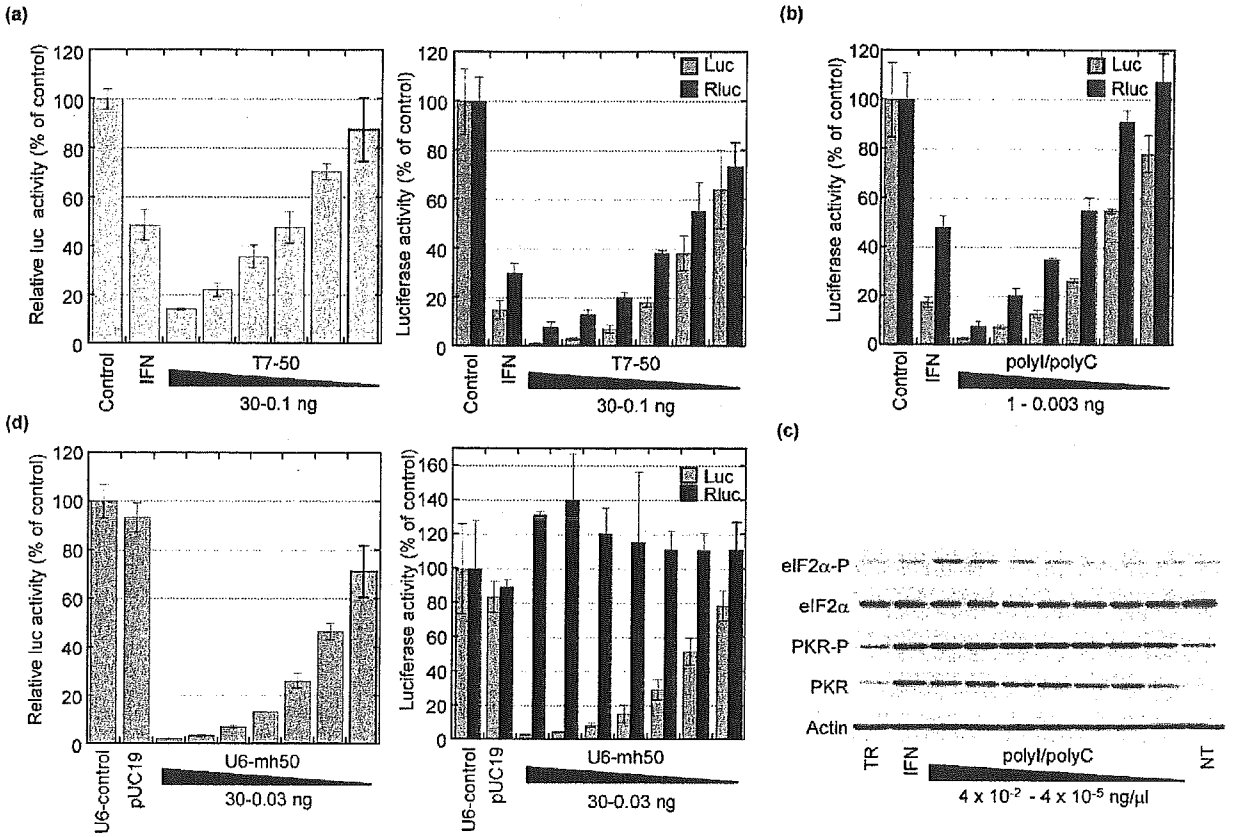

Fig. 1 The nonspecific effects in HeLa S3 cells transfected with dsRNAs of longer than 30 bp. (a) HeLa S3 cells in each well of a 48-well plate were transfected with 25 ng of firefly luciferase expression plasmid, 2.5 ng of *Renilla* luciferase expression plasmid, and 0.1, 0.3, 1, 3, 10 or 30 ng of an *in vitro* transcribed 50 bp dsRNA (4.0×10^{-4}–1.2×10^{-1} μg ml^{-1}), as indicated. The left panel shows the activity of firefly luciferase (luc), normalized by reference to that of *Renilla* luciferase (Rluc). The right panel shows the actual activities of the firefly and *Renilla* enzyme (as percentages of the control). Control: cells were transfected with luciferase expression plasmids; IFN: luciferase expression plasmids plus 1000 U per ml interferon α. Each experiment was performed in triplicate and results are shown as the mean ± SD. (b) Nonspecific effects of polyI/polyC on the luciferase activities in HeLa S3 cells. HeLa S3 cells were treated as described in (a), with the exception that 0.003, 0.01, 0.03, 0.1, 0.3 or 1 ng of polyI/polyC (1.2×10^{-5}–4.0×10^{-3} μg ml^{-1}) was used instead of the *in vitro* transcribed 50 bp dsRNA. Each experiment was performed in triplicate and results are shown as the mean ± SD. (c) Induction of PKR pathway genes in HeLa S3 cells. HeLa S3 cells were transfected with the indicated amounts of polyI/polyC and levels of each protein were analyzed 48 h after transfection by Western blotting. About 20% of HeLa S3 cells underwent apoptosis at a concentration of 0.5 ng of polyI/polyC per well of a 12-well plate, a level that corresponds to the concentration of 4×10^{-4} μg ml^{-1}. With increases in the concentration of polyI/polyC, more than 99% of cells succumbed to apoptotic cell death (data not shown). Since the level of eIF2α relative to the level of the actin control at each dose of polyI/polyC was constant during the experiments, eIF2α was used as a control for loading of equal amounts of cellular proteins in subsequent experiments. TR: Cells were treated with Lipofectamine™ 2000 only; IFN: Lipofectamine™ 2000 plus 1000 U per ml interferon α; and NT: no treatment. -P denotes the phosphorylated form. (d) HeLa S3 cells were treated as described in (a), with the exception that a U6 promoter-driven 50-bp modified hairpin-RNA expression vector (mhRNA) was used instead of the *in vitro* transcribed 50-bp dsRNA.

clear, as shown in the right panel in Fig. 1a, that levels of the control Rluc activity were suppressed similarly to those of the firefly enzyme. dsRNA analogue polyI/polyC and IFN had similar suppressive effects on the activities of luciferase (Fig. 1b), and activated PKR pathway genes (Fig. 1c) in HeLa S3 cells. Thus, as reported previously,[4] there were clear nonspecific effects on the expression of both the luc and Rluc genes, which were not targets of the transfected dsRNA.

In the course of our studies of RNAi, we became aware that somewhat longer hairpin-RNAs, which were transcribed endogenously, seemed to cause RNAi without significant cytotoxicity, such as decreased rates of proliferation, suggesting that some difference might exist between the responses of cells to intracellularly transcribed dsRNA and exogenous dsRNA (Fig. 1d and data not shown). Therefore, we decided to re-examine in detail the characteristics of RNAi caused by long hairpin-RNA expression vectors.

Conventional tRNA-driven short hairpin RNA vector (shRNA) induces the IFN response, whereas modified hairpin RNA (mhRNA) does not

It has been demonstrated that pol III promoters, such as U6, H1, and $tRNA^{Val}$ promoters, are suitable for the transcription of short sequences and for application to RNAi.[5–12] For transcription, U6 and H1 promoters require elements in the 5′

flanking region, allowing the transcription of hairpin RNAs with no extra sequences.[13] By contrast, transcripts driven by the $tRNA^{Val}$ promoter are tRNA-attached hairpin-RNAs since tRNA promoters are internal promoters.[12,14]

As shown in Fig. 2, we constructed $tRNA^{Val}$ promoter-driven hairpin-RNA expression vectors, and examined the RNAi and nonspecific effects of tRNA-driven hairpin-RNAs. In our hairpin-RNA expression vectors, we included multiple point mutations in the sense strand of each hairpin in order to improve the stability of each plasmid in *E. coli*, as described elsewhere.[15] Thus, we constructed both the conventional short hairpin RNA without mismatches (shRNA) and the corresponding modified hairpin RNA with mismatches within the sense strand (mhRNA), and examined the RNAi and nonspecific effects of both types of hairpin RNAs. Specifically, we constructed 50-bp and 100-bp conventional hairpin-RNA expression plasmids (tRNA-sh50c and tRNA-sh100c; the lower case 'c' refers to a conventional and perfectly complementary dsRNA region), in which the stem region of each sense strand had no mutations, and the corresponding mhRNAs with mutations within the sense strand (tRNA-mh50 and tRNA-mh100). To facilitate detection of the IFN response, we transfected HeLa S3 cells with each plasmid at a concentration 10 times higher (300 ng per well of a 48-well plate) than that of the highest dose shown in Fig. 1a.

Fig. 2b shows the expression levels of the non-target Rluc, measuring nonspecific suppression by each construct. In the case of perfectly matched tRNA constructs, Rluc activity tended to decrease (Fig. 2b). However, the nonspecific effect was significantly reduced by the mutations within the sense strand, as demonstrated by the corresponding mhRNA (Fig. 2b, compare tRNA-sh100c with tRNA-mh100). The results of assays of transient luciferase activity indicated that perfectly matched dsRNA expression plasmids seemed to have nonspecific effects on Rluc activities, and the presence of mutations within the sense strand could attenuate the nonspecific effect.

Next, we examined the expression of genes in the PKR pathway 48 h after transfection of HeLa S3 cells with various tRNA-driven hairpin-RNA expression vectors. In the case of the tRNA-mh50 vector, levels of PKR and phosphorylated PKR were similar to those obtained with the control tRNA vector (Fig. 2c). By contrast, with the tRNA-driven 50-bp or 100-bp dsRNA with a perfectly complementary sequence, levels of phosphorylated PKR and/or PKR were elevated (Fig. 2c, compare tRNA-mh50 with tRNA-sh50c, and tRNA-mh100 with tRNA-sh100c). The accumulation of phosphorylated PKR seemed to be a function of increasing PKR protein concentration, which itself is the source of nonspecific effects in the cell.[16] In the course of our experiments, a significant fraction of cells transfected with the conventional tRNA-sh50c vector or the tRNA-sh100c vector underwent apoptosis (data not shown).

Induction of the 2′,5′-*OAS* gene, the product of which is directly activated with binding to dsRNAs as is PKR,[3] has been reported as a representative indicator of the IFN response.[17–19] IFN-β is the primary signal of the IFN response[3]. Therefore, to ascertain any possible levels of activation of the IFN pathway in cells transfected with the mhRNA vectors, we examined levels of 2′,5′-*OAS* and *IFN-β* mRNAs by a real-time RT-PCR assay (Fig. 2d). Using this very sensitive quantitative assay, we did not detect significant activation of the 2′,5′-*OAS* and *IFN-β* genes in the case of mhRNA vectors (Fig. 2d). In contrast, both genes were induced in cells transfected with the conventional tRNA-sh50c at a significantly higher level than in cells transfected with the tRNA-control vector or the tRNA-mh50 vector (Fig. 2d).

Taken together, all the data demonstrate that the non-specific interferon responses caused by long dsRNAs could be avoided by a simple introduction of mismatches into the sense strand of endogenously transcribed dsRNAs.

Various tRNA-driven modified hairpin-RNA (mhRNA) expression vectors cause specific RNAi in HeLa S3 cells

It was reported recently that even siRNAs of 21 bp can induce the IFN response, with the extent of induction of the response depending on the sequence and/or dose of the siRNA.[17–19] Therefore, we constructed several tRNA-driven modified hairpin-RNA expression vectors and 21-bp mhRNA vectors that included part of the antisense sequence of the luc gene. In each vector, we varied the sequence and/or length of dsRNA region, as shown in Fig. 2e. With all the vectors that we constructed, including the tRNA-mh100 vector, we observed significant reductions in the activity of firefly luc without any effects on Rluc activity (Fig. 2f). Thus, in our transient expression assay, there were no nonspecific effects, irrespective of the sequence, high dose (300 ng), and length of the stem region as long as mutations were introduced into the sense strand.

Generality of the usefulness of mhRNA

In order to examine the generality of the avoidance of nonspecific effects by the introduction of mutations within the sense strand of long hairpin RNA, we connected the hairpin sequences that we had used in our previous tRNA experiments, as shown in Fig. 2e, directly downstream of a U6 promoter. Using these various U6 constructs with multiple C-to-T and A-to-G mutations within the sense strand, we performed transient luciferase assays similar to the ones for which results are shown in Fig. 2b,f. As shown in Fig. 3a, U6-driven transcripts did not affect the activity of Rluc. Moreover, increases in *OAS* and *IFN-β* mRNAs were not detected in cells transfected with the same U6-driven transcripts by a real-time RT-PCR experiment (data not shown). Therefore, the cellular response to U6-driven dsRNA seemed to be very limited.

The luciferase activity in HeLa S3 cells transfected with the various U6 constructs exhibited similar tendencies to those observed above (Fig. 3b). However, Western blotting analysis revealed that levels of PKR and phosphorylated PKR induced by the conventional U6-driven 50-bp or 100-bp perfectly matched hairpin-RNAs were consistently higher than those induced by the corresponding mhRNA constructs (Fig. 3c, compare U6-mh100 with U6-sh100c). Sledz *et al.* reported that nonspecific effects caused by siRNA or siRNA vector[17–19] were mediated by PKR, because such nonspecific siRNA-induced signalling was not observed in *PKR* null cells.[19] Therefore, it would be preferable to include the mutation in the

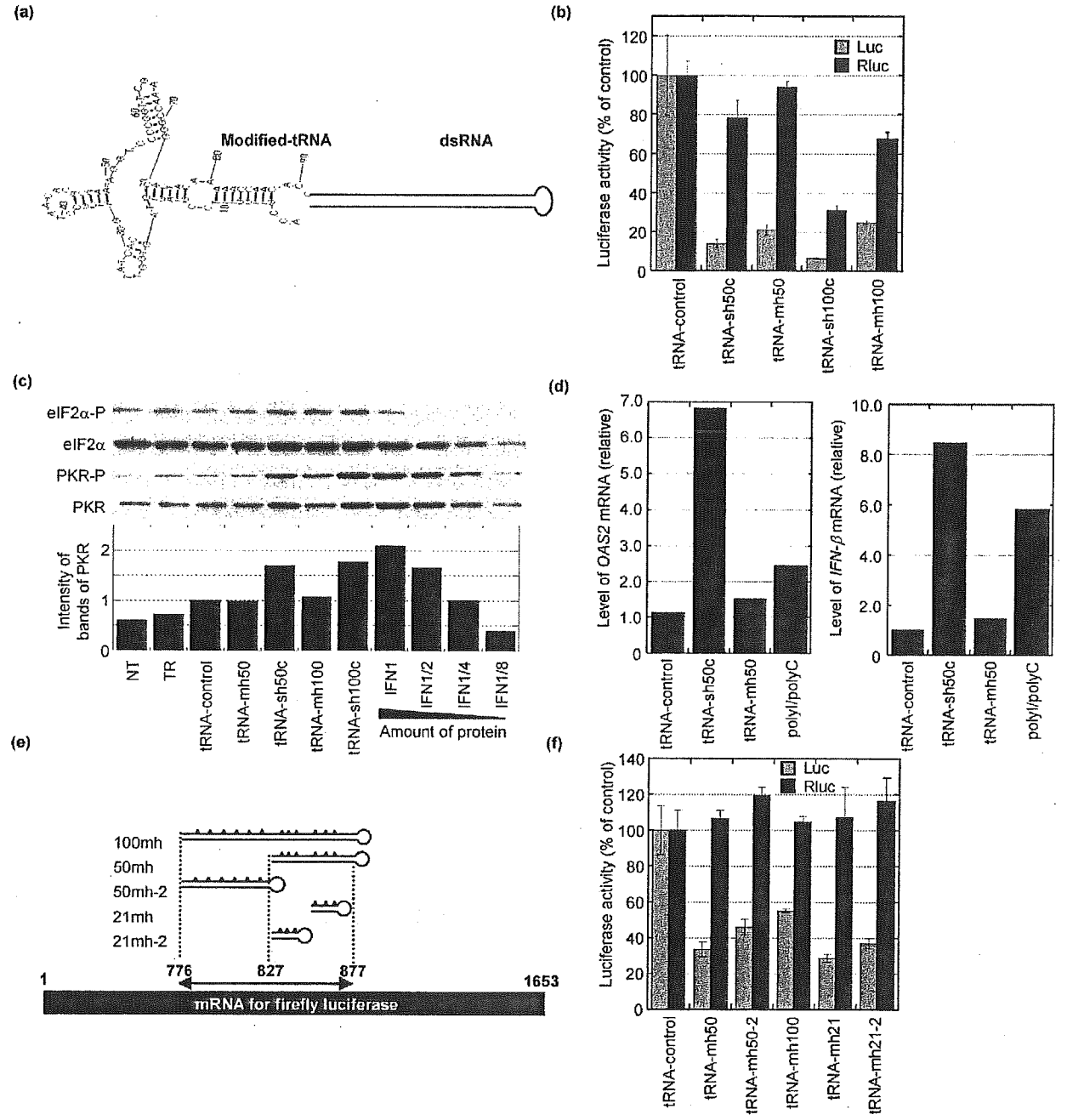

Fig. 2 RNAi and the IFN response in HeLa S3 cells transfected with tRNA-hairpin vectors. (a) Schematic representation of the $tRNA^{Val}$-hairpin-RNAs. The secondary structure of the modified tRNA was predicted using the computer program "RNA structure 3.71" (http://www.bioinfo.rpi.edu/~zukerm/). Each hairpin was attached immediately downstream of the indicated modified-$tRNA^{Val}$ sequence. (b) RNAi effects of tRNA promoter-driven perfectly complementary or modified 50-bp and 100-bp hairpin-RNA vectors targeted against the firefly gene for luciferase. HeLa S3 cells were treated as described in the legend to Fig. 1d with the exception that the amount of the U6-hairpin vector was 300 ng. (c) The attenuation of accumulation of PKR pathway genes in HeLa S3 cells transfected with tRNA promoter-driven modified 50-bp or 100-bp hairpin-RNA expression vectors (1.2 μg). HeLa S3 cells were treated as described in the legend to Fig. 1c. Total proteins from cells treated with IFN were serially diluted with lysis buffer as shown in figure, to facilitate the comparison of intensity of each band. The level of PKR is graphically shown using the Scion Image program. (d) The effects of tRNA-driven hairpin RNA expression vectors on the levels of *OAS2* and *IFN-β* mRNA in HeLa S3 cells. Cells in each well of a 6-well plate were transfected with 3 μg of vectors or 2.25 ng of polyI/polyC (1.3×10^{-3} μg ml^{-1}). *OAS2* and *IFN-β* mRNA were detected by a real-time RT-PCR method. The level of the target RNA was normalized to the amount of *GAPDH* mRNA. Each experiment was performed in duplicate and results are shown as the mean. (e) Schematic representation of the position of each hairpin RNA relative to the sequence of the target mRNA. The Δs indicate mutation within sense strand of hairpin. (f) Effects of various tRNA-driven modified hairpin-RNA expression vectors targeted against the firefly gene for luciferase. HeLa S3 cells were treated as described in (b).

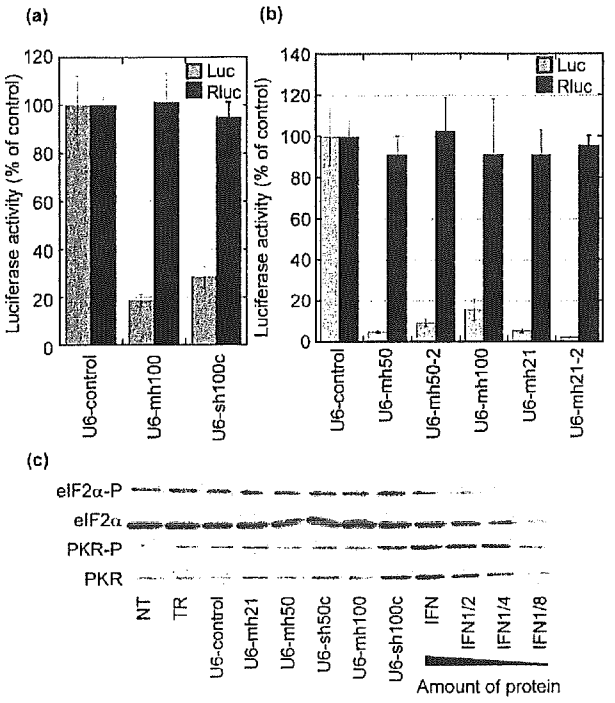

Fig. 3 RNAi and the IFN response in HeLa S3 cells transfected with U6-driven hairpin-RNA expression vectors. (a) Effects of U6 promoter-driven perfectly complementary or modified and 100-bp hairpin-RNA expression vectors targeted against the firefly gene for luciferase. HeLa S3 cells were treated as described in the legend to Fig. 2b. (b) Effects of various U6-driven hairpin-RNA expression vectors with mutation in the sense strand targeted against the firefly gene for luciferase. HeLa S3 cells were treated as described in the legend to Fig. 2b with the exception that U6-hairpin vector was transfected. (c) The effects of U6-driven hairpin-RNA expression vectors on the levels of products of PKR pathway genes. HeLa S3 cells were transfected with various U6-driven hairpin-RNA expression vectors and levels of each protein were analyzed 48 h after transfection by Western blotting. Total proteins from cells treated with IFN were serially diluted with lysis buffer, as shown in the Figure, to facilitate the comparison of intensity of each band. -P denotes the phosphorylated form.

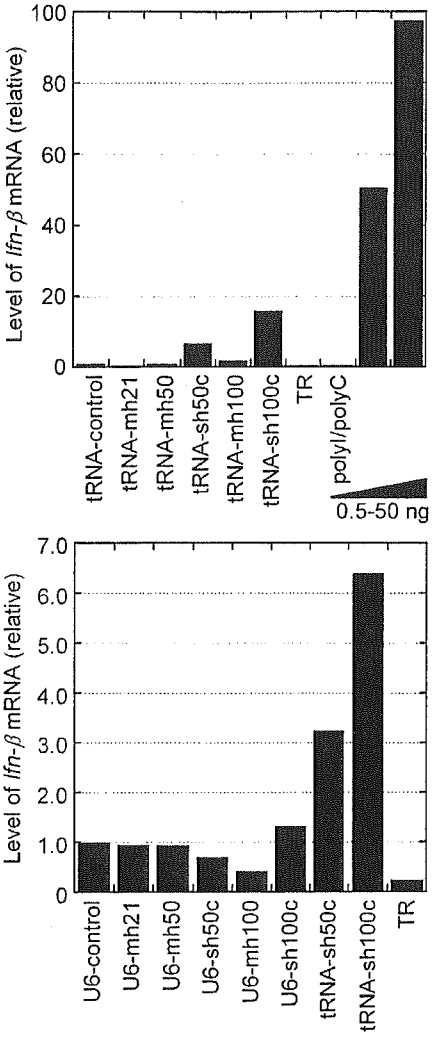

Fig. 4 The effects of U6- and tRNA-driven hairpin RNA expression vectors on the levels of *Ifn-β* mRNA in MEF cells. MEF cells in each well of a 24-well plate were transfected with 0.6 μg vector or 0.5, 5 or 50 ng of polyI/polyC (1.4×10^{-3}–1.4×10^{-1} μg ml^{-1}). Twenty-four hours after transfection, total RNA was isolated and levels of *Ifn-β* mRNA were detected by real-time RT-PCR. The level of *Ifn-β* mRNA was normalized to the amount of 18S ribosomal RNA.

sense strand for the avoidance of the induction of IFN pathway genes.

To further investigate whether inhibiting the IFN response using long mhRNA is general or not in mammalian systems, we detected *Ifn-β* by real-time RT-PCR in non-transformed primary mouse embryo fibroblast (MEF) cells that had been transfected with the mhRNA vectors. Even in MEF cells, the inhibitory effects on induction of *Ifn-β* mRNA were similarly observed as in HeLa S3 cells (Fig. 4).

Therefore, our finding that modified hairpin-RNA (mhRNA) vectors can circumvent the IFN response represents a general phenomenon.

Multiple siRNA species can be generated in HeLa S3 cells transfected with pol III-driven 50-bp hairpin-RNA expression vectors

RNAi is likely to involve multiple species of siRNA in cells transfected with longer hairpin-RNA expression vectors. To detect siRNAs generated from each sequence of longer constructs, we performed Northern blotting with mh21 and mh21-2 probes and a mh50-specific antisense probe (Fig. 5). The mh21 probe and mh21-2 probe did not overlap each other.

In cells transfected with the U6-mh50 vector, we detected at least two kinds of siRNA (Fig. 5, U6-mh50). Thus, in cells transfected with the 50-bp hairpin-RNA expression vector, transcription of the hairpin-RNA sequence extended to its 3′ ends (Fig. 5, middle panel), and each longer hairpin-RNA generated multiple species of siRNA, probably *via* the action of a Dicer-like enzyme, and contributed to the suppressive effect on the expression of the target gene. In some cases, these multiple species of siRNAs may become important, as demonstrated below.

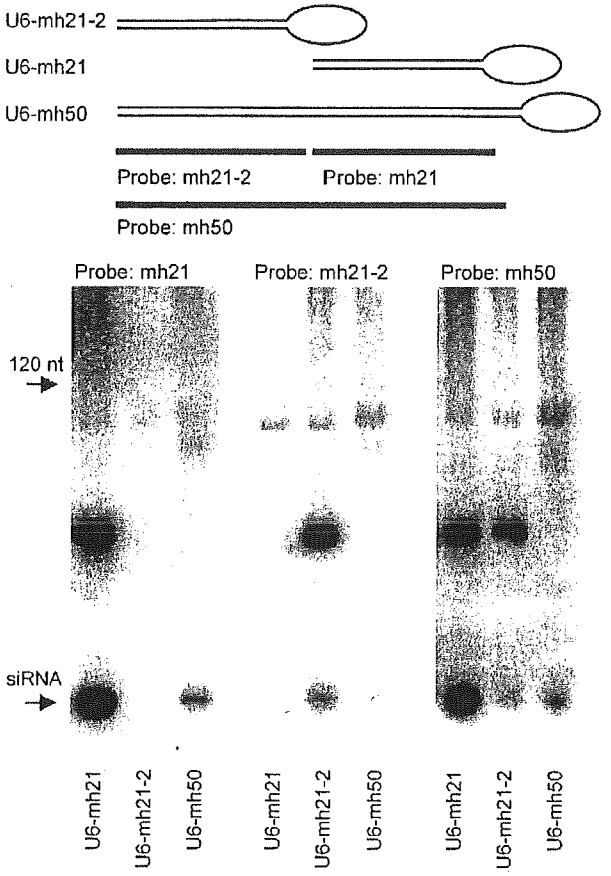

Fig. 5 Detection by Northern blotting of multiple species of siRNA generated from 50-bp hairpin RNA. RNAs from cells transfected with U6-driven modified hairpin-RNA expression vectors were examined with siRNA-specific probes, as indicated.

Long mhRNA expression vectors can effectively suppress the replication of hepatitis C virus

Since multiple siRNA species are generated in cells transfected with a long mhRNA vector, this might be useful for suppressing RNA viruses, which escape by mutating their RNA genome from the RNAi effect by conventional short siRNA. To evaluate if the long mhRNA vector might be addressed simultaneously against the wild type and its mutated variants of hepatitis C virus (HCV), we fused a part of a common NS5B region derived from a Seeger clone or a 9 point-mutated NS5B region derived from a Wakita clone, to a luc sequence (Seeger-luc or Wakita-luc), and examined the RNAi effects caused by 51- and 21-bp hairpin-RNA vectors. Two 21 bp siRNA vectors targeted to the sequence of a Seeger clone reduced Seeger-luc activity but did not affect Wakita-luc activity (Fig. 6a, U6-sh21c-A and -B). In contrast, a 51-bp mhRNA vector targeted to Seeger-luc efficiently suppressed both Seeger- and Wakita-luc activities (Fig. 6a, U6-mh51), suggesting that viruses harboring several mutations could not escape from RNAi caused by a long mhRNA vector.

Next, to examine the effect of the mhRNA vector on suppression for the replication of raw HCV, we established an HuH-7 cell line carrying an HCV replicon that harbors a

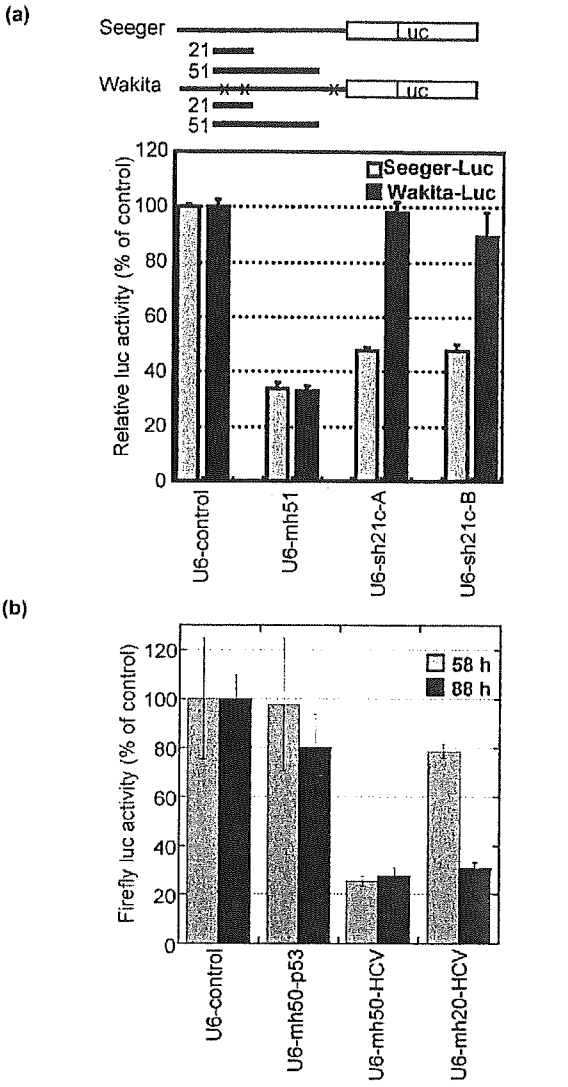

Fig. 6 Suppression of hepatitis C virus (HCV) using 50-bp mhRNA vectors. (a) 293T cells were transfected with a pSeeger-luc or pWakita-luc that harbors a part of HCV sequence fused to a firefly luc gene, an Rluc expression plasmid, and a U6 promoter-driven 21 bp (U6-sh21c-A or -B) or 51 bp (U6-mh51) hairpin RNA expression plasmid targeted to the Seeger sequence. (b) The HuH-7 cells carrying an HCV replicon were transfected with a U6 promoter-driven 20-bp (U6-mh20-HCV) or 50-bp (U6-mh50-HCV) hairpin-RNA expression plasmid targeted to HCV sequence. Control U6 vector or 50 bp mhRNA vector targeted to p53 (U6-mh50-p53) were used as negative controls.

neomycin phosphotransferase (*neo*) gene as a selection marker, according to a previous report.[20] Moreover, the replicon RNA derived from an infectious HCV clone, genotype 1b,[21] was modified by substituting the *neo* gene with a fusion of luc and *neo* genes, which enables the quantification of HCV replication levels by that of luc activity. We transfected HuH-7 cells carrying the HCV replicon with mhRNA vectors, which did not induce IFN-β (data not shown), against HCV sequence. Then, at both 58 and 88 h after transfection, the viral replication was estimated by measuring luc activity. As shown in Fig. 6b, replication of HCV in cells transfected with

a 50 bp mhRNA vector was suppressed more rapidly and effectively than with a 21 bp mhRNA vector. Rapid accumulation of a specific siRNA species with strong activity may result in differences observed at the earlier time point, or the Dicer-substrate (longer mhRNA) may be more active at a lower concentration (at the earlier time point), as recently described by the groups of Rossi[22] and Hannon.[23]

Collectively, we have shown that the long mhRNA vector can avoid the IFN pathway and simultaneously generate multiple species of siRNA. Therefore, long mhRNA vectors might be useful in attempts to knock down family genes at once, or to destroy RNA viruses, such as HCV or HIV, which might escape from RNAi by mutating their RNA genomes.[24–26]

Discussion

In this study, we showed that pol III promoter-driven 50-bp and 100-bp modified hairpin-RNA expression vectors can cause RNAi without any nonspecific effects in HeLa S3 cells, which had been shown previously to be sensitive to long dsRNA.[4] For all applications of vector-based RNAi, we recommend the inclusion of multiple mutations within the sense strand of the dsRNA region, to generate G : U wobble base-pairing (Fig. 7). The presence of point mutations assures the stability of plasmids in *E. coli*,[15] allows sequencing without major difficulties even in the case of long (stem length of 30 to >100 bp) hairpin-RNA expression vectors, and prevents loss of RNAi activity (Fig. 3a). Moreover, avoidance of induction of the IFN response was observed, at least in HeLa S3 cells, HEK293 cells and non-transformed mouse embryonic fibroblast cells (Fig. 2b–d, 3c, 4 and data not shown). It should also be mentioned that the phenotype of RNAi-based knockdown mice could be maintained for progeny mice when mhRNA was used (TY, unpublished data), but the phenotype was lost with the conventional completely matched shRNA.

Cells transfected with *in vitro* synthesized 50 bp dsRNA with mutations within the sense strand targeted against the luc gene exhibited nonspecific reduction in Rluc activity (Fig. 1a), while cells transfected with pol III-driven mhRNA vectors transcribing the same sequence did not (Fig. 1d). These observations suggest that systems exist that allow cells to recognize the origin of a dsRNA. Recently, Kim *et al.* reported that one such difference is the existence of a 5′ triphosphate.[27] Thus, the *in vitro*-synthesized dsRNAs might be recognized directly by a receptor for exogenous RNAs, such as a Toll-like receptor 3 or Toll-like receptor 7, and might then activate downstream genes.[28–30]

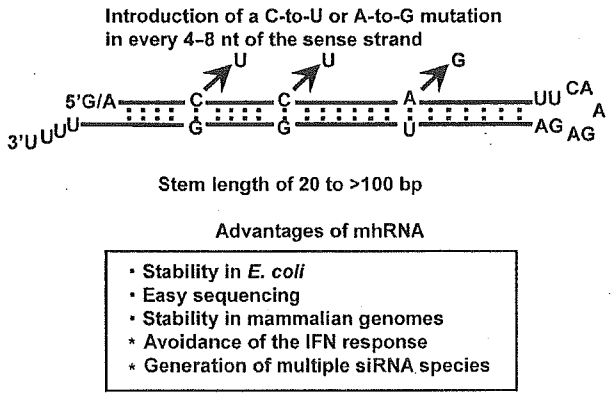

Fig. 7 Schematic representation and several of the advantages of the mhRNA system for induction of RNAi.

We also showed that the levels of RNAi effects and non-specific effects depended on the promoters for hairpin RNAs and/or the presence of the 5′ extra sequence of the hairpin RNA. These differences would be due to variations of localization of transcripts and/or of mode of processing. Thus far, it has been reported that U6-, VA1-, and tRNA-based siRNA vectors can cause various levels of RNAi activity and of IFN response.[12,17,18] Our methodology, which uses mhRNA vectors, with no extra 5′ sequence and with mutation within the sense strand, might be useful for generating effective RNAi vectors.[15]

We have demonstrated that long mhRNA vectors can be used for efficient suppression of the replication of the HCV virus, irrespective of the presence of mutation in its genome (Fig. 6). Therefore, it might be useful to apply our long mhRNA to the destruction of such RNA viruses since several mutations in the genome of a virus would be required before it could escape from the effects of RNAi.[24,25] However, it has not yet been determined that viral variants in chronic patients can be blocked by RNAi. In other applications, the efficacy of longer dsRNA transcripts would be less seriously affected by the site dependence of RNAi activity. Moreover, longer dsRNA could be used for the simultaneous suppression of redundant genes or of genes for members of a same protein family that are relatively conserved in terms of sequence. More recently, we and others have shown that multiple siRNAs could suppress gene expression at a transcriptional level.[31–34] Therefore, our long mhRNA system might better regulate the gene expression not only at a post-transcriptional level but also at a transcriptional level.

Our finding that mhRNA can both facilitate the exploitation of RNAi in response to long dsRNAs and also allow avoidance of the IFN response in mammalian cells should contribute firstly to the engineering of effective long hairpin-RNA expression vectors against viruses, and secondly to the elucidation of mechanisms of cytotoxicity due to long dsRNA and RNAi and to the biology of microRNAs.

Materials and methods

Construction of plasmids

Plasmids containing a human U6 promoter[15] and a $tRNA^{Val}$ promoter[12,14,35] were described previously. A series of long hairpin-RNA expression vectors was constructed by inserting antisense sequence between the U6 or tRNA promoter, and the corresponding sense sequence, into *Bsp* MI site. Two luciferase expression vector harboring HCV sequences, pSeeger-Luc and pWakita-Luc, were constructed by subcloning a part of the HCV genome NS5B region sequence of a Seeger clone (GenBank Accession No. AF139594, 9069-9389) or a Wakita clone (GenBank Accession No. AB047639, 9123-9443) into *Nco* I site of pGL3-Control (Promega, Madison,

WI). Sequences downstream of the U6 or tRNA promoter are shown in the electronic supplementary information (Table S1).

All the plasmids designed to produce long hairpin-RNAs without mutations were amplified in *E. coli* SURE II (Stratagene, La Jolla, CA), as described previously.[36] Other plasmids were amplified in *E. coli* DH5α.

Culture and transfection of cells

HeLa S3 cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical, St. Louis, MO), supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin at 37 °C in an atmosphere of 5% CO_2 in air. All transfections of HeLa S3 cells with nucleic acids were carried out using Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Interferon from human leukocyte (Sigma Chemicals) was used for the positive control of the IFN response at the final concentration of 1000 U ml^{-1} in the medium. Luciferase assays were performed as described previously,[7] with some modifications. In most cases, 24 h after 2.5 × 10^4 HeLa S3 cells had been plated, cells were transfected with 2.5 ng of *Renilla* luciferase (Rluc) expression vector (pRL-RSV), 25 ng of firefly luciferase (luc) expression vector (pGL3-control) and an appropriate amount of plasmid vector or polyI/polyC (Amersham Biosciences, Piscataway, NJ) in individual wells of 48-well plates. Luciferase activities were determined 24 h after transfections with the Dual Luciferase Assay System (Promega). Cells were lysed and luminescence was detected with a luminometer. Firefly luciferase activity was adjusted by reference to the activity of Rluc to normalize the efficiency of transfections.

The human embryonic kidney cell line 293T were maintained in DMEM (Sigma), supplemented with 10% FCS. Fifty ng pSeeger-Luc or pWakita-Luc were transiently transfected with 0.2 μg of each siRNA-expression vector and 30 ng of pRL-RSV to 293T cells with Lipofectamine plus™ (Invitrogen). Twenty-four hours after the transfection, cells were harvested and luciferase activities were quantified using a Bright-Glo Luciferase Assay System (Promega).

The HuH-7 cell line carrying an HCV replicon was established as previously described.[20,21] Cells were maintained in DMEM (Invitrogen) supplemented with 10% fetal calf serum, nonessential amino acids, 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. G418 (Geneticin; Invitrogen) was added at a final concentration of 500 μg ml^{-1} to cell lines carrying an HCV replicon. The following day after 5 × 10^3 HCV replicon cells were plated in 96-well cluster plates, transfections were carried out by using Lipofectamine™ 2000 reagent. The luc assay was performed using the Bright-Glo luciferase assay system (Promega). Luciferase activities were quantified using a luminometer (Mithras LB940, Berthold). Assays were performed in triplicate, and the results were shown as averages ± SD as percentages of controls.

The primary mouse embryonic fibroblast cells derived from BALB/c mouse were maintained in DMEM supplemented with 10% FBS, 1000 mg l^{-1} glucose, and 1% penicillin–streptomycin. Transfections of plasmids, which were prepared using EndoFree Plasmid Kit (Qiagen GmbH, Hilden, Germany), were carried out by using Lipofectamine™ 2000 reagent.

Preparation of duplexes of RNA oligonucleotides

Using T7 RNA polymerase, we prepared sense RNAs with point mutations and antisense RNAs of 50 nt in length, targeted against the firefly gene for luciferase. The sequences are shown in the electronic supplementary information (Table S2). We purified these RNAs by standard methods. Duplexes were generated by annealing a mutated sense RNA and an antisense RNA, and formation of duplexes was confirmed by staining with ethidium bromide after Native-PAGE (6% polyacrylamide).

Western blotting analysis

From 24–36 h after the passage of 1.8–2.1 × 10^5 HeLa S3 cells, cells were transfected with 1.2 μg of the indicated plasmid in individual wells of 12-well plates. Then, 48 h after transfection, cells were lysed in a Triton X-100-based buffer [1% (*v/v*) Triton X-100, 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 50 mM NaF, 0.2 mM sodium ortho vanadate] with protease inhibitors (complete cocktail; Roche Applied Science, Mannheim, Germany), and each resulting lysate was sonicated and cleared by centrifugation. The amount of protein was measured with a DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as the standard. Total proteins (7.5 μg per lane) were resolved by SDS-PAGE (12% polyacrylamide) and bands of proteins were transferred to a poly(vinylidene) fluoride (PVDF) membrane (Immobilon-P™; Millipore, Bedford, MA). The membrane was blocked with 5% skim milk and probed with antibody against PKR (1 : 10000; Santa Cruz Biotechnology, Santa Cruz, CA), phospho-PKR(Thr451) (1 : 1000; Cell Signaling Technology, Beverly, MA), eIF2α (1 : 20000; Santa Cruz Biotechnology), phospho-eIF2α(Ser51) (1 : 1000; Cell Signaling Technology), or actin (1 : 10000; Sigma Chemical). After incubation of the membrane with horseradish peroxidase-conjugated antibodies raised in donkey against rabbit IgG (1 : 1000; Amersham Biosciences), products of immunoreactions were visualized with ECL plus™ reagent (Amersham Biosciences).

Northern blotting analysis

Northern blotting was performed as described previously,[7] with some modifications. Twenty four hours after the passage of 3.4 × 10^5 HeLa S3 cells in individual wells of 6-well plates, cells were transfected with 3 μg of the indicated plasmid per well. Then, 24 h after transfection, total RNA was isolated from cells in each well with Isogen™ reagent (Wako, Tokyo, Japan) according to the manufacturer's protocol. The RNA (7.5 μg per lane) was fractionated by electrophoresis on an 8% (*w/v*) polyacrylamide–urea gel (8.3 M urea) and transferred to a nylon membrane (Hybond-XL™; Amersham Biosciences). Each blot was incubated for 1 h at 36 °C in 30% formamide, 10% dextran sulfate, 5 × SSPE, 0.5% SDS, 5 × Denhardt's solution, and 0.1 mg ml^{-1} salmon testes

DNA, and then the RNAs were allowed to hybridize with a ^{32}P-labelled probe at 36 °C for 3 h. The membrane was washed in 2 × SSPE twice for 15 min at 36 °C and analyzed with a Bio-Image Analyzer (BAS1000; Fuji Photo Film, Tokyo, Japan). Probes were as follows. mh21: 5′-AAG TGC GCT GCT GGT GCC AAC CC-3′; mh21-2: 5′-AAG CCT TCA GGA TTA CAA GAT TC-3′; and mh50: 5′-AAG CCT TCA GGA TTA CAA GAT TCA AAG TGC GCT GCT GGT GCC AAC CCT ATT C-3′.

Real-time RT-PCR analysis

For quantitation of 2′,5′-*OAS* (*OAS2*) or *IFN-β* mRNA, 1 μg of total RNA isolated from cells with Isogen® reagent were reverse transcribed using SuperScript® III reagent (Invitrogen). Quantitative real-time RT-PCR was performed using a LightCycler instrument (Roche). Primers for human *OAS2*, *IFN-β*, and *GAPDH* were purchased from Roche. Quantitation of *Ifn-β* mRNA in MEF cells was performed as described previously.[21] Primers for *Ifn-β*, 18S ribosomal RNA were purchased from Applied Biosystems (Foster City, CA).

Acknowledgements

We thank Prof. G. M. Blackburn at The University of Sheffield for helpful comments; K. Ito at The Tokyo Medical and Dental University for technical assistance; Dr N. Sakamoto at Tokyo Medical and Dental University for kindly providing HCV DNA; C. Matsuda at Tokyo Metropolitan Institute of Medical Science for technical assistance. This work was supported by a grant from Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists, by grants from the National Institute of Advanced Industrial Science and Technology (AIST), Japan, and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Culture (MEXT) of Japan.

References

1 D. M. Dykxhoorn, C. D. Novina and P. A. Sharp, *Nat. Rev. Mol. Cell Biol.*, 2003, **4**, 457–467.
2 G. J. Hannon, *Nature*, 2002, **418**, 244–251.
3 G. R. Stark, I. M. Kerr, B. R. Williams, R. H. Silverman and R. D. Schreiber, *Annu. Rev. Biochem.*, 1998, **67**, 227–264.
4 S. M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber and T. Tuschl, *Nature*, 2001, **411**, 494–498.
5 T. R. Brummelkamp, R. Bernards and R. Agami, *Science*, 2002, **296**, 550–553.
6 P. J. Paddison, A. A. Caudy, E. Bernstein, G. J. Hannon and D. S. Conklin, *Genes Dev.*, 2002, **16**, 948–958.
7 M. Miyagishi and K. Taira, *Nat. Biotechnol.*, 2002, **20**, 497–500.
8 N. S. Lee, T. Dohjima, G. Bauer, H. Li, M. J. Li, A. Ehsani, P. Salvaterra and J. Rossi, *Nat. Biotechnol.*, 2002, **20**, 500–505.
9 C. P. Paul, P. D. Good, I. Winer and D. R. Engelke, *Nat. Biotechnol.*, 2002, **20**, 505–508.
10 G. Sui, C. Soohoo, E. B. Affar, F. Gay, Y. Shi and W. C. Forrester, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 5515–5520.
11 J. Y. Yu, S. L. DeRuiter and D. L. Turner, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 6047–6052.
12 H. Kawasaki and K. Taira, *Nucleic Acids Res.*, 2003, **31**, 700–707.
13 J. Ohkawa and K. Taira, *Hum. Gene Ther.*, 2000, **11**, 577–585.
14 S. Koseki, T. Tanabe, K. Tani, S. Asano, T. Shioda, Y. Nagai, T. Shimada, J. Ohkawa and K. Taira, *J. Virol.*, 1999, **73**, 1868–1877.
15 M. Miyagishi and K. Taira, *Oligonucleotides*, 2003, **13**, 325–333.
16 S. B. Lee and M. Esteban, *Virology*, 1994, **199**, 491–496.
17 A. J. Bridge, S. Pebernard, A. Ducraux, A. L. Nicoulaz and R. Iggo, *Nat. Genet.*, 2003, **34**, 263–264.
18 S. Pebernard and R. D. Iggo, *Differentiation*, 2004, **72**, 103–111.
19 C. A. Sledz, M. Holko, M. J. de Veer, R. H. Silverman and B. R. Williams, *Nat. Cell Biol.*, 2003, **5**, 834–839.
20 V. Lohmann, F. Korner, J. Koch, U. Herian, L. Theilmann and R. Bartenschlager, *Science*, 1999, **285**, 110–113.
21 K. Tsukiyama-Kohara, S. Tone, I. Maruyama, K. Inoue, A. Katsume, H. Nuriya, H. Ohmori, J. Ohkawa, K. Taira, Y. Hoshikawa, F. Shibasaki, M. Reth, Y. Minatogawa and M. Kohara, *J. Biol. Chem.*, 2004, **279**, 14531–14541.
22 D. H. Kim, M. A. Behlke, S. D. Rose, M. S. Chang, S. Choi and J. J. Rossi, *Nat. Biotechnol.*, 2005, **23**, 222–226.
23 D. Siolas, C. Lerner, J. Burchard, W. Ge, P. S. Linsley, P. J. Paddison, G. J. Hannon and M. A. Cleary, *Nat. Biotechnol.*, 2005, **23**, 227–231.
24 A. T. Das, T. R. Brummelkamp, E. M. Westerhout, M. Vink, M. Madiredjo, R. Bernards and B. Berkhout, *J. Virol.*, 2004, **78**, 2601–2605.
25 L. Gitlin, S. Karelsky and R. Andino, *Nature*, 2002, **418**, 430–434.
26 A. J. Leslie, K. J. Pfafferott, P. Chetty, R. Draenert, M. M. Addo, M. Feeney, Y. Tang, E. C. Holmes, T. Allen, J. G. Prado, M. Altfeld, C. Brander, C. Dixon, D. Ramduth, P. Jeena, S. A. Thomas, A. St John, T. A. Roach, B. Kupfer, G. Luzzi, A. Edwards, G. Taylor, H. Lyall, G. Tudor-Williams, V. Novelli, J. Martinez-Picado, P. Kiepiela, B. D. Walker and P. J. Goulder, *Nat. Med.*, 2004, **10**, 282–289.
27 D. H. Kim, M. Longo, Y. Han, P. Lundberg, E. Cantin and J. J. Rossi, *Nat. Biotechnol.*, 2004, **22**, 321–325.
28 L. Alexopoulou, A. C. Holt, R. Medzhitov and R. A. Flavell, *Nature*, 2001, **413**, 732–738.
29 K. Kariko, P. Bhuyan, J. Capodici and D. Weissman, *J. Immunol.*, 2004, **172**, 6545–6549.
30 V. Hornung, M. Guenthner-Biller, C. Bourquin, A. Ablasser, M. Schlee, S. Uematsu, A. Noronha, M. Manoharan, S. Akira, A. de Fougerolles, S. Endres and G. Hartmann, *Nat. Med.*, 2005, **11**, 263–270.
31 D. Castanotto, S. Tommasi, M. Li, H. Li, S. Yanow, G. P. Pfeifer and J. J. Rossi, *Mol. Ther.*, 2005, **12**, 179–183.
32 H. Kawasaki and K. Taira, *Nature*, 2004, **431**, 211–217.
33 K. V. Morris, S. W. Chan, S. E. Jacobsen and D. J. Looney, *Science*, 2004, **305**, 1289–1292.
34 A. H. Ting, K. E. Schuebel, J. G. Herman and S. B. Baylin, *Nat. Genet.*, 2005, **37**, 906–910.
35 Y. Kato, T. Kuwabara, M. Warashina, H. Toda and K. Taira, *J. Biol. Chem.*, 2001, **276**, 15378–15385.
36 H. Akashi, M. Miyagishi and K. Taira, *Antisense Nucleic Acid Drug Dev.*, 2001, **11**, 359–367.

Supplementary Table 1. Sequences downstream of the U6 or tRNA promoter. Sense mutations are indicated in lower cases.

mh21	5'-GTG CGt TGt TGG TGt tAA tCC TTC AAG AGA GGG TTG GCA CCA GCA GCG CAC TTT TT-3'
mh21-2	5'-GCC TTt AGG ATT AtA AGg TTC TTC AAG AGA GAA TCT TGT AAT CCT GAA GGC TTT TT-3'
mh50	5'-GCC TTt AGG ATT AtA AGg TTC AAA GTG tGC TGt TGG TGt CAA CtC TAT TCT TCA AGA GAG AAT AGG GTT GGC ACC AGC AGC GCA CTT TGA ATC TTG TAA TCC TGA AGG CTT TTT-3'
mh50-2	5'-GAT TTC GgG TtG TCT TgA TGT ATg GgT TTG gAG AgG AGt TGT TTC TGg GGT TCA AGA GAC CTC AGA AAC AGC TCT TCT TCA AAT CTA TAC ATT AAG ACG ACT CGA AAT CTT TTT-3'
mh100	5'-GAT TTC GgG TtG TCT TgA TGT ATg GgT TTG gAG AgG AGt TGT TTC TGg GGA GtC TTt AGG ATT AtA AGg TTC AAA GTG tGC TGt TGG TGt CAA CtC TAT TCT TCA AGA GAG AAT AGG GTT GGC ACC AGC AGC GCA CTT TGA ATC TTG TAA TCC TGA AGG CTC CTC AGA AAC AGC TCT TCT TCA AAT CTA TAC ATT AAG ACG ACT CGA AAT CTT TTT-3'
sh21c-A	5'-GAA TCC CGG CTG CGT CCC AGT TAT ACA AGA GAC TGG GAC GCA GCC GGG ATT TTT-3'
sh21c-B	5'-GCT GCG TCC CAG TTG GAC TTA TTC AAG AGA TAA GTC CAA CTG GGA CGC AGC TTT TT-3'
mh51	5'-AgG Att AAG CTt AAA CTt ACT CtA ATC tCG GtT GtG TCC tAG

	TTG GgC TTA TTC AAG AGA TAA GTC CAA CTG GGA CGC AGC CGG GAT TGG AGT GAG TTT GAG CTT GGT CTT TTT-3'
mh20-HCV	5'-GTC TtG TAG Att GTG tAT tAT AGA ATT ACA TCA AGG GAG ATT GAT GCA CGG TCT ACG AGA CTT TTT-3'
mh50-p53	5'-CAT tAC AtT GGA gGA tTC CAG TGG TgA TCT AtT GGG gCG GAg tAG CTT TGG TGT GCT GTC CCA AAG CTG TTC CGT CCC AGT AGA TTA CCA CTG GAG TCT TCC AGT GTG ATG TTT TT-3'
mh50-HCV	5'-GAG TGt tCt GGG AGG TtT CGT AGA tCG TGt ATC gTG AGt ACA AgT tCT AAG TGT GCT GTC CTT AGG ATT TGT GCT CAT GAT GCA CGG TCT ACG AGA CCT CCC GGG GCA CTC TTT TT-3'

Supplementary Table 2. The sequence of RNA oligonucleotides. Mutations within the sense strand are indicated in lower cases.

mutated sense strand of 50-bp dsRNA	5'-GGG GCC UUu AGG AUU AuA AGg UUC AAA GUG uGC UGu UGG UGu CAA CuC UAU UCU U-3'
antisense strand of 50-bp dsRNA	5'-GGG GAG AAU AGG GUU GGC ACC AGC AGC GCA CUU UGA AUC UUG UAA UCC UGA AGG C-3'

Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Biochemical and Biophysical Research Communications 340 (2006) 263–267
BBRC
www.elsevier.com/locate/ybbrc

In vivo delivery of small interfering RNA targeting brain capillary endothelial cells

Taro Hino [a,1], Takanori Yokota [a,*], Shingo Ito [b], Kazutaka Nishina [a], Young-Sook Kang [b,c], Shinobu Mori [b], Satoko Hori [b], Takashi Kanda [a], Tetsuya Terasaki [b], Hidehiro Mizusawa [a,1]

[a] *Department of Neurology and Neurological Science, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan*
[b] *Department of Molecular Biopharmacy and Genetics, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan*
[c] *College of Pharmacy, Sookmyung Women's University, Seoul, Republic of Korea*

Received 24 November 2005
Available online 9 December 2005

Abstract

Brain capillary endothelial cells (BCECs) play an important role in blood–brain barrier (BBB) functions and pathophysiologic mechanisms in brain ischemia and inflammation. We try to suppress gene expression in BCECs by intravenous application of small interfering RNA (siRNA). After injection of large dose siRNA with hydrodynamic technique to mouse, suppression of endogenous protein and the BBB function of BCECs was investigated. The brain-to-blood transport function of organic anion transporter 3 (OAT3) that expressed in BCECs was evaluated by Brain Efflux Index method in mouse. The siRNA could be delivered to BCECs and efficiently inhibited endogenously expressed protein of BCECs. The suppression effect of siRNA to OAT3 is enough to reduce the brain-to-blood transport of OAT3 substrate, benzylpenicillin at BBB. The in vivo siRNA-silencing method with hydrodynamic technique may be useful for the study of BBB function and gene therapy targeting BCECs.

Keywords: Small interfering RNA; Blood–brain barrier; Organic anion transporter 3; Brain ischemia; Brain inflammation; Drug delivery system

In brain ischemia and inflammation, the brain capillary endothelial cells (BCECs) have no longer been regarded as an inert vascular lining that is injured and morphologically changed, but actively play many important roles of these pathophysiologic mechanisms. The inhibition of signaling molecule in BCECs of vascular endothelial growth factor (VEGF)-induced vasogenic edema can reduce an ischemic lesion [1]. The inflammatory cell adhesion molecules expressed in BCECs induced by ischemia, such as intercellular adhesion molecule (ICAM) and E-selectin, can be a target molecule [2,3] for the therapy of these diseases. Because leukocytes activation and adhesion to BCECs are believed to contribute to additional, secondary neuronal injury after reperfusion [4] and initiate immune-mediated encephalopathy such as multiple sclerosis [5]. Endothelial nitric oxide synthases expressed in BCECs are also a possible target molecule. In cerebral ischemia, nitric oxide is increased and works as a prooxidant via peroxynitrite [6]. Therefore, BCECs are an important platform in the cerebral ischemia and inflammation, and express many constitutively or transiently expressed molecules which might be a therapeutic target for these pathologies.

RNA interference is a powerful tool for post-transcriptional gene silencing. Recently, we showed an in vitro model whose function of the transporter protein expressed in BCECs is inhibited by siRNA [7]. Here, we try to introduce siRNA by hydrodynamic, intravenous injection method from mouse tail vein and investigate the siRNA effect on brain-to-blood transport function by inhibiting organic anion transporter 3 (OAT3) with Brain Efflux Index method.

* Corresponding author. Fax: +81 3 5803 0169.
E-mail address: tak-yokota.nuro@tmd.ac.jp (T. Yokota).
[1] 21st century COE Program on Brain Integration and it's Disorders.

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.11.173

Materials and methods

Effect of siRNA on expression of recombinant OAT3 in culture cells. The mOAT3cDNA was subcloned from pGEM-HEN/Roct (OAT3) [8] into the *Renilla* luciferase expression vector, psiCHECK-1 (Promega).

Human embryonic kidney 293 (HEK293) cells were transfected with 80 ng of *Renilla* luciferase-fused OAT3 expression vector, 20 ng of *firefly* luciferase expression vector (pGL3; Promega), and 25 nM siRNA in each well of 24-well plates. *Renilla* luciferase activity was normalized with *firefly* luciferase activity. The luciferase activities were analyzed after 24 h after transfection using the Dual Luciferase System (Promega).

Effect of siRNA on uptake of OAT3 substrate in culture cells. The mOAT3 cDNA was subcloned into the pcDNA3 vector. HEK293 cells in 6-well plates were transfected by 0.5 μg of pcDNA3/OAT3 or vector alone with 100 nM siRNA using the Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, the cells were passaged into the 24-well plates, and after another 24 h the cells were washed with phosphate-buffered saline (PBS). The uptake study was initiated at 37 °C by applying 200 μl PBS containing 0.5 μCi [^{3}H]benzylpenicillin to estimate the volume of adherent water. After incubation for 2 min, the radioactivities of ^{3}H in the cells were measured. The uptake of [^{3}H]benzylpenicillin was expressed as the ratio to control siRNA (shuffle sequence).

Animals. Adult male of Institute of Cancer Research (ICR) mice, weighing 35–42 g and age 9–10 weeks, were purchased from Charles River Laboratories. All experiments were approved by the Animal Experiment Committee of Tokyo Medical and Dental University.

In vivo transduction of siRNA with hydrodynamic injection method. Hydrodynamic injection method has been performed according to a previously reported method in mice [9]. The 50 μg siRNA in a volume equivalent to 5–10% of the body weight was rapidly injected in 3–5 s into the mouse tail vein. For comparison, the same amount of siRNA in 0.2 ml PBS was injected slowly in more than 60 s into the mouse tail vein as a regular intravenous injection method.

Brain small vascular fractionation and Western blot analysis. Mice brains were harvested 24 h after application of 50 μg siRNA SOD1 with the hydrodynamic or regular injection method. The total brain homogenate [10] and the brain vascular fraction of small vessels were prepared using a modified method reported previously [11]. Briefly, brains were homogenized in Dulbecco's modified Eagle's medium (DMEM). The homogenates were dissociated further with 0.005% (wt/vol) dispase (grade 1; Roche Diagnostic) at 37 °C for 2 h. After centrifugation (800*g*, 5 min), the pellets were suspended with a dextran solution (M_w 70,000; 15% wt/vol; Sigma) and centrifuged (4 °C, 4500*g* for 10 min). The pellets were resuspended with 0.05 M PBS for 10 min. After centrifugation (800*g*, 5 min), the final pellets of small vessels were resuspended in lysis buffer (20 mM Tris–HCl, 0.1% SDS, and 1% Triton).

Fractionated mouse brain tissues and mouse brain capillary endothelial cell line [12] cells were homogenized in buffer containing 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 4% Chaps, 1 mM phenylmethylsulfonyl fluoride (PMSF), and a protease-inhibitor cocktail (Complete-Mini; Roche Diagnostic). The 2.5 μg samples were separated with 7.5% SDS–polyacrylamide mini-gel (Bio-Rad) and transferred to a polyvinylidene difluoride membrane. The membrane was probed with anti-glucose-transporter-1 antibodies (Alpha Diagnostic International) or anti-SOD1 antibodies (Stressgen Biotechnologies) and visualized by using an ECL Western blot system (Amersham–Pharmacia).

Assay for efflux function of OAT3 in vivo. Fifty micrograms of siRNA OAT3 or control siRNA was delivered to brain capillary endothelial cells with hydrodynamic injection via the tail vein. After 36 h, the in vivo brain efflux experiments were carried out using Brain Efflux Index (BEI) method as described previously [13]. Each mouse was anesthetized intramuscularly with a mixture of ketamine (125 mg/kg) and xylazine (1.22 mg/kg), then mounted on a stereotaxic frame (SRS-6; Narishige), to hold the head in position. Using a dental drill, a bore hole was made 3.8 mm lateral to the bregma. Then, extracellular fluid buffer (122 mM NaCl, 25 mM $NaHCO_3$, 3 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.4 mM K_2HPO_4, 10 mM D-glucose, and 10 mM Hepes, pH 7.4) containing 96 nCi [^{3}H]benzylpenicillin and 4.8 nCi [^{14}C]inulin was injected over a period 1 min using a 5.0-μl microsyringe (Hamilton Reno) fitted with a fine needle at a depth of 2.5 mm from the surface of the scalp, i.e., the secondary somatosensory cortex 2 (S2) region. The needle was left in this configuration for an additional 4 min to prevent reflux of the injected solution along the injection track, before being slowly retracted. After 40 min, the whole brain was subsequently removed and the left cerebrum was isolated. After weighing each of these, tissue samples were solubilized in 2 N NaOH at 60 °C for 1 h and then mixed with Hionic-fluor (Packard). The radioactivity in each sample was assayed in a liquid scintillation counter equipped with an appropriate crossover correction for ^{3}H and ^{14}C (LS-6500; Beckman).

The BEI was defined by Eq. (1) and the percentage of substrate remaining in the ipsilateral cerebrum was determined from Eq. (2).

$$\mathrm{BEI}(\%) = \frac{\text{test substrate undergoing efflux at the BBB}}{\text{test substrate injected into the brain}} \times 100 \quad (1)$$

$$100\text{-BEI}(\%) = \frac{(\text{amount of test substrate in the brain}/\text{amount of reference in the brain})}{(\text{concentration of test substrate injected}/\text{concentration of reference injected})} \times 100. \quad (2)$$

The percentage of [^{3}H]benzylpenicillin remaining in the brain is given by (100-BEI).

The data were used when the remaining amount of [^{14}C]inulin in the brain was more than 15% of the injected amount. No significant difference was observed in the remaining percentage of [^{14}C]inulin, which is a non-permeable marker, among all samples (#1, 39.7 ± 3.5%; #2, 27.5 ± 3.4%; #3, 31.4 ± 2.9%; #2 shuffle, 28.9 ± 2.2%) (ANOVA), showing that the hydrodynamic injection of siRNA did not damage the integrity of BBB.

Data analysis. All data represent means ± SEM. An unpaired, two-tailed Student's *t* test was used to determine the significance of differences between two group means. (The difference is certified when $P < 0.05$.)

Results

siRNA directed against the OAT3 and SOD1 genes

Sense sequences of the siRNA designed to OAT3 and SOD1 genes are described as follows. The siRNA of shuffle sequence of siRNA OAT3 #2 and siRNA against unrelated gene, GBV-B virus, were used as negative controls. Uppercase letters at 3′ end indicate deoxyribonucleotides.

siRNA OAT3 #1:	5′-ucuacaacagcaccagagaTT-3′
siRNA OAT3 #2:	5′-ccauuaucuugaauguggaTT-3′
siRNA OAT3 #3:	5′-aaacaaagcaggagccagaTT-3′
siRNA-shuffle sequence:	5′-agugguaaugucuauauccTT-3′
siRNA-unrelated control:	5′-agugguaaugucuauauccTT-3′
siRNA SOD1:	5′-gguggaaaugaagaaaguaTT-3′

Effect of siRNA on expression and function of recombinant OAT3 in culture cells

siRNA OAT3 #2 most effectively reduced the expression of OAT3 in HEK293 cells by 86.2% on luciferase activity compared with control siRNA with shuffle sequence of siRNA OAT3 #2 (Fig. 1). siRNA OAT3 #1 and #3 were moderately effective.

To investigate the inhibition effect of siRNA OAT3 to its efflux function in vitro, we measured uptake of OAT3 substrate, [^{3}H]benzylpenicillin. After expression of OAT3

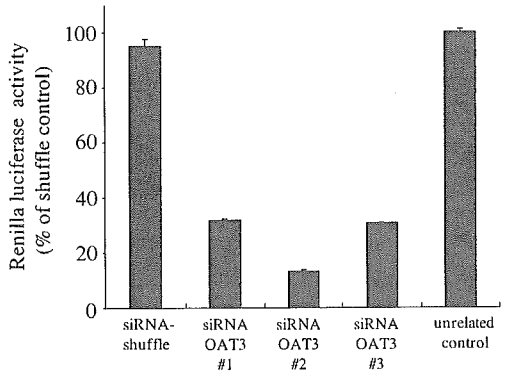

Fig. 1. Effect of siRNAs directed against the OAT3 in vitro. HEK293 cells were transfected with *Renilla* luciferase-fused OAT3 expression vector, *firefly* luciferase expression vector, and 25 nM siRNA. Reduction effect of *Renilla* luciferase activity relative to *firefly* luciferase activity was analyzed. Negative controls were the siRNA with randomized sequence of siRNA OAT3 #2 (siRNA-shuffle) and the siRNA against unrelated gene. Data were averaged from three experiments with SEM indicated.

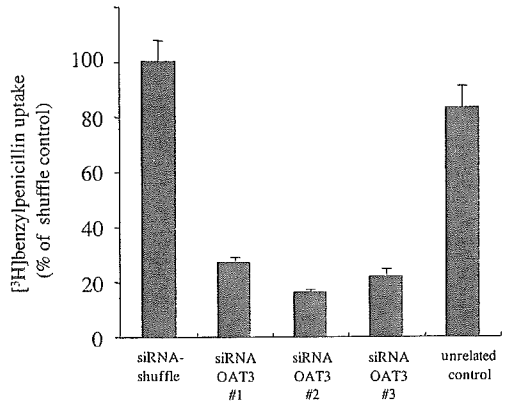

Fig. 2. Effect of siRNAs on uptake of OAT3 substrate in culture cells. Effect of siRNAs OAT3 on the OAT-3-mediated [^{3}H]benzylpenicillin uptake in HEK293 cells. After expression of OAT3 to the cells, [^{3}H]benzylpenicillin uptake was performed at 2 min, reflecting the initial uptake phase. All siRNAs were used at a concentration of 100 nM. Each value represents the mean ± SEM ($n = 4$). The increased uptake by expression of OAT3 was significantly reduced by siRNA OAT3 compared to siRNA-shuffle and siRNA-unrelated control. ($p < 0.0001$).

to HEK293 cells, the uptake mediated OAT3 was increased, and siRNA OAT3 #2 significantly inhibited the increased uptake of the substrate in HEK293 cells, compared with siRNA-shuffle and siRNA-unrelated control (Fig. 2).

In vivo delivery of siRNA to brain endothelial cells

We biochemically investigated an inhibitory effect of siRNA on expression of endogenous protein in BCECs using brain vascular fraction of small vessels from mouse brain.

For detection of endogenous protein in BCECs, we used SOD1 and siRNA to SOD1, because we have confirmed the efficient in vivo effect of this siRNA to endogenous mouse SOD1 in the siRNA-overexpressed transgenic mouse [14].

Western blot of the mouse brain small vascular fraction showed a reduction of endogenous mouse SOD1 level after hydrodynamic injection of siRNA SOD1 (Fig. 3A, left), whereas SOD1 level in the total homogenate of brain did not change (data not shown). There was a potentially more significant level of reduction on a per-BCEC basis, because the brain small vascular fraction contained proteins from cells other than BCECs such as pericytes and astrocytes [15]. We roughly estimated the content of BCECs in the brain small vascular fraction by performing a Western blot analysis with antibody to glucose-transporter-1 (GLUT-1) which specifically expressed in BCECs (Fig. 3B). The band intensity of GLUT-1 in the brain small vascular fraction was 4.1 (± 0.58) times more than that in mouse brain capillary endothelial cell lines which we previously established [12] (Fig. 3B). Since the cell line contains more than 1/8 of GLUT-1 [12], around 50% protein of the brain small vascular fraction that we made was supposed to come from brain endothelial cells.

In contrast, there was not obvious reduction of SOD1 level in the small vascular fraction after a regular intrave-

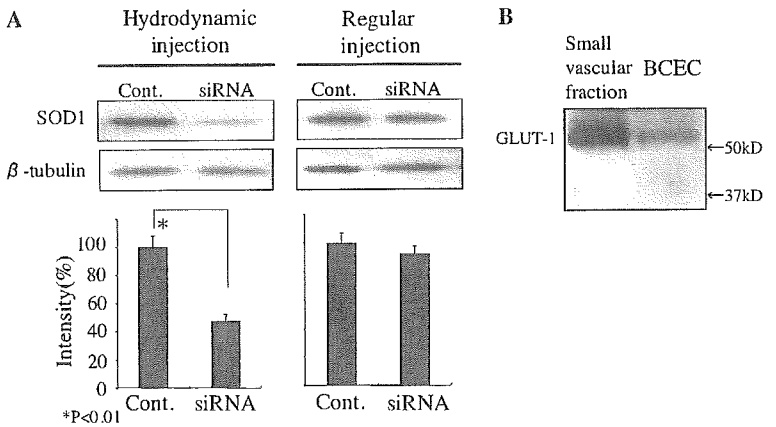

Fig. 3. Western blot analysis of mouse brain capillary-rich fraction. (A) The mouse brain small vascular fraction was examined on Western blot analysis after hydrodynamic (left) and regular (right) injection of 50 μg siRNA SOD1. The lower panels indicate percentages of signal intensities of SOD1 normalized with that of tubulin. (B) Western blot analysis with 2.5 μg protein of anti-GLUT-1 antibody of the mouse brain small vascular fraction (left) and mouse brain capillary endothelial cell lines (right). Signal intensity of GLUT-1 in the mouse brain small vascular fraction is 4.1 (± 0.58) times more than that in mouse brain capillary endothelial cell lines. BCEC, brain capillary endothelial cell line cells.

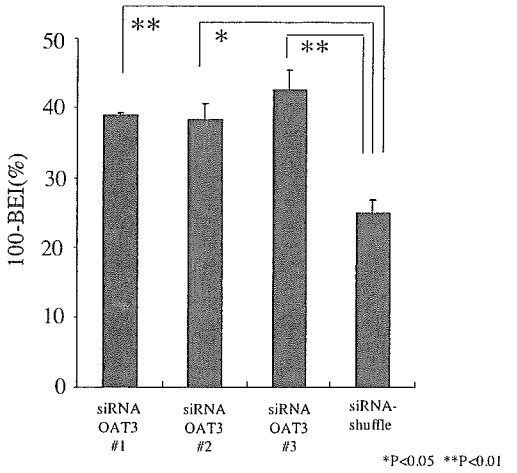

Fig. 4. Effect of siRNA on transport function of OAT3 by BEI. The 50 μg siRNA dissolved in the 5–10% volume PBS of mouse body weight was rapidly injected into the tail vein 36 h before the BEI assay. The residual radioactivity of OAT3 substrate, [^{3}H]benzylpenicillin in the brain, was measured at 40 min after intracerebral injection.

nous injection (Fig. 3A, right). These results indicate that hydrodynamic injection method is effective for delivery of siRNA to brain capillary endothelial cells.

In vivo effect of siRNA on transporter function of OAT3 in vivo

The in vivo inhibitory effect of siRNA OAT3 on the brain-to-blood efflux transport was examined with BEI method with intracerebral injection of OAT3 substrate, [^{3}H]benzylpenicillin. We intravenously injected siRNA OAT3 #2 to 11 mice, siRNA OAT3 #1 and #3 to 3 mice each, and siRNA-shuffle (control) to 7 mice with hydrodynamic method. Transport function of OAT3 was evaluated by BEI method at 36 h after the injection of siRNA. The results of 100-BEI, percentage of OAT3 substrate remaining in the brain after injection, are shown in Fig. 4. The value of 100-BEI of siRNA OAT3 #2 is significantly higher than that of siRNA-shuffle by 26.4%. Those of siRNA OAT3 #1 and #3 were also similarly higher than that of control. The results that plural different siRNAs to the OAT3 gene similarly increased 100-BEI value indicated that these siRNA influences were not "off-target effect" on molecules other than OAT3 in the brain. Taken together, these results suggested that in vivo applied-siRNA to OAT3 could suppress the brain-to-blood efflux function of OAT3.

Discussion

This is the first report of successful in vivo inhibition of endogenous gene in BCECs by systemic intravenous injection of siRNA. Furthermore, we demonstrated that our gene silencing effect was enough to suppress the transport function of OAT3 endogenously expressed in BCECs at BBB. We could deliver siRNA to BCECs by hydrodynamic injection method, but not by regular intravenous injection from the mouse tail vein. It has been thought that a rapid injection of a large bolus of solution develops a high pressure in the inferior vena cava, causing retrograde movement of the solution to the abdominal organs including liver and kidneys. Such a sharp increase in venous pressure enlarges the liver fenestrae and promotes membrane permeability of the hepatocytes, making siRNA enter the cells [16]. Since BCECs are circulated from the tail vein via lung capillary, the phasic hydrodynamic pressure in the inferior vena cava should decrease in the lung. However, rapid loading of extremely large volume of solution, 40–80% of circulating plasma volume should considerably increase hydrostatic pressure in the carotid artery due to volume overload. In addition, the rapid injection of large volume solution prevents the solution from being mixed with the serum containing RNase and keeps the concentration of siRNA extremely high when it is delivered to BCECs.

This in vivo knockdown method with siRNA to BCECs is expected to be a powerful tool for investigating function of BBB. The BBB is formed by the tight intercellular junctions of BCECs and regulates CNS homeostasis and drug delivery by restricting the transfer of substances between the circulating blood and the brain [17]. We have developed Brain Efflux Index as a reliable in vivo method of analyzing efflux transport at the BBB [18]. The efflux function of a transporter protein expressed in BCECs, such as OAT3, can be well evaluated by combining in vivo knockdown method with siRNA and BEI method.

Since synthetic siRNA does not work in the cells for no more than six days [19], long-term silencing of the target gene is necessary for investigating other functions of BCECs in the pathophysiology of atherosclerosis and Alzheimer's disease. Long-standing gene suppression can be achieved in vivo with adenovirus and adeno-associated virus (AAV) vectors expressing short hairpin RNA (shRNA) [20,21]. Actually, with the adenovirus expressing shRNA to SOD1 gene (2.0×10^9 pfu), we could efficiently suppress the endogenous SOD1 level of brain capillary-rich fraction by regular intravenous injection into mouse tail vein (unpublished data). For the evaluation of BCEC function, however, the AAV may be better than adenovirus, because BBB function should be less affected due to limited local immune response to the AAV capsid [22].

The hydrodynamic injection does not cause marked injury to organs in the animals [23], but it is hard to be clinically applied to patients because of its extremely high hydrostatic pressure and volume overload. Possible alternate is a regional delivery of large dose siRNA into carotid artery, but development of less invasive systemic delivery system in vivo is necessary for a therapeutic application of siRNA. Novel cationic liposomes have been reported to transduce efficiently siRNA into the liver [24] as well as tumor tissue [25]. These siRNAs formulated with cationic liposomes also induce interferons and cytokines in vivo through toll-like receptors [26,27] which should change the BBB function. Recently, the lipid-conjugated siRNA

at the 5′-end of the sense strand enhanced cellular uptake and gene silencing [28]. Combined with chemical modification of 2′-O-methylation and phosphorothioate to stabilize siRNA, substantial gene silencing in the liver and jejunum was achieved by a regular intravenous injection into the mouse tail vein [29]. Now, we are trying to use these new siRNA delivery methods to achieve more effective, stable, and safe gene suppression in BCECs for a clinical application.

Acknowledgments

We thank Dr. Tadashi Kanouchi and Miss Tsubura Takahashi for their help. This work was supported by grants from the Ministry of Education, Science and Culture of Japan and from the Ministry of Health, Labor and Welfare of Japan.

References

[1] R. Paul, Z.G. Zhang, B.P. Eliceiri, Q. Jiang, A.D. Boccia, R.L. Zhang, M. Chopp, D.A. Cheresh, Src deficiency or blockade of Src activity in mice provides cerebral protection following stroke, Nat. Med. 7 (2001) 222–227.

[2] C.J. Frijns, L.J. Kappell, Inflammatory cell adhesion molecules in ischemic cerebrovascular disease, Stroke 33 (2002) 2115–2122.

[3] J.J. Archelos, S. Jung, M. Mäurer, M. Schmied, H. Lassmann, T. Tamatani, M. Miyasaka, K.V. Toyka, H.P. Hartung, Inhibition of experimental autoimmune encephalomyelitis by an antibody to the intercellular adhesion molecule ICAM-1, Ann. Neurol. 34 (1993) 145–154.

[4] G.J. del Zoppo, T. Mabuchi, Cerebral microvessel responses to focal ischemia, J. Cereb. Blood Flow Metab. 23 (2003) 879–894.

[5] D.H. Miller, O.A. Khan, W.A. Sheremata, L.D. Blumhardt, G.P. Rice, M.A. Libonati, A.J. Willmer-Hulme, C.M. Dalton, K.A. Miszkiel, P.W. O'Connor, International Natalizumab Multiple Sclerosis Trial Group, A controlled trial of natalizumab for relapsing multiple sclerosis, N. Engl. J. Med. 348 (2003) 15–23.

[6] P.H. Chan, Reactive oxygen radicals in signaling and damage in the ischemic brain, J. Cereb. Blood Flow Metab. 21 (2001) 2–14.

[7] S. Hori, S. Ohtsuki, M. Ichinowatari, T. Yokota, T. Kanda, T. Terasaki, Selective gene silencing of rat ATP-binding cassette G2 transporter in an in vitro blood-brain barrier model by short interfering RNA, J. Neurochem. 93 (2005) 63–71.

[8] S. Ohtsuki, T. Kikkawa, S. Mori, S. Hori, H. Takanaga, M. Otagiri, T. Terasaki, Mouse reduced in osteosclerosis transporter function as an organic anion transporter 3 and is localized at albuminal membrane of blood-brain barrier, J. Pharmacol. Exp. Ther. 309 (2004) 1273–1281.

[9] F. Liu, Y.K. Song, D. Liu, Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA, Gene Ther. 6 (1999) 1258–1266.

[10] T. Yokota, K. Igarashi, T. Uchihara, K. Jishage, H. Tomita, A. Inaba, Y. Li, M. Arita, H. Suzuki, H. Mizusawa, H. Arai, Delayed-onset ataxia in mice lacking alpha-tocopherol transfer protein: model for neuronal degeneration caused by chronic oxidative stress, Proc. Natl. Acad. Sci. USA 98 (2001) 15185–15190.

[11] T. Kanda, H. Yoshino, T. Ariga, M. Yamawaki, R.K. Yu, Glycosphingolipid antigens in cultured microvascular bovine brain endothelial cells: sulfoglucuronyl paragloboside as a target of monoclonal IgM in demyelinative neuropathy, J. Cell Biol. 126 (1994) 235–246.

[12] K. Hosoya, K. Tetsuka, K. Nagase, M. Tomi, S. Saeki, S. Ohtsuki, T. Terasaki, Conditionally immortalized brain capillary endothelial cell lines established from a transgenic mouse harboring temperature-sensitive Simian Virus 40 large T-antigen gene, AAPS Pharmsci. 2 (2000), [article 27].

[13] A. Kakee, T. Terasaki, Y. Sugiyama, Brain efflux index as a novel method of analyzing efflux transport at the blood-brain barrier, J. Pharmacol. Exp. Ther. 277 (1996) 1550–1559.

[14] Y. Saito, T. Yokota, T. Mitani, K. Ito, M. Anzai, M. Miyagishi, K. Taira, H. Mizusawa, Trangenic small interfering RNA halts amyotrophic lateral sclerosis in a mouse model, J. Biol. Chem. 2005 (in press).

[15] T. Kanda, T. Ariga, H. Kubodera, H.L. Jin, K. Owada, T. Kasama, M. Yamawaki, H. Mizusawa, Glycosphingolipid composition of primary cultured human brain microvascular endothelial cells, J. Neurosci. Res. 78 (2004) 141–150.

[16] G. Zhang, X. Gao, Y.K. Song, R. Vollmer, D.B. Stolz, J.Z. Gasiorowski, D.A. Dean, D. Liu, Hydroporation as the mechanism of hydrodynamic delivery, Gene Ther. 11 (2004) 675–682.

[17] K. Hosoya, S. Ohtsuki, T. Terasaki, Recent advances in the brain-to-blood efflux transport across the blood-brain barrier, Int. J. Pharm. 248 (2002) 15–29.

[18] T. Terasaki, S. Ohtsuki, S. Hori, H. Takanaga, E. Nakashima, K. Hosoya, New approaches to in vitro models of blood-brain barrier drug transport, Drug Discov. Today 8 (2003) 944–954.

[19] S.M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl, Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells, Nature 411 (2001) 494–498.

[20] H. Xia, Q. Mao, H.L. Paulson, B.L. Davidson, siRNA-mediated gene silencing in vitro and in vivo, Nat. Biotechnol. 20 (2002) 1006–1010.

[21] H. Xia, Q. Mao, S.L. Eliason, S.Q. Harper, I.H. Martins, H.T. Orr, H.L. Paulson, L. Yang, R.M. Kotin, B.L. Davidson, RNAi suppresses polyglutamine-induced neurodegeneration in a model of spinocerebellar ataxia, Nat. Med. 10 (2004) 816–820.

[22] M.G. Kaplitt, P. Leone, R.J. Samulski, X. Xiao, D.W. Pfaff, K.L. O'Malley, M.J. During, Long-term gene expression and phenotypic correction using adeno-associated virus vectors in the mammalian brain, Nat. Genet. 8 (1994) 148–154.

[23] J.E. Knapp, D. Liu, Hydrodynamic delivery of DNA, Methods Mol. Biol. 245 (2004) 245–250.

[24] D.R. Sorensen, M. Leirdal, M. Sioud, Gene silencing by systemic delivery of synthetic siRNAs in adult mice, J. Mol. Biol. 327 (2003) 761–766.

[25] J. Yano, K. Hirabayashi, S. Nakagawa, T. Yamaguchi, M. Nogawa, I. Kashimori, H. Naito, H. Kitagawa, K. Ishiyama, T. Ohgi, T. Irimura, Antitumor activity of small interfering RNA/cationic liposome complex in mouse models of cancer, Clin. Cancer Res. 10 (2004) 7721–7726.

[26] A.D. Judge, V. Sood, J.R. Shaw, D. Fang, K. McClintock, I. MacLachlan, Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA, Nat. Biotechnol. 23 (2005) 457–462.

[27] V. Hornung, M. Guenthner-Biller, C. Bourquin, A. Ablasser, M. Schlee, S. Uematsu, A. Noronha, M. Manoharan, S. Akira, A. de Fougerolles, S. Endres, G. Hartmann, Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7, Nat. Med. 11 (2005) 263–270.

[28] C. Lorenz, P. Hadwiger, M. John, H.P. Vornlocher, C. Unverzagt, Steroid and lipid conjugates of siRNAs to enhance cellular uptake and gene silencing in liver cells, Bioorg. Med. Chem. Lett. 14 (2004) 4975–4977.

[29] J. Soutschek, A. Akinc, B. Bramlage, K. Charisse, R. Constien, M. Donoghue, S. Elbashir, A. Geick, P. Hadwiger, J. Harborth, M. John, V. Kesavan, G. Lavine, R.K. Pandey, T. Racie, K.G. Rajeev, I. Rohl, I. Toudjarska, G. Wang, S. Wuschko, D. Bumcrot, V. Koteliansky, S. Limmer, M. Manoharan, H.P. Vornlocher, Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs, Nature 432 (2004) 173–178.

—Mini Review—

RNA Interference as a Tool for Producing Knockdown Mice

Tasuku Mitani[1,2]* and Takanori Yokota[3]

[1]Institute of Advanced Technology, Kinki University,
[2]Gene Control Corporation, 14-1 Minami-Akasaka, Kainan, Wakayama 642-001, Japan
[3]Department of Neurology and Neurological Science, Graduate School, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan

Abstract: *Analysis at the individual level using genetically-engineered mice has allowed conclusions to be reached regarding the actual function of a target gene. In recent years, the "knockdown" method using RNA interference (RNAi) has been established as a powerful tool for analyzing gene function. In this review, we focus on RNAi knockdown technology for producing genetically-engineered mice and describe the value of this approach from the perspective of both basic research and therapeutic potential. First, we introduce the basic mechanism of RNAi and development of knockdown animals from worms to mice. Next, we describe strategies to produce knockdown mice using DNA-based expression vectors introduced into zygotes or embryonic stem cells. Finally, we refer to the trends of research for clinical application. By way of illustration, we show the production of knockdown mice for treatment of neurodegenerative disease and mention the prospect of therapeutic potential of RNAi technology.*
Key words: *RNAi, dsRNA, siRNA, Knockdown, ES cells*

Introduction

The decoding of the human genome has been a turning point in the study of life sciences. Analysis at the individual level using genetically-engineered mice has allowed conclusions to be reached regarding the actual function of a target gene on the basis of phenotypic analysis. Genetic information from mice has become more and more important, because decoding of the mouse genome has made it possible to apply such information to humans, and data from genetically-engineered mice provide findings for the target gene that cannot be obtained from *in vitro* experiments.

To date, genetically-engineered mice are classified into "transgenic mice" and "gene-targeted mice", such as knock-out mice or knock-in mice. The former are produced by microinjection of a DNA fragment into the pronucleus of fertilized eggs [1], and the latter are based on gene targeting that destroys or alters the object gene by exchange with an exogenous targeting vector sequence, using homologous recombination in embryonic stem cells [2]. In recent years, the "knockdown" method using RNA interference (RNAi) has been established in lower animals [3–5] and has also been applied to mammalian cultured cells [6–10] as a powerful tool for analyzing gene function. More recently, production of "knockdown mice" by RNAi has become possible [11, 12].

In this review, we focus on RNAi knockdown for producing genetically-engineered mice and describe the value of this approach from the perspective of both basic research and therapeutic potential.

The development of Knockdown Animals by RNAi from Worms to Mice

RNA interference (RNAi) is a sequence-specific post-transcriptional gene silencing mechanism, which is triggered by double-stranded RNA (dsRNA) and consequently induces degradation of mRNAs with the same sequence of the sense strand of dsRNA (Fig. 1) [13–25]. In 1998, Fire *et al.* first showed this phenomenon in the nematode worm *Caenorhabditis elegans*. When dsRNA was injected into the worm the corresponding gene products disappeared from both the somatic cells of the organism and its F1 progeny [3]. This discovery has led to a new technology allowing rapid reverse genetic analysis, and similar effects were

Received: September 7, 2005
Accepted: September 20, 2005
*To whom correspondence should be addressed.
e-mail: mitani@waka.kindai.ac.jp

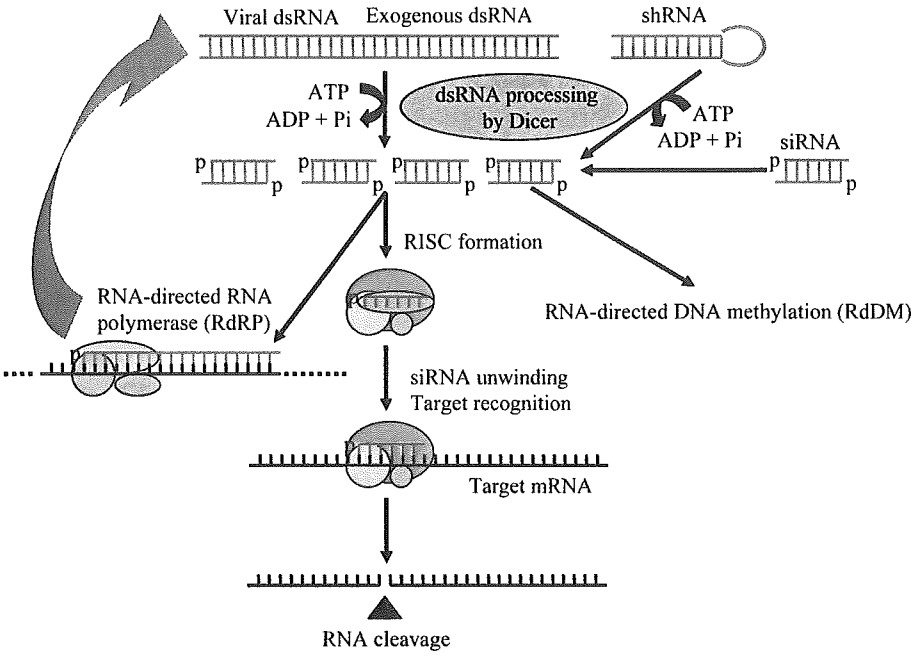

Fig. 1. A model for the mechanism of RNAi. dsRNAs or shRNAs are cleaved by RNase III-type enzymes such as Dicer into 21–25 nt siRNAs. These siRNAs or the introduced siRNAs are then incorporated into a protein complex termed RNA-induced silencing complex (RISC). The unwinding siRNA guides the RISC to homologous region of the target mRNA, ensuring its degradation. The unwinding siRNAs can also serve as primers on the target RNA in association with RNA-dependent RNA polymerases (RdRPs) to subsequently generate secondary siRNAs. Furthermore, besides this post-transcriptional gene silencing (PTGS), siRNAs mediate RNA-directed DNA methylation (RdDM) for silencing the target gene.

immediately demonstrated in fungi such as *Neurospora crassa* [26], and other animals such as *Tripanosoma brucei* [27], *Drosophila melanogaster* [28–31] Zebrafish [32–34] and *Xenopus laevis* [35].

Similar phenomena, referred to as post-transcriptional gene silencing (PTGS) in plants and quelling in fungi, have actually been known for many years [36–40]. Since the RNAi effect was subsequently shown in a wide range of eukaryotes, it was anticipated that a similar effect would occur in mammals. However, gene silencing by RNAi in cultured mammalian cells has been hampered by an antiviral response via dsRNA-triggered pathways that mediate non-specific suppression of gene expression (Fig. 2) [41–46]. dsRNA induces type I interferon (IFN) synthesis, which activates two classes of IFN-induced enzymes: PKR, a dsRNA-dependent protein kinase, which phosphorylates eIF-2α and induces inhibition of translation [42, 43, 45, 46]; and 2',5'-oligoadenylate synthetase, whose products activate the ribonuclease RNase L [43–45]. These pathways induce cell death via apoptosis. Surprisingly, dsRNA can induce sequence-specific gene silencing only in mammalian embryos, because of their lack of such IFN responses against dsRNA. Indeed, microinjection of dsRNA into mouse zygotes results in the RNAi phenomenon for both exogenous transgenes such as green fluorescent protein (*GFP*) [47] and endogenous genes such as c-mos, with phenotypes equivalent to the gene-deficient mice [47, 48]. Tuschl *et al.* first demonstrated that RNA duplexes of 21–23 nt (short interfering RNA: siRNA), which were designed to mimic the naturally processed products from long dsRNA produced by the RNase III enzyme, Dicer, could evade the antiviral response via IFN-induced pathways and induce sequence-specific silencing *in vitro* in Drosophila embryo extracts [49–51]. These results were successfully extended to cultured mammalian somatic cells [6, 52] making the use of siRNA possible as a sequence-specific "gene knockdown" approach, and it has rapidly become popular as a convenient tool for analyzing gene function. However, siRNA can only transiently suppress the expression of target genes,

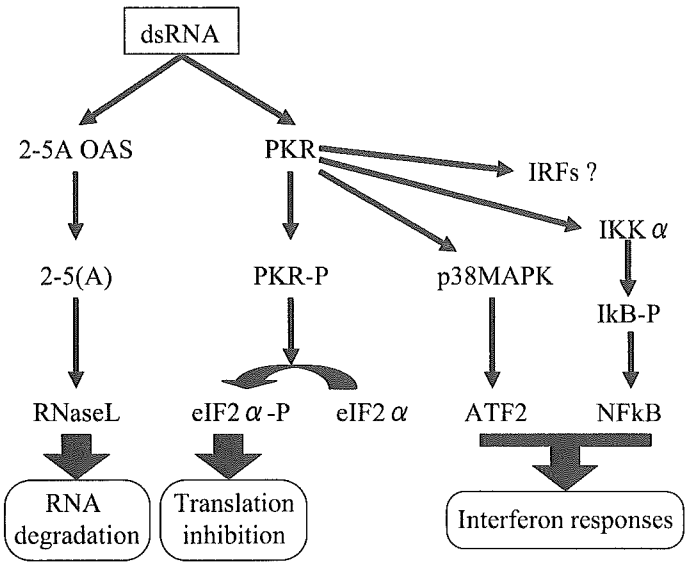

Fig. 2. Model for the diverse actions triggered by dsRNA in mammalian cells. dsRNA activates latent 2'-5'-oligoadenylate synthetase (2–5 OAS) and this enzyme activates RNase L via 2'-5'-oligoadenylates (2–5(A)). Activated RNase L non-specifically degrades single-stranded RNAs. dsRNA also activates protein kinase R (PKR). Activated PKR inhibits protein synthesis by phosphorylation of transcriptional initiation factor eIF2α. Moreover, PKR can function as a signal-transducing kinase interacting with IkB kinase (IKK) and p38 MAPK to mediate the antiviral actions of type I interferons.

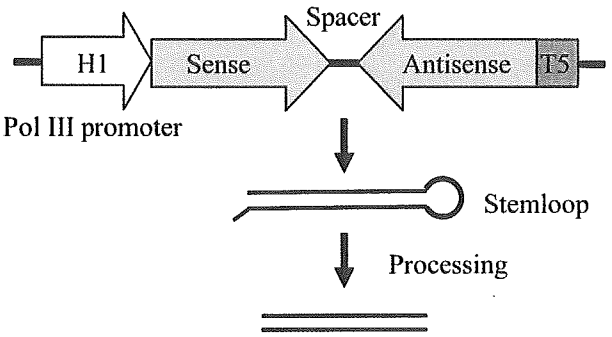

Fig. 3. Strategy for generating siRNA from DNA template *in vivo*. The 19–23 nt long of the sense and antisense regions that target the gene of interest are separated by a spacer of 6–8 nt and are followed by the transcriptional termination signal of five thymidines (T5). The shRNA is formed by folding back into a loop structure and is consequently processed into siRNA.

which seriously restricts its application in the analysis of mammalian organisms. To circumvent this limitation, on the basis of an *in vivo* RNAi expression strategy [53], a mammalian expression vector that synthesizes siRNA-like transcripts has been designed [54, 55]. The transcripts are synthesized under the control of RNA polymerase III (Pol III)-dependent promoters, such as H1 or U6, that effectively express small RNAs lacking poly adenosine tails in a fold-back stem-loop structure, named the short hairpin RNA (shRNA), which are subsequently processed into active siRNAs (Fig. 3). This DNA vector-based approach gave rise to stable suppression of target gene expression. Subsequently, the production of knockdown mice using shRNA expression vectors has been examined.

Generation of Knockdown Mice using a Conventional Transgenic Method: Microinjection of a shRNA-expression Vector into Fertilized Eggs

Hasuwa *et al.* produced transgenic mice by introducing a shRNA-expression vector specific for enhanced green fluorescent protein (*EGFP*) [56]. These mice were mated with *EGFP*-transgenic mice, "green mice", and the double transgenic individuals showed dramatic silencing of *EGFP* throughout their lifetime. This experiment first proved that stable gene

silencing by RNAi was possible in mammalian organisms. However, the RNAi effect disappeared in some F1 progeny derived from founders that showed effective gene silencing, owing to fade-away of shRNA expression, and this observation identified a serious problem that needs to be resolved to allow RNAi technology to be used in mice [57]. Hence, future development of a method for heritable and stable gene silencing in organisms is required to establish stable knockdown mouse strains as a powerful tool for reverse genetics.

To date, suppression of endogenous genes in mammalian organisms by microinjection of a shRNA-expression vector into zygotes has been unsuccessful [11], and we were also unable to produce knockdown mice by this method, as discussed below. Carmell *et al.* constructed shRNA expression vectors directed against a variety of targets with well-known phenotypes, such as tyrosinase (albino), myosin VIIa (shaker), *Bmp-5* (crinkled ears), homogentisate 1,2-dioxygenase (urine turns black upon exposure to air), *Hoxa10* (limb defects), *Hairless* (hair loss) and melanocortin 1 receptor (yellow), and injected these vectors into fertilized mouse eggs. Despite the presence of transgenes in some animals, virtually none of the animals showed a distinct or reproducible phenotype associated with the particular target gene that was expected based on the phenotype of a null mutant or knockout mouse.

Recently, Chang *et al.* produced a knockdown mice using RNAi directed against an endogenous gene, *ABCA1*, which is a member of the ABC transporter protein family, using inducible regulation of RNAi by a combination of Cre-*loxP*- and tetracycline systems in a time- or tissue-dependent manner [58]. In their study, knockdown mice were produced by a conventional transgenic method. This is one of the rare cases reported to date in which a shRNA expression vector has been used in the successful silencing of an endogenous gene, and the reason for the success in this particular case is still unclear. In addition to such conventional methods, viral vectors can also be used to produce transgenic mice [59, 60]. Lentiviral vectors capable of introducing exogenous DNA into zygotes by mixing with zona-free eggs have been developed [60]. Using this strategy, it has been demonstrated that lentiviral vectors expressing siRNA against *GFP*, as illustrated in Fig. 4a, can reduce fluorescence in *GFP*-transgenic embryos and in mice after transduction of eggs [61]. Notably, it has been observed that the lentiviral vector avoids transcriptional shut-off from integrated viral DNA, which might account for the appearance of siRNA expression and the resulting down-regulation of a specific gene, not only in embryos but also in organisms after birth. This methodology has been applied to conditional gene silencing, as described below [58, 62–64].

Generation of Knockdown Mice by Introduction of a siRNA-expression Vector into Embryonic Stem Cells

Theoretically, embryonic stem cells (ES cells) should be suitable for production of knockdown mice. Similar to gene targeting, a shRNA expression vector is introduced into ES cells. Stable integrants that effectively silence the target gene are injected into host blastocysts, and the resulting chimeric mice are mated with wild type mice to produce F1 progeny with transgenes, which show gene silencing of the target gene. Since Carmell *et al.* failed to make knockdown mice by the microinjection method using mouse zygotes, as described above [11], they examined this strategy as an alternative. A shRNA expression vector directed against a novel gene, *Neil1*, was introduced into ES cells, and *Neil1* knockdown mice were successfully produced using stable integrants with strong silencing of the target gene. *Neil1* is a member of a newly discovered family of mammalian DNA *N*-glycosylases, related to the Fpg/Nei family of proteins from *Escherichia coli*, and has been proposed to have a role in DNA repair. Germline transmission of the shRNA expression transgene from chimeras to F1 progeny was achieved and a consequent reduction of the level of Neil1 protein was confirmed. It should be noted that suppression of the target gene expression in F1 mice was similar to that in the ES cells used. These results indicate that the use of ES cells provides a significant advantage in that the knockdown level of the target gene can be practically evaluated in ES cells in culture prior to the production of knockdown mice, which is quite difficult using the microinjection method.

Cells with a shRNA transgene exhibit dominantly gene silencing, and because of this the tetraploid aggregation method [65], in which the embryos are completely derived from ES cells, may make it possible to assess embryonic phenotypes directly. In tetraploid embryos aggregated with ES cells, the host tetraploid cells contribute to the extra-embryonic tissues, including the trophoblast tissue of the placenta and the extra-embryonic endoderm component of the yolk sac, but they are rigorously excluded from the embryo proper,

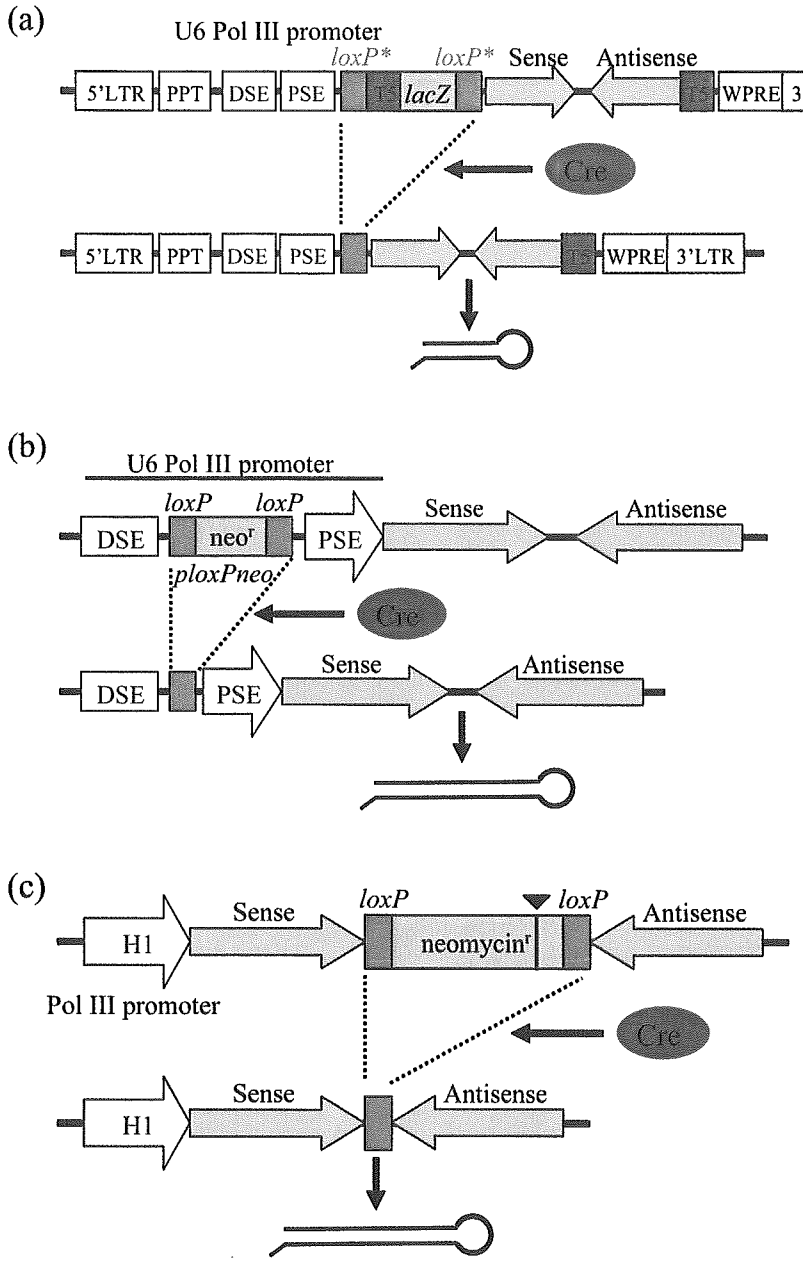

Fig. 4. Diagram of the siRNA expression vector using Cre-*loxP* system. (a) Lentiviral vector for expressing siRNA is inserted with a DNA stuffer sequence (T5/*lacZ*) flanked by two modified *loxP** sites. The *loxP** sequence contains two mutations, which generate the intercalated *loxP* sequence acting as a U6 TATA box after the loop-out of the stuffer sequence by Cre recombinase [63]. (b) A neomycin cassette flanked by two *loxP* sites (*ploxPneo*) separates a Proximal Site Enhancer (PSE) with a Distal Site Enhancer (DSE) of a U6 promoter. Cre recombinase eliminates *ploxP*, allowing the siRNA expression [67, 68]. (c) A neomycin cassette with a Pol III stop codon of 5 thymidines (T5) (arrowhead) is flanked by two *loxP* sites. This cassette is inserted downstream of the sense strand of the inverted repeat sequence. In the presence of Cre recombinase, the neomycin cassette is eliminated and the resulting intercalated *loxP* sequence acts as a loop to fold back the antisense strand [69].