs conferring strong and moderate RNAi activities*

When the target sequences of the synthetic siRNA duplexes used previously for induction of mammalian RNAi are aligned, it appears that: (i) the target sequences of the siRNAs conferring a strong RNAi activity possess C or G residues at the 5′-ends, and tend to be AU-rich around the 3′-ends; in contrast, (ii) the sequences of the siRNAs conferring a moderate RNAi activity tend to be GC-rich around the 3′-ends, and contain A or U residues at the 5′-ends in some cases (Fig. 2). Together with the results presented here, it may be possible that the dissociation of siRNA duplexes from the 3′-ends of the ss-siRNA elements, and the ease of the dissociation due to AU-rich sequences around the 3′-ends could contribute to the enhancement of RNAi activity, and it may be that a possible helicase activity in RISCs could participate in the dissociation of siRNA duplexes. Most recently, while this work is reviewed, two papers concerning the asymmetrical feature of siRNA duplexes have just been published, and these suggest that functional siRNA duplexes can be characterized by a low base-pairing stability at the 5′-ends of the as-siRNA elements [20,21]. Consequently, the present observations and possibilities described above appear to be compatible with those recent observations.

The alignments of the siRNA target sequences indicate another possible feature common to the sequences of the siRNAs conferring a strong RNAi activity – A or U residues tend to be present at position 12 from the 3′-ends of the target sequences (Fig. 2). This tendency, i.e. a low internal stability around position 12 from the 5′-ends of functional as-siRNA elements, has also been observed in the recent study [21]. Based on the feature, fork-siRNA duplexes carrying 2-nt mismatches at the 3′-ends and an additional mismatch at position 12 from the 3′-ends in the ss-siRNA elements were constructed, and the effect of the resultant siRNA duplexes, the La2-3′m2m12 and La21-3′m2m12 duplexes (Table 1), on the

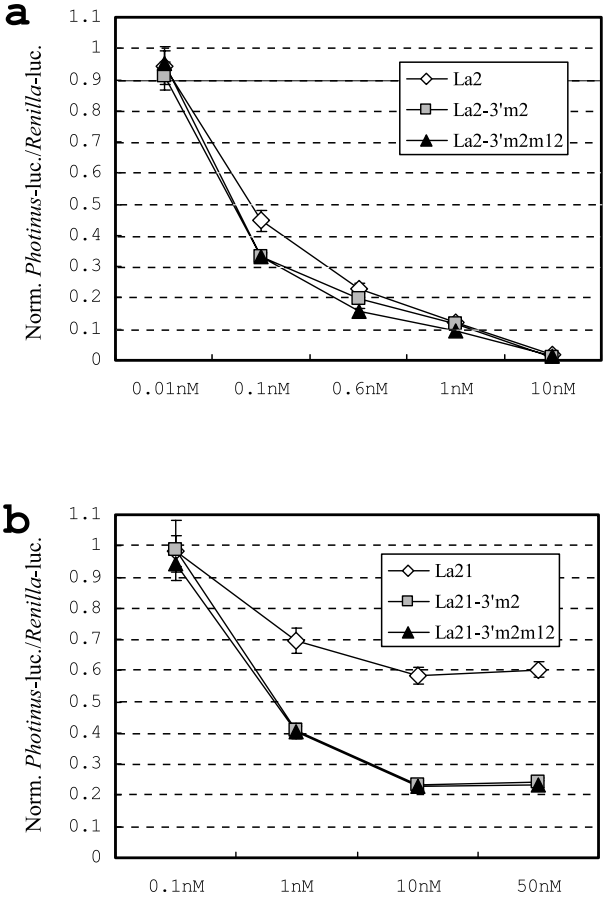

Fig. 3. Dose-dependent inhibition of target luciferase in HeLa cells. The pGL3-control and phRL-TK plasmids were cotransfected with an increasing amount of each siRNA duplex, a: from 0.01 to 10 nM and b: from 0.1 to 50 nM. Used siRNA duplexes are indicated. 24 h after transfection, dual luciferase assay was carried out. Data are presented as normalized ratios of target (*Photinus*) luciferase activity to control (*Renilla*) luciferase activity as in Fig. 1.

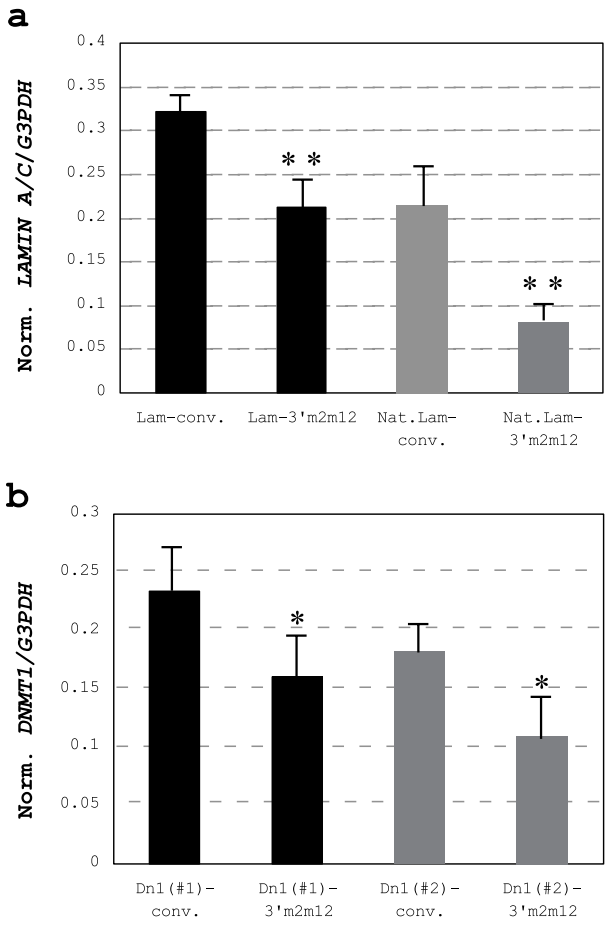

Fig. 4. Gene silencing of endogenous genes with fork-siRNA duplexes. Fork-siRNA and conventional siRNA duplexes targeting the *LAMIN A/C* and *DNMT1* genes were designed (Table 1). The target sequences of the siRNA duplexes in each of the genes are as follows (nucleotide positions relative to the start codons in the mRNAs are shown): Lam-siRNA duplexes, 829–851 (human *LAMIN A/C*); Nat.Lam-siRNA duplexes, 608–630 (human *LAMIN A/C*); Dn1(#1)-siRNA duplexes, 70–89 (human *DNMT1*); Dn1(#2)-siRNA duplexes, 185–203 (human *DNMT1*). The Nat.Lam-conv. siRNA duplex was used in a previous study [15]. The synthetic siRNA duplexes (100 nM each) were introduced into HeLa cells using jetSI transfection reagent (Polyplus transfection), and 48 h after transfection, total RNA was isolated and subjected to cDNA synthesis with a RT. The expression levels of the target genes were examined by means of a real-time PCR using the synthesized cDNAs as templates. The expression levels of either *LAMIN A/C* or *DNMT1* are normalized to that of *G3PDH*, and the resultant expression levels in the presence of either fork-siRNA or conventional siRNA duplex are normalized to the expression levels determined in the presence of non-silencing siRNA duplexes (Qiagen). The resultant normalized ratios are indicated. Data are averages of at least four independent experiments. Error bars represent standard errors. Statistical analyses were carried out by using Student's *t*-test: * and ** represent $P<0.05$ and $P<0.01$ versus the conventional siRNA duplexes, respectively.

suppression of the expression of the *P. luciferase* gene was tested. The results suggest that the La2-3′m2m12 and La21-3′m2m12 duplexes likely confer a slight further enhancement in RNAi activity compared with the La2-3′m2 and La21-3′m2 duplexes, respectively (Figs. 1 and 3): the difference in the level of gene silencing between the La2-3′m2m12 and La2-3′m2 duplexes is statistically significant ($P<0.01$), but the difference between the La21-3′m2m12 and La21-3′m2 duplexes could not attain statistical significance, although the average level of expression of *P. luciferase* in the presence of the La21-3′m2m12 duplex was lower than that in the presence of the La21-3′m2 duplex. In addition, from the results of Fig. 3 it appears that the introduction of base substitutions at the 3′-ends of ss-siRNA elements into siRNA duplexes conferring a moderate RNAi activity such as the La21 siRNA duplex is more effective in enhancement of RNAi activity than that into siRNA duplexes conferring a strong RNAi activity.

3.3. Gene silencing of endogenous genes by fork-siRNA duplexes

The effect of fork-siRNA duplexes on the suppression of the expression of endogenous genes was then examined. I chose two endogenous genes, *LAMIN A/C* and *DNMT1*, and constructed both conventional and fork-siRNA duplexes targeting the genes (Table 1). The duplexes were transfected into HeLa cells, and the expression levels of the target genes were examined 48 h after transfection by means of a real-time reverse transcription (RT)-PCR using a SYBR green PCR kit. As shown in Fig. 4, while the conventional siRNA duplexes suppress the expression of the cognate genes, the fork-siRNA duplexes can consistently induce a more efficient silencing of the expression of the cognate genes than the conventional siRNA duplexes: the differences in the suppression of the expression of the cognate genes between the fork- and conventional siRNA duplexes are statistically significant ($P<0.05$). In addition, the enhancement of the RNAi activity by the fork-siRNA duplexes appears to occur regardless of the siRNA target sequences, which agrees with the results with the exogenous gene, *P. luciferase* as shown in Fig. 1. Taking all the data together, the fork-siRNA duplexes appear to reinforce RNAi so that it can become a still more powerful tool for suppressing the expression of genes, and enable us to regulate the levels of RNAi activity in mammalian cells.

Finally, based on the observations presented here and in the recent studies [20,21], I would like to propose the following hypothetical model (Fig. 5): the directionality of the dissociation of siRNA duplex from one of the ends may confer the orientation of the siRNA duplex in RISC, by which the siRNA element unwound from the 5′-end could be determined and function as a sequence-specific RNAi mediator in RISC. Therefore, the ease of unwinding of siRNA duplexes from one of the ends would influence the determination of the siRNA elements to function as sequence-specific RNAi mediators, and enhance an unequal incorporation of siRNA elements as RNAi mediators into RISCs, and these may account for the reason why the fork-siRNA duplexes can induce a more efficient RNAi activity than the conventional siRNA duplexes. In addition, when the dissociation of siRNA duplexes from the 5′-ends of the ss-siRNA elements occurs, the ss-siRNA elements could remain and work as RNAi mediators in RISCs, and the resultant RISCs might participate in off-target gene silencing or result in incompetence. This possible pathway may help account for the results using the La2-5′m2 and La21-5′m2 duplexes (Fig. 1b and c). In order to elucidate these possibilities, more extensive studies must be conducted.

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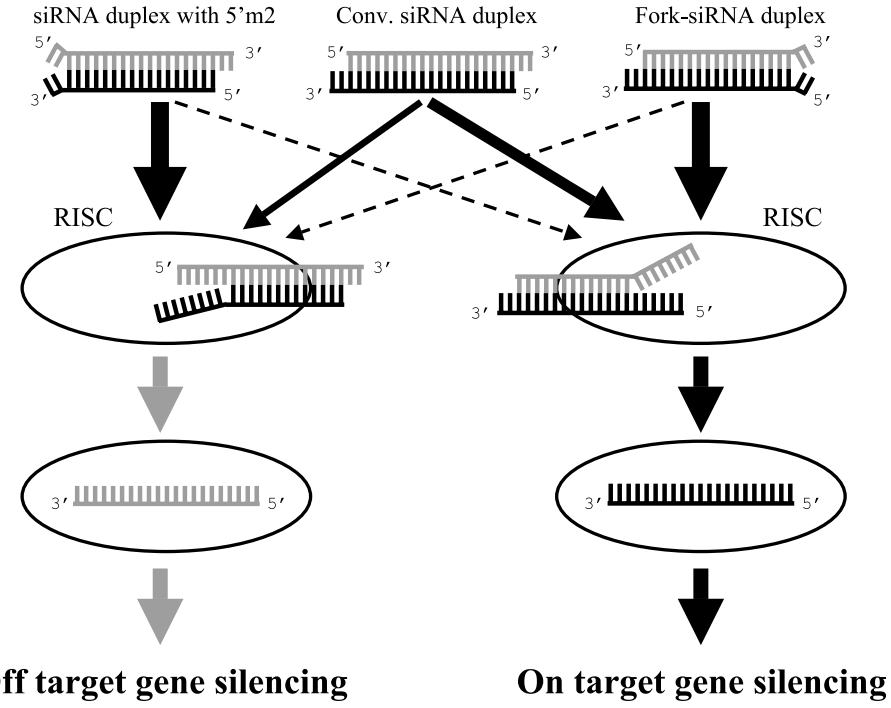

Fig. 5. Proposed model for the incorporation of siRNA into RISC. Conventional and fork-siRNA duplexes and RISCs are schematically drawn, and ss- and as-siRNA elements are colored in gray and black, respectively. When siRNA duplexes are incorporated into RISCs, they would be dissociated from one of the ends by a possible helicase activity in the RISCs, and the resultant siRNA elements unwound from the 5′-ends could be determined and remain as sequence-specific RNAi mediators in the RISCs. When the as-siRNA elements are determined as RNAi mediators in RISCs, the RISCs can contribute to a proper gene silencing against cognate genes (on-target gene silencing). In contrast, if the ss-siRNA elements were determined as RNAi mediators in RISCs, the resultant RISCs would participate in off-target gene silencing or result in incompetence. Thick, moderate, thin and dotted arrows from the indicated siRNA duplexes schematically represent the levels of possibility (from high to low).

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