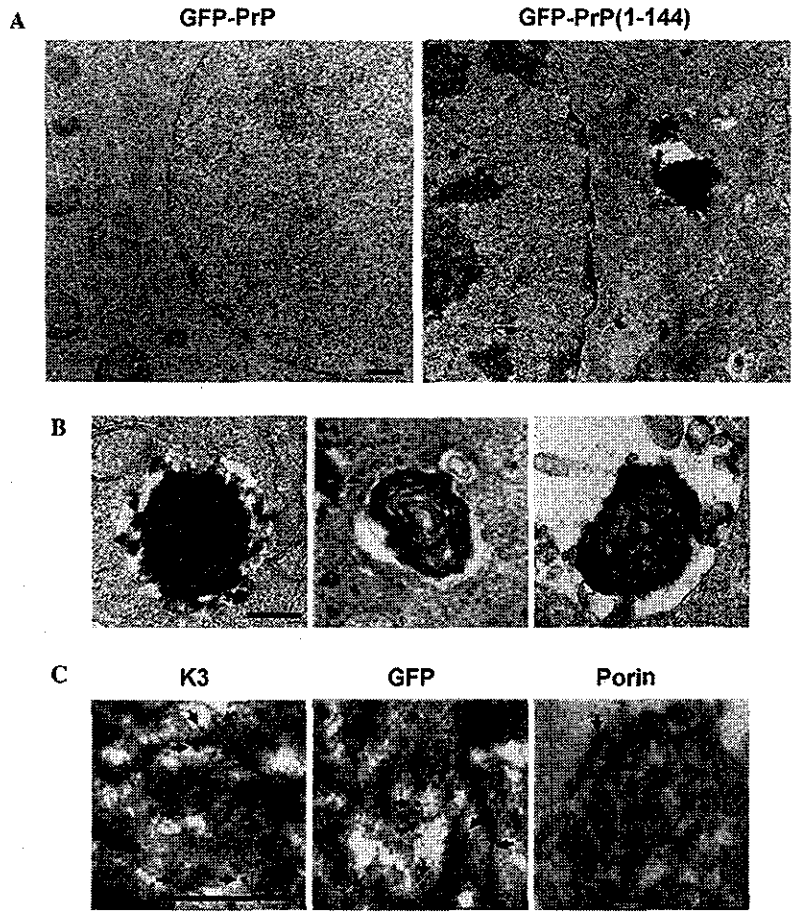

Fig. 3. GFP-PrP(1–144)-related electron-dense deposits. Scale bars = 0.1 μm. (A) Electron microscopy (30,000×) detects numerous electron-dense deposits in N2a cells transfected with GFP-PrP(1–144), whereas full-length GFP-PrP induces no deposit. (B) Some vesicles contain myelin-like figures. (C) Immunoelectron microscopy (30,000×) detects GFP-PrP(1–144) with anti-PrP antibody K3 (10 nm golds, left panel) or anti-GFP antibody (10 nm golds, middle panel) within the electron dense deposits of N2a cells. Anti-porin antibody (20 nm golds) also stains the deposits (right panel).

chondria-mediated apoptosis. Consequently, such neurotoxic property may contribute to a common pathogenic mechanism shared in various PrP-related disorders.

Deposition of numerous electron-dense deposits immunostained with anti-PrP antibody is another characteristic in GFP-PrP(1–144)-transfected cells, and has not been reported in other studies so far. The relevance of such electron-dense deposits with PrP amyloid deposits, a characteristic feature of human GSS with Y145STOP, is an intriguing question. These amyloid plaques were composed of COOH-terminal truncated PrP [12], but have not transmitted to mice [17]. Of note, both the electron-dense deposits in Y145STOP-transfected N2a cells and PrP^{Sc} in scrapie-infected N2a cells were found in the similar vacuolar compartment resembling secondary lysosomes [23], suggesting that both deposits may share a similar resistance to such a harsh lysosomal condition.

The Y145STOP mutation has been widely investigated in terms of its biochemical property. Peptides encompassing PrP(89–143) when mixed with PrP^{C} produced fibrous aggregates and displayed a high β-sheet content, although no prion infectivity was observed [24,25]. Recently, Kundu et al. [26] reported a spontaneous conversion of the recombinant polypeptide, human PrP(23–144), from a monomeric unordered state to a fibrillar form, in which human PrP residues within the 138–141 region are essential. Interestingly, this conversion has characteristics of a nucleation-dependent polymerization. Whether the numerous electron-dense deposits may serve as a seed for the growth of amyloid plaques with Y145STOP awaits further investigations.

Our current observations may provide clues as to the yet unknown underlying mechanism concerning the heritable human prion disease with Y145STOP at least in part. At the same time, the prion disease with Y145STOP has untransmitted to mice [17]. How this relates to the puzzle in prion biology, the discrepancy between the infectious and neurotoxic properties of PrP [27], remains to be further examined.

Acknowledgments

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RNAi induction and activation in mammalian muscle cells where *Dicer* and *eIF2C* translation initiation factors are barely expressed

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Abstract

Dicer plays an important role in the course of RNA interference (RNAi), i.e., it digests long double-stranded RNAs into 21–25 nucleotide small-interfering RNA (siRNA) duplexes functioning as sequence-specific RNAi mediators. In this study, we investigated the expression levels of *Dicer* and *eIF2C1~4*, which, like *Dicer*, appear to participate in mammalian RNAi, in various mouse tissues. Results indicate that the levels of *eIF2C1~4* as well as *Dicer* are lower in skeletal muscle and heart than in other tissues. To see if RNAi could occur under such a condition with low levels of expression of *Dicer* and *eIF2C1~4*, we examined RNAi activity in mouse skeletal muscle fibers. The results indicate that RNAi can be induced by synthetic siRNA duplexes in muscle fibers. We further examined RNAi activity during myogenic differentiation of mouse C2C12 cells. The data indicate that although the expression levels of *Dicer* and *eIF2C1~4* decrease during the differentiation, RNAi can be induced in the cells. Altogether, the data presented here suggest that muscle cells retain the ability to induce RNAi, although *Dicer* and *eIF2C1~4* appear to be barely expressed in them.

Keywords: RNA interference; Dicer; eIF2C translation initiation factors; Muscle; C2C12 cell

RNA interference (RNAi) is the process of a sequence-specific post-transcriptional gene silencing triggered by double-stranded RNAs (dsRNAs) homologous to the silenced genes. This intriguing gene silencing has been found in various species including flies, worms, protozoa, vertebrates, and higher plants (reviewed in [1–4]). DsRNAs introduced or generated in cells are digested by an RNase III enzyme, Dicer, into 21–25 nucleotide (nt) RNA duplexes [5–8] and the resultant duplexes, referred to as small-interfering RNA (siRNA) duplexes, function as essential sequence-specific RNAi mediators in the RNA-induced silencing complexes (RISCs) [5,7]. Thus, Dicer appears to play an important role in the process of RNAi induction.

In mammalian cells except for a part of undifferentiated cells [9–12], long dsRNAs (>30 bp) can 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, instead of induction of RNAi [14]. In contrast, chemically synthesised siRNA duplexes can induce the sequence-specific RNAi activity in mammalian cells without triggering the rapid and non-specific RNA degradation and translation inhibition [15]. Together, it is likely that RNAi activity induced by the long dsRNAs could be masked by those rapid responses to the long dsRNAs in most of mammalian cells.

It may be of interest to examine the role of Dicer in differentiated mammalian cells possessing the rapid responses to long dsRNAs. Mammalian dicer has been identified and found to be a large multi-domain

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polypeptide (~215 kDa) characterised by containing a putative DExH/DEAH RNA helicase/ATPase domain, a PAZ domain, two RNase domains, and a dsRNA-binding domain [16–20]. The expression of *Dicer* appears to be ubiquitous, but the level of its expression varies among tissues. Of the tissues examined previously, skeletal muscle appeared to express *Dicer* at a low level, i.e., the *Dicer* transcript appeared to be barely detectable at least using RT-PCR [16,17].

In this study, we investigated not only RNAi activity but also the expression levels of *Dicer* and *eIF2C1~4*, which, like *Dicer*, appear to participate in mammalian RNAi [21,22], in mouse skeletal muscle fibers, and muscle cells that differentiated from mouse C2C12 cells. The results indicate that RNAi can be induced by synthetic siRNA duplexes in those cells although the expression levels of *Dicer* and *eIF2C1~4* are lower than those in other tissues and undifferentiated C2C12 cells.

Materials and methods

Preparation and culture of muscle fibers isolated from extensor digitorum longus in mice. Isolation of muscle fibers from mice was carried out as described previously [23]. Briefly, extensor digitorum longus (EDL) was isolated from mice (ICR mouse strain), treated with 0.5% type 1 collagenase (Washington biochemical) in Dulbecco's modified Eagle's medium (DMEM) (Sigma), and incubated at 37 °C for 90 min. After incubation, the EDL was dissociated into single muscle fibers by gently pipetting, and dissociated single fibers were plated on matrigel-coated 24-well culture plates (approximately 100 fibers/well). The muscle fibers were cultured at 37 °C in DMEM supplemented with 10% horse serum (Invitrogen) in a 5% CO_2-humidified chamber. Two–three hours after starting culture, transfection was carried out.

Cell culture. C2C12 cells were grown at 37 °C in DMEM supplemented with 15% fetal calf serum (Sigma), 100 U/ml penicillin (Invitrogen), and 100 μg/ml streptomycin (Invitrogen) in a 5% CO_2-humidified chamber. For induction of myogenic differentiation, cells were cultured at 37 °C in DMEM supplemented with 5% horse serum (Invitrogen) in a 5% CO_2-humidified chamber [24]. The medium was changed everyday.

Synthetic oligonucleotides. RNA and DNA synthetic oligonucleotides were obtained from PROLIGO and SIGMAGENOSIS, respectively. The La2 siRNA duplex described previously was used in this study, and preparation of RNA duplexes was performed as described previously [25].

Transfection and luciferase assay. Reporter plasmids and siRNA duplexes were cotransfected into isolated single muscle fibers and undifferentiated and differentiated C2C12 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturers' instructions. When undifferentiated C2C12 cells were used, the day before transfection, the cells were trypsinised, diluted with the fresh medium without

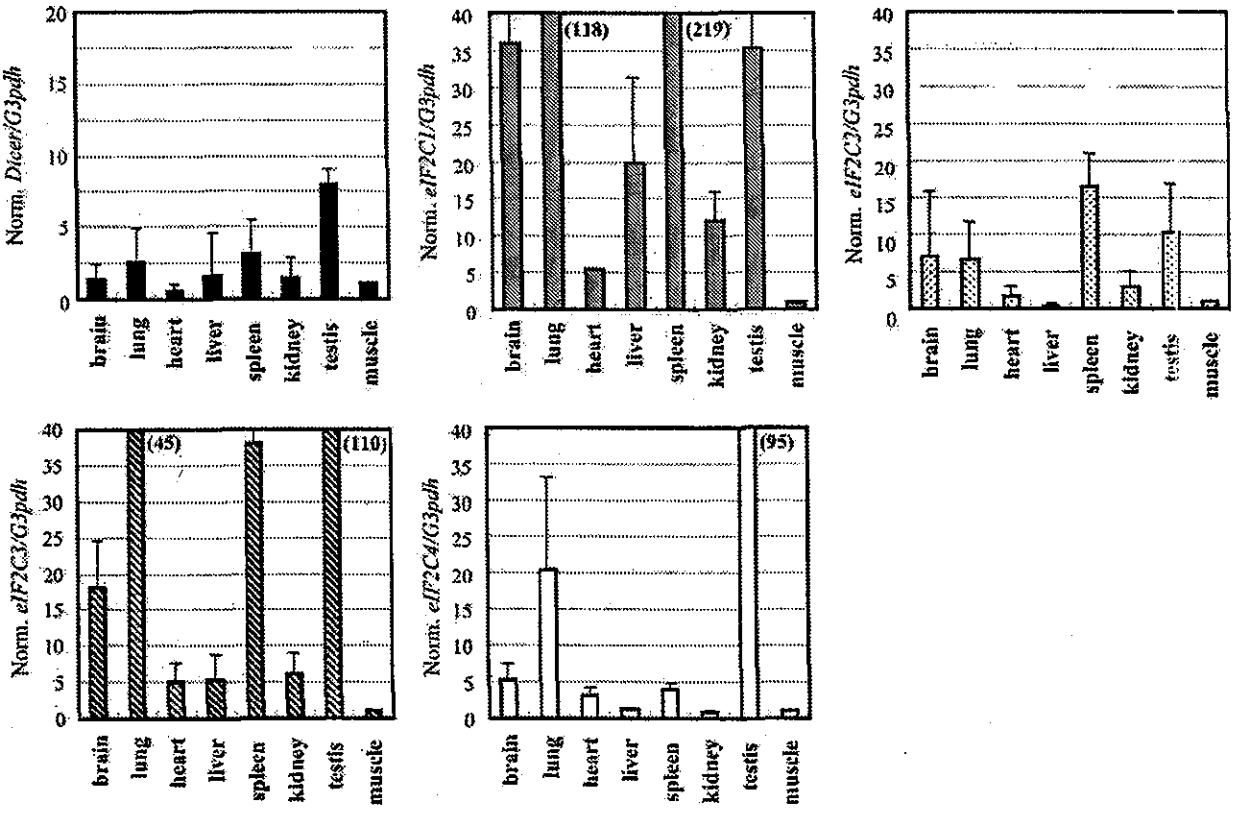

Fig. 1. Expression profiles of *Dicer* and *eIF2C1~4* in various mouse tissues. Total RNA was extracted from indicated tissues and subjected to cDNA synthesis with oligo(dT) primer and a reverse transcriptase. The expression levels of *Dicer* and *eIF2C1~4* were examined by means of a real-time PCR using the synthesised cDNAs as templates. The expression levels of the genes are normalised to that of the *G3pdh* gene examined as a control, and plotted when the expression level of either *Dicer* or *eIF2C1~4* in skeletal muscle is given as 1. Figures in parentheses indicate the averaged expression levels which are over the plotted areas. Data are averages of three independent experiments. Error bars represent standard deviations.

antibiotics, and seeded into 24-well culture plates (approximately 5×10^4 cells/well). Before the transfection, the culture medium was replaced with 0.5 ml OPTI-MEM I (Invitrogen), and to each well, 0.25 μg pGL3-control plasmid (Promega), 0.05 μg pRL-SV40 plasmid (Promega), and 0.2 μg siRNAs were applied. After 4-h incubation, 0.5 ml of the fresh culture medium without antibiotics was added, and further incubation at 37 °C was carried out. In the case of transfection into the isolated muscle fibers, the transfection mixture was directly applied into wells, and further incubation at 37 °C was carried out. When a short-hairpin expression plasmid, pRNA-U6.1/Neo/siRNA (GenScript), was used instead of synthetic siRNAs, 0.1 μg pGL3-control and 0.05 μg phRL-TK (Promega) together with various amounts of pRNA-U6.1/Neo/siRNA were introduced into C2C12 cells. The expression of luciferase was examined using a Dual-Luciferase reporter assay system (Promega) according to the directions provided by the manufacturer.

RT-PCR. Total RNA was extracted from the cultured cells and various mouse tissues using Trizol reagent (Invitrogen). Reverse-transcription (RT) for synthesizing the first-strand cDNAs was carried out using oligo(dT) primer and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions, and 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 or a TaqMan Universal PCR Master Mix together with Assays-on-Demand Gene Expression products (Applied Biosystems) according to the manufacturer's instructions. For plotting a standard curve, the 1, 5, 25, 125, and 625-fold diluted brain cDNA samples, which were prepared from a brain tissue (total RNA) and designated as standards, were used in every real-time PCR. Expression levels of the genes examined were normalised to that of the control *G3pdh* gene. The PCR primers used in the real-time PCR were as follows:

G3pdh-F; 5′-TCTTCACCACCATGGAGAAG-3′
G3pdh-R; 5′-TCATGGATGACCTTGGCCAG-3′
Dicer-F; 5′-GCAGGCTTTTACACACGCCT-3′
Dicer-R; 5′-GGGTCTTCATAAAGGTGCTT-3′
eIF2C2-F; 5′-AGATGAAGAGGAAGTACCGT-3′
eIF2C2-R; 5′-CAGAACCAGCTTGTGCCTGT-3′

The Assays-on-Demand Gene Expression products used (the Assay ID numbers) were as follows:

eIF2C1; Mm00462977m1, *eIF2C3*; Mm00462959m1, *eIF2C4*; Mm00462659m1.

5-Bromodeoxyuridine incorporation assay. Cells were metabolically labeled in the culture medium containing 10 μM of 5-bromodeoxyuridine (BrdU) (Sigma) for 20 h, and rinsed with phosphate-buffered saline solution (PBS) followed by fixation with 70% ethanol containing 0.5 M HCl at −20 °C for 1 h. The resultant cells were incubated with anti-BrdU antibody (Oxford biotechnology) at 4 °C overnight. The BrdU-antibody complexes were visualised with Alexa488 conjugated secondary antibody (Invitrogen) and examined using a ZEISS (Axiovert) microscope.

Results and discussion

Expression profiles of Dicer and eIF2C1~4 in various mouse tissues

Previous studies suggested that *Dicer* and *eIF2C* translation initiation factors (*eIF2C1~4*) homologous to the *Ago* genes in *Drosophila* [26,27] contributed to mammalian RNAi [21,22]. *Dicer* appears to be expressed ubiquitously, but its expression level varies among tissues [16,17]. Since little is known about the expression levels of *eIF2C1~4* among tissues, we first examined the levels of expression of *eIF2C1~4* and *Dicer* in various tissues. Total RNA was extracted from mouse tissues and subjected to cDNA synthesis with oligo(dT) primer and reverse transcriptase. The resultant cDNAs were examined by a real-time PCR. The results are shown in Fig. 1. The expression level of *Dicer* in either skeletal muscle or heart appears to be lower than those in other tissues, which agrees with the previous observations [16,17]. It should be noted that the expression levels of *eIF2C1~4* in either skeletal muscle or heart, like the expression profile of *Dicer*, are also significantly lower than those in the other tissues examined. Consequently, the observations suggest that

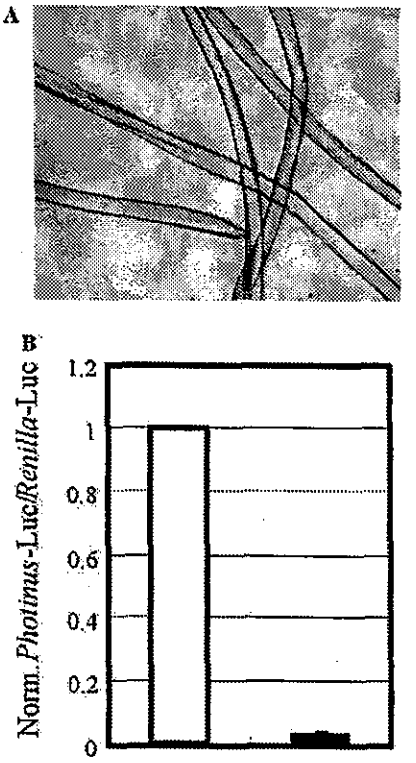

Fig. 2. RNAi induction by synthetic siRNA duplexes in muscle fibers prepared from mouse extensor digitorum longus. (A) Photograph of isolated muscle fibers. Isolation of muscle fibers from mouse extensor digitorum longus was carried out as described in Materials and methods. (B) RNAi activity in isolated muscle fibers. The La2 siRNA duplex against the *Photinus* luciferase gene [25] or a non-silencing siRNA duplex (Qiagen) together with pGL3-control and pRL-SV40 plasmids carrying *Photinus* and *Renilla* luciferase reporter genes, respectively, were cotransfected into the isolated muscle fibers. Twenty-four hours after transfection, cell lysate was prepared and dual luciferase assay was carried out. Ratios of normalised target (*Photinus*) luciferase activity to control (*Renilla*) luciferase activity are indicated: the ratios of luciferase activity determined in the presence of the La2 siRNA duplex are normalised to the ratios obtained in the presence of the non-silencing siRNA duplex. Open and solid bars indicate the data in the presence of the non-silencing siRNA and La2 siRNA duplexes, respectively. Data are averages of at least three independent experiments. Error bars represent standard deviations.

skeletal and cardiac muscle cells express either *Dicer* or *eIF2C1~4* at a low level.

RNAi activity in muscle fibers isolated from mouse extensor digitorum longus

The observations described above raised the question whether RNAi could occur in muscle, i.e., whether RNAi could be induced under a condition with a low level of expression of either *Dicer* or *eIF2C1~4*. In order to address the question, we isolated mouse muscle fibers from extensor digitorum longus of ICR mice (Fig. 2A), and introduced synthetic 21-nt siRNA duplex targeting the exogenous reporter gene, *Photinus* luciferase, together with a pGL3-control plasmid carrying the *Photinus luciferase* gene and a pRL-SV40 plasmid carrying the *Renilla luciferase* gene as a control into the isolated muscle fibers. For realizing an efficient RNAi induction, we used the La2 siRNA duplex having the potential for inducing a strong RNAi activity in cultured mammalian cells [25]. As shown in Fig. 2B, the results indicate that the La2 siRNA duplex can induce a strong gene silencing of the *Photinus luciferase* gene in the muscle fibers. This result suggests that RNAi can be induced by synthetic siRNA duplexes in skeletal muscle which barely expresses either *Dicer* or *eIF2C1~4*.

RNAi activity during myogenic differentiation of mouse C2C12 cells

To further examine the properties of RNAi in muscle cells and during myogenic differentiation, we investigated RNAi activity in C2C12 cells, a mouse myoblast cell line, which can be induced by changing culture conditions (detailed in Materials and methods) to differentiate into contractile myotubes [24]. First, we

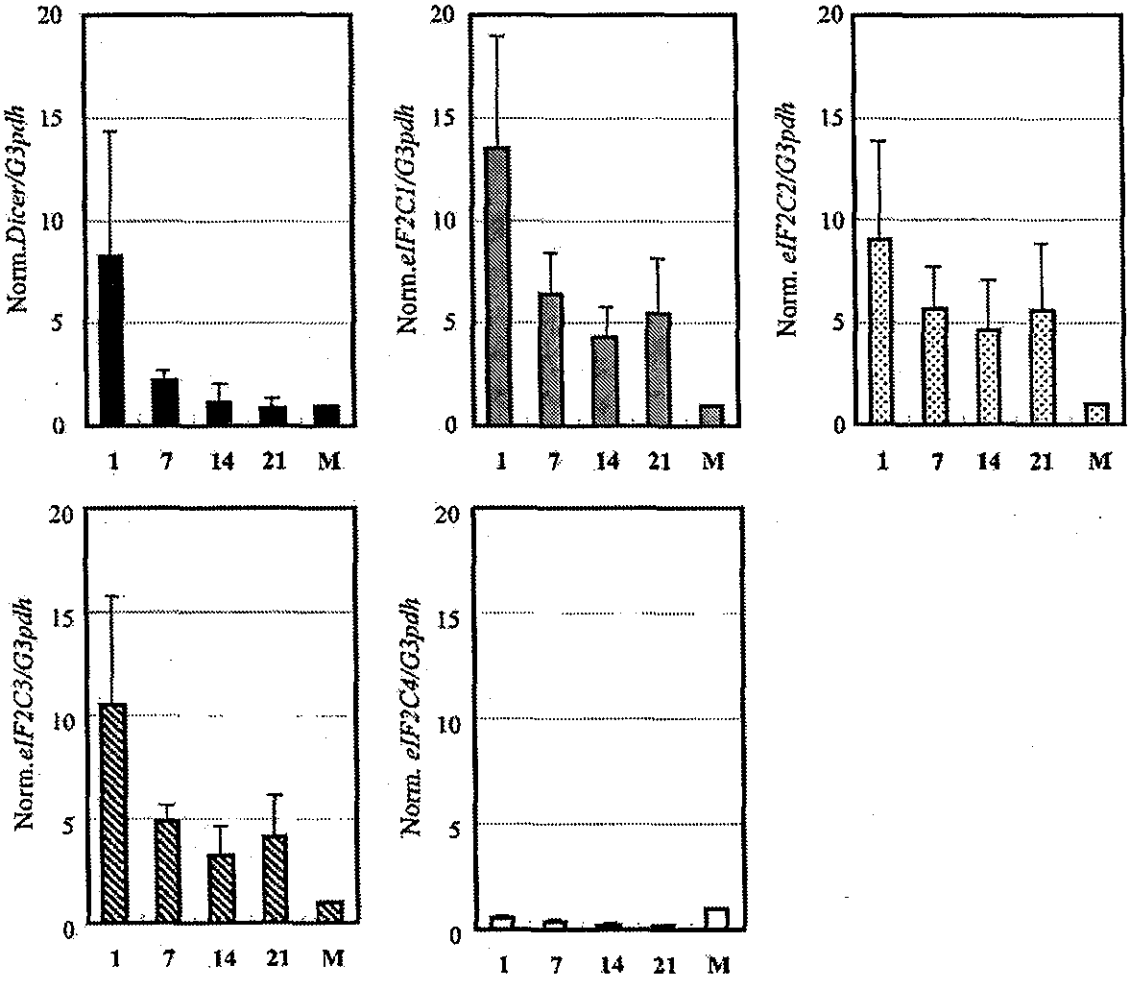

Fig. 3. Expression profiles of *Dicer* and *eIF2C1~4* during myogenic differentiation of mouse C2C12 cells. Total RNA was extracted from C2C12 cells at various days (indicated) after induction of myogenic differentiation of the cells (day 1 indicates undifferentiated C2C12 cells), and subjected to RT-PCR to examine the expression levels of *Dicer* and *eIF2C1~4* as in Fig. 1. The expression levels of the genes are normalised and plotted as in Fig. 1. M indicates skeletal muscle. Data are averages of three independent experiments. Error bars represent standard deviations.

examined the expression profiles of *Dicer* and *eIF2C1~4* during the myogenic differentiation of C2C12 cells and compared them with those of skeletal muscle examined above. As shown in Fig. 3, the expression profiles reveal that the level of expression of either *Dicer* or *eIF2C1~3* is gradually decreased during the myogenic differentiation of C2C12 cells, and that the *eIF2C4* gene is expressed at a low level in either C2C12 myoblast or myotube.

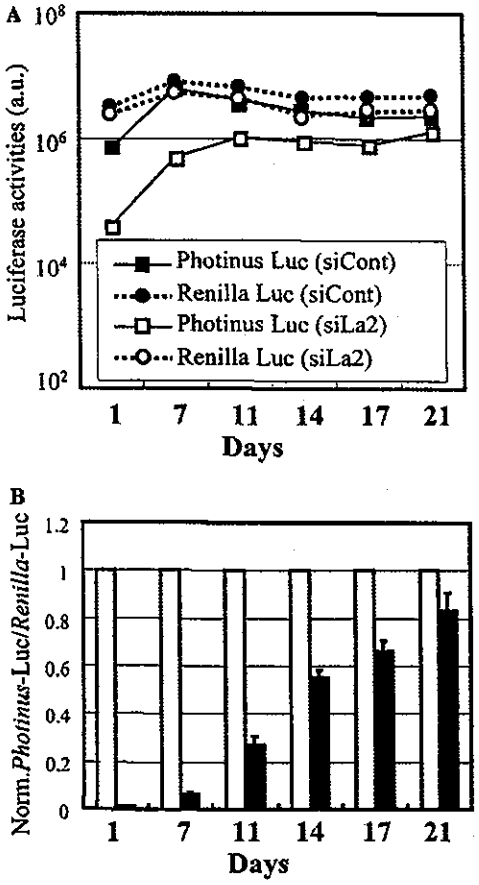

Fig. 4. Persistence of RNAi activity during myogenic differentiation of mouse C2C12 cells. The La2 siRNA duplex or a non-silencing siRNA duplex (Qiagen) together with pGL3-control and pRL-SV40 plasmids were cotransfected into C2C12 cells as in Fig. 2. Before transfection, the culture medium (DMEM containing 15% fetal calf serum) was replaced with DMEM containing 5% horse serum for induction of the myogenic differentiation of C2C12 cells. RNAi activity was examined 24 h after transfection (day 1), and thereafter examined at various days (indicated) up to 3 weeks after the transfection. (A) Absolute *Photinus* and *Renilla* luciferase expressions. The expression levels are plotted in arbitrary luminescence units (a.u.). (B) Ratios of normalised target to control luciferase. Ratios of normalised target (*Photinus*) luciferase activity to control (*Renilla*) luciferase activity are indicated as in Fig. 2. Open and solid bars indicate the data in the presence of the non-silencing siRNA and La2 siRNA duplexes, respectively. Data are averages of at least three independent experiments. Error bars represent standard deviations.

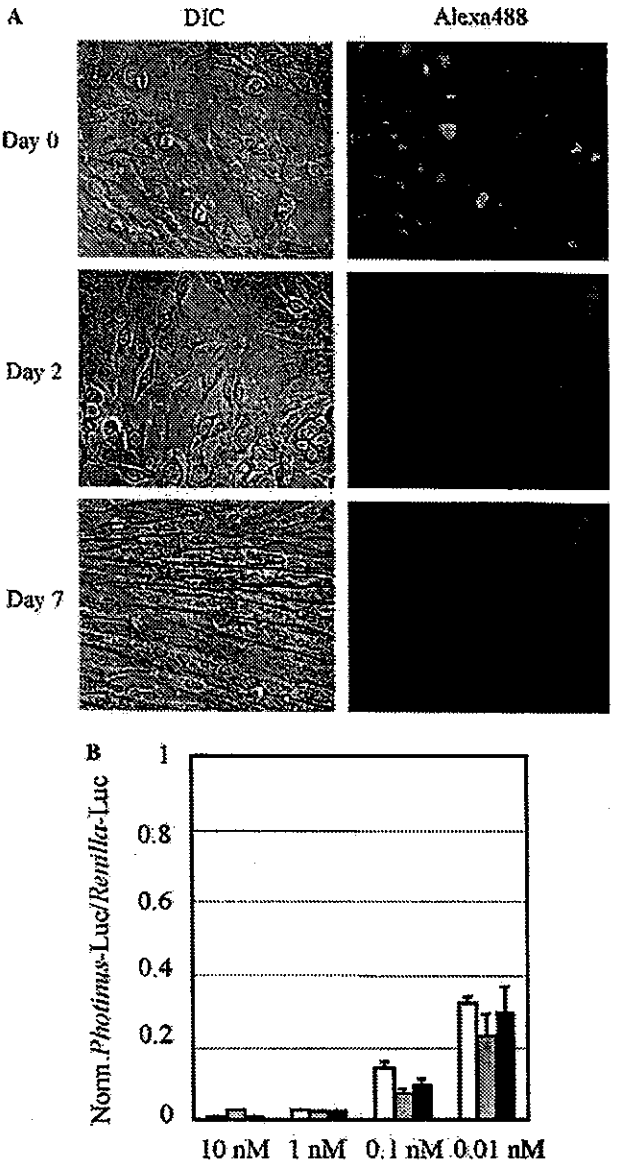

Fig. 5. Cell-cycle arrest and RNAi activity during myogenic differentiation of C2C12 cells. Myogenic differentiation of C2C12 cells was induced by changing the culture medium from DMEM containing 15% fetal calf serum to DMEM containing 5% horse serum. (A) Cell-cycle arrested C2C12 cells. Metabolically labeling of the cells with BrdU was carried out at indicated days after the differentiation. Day 0 indicates undifferentiated C2C12 cells. BrdU incorporated into the cells was visualised with an anti-BrdU antibody and an Alexa488 conjugated secondary antibody. The cells were examined by a florescent microscope. Left (DIC, differential interference contrast) and right (Alexa488, florescence image) panels are identical in visual field. (B) RNAi activity during the differentiation. The reporter plasmids carrying the *Photinus* and *Renilla luciferase* genes were cotransfected with a decreasing amount of the La2 siRNA or non-silencing siRNA duplexes (Qiagen), from 10 to 0.01 nM, into either undifferentiated or differentiated C2C12 cells. Ratios of normalised target (*Photinus*) luciferase activity to control (*Renilla*) luciferase activity are indicated as in Fig. 2. Open, dotted, and solid bars indicate the data in C2C12 cells that differentiated for 0 (undifferentiated), 2, and 7 days, respectively. Data are averages of at least three independent experiments. Error bars represent standard deviations.

Next we examined RNAi activity during the myogenic differentiation of C2C12 cells. The La2 siRNA duplex together with pGL3-control and pRL-SV40 plasmids was cotransfected into undifferentiated C2C12 cells, and simultaneously myogenic differentiation of the cells was carried out by changing culture medium as described above (see Materials and methods). As a result, a strong RNAi activity was detected by day 7 after RNAi induction (Fig. 4), when morphological changes of C2C12 cells into myotubes appeared to be completed (Fig. 5A); thereafter, the cells gradually lost the RNAi activity and lost most of the activity by day 21 after the induction (Fig. 4).

Because proliferating mammalian cells gradually lose RNAi activity with an increase in the number of cell divisions [12,28,29], we investigated whether cell division occurred in C2C12 cells during the differentiation by means of a BrdU incorporation assay. As shown in Fig. 5A, while the incorporation of BrdU into nuclei could be observed in undifferentiated C2C12 cells, few or no BrdU-positive cells were detectable at day 2 and 7 after induction of the differentiation. In addition, from the data of Fig. 5B, the nature of RNAi activity during the differentiation appears to remain unchanged. Consequently, these observations suggest that C2C12 cells differentiated over 2 days are probably cell-cycle arrested cells, and thus that the decrease in RNAi activity during the myogenic differentiation of C2C12 cells is not caused by cell division.

We further examined RNAi activities in C2C12 myotubes that differentiated for 14 and 21 days. The results indicate that RNAi activities induced by synthetic siRNA duplexes are detectable in those differentiated C2C12 myotubes (Fig. 6), although the transfection efficiency of siRNA and plasmid DNA into the cells seemed to become lower as the culture was long. Taking all the data together, it is conceivable that the decrease in RNAi activity during the myogenic

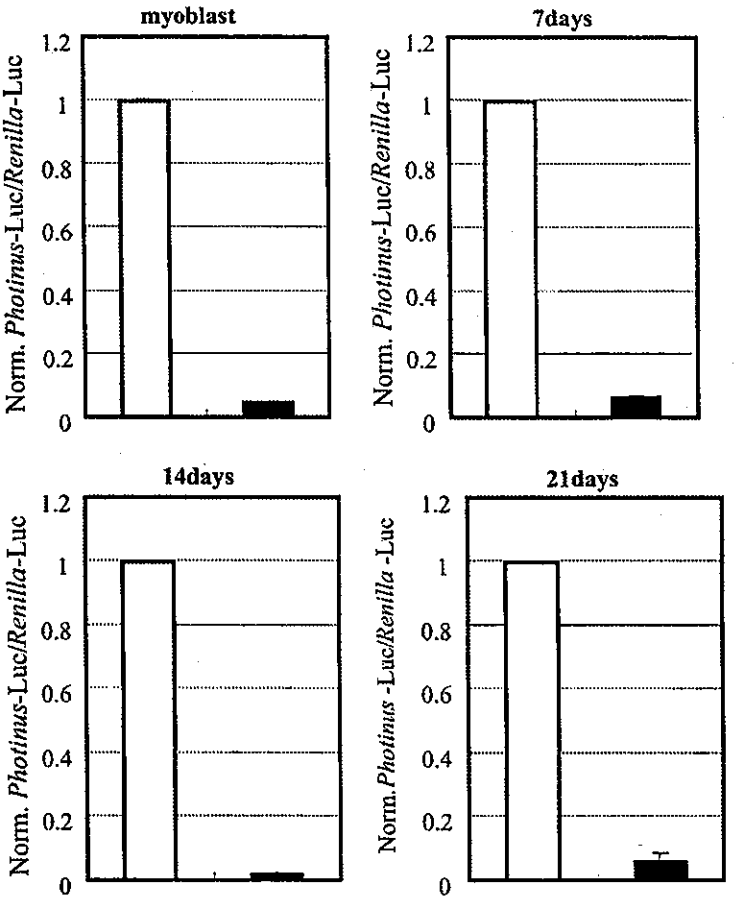

Fig. 6. RNAi induction after myogenic differentiation of C2C12 cells. Myogenic differentiation of C2C12 cells was performed as in Fig. 5. RNAi induction was carried out as in Fig. 2 at indicated days after induction of the myogenic differentiation, and each RNAi activity was examined 24 h after RNAi induction. Ratios of normalised target (*Photinus*) luciferase activity to control (*Renilla*) luciferase activity are indicated as in Fig. 2. Open and solid bars indicate the data in the presence of the non-silencing siRNA and La2 siRNA duplexes, respectively. Data are averages of at least three independent experiments. Error bars represent standard deviations.

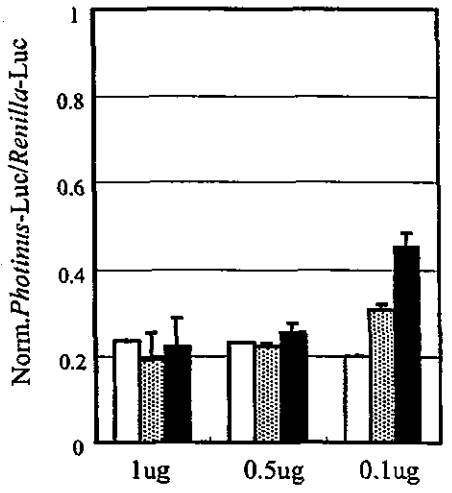

Fig. 7. RNAi induction by short-hairpin RNAs during myogenic differentiation of C2C12 cells. The pRNA-U6.1/Neo/siFluc plasmid (GenScript), which can express a short-hairpin RNA (shRNA) against *Photinus luciferase*, and pRNA-U6.1/Neo empty vector (GenScript) as a control were used. The pGL3-control and phRL-TK plasmids together with a decreasing amount of each of the pRNA-U6.1/Neo/siFluc and pRNA-U6.1/Neo (a negative control) plasmids, from 1 to 0.1 μg, were cotransfected into C2C12 cells. The expression of luciferase was examined 24 h after the transfection. Ratios of normalised target (*Photinus*) luciferase activity to control (*Renilla*) luciferase activity are indicated as in Fig. 2. Open, dotted, and solid bars indicate the data in C2C12 cells that differentiated for 0 (undifferentiated), 2, and 7 days, respectively. Data are averages of at least three independent experiments. Error bars represent standard deviations.

differentiation of C2C12 cell may be caused by losing the stability of functional RISCs in the differentiated C2C12 myotubes.

RNAi induction by short-hairpin RNAs in C2C12 cells

Because Dicer appears to be required for the process of short-hairpin RNAs (shRNAs) into siRNA duplexes, it may be of interest to see if shRNAs can induce RNAi in C2C12 myotubes which barely express *Dicer*. To examine this, we introduced a shRNA expression plasmid against *Photinus* luciferase, pRNA-U6.1/Neo/siRNA, together with the reporter plasmids carrying the *Photinus* and *Renilla* luciferase genes into C2C12 myoblast and myotubes. The results indicate that the shRNA expression plasmid, or shRNAs can induce RNAi in either C2C12 myoblast or myotube (Fig. 7), suggesting that the Dicer protein could be present in those cells. An interesting point to note is that a decrease in the RNAi activity induced by 0.1 μg pRNA-U6.1/Neo/siRNA was observed in C2C12 myotubes that differentiated for 7 days. This may be caused by a possible decrease in the amounts of Dicer and eIF2C1~4 in the cells. To further evaluate the results and a possible relationship between the quantitative level of either Dicer or eIF2C1~4 and RNAi activity, more extensive studies must be conducted.

Integrity of mammalian RNAi

Our previous study has demonstrated that RNAi activity induced by synthetic siRNA duplexes in post mitotic neurons persists for at least 3 weeks, i.e., a long-lasting RNAi activity occurs in mammalian neurons [29]. Our present and previous studies, therefore, suggest that there is a significant difference in the duration of RNAi activity between muscle and neuron, both of which are terminally differentiated and cell cycle-arrested cells. Since neither muscle nor neuron probably undergoes a decrease in the number of functional RISCs by cell division, it may be possible that the stability of functional RISCs could differ between muscle and neuron.

The present observations further suggest the possibility that a little amount of either Dicer or eIF2C1~4 might be sufficient for activation of mammalian RNAi. This seems to be an important point for understanding mammalian RNAi, and further studies on the contribution of either Dicer or eIF2C1~4 to mammalian RNAi must be conducted.

Finally, all the data presented here lead us to the possibility that RNAi may be applicable for a creation of possible model cells and/or model animals for inherited muscular diseases, for example, muscular dystrophy.

Acknowledgments

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Influence of assembly of siRNA elements into RNA-induced silencing complex (RISC) by fork-siRNA duplex carrying nucleotide mismatches at the 3'- or 5'-end of the sense-stranded siRNA element

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Abstract

RNA interference (RNAi) is a powerful method for suppressing the expression of a gene of interest, and can be induced by 21-25 nucleotide small interfering RNA (siRNA) duplexes homologous to the silenced gene, which function as sequence-specific RNAi mediators in RNA-induced silencing complexes (RISCs). In the previous study, it was shown that fork-siRNA duplexes, whose sense-stranded siRNA elements carried a few nucleotide mismatches at the 3'-ends against the antisense-stranded siRNA elements, could enhance RNAi activity more than conventional siRNA duplexes in cultured mammalian cells. In this study, we further characterized fork-siRNA duplexes using reporter plasmids carrying target sequences complementary to the sense- or antisense-stranded siRNA elements in the untranslated region of *Renilla luciferase*. The data presented here suggest that nucleotide mismatches at either the 3'- or 5'-end of the sense-stranded siRNA elements in fork-siRNA duplexes could influence assembly of not only the antisense-stranded siRNA elements but also the sense-stranded elements into RISCs. In addition, we further suggest the possibility that there could be a positional effect of siRNA duplex on RNAi activity.

Key words: RNA interference (RNAi), fork-siRNA, mismatch, RNAi mediator, RISC

Introduction

RNA interference (RNAi) is the process of a sequence-specific post-transcriptional gene silencing triggered by double-stranded RNAs (dsRNAs) homologous to the silenced

gene (reviewed in [1-4]). DsRNAs introduced or generated in cells are subjected to digestion with an RNase III enzyme, Dicer, into 21-25 nucleotide (nt) RNA duplexes [5-8], and the resultant RNA duplexes, referred to as small interfering RNA (siRNA) duplexes, can be associated with the RNA-induced silencing complexes (RISCs) and function as sequence-specific RNAi mediators in the complexes [5, 7]. In terms of rapid and potent induction of RNAi by exogenous dsRNAs, RNAi has become a powerful reverse genetic tool for suppressing the expression of a gene of interest in various species including mammals.

In mammals, direct introduction of chemically synthesized 21-25 nt siRNA duplexes into cells is often used for induction of RNAi [9-12], although different siRNAs induce different levels of RNAi activities [10, 13]. In previous studies, where the effect of various types of synthetic siRNAs on the induction of mammalian RNAi was tested, an improvement of the siRNA duplexes for enhancing RNAi activity was found [14]. The improved siRNA duplexes, named 'fork-siRNA duplexes', possess mismatched sequences at their termini due to introduction of base substitutions into the 3'-ends of the sense-stranded siRNA elements: one of the ends of the fork-siRNA duplex, on the 3'-end of the sense-stranded siRNA element, remains open (unannealed). In addition to the properties of fork-siRNA duplex, recent studies suggested that siRNA duplexes conferring a strong RNAi activity could be characterized by a low base-stability due to AU-rich sequences around the 3'-ends of the sense-stranded siRNA elements, i.e., the functional siRNA duplexes have the asymmetrical features of the AU (or GC) content in their sequences [14-16]. Based on these observations, a model was proposed: the ease of unwinding from one of the ends of the siRNA duplex could determine the orientation of the duplex, and the siRNA element unwound from the

5'-end could be determined and function as a sequence-specific RNAi mediator in RISC [14].

In order to further characterize fork-siRNA duplexes, we report herein the construction of reporter plasmids carrying target sequences complementary to the sense- and antisense-stranded siRNA elements, and examination of the levels of gene silencing depending upon the sense- and antisense-stranded siRNA elements functioning as RNAi mediators in RISCs. The data presented here suggested that the structural features of fork-siRNA duplexes could influence incorporation of their siRNA elements into RISCs.

Materials and Methods

Preparation of oligonucleotides

RNA and DNA synthetic oligonucleotides were obtained from PROLIGO and INVITROGEN, respectively. For preparation of duplexes, sense- and antisense-stranded oligonucleotides (20 μM each) were mixed in an annealing buffer (30 μM HEPES pH7.4, 100 μM potassium acetate, 2 μM magnesium acetate), heat-denatured at 90 °C for 3 min, and annealed at 37 °C overnight. The siRNA duplexes used in this study were as follows: the La21-conv. siRNA duplex (a conventional siRNA duplex), La21-3'm2 siRNA duplex (a fork-siRNA duplex carrying two-base mismatches at the 3'-end of the sense-stranded siRNA element), La21-5'm2 siRNA duplex (a fork-siRNA duplex carrying two-base mismatches at the 5'-end of the sense-stranded siRNA element), and La21-ss19 siRNA duplex (a siRNA duplex containing 19 nt sense-stranded siRNA element) (Fig. 1B) [14]. Note that these La21 siRNA duplexes possess the same antisense-stranded siRNA elements. Non-silencing

siRNA duplex (Qiagen) was used as a negative control.

Construction of reporter plasmids

In order to insert target sequence complementary to the sense- or antisense-stranded La21 siRNA element [10] against *Photinus luciferase* in the 3' untranslated region (UTR) of the *Renilla luciferase* transcripts, the phRL-TK plasmid (Promega) carrying the *Renilla luciferase* gene was digested with *Xba* I and *Not* I, and subjected to ligation with synthetic oligonucleotide duplexes carrying the La21 siRNA target sequences. The sequences of the synthesized oligonucleotides were as follows:

sLa21Fw; 5'-CTAGCATGCAACCGCTGGAGAGCAACTGCA-3'

asLa21Fw; 5'-GGCCTGCAGTTGCTCTCCAGCGGTTGCATG-3'

sLa21Rv; 5'-CTAGCATGCAGCAGTTGCTCTCCAGCGGTA-3'

asLa21Rv; 5'-GGCCTACCGCTGGAGAGCAACTGCTGCATG-3'

The resultant plasmids possessing the target sequences for the antisense- and sense-stranded La21 siRNA elements were named 'phRL-La21Fw' and 'phRL-La21Rv', respectively.

We further constructed reporter plasmids carrying two target sites of the La21 siRNA duplex. The *Hind* III – *Xba* I fragment encoding the *Photinus luciferase* gene was isolated from the pGL3-control plasmid (Promega), and substituted for the *Hind* III – *Xba* I regions carrying *Renilla luciferase* in the phRL-La21Fw and phRL-La21Rv plasmids. The resultant plasmids derived from the phRL-La21Fw and phRL-La21Rv plasmids were named 'pGL3-TK-La21Fw' and 'pGL3-TK-La21Rv', respectively, and possessed two La21 siRNA duplex target sites, one in the Photinus luciferase coding region, and the other in the 3' UTR. We also constructed 'pGL3-TK' plasmid by

substitution of *Photinus luciferase* for *Renilla luciferase* in the phRL-TK plasmid using the same procedure described above.

Cell culture, transfection, and luciferase and β-Galactosidase assays

HeLa cells were grown as described previously [10]. The day before transfection, cells were trypsinized, diluted with fresh medium without antibiotics, and seeded into 24-well culture plates (approximately 0.5 x 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 of siRNA duplexes, 0.05 μg of phRL-La21Fw or phRL-La21Rv plasmid and 0.1 μg of pSV-β-Galactosidase control vector (Promega) as a control were applied. Twenty-four hours after transfection, cell lysate was prepared and the expression levels of luciferase and β-galactosidase were examined by a Dual-Luciferase reporter assay system (Promega) and a Beta-Glo assay system (Promega), respectively, according to the manufacturer's instructions. In the case of transfection with pGL3-TK, pGL3-Tk-La21Fw or pGL3-TK-La21Rv, 0.24 μg of siRNA duplexes, 0.1 μg of any one of the pGL3-TK, pGL3-Tk-La21Fw and pGL3-TK-La21Rv plasmids and 0.05 μg of the phRL-TK plasmid as a control were applied into HeLa cells. Twenty-four hours after transfection, a Dual-Luciferase reporter assay was conducted.

Results and Discussion

Influence of assembly of siRNA elements into RISCs by fork-siRNA duplexes

In the previous study, it was shown that fork-siRNA duplexes carrying nucleotide mismatches at the 3'-ends of the sense-stranded siRNA elements could enhance RNAi

activity more than conventional siRNA duplexes [14]. This suggests the possibility of greater occurrence of assembly of the antisense-stranded siRNA elements rather than the sense-stranded elements into RISCs in fork-siRNA duplexes over that in conventional duplexes. Accordingly, we attempted to examine whether fork-siRNA duplexes could influence incorporation of their siRNA elements into RISCs. To address this, we constructed two reporter plasmids, phRL-La21Rv and phRL-La21Fw, carrying the target sequences for the sense- and antisense-stranded La21 siRNA elements, respectively, in the 3' untranslated region (UTR) of *Renilla luciferase* (Fig. 1A). This is because the previous study showed that the La21-conv., La21-3'm2, and La21-5'm2 siRNA duplexes (Fig. 1B) could confer different levels of RNAi activities, although they possessed the same antisense-stranded siRNA element [14]. In addition to the previous results, the result with a newly designed siRNA duplex, the La21-ss19 siRNA duplex (Fig. 1B, C), also supported the idea that the forked terminus of siRNA duplex could influence RNAi activity. Accordingly, we decided to use the sequences of the sense- and antisense-stranded La21 siRNA elements as targets in this study. Using the phRL-La21Fw and -La21Rv plasmids and a series of the La21 siRNA duplexes, the levels of gene silencing depending upon the sense- and antisense-stranded La21 siRNA elements were investigated.

First we examined if the sense-stranded siRNA elements, like the antisense-stranded siRNA elements, could have potential for functioning as sequence-specific RNAi mediators in RISCs. To see this, the La21-conv. siRNA duplex together with phRL-La21Rv, phRL-La21Fw or phRL-TK and pSV-β-Galactosidase control vector as a control were cotransfected into HeLa cells, and the levels of the expression of *Renilla luciferase* were examined. As a result,

significant suppression of the expression of *Renilla luciferase* was detectable in the presence of either phRL-La21Rv or phRL-La21Fw, whereas little or no suppression was seen in the presence of phRL-TK as a negative control (Fig. 2). Therefore, these results strongly suggest that either the sense- or antisense-stranded La21 siRNA element can be incorporated into RISC and function as a sequence-specific RNAi mediator in the complex.

We next examined the RNAi activities directed by the sense- and antisense-stranded siRNA elements derived from the La21-3'm2, and La21-5'm2 siRNA duplexes (fork-siRNA duplexes) as well as the La21-conv. siRNA duplex. As shown in Fig. 3A, when the phRL-La21Fw plasmid was used, ~ 86%, 95%, and 72% gene silencing mediated by the antisense-stranded La21 siRNA elements derived from the La21-conv., La21-3'm2, and La21-5'm2 siRNA duplexes, respectively, were observed. Although the levels of the gene silencing with phRL-La21Fw as a reporter plasmid increased further than those with the pGL3-control plasmid carrying *Photinus luciferase* in the previous study [14] (further discussion below), the effects of mismatches at the 3'- and 5'-ends of the sense-stranded elements in the La21 fork-siRNA duplexes on RNAi activity appeared to remain unchanged in the experiments using either phRL-La21Fw or pGL3-control.

When the phRL-La21Rv plasmid was used, i.e., when the levels of the RNAi activity directed by the sense-stranded La21 siRNA elements were examined, significant differences in the level of RNAi activity among the La21 siRNA duplexes used were observed: while ~ 92% and 89% suppression of the expression of *Renilla luciferase* were detectable in the presence of the La21-conv., and La21-5'm2 siRNA duplexes, respectively, the gene silencing mediated by the sense-stranded element

derived from the La21-3'm2 duplex appeared to confer ~ 20% inhibition of the expression of *Renilla luciferase* (Fig. 3B), suggesting that the degree of assembly of the sense-stranded siRNA element into RISC in the La21-3'm2 siRNA duplex could be much lower than those in the La21-conv., and La21-5'm2 siRNA duplexes. Taking all the data together, these observations suggest that nucleotide mismatches at the ends of fork-siRNA duplexes can influence assembly of not only the antisense-stranded siRNA elements but also the sense-stranded siRNA elements into RISCs.

The previous *in vitro* RNAi reaction with *Drosophila* embryo lysate has demonstrated that single nucleotide mismatch around the termini of siRNA duplex can affect target-RNA cleavages directed by the sense- and antisense-stranded siRNA elements [15]. The results of our present study using cultured human cells consistently agree with those in the previous study. Therefore, it appears that the effect of low base-pairing stabilities due to either AU-rich or nucleotide mismatches around the termini of siRNA duplexes on RNAi activity is likely common among various species. In addition, such low base-pairing stability could contribute to ready unwinding of the duplex from that end by a possible helicase activity in RISCs.

Another important point to note in this study is that the sense-stranded siRNA elements have potential for functioning as sequence-specific RNAi mediators in RISCs. As previously suggested [14], this indicates that off-target gene silencing mediated by the sense-stranded siRNA elements could occur in RNAi induction by siRNA duplexes. Our present data also indicated a possible avoidance of such off-target gene silencing: fork-siRNA duplexes carrying nucleotide mismatches at the 3'-end of the sense-stranded elements could reduce such off-target silencing. Therefore, fork-siRNA duplexes may provide us with not only an increase in RNAi activity but also decrease in

off-target gene silencing directed by the sense-stranded siRNA elements.

Positional effect of siRNA target site on RNAi activity

The results shown in Fig. 3A led us to the possibility that the position of an siRNA target site on a silenced gene transcript could influence its RNAi activity, i.e., there could be a positional effect of the siRNA target site on RNAi activity. To examine this possibility, we constructed two reporter plasmids carrying *Photinus luciferase*, pGL3-TK-La21Rv and pGL3-TK-La21Fw, whose 3' UTRs contained the target sequences complementary to the sense- and antisense-stranded La21 siRNA elements, respectively (Fig. 1A). Thus, the resultant *Photinus luciferase* transcripts derived from pGL3-TK-La21Fw and pGL3-TK-La21Rv possess two target sites: one site complementary to the antisense-stranded La21 siRNA element is in the luciferase coding region, and the other complementary to the sense- or antisense-stranded siRNA element is in its 3' UTR.

The La21-conv., La21-3'm2, or La21-5'm2 siRNA duplexes together with the pGL3-TK (carrying one target site in the luciferase coding region), pGL3-TK-La21Rv or pGL3-TK-La21Fw plasmid (Fig. 1A) and the phRL-TK plasmid as a control were cotransfected into HeLa cells, and the levels of RNAi activity were examined by a dual-luciferase assay. When the pGL3-TK plasmid was used, results similar to those in the previous study using the pGL3-control plasmid encoding *Photinus luciferase* driven by the SV40 promoter [14] were observed (Fig. 4). When the pGL3-TK-La21Fw and pGL3-TK-La21Rv plasmids were used, the levels of RNAi activity, other than those in the presence of the pGL3-TK-La21Rv plasmid and the La21-3'm2 siRNA duplex, appeared to increase more greatly in both the