Table 2
Alignment of HEAT repeats

Repeat*	Species**	AA position		Fragment†	Score	E-value
		From	To			
HEAT_AAA	Hs – Htt	124	162	QKLLGIAMELFLLCSDDAESDVRMVADECLNKVIKALMD	1510	1.26E–06
	Cj – Htt	112	150	QKLLGIAMELLLLCSDDAESDVRMVADECLNKVIKALMD	1590	5.48E–07
HEAT_AAA	Hs – Htt	205	243	RPYLVNLLPCLTRTSKRPEESVQETLAAAVPKIMASFGN	1990	1.03E–04
	Cj – Htt	193	231	RPYLVNLLPCLTRTSKRPEESVQETLAAAVPKIMASFGN	1990	2.37E–08
HEAT_AAA	Hs – Htt	247	285	DNEIKVLLKAFIANLKSSSPTIRRTAAGSAVSICQHSRR	1590	5.48E–07
	Cj – Htt	235	273	DNEIKILLKAFIANLKSSSPTIRRTAAGSAVSICQHSRR	1590	1.97E–06
HEAT_AAA	Hs – Htt	317	355	LLTLRYLVPLLQQQVKDTSLKGSFGVTRKEMEVSPSAEQ	1620	1.11E–06
	Cj – Htt	305	343	LLTLRYLVPLLHQQVKDTSLKGSFGVTRKEMEVSPSAEQ	1570	1.46E–07
HEAT_ADB	Hs – Htt					
	Cj – Htt	734	771	YPEEQYVSDILNYIDHGDPQVRGATAILCGTLVCSILS	1450	3.29E–07
HEAT_AAA	Hs – Htt	803	841	TFSLADCIPLLRKTLKDESSVTCKLACTAVRNCVMSLCS	1500	5.78E–07
	Cj – Htt	791	829	TFSLADCVPLLRKTLKDESSVTCKLACTAVRHCVMSLCS	1449	2.90E–06
HEAT_AAA	Hs – Htt	904	942	KLQERVLNNVVIHLLGDEDPRVRHVAAASLIRLVPKLFY	1930	6.69E–08
	Cj – Htt	892	930	TLQERVLTSVVIHLLGDEDPRVRHVAAASLIRLVPKLFY	2150	2.51E–05
HEAT_AAA	Hs – Htt	984	1025	RIYRGYNLLPSITDVTMENNLSRVIAAVSHELITSTTRALTF	1370	9.05E–06
	Cj – Htt	972	1013	RIYRGYNLLPSIIDVTMENNLSRVIAAVSHELITSTTRALTF	1410	2.71E–06
HEAT_AAA	Hs – Htt	1425	1463	RLFEPLVIKALKQYTTTTCVQLQKQVLDLLAQLVQLRVN	1370	5.62E–06
	Cj – Htt	1413	1451	RLFEPLVIKALKQYTTTTSVQLQKQVLDLLAQLVQLRVN	1580	3.20E–07
HEAT_AAA	Hs – Htt	2798	2836	DDTAKQLIPVISDYLLSNLKGIAHCVNIHSQQHVLVMCA	1430	3.51E–06
	Cj - Htt	2785	2823	DDTAKQLIPVISDYLLSSLKGLAHCVNTHSQQHVLVMCA	1430	3.29E–06

* HEAT_AAA and HEAT_ADB indicate subsets of HEAT repeats representing PP2A and adaptin families, respectively.
** Hs-Htt and Cj-Htt indicate the human and common marmoset Htt proteins, respectively.
†Amino acids which are different from the sequence of Hs-Htt are indicated in red.

cDNA have significant sequence homology to that of other species' *Htt* genes. In addition, it should be noted that *Htt* sequences between the human and common marmoset (colored in yellow in Table 1) appear to be particularly conserved as compared with sequence conservation within non-primate *Htt* genes, suggesting that the *Htt* gene is highly conserved in primates.

Fig. 2 shows the alignment of amino acid sequences encoded by *Htt* exon 1 and its corresponding region in various species. From the alignment, *Cj-Htt* appears to possess a short polyglutamine tract of nine glutamines compared with that of the human and chimpanzee *Htt* genes; but other than the polyglutamine tract, the exon 1 corresponding region in *Cj-Htt* exhibits high sequence homology to the human *Htt* exon 1. It is also interesting that polyproline region adjacent to the polyglutamine tract has differences between primates and non-primates: amino acid substitutions and deletions are observed, and the lack of the polyproline region in the *Gallus gallus Htt* exon 1 is particularly remarkable. These differences may influence folding and aggregation of the Htt protein, and might represent adaptive evolution of *Htt* to each species. The difference in the exon 1 among various species may provide us with a hint for understanding the expansion of the polyglutamine tract in Huntington's disease in human.

Other than the exon 1 region, we also investigated the HEAT repeats possessing tandem arrayed bihelical structure, which appear to wrap around target substrates (Andrade and Bork, 1995; Neuwald and Hirano, 2000), and found that the HEAT repeats are also conserved in the Cj-Htt protein (Table 2). In addition, it may be interesting that HEAT_ADB, a subset of HEAT repeats representing adaptin family, is present in Cj-Htt, but not in Hs-Htt.

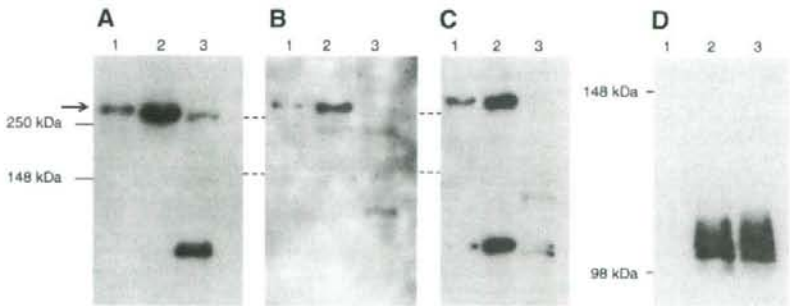

Fig. 3. Assessment of anti-human Htt antibodies against the common marmoset Htt polypeptide. Cell lysate derived from the common marmoset cell line (HSCJ-110) (lane 1), brain tissue (lane 2), and mouse brain tissue as a control (lane 3) was examined by Western blotting with anti-human Htt antibodies. Tested antibodies were as follows: (A) MAB2166 (Chemicon), (B) MAB2170 (Chemicon), and (C) ab7666 (Abcam). Arrow indicates the signals of Htt proteins. The same results as those of HSCJ-110 were also obtained when HSCJ-002 and-009 were used (data not shown). After detection of signals, blotted membranes were subjected to antibody removal and then incubated with anti-APP antibody [MAB348 (Chemicon)] (D) followed by the same procedure as in the anti-human Htt antibodies described above.

3.2. Detection of Cj-Htt gene products

It is important to be able to properly detect the *Cj-Htt* gene and its gene products. Since the nucleotide and predicted amino acid sequences of the *Cj-Htt* cDNA showed significantly high sequence homologies to those of the human *Htt* gene, we tested whether commercially-available TaqMan probe and antibodies against human *Htt* gene products (mRNA and protein) were also able to detect *Cj-Htt* gene products. As a result, the TaqMan probe (Fig. 5A) appears to be able to detect *Cj-Htt* mRNA. In addition, newly designed PCR primers, which are perfectly matched to *Cj-Htt*, also appear to be able to detect *Cj-Htt* mRNA (Fig. 5B).

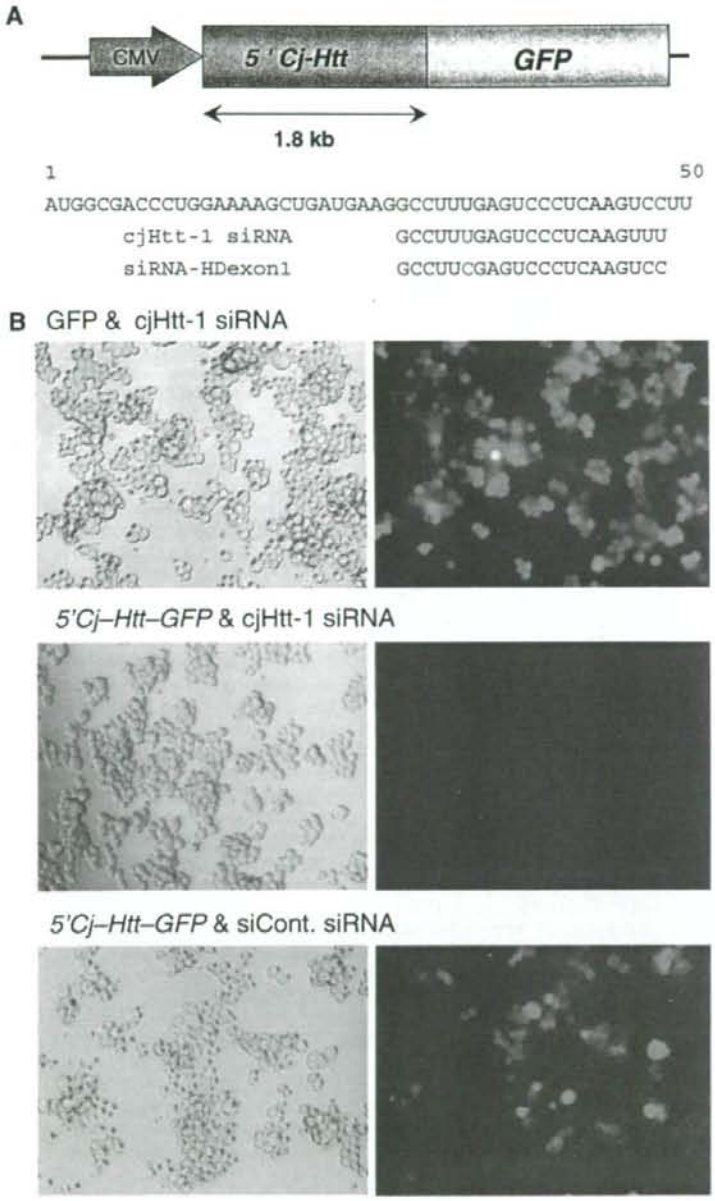

Fig. 4. Gene silencing against the 5'*Cj-Htt-GFP* fusion gene. (A) Schematic drawing of the 5'*Cj-Htt-GFP* fusion gene and designed cjHtt-1 siRNA. The fusion gene is composed of the 5' terminal region of the Cj-Htt ORF and GFP, and driven by the Human cytomegalovirus promoter. The *Cj-Htt* sequence from the first ATG to position 50 is shown together with sequences of cjHtt-1 siRNA and siRNA-HDexon1 targeting human *Htt*. A variant nucleotide between the siRNAs is indicated in red. (B) Effect of cjHtt-1 siRNA on gene silencing. Reporter genes [5'*Cj-Htt-GFP* or *GFP* (empty reporter)] and siRNAs [cjHtt-1 or siCont (non-silencing siRNA)] were introduced into mouse neuroblastoma Neuro2a (N2a) cells. Two days after transfection, the cells were examined by a fluorescent microscope. Left (differential interference contrast) and right (fluorescence image) panels are identical in the visual field.

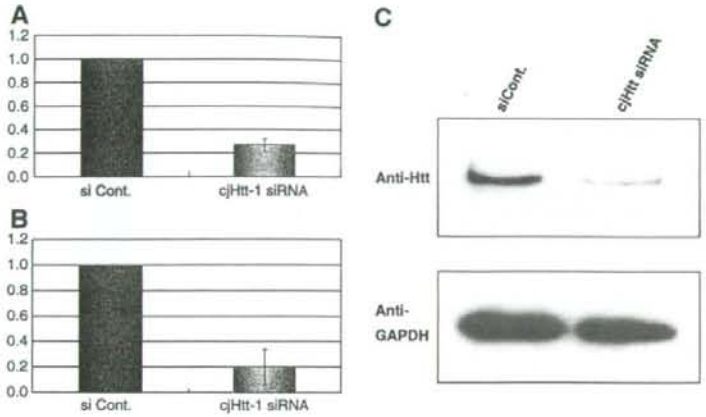

Fig. 5. Inhibition of expression of endogenous *Cj-Htt* by RNAi. The cjHtt-1 siRNA was introduced into HSCj-009 cells by means of electroporation. Two days after transfection, total RNA and cell lysate were prepared and examined by RT-real time PCR and Western blotting, respectively. Total RNA was subjected to cDNA synthesis as in Fig. 1. The resultant cDNA was examined by real time PCR with a TaqMan probe for the human *Htt* gene (A) and newly designed PCR primers [cjHtt(1139)-F and cjHtt(1254)-R] (B). The expression of *Gapdh* as a control was also examined using Perfect Real Time Primers for the human *GAPDH* gene (TAKARA BIO). The expression level of *Cj-Htt* was normalized against that of *Gapdh*, and the ratios of *Cj-Htt* expression level in the presence of cjHtt-1 siRNA were normalized against the ratio obtained in the presence of the siControl siRNA (siCont.). Data are means of at least three independent determinations. Error bars represent standard deviations. (C) Western blot. Cell lysate was examined by Western blotting with anti-human Htt antibody (MAB2166; Chemicon) as in Fig. 3. After detection of signals, the expression of GAPDH as a control was also examined by anti-GAPDH antibody (AM4300; Ambion).

Western blot analyses indicate that a polypeptide of approximately 350 kDa, which is almost the same as the molecular weight estimated from the amino acid sequence (346 kDa) in the *Cj-Htt* cDNA, can be detected in the common marmoset specimens by the antibodies tested, suggesting that the Cj-Htt protein is detectable with the antibodies (Figs. 3A–C). The 350 kDa mouse Htt protein was detected by the MAB2166 antibody (Fig. 3A), but hardly with the other antibodies (Figs. 3B and C). This may be caused by possibly low expression level of mouse Htt in the brain tissue, and/or by difference in the epitope sequences between the common marmoset and mouse Htt proteins. Other than the 350 kDa band, a few bands migrated faster than the 350 kDa band were also observed. They may be degradation products of the Htt protein, and different cells and/or species might have different degradation of the protein. To clarify these, further studies need to be carried out.

In addition to the Htt protein, we also examined the expression of amyloid precursor protein (App) with the 22C11 antibody, which can recognize the same amino acid sequence at positions 66–81 of either the human or mouse App. As a result, the App signal was able to be detected in either the common marmoset or mouse brain tissue, but not in the common marmoset immortalized peripheral blood mononuclear cells (PBMCs) (Fig. 3D), suggesting little or no expression of App in PBMCs and availability of the antibody for detection of the Cj-App protein.

3.3. Gene silencing against the Cj-Htt gene

To verify the data presented here and tools for the detection of *Cj-Htt*, we carried out gene silencing against the expression of endogenous *Cj-Htt* by means of RNA interference (RNAi), and assessed the knockdown potency of designed siRNA targeting *Cj-Htt* using the tools evaluated above. Based on a previous study where a competent siRNA duplex, siRNA-HDexon 1, conferring strong inhibition against the expression of the human *Htt* gene was used (Liu et al., 2003), we chemically synthesized an siRNA duplex, cjHtt-1 siRNA, corresponding to the siRNA-HDexon 1 duplex; note that there is one nucleotide change between cjHtt-1 siRNA and siRNA-HDexon 1 (Fig. 4A).

To examine the effect of the siRNA duplex on gene silencing, we constructed a reporter plasmid carrying the 5′ terminal region of *Cj-Htt* cDNA linked with the *GFP* reporter gene (the 5′*Cj-Htt-GFP* fusion gene). The reporter plasmid and the siRNA were cotransfected into mouse Neuro2a cells, and the expression of the 5′*Cj-Htt-GFP* fusion gene was examined by a fluorescent microscope. As shown in Fig. 4B, the data indicated that the cjHtt-1 siRNA duplex was able to induce strong RNAi activity against the fusion gene expression.

Next, we introduced the cjHtt-1 siRNA duplex into immortalized common marmoset mononuclear cells by means of electroporation, and two days after transfection, the expression levels of the endogenous *Cj-Htt* mRNA and protein were examined by RT-real time PCR and Western blotting, respectively. As shown in Fig. 5, the results consistently indicated that *Cj-Htt* mRNA (A and B) and protein (C) levels markedly decreased in the presence of the cjHtt-1 siRNA duplex, i.e., potent RNAi knockdown against the endogenous *Cj-Htt* gene was induced by the siRNA duplex. Finally, the data presented here also indicate that proper detection of the newly identified *Cj-Htt* gene and its products can be performed by means of the methods and tools assessed in this study.

In conclusion, we described for the first time the common marmoset *Htt* gene, and also detection methods and tools for the gene and its gene products. The data presented here may assist us in promoting a non-human primate HD model with the common marmoset.

Acknowledgments

We would like to thank Dr. R.C. Desrosiers for kindly providing HS viruses. We also thank Drs. K. Nakamura, T. Kabuta, M. Suzuki, and C. Konya for their helpful advice and discussion. Finally, we would like to thank Dr I. Kanazawa for his encouragement and helpful advice. This work was supported by research grants from the Ministry of Health, Labour and Welfare of Japan.

References

Akari, H., et al., 1996. In vitro immortalization of Old World monkey T lymphocytes with Herpesvirus saimiri: its susceptibility to infection with simian immunodeficiency viruses. Virology 218, 382–388.

Andrade, M.A., Bork, P., 1995. HEAT repeats in the Huntington's disease protein. Nat. Genet. 11, 115–116.

Deisboeck, T.S., et al., 2003. Development of a novel non-human primate model for preclinical gene vector safety studies. Determining the effects of intracerebral HSV-1 inoculation in the common marmoset: a comparative study. Gene Ther. 10, 1225–1233.
Doods, H., et al., 2000. Pharmacological profile of BIBN4096BS, the first selective small molecule CGRP antagonist. Br. J. Pharmacol. 129, 420–423.
Gilliam, T.C., et al., 1987. A DNA segment encoding two genes very tightly linked to Huntington's disease. Science 238, 950–952.
Gusella, J.F., et al., 1983. A polymorphic DNA marker genetically linked to Huntington's disease. Nature 306, 234–238.
Huntington's-Disease, C.R.G.o., The Huntington's Disease Collaborative Research Group, 1993. A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. Cell 72, 971–983.
Kazemi-Esfarjani, P., Benzer, S., 2000. Genetic suppression of polyglutamine toxicity in Drosophila. Science 287, 1837–1840.
Kendall, A.L., Rayment, F.D., Torres, E.M., Baker, H.F., Ridley, R.M., Dunnett, S.B., 1998. Functional integration of striatal allografts in a primate model of Huntington's disease. Nat. Med. 4, 727–729.
Liu, W., Goto, J., Wang, Y., Murata, M., Wada, K., Kanazawa, I., 2003. Specific inhibition of Huntington's disease gene expression by siRNAs in cultured cells. Proc. Jpn. Acad. 79, 293–298.
Lunkes, A., Mandel, J.L., 1998. A cellular model that recapitulates major pathogenic steps of Huntington's disease. Hum. Mol. Genet. 7, 1355–1361.
Mangiarini, L., et al., 1996. Exon 1 of the HD gene with an expanded CAG repeat is sufficient to cause a progressive neurological phenotype in transgenic mice. Cell 87, 493–506.
Neuwald, A.F., Hirano, T., 2000. HEAT repeats associated with condensins, cohesins, and other complexes involved in chromosome-related functions. Genome Res. 10, 1445–1452.
Palfi, S., et al., 2007. Expression of mutated huntingtin fragment in the putamen is sufficient to produce abnormal movement in non-human primates. Mol. Ther. 15, 1444–1451.
Sakai, T., Hohjoh, H., 2006. Gene silencing analyses against amyloid precursor protein (APP) gene family by RNA interference. Cell Biol. Int. 30, 952–956.
t'Hart, B.A., Vervoordeldonk, M., Heeney, J.L., Tak, P.P., 2003. Gene therapy in nonhuman primate models of human autoimmune disease. Gene Ther. 10, 890–901.
von Horsten, S., et al., 2003. Transgenic rat model of Huntington's disease. Hum. Mol. Genet. 12, 617–624.
Walker, F.O., 2007. Huntington's disease. Lancet 369, 218–228.
Yang, S.H., et al., 2008. Towards a transgenic model of Huntington's disease in a non-human primate. Nature 453, 921–924.

OPEN ACCESS Freely available online

PLoS one

Enhancement of Allele Discrimination by Introduction of Nucleotide Mismatches into siRNA in Allele-Specific Gene Silencing by RNAi

Yusuke Ohnishi[1,2], Yoshiko Tamura[1], Mariko Yoshida[1], Katsushi Tokunaga[2], Hirohiko Hohjoh[1]*

1 Department of Molecular Genetics, National Institute of Neuroscience, NCNP, Kodaira, Tokyo, Japan, 2 Department of Human Genetics, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

Abstract

Allele-specific gene silencing by RNA interference (RNAi) is therapeutically useful for specifically inhibiting the expression of disease-associated alleles without suppressing the expression of corresponding wild-type alleles. To realize such allele-specific RNAi (ASP-RNAi), the design and assessment of small interfering RNA (siRNA) duplexes conferring ASP-RNAi is vital; however, it is also difficult. In a previous study, we developed an assay system to assess ASP-RNAi with mutant and wild-type reporter alleles encoding the *Photinus* and *Renilla luciferase* genes. In line with experiments using the system, we realized that it is necessary and important to enhance allele discrimination between mutant and corresponding wild-type alleles. Here, we describe the improvement of ASP-RNAi against mutant alleles carrying single nucleotide variations by introducing base substitutions into siRNA sequences, where original variations are present in the central position. Artificially mismatched siRNAs or short-hairpin RNAs (shRNAs) against mutant alleles of the human *Prion Protein* (*PRNP*) gene, which appear to be associated with susceptibility to prion diseases, were examined using this assessment system. The data indicates that introduction of a one-base mismatch into the siRNAs and shRNAs was able to enhance discrimination between the mutant and wild-type alleles. Interestingly, the introduced mismatches that conferred marked improvement in ASP-RNAi appeared to be largely present in the guide siRNA elements, corresponding to the 'seed region' of microRNAs. Due to the essential role of the 'seed region' of microRNAs in their association with target RNAs, it is conceivable that disruption of the base-pairing interactions in the corresponding seed region, as well as the central position (involved in cleavage of target RNAs), of guide siRNA elements could influence allele discrimination. In addition, we also suggest that nucleotide mismatches at the 3'-ends of sense-strand siRNA elements, which possibly increase the assembly of antisense-strand (guide) siRNAs into RNA-induced silencing complexes (RISCs), may enhance ASP-RNAi in the case of inert siRNA duplexes. Therefore, the data presented here suggest that structural modification of functional portions of an siRNA duplex by base substitution could greatly influence allele discrimination and gene silencing, thereby contributing to enhancement of ASP-RNAi.

Citation: Ohnishi Y, Tamura Y, Yoshida M, Tokunaga K, Hohjoh H (2008) Enhancement of Allele Discrimination by Introduction of Nucleotide Mismatches into siRNA in Allele-Specific Gene Silencing by RNAi. PLoS ONE 3(5): e2248. doi:10.1371/journal.pone.0002248

Editor: Luis M. Corrochano, University of Sevilla, Spain

Received November 1, 2007; **Accepted** April 14, 2008; **Published** May 21, 2008

Funding: This work was supported in part by research grants from the Ministry of Health, Labor, Welfare in Japan, and from Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists, and also by a Grant-in-Aid from the Japan Society for the Promotion of Science.

Competing Interests: The corresponding author has a pending patent on the method used in this study.

* E-mail: hohjohh@ncnp.go.jp

Introduction

RNA interference (RNAi) is the process of sequence-specific posttranscriptional gene silencing induced by double-stranded RNAs (dsRNAs) homologous to the silenced gene, and it is currently used as a powerful tool to suppress the expression of genes of interest [1,2]. Introduced or generated dsRNAs are subjected to digestion by an RNase III enzyme, Dicer, into 21–25 nucleotide (nt) RNA duplexes [3]. The resultant RNA duplexes, referred to as small interfering RNA (siRNA) duplexes, are unwound, and single-stranded siRNA elements can then be incorporated into RNA-induced silencing complexes (RISCs) to function as sequence-specific mediators. These are referred to as guide siRNA elements [1,2,4].

In mammals, RNAi can be induced by direct introduction of chemically synthesized siRNA duplexes into cells, or by generation of siRNA duplexes using short-hairpin RNA (shRNA) expression vectors. Applications are expanding in various fields of science, with the potential therapeutic use of RNAi in medical science and pharmacogenesis being particularly promising [1,5–8].

Allele-specific gene silencing by RNAi (allele-specific RNAi: ASP-RNAi) is an advanced application of RNAi techniques that allows the expression of an allele of interest to be specifically inhibited [9]. ASP-RNAi would thus be therapeutically very useful, as it can specifically suppress the expression of alleles causing disease without inhibiting the expression of corresponding wild-type alleles [10–17]. To induce such ASP-RNAi, it is necessary to design siRNAs that exhibit strong allele-specific gene silencing; thus, siRNAs must be designed such that they are able to carry nucleotide variations characterizing target disease alleles and to discriminate the target alleles from corresponding wild-type alleles. In addition, qualitative and quantitative evaluation of such designed siRNAs on allele-specific gene silencing is required.

In a previous study, we developed an assay system to assess ASP-RNAi with mutant and wild-type reporter alleles encoding

the *Photinus* and *Renilla luciferase* genes. In this system, the effects of designed siRNAs and short-hairpin RNAs (shRNAs) against mutant alleles in allele-specific gene silencing, as well as off-target silencing against wild-type alleles, can be simultaneously examined [17]. With amyloid precursor protein (*APP*) variants (the Swedish- and London-type variants) related to familial Alzheimer's disease [18,19] as model disease alleles, we were able to determine competent siRNA duplexes conferring ASP-RNAi [17]. Previous observations have also suggested that enhanced discrimination of target mutant alleles carrying single nucleotide variations from wild-type allele RNAs is required for ASP-RNAi.

In the present study, we describe the improvement of ASP-RNAi against mutant alleles carrying single nucleotide variations. We introduced base substitutions into siRNA and shRNA sequences, and examined the effects of the resultant mismatched siRNAs and shRNAs on ASP-RNAi by means of our assay system. The results presented here suggest that base substitutions introduced into certain portions of siRNA and shRNA sequences may contribute to enhancement of ASP-RNAi.

Results

ASP-RNAi against *PRNP* alleles carrying single nucleotide variations

In a previous study, we established an assessment system for siRNA duplexes conferring ASP-RNAi [17]. This system depends on two reporter alleles encoding the *Photinus* and *Renilla luciferase* genes carrying mutant and wild-type allelic sequences in their 3′-UTRs. Briefly, using this system, the effects of test siRNA duplexes against mutant alleles in allele-specific silencing, as well as off-target silencing against wild-type alleles, can be examined under heterozygous conditions generated by cotransfecting the reporter alleles and siRNA duplexes into cultured human cells.

In this study, we focused on the human *Prion Protein (PRNP)* gene, which is known to possess a number of single nucleotide variations [20,21]. We selected three *PRNP* variants, which are also followed by amino acid substitutions (*P102L*, *P105L*, and *D178N*) and appear to be associated with susceptibility to various prion diseases such as Gerstmann-Sträussler-Scheinker disease (GSS) and fatal familial insomnia (FFI) [22–25]. We constructed three mutant reporter alleles, designated the *PRNP-P102L*, *PRNP-P105L*, and *PRNP-D178N* alleles, and their corresponding wild-type reporter alleles (Figure 1A). The reporter alleles, synthetic siRNA duplexes against the mutant alleles (supplementary Table s1 and supplementary Figure s1), and the *beta-galactosidase* gene (control), were cotransfected into HeLa cells; thus, the transfected cells were artificially heterozygous with the mutant and wild-type reporter alleles. The effects of the designed siRNA duplexes on suppression of both the mutant and wild-type alleles were then simultaneously examined. As shown in Figure 1, the siRNA duplexes other than siPrnp102(T7), siPrnp102(T8), siPrnp105(T7) and siPrnp105(T9) were not able to induce significant ASP-RNAi. Of the four siRNAs just listed, the siPrnp105(T9) duplex appears to confer ASP-RNAi.

To realize ASP-RNAi against any target alleles, it is important and necessary to establish techniques for enhancement of allele discrimination followed by specific digestion against the target alleles. To address this, we selected siRNA duplexes possessing strong knockdown potency as candidates for improvement. This is because the only apparent failure of such siRNAs is being unable to discriminate target alleles from non-target ones, i.e., ASP-RNAi may be improved just by reinforcing allele discrimination. Since RNAi activity appears to be influenced by nucleotide mismatches

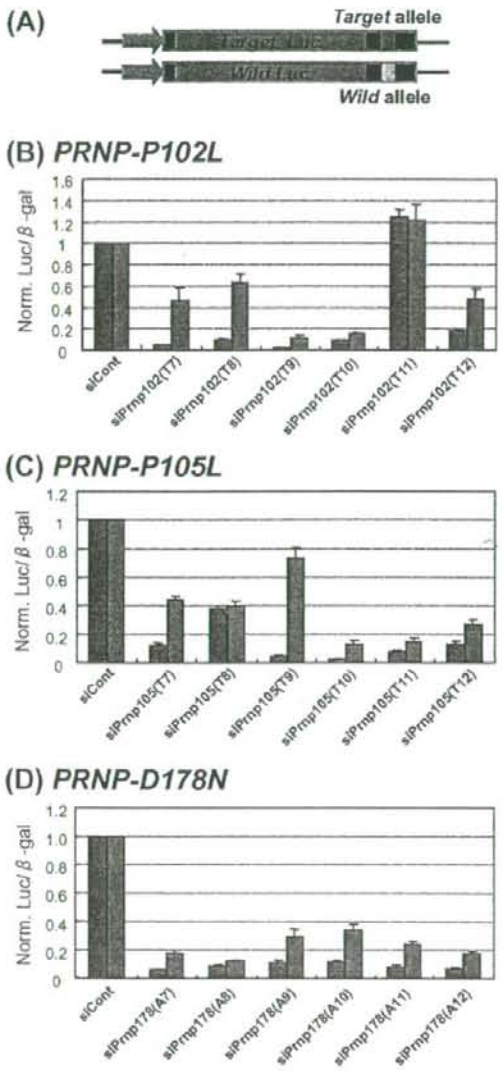

Figure 1. Assessment of ASP-RNAi with reporter alleles. (A) Schematic drawing of reporter alleles. Expression alleles were constructed by inserting synthetic oligonucleotides of mutant and wild-type allelic sequences into the 3′-UTRs of the reporter genes driven by the same TK promoter (indicated by arrows). Assessment of designed siRNA duplexes against the mutant allele was carried out as described in Materials and Methods. Effects of designed siRNA duplexes against the *PRNP-P102L* (B), *PRNP -P105L* (C), and *PRNP -D178N* (D) mutants on ASP-RNAi. Reporter alleles, synthetic siRNA duplexes against the mutant alleles (indicated) and the *β-galactosidase* gene (control) were cotransfected into HeLa cells. Twenty-four hours after transfection, expression levels of the reporter genes were examined. Levels of either mutant allele (pink boxes) or wild-type allele (blue boxes) luciferase activity were normalized against the levels of β-galactosidase activity, and the ratios of mutant and wild-type luciferase activities in the presence of siRNA duplexes were normalized against the control ratios obtained in the presence of siControl duplex (siCont). Data are averages of at least three independent determinations. Error bars represent standard deviations.
doi:10.1371/journal.pone.0002248.g001

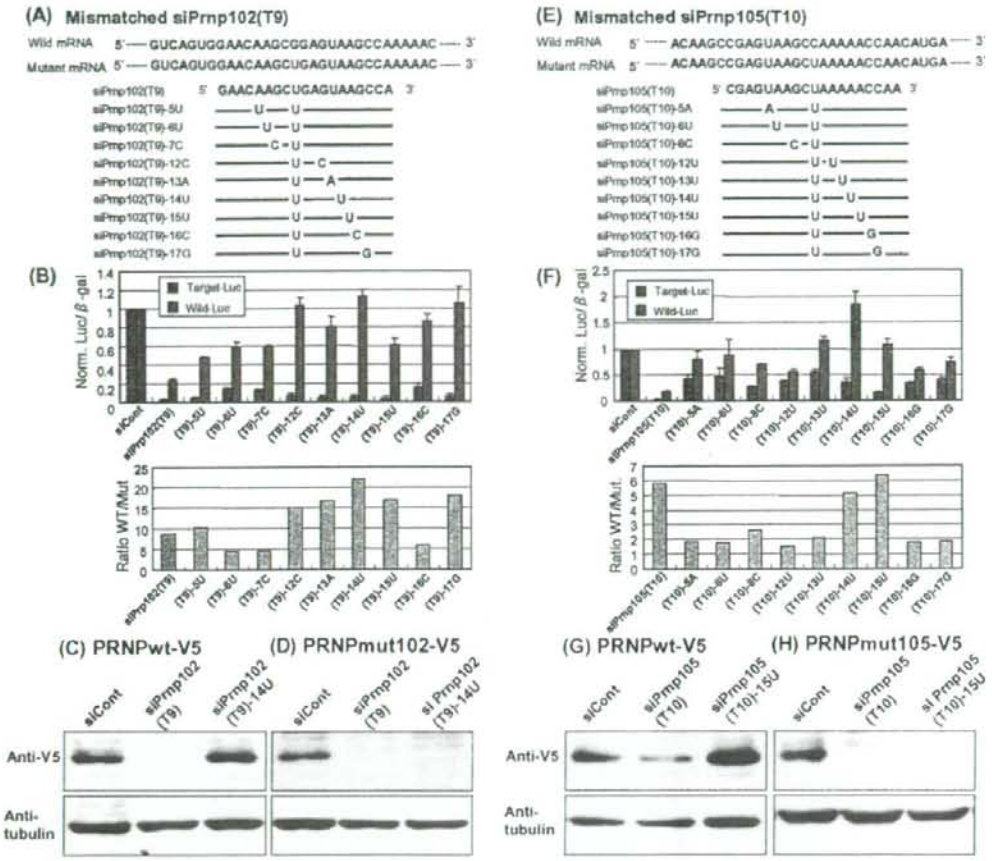

Figure 2. Assessment of mismatched siPrnp102(T9) and siPrnp105(T10) duplexes on ASP-RNAi. (A, E) Nucleotide sequences of wild-type and mutant *PRNP* mRNAs and designed siRNAs. The wild-type and mutant *PRNP* mRNA sequences around the P102L (A) and P105L (E) variations are shown and the variations are indicated in red. Designed siRNAs (indicated) are represented based on the sequence of the sense-strand (passenger) siRNA element; mismatched nucleotides (introduced base substitutions) and the original variations are indicated in blue and red, respectively. The same sequences as the siPrnp102(T9) or siPrnp105(T10) are represented by thin lines. (B, F) Effects of mismatched siPrnp102(T9) and siPrnp105(T10) on ASP-RNAi. Mismatched siPrnp102(T9) (B) and siPrnp105(T10) (E) duplexes (indicated) were examined as in Figure 1. The ratio of wild-type allele-luciferase activity against the mutant allele-luciferase activity (WT/Mut) was also examined to evaluate the improvement in ASP-RNAi. Data are averages of at least three independent determinations. Error bars represent standard deviations. Expression of the wild-type (C, G) and mutant [P102L (D) and P105L (H)] PRNP polypeptides (PRNPmut102-V5 and PRNPmut102-V5) in the presence of indicated siRNA duplexes was investigated by Western blotting using anti-V5 antibody. Expression of α-tubulin was examined as the control.
doi:10.1371/journal.pone.0002248.g002

between siRNAs and their target RNAs [11,26], we introduced a one-base substitution into the selected siRNAs (Figures 2A and E; supplementary Table s2) and examined the effects of such mismatched siRNA duplexes using our assessment system. It should be noted that the resultant mismatched siRNAs give rise to one nucleotide mismatches against the target mutant allele, and more importantly, two nucleotide mismatches against the wild-type allele (the artificially introduced mismatch and the original variation). Accordingly, we expected that such mismatched siRNAs would exhibit better discrimination between target mutant and wild-type alleles, and only suppress the expression of the mutant alleles.

Effects of mismatched siRNA duplexes on ASP-RNAi against *PRNP-P102L* and *PRNP-P105L* alleles

We selected the siPrnp102(T9) and siPrnp105(T10) duplexes from the first screening of siRNA duplexes against the *PRNP* mutant alleles (Figure 1), and introduced base substitutions into the siRNAs (Figures 2A and E). The resultant mismatched siRNAs were investigated using the assessment system to determine whether they could improve ASP-RNAi. To more precisely determine allele-specific gene silencing, we examined the ratio of the wild-type allele expression to the mutant allele expression. Figures 2B and F show the results of the assessment of the mismatched siPrnp102(T9) and siPrnp105(T10) duplexes, respec-

tively. The results indicate that: (i) introduction of a one-base substitution into the siRNAs was able to influence allele discrimination and RNAi activity, (ii) different nucleotide mismatches appeared to yield different levels of discrimination and inhibition against either the target mutant or wild-type allele, and (iii) some base substitutions appeared to confer marked allele discrimination, resulting in enhancement of ASP-RNAi. It may be of interest that the expression level of the wild-type allele in the presence of siPrnp105(T10)-14U was markedly increased, which may have been caused by the unusual expression level of the control *beta-galactosidase* gene, for as yet unknown reasons. Examination of the ratio of wild-type expression to mutant expression revealed that the siPrnp102(T9)-12C, -13A, -14U and -17G duplexes (Figure 2B) and the siPrnp105(T10)-14U and -15U duplexes (Figure 2F) appear to have markedly improved ASP-RNAi activity. In addition, when HEK293 cells were used instead of HeLa cells, similar results were obtained (data not shown).

To further confirm the results, we examined the effects of the siRNAs on the recognition and inhibition of the *bona fide* wild-type and mutant *PRNP* alleles using their full-length cDNAs. The pPRNPwt-V5 and pPRNPmut102(or mut105)-V5 expression plasmids carrying the wild-type and mutant cDNAs, respectively, were subjected to cotransfection with the siRNAs into HeLa cells, and the expression of the PRNPwt-V5 or the PRNPmut102(or mut105)-V5 polypeptide then examined by Western blotting. The results indicate that while the signal of PRNPwt-V5 in the presence of siPrnp102(T9) or siPrnp105(T10) was reduced, the signal intensity of PRNPwt-V5 was increased in the presence of either siPrnp102(T9)-12C, siPrnp102(T9)-14U or siPrnp105(T10)-15U (Figures 2C and G; supplementary Figure s2A). As expected, the mismatched siRNAs still hold a strong knockdown potency against the mutant *PRNPs* (Figures 2D and H; supplementary Figure s2B). These observations agree with the results for the reporter alleles described above.

Effects of mismatched siRNA duplexes on ASP-RNAi against *PRNP* mutant alleles

From the first screening (Figures 1C and D), we also selected the siPrnp102(T10) and siPrnp178(A9) duplexes and introduced base substitutions into the siRNAs (supplementary Figures s3A and D). Assessment of the mismatched siRNAs suggested that, similar to Prnp102(T9) and Prnp105(T10), introduction of a one-base substitution into the siRNAs influenced ASP-RNAi activity. Furthermore, the ratios of wild-type allele expression to mutant allele expression indicated that the siPrnp102(T10)-13C and siPrnp178(A9)-13C duplexes have significantly improved ASP-RNAi activity (supplementary Figures s3B and E). Western blot analysis indicated that while the PRNPwt-V5 signal was reduced in the presence of either siPrnp102(T10) or siPrnp178(A9), the signal was increased in the presence of the mismatched siPrnp102(T10)-13C or siPrnp178(A9)-13C duplexes (supplementary Figures s3C and F); thus, the data is also compatible with the results for the reporter alleles described above.

Effects of shRNA expression plasmids on ASP-RNAi

As short-hairpin RNA (shRNA) expression vectors appear to be useful for long-term gene silencing [16,27,28], their potential use in ASP-RNAi is also of interest. We constructed shRNA expression plasmids for production of the siPrnp102(T9), (T9)-12C, (T9)-13A, (T9)-14U and (T9)-15U duplexes in cells [designated sh102(T9), sh102(T9)-12C, sh102(T9)-13A, sh102(T9)-14U, sh102(T9)-15U plasmids, respectively]. The shRNA expression plasmids were examined by the assessment system and Western blotting as described above. As shown in Figure 3, the results

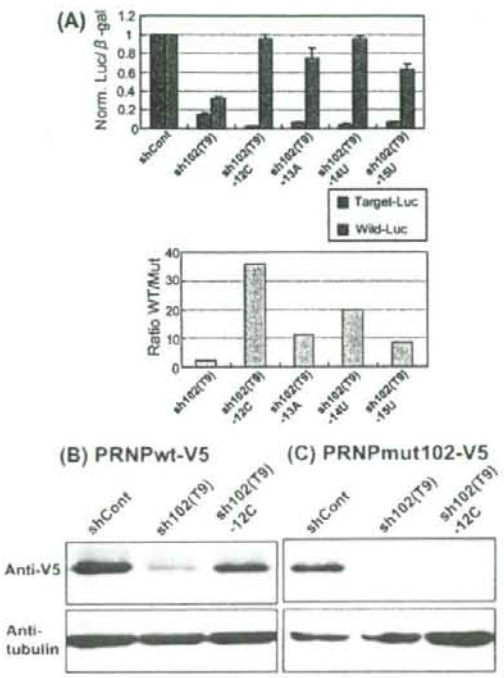

Figure 3. ASP-RNAi with mismatched shRNA expression plasmids against the *PRNP-P102L* mutant. (A) sh102(T9), sh102(T9)-12C, sh102(T9)-14U, sh102(T9)-15U and shCont shRNA expression plasmids for production of siPrnp102(T9), (T9)-12C, (T9)-14U and (T9)-15U duplexes and siControl duplex, respectively, were constructed with reporter alleles and their effects on ASP-RNAi were examined as in Figure 1. Western blot analysis of the wild-type (B) and P102L (C) PRNP polypeptides was carried out as in Figure 2.
doi:10.1371/journal.pone.0002248.g003

indicate that while the sh102(T9) plasmid induced strong gene silencing against either the mutant or wild-type allele, the sh102(T9)-12C, -13A, -14U and -15U plasmids were able to confer allele-specific gene silencing, which agrees with the data for synthetic siRNA duplexes (Figure 2). It is noteworthy that the mismatched shRNA expression plasmids appear to enhance ASP-RNAi to a greater degree than the corresponding synthetic siRNA duplexes; of the mismatched plasmids, the sh102(T9)-12C plasmid, appears to induce strong ASP-RNAi. It is possible that mismatched shRNA expression plasmids are superior to mismatched siRNA duplexes for induction of ASP-RNAi.

Effects of structural modification of siRNA duplexes on ASP-RNAi

In a previous study, we experienced difficulty in inducing ASP-RNAi against the London-type *amyloid precursor protein* (*APP*) mutants, in which single nucleotide substitutions followed by amino acid substitutions (*V717I, V717L, V717G*) were present [17]. While a few designed siRNAs targeting the mutants appeared to discriminate between the mutant and the corresponding wild-type alleles to some degree, most of the siRNAs resulted in weak gene silencing. This is in contrast to the knockdown potency of siRNAs targeting the *PRNP* mutants described above (Figure 1). To

improve such ASP-RNAi, it is necessary to design siRNAs such that they can gain knockdown potency against the mutants, and structural modification may also be applicable for achieving such improvements.

Previous studies showed that fork-siRNA duplexes (F-siRNA duplexes) carrying two nucleotide mismatches at the 3′-ends of the sense-strand siRNA elements are able to enhance RNAi activity to a greater degree than conventional siRNA duplexes [29,30]. Accordingly, we investigated whether F-siRNA duplexes improve ASP-RNAi activity against the London-type *APP* mutants (Figure 4 and supplementary Table s5). The results indicate that several F-siRNA duplexes [F-siAPP(A11), (T11), (G11) and (G12) duplexes] were able to enhance ASP-RNAi to some degree (Figures 4B-D).

In relation to the improvement of ASP-RNAi against the London-type *APP* mutants, we further observed that F-siRNA duplexes, F-siAPP(T12/C13), targeting the Swedish *APP* mutant carrying double nucleotide mutations, were able to markedly improve ASP-RNAi activity, although the conventional siAPP(T12/C13) duplexes induced little or no RNAi activity (Figure 4E).

Different effects of miR-196a and miR-196b on recognition of target *HOXB8*

From the data of the mismatched siPrnp duplexes (Figures 2 and supplementary Figures s2 and s3) and shPrnp RNAs (Figure 3), it appears that the base substitutions conferring marked improvement are largely present in the region of siRNAs corresponding to the seed region of microRNA. This suggests that the region corresponding to the seed region, as well as the central position (where the original variations are present), of guide siRNAs may play a role in allele discrimination in allele-specific gene silencing (details in Discussion). Based on this, it is of particular interest to determine whether the two regions of *bona fide* microRNA (miRNA) can contribute to the regulation of gene expression. From a previous study [31] and an miRNA database, we focused on miR-196a and miR-196b, both of which are nearly complementary to part of the 3′-UTR sequence of *HOXB8* mRNA. Note that one and two mismatches are present in the predicted base-pairing of *HOXB8* with miR-196a and miR-196b, respectively (Figure 5A). In addition, one mismatch, which can form a G:U wobble base-pair, is present in the seed region of both miRNAs, while the other mismatch (U vs. C) is present in the central position of miR-196b. We examined whether the mismatches in miR-196a and miR-196b participate in the recognition of their target, *HOXB8*.

To address this, we constructed a reporter plasmid encoding the *Renilla luciferase* gene with a part of the *HOXB8* sequence in its 3′-UTR. The resultant reporter gene was cotransfected with either synthetic miR-196a or miR-196b duplex into HeLa cells, and the expression levels of the reporter gene then examined. The results (Figure 5B) indicate that miR-196a induced potent inhibition of the expression of the target reporter gene, whereas miR-196b conferred moderate levels of suppression against target reporter gene expression, thus suggesting different levels of recognition against *HOXB8* between miR-196a and miR-196b. Consequently, the evidence suggests that the mismatches in miR-196a and miR-196b probably influence the recognition of their target *HOXB8* mRNA.

Discussion

Enhancement of ASP-RNAi by mismatched siRNAs

In order to realize and control ASP-RNAi, it is necessary to design competent siRNAs or shRNAs possessing strong allele discrimination between target mutant and wild-type alleles, thereby inducing allele-specific gene silencing. In the case of ASP-RNAi, designed siRNAs perfectly match target mutant alleles, but do not correspond with wild-type alleles, i.e., mismatched base pairing(s) will occur at variation site(s) between the siRNAs and wild-type alleles (Figure 6A; wild-type mRNA).

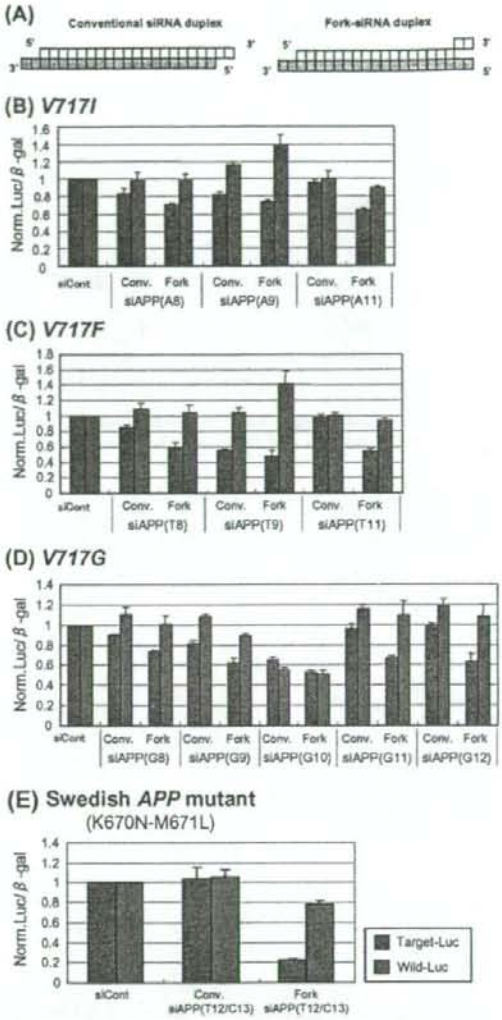

Figure 4. Effects of forked siRNA duplexes on APS-RNAi against *APP* mutants. (A) Schematic drawing of conventional and forked siRNA duplexes. Each box represents a ribonucleotide, and antisense-stranded siRNA elements are indicated by gray boxes. (B)-(E) Comparison of ASP-RNAi activities between conventional (Conv.) and forked (Fork) siRNA duplexes. Assessment of conventional and forked siRNA duplexes against the London *V717I* (B), *V717F* (C), and *V717G* (D) *APP* mutants and the Swedish (E) *APP* mutant was carried out as described previously [17]. The ratios of mutant and wild-type luciferase activities in the presence of siRNA duplexes were normalized against the control ratio obtained in the presence of siControl duplex. Data are averages of at least three independent determinations. Error bars represent standard deviations.
doi:10.1371/journal.pone.0002248.g004

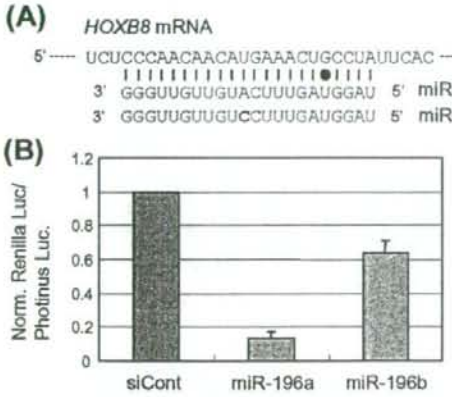

Figure 5. Different knockdown potencies against *HOXB8* between miR-196a and miR-196b. (A) Nucleotide sequences of *HOXB8* mRNA, miR-196a and miR-196b. The *HOXB8* mRNA sequence, which is nearly complementary to miR-196a, together with mature miR-196a and miR-196b are aligned. Perfect base-pairing and G:U wobble base-pairing are indicated by the horizontal bars and dot, respectively. The mismatched base in miR-196b is indicated in red. (B) Effects of miR-196a and miR-196b on gene silencing against *HOXB8*. The miR-196a and miR-196b duplexes were chemically synthesized, as described previously [31]. The synthetic miRNA duplexes, together with a reporter plasmid carrying a part of the *HOXB8* gene (see Materials and Methods), were cotransfected into HeLa cells and the expression of reporter gene was examined. Ratios of normalized target (*Renilla*) luciferase activity to control (*Photinus*) luciferase activity are shown. Data are averages of at least three independent experiments. Error bars represent standard deviations.
doi:10.1371/journal.pone.0002248.g005

Previous studies in which the effects of single-nucleotide mismatches in siRNAs on RNAi activities were systematically examined suggest that nucleotide mismatches are able to influence RNAi activity [26,32]. Our present and previous studies indicate that some, but not all, of the designed siRNA duplexes targeting mutant alleles apparently discriminate the mutant alleles from the wild-type alleles. Since different siRNAs confer different levels of RNAi activity depending upon their thermodynamic properties, the intrinsic knockdown potency may also affect allele discrimination between target and non-target alleles.

In the present study, we observed an improvement in ASP-RNAi when siRNAs induced double knockdown of mutant and wild-type alleles: introduction of a one-base substitution into such siRNAs carrying the original variations around the central position appeared to influence allele discrimination and inhibition of target mutant alleles, although different base substitutions conferred different levels of discrimination and inhibition (Figure 6B). This phenomenon may be associated with the thermodynamic properties of the modified siRNA duplexes. Interestingly, the base substitutions conferring marked ASP-RNAi appeared to be largely present in the region of guide siRNAs, corresponding to the seed region of microRNAs. Since such siRNAs exhibit one and two mismatches against mutant and wild-type alleles, respectively, we suggest that disruption of base-pairing interaction in the seed region, as well as the central position, of the guide siRNAs reduces recognition and/or silencing activity against wild-type alleles, and that a one-base mismatch in the seed region of the guide siRNAs against the target mutant alleles hardly affects gene silencing, i.e., potent RNAi activity against the mutant alleles may remain unchanged.

In addition to the improvement in siRNAs conferring double knockdown of mutant and wild-type alleles, we also observed an enhancement of allele discrimination in ASP-RNAi when siRNAs induced weak knockdown potency, which is in contrast to that of the siRNAs described above. In that case, forked siRNA duplexes carrying two nucleotide mismatches at the 3′-ends of the sense-stranded siRNA elements may allow enhanced allele discrimination between target mutant and wild-type alleles. Since forked siRNA duplexes appear to increase the assembly of antisense-strand (guide) siRNA elements into RISCs, the ease of incorporation of antisense-strand siRNA elements into RISCs may be a key factor for improvement of ASP-RNAi activity.

Altogether, the evidence presented here suggests that structural modification by introduction of base substitution into siRNA or shRNA sequences could influence allele discrimination and silencing activity in ASP-RNAi.

Mismatches in the seed region and the central position of miRNAs affect gene silencing

It is noteworthy that destabilization of base-pairing interaction in the seed region, as well as in the central position, of *bona fide* miRNAs appears to also influence recognition and/or suppression of target RNAs. In the case of miR-196a and miR-196b presented in this study, the miRNAs appear to induce different levels of suppression against *HOXB8* RNA. Accordingly, it is conceivable that the difference in gene silencing involving miRNAs could yield complex regulation of gene expression. Since there are various base-pairing interactions between miRNAs and their target RNAs, such interactions may contribute in the generation of various and complex gene regulatory events. More extensive research is required to further evaluate this possibility.

Properties of siRNA duplexes conferring ASP-RNAi

Based on the assessed siRNA duplexes efficacy of ASP-RNAi, it appears that different siRNA duplexes induce different levels of ASP-RNAi, suggesting that allele-specific knockdown potencies are most likely dependent on the designed siRNA sequences. Previous studies have suggested that functional siRNA duplexes can be characterized by low base-pairing stability at their 3′-ends [29,33–35], i.e., an asymmetrical feature of base-pairing stability occurs between both ends of functional siRNA duplexes. From the alignment of the siRNA sequences examined in the present and previous studies, it was observed that the siRNA duplexes conferring little or no gene silencing possessed symmetrical base-pairing stability and contained G or C residues at many 3′-ends of their sense-stranded elements (supplementary Table s7A): these features are distinct from those of functional siRNA duplexes. In contrast to the above siRNA duplexes, most of the siRNA duplexes triggering strong RNAi activity, including ASP-RNAi, tended to have asymmetrical base-pairing stability (supplementary Table s7B). It may be of interest to elucidate the properties of siRNA duplexes that confer strong ASP-RNAi, other than the asymmetrical feature. To address this, extensive examination of the knockdown potency of siRNA duplexes targeting various mutant alleles is required.

In conclusion, in order to realize ASP-RNAi against target mutant alleles carrying nucleotide variations, the design and evaluation of competent siRNA duplexes conferring ASP-RNAi is vital; but designed siRNAs do not always confer potent ASP-RNAi activity. The evidence presented here suggests that structural modification of siRNA duplexes by base substitutions may improve ASP-RNAi. The key regions in an siRNA duplex for such modifications are the central position, the seed region and the 3′-end of the sense-strand siRNA element, which appear to be related to target RNA cleavage, target RNA recognition and assembly of the antisense-strand (guide)

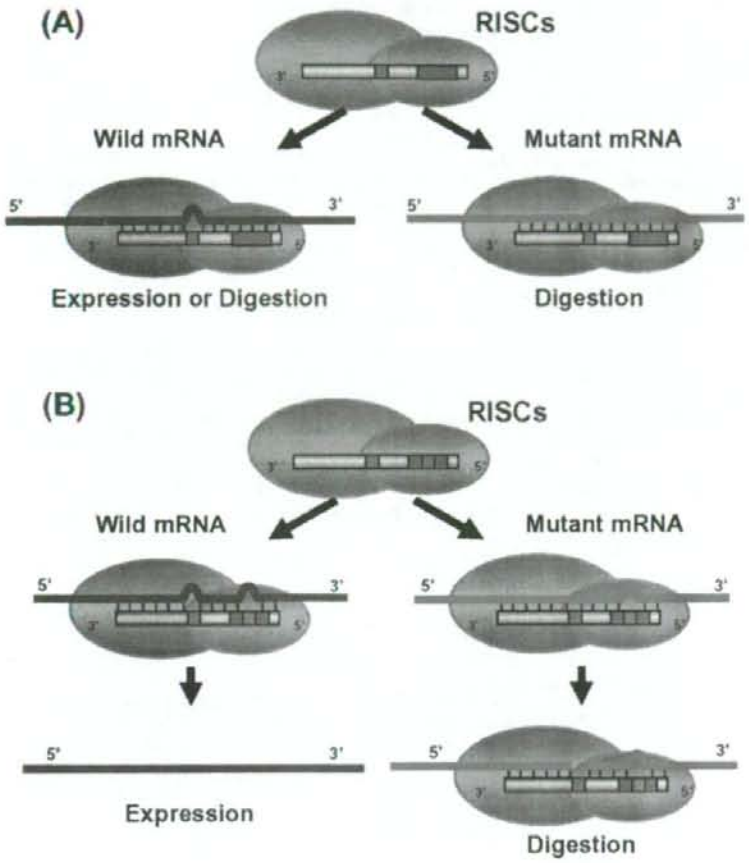

Figure 6. Schematic summary of allele-specific gene silencing with mismatched siRNA. Guide siRNA elements in RISCs are indicated by gray bars, in which nucleotide variation and the seed region are indicated by red and blue boxes, respectively. Introduced base substitutions are indicated by green boxes. Mutant and wild-type allelic transcripts are indicated by red and blue lines, respectively. (A) When designed siRNA targeting the mutant allele has potential for potent gene silencing, not only the mutant but also the wild-type alleles may be inhibited by RNAi mediated by the siRNA, i.e., double knockdown against both alleles may occur. (B) When a one-base substitution is introduced into the seed region of an siRNA conferring double knockdown, the resultant (guide) siRNA generates one and two mismatches against the mutant and wild-type alleles, respectively, thereby possibly gaining the ability to induce ASP-RNAi.
doi:10.1371/journal.pone.0002248.g006

siRNA element into RISCs, respectively. Therefore, structural modification of such functional portions of siRNA duplexes may greatly influence allele discrimination and gene silencing activity, thereby conferring improvement of ASP-RNAi.

Materials and Methods

Preparation of oligonucleotides

RNA and DNA oligonucleotides were obtained from TAKARA BIO, and INVITROGEN or BEX, respectively. For preparation of duplexes, sense- and antisense-strand oligonucleotides (20 μM each) were mixed and annealed, as described previously [29]. Sequences of synthesized RNA and DNA oligonucleotides are shown in supplementary Tables s1, s2, s3, s4, s5 and s6. Non-silencing siRNA duplex (siControl; Qiagen) was used as the negative control.

Cell culture

HeLa and HEK293 cells were grown at 37°C in Dulbecco's modified Eagle's medium (Wako) supplemented with 10% fetal bovine serum (Sigma), 100 U/ml penicillin and 100 μg/ml streptomycin (Wako) in a 5% CO_2-humidified chamber. HEK293 cells (Registry No. JCRB9068) were obtained from the Health Science Research Resources Bank.

Construction of reporter alleles and shRNA expression plasmids

Reporter alleles were constructed as described previously [17]. Briefly, phRL-TK (Promega) and pGL3-TK [30] plasmids encoding the *Renilla* and *Photinus luciferase* genes, respectively, were digested with XbaI and NotI, and subjected to ligation with synthetic oligonucleotide duplexes corresponding to the *PRNP*-

P102L, *PRNP-P105L* and *PRNP-D178N* alleles, as well as wild-type alleles (oligonucleotide sequences are indicated in supplementary Table s3). The resultant plasmids carry allelic *PRNP* sequences in the 3′-untranslated regions (UTRs) of the *luciferase* genes (Figure 1A). The London-type *APP* mutant and wild-type *APP* reporter alleles described in a previous paper [17] were also used. For construction of shRNA expression vectors, the GeneSilencer shRNA Vector (Gene Therapy System, Inc.) was used, and synthetic oligonucleotide duplexes were inserted into the vector according to the manufacturer's instructions (oligonucleotide sequences are indicated in Table s4). To construct a reporter plasmid carrying part of the *HOXB8* sequence, psiCHECK-2 vector (Promega) was digested with XhoI and PmeI, and subjected to ligation with synthetic *HOXB8* oligonucleotide duplex (supplementary Table s3). With regard to expression plasmids carrying a full-length cDNA of the human *PRNP* linked with the V5-tag sequence, the wild-type *PRNP* cDNA was amplified by RT-PCR with human brain total RNA (Ambion), trimmed with EcoRI and NotI, and inserted into the pTracer-EF/Bsd vector (Invitrogen); the resultant plasmid was designated 'pPRNPwt-V5'. Using this plasmid, mutant plasmids carrying the *PRNP P102L* and *P105L* were constructed using the GeneEditor *in vitro* Site-Directed Mutagenesis System (Promega) and the QuikChange Site-Directed Mutagenesis Kit (Stratagene), respectively, according to the manufacturer's instructions; the resultant mutant expression plasmids were designated 'pPRNPmut102-V5' and 'pPRNPmut105-V5'. The PCR primers for *PRNP* cDNA synthesis and oligonucleotides used for the mutagenesis were as follows:

For *PRNP* cDNA synthesis;

Forward PCR primer; 5′-TTCCGAATTCGCCACCATGGC-GAACCTTGGCTGCT-3′

Reverse PCR primer; 5′-ACATTGCGGCCGCTCCCAC-TATCAGGAAGATGAGG-3′

For site-directed mutagenesis:

PRNP P102L oligo DNA;

5′-pGTGGAACAAGCTGAGTAAGCCAA-3′

PRNP P105L oligo DNAs;

Forward;

5′-GGAACAAGCCGAGTAAGCTAAAAACCAACAT-GAAGCACATGGC-3′

Reverse;

5′- GCCATGTGCTTCATGTTGGTTTTTAGCTTACTC-GGCTTGTTCC-3′

Transfection and reporter assay

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). Next, 0.24 μg (40 nM) of siRNA duplexes or 0.1 μg of shRNA vectors together with 0.2 μg of pGL3-TK-backbone plasmid, 0.05 μg of phRL-TK-backbone plasmid and 0.1 μg of pSV-β-Galactosidase control vector (Promega) as a control were applied to each well using Lipofectamine 2000 transfection reagent (Invitrogen) as described previously [30]. Twenty-four hours after transfection, cell lysate was prepared and expression levels of luciferase and β-galactosidase were examined using the Dual-Luciferase reporter assay system (Promega) and Beta-Glo assay system (Promega), respectively, according to the manufacturer's instructions. The luminescent signals were measured using a TD-20/20 luminometer (Promega).

Western blotting

The pPRNPwt-V5 or pPRNPmut102(or mut105)-V5 plasmid (0.1 μg) was cotransfected with siRNA duplex (0.24 μg) or shRNA expression plasmid (0.1 μg) into HeLa cells. Forty-eight hours after transfection, cell lysate was prepared and examined by Western blotting. Equal amounts of cell lysate were separated by SDS-PAGE and electrophoretically blotted onto PVDF membranes (Millipore). Membranes were blocked for 1 h in blocking solution [5% non-fat milk in washing buffer (0.1% Tween-20 in PBS)] and incubated with anti-V5 antibody (Invitrogen) or anti-α-tubulin antibody DM1A (Sigma), followed by washing in PBS containing 0.1% Tween-20 and further incubation with horseradish peroxidase-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories). Antigen-antibody complexes were visualized using Immobilon Western reagent (Millipore). The experiments were duplicated at least twice independently.

Supporting Information

Figure S1 Wild-type and mutant PRNP mRNAs and designed sense-strand siRNAs. The wild-type and mutant PRNP mRNA sequences around the P102L (A), P105L (B), and D178N (C) variations, respectively, are shown and the nucleotide variations are indicated in red. Designed siRNAs (indicated) are represented by thin lines and only the variations are indicated in red.
Found at: doi:10.1371/journal.pone.0002248.s001 (0.26 MB TIF)

Figure S2 Expression of the wild-type (A) and P102L (B) PRNP polypeptides in the presence of indicated siRNAs was examined by Western blotting as in Figure 2. Expression of alpha-tubulin was also examined as the control.
Found at: doi:10.1371/journal.pone.0002248.s002 (0.99 MB TIF)

Figure S3 Assessment of mismatched siPrnp102(T10) and siPrnp178(A9) on ASP-RNAi. (A, D) Nucleotide sequences of wild-type and mutant PRNP mRNAs and designed siRNAs are indicated as in Figure 2. (B, E) Assessment of mismatched siRNAs was carried out and the results are shown as in Figure 2. (C, F) Western blot analysis of the wild-type PRNP polypeptide (PRNPwt-V5) in the presence of indicated siRNAs was carried out as in Figure 2. The PRNPwt-V5 gene was driven by the EF-1α promoter instead of the CMV promoter in this experiment. Expression of α-tubulin was also examined as the control.
Found at: doi:10.1371/journal.pone.0002248.s003 (0.61 MB TIF)

Table S1
Found at: doi:10.1371/journal.pone.0002248.s004 (0.04 MB DOC)

Table S2
Found at: doi:10.1371/journal.pone.0002248.s005 (0.05 MB DOC)

Table S3
Found at: doi:10.1371/journal.pone.0002248.s006 (0.03 MB DOC)

Table S4
Found at: doi:10.1371/journal.pone.0002248.s007 (0.03 MB DOC)

Table S5
Found at: doi:10.1371/journal.pone.0002248.s008 (0.04 MB DOC)

Table S6
Found at: doi:10.1371/journal.pone.0002248.s009 (0.03 MB DOC)

Table S7
Found at: doi:10.1371/journal.pone.0002248.s010 (0.11 MB DOC)

Author Contributions

Conceived and designed the experiments: HH YO. Performed the experiments: HH YO YT MY. Analyzed the data: HH YO YT. Contributed reagents/materials/analysis tools: KT HH. Wrote the paper: HH YO.

References

1. Dykxhoorn DM, Novina CD, Sharp PA (2003) Killing the messenger: short RNAs that silence gene expression. Nat Rev Mol Cell Biol 4: 457–467.
2. Meister G, Tuschl T (2004) Mechanisms of gene silencing by double-stranded RNA. Nature 431: 343–349.
3. Bernstein E, Caudy AA, Hammond SM, Hannon GJ (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 409: 363–366.
4. Matranga C, Tomari Y, Shin C, Bartel DP, Zamore PD (2005) Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. Cell 123: 607–620.
5. Paddison PJ, Silva JM, Conklin DS, Schlabach M, Li M, et al. (2004) A resource for large-scale RNA-interference-based screens in mammals. Nature 428: 427–431.
6. Caplen NJ (2004) Gene therapy progress and prospects. Downregulating gene expression: the impact of RNA interference. Gene Ther 11: 1241–1248.
7. Karagiannis TC, El-Osta A (2005) RNA interference and potential therapeutic applications of short interfering RNAs. Cancer Gene Ther 12: 787–795.
8. Bonini NM, La Spada AR (2005) Silencing polyglutamine degeneration with RNAi. Neuron 48: 715–718.
9. Victor M, Bei Y, Gay F, Calvo D, Mello C, et al. (2002) HAT activity is essential for CBP-1-dependent transcription and differentiation in Caenorhabditis elegans. EMBO Rep 3: 50–55.
10. Wood M, Yin H, McClorey G (2007) Modulating the expression of disease genes with RNA-based therapy. PLoS Genet 3: e109.
11. Miller VM, Xia H, Marrs GL, Gouvion CM, Lee G, et al. (2003) Allele-specific silencing of dominant disease genes. Proc Natl Acad Sci U S A 100: 7195–7200.
12. Miller VM, Gouvion CM, Davidson BL, Paulson HL (2004) Targeting Alzheimer's disease genes with RNA interference: an efficient strategy for silencing mutant alleles. Nucleic Acids Res 32: 661–668.
13. Rodriguez-Lebron E, Paulson HL (2006) Allele-specific RNA interference for neurological disease. Gene Ther 13: 576–581.
14. Maxwell MM, Pasinelli P, Kazantsev AG, Brown RH Jr (2004) RNA interference-mediated silencing of mutant superoxide dismutase rescues cyclosporin A-induced death in cultured neuroblastoma cells. Proc Natl Acad Sci U S A 101: 3178–3183.
15. Denovan-Wright EM, Davidson BL (2006) RNAi: a potential therapy for the dominantly inherited nucleotide repeat diseases. Gene Ther 13: 525–531.
16. Xia H, Mao Q, Eliason SL, Harper SQ, Martins IH, et al. (2004) RNAi suppresses polyglutamine-induced neurodegeneration in a model of spinocerebellar ataxia. Nat Med 10: 816–820.
17. Ohnishi Y, Tokunaga K, Kaneko K, Hohjoh H (2006) Assessment of allele-specific gene silencing by RNA interference with mutant and wild-type reporter alleles. Journal of RNAi and Gene Silencing 2: 154–160.
18. Mullan M, Crawford F, Axelman K, Houlden H, Lilius L, et al. (1992) A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of beta-amyloid. Nat Genet 1: 345–347.
19. Goate A, Chartier-Harlin MC, Mullan M, Brown J, Crawford F, et al. (1991) Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. Nature 349: 704–706.
20. Collinge J (1997) Human prion diseases and bovine spongiform encephalopathy (BSE). Hum Mol Genet 6: 1699–1705.
21. Jackson GS, Collinge J (2001) The molecular pathology of CJD: old and new variants. Mol Pathol 54: 393–399.
22. Prusiner SB (1998) Prions. Proc Natl Acad Sci U S A 95: 13363–13383.
23. Hsiao K, Baker HF, Crow TJ, Poulter M, Owen F, et al. (1989) Linkage of a prion protein missense variant to Gerstmann-Straussler syndrome. Nature 338: 342–345.
24. Yamada M, Itoh Y, Inaba A, Wada Y, Takashima M, et al. (1999) An inherited prion disease with a PrP P105L mutation: clinicopathologic and PrP heterogeneity. Neurology 53: 181–188.
25. Chen SG, Parchi P, Brown P, Capellari S, Zou W, et al. (1997) Allelic origin of the abnormal prion protein isoform in familial prion diseases. Nat Med 3: 1009–1015.
26. Du Q, Thonberg H, Wang J, Wahlestedt C, Liang Z (2005) A systematic analysis of the silencing effects of an active siRNA at all single-nucleotide mismatched target sites. Nucleic Acids Res 33: 1671–1677.
27. Raoul C, Abbas-Terki T, Bensadoun JC, Guillot S, Haase G, et al. (2005) Lentiviral-mediated silencing of SOD1 through RNA interference retards disease onset and progression in a mouse model of ALS. Nat Med 11: 423–428.
28. Singer O, Marr RA, Rockenstein E, Crews L, Coufal NG, et al. (2005) Targeting BACE1 with siRNAs ameliorates Alzheimer disease neuropathology in a transgenic model. Nat Neurosci 8: 1343–1349.
29. Hohjoh H (2004) Enhancement of RNAi activity by improved siRNA duplexes. FEBS Lett 557: 193–198.
30. Ohnishi Y, Tokunaga K, Hohjoh H (2005) Influence of assembly of siRNA elements into RNA-induced silencing complex by fork-siRNA duplex carrying nucleotide mismatches at the 3'- or 5'-end of the sense-stranded siRNA element. Biochem Biophys Res Commun 329: 516–521.
31. Yekta S, Shih IH, Bartel DP (2004) MicroRNA-directed cleavage of HOXB8 mRNA. Science 304: 594–596.
32. Schwarz DS, Ding H, Kennington L, Moore JT, Schelter J, et al. (2006) Designing siRNA that distinguish between genes that differ by a single nucleotide. PLoS Genet 2: e140.
33. Ui-Tei K, Naito Y, Takahashi F, Haraguchi T, Ohki-Hamazaki H, et al. (2004) Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference. Nucleic Acids Res 32: 936–948.
34. Schwarz DS, Hutvagner G, Du T, Xu Z, Aronin N, et al. (2003) Asymmetry in the assembly of the RNAi enzyme complex. Cell 115: 199–208.
35. Khvorova A, Reynolds A, Jayasena SD (2003) Functional siRNAs and miRNAs exhibit strand bias. Cell 115: 209–216.

Orphan nuclear receptor NR4A2 expressed in T cells from multiple sclerosis mediates production of inflammatory cytokines

Yoshimitsu Doi*, Shinji Oki*†, Tomoko Ozawa*, Hirohiko Hohjoh‡, Sachiko Miyake*, and Takashi Yamamura*†

Departments of *Immunology and ‡Molecular Genetics, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan

Communicated by Tadamitsu Kishimoto, Osaka University, Osaka, Japan, April 11, 2008 (received for review March 11, 2008)

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) mediated by Th17 and Th1 cells. DNA microarray analysis previously showed that NR4A2, an orphan nuclear receptor, is strongly up-regulated in the peripheral blood T cells of MS. Here, we report that NR4A2 plays a pivotal role for mediating cytokine production from pathogenic T cells. In experimental autoimmune encephalomyelitis (EAE), an animal model of MS, NR4A2, was selectively up-regulated in the T cells isolated from the CNS. Strikingly, a forced expression of NR4A2 augmented promoter activities of IL-17 and IFN-γ genes, leading to an excessive production of these cytokines. Conversely, treatment with siRNA for NR4A2, resulted in a significant reduction in the production of IL-17 and IFN-γ. Furthermore, treatment with NR4A2 siRNA reduced the ability of encephalitogenic T cells to transfer EAE in recipient mice. Thus, NR4A2 is an essential transcription factor for triggering the inflammatory cascade of MS/EAE and may serve as a therapeutic target.

IL-17 | interferon-γ | EAE | Th17 | siRNA

Multiple sclerosis (MS) is a chronic disease of the central nervous system (CNS), accompanying multiple foci of inflammatory lesions. MS is thought to have an autoimmune pathogenesis, involving autoimmune T cells reactive to myelin antigens (1). Development of the CNS inflammation is triggered by proinflammatory cytokines produced by the autoimmune T cells, which penetrate into the CNS parenchyma after being activated in the periphery (2, 3). Although the precise mechanism for the peripheral T cell activation remains obscure, studies indicated possible roles for cross-reactive peptides, cytokines, or superantigen (4).

Experimental autoimmune encephalomyelitis (EAE) is a prototype autoimmune disease model (5) that can be induced in laboratory animals by active immunization with myelin antigens (mAg) or by passive transfer of mAg-specific T cells. Because Th1 cell clones reactive to mAg are capable of inducing clinical and pathological manifestations of EAE in naive mice, it has long been believed that Th1 cells producing IFN-γ play a central role in the pathogenesis of EAE and MS. This postulate is also supported by the past experience that clinical application of IFN-γ treatment for MS turned out to worsen the disease (6). Furthermore, treatment with a peptide analogue of myelin basic protein (MBP) resulted in disease exacerbation along with an expansion of MBP-reactive Th1 cells (7). These results have been repeatedly mentioned to support the Th1-mediated pathogenesis of MS. However, this dogma has recently been challenged. Namely, despite an obvious reduction of Th1 cells, mice deficient for IFN-γ or IFN-γ receptor (8) or for IL-12 signaling were susceptible to EAE (9, 10). Subsequent studies have clarified that IL-23 rather than IL-12 is essential for EAE induction. Lately, the IL-23-dependent pathogenic T cells were identified as Th17 cells, a novel helper T cells producing IL-17 (11, 12). Currently, it is widely appreciated that Th17 cells are crucial in the development of autoimmune diseases either independently or collaboratively with Th1 cells (13).

DNA microarray analysis revealed an up-regulation of IL-17 in the brain lesions of MS (14). More recently, a pathological study has demonstrated that IL-17 secreting T cells are present in active lesions of MS (15). Gene expression profiling provided a number of potential candidate molecules that might be appropriate as a therapeutic target (14, 16). We recently characterized gene signature of peripheral blood T cells from Japanese MS patients and found that a nuclear orphan receptor NR4A2 is most significantly overexpressed in MS (17). NR4A2 mutations are reported to cause familial Parkinson's disease, reflecting its essential role in the development and survival of substantia nigra neurons (18). In contrast, much less attention has been paid onto its role in T cells. NR4A family members (NR4A1 and -3) were shown to mediate apoptotic processes of mature (19, 20) and immature T cells (21, 22). However, these studies do not give insights into an overexpressed NR4A2 in MS. Here, we report that NR4A2 is a transcription factor regulating the expression of key cytokines in the pathogenesis of MS, including IL-17. Furthermore, we revealed that silencing NR4A2 expression by specific siRNA effectively prevents the production of the cytokines, thereby inhibiting their pathogenic potentials to mediate EAE.

Results

Up-Regulation of NR4A2 in Peripheral Blood T Cells of MS. We analyzed gene expression profiles of peripheral blood T cells from MS and control subjects (17, 23). Comparison of the patients and healthy donors has revealed that 286 of 1,263 genes are differentially expressed between MS and controls. Among genes up-regulated in MS, NR4A2 was most significantly overexpressed in MS in statistical P values and an increase ratio (3.6-fold). To consolidate the overexpression of NR4A2 in MS, we performed quantitative RT-PCR for NR4A2 expression, using the same samples previously analyzed. Expression of NR4A2 in T cells from MS increased ~5-fold on average compared with healthy donors (Fig. 1; $P < 0.01$).

T Cell Expression of NR4A2 in EAE. NR4A2 is a transcription factor of steroid/thyroid receptor family implicated in various cellular responses such as steroidogenesis, neuronal development, atherogenesis, and cell cycle regulation (24). However, its role in

Author contributions: Y.D., S.O., H.H., S.M., and T.Y. designed research; Y.D., S.O., and T.O. performed research; Y.D., S.O., S.M., and T.Y. analyzed data; and S.O. and T.Y. wrote the paper.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

†To whom correspondence may be addressed. E-mail: soki@ncnp.go.jp or yamamura@ncnp.go.jp.

This article contains supporting information online at www.pnas.org/cgi/content/full/0803454105/DCSupplemental.

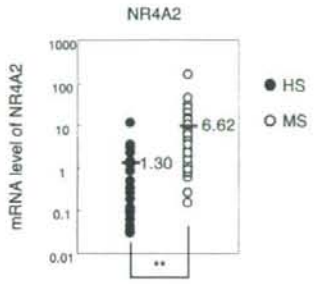

Fig. 1. Quantitative analysis of NR4A2 transcription between MS and controls. CD3+ T cells were isolated from PBMC of 57 MS patients and of 19 healthy donors, and total RNA was extracted. cDNA was synthesized and the expression levels of NR4A2 transcript were analyzed by quantitative RT-PCR. Each sample was normalized to GAPDH to adjust for variations. Open circles, MS patients; filled circles, healthy controls. Bars indicate mean values of each group. The statistical difference was determined by two-sided Student *t* test (**, $P < 0.01$).

T cell-mediated autoimmune diseases is unknown. Therefore, we explored the functional involvement of NR4A2 in EAE induced in C57BL/6 (B6) mice by immunization with MOG_{35-55}. $CD3^+$ T cells were isolated from SPL, dLN, and PBMC after EAE induction and the expression levels of NR4A2 gene were measured by quantitative RT-PCR (Fig. 2*a Right*). NR4A2 expression was detectable in PBMC-T cells on days 14, 21, and 28, showing a maximum value on day 21, which was well correlated with the clinical severity of EAE (Fig. 2*a Left*). NR4A2 expression in SPL-T cells and dLN-T cells was also correlated with the severity of EAE, but only marginally.

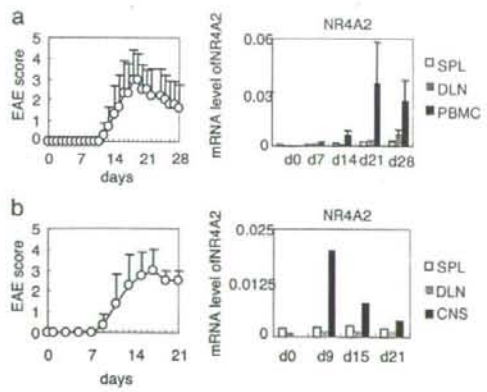

Fig. 2. Kinetic analysis of NR4A2 expression in the disease course of EAE. (*a*) (*Left*) EAE was induced in B6 mice by immunization with MOG_{35-55} in CFA. Mice were killed on days 7, 14, 21 and 28 after immunization, and T cells were isolated from dLN, SPL, or PBMC, using anti-CD3 magnetic beads. (*Right*) Total RNAs were isolated from the T cell populations, and the expression levels of NR4A2 were determined by quantitative RT-PCR. One representative data from three independent experiments is shown, and data are expressed as mean ± SEM ($n = 5$ for each). (*b*) EAE induced in B6 mice with MOG_{35-55}. Clinical scores were expressed as mean ± SEM ($n = 4$). Here, we determined NR4A2 expression in $CD3^+$ T cells isolated by using EPICS ALTRA cell sorter. The lymphoid cells (SPL, dLN, and CNS) were pooled from four mice on days 0, 9, 15, and 21 and used for cell sorting and RT-PCR analysis. The purity of the CNS-derived $CD3^+$ T cells was >93%.

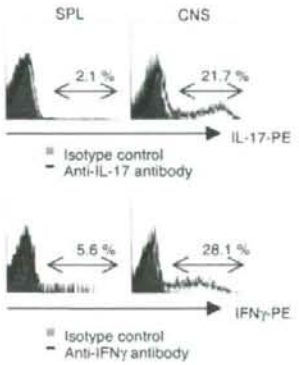

Fig. 3. Accumulation of IL-17 or IFN-γ-producing inflammatory T cells in the CNS. Mononuclear cells were isolated from spleen or CNS on day 17 after immunization and stimulated with PMA (20 ng/ml) and ionomycin (1 μg/ml) in the presence of 2 mM monensin for 4 h. Production of IL-17 and IFN-γ was analyzed for the gated $CD4^+$ T cell population by intracellular cytokine staining. Black line represents samples stained with either anti-IL-17 or anti-IFN-γ Ab, and the filled histogram represents samples stained with isotype control. Given values show the percentage of cytokine producing-T cells present in each panel.

In the course of EAE, mAg-primed T cells would accumulate into the CNS and produce inflammatory cytokines, leading to the formation of inflammatory lesions (25). We next examined a kinetic change of NR4A2 in the T cells infiltrating into the CNS. As assessed by quantitative RT-PCR, remarkable expression of NR4A2 was observed in the CNS-T cells on day 9, when an early EAE sign became evident (Fig. 2*b*). The expression level decreased gradually thereafter, but was still significant until day 21. These results suggest that the CNS-T cells also express NR4A2, but the expression kinetics significantly differed from that of PBMC-T cells.

Accumulation of IL-17- and IFN-γ-Producing T Cells in the CNS of EAE. Th1 cells specific for mAg have long been thought to induce EAE through their production of IFN-γ. However, recent studies indicate that Th17 rather than Th1 cells may play a central role (13). To make this point clear in our experimental setting, we examined the ability of the CNS-T cells to produce IFN-γ and IL-17. Mononuclear cells were recovered from the CNS and SPL on day 17, and stimulated with PMA and ionomycin (P/I). After immunostaining, expression of IL-17 or IFN-γ in the $CD4^+$ T cells was analyzed by flow cytometry. Major proportions of the CNS-T cells were found to produce IL-17 (21.7% of the cells) or IFN-γ (28.1%) after stimulation (Fig. 3). In contrast, spleen cells contained a lower number of cells producing these cytokines.

Transcriptional Up-Regulation of IL-17 and IFN-γ After Introduction of NR4A2. The concomitant expression of inflammatory cytokines and NR4A2 has guided us to investigate whether NR4A2 directly affects cytokine gene expression as a transcription factor, using luciferase reporter plasmids containing the promoter fragment of IL-17, IFN-γ, or IL-2. NR4A2 gene transduction would result in a twofold augmentation of IL-17 promoter activity and, for IFN-γ, an even higher (5-fold) induction (Fig. 4*a*). A significant induction of IL-2 promoter activity was also noted. Intriguingly, an introduction of NR4A2 plasmid

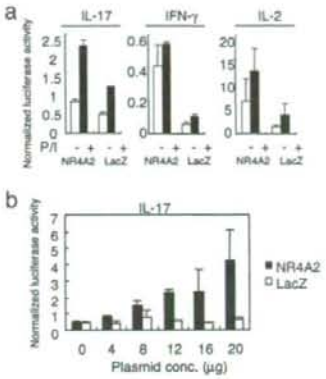

Fig. 4. Promoter activities of cytokine genes in the presence of NR4A2. (*a*) The effect of NR4A2 expression on IL-17, IFN-γ, and IL-2 promoter activity. A reporter plasmid containing promoter of cytokine gene (10 μg) and *Renilla* luciferase plasmid (100 ng) were introduced into EL4 cells by electroporation, together with pcDNA4-NR4A2 or pcDNA4-LacZ (10 μg). Cells were stimulated for 18 h with P/I. Luciferase activity was determined for each cell lysate after normalization to the *Renilla* luciferase activity. One representative data from three independent experiments is shown. Data are expressed as mean ± SD. (*b*) The effect of NR4A2 expression on basal promoter activity of IL-17 gene. EL4 cells transfected with pcDNA4-NR4A2 or pcDNA4-LacZ together with IL-17 reporter plasmid and *Renilla* luciferase plasmid as desribed in a were cultured for 18 h without stimulation. One representative data from three independent experiments is shown. Data are expressed as mean ± SD.

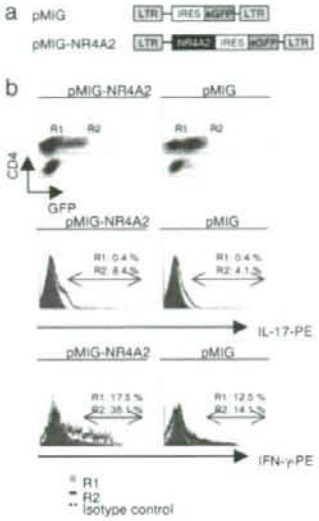

Fig. 5. The effect of retrovirally transduced NR4A2 on cytokine production by primary murine CD4+ T cells. (*a*) DNA fragments encoding wild-type NR4A2 were cloned into the pMIG(W) bicistronic retroviral vector. LTR, long terminal repeat; IRES, internal ribosome entry site; eGFP, enhanced green fluorescence protein b. (*b*) Splenic CD4+ T cells were infected with retrovirus encoding NR4A2 or control retrovirus, and CD4+ GFP− T cells and CD4+ GFP+ T cells were gated as R1 and R2, respectively. Forced expression of NR4A2 increased the number of CD4+ T cells producing IL-17 or IFN-γ. The histogram shows intracellular cytokine staining on the gated cells (R1 or R2). Black line represents cells in R2 gate (GFP+) stained with either anti-IL-17 or anti-IFN-γ Ab, and the filled histogram represents cells in R1 gate (GFP−) stained with isotype control. Given values show the percentage of cytokine producing-T cells present.

without P/I stimulation also augmented basal promoter activity of IL-17 genes in a dose dependent manner (Fig. 4*b*). Similarly, basal promoter activity of IFN-γ was promoted (data not shown).

Retroviral Transduction of NR4A2 Gene Enhances Expression of Inflammatory Cytokine in Primary T Cells. The results obtained in EL4 lymphoma cells need to be verified in more physiological settings. Next, we examined whether forced expression of NR4A2 may affect the expression of cytokines in primary rodent T cells. Bicistronic retroviral vector containing NR4A2 gene fragment (pMIG-NR4A2) or empty vector (pMIG) were used for production of retroviruses (Fig. 5*a*). We infected the B6 T cells with either of the retroviruses as described in ref. 26 and compared the cytokine production between GFP-positive (infected) and GFP-negative (uninfected) CD4+ T cells by intracellular cytokine staining (Fig. 5*b Top*). CD4+ T cells infected with pMIG-NR4A2-introduced retrovirus showed a twofold enhancement of IL-17 expression (8.4%) compared with those infected with control retrovirus (4.1%) after stimulation with P/I. In contrast, IL-17 production by uninfected T cells in either panel was almost equivalent (Fig. 5*b Middle*). Furthermore, one-third of the CD4+ T cells infected with pMIG-NR4A2-introduced retrovirus showed a massive IFN-γ expression (35.1%) compared with control retrovirus (14.1%) (Fig. 5*b Bottom*).

Silencing of NR4A2 Gene Expression Results in a Reduced Production of IL-17 and IFN-γ. Reporter gene analysis and retroviral transduction experiments demonstrated that T cell production of IL-17 and IFN-γ is controlled by NR4A2 (Figs. 4 and 5). We further explored whether silencing of NR4A2 gene may affect the production of inflammatory cytokines by CD4+ T cells. An NR4A2-specific siRNA was selected from three siRNAs based on the inhibitory efficacy. The targeting sequence of the NR4A2 siRNA is completely conserved between mice and human. Therefore, we could apply it to human T cells and study whether NR4A2 could be a therapeutic target in human MS. In a preparatory experiment, using FITC-labeled siRNA, the transfection efficiency was found to be 95%. We purified CD4+ T cells from human PBMC and transfected them with the NR4A2 siRNA or control RNA, using nucleofector II. The cells were stimulated with immobilized anti-CD3 Ab. As shown in Fig. 6*a*, silencing NR4A2 gene expression resulted in a 50% reduction of IL-17 and IFN-γ production. However, production of TNF-α, IL-4, or IL-5 was not changed significantly after siRNA treatment (Fig. 6*b*). Intriguingly, the siRNA treatment also induced a modest reduction of IL-10 production. The molecular mechanism of this inhibition is not clarified yet. Because silencing of NR4A2 expression rather selectively inhibited the expression of inflammatory cytokines, it is arguable that NR4A2 may be a good target for therapeutic intervention of MS. In this line, we next examined whether the NR4A2 siRNA is effective for inhibiting a production of inflammatory cytokines in MS. For this aim, CD4+ T cells were isolated from pairs of an MS patient and an age- and sex-matched healthy donor and were stimulated with anti-CD3 Ab after being transfected with the NR4A2 siRNA or control RNA. We found that the siRNA treatment significantly reduced the production of IL-17 and IFN-γ by T cells from MS or healthy donors [supporting information (SI) Fig. S1]. Again we observed some reduction of IL-10 after siRNA treatment. However, the siRNA showed little effect on

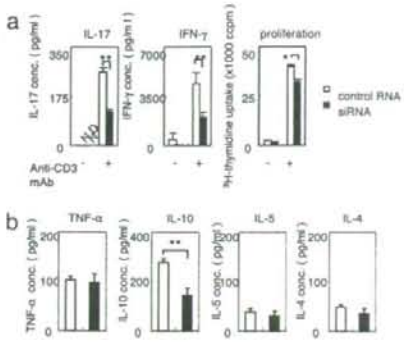

Fig. 6. The effect of NR4A2 gene silencing on T cell cytokine production. (*a*) Specific inhibition of T cell production of IL-17 and IFN-γ by siRNA treatment. Human CD4⁺ T cells derived from PBMC were transfected with siRNA or control RNA and stimulated by immobilized anti-CD3 Ab for 48 h. Cytokine levels in the culture supernatant were determined by ELISA or a CBA human Th1/2 cytokine kit. Proliferation rate was measured by ^{3}H-TdR uptake. (*b*) Effect of siRNA treatment for T cell production of TNF-α, IL-10, IL-5, and IL-4 after stimulation with immobilized anti-CD3 Ab. The data are expressed as mean ± SD (•, $P < 0.05$; ••, $P < 0.01$; Mann–Whitney *U* test).

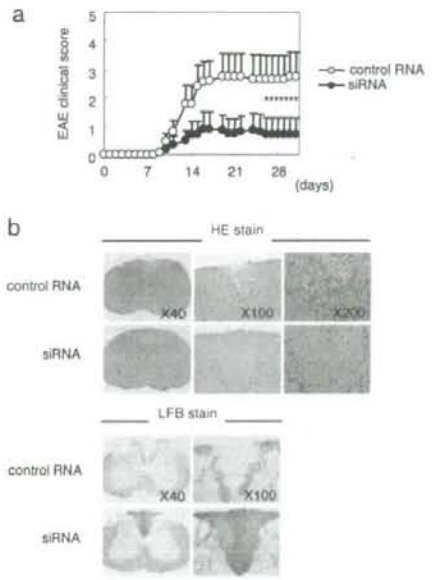

Fig. 7. The effect of T cell silencing of NR4A2 expression on passive EAE. (*a*) Inguinal and popliteal LNs cells were collected from female SJL/J mice 10 days after immunization with $PLP_{139-151}$, and were transfected with siRNA for NR4A2 or control RNA, using HVJ-E vector kit. The cells were cultured in complete media for 8 h. Then the media were replaced with fresh complete media containing 35 μg/ml $PLP_{139-151}$, and the cells were stimulated for another 3 days. After expansion, cells were harvested and transferred i.p. (5×10^6 cells per mouse) into 3Gy-irradiated naïve SJL/J mice ($n = 10$) followed by i.p. injection of PT. Mean ± SEM clinical scores were indicated. (•, $P < 0.05$ by Mann–Whitney *U* test.) (*b*) Histological analysis of spinal cords removed on day 31 after adoptive transfer of $PLP_{139-151}$-reactive T cells. Sections obtained from cervical cord regions were stained with HE or LFB. Infiltration of mononuclear cells and demyelination of the cervical cord regions were analyzed for mice injected with $PLP_{139-151}$-reactive T cells pretreated with control RNA or siRNA for NR4A2.

production of TNF-α, IL-5, and IL-4 from T cells used for assays (Table S1).

Amelioration of EAE by Silencing of NR4A2. Finally, we investigated the therapeutic implication of the siRNA experiments in a model of passively induced EAE, induced by adoptive transfer of mAg-activated LN cells. We prepared lymphoid cells from dLN of SJL/J mice 10 days after immunization with $PLP_{139-151}$. The dLN cells were transfected with the NR4A2 siRNA or control RNA and stimulated with $PLP_{139-151}$ *in vitro*. Three days later, the cultured cells enriched in lymphoblasts were transferred to irradiated naïve SJL/J mice. In addition to evaluating clinical manifestations, histology was assessed by hematoxylin-eosin (HE) and luxol fast blue (LFB) staining of paraffin-embedded spinal cord sections. Notably, severity of clinical (Fig. 7*a*) and histological EAE on day 31 (Fig. 7*b*) was significantly prevented in siRNA-treated group compared with control RNA-treated group (Fig. 7*b*). These results suggest that modulation of NR4A2 expression by specific siRNAs or other chemical compounds might be a promising treatment for active MS that are harboring potent encephalitogenic T cells.

Discussion

Although mAg-specific T cell clones isolated from the peripheral blood has been widely used to gain insights into the pathogenesis of MS (27), analysis of polyclonal T cells has been undervalued for a long time. However, it was recently demonstrated that peripheral T cells from MS and healthy subjects significantly differ in surface phenotype or gene expression profiling (17, 23, 28). Using cDNA microarray, we have identified NR4A2 as a gene most significantly up-regulated in the peripheral T cells of MS (17). We conducted the present study to clarify the implication of this interesting observation. Inspired by the recent discovery that retinoid-related orphan receptor γt (RORγt) is essential for Th17 cell differentiation (29) and that retinoic acids play a regulatory role in Th17 cell differentiation (30), we have focused our efforts to explore the possible role of NR4A2 in cytokine regulation. Reporter gene analysis and retroviral transduction of NR4A2 clearly demonstrated that T cell production of inflammatory cytokines, including IL-17 and IFN-γ, is regulated by NR4A2, whereas silencing of NR4A2 by a specific siRNA prevents expression of these cytokines. Furthermore, treatment with the siRNA reduced the ability of pathogenic T cells to adoptively transfer EAE. These results have identified a previously uncharacterized role for NR4A2 in the regulation of T cell production of inflammatory cytokines.

NR4A2 is a member of the orphan nuclear NR4A subfamily that consists of NR4A1 (also referred to as Nur77), NR4A2 (Nurr1), and NR4A3 (NOR-1) (24). The NR4A members share a highly conserved zing finger DNA binding domain and a less conserved putative ligand-binding domain. All these members bind to the DNA sequence NBRE (AAAGGTCA) or NurRE to activate target gene expression. NR4A1 and NR4A2 can also heterodimerize with retinoic X receptor (RXR) and activate gene expression through DR5 (24). They exert pleiotropic functions and are classified as immediate early genes induced by physiological and physical stimuli. Studies of gene-targeted mice have shown that NR4A1 and NR4A3 play a critical role in T cell apoptosis during the thymocyte development (20–22, 31). In contrast, developing thymocytes in NR4A2 deficient mice ap-

pear to be normal (21, 32), which distinguishes NR4A2 from other NR4A members.

Involvement of orphan nuclear receptor in T cell differentiation has recently attracted broad attention, because RORγt, a splice variant of RORγ, was found to play an essential role in the development of Th17 cells (29). RORγ/RORγt were reported to play an essential function in survival of $CD4^+CD8^+$ thymocytes (33, 34) and in the generation of fetal lymphoid tissue inducer (LTi) cells (35). It is particularly intriguing that the consensus binding sequence for RORγ [$(A/T)_5AGGTCA$] overlaps with that for NR4A (NBRE; AAAGGTCA), which has encouraged us to explore the functional role of NR4A2 in the production of IL-17 and IFN-γ. Although the molecular mechanism of cytokine production through the induced expression of NR4A2 is not clear yet, NR4A2 and RORγt may have an overlapping role in regulating the development and effector functions of Th17 cells.

NR4A2 expression in the CNS-infiltrating T cells showed a peak value at a very early phase of EAE (day 9–12) (Fig. 2*b*). We speculate that this probably coincides with the entry of encephalitogenic cells into the CNS (2, 3). Consistently, a similar kinetic change was found in expression of T-bet and RORγt in the CNS-T cells (data not shown). In contrast, up-regulation of NR4A2 in peripheral blood T cells was significantly delayed. This is likely to result from a late activation of peripheral T cells after peripheral recruitment of antigen presenting cells engulfing myelin and/or peripheral dispersion of myelin protein or its fragments.

By applying a specific siRNA, we showed that blocking NR4A2 expression is effective for inhibiting production of IL-17 and IFN-γ from T cells from healthy donors and MS patients. Therapeutic implication was further demonstrated by using an adoptive transfer EAE model. Because Th17 cells were identified as a major player in autoimmunity (12, 15), it is sometimes argued that Th17 cells would be a sole potent inducer of autoimmune inflammation. However, T-bet-deficient mice and Stat4-deficient mice that obviously lack Th1 cells would resist against induction of EAE, although they maintain a large number of Th17 cells (36, 37). This suggests that both Th1 and Th17 cells are required for induction of full-blown EAE (38). In this context, the ability of the NR4A2 siRNA to inhibit production of both IL-17 and IFN-γ suggests the advantage of NR4A2 targeting therapy in controlling autoimmune inflammation.

Materials and Methods

EAE Induction. Active EAE was induced with myelin oligodendrocyte glycoprotein (MOG) amino acids 35–55 (MOG_{35-55}; MEVGWYRSPFSRVVHLYRNGK) as described in ref. 39. Female B6 mice were immunized s.c. with 100 μg of MOG_{35-55} mixed with 1 mg of heat-killed *Mycobacterium tuberculosis* H37RA emulsified in Freund's adjuvant (CFA). Pertussis toxin (PT) (200 ng) was injected i.p. on days 0 and 2 after immunization. Clinical signs were scored daily as follows: 0, no clinical signs; 1, loss of tail tonicity; 2, flaccid tail; 3, partial hind limb paralysis; 4, total hind limb paralysis; and 5, fore and hind limb paralysis.

Quantitative RT-PCR. DNase-treated total RNAs were processed for cDNA synthesis, using random hexamer primers and SuperScript II reverse transcriptase (Invitrogen). cDNAs were amplified by PCR on Light Cycler ST300 (Roche Diagnostics) by using a Light Cycler-FastStart DNA Master SYBR Green I kit (Roche). Values for each gene were normalized to those of a housekeeping gene GAPDH to adjust for variations between different samples. Forward primer for amplifying human NR4A2 gene was 5′-CGACATTTCTGCCTTCTCC-3′ and reverse primer 5′-GGTAAAGTGTCCAGGAAAAG-3′. Mouse NR4A2 forward primer was designed as 5′-GCATACAGGTCCAACCCAGT-3′ and reverse primer 5′-AATGCAGGAGAAGGCAGAAA-3′. To evaluate silencing efficacy of *NR4A2*-specific siRNAs, expression of NR4A2 gene was quantified by RT-PCR, using the primers to flank the siRNA target sequence (forward, 5′-TGCCACCACTTCTCTCCCCA-3′; reverse, 5′-GCGGCATCATCTCCTCAGAC-3′).

Luciferase Assays. Ten million of EL4 thymoma cells suspended in 500 μl of cold PBS and transfected with 4–20 μg of pcDNA4-NR4A2 or pcDNA4-LacZ in the presence of 10 μg of reporter plasmid, 100 ng of Renilla luciferase plasmid, and 5 μg of DEAE-DEXTRAN (Sigma) by electroporation (250 V, 975 μF, time constant = 30–34 ms) with a GenePulser electroporator II (Bio-Rad). Six hours later, cells were stimulated with 20 ng/ml PMA and 1 μg/ml ionomycin for 24 h, followed by analysis for luciferase activity. The data were normalized for internal controls of Renilla luciferase activity.

Retroviral Infection. Mouse $CD4^+$ T cells purified by AutoMACS using mouse CD4 T isolation kit (Miltenyi Biotec) were stimulated with immobilized anti-CD3 Ab and soluble anti-CD28 Ab in complete medium supplemented with IL-2 (100 units/ml) for 24–48 h before infection. The primed $CD4^+$ T cells were infected twice with retroviruses produced by 293T cells cotransfected with pMIG retroviral vector and pCL-Eco packaging vector. The T cells were cultured in the presence of 30 units/ml of IL-2 for 3 days and were then subjected to intracellular cytokine staining.

Silencing Effects of NR4A2 siRNA on Passive EAE. To evaluate an effect of NR4A2 siRNA, an adoptive transfer EAE model in SJL/J mice was applied, because consistent disease could be induced relatively easily. Female SJL/J mice (8–12 weeks old) (Charles River Laboratories) were immunized s.c. with 100 μg of proteolipid protein (PLP) amino acids 139–151 ($PLP_{139-151}$; HSLGKWLGHPDKF) and 1 mg of heat-killed *M. tuberculosis* H37RA in CFA. Inguinal and popliteal LNs harvested 10 days after immunization were transfected with siRNAs, using hemaggultinating Virus of Japan envelope (HVJ-E) vector kit (GENOMEONE; Ishihara Sangyo). Eight hours later, the cells were stimulated with $PLP_{139-151}$ peptide (35 μg/ml). After 3 days, collected cells were injected i.p. (5×10^6 cells per body) into irradiated mice (3 Gy/body) with intrapelitoneal injection of PT. For conventional histological analysis of EAE, paraffin-embedded spinal cords were stained with either HE or LFB.

Statistics. For statistical analysis, a nonparametric Mann–Whitney *U* test or Student *t* test was used. $P < 0.05$ was considered statistically significant.

Supporting Information. For further details, see *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Mayumi Fujita for EAE induction, Miho Mizuno, Chiharu Tomi, and Yuki Kikai for excellent technical assistance. This work was supported by grants from the Ministry of Health, Labour and Welfare of Japan.

1. Sospedra M, Martin R (2005) Immunology of multiple sclerosis. *Annu Rev Immunol* 23:683–747.
2. Hickey WF, Hsu BL, Kimura H (1991) T-lymphocyte entry into the central nervous system. *J Neurosci Res* 28:254–260.
3. Kawakami N, *et al.* (2005) Live imaging of effector cell trafficking and autoantigen recognition within the unfolding autoimmune encephalomyelitis lesion. *J Exp Med* 201:1805–1814.
4. Hur EM, *et al.* (2007) Osteopontin-induced relapse and progression of autoimmune brain disease through enhanced survival of activated T cells. *Nat Immunol* 8:74–83.
5. Gold R, Linington C, Lassmann H (2006) Understanding pathogenesis and therapy of multiple sclerosis via animal models: 70 years of merits and culprits in experimental autoimmune encephalomyelitis research. *Brain* 129:1953–1971.
6. Panitch HS, Hirsch RL, Schindler J, Johnson KP (1987) Treatment of multiple sclerosis with gamma interferon: Exacerbations associated with activation of the immune system. *Neurology* 37:1097–1102.
7. Bielekova B, *et al.* (2000) Encephalitogenic potential of the myelin basic protein peptide (amino acids 83–99) in multiple sclerosis: Results of a phase II clinical trial with an altered peptide ligand. *Nat Med* 6:1167–1175.
8. Willenborg DO, Fordham S, Bernard CC, Cowden WB, Ramshaw IA (1996) IFN-gamma plays a critical down-regulatory role in the induction and effector phase of myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis. *J Immunol* 157:3223–3227.
9. Becher B, Durell BG, Noelle RJ (2002) Experimental autoimmune encephalitis and inflammation in the absence of interleukin-12. *J Clin Invest* 110:493–497.
10. Zhang GX, *et al.* (2003) Induction of experimental autoimmune encephalomyelitis in IL-12 receptor-beta 2-deficient mice: IL-12 responsiveness is not required in the pathogenesis of inflammatory demyelination in the central nervous system. *J Immunol* 170:2153–2160.
11. Cua DJ, *et al.* (2003) Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* 421:744–748.
12. Langrish CL, *et al.* (2005) IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med* 201:233–240.
13. Bettelli E, Korn T, Kuchroo VK (2007) Th17: The third member of the effector T cell trilogy. *Curr Opin Immunol* 19:652–657.
14. Lock C, *et al.* (2002) Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. *Nat Med* 8:500–508.

15. Tzartos JS, *et al.* (2008) Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis. *Am J Pathol* 172:146–155.
16. Lock CB, Heller RA (2003) Gene microarray analysis of multiple sclerosis lesions. *Trends Mol Med* 9:535–541.
17. Satoh J, *et al.* (2005) Microarray analysis identifies an aberrant expression of apoptosis and DNA damage-regulatory genes in multiple sclerosis. *Neurobiol Dis* 18:537–550.
18. Le WD, *et al.* (2003) Mutations in NR4A2 associated with familial Parkinson disease. *Nat Genet* 33:85–89.
19. Liu ZG, Smith SW, McLaughlin KA, Schwartz LM, Osborne BA (1994) Apoptotic signals delivered through the T-cell receptor of a T-cell hybrid require the immediate-early gene nur77. *Nature* 367:281–284.
20. Woronicz JD, Calnan B, Ngo V, Winoto A (1994) Requirement for the orphan steroid receptor Nur77 in apoptosis of T-cell hybridomas. *Nature* 367:277–281.
21. Cheng LE, Chan FK, Cado D, Winoto A (1997) Functional redundancy of the Nur77 and Nor-1 orphan steroid receptors in T-cell apoptosis. *EMBO J* 16:1865–1875.
22. Calnan BJ, Szychowski S, Chan FK, Cado D, Winoto A (1995) A role for the orphan steroid receptor Nur77 in apoptosis accompanying antigen-induced negative selection. *Immunity* 3:273–282.
23. Satoh J, *et al.* (2006) T cell gene expression profiling identifies distinct subgroups of Japanese multiple sclerosis patients. *J Neuroimmunol* 174:108–118.
24. Maxwell MA, Muscat GE (2006) The NR4A subgroup: Immediate early response genes with pleiotropic physiological roles. *Nucl Recept Signal* 4:e002.
25. Hofstetter HH, Toyka KV, Tary-Lehmann M, Lehmann PV (2007) Kinetics and organ distribution of IL-17-producing CD4 cells in proteolipid protein 139–151 peptide-induced experimental autoimmune encephalomyelitis of SJL mice. *J Immunol* 178:1372–1378.
26. Oki S, Chiba A, Yamamura T, Miyake S (2004) The clinical implication and molecular mechanism of preferential IL-4 production by modified glycolipid-stimulated NKT cells. *J Clin Invest* 113:1631–1640.
27. Meinl E, *et al.* (1993) Myelin basic protein-specific T lymphocyte repertoire in multiple sclerosis. Complexity of the response and dominance of nested epitopes due to recruitment of multiple T cell clones. *J Clin Invest* 92:2633–2643.
28. Inoges S, *et al.* (1999) Cytokine flow cytometry differentiates the clinical status of multiple sclerosis (MS) patients. *Clin Exp Immunol* 115:521–525.
29. Ivanov II, *et al.* (2006) The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 126:1121–1133.
30. Mucida D, *et al.* (2007) Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science* 317:256–260.
31. Zhou T, *et al.* (1996) Inhibition of Nur77/Nurr1 leads to inefficient clonal deletion of self-reactive T cells. *J Exp Med* 183:1879–1892.
32. Zetterstrom RH, *et al.* (1997) Dopamine neuron agenesis in Nurr1-deficient mice. *Science* 276:248–250.
33. He YW, Deftos ML, Ojala EW, Bevan MJ (1998) RORγ t, a novel isoform of an orphan receptor, negatively regulates Fas ligand expression and IL-2 production in T cells. *Immunity* 9:797–806.
34. Kurebayashi S, *et al.* (2000) Retinoid-related orphan receptor gamma (RORgamma) is essential for lymphoid organogenesis and controls apoptosis during thymopoiesis. *Proc Natl Acad Sci USA* 97:10132–10137.
35. Eberl G, *et al.* (2004) An essential function for the nuclear receptor RORγ(t) in the generation of fetal lymphoid tissue inducer cells. *Nat Immunol* 5:64–73.
36. Bettelli E, *et al.* (2004) Loss of T-bet, but not STAT1, prevents the development of experimental autoimmune encephalomyelitis. *J Exp Med* 200:79–87.
37. Chitnis T, *et al.* (2001) Effect of targeted disruption of STAT4 and STAT6 on the induction of experimental autoimmune encephalomyelitis. *J Clin Invest* 108:739–747.
38. Bettelli E, Oukka M, Kuchroo VK (2007) T(H)-17 cells in the circle of immunity and autoimmunity. *Nat Immunol* 8:345–350.
39. Croxford JL, Miyake S, Huang YY, Shimamura M, Yamamura T (2006) Invariant V(alpha)19i T cells regulate autoimmune inflammation. *Nat Immunol* 7:987–994.

Mol Biol Rep
DOI 10.1007/s11033-008-9330-4

Variation of gene silencing involving endogenous microRNA in mammalian cells

Yoshiko Tamura · Mariko Yoshida · Yusuke Ohnishi · Hirohiko Hohjoh

Received: 22 May 2008 / Accepted: 30 July 2008

Abstract MicroRNAs (miRNAs) are small noncoding RNA and play a role in gene expression regulation by inhibiting translation of their target messenger RNAs (mRNAs). In this study, we investigated the effects of endogenous *let-7* miRNA on the expression of target genes in various mammalian cells by means of two types of reporter plasmids possessing target sequences for *let-7*: one carries perfectly matched target sequence for *let-7* in the 3′-untranslated region of the *luciferase* reporter gene to monitor RNA interference (RNAi) activity and the other has three bulged binding sites for *let-7* to monitor translation-inhibition activity. The results indicate that different cells have different levels of gene silencing against the target reporter genes. The data presented here suggest that not only microRNA level but also target transcript level likely participate in the generation of a variety of gene silencing.

Keywords MicroRNA · *Let-7* · Gene silencing · Variation · Target gene expression

Y. Tamura · M. Yoshida · Y. Ohnishi · H. Hohjoh (✉)
National Institute of Neuroscience, NCNP, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan
e-mail: hohjohh@ncnp.go.jp

Y. Ohnishi
Department of Human Genetics, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

Introduction

MicroRNAs (miRNAs) are small noncoding RNA, with a typical length of 19–23 nt, which are processed from longer transcripts by digestion with a microprocessor complex containing Drosha and Pasha in the nucleus and Dicer in the cytoplasm [1–3]. Hundreds of miRNA genes have been found in various species [3–5], and their tissue- or organ-specific expression has been detected [5–7]. After Dicer processing, miRNA duplexes undergo strand selection and then the single-stranded mature miRNA elements are incorporated into the RNA-induced silencing complex (RISC) and function as mediators in suppression of gene expression [8–10]. There are two types of gene silencing involving endogenous miRNAs: one is the inhibition of translation of target mRNAs carrying partially complementary fragments to miRNAs in their 3′ untranslated regions (3′UTRs) [11–15], and the other is the digestion of target RNAs which are perfectly or nearly complementary to miRNAs, such as RNA interference (RNAi) [8, 11, 16]. The gene silencing involving miRNAs play an important role in regulation of gene expression in development, differentiation and proliferation [4, 7, 17–22]. Recent studies have further suggested significant association of miRNAs with various cancers [23–26].

While many findings and data on miRNAs themselves are accumulating, little is known about their target gene expression. It is of interest and importance to realize the entity of regulation of gene expression involving gene silencing mediated by endogenous miRNAs. In this report, we investigated the effects of endogenous *let-7* miRNA on the expression of target genes in various mammalian cells by using reporter plasmids carrying target sequences for *let-7*. The data indicated that different cells had different levels of gene silencing involving endogenous *let-7*.