Table 2 Proteomics characteristics of oxidatively modified proteins identified in *gad* mouse brain

Identified protein	GI accession no.	No. of peptides	Sequence	Sequence coverage (%)	MOWSE score	*p*
Phosphoglycerate mutase	gil8248819	5	LVLIR KAMEAVAAQGK + Oxidation(M) VLIAAHGNSLR NLKPIKPMQFLGDEETVR + Oxidation(M) HLEGLSEEAIMELNLPTGIPIVYELDK + Oxidation(M)	28	228	1.60×10^{-23}
Thioredoxin peroxidase (peroxiredoxin 2)	gil2499469	9	GVLR EYFSK SVDEALR GLFIIDAK NDEGIAYR SLSQNYGVLK SAPDFTATAVVDGAFK EGGLGPLNIPLLADVTK KEGGLGPLNIPLLADVTK	38	330	1.00×10^{-33}
Rab GDP dissociation inhibitor α	gil21903424	8	VVGVK LYSESLAR VVEGSFVYK FLMANGQLVK + Oxidation(M) KQNDVFGEADQ FQMLEGPPESMGR + 2Oxidation(M) NPYYGGESSSITPLEELYK YIAIASTTVETAEPEKEVEPALELLEPIDQK	23	388	1.60×10^{-39}
NF-L	gil20876600	20	DLR YVETPR LENELR FASFIER LLEGEETR KGADEAALAR ALYEQEIR YEEEVLSR LAAEDATNEK FTVLTESAAK AQLQDLNDR EYQDLLNVK VHELEQQNK MALDIEIAAYR + Oxidation(M) NMQNAEEWFK + Oxidation(M) IDSLMDEIAFLK + Oxidation(M) QNADISAMQDTINK + Oxidation(M) RIDSLMDEIAFLK + Oxidation(M) SAYSGLQSSSYLMSAR + Oxidation(M) SFPAYYTSHVQEEQTEVEETIEATK	35	864	3.90×10^{-87}

Oxidation (M); Oxidation of methionine.

nitration in ALS, neurofilament aggregates also bear advanced glycation endproducts in ALS conglomerates, indicating the susceptibility of neurofilament-L to oxidative insult.

The present finding of oxidized NF-L in *gad* mouse brain, a model for neuron motor disease, confirms previous data that provided evidence for the oxidation-related involvement of NF-L in motor neuron degeneration and established a common pattern for degeneration. If the results shown here for cortical tissue can be extended to spinal cord and motor neurons, the findings may be relevant to molecular mechanisms of neurodegeneration in ALS. Oxidation of NF-L implies modification of hydrophobic interactions that are responsible for the association of the different subunits. In fact, it has been demonstrated that the structure of neurofilament proteins is modified from α-helix to β-sheet

and random coil following free radical damage (Gelinas *et al.* 2000), directly relating oxidative damage of neurofilaments to the axonal degeneration in motor neurons. The consequences of neurofilament disassembly are deleterious to neuronal survival: the axonal antegrade and retrograde transport, which is essential for organelles, especially mitochondria which do not self-sufficiently synthesize most of the proteins necessary for their function, would be compromised.

Reduction of the fixed ubiquitin pool and decreased proteasomal function might lead to accumulation of oxidized proteins, which, in turn, might activate microglia and trigger an inflammatory response with production of free radicals. Such events, which might lead to neurodegeneration, might be exacerbated by the oxidation of thioredoxin peroxidase, which was also a target of oxidation in this *gad* mouse model of neurodegeneration. Thioredoxin peroxidases or peroxiredoxins are small redox proteins that act as antioxidants and catalyze the elimination of hydroperoxides through the reducing system thioredoxin/thioredoxin reductase. This enzyme is considered a strong defense against oxidative stress. Based on the loss of activity of other oxidatively modified brain proteins (Hensley *et al.* 1995; Butterfield *et al.* 1997), the finding of the present study that thioredoxin peroxidase is a target of protein oxidation in *gad* mouse brain implies harmful consequences for neurons in this UCH L-1-altered mouse, owing to a dramatic decrease in cellular antioxidant capability. Peroxiredoxin I/thioredoxin peroxidase 2 is up-regulated in ALS (Allen *et al.* 2003). Moreover, in oxidative stress induced by Fe^{2+} (Drake *et al.* 2002) or H_2O_2 (Mitsumoto *et al.* 2001) variant forms of peroxiredoxins have been identified. These investigations by others, coupled with the present finding that peroxiredoxin is oxidized in the *gad* mouse, are consistent with the existence of common downstream events in oxidative stress-induced neurodegeneration.

An oxidative stress-based mechanism of peroxiredoxin enzyme inactivation can be speculated: the enzyme bears two cysteine residues that are crucial for the antioxidant activity of thioredoxin peroxidase. Products of free radical-induced lipid peroxidation, such as 4-hydroxy-2-nonenal, can introduce carbonyls to proteins by covalent Michael addition to -SH groups of protein side chains (Butterfield and Stadtman 1997; Lauderback *et al.* 2001). Thus, it is conceivable that these cysteine proteins in peroxiredoxin are susceptible to attack by the excess 4-hydroxy-2-nonenal in neurodegenerative disorders associated with oxidative stress, including AD. Michael addition of 4-hydroxy-2-nonenal to brain proteins changes their structure and activity (Esterbauer *et al.* 1991; Subramaniam *et al.* 1997; Lauderback *et al.* 2001).

Phosphoglycerate mutase is the glycolytic enzyme responsible for the interconversion of 3-phosphoglycerate to 2-phosphoglycerate. Glycolytic enzymes and creatine kinase BB are oxidatively modified in AD brain (Castegna *et al.* 2002a, 2002b, 2003; Butterfield and Castegna 2003; Butterfield *et al.* 2003), and this oxidation might contribute to the reduced glucose metabolism observed in AD brain (Messier and Gagnon 1996), especially in the temporoparietal and frontal areas (Mielke *et al.* 1996). Although there is no evidence of involvement of energy metabolism depletion in the *gad* brain, the present finding that phosphoglycerate mutase was significantly more oxidized in *gad* brain than in control brain suggests a possible lower energy metabolism that might exacerbate the consequences of a lack of ATP necessary to carry out normal cellular function. Future studies should address this possibility.

Rab GDP dissociation inhibitor α and ATP synthase were detected as a single spot on the *gad* brain map, and the spot corresponding to these proteins showed increased carbonyl immunoreactivity. It is not possible at the present time to distinguish the extent of oxidation for the two proteins, the pI and molecular mass of which are practically identical. This result illustrates a limitation of 2D PAGE in separating proteins of similar size and charge distribution. Additional studies are required, perhaps using different isoelectric focusing strips with wider pH gradients, to separate the mixture into two detectable spots.

As seen in the present study, oxidative stress may be a source of protein damage by a vicious cycle, in which targets of oxidation may lead to further damage, as in the *gad* mouse. Further study of these connections is warranted. The present study demonstrates how the powerful tools of proteomics can provide insights into potential mechanisms of neurodegenerative disease. Continued use of proteomics analysis for the brain proteome in oxidative stress-related neurodegenerative disorders, and animal and culture models thereof, is in progress.

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Specific inhibition of Huntington's disease gene expression by siRNAs in cultured cells

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Abstract: Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by an abnormally expanded CAG repeats in exon 1 of HD gene, and consequently its gene product, huntingtin, contains an abnormally long glutamine tract. It is generally accepted that the mutant huntingtin selectively kills striatal neurons. As a first step for the development of a radical therapy toward HD, here we have investigated the effects of siRNAs directed against the HD gene in order to knock down its expression in cultured cells. Results showed that, although efficiencies of three siRNA molecules tested were slightly different from each other with cell types and origins, one siRNA (named siRNA-HDexon1), targeted against a region at immediately upstream of CAG repeats, can efficiently and specifically inhibit the expression of huntingtin exon 1-EGFP fusion construct, whereas the other two had moderate or minor effects. The siRNA-HDexon1 did also efficiently suppress the endogenous huntingtin expression in cells of human origin.

Key words: Huntington's disease; huntingtin; RNAi; siRNA; cell culture.

Introduction. Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder clinically characterized by the impairments of motor, mind and cognitive functions and pathologically by a selective neuronal loss, most prominently of medium-spiny neurons in the striatum.[1),2)] The disease is caused by an expansion of a trinucleotide repeat (CAG: a codon for glutamine) in exon 1 of the gene coding for the huntingtin protein,[3)] function of which is not yet fully elucidated.[4)] The normal alleles carry 6 to 34 CAG repeats, whereas mutant alleles possess 36 to more than 100 CAG repeats at the HD locus (4p 16.3). Therefore, the mutant huntingtin possesses abnormally long glutamine tract (poly Q).[5)]

Bates and her colleagues[6)] found that when exon 1 of the huntingtin gene with abnormally expanded CAG repeats was transgened to mice, abnormal behaviors appeared and ubiquitinated nuclear inclusion bodies composed of poly Q aggregations were detected in the striatal and cortical neurons.[7)] This also occurred in patients with HD, suggesting that the continuous expression of the mutant huntingtin and consequently accumulation of incompletely processed proteins and/or co-aggregation of the mutant huntingtin with other essential proteins in neurons may play a critical role in the disease development and progression.[8)-11)] Moreover, according to Yamamoto's report,[12)] a conditional knock-out of the abnormal HD gene in the adult mice would lessen the degree of the disease. All of these findings suggest that the expression of the abnormal huntingtin may cause the disease in a gain-of-function fashion. Therefore, it is reasonable to suppose that the suppression of the HD gene expression may lessen the accumulation of mutant huntingtin, thereby delaying the onset of the disease and slowing its progression.

In this respect, it is worthy to note that RNA interference (RNAi), the process of the posttranscriptional gene silencing mediated by double-stranded RNAs (dsRNAs) first discovered in *C. elegance*,[13)] has rapidly emerged as an important tool for regulating the gene expression in a wide range of species.[14)] Particularly in mammalian cells, the inhibition of the specific gene

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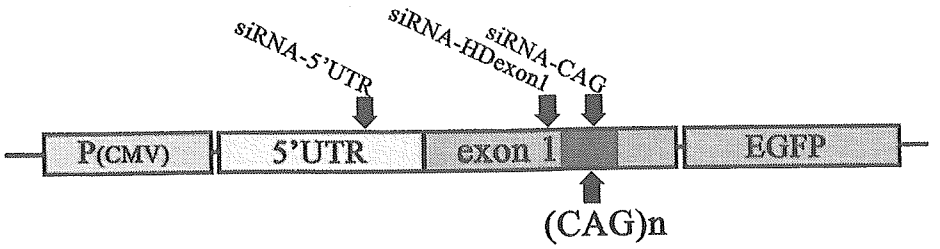

Fig. 1. A schematic drawing of the constructs used in the experiments and target positions of siRNAs. Construct expression vector contains human HD gene exon 1. Cytomegalovirus promotor [P(CMV)] was ligated at the 5' end of the exon 1 gene, and a set of the sequence for GFP [EGFP] ligated at the 3' end as a reporter. Two groups of constructs were made in terms of the length of CAG repeats: normal HD exon 1 containing 16-32 CAG repeats and mutant HD exon 1 containing 41-151 CAG repeats (see Table I). Positions of siRNAs tested are indicated with black arrows.

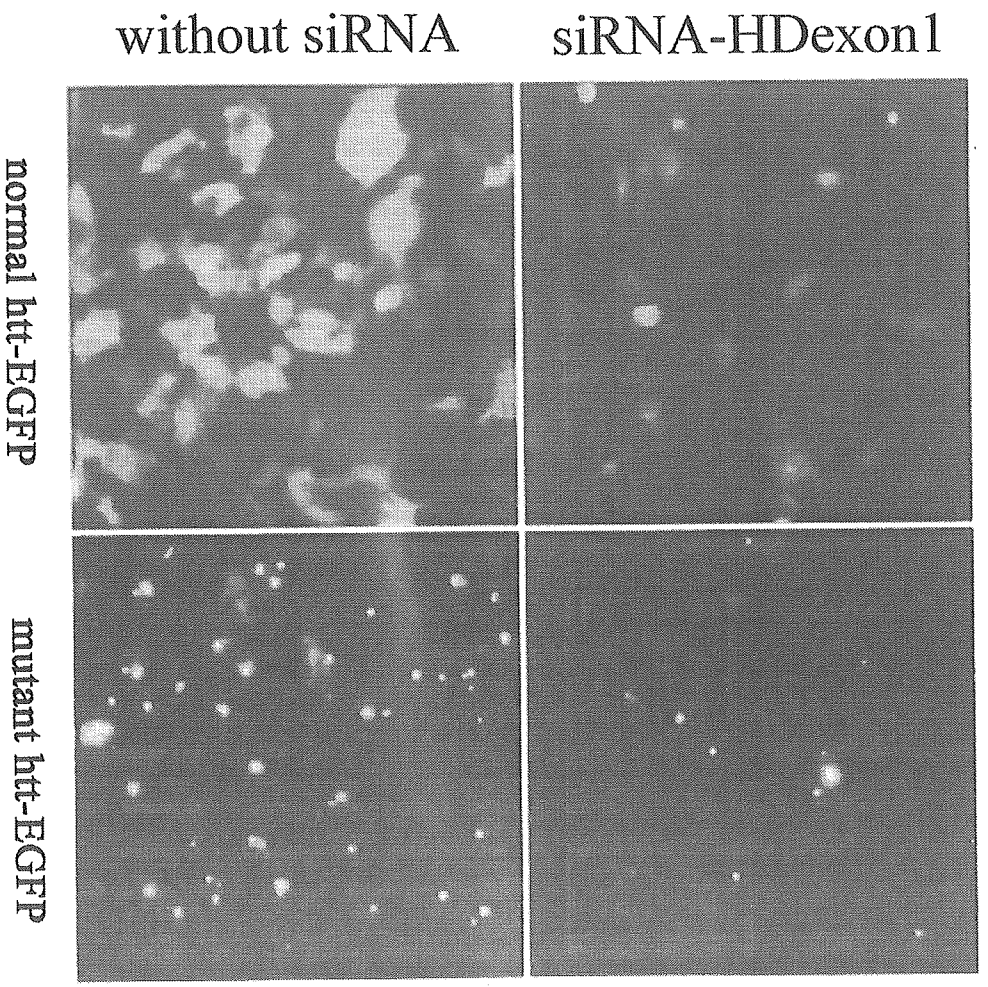

Fig. 3. Fluorescence images of COS-7 cells transfected with constructs ligated with EGFP sequence. Upper panels are representative fluorescence images of COS-7 cells transfected with the construct inserted with HD exon 1 containing normal sized CAG repeat (17Q). Lower panels are cells transfected with the construct inserted with HD exon 1 containing expanded CAG repeat (72Q). Left panels are cells transfected with HD gene construct alone. Right panels are cells co-transfected with HD gene construct and siRNA-HDexon1 (40 nM).

1) siRNA-5'UTR

5'-GAUGGACGGCCGCUCAGGUUU-3'
3'-UUCUACCUGCCGGCGAGUCCA-5'

2) siRNA-HDexon1

5'-GCCUUCGAGUCCCUCAAGUCC-3'
3'-UCCGGAAGCUCAGGGAGUUCA-5'

3) siRNA-CAG

5'-GCAGCAGCAGCAGCAGCAGCA-3'
3'-GUCGUCGUCGUCGUCGUCGUC-5'

Fig. 2. Nucleotide sequences of the three siRNAs tested.

expression is mediated by short inhibitory RNAs (siRNAs), which are dsRNAs composed of 21-23 nucleotides (nt) with 2 nt overhang on 3'-terminus of each strand, whereas dsRNAs of longer than 30 nt induced a global suppression of the gene expression by a mechanism related to interferon response.[15),16)] Recently, siRNAs targeted against a great variety of genes have been used to knock down the expression of the homologous genes successfully at least in cultured mammalian cells. Therefore, it is reasonable to expect a potential of therapeutic use of siRNAs for inheritable diseases which are caused by the mechanism of a gain-of-function. Based on this assumption, we have developed siRNAs for suppressing the HD gene expression in cultured cells which were transfected with the construct for the human HD exon1-EGFP fusion proteins, hoping that this could be the first step toward a development of siRNAs as a therapeutic tool for HD.

Materials and methods. *Preparation of siRNAs.* 21 nt RNAs were chemically synthesized, deprotected and HPLC purified (Xeragon, USA). Double stranded siRNA was prepared by denature and annealing 20 mM sense- and antisense-strand RNAs in annealing buffer (100 mM Potassium acetate, 2 mM Magnesium acetate, 30 mM HEPES, adjusted to pH 7.4 by 0.1 N KOH, Stored at 4 °C). The reaction mixture was heated at 95 °C for 5 minutes, then gradually cooled down to 37 °C, and incubated for 1.5 hours, then 6-20 hours at room temperature. Annealed siRNAs were stored at −20 °C or −80 °C until use. We have searched using BLAST through the HD mRNA upstream of CAG repeats and found two sites that bare unique sequences as valid targets of siRNA and homologous siRNAs have been made: a) siRNA directed against 5'-untranslated region (siRNA-5'-UTR), and b) siRNA against a section in ORF at immediate upstream of CAG repeats (siRNA-HDexon1). We also made the siRNA-CAG directed against the CAG repeats itself, for the only difference between normal and mutant HD gene is the lengths of the CAG repeats (see Fig. 1). Sequences of above three siRNAs are shown in Fig. 2.

Table I. Constructs of fused htt-EGFP containing various poly Q expansions.

	Normal	Mutant
with 5'-UTR	pd1EGFP-U-16Q pd1EGFP-U-31Q	pd1EGFP-U-41Q pd1EGFP-U-69Q
Exon 1 only	pd1EGFP-17Q pd1EGFP-22Q pd1EGFP-32Q	pd1EGFP-49Q pd1EGFP-72Q pd1EGFP-151Q

Constructs. We have chosen pd1-EGFP N1(Clontech, USA) as parent vector because the reporter gene is destabilized EGFP, it has a fast turning over and because of its character of much less stable than EGFP, it is sensitive in reflecting changes of expression level. Two groups of expression constructs were made: one with 5'UTR and exon 1, in order to test siRNA-5'UTR, and the other with only HD exon 1 (Table I). The constructs were generated by cloning human HD partial 5'-UTR and full length of exon 1 into pd1EGFP-N1(de-stabled EGFP, Clontech), fused in-frame with EGFP. For the constructs with 5'UTR, an ATG initiation codon was introduced at the starting region of 5'-UTR in order to avoid mis-translation. The vector with partial 5'-UTR is marked with "U" before the number of poly-Q. The vectors without "U" were inserted with the translated region of HD exon 1. DNA fragments of HD exon 1 containing different CAG repeat lengths were generated following a method using polymerase chain reaction (PCR)-mediated mutagenesis[17)] and were used to construct expression vectors to represent both normal and mutant huntingtin (see Table I).

Cell cultures. The following three cell lines, established from different kind of mammals, were used: COS-7 (African green monkey fibroblasts); SH-SY5Y (human neuroblastoma); Neuro-2A (mouse neuroblastoma). Cells were grown in Minimum Essential Medium-Alpha Medium (Gibco BRL) (for COS-7), or in Dulbecco's Modified Eagle's Medium (Gibco BRL) (for SH-SY5Y and Neuro-2A) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Mitsubishi Kasei)

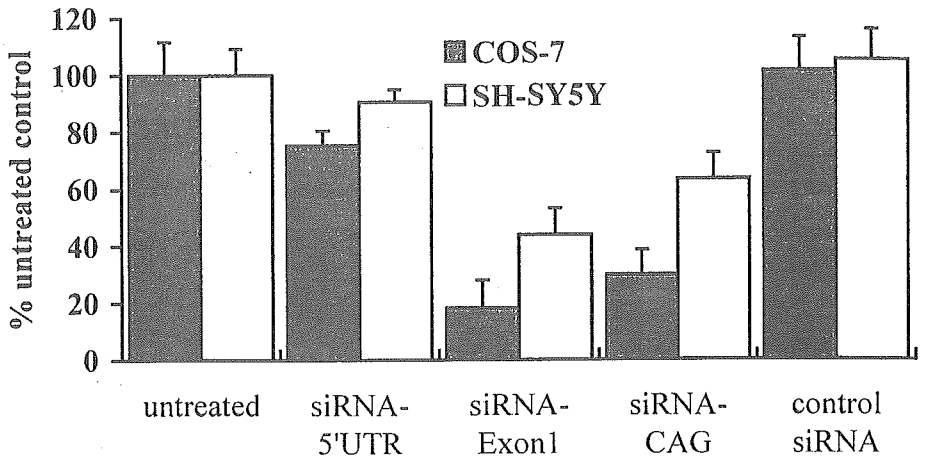

Fig. 4. Quantitative representation of the effects of siRNAs (40 nM) on the strength of fluorescence. Relative strength of GFP fluorescence was measured with untreated control as a reference. Constructs used in this figure contained normal sized CAG repeat (17Q). Vertical axis indicates %. Untreated means without co-transfection of any siRNAs. The efficacy of siRNAs differs depending on target positions and cell types.

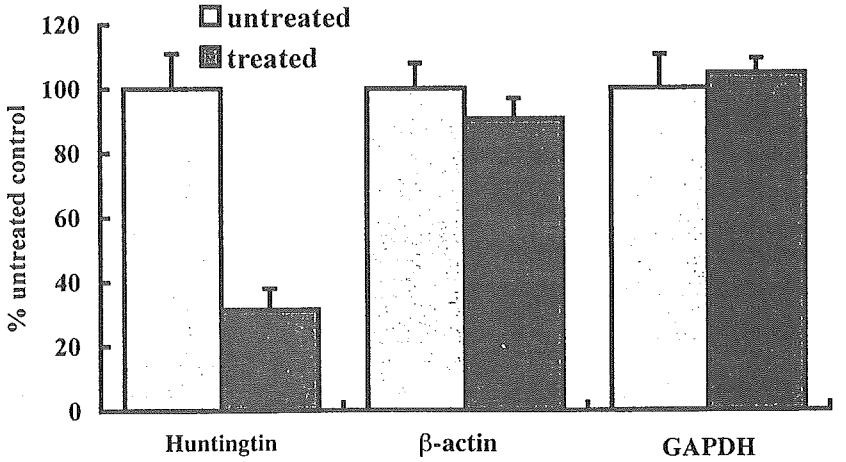

Fig. 5. Quantitative effects of siRNA-HDexon1 on endogenous mRNA levels of huntingtin, β-actin and GAPDH in human cells (SH-SY5Y). Amounts of mRNAs of endogenous huntingtin, β-actin and GAPDH were measured by quantitative RT-PCR and expressed as relative values. Vertical axis indicates %.

and antibiotics (10 units/ml of penicillin and 50 μg/ml of streptomycin; Meiji).

Co-transfection. 96-well plate was used to perform co-transfection experiments of HD gene expression constructs and siRNAs. Subcultures were prepared 24 hours before transfection and maintained in medium with 10% FBS but without antibiotics. Lipofectamine 2000 (Invitrogen, USA) was used as transfection reagent in order to introduce the construct plasmid and siRNA following manufacturer's instructions.

Assessment of siRNA efficacy. 24 to 48 hours after co-transfection, cultured plates were observed under fluorescence microscopy to examine the expression level and the distribution of fused huntingtin (htt)-EGFP proteins. To perform a quantitative evaluation of the effect of siRNAs, the GFP fluorescence was measured (excitation at 485 nm, emission at 538 nm) using Wallac 1420 ARVO sx (PerkinElmer, USA), or FluoreScan II.

Quantitative RT-PCR. Cells were harvested 48

hours after co-transfection. Total RNA was isolated using Trizol reagent (Invitrogen, USA) following manufacturer's instructions. Quantitative estimation of transgenic HD exon 1-EGFP mRNAs were obtained by real-time RT-PCR using LightCycler (Roche, USA). To test the efficacy of siRNA suppressing human endogenous HD gene expression, the cultured cells of human origin (SH-SY5Y) were used because the sequences of human and murine HD gene are slightly different. The endogenous HD transcripts were estimated using the same method of quantitative RT-PCR. Expression levels of GAPDH and β-actin of each sample was estimated as controls to know whether the effects of siRNAs used are specific to HD gene or not.

Results and discussion. Transient transfection of the htt-EGFP constructs into three types of cultured cells successfully expressed the htt-EGFP fused proteins (Fig. 3, upper left panel). Cells transfected with constructs with mutant htt (see Table I) were observed to form inclusions in nucleus and/or cytoplasm (Fig. 3, lower left panel). When those cultured cells were co-transfected with various concentrations of siRNAs and observed 24 to 48 hours after tranfection, an expression of htt-EGFP was significantly reduced (Fig. 3, right panels). When siRNAs were co-transfected with parent plasmid (without htt insert) or an indifferent siRNA as control was used, no suppression of GFP fluorescence was observed (data not shown).

In the dose-finding experiments using COS-7 and siRNA-HDexon1, we found that the lowest amount of siRNA needed for the apparent gene suppression was 5 nM in cell culture. The power of suppression increased with the siRNAs concentration and a concentration of 40 nM was enough to induce a maximal suppression efficiency (data not shown).

Concerning the effect of siRNAs judged by a relative strength of GFP fluorescence, siRNA-HDexon1 was strongest among the three (Fig. 4), knocking down over 80% of the target gene expression, while the other two siRNAs showed moderate or minor efficacy. Although we found that the siRNA-CAG induced a significant suppression of EGFP expression (Fig. 4), the suppression also occurred in the control experiment (data not shown). Therefore, the effects of siRNA-CAG seemed to be non-specific.

We next tested the efficacy and specificity of siRNA-HDexon1 on endogenous HD gene expression using SH-SY5Y (human origin) cells. A quantitative RT-PCR measure of HD mRNA by LightCycler showed that nearly 70% of endogenous HD gene expression was inhibited successfully 48 hours after the siRNA-HDexon1 was transfected, whereas mRNA levels of both GAPDH and β-actin did not change significantly (Fig. 5), suggesting that siRNA-HDexon1 specifically knocked down the HD gene expression. These findings suggest that, although the effects were slightly different between cells used in the present study, siRNA-HDexon1 was the best tool for the HD gene suppression *in vitro* in terms of both efficacy and specificity.

Based on knowledge of the HD gene abnormality and pathophysiology of specific neuronal cell death, there are several possible directions to prevent the disease; to prevent the occurrence of toxic truncated huntingtin molecules, to prevent the transport of truncated huntingtin into nucleus for forming nuclear aggregates and/or inclusions, to prevent the inclusion body formation, or to salvage the life threatening general gene depression by histone deacetylase inhibition.[18] These ideas are mostly related to the prevention of formation of aggregates or inclusion bodies in cytoplasm and/or nucleus, and are possible to apply not only to HD but also to other CAG repeat diseases such as Machado-Joseph disease or SCA1 and so fourth. Therefore, mechanism of those procedures is not specific to HD. A most plausible method to prevent HD would be to reduce or abolish the expression of the HD gene in neurons, because the mutant huntingtin seems to kill particular neurons with a gain-of-function mechanism. Indeed, according to our unpublished data of a single neuron analysis (Jeong, Goto, and Kanazawa), the expressed ratio of mutant to normal huntingtin of the striatal remaining neurons in HD patients was 10-15% more than that of Purkinje cells which are less affected in HD. In order to reduce the particular gene expression, an antisense technology may be applicable using either single stranded RNA or other substitutive chemicals or drugs blocking the translation of mRNA.[19] In fact, the antisense mediated down-regulation of huntingtin expression had been proved feasible[20),21)] in cultured cells. However, the results were not satisfactory because of low efficacy (μM level of RNA was needed) and instability of single stranded RNA molecules.

In this respect, RNAi technology using double stranded RNA (dsRNA) instead of single stranded RNA seems hopeful. Since the first discovery of RNAi in *C. elegans* by Fire and his colleagues,[13] a flood of reports confirmed the powerful nature of RNAi not only in *C. elegans* but also in many other organisms. Indeed the efficiency of dsRNAs is much higher than that of antisense RNA, and dsRNAs are much more stable than sin-

gle stranded RNAs.[22] Moreover, particularly for mammalian cells, it was recently demonstrated that short dsRNA (composed of 21-23nt), rather than long dsRNA, should be used in order to avoid non-specific shut-down response of the unrelated genes.[15),16)] Therefore, RNAi using siRNAs is regarded to be a most promising tool to suppress the specific target gene expression in mammalian cells including humans. The present study demonstrated one of the siRNAs we designed (siRNA-HDexon1) effectively and specifically suppressed the HD gene expression in cultured cells. Further works are needed to elucidate the effectiveness of siRNA-HDexon1 *in vivo*.

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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-

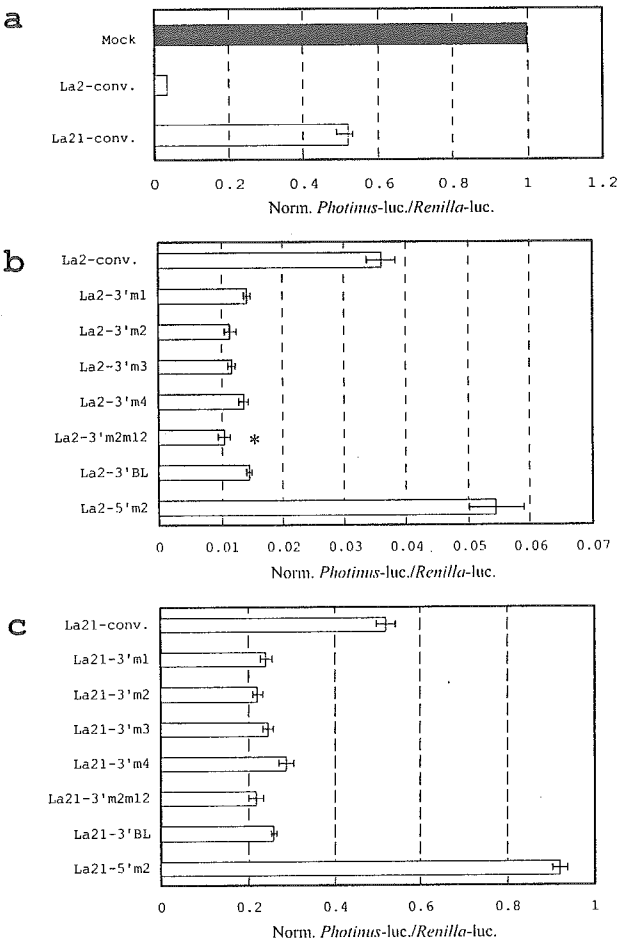

Fig. 1. Silencing of the expression of exogenous reporter gene with various types of synthetic siRNA duplexes. a: Gene silencing of *P. luciferase* with conventional 21-nt siRNA duplexes. The La2-conv. or La21-conv. siRNA duplex against the *P. luciferase* gene together with pGL3-control and phRL-TK 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' G G A A G A C G C C A A A A A C A U A U U 3' 3' U U C C U U C U G C G G U U U U U G U A U 5'
La2-3'm1	5' G G A A G A C G C C A A A A A C A U U 3' 3' U U C C U U C U G C G G U U U U U G U A U 5'
La2-3'm2	5' G G A A G A C G C C A A A A A C A A U 3' 3' U U C C U U C U G C G G U U U U U G U A U 5'
La2-3'm3	5' G G A A G A C G C C A A A A A C U A U 3' 3' U U C C U U C U G C G G U U U U U G U A U 5'
La2-3'm4	5' G G A A G A C G C C A A A A A U U A U 3' 3' U U C C U U C U G C G G U U U U U G U A U 5'
La2-5'm2	5' U U A A G A C G C C A A A A A C A U A U U 3' 3' U U C C U U C U G C G G U U U U U G U A U 5'
La2-3'm2ml2	5' G G A A G A C U C C A A A A A C A A U 3' 3' U U C C U U C U G C G G U U U U U G U A U 5'
La2-3'BL	5' G G A A G A C G C C A A A A A C A U A 3' 3' U U C C U U C U G C G G U U U U U G U A U 5'
La21-conv.	5' A C C G C U G G A G A G C A A C U G C U U 3' 3' U U U G G C G A C C U C U C G U U G A C G 5'
La21-3'm1	5' A C C G C U G G A G A G C A A C U G U 3' 3' U U U G G C G A C C U C U C G U U G A C G 5'
La21-3'm2	5' A C C G C U G G A G A G C A A C U U U 3' 3' U U U G G C G A C C U C U C G U U G A C G 5'
La21-3'm3	5' A C C G C U G G A G A G C A A C A U U 3' 3' U U U G G C G A C C U C U C G U U G A C G 5'
La21-3'm4	5' A C C G C U G G A G A G C A A U A U U 3' 3' U U U G G C G A C C U C U C G U U G A C G 5'
La21-5'm2	5' U A C G C U G G A G A G C A A C U G C U U 3' 3' U U U G G C G A C C U C U C G U U G A C G 5'
La21-3'm2ml2	5' A C C G C U G U A G A G C A A C U U U 3' 3' U U U G G C G A C C U C U C G U U G A C G 5'
La21-3'BL	5' A C C G C U G G A G A G C A A C U G C 3' 3' U U U G G C G A C C U C U C G U U G A C G 5'
Lam-conv.	5' U G C U G A G A G G A A C A G C A A C C U 3' 3' A G A C G A C U C U C C U U G U C G U U G 5'
Lam-3'm2ml2	5' U G C U G A G U G G A A C A G C A U U 3' 3' A G A C G A C U C U C C U U G U C G U U G 5'
Nat.Lam-conv.	5' C U G G A C U U C C A G A A G A A C A U U 3' 3' U U G A C C U G A A G G U C U U C U U G U 5'
Nat.Lam-3'm2ml2	5' C U G G A C U A C C A G A A G A A U U 3' 3' U U G A C C U G A A G G U C U U C U U G U 5'
Dnl(#1)-conv.	5' G U C C G C A G G C G G C U C A A A G A U U 3' 3' U U C A G G C G U C C G C C G A G U U U C U 5'
Dnl(#1)-3'm2ml2	5' G U C C G C A G U C G G C U C A A A U U 3' 3' U U C A G G C G U C C G C C G A G U U U C U 5'
Dnl(#2)-conv.	5' G U G A C U U G G A A A C C A A A U U U U 3' 3' U U C A C U G A A C C U U U G G U U U A A 5'
Dnl(#2)-3'm2ml2	5' G U G A C U U U G A A A C C A A A A A A 3' 3' U U C A C U G A A C C U U U G G U U U A A 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

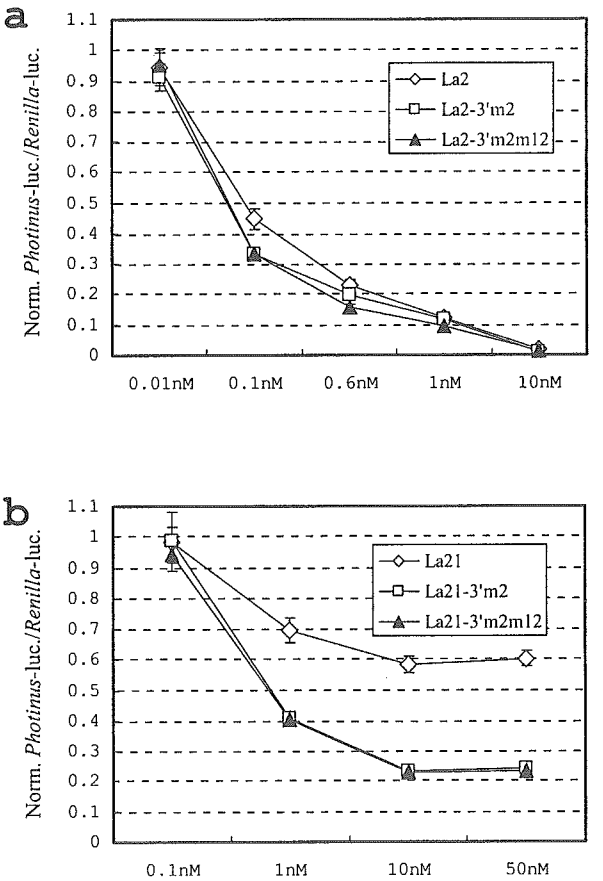

Fig. 3. Dose-dependent inhibition of target luciferase in HeLa cells. The pGL3-control and phRL-TK plasmids were cotransfected with an increasing amount of each siRNA duplex, a: from 0.01 to 10 nM and b: from 0.1 to 50 nM. Used siRNA duplexes are indicated. 24 h after transfection, dual luciferase assay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

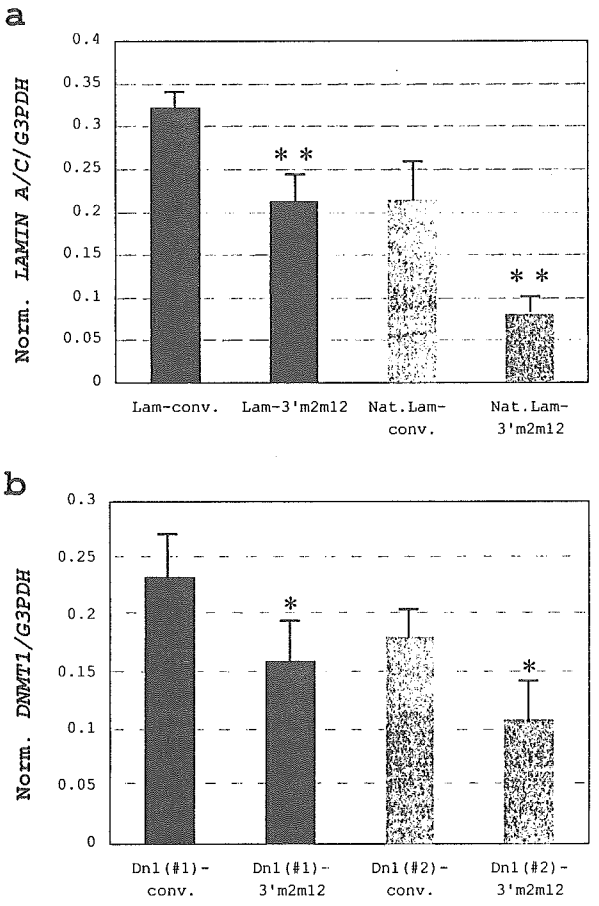

Fig. 4. Gene silencing of endogenous genes with fork-siRNA duplexes. Fork-siRNA and conventional siRNA duplexes targeting the *LAMIN A/C* and *DNMT1* genes were designed (Table 1). The target sequences of the siRNA duplexes in each of the genes are as follows (nucleotide positions relative to the start 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.

Acknowledgements: I would like to thank K. Omi for his helpful advice on statistical analyses. This work was supported in part by research grants from the Ministry of Health, Labor, Welfare in Japan, and by a Grant-in-Aid from the Japan Society for the Promotion of Science.

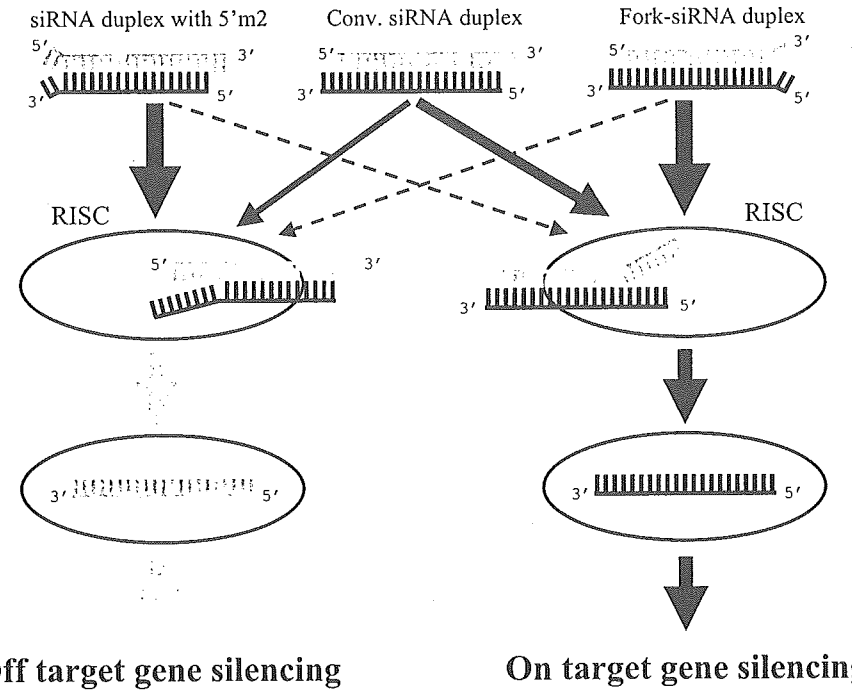

Fig. 5. Proposed model for the incorporation of siRNA into RISC. Conventional and fork-siRNA duplexes and RISCs are schematically drawn, and ss- and as-siRNA elements are colored in gray and black, respectively. When siRNA duplexes are incorporated into RISCs, they would be dissociated from one of the 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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Long-lasting RNAi activity in mammalian neurons☆

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Abstract The effect of RNA interference (RNAi) induced by synthetic small interfering RNAs (siRNAs) on proliferating mammalian cells appears to last for approximately 3–7 days after its induction. Here we show that the RNAi activity induced by a synthetic 21-nucleotide siRNA duplex in postmitotic neurons, mouse primary hippocampal neurons and neurons that differentiated from mouse embryonal carcinoma P19 cells persists for at least 3 weeks, suggesting long-lasting RNAi activity in mammalian neurons. In addition, we also show that an apoptotic (or antiviral) pathway triggered by long dsRNAs is generated during neuronal differentiation of P19 cells, by which the sequence-specific RNAi activity involving long dsRNA appears to be masked.

Key words: RNA interference; Mammalian neuron; Synthetic small interfering RNA; Long double-stranded RNA; Apoptosis

1. Introduction

Double-stranded RNAs (dsRNAs) induce sequence-specific posttranscriptional gene silencing or RNA interference (RNAi) in various kinds of 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-like 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 sequence-specific mediators of RNAi in the RNA-induced silencing complexes (RISCs) [7,8]. In mammalian cells except for a part of undifferentiated cells [9–12], however, 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], instead of induction of RNAi: these rapid responses to long dsRNAs may mask the sequence-specific RNAi activity in differentiated mammalian cells. Elbashir et al. [15] have demonstrated that synthetic 21-nt siRNA duplexes can induce 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 has not only allowed us to analyze the molecular mechanism of mammalian RNAi, but also provided us with a powerful reverse genetic tool for suppressing the expression of a gene of interest in various mammalian cells [16].

The persistence of RNAi activity appears to be an important parameter when considering the effect of RNAi on the regulation of the expression of genes in cells, particularly in mammalian cells [17,18]. Previous studies indicated that the RNAi activity induced by synthetic siRNA duplexes persisted for approximately 3–7 days in cultured mammalian cells [12,19,20]. However, it should be noted that these observations were obtained from experiments using proliferating mammalian cells. Therefore, it is conceivable that whenever cell division occurs, the number of RISCs carrying siRNA duplexes decreases in those cells.

A neuron is known as a terminally differentiated and cell cycle-arrested cell. RNAi activity in mammalian neurons has been reported [21,22], but the duration and features of RNAi in neurons are still unknown. In this study, we investigated RNAi activity induced by synthetic siRNA duplexes in mouse primary hippocampal neurons and neurons that differentiated from mouse embryonal carcinoma P19 cells by treatment with retinoic acid. The results indicated that the RNAi activity in the neurons persisted for at least 3 weeks, whereas undifferentiated P19 cells studied as proliferating cells lost the RNAi activity by day 5 after its induction.

2. Materials and methods

2.1. Cell culture

Primary culture of dissociated hippocampal neurons was carried out as described previously [23,24] with minor modifications. Briefly, mouse E17 embryonic hippocampal tissue (ICR mouse strain) was isolated, treated with 90 U/ml papain (Worthington) at 37°C for 15 min, and gently dissociated with pipetting. The dissociated hippocampal neurons were seeded into polyethyleneimine-coated 24-well culture plates at a density of approximately $5 \times 10^5/cm^2$. The cells were grown at 37°C in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (Invitrogen) supplemented with 5% fetal bovine serum (Invitrogen), and 5% heat-inactivated horse serum (Invitrogen) in a 5% CO_2-humidified chamber. Transfection as described below was carried out 4 h after seeding the neurons onto culture plates. After a 2-day incubation, 10 μM cytosine arabinoside (Ara-C) (Sigma) was added, and further incubation at 37°C was carried out.

P19 mouse embryonic carcinoma cells were grown at 37°C in α-MEM (Sigma) supplemented with 10% fetal bovine serum (Sigma), 100 U/ml penicillin (Sigma) and 100 μg/ml streptomycin (Sigma) in a

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Abbreviations: RNAi, RNA interference; siRNA, small interfering RNA; dsRNA, double-stranded RNA; PKR, interferon-inducible, dsRNA-activated protein kinase; nt, nucleotide

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5% CO_2-humidified chamber. For induction of neuronal differentiation of P19 cells, the cells were cultured in the presence of 5×10^{-7} M all-*trans*-retinoic acid (RA) (Sigma) in bacterial-grade Petri dishes for 4 days. After a 4-day incubation, aggregated cells were collected, dissociated into single cells by treatment with trypsin–EDTA and seeded into poly-L-lysine-coated 24-well culture plates at a density of 1×10^5 cells/cm^2 in the normal medium described above. After a 1-day incubation, the medium was changed to the Neurobasal (Invitrogen) medium containing B27 supplement (Invitrogen), 0.5 mM glutamine, and 10 μM Ara-C (Sigma); thereafter, the medium was changed every 2 days. Transfection was carried out over 240 h after RA treatment.

2.2. Synthetic oligonucleotides

RNA and DNA synthetic oligonucleotides were obtained from Proligo and Sigmagenosis, respectively. Preparation of RNA duplexes was performed as described previously [25]. The sequences of siRNA synthesized newly were as follows: ssRLa1: 5′-UGGCUUCCAAGGUGUACGAUU-3′, asRLa1: 5′-UCGUACACCUUGGAAGCCAUU-3′.

The GFP-22 and non-silencing siRNAs and the *silencer* GAPDH siRNA were purchased from Qiagen and Ambion, respectively.

2.3. In vitro transcription

For preparation of a 200-bp-long dsRNA against the *Photinus luciferase* gene, a plasmid containing the region from positions 434 to 633 in the pGL3-control vector (Promega) was constructed. Briefly, the region was amplified by polymerase chain reaction (PCR) with the following primers: 5′-TGGAGGTACCTTACGCTGAGTACTTCG-3′ and 5′-GCGACCGCGGCATACTGTTGAGCAATTC-3′, and the resultant PCR product was digested with *Kpn*I and *Sac*II and inserted into the pBluescript vector (Stratagene). In vitro transcription with the plasmid as a template was carried out using MEGAscript T7 and T3 kits (Ambion). Duplex formation was carried out as described previously [25].

2.4. Transfection and luciferase assay

Reporter plasmids and siRNA duplexes were cotransfected into primary hippocampal neurons and undifferentiated P19 cells using Lipofectamine 2000 (Invitrogen), and into differentiated neuronal P19 cells using NeuroPORTER transfection reagents (Gene Therapy Systems) according to the manufacturers' instructions with minor modifications. In the case of introduction of just siRNA duplexes into the neurons, the transfection was carried out using GeneSilencer siRNA transfection reagent (Gene Therapy Systems) according to the manufacturer's instructions. Before the transfection, the culture medium was replaced with 0.5 ml of the fresh medium without antibiotics, and to each well (24-well culture plates), 0.25 μg of pGL3-control plasmid (Promega), 0.05 μg phRL-TK plasmid (Promega), and 0.2 μg of siRNAs were applied. In the case of targeting the *GFP* gene, 0.25 μg of pEGFP-C1 (BD Biosciences), 0.25 μg of pDsRed2-C1 (BD Biosciences), 0.2 μg of GFP-22 siRNA (Qiagen) were applied. The cells were incubated for 4 h at 37°C. After the 4-h incubation, 0.5 ml of the fresh culture medium without antibiotics was added, and further incubation at 37°C was carried out. For medium change, a half volume of the medium was changed with the fresh medium at 10 days after transfection in primary hippocampal neurons, and every 2 days in differentiated P19 cells. When transfection efficiency was examined using the pEGFP-C1 plasmid and Cy3-labeled siRNA duplexes, it appeared that the present procedures yielded approximately 5–8% and 60–70% transfected neurons using the plasmid and siRNA duplexes, respectively (data not shown).

In order to maintain the exponential cell growth of undifferentiated P19 cells, the cells were collected 24 h after transfection, counted using a hemocytometer, and divided into two portions. One portion was subjected to preparation of cell lysate followed by luciferase expression assay, and other portion was diluted and grown in 6-well culture plates containing the medium without antibiotics. Thereafter, the number of cells was counted when the luciferase assay was carried out. The expression of luciferase was examined using a Dual-Luciferase reporter assay system (Promega) according to the directions provided by the manufacturer.

2.5. Reverse transcription (RT) PCR

Total RNA was isolated from the cultured cells using Trizol reagent (Invitrogen). 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 subjected to a real-time PCR using the LightCycler thermal cycler system (Roche) with a LightCycler FastStart DNA Master SYBR Green I kit (Roche) according to the manufacturer's instructions. PCR primers were designed so that they can span exon–intron junctions on the transcripts of genes. The sequences of the primers are available upon request. The expression levels of the genes examined were normalized to that of the control *Gapdh* gene.

2.6. TdT-mediated dUTP biotin nick end labeling (TUNEL)

Genomic DNA breaks were examined by TUNEL using an Apoptosis Screening Kit (Wako) according to the manufacturer's instructions. The assay was carried out 48 h after transfection with dsRNAs.

3. Results

3.1. Long-lasting RNAi activity in primary hippocampal neurons

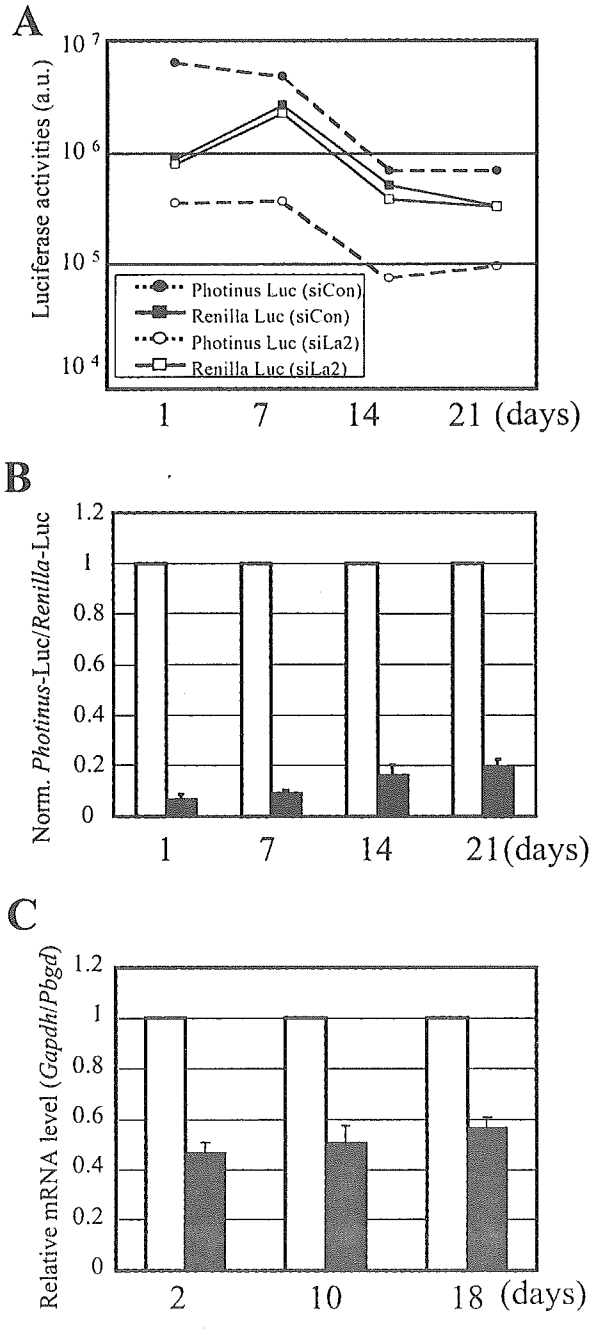
In order to induce RNAi in postmitotic neurons, we prepared primary hippocampal neurons from E17 mouse embryos, and introduced synthetic 21-nt siRNAs targeting the exogenous reporter gene, *Photinus luciferase*, together with a pGL3-control plasmid carrying the *Photinus luciferase* gene and a phRL-TK plasmid carrying the *Renilla luciferase* gene as a control into the neurons. 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. 1, the La2 siRNA duplex can specifically and strongly inhibit the expression of *Photinus luciferase* in the primary hippocampal neurons, which agrees with the previous study using cultured mammalian cells, HeLa and NTera2D1 cells [25]. The most important point to note in this experiment is that the duration of the RNAi activity in the neurons was quite a long, i.e. a strong RNAi activity lasted for at least 3 weeks after induction of RNAi. To further confirm this, we examined RNAi activities induced by the RLa1 siRNA duplex targeting the *Renilla luciferase* gene present in phRL-TK plasmid (see supplementary Fig. s1A), the GFP-22 siRNA duplex (Qiagen) targeting the *GFP* gene present in pEGFP-C1 plasmid (see supplementary Fig. s1B) and the *silencer* GAPDH siRNA duplex (Ambion) targeting the endogenous *Gapdh* gene (Fig. 1C) in the primary hippocampal neurons. Consistently, all the results demonstrate that long-lasting RNAi activity occurs in the neurons. We also add that little or no morphological change of the primary hippocampal neurons used was observed during the experiments (see supplementary Fig. s1C). To our knowledge, this is the first report on the longest duration of RNAi activity in the former RNAi activities induced by synthetic siRNA duplexes in mammalian cells.

3.2. Long-lasting RNAi activity in P19 neurons

To further confirm such a long-term effect of RNAi in other neurons and to examine the properties of RNAi during neuronal differentiation, we investigated RNAi in P19 cells, a mouse embryonal carcinoma cell line, which can be induced by RA to differentiate into neuroectodermal derivatives including neuron and glia [26], and in this study, we used P19 cells that differentiated into neurons over 240 h after RA treatment and a 3-day treatment with Ara-C as terminally differentiated and cell cycle-arrested neurons (provisionally named 'P19 neurons'). Additionally, the expression profile of genes related to neuronal differentiation in P19 neurons

appears to be similar to that in E17 hippocampal neurons (see supplementary Fig. s2).

We introduced the La2 siRNA duplex together with the pGL3-control and phRL-TK plasmids into P19 neurons and undifferentiated P19 cells as in the primary hippocampal neurons (Fig. 1). As shown in Fig. 2, the La2 siRNA duplex can specifically inhibit the expression of *Photinus luciferase* in either P19 neurons or undifferentiated P19 cells, suggesting that there is no significant difference in RNAi activity between P19 neurons and undifferentiated P19 cells. Next we examined the duration of RNAi activity in P19 neurons and undifferentiated P19 cells. The expression levels of the *luciferase* genes were examined at various hours after induction of RNAi. When undifferentiated P19 cells were examined, a strong RNAi activity was detected for 2 days after RNAi induction; thereafter, the cells gradually lost the activity and lost it completely by day 5 (120 h) after the induction (Fig. 3A). Note that the RNAi activity is being lost with an increase in the number of the cells (Fig. 3B). Therefore, these observations agree with results of previous studies using proliferating mammalian cells [12,19]. In contrast to the results of undifferentiated P19 cells, P19 neurons, like primary hippocampal neurons, revealed a long-lasting RNAi activity. When RNAi was induced in P19 neurons, a strong RNAi activity lasted for as long as 18 days after the induction (Fig. 3C). We must mention that the maintenance of P19 neurons for as long as 18 days after RNAi induction was a limit in culture under our current condition. Taken all together, our present data suggest that once RNAi is induced in mammalian neurons, a long-lasting RNAi activity occurs.



3.3. Significant difference in response to long dsRNAs between undifferentiated P19 cells and P19 neurons

Undifferentiated P19 cells appear to allow long dsRNAs to trigger sequence-specific gene silencing, i.e. RNAi [11]. In this study, we have shown that synthetic 21-nt siRNA duplexes can induce RNAi in either undifferentiated or differentiated P19 cells (Figs. 2 and 3): an authentic RNAi pathway occurs in either of the cells. These observations raise the question whether P19 neurons (differentiated P19 cells), like undifferentiated P19 cells, allow long dsRNAs to induce the sequence-specific RNAi activity. To address this, a 200-bp-long dsRNA against *Photinus luciferase* (detailed in Section 2) was prepared and cotransfected with the pGL3-control and phRL-TK plasmids into either differentiated or undifferentiated P19 cells. When the long dsRNAs were introduced into undifferentiated P19 cells, consistently, sequence-specific gene silencing of *Photinus luciferase* was observed without induction of cell death (Fig. 4A). In contrast, when P19 neurons were used, excessive cell death was observed the day after

←

Fig. 1. Persistence of RNAi activity in postmitotic neurons. The La2 siRNA duplex against the *Photinus luciferase* gene [25] or a non-silencing siRNA duplex (Qiagen) together with pGL3-control and phRL-TK plasmids carrying *Photinus* and *Renilla luciferase* reporter genes, respectively, were cotransfected into mouse primary hippocampal neurons. RNAi activity was examined every week up to 3 weeks after RNAi induction. A: Absolute *Photinus* and *Renilla* luciferase expressions. The expression levels are plotted in arbitrary luminescence units (a.u.). B: Ratios of normalized 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 normalized 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. C: Gene silencing of the endogenous *Gapdh* gene. The *silencer* GAPDH siRNA duplex (40 nM) (Ambion) was transfected into primary hippocampal neurons using GeneSilencer siRNA transfection reagent (Gene Therapy Systems). Total RNA was extracted from the neurons at indicated days after transfection and subjected to cDNA synthesis with reverse transcriptase followed by real-time PCR. The expression level of *Gapdh* was normalized to that of the *hydroxymethylbilane synthase* (*Pbgd*) gene as a control, and the resultant expression level in the presence of the silencer GAPDH siRNA duplex was normalized to the expression level determined in the presence of non-silencing siRNA duplex (Qiagen). The resultant normalized ratios are indicated. Data are averages of three independent experiments. Error bars represent standard deviations.

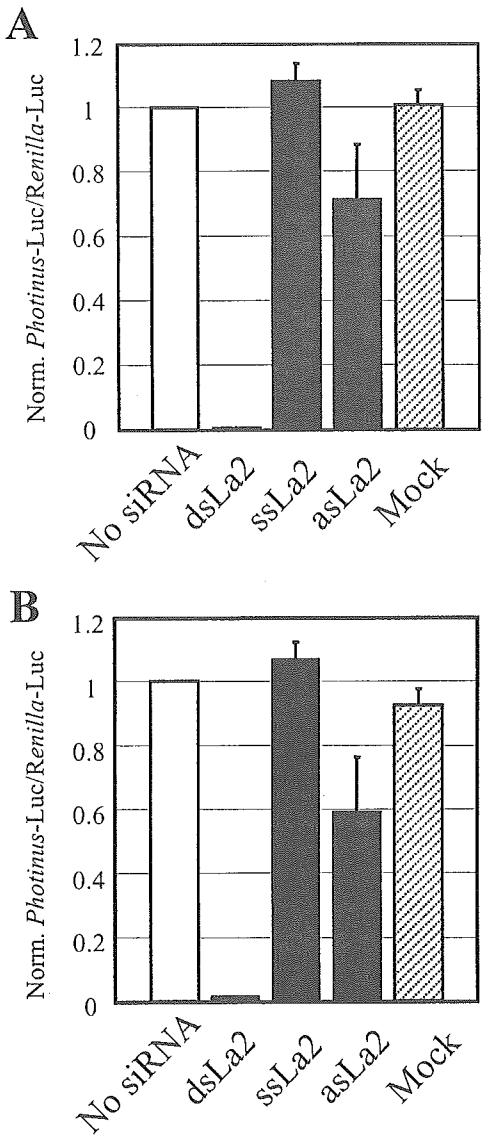


Fig. 2. RNAi activities induced by synthetic siRNAs in undifferentiated (A) and differentiated (B) P19 cells. The La2 siRNAs [25] or a non-silencing siRNA duplex (Mock) (Qiagen) together with pGL3-control and phRL-TK plasmids were cotransfected into either undifferentiated or differentiated (neurons) P19 cells. Prefixes attached to the name of La2 siRNA, i.e. ds, ss, and as, represent double-stranded (duplex), and sense- and antisense-stranded siRNAs, respectively. Twenty-four hours after transfection, cell lysate was prepared and dual luciferase assay was carried out. Ratios of normalized target (*Photinus*) luciferase activity to control (*Renilla*) luciferase activity are indicated: the ratios of luciferase activity determined in the presence of the siRNAs are normalized to the ratios obtained for a control in the absence of siRNA (No siRNA). Data are averages of at least three independent experiments. Error bars represent standard deviations.

transfection of the long dsRNAs. Note that although the observed ratio of normalized target (*Photinus*) luciferase activity to control (*Renilla*) luciferase activity was below 0.2 in the presence of the long dsRNAs in P19 neurons (Fig. 4A), the expression level of the control (*Renilla*) luciferase was significantly lower than those in the presence of synthetic 21-nt siRNA duplexes (Fig. 4B), which appeared to be due to excessive cell death triggered by the long dsRNAs. To further

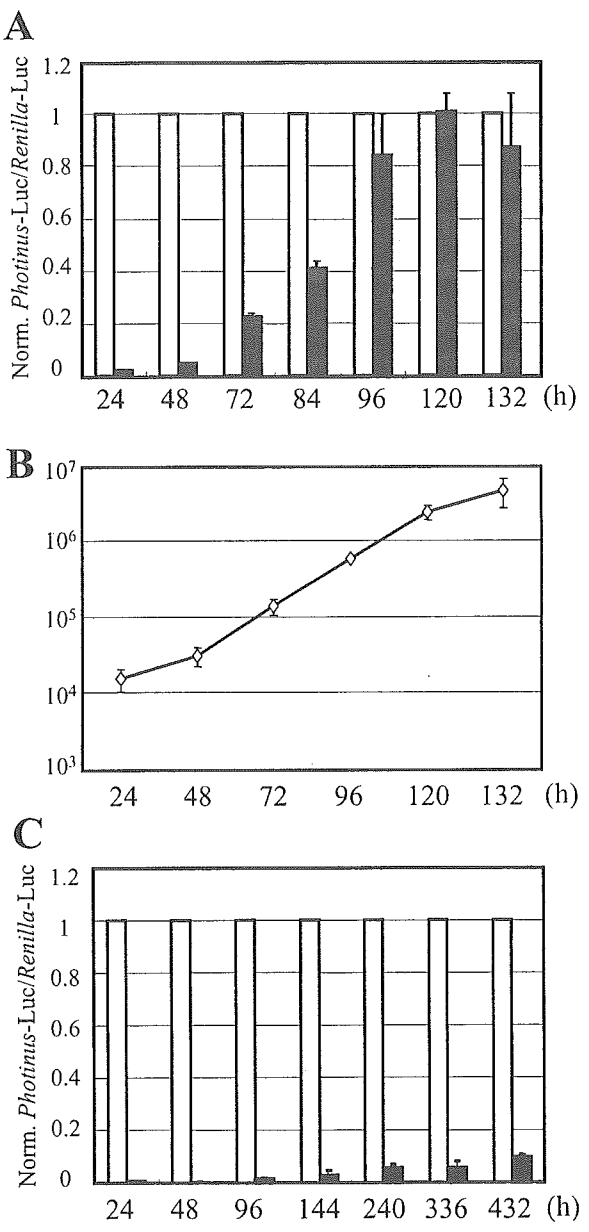


Fig. 3. Persistence of RNAi activity in undifferentiated and differentiated P19 cells. A: RNAi activity in undifferentiated P19 cells. RNAi activity was examined every 24 h up to 132 h after RNAi induction. Ratios of normalized target (*Photinus*) luciferase activity to control (*Renilla*) luciferase activity are indicated as in Fig. 1. 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. B: Growth of undifferentiated P19 cells. The dual luciferase assay was carried out as in A, and the number of cells was counted immediately before preparation of cell lysate. Data indicate the mean number of cells at each time after RNAi induction. Error bars represent standard deviations. C: RNAi activity in P19 neurons. RNAi activities were examined up to 432 h (18 days) after RNAi induction. Ratios of normalized target (*Photinus*) luciferase activity to control (*Renilla*) luciferase activity are indicated as in A. 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.

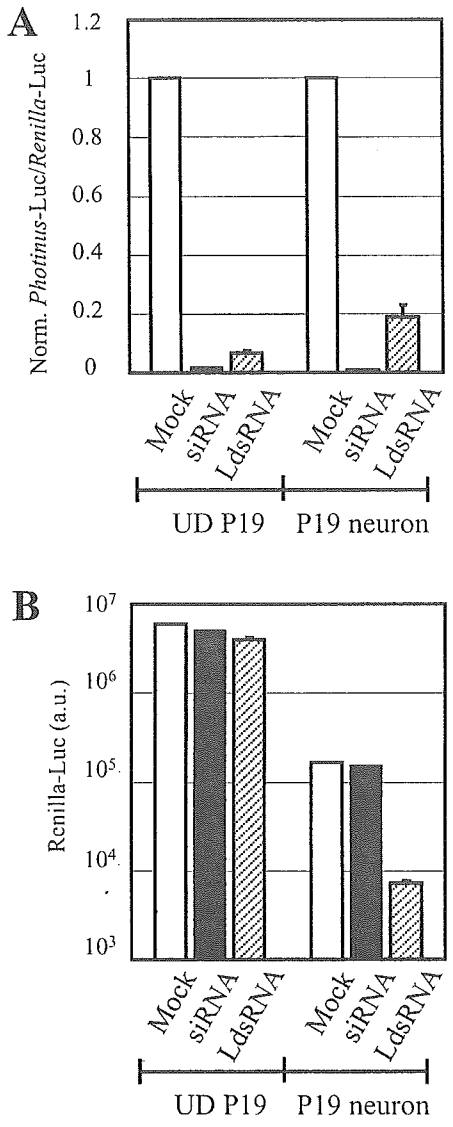


Fig. 4. Response to long dsRNAs in undifferentiated and differentiated P19 cells. A: Induction of RNAi by long dsRNAs. Two-hundred-bp-long dsRNA targeting *Photinus* (LdsRNA) and the La2 (siRNA) and non-silencing (Mock) siRNA duplex as a positive and negative control, respectively, were cotransfected with pGL3-control and phRL-TK plasmids into either undifferentiated (UD P19) or differentiated (P19 neuron) P19 cells. Twenty-four hours after transfection, dual luciferase assay was carried out. Ratios of normalized target (*Photinus*) luciferase activity to control (*Renilla*) luciferase activity are indicated as in Fig. 1. Data are averages of at least three independent experiments. Error bars represent standard deviations. Note that excessive cell death was observed in P19 neurons, but not in undifferentiated P19 cells after transfection with long dsRNAs. B: Absolute *Renilla* luciferase expression. The expression levels of *Renilla* luciferase were plotted in arbitrary luminescence units (a.u.).

confirm the induction of cell death (apoptosis) by the long dsRNAs, we examined the cells transfected with the long dsRNAs and siRNAs by using a TUNEL assay. As shown in Fig. 5, the results consistently indicate that while neither the siRNAs nor the long dsRNAs can induce apoptosis in undifferentiated P19 cells, either P19 neurons or primary hippocampal neurons undergo apoptosis in the presence of the long dsRNAs, but not in the presence of siRNA duplexes. We next examined the levels of expression of the *PKR* and *RNase L* genes involved in such an apoptotic pathway [13,14,27]. The results of a real-time RT-PCR of the genes indicate that both the genes were rapidly expressed in the course of neuronal differentiation of P19 cells (Fig. 6), and that the expression

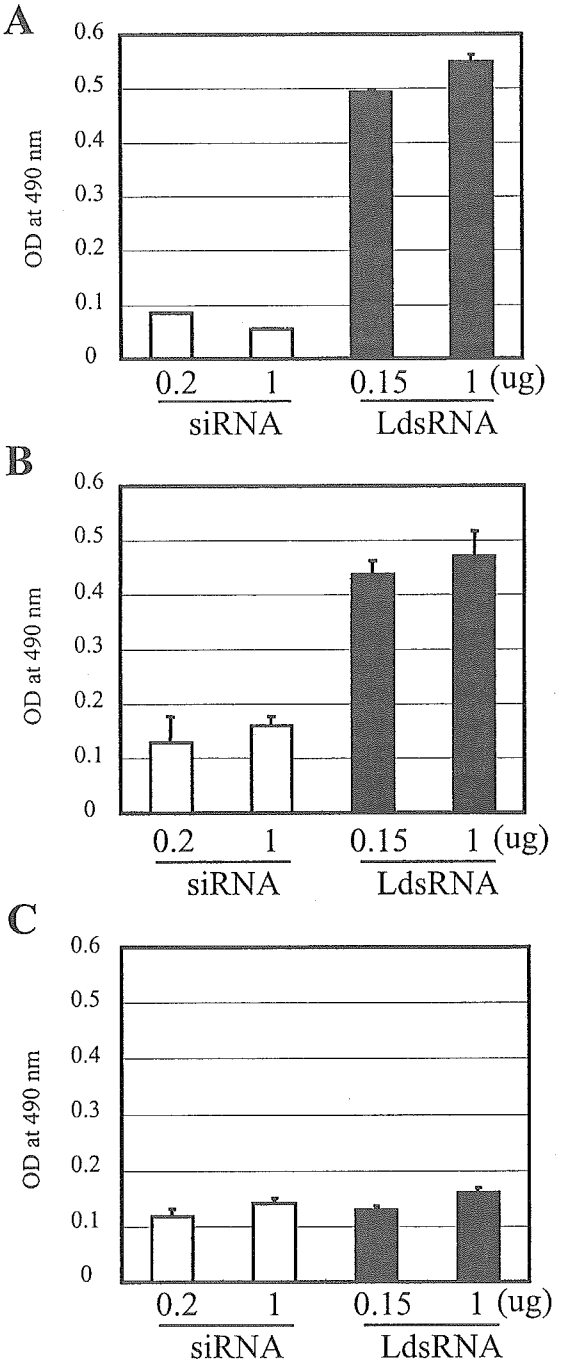


Fig. 5. Apoptosis induced by long dsRNAs in postmitotic neurons. The La2 siRNA duplex (siRNA) and 200-bp-long dsRNAs (LdsRNA) were transfected into primary hippocampal neurons (A), P19 neurons (B) and undifferentiated P19 cells (C). The amounts of dsRNA transfected are indicated. Forty-eight hours after transfection, double-stranded DNA breaks were examined by TUNEL. The absorbance was measured at 490 nm with a spectrophotometer. Data are averages of at least three independent experiments. Error bars represent standard deviations.