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Gene silencing in primary and metastatic tumors by small interfering RNA delivery in mice: Quantitative analysis using melanoma cells expressing firefly and sea pansy luciferases

Yuki Takahashi, Makiya Nishikawa, Naoki Kobayashi, Yoshinobu Takakura*

Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan
Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan

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Abstract

Silencing of oncogenes or other genes contributing to tumor malignancy or progression by RNA interference (RNAi) offers a promising approach to treating tumor patients. To achieve RNAi-based tumor therapy, a small interfering RNA (siRNA) or siRNA-expressing vector needs to be delivered to tumor cells, but little information about its in vivo delivery has been reported. In this study, we examined whether the expression of the target gene in tumor cells can be suppressed by the delivery of RNAi effectors to primary and metastatic tumor cells. To quantitatively evaluate the RNAi effects in tumor cells, mouse melanoma B16-BL6 cells were stably transfected with both firefly (a model target gene) and sea pansy (an internal standard gene) luciferase genes to obtain B16-BL6/dual Luc cells. The target gene expression in subcutaneous primary tumors of B16-BL6/dual Luc cells was significantly suppressed by direct injection of the RNAi effectors followed by electroporation. The expression in metastatic hepatic tumors was also significantly reduced by an intravenous injection of either RNAi effector by the hydrodynamics-based procedure. These results indicate that the both RNAi effectors have a potential to silence target gene in tumor cells in vivo when successfully delivered to tumor cells.

Keywords: RNAi; Gene delivery; Hydrodynamics-based procedure; Hepatic tumor; Luciferase

1. Introduction

RNA interference (RNAi) is a post-transcriptional gene silencing event in which short double-stranded RNA (siRNA) degrades target mRNA in a sequence-specific manner [1–3]. After the discovery that the use of short double-stranded RNA can induce RNAi in

* Corresponding author. Tel.: +81 75 753 4615; fax: +81 753 4614.
E-mail address: takakura@pharm.kyoto-u.ac.jp (Y. Takakura).

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mammalian cells without a sequence-nonspecific response [4,5], RNAi has been widely used as an experimental tool to suppress specific gene expression for research involving gene function. This is because RNAi is attractive for its speed, usefulness, and lower cost, compared with conventional strategies to suppress gene function, such as gene knock-out by homologous recombination, and is generally more powerful than antisense strategies [6,7]. Moreover, RNAi is expected to be used as a therapeutic tool in the treatment of various diseases, such as cancer, viral infections, and neurodegenerative disorders [8,9].

Tumor cells have at least two major abnormalities: dysregulation of the cell cycle leading to uncontrolled growth, and resistance to death resulting from abnormalities in one or more genes that mediate apoptosis. Therefore, silencing these genes by RNAi offers a therapeutic treatment for cancer patients. In the last 5 years, there have been a number of reports in the literature describing how induction of RNAi suppresses these genes and decreases the malignancy of tumor cells in vitro. However, a few reports have described the successful treatment of tumors by the induction of RNAi in vivo [10–13]. Poor delivery of RNAi effectors may be the major reason for the limited success in tumor therapy by RNAi in vivo in contrast to the many successes in vitro. One reason for this is that RNAi effectors are water soluble macromolecules and so they have difficulty in crossing cellular membranes and their gene silencing effect is limited in the cells that have received RNAi effectors [14,15]. Regarding the delivery of RNAi effectors, the authors reporting successful in vivo tumor therapy administrated naked siRNA, siRNA or siRNA-expressing vector complexed with a cationic carrier. Duxbury et al. [13] and Verma et al. [10] did not evaluate the delivery of siRNA to tumor cells in detail but they examined the therapeutic effects produced by silencing the target genes. To inhibit tumor growth by siRNA targeting to vascular endothelial growth factor, Filleur et al. [11] investigated the effect of the routes of administration of siRNA on the gene silencing efficiency in subcutaneous tumors. Zhang et al. [12] administered siRNA-expressing plasmid DNA (pDNA) encapsulated in liposomes coated with antibody targeting the brain tumor and succeeded in suppressing gene expression in the tumor. However, the delivery of RNAi effectors to tumor cells and intratumoral gene silencing in vivo has received little attention in any of these previous studies.

RNAi can be induced by the delivery of siRNA or siRNA-expressing vector, which works as a platform to produce siRNA within the target cell for a relatively long period. Some groups have developed vector-based siRNA expression systems [16–19], which showed effective RNAi induction. The molecular weight of siRNA-expressing vector is several hundred-fold greater than that of siRNA and must be delivered to the nucleus of target cells, unlike siRNA which can work if it is present in the cytoplasm [14]. Therefore, siRNA-expressing vector is more problematic than siRNA as far as delivery to the target cells is concerned. However, siRNA-expressing vector has the advantages of a sustained effect and ease in regulating its function compared with siRNA. Moreover, siRNA-expressing vectors that have an on-off switch for siRNA have also been reported [20]. Both RNAi effectors have advantages and disadvantages, and so should be used as the situation demands.

In either case of siRNA or siRNA-expressing vectors, the delivery of RNAi effectors to the tumor cells is the key factor for treating tumor patients with RNAi, because the gene silencing effect is limited in the cells that receive RNAi effectors. To improve RNAi-based tumor therapy, efficient RNAi effector delivery systems and delivery methods need to be developed. We considered that a model system in which the gene silencing effect can be sensitively evaluated may be a powerful tool for achieving successful in vivo RNAi-based tumor therapy. As used in previous studies involving the simultaneous administration of target gene and RNAi effectors [21], we think that clones of tumor cells stably expressing reporter genes, such as luciferase and green fluorescent protein, will be useful for examining whether siRNA or siRNA-expressing vector is effective in reducing target gene expression in tumor cells in vivo. Some research groups have introduced marker genes to detect tumor cells in vivo [22,23]. We selected firefly (model target gene of RNAi) and sea pansy luciferases (indicator of tumor cell number) for detecting in vivo RNAi, because (i) these luciferases can be detected with a high degree of sensitivity and (ii) they can be simultaneously and quantitatively measured using a simple luminometric assay. Therefore, mouse melanoma B16-BL6 cells were stably

transfected with firefly and sea pansy luciferases to obtain B16-BL6/dual Luc cells. This cell clone has advantages in evaluating in vivo intratumoral gene silencing effect in that we can investigate the effect easily, sensitively, and quantitatively. A subcutaneous primary tumor model and a hepatic metastasis model were selected as the targets of the delivery. There are no literature reports about gene silencing in metastatic tumors in the liver. As delivery methods, we used naked siRNA or pDNA without any viral or cationic carriers. We investigated whether the gene transfer methods reported to result in high transgene expression in the liver or muscle can deliver RNAi effectors into tumor cells inoculated in the liver or under the skin. Our data suggest one solution to the question of how the in vivo intratumoral delivery of siRNA can be achieved.

2. Materials and methods

2.1. Plasmid DNA

pCMV-Luc encoding firefly luciferase$^+$ and neomycin-resistant gene was used to construct the cell line stably expressing firefly luciferase. The pDNA was constructed by subcloning the Hind III /Xba I firefly luciferase$^+$ cDNA fragment from pGL3-control (Promega, Madison, WI, USA) vector into the polylinker of the pcDNA3 vector [24]. pCMV-RL/Hygro encoding sea pansy *Renilla reniformis* luciferase and hygromycin-resistant gene was transfected to obtain the cell line stably expressing sea pansy luciferase. The pDNA was constructed by subcloning the Nhe I/BamH I sea pansy luciferase cDNA fragment from pRL-CMV vector (Promega) into the multicloning site of the pcDNA3.1/Hygro vector (Invitrogen, Carlsbad, CA, USA). pEGFP-F encoding farnesylated EGFP (enhanced green fluorescent protein), a modified form to bind to the plasma membrane, and pDsRed2-N1 encoding red fluorescent protein Dsred2 were purchased from BD Biosciences Clontech (Palo Alto, CA, USA). We used pEGFP-F for the genetic labeling of tumor cells to avoid effusion of the gene product following the hydrodynamics-based injection, since the unmodified EGFP might diffuse into the circulation following a large volume injection [25]. Each pDNA was amplified in the DH5α strain of *Escherichia coli* and purified using a QIAGEN Endofree Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany).

2.2. Construction of stably expressing cell lines

Murine melanoma cell line B16-BL6 [26] and genetically labeled B16 cell lines were cultured in Dulbecco's modified Eagle's minimum essential medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin/L-glutamine (PSG) at 37 °C and 5% CO_2. B16-BL6 plated on 6-well plates was transfected with pCMV-Luc using Lipofectamine 2000 (Invitrogen) to obtain B16-BL6 expressing the firefly luciferase, B16-BL6/Luc as reported previously [27]. B16-BL6/Luc plated on 6-well plates was then transfected with pCMV-RL/Hygro using Lipofectamine 2000, and the cells were selected using 1 mg/ml of hygromycin for 14–20 days to obtain a clone expressing the highest sea pansy luciferase activity, B16-BL6/dual Luc. The selected clone continuously expressed the firefly and sea pansy luciferases of activities to about 3 and 10 RLU/cell, respectively. In addition, B16-BL6/EGFP-F that stably expresses EGFP-F was also prepared by the same procedure as B16-BL6/Luc using pEGFP-F. The EGFP-F-expressing cell clones were screened and selected by confocal microscopic observations (MRC-1024; Bio-Rad, Hercules, CA, USA).

2.3. siRNA and siRNA-expressing pDNA

siRNA-expressing pDNAs driven by human U6 promoter were constructed from piGENE hU6 vector (iGENE Therapeutics, Tsukuba, Japan) according to the manufacturer's instructions. pU6-siGL3 transcribes a single-stranded RNA 5′-GUG CGU UGU UGG UGU UAA UCC UUC AAG AGA GGG UUG GCA CCA GCA GCG CAC UUU U-3′, which forms stem-loop-structured siRNA, targeted to firefly luciferase$^+$ mRNA (targeted sequence: GTG CGC TGC TGG TGC CAA CCC), with loop sequences of UUCAAGAGA [17]. pU6-siGL2 produces siRNA whose target sequence is not found in this study. piGENE hU6 vector, which transcribes the non related sequence of RNA 5′-GUG AGC AGG UGU AAA GCC ACC AUG GAA GAC ACC UGC CAA CUU UU-3′ with partial duplex formation, was used as a

control pDNA throughout the present study. Synthetic siRNA targeted to the mRNA of pGL3 firefly luciferase$^+$ (target sequence: same as pU6-siGL3) was purchased from Takara Bio (Otsu, Japan).

2.4. In vitro transfection

B16-BL6/dual Luc cells were plated on 24-well culture plates (at a density of 1×10^5 cells/well) or on 6-well culture plates (at a density of 5×10^5 cells/well). After an overnight incubation, transfection of pDNA or siRNA was performed using Lipofectamine 2000 at a final concentration of 2 μg pDNA/ml or 40 pmol siRNA/ml according to the manufacturer's instructions. In brief, 1 μg pDNA or 60 pmol siRNA was mixed with 3 μg Lipofectamine 2000, and the resulting complex was added to the cells. At 24 h after transfection (unless otherwise mentioned), the cells were lysed and the luciferase activity as well as the amount of mRNA were measured.

2.5. mRNA quantification

Total RNA was isolated using MagExtractor MFX-2100 and MagExtractor RNA kit (TOYOBO, Osaka, Japan) following manufacturer's protocol. Before reverse transcription, the total RNA was treated with DNase I (Takara Bio). Reverse transcription was performed using a SuperScript II (Invitrogen) and dT-primer following the manufacturer's protocol. For quantitative mRNA expression analysis, real time PCR was carried out with total cDNA using a LightCycler instrument (Roche Diagnostics, Basle, Switzerland). The oligodeoxynucleotide primers used for amplification were GL3-fw: 5′-CGC ATG CCA GAG ATC CTA TT-3′, GL3-rev: 5′-TAC CTG GCA GAT GGA ACC TC-3′ yielding an expected amplification product of 348bp specific for firefly luciferase cDNA, and RL-fw: 5′-GAT AAC TGG TCC GCA GTG GT-3′, RL-rev: 5′-ACC AGA TTT GCC TGA TTT GC-3′ yielding an expected amplification product of 223bp specific for sea pansy luciferase. Amplification products were detected on-line via intercalation of the fluorescent dye SYBR green (LightCycler-FastStart DNA Master SYBR Green I kit, Roche Diagnostics). The cycling conditions for GL3 and RL were as follows: initial enzyme activation at 95 °C for 10 min, followed by 55 cycles at 95 °C for 10 s, 60 °C for 5 s, and 72 °C for 20 s. All cycling reactions were performed in the presence of 3.5 mM $MgCl_2$. Gene-specific fluorescence was measured at 72 °C.

2.6. Animals

Seven-week-old male C57/BL6 mice, purchased from Shizuoka Agricultural Cooperative Association (Shizuoka, Japan) were used for all experiments. All animal experiments were conducted in accordance with the principles and procedures outlined in the US National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocols for animal experiments were approved by the Animal Experimentation Committee of Graduate School of Pharmaceutical Sciences of Kyoto University.

2.7. Subcutaneous primary tumor model

Genetically-labeled B16-BL6 cells in the exponential growth phase were harvested by trypsinization and suspended in Hanks' balanced salt solution (HBSS, Nissui Pharmaceutical). The tumor cells (2×10^5 cells) were injected into the footpad of syngeneic C57/BL6 mice. At indicated times after tumor inoculation, mice received an intratumoral injection of the described amount of pDNA or siRNA dissolved in 50 μl saline (unless stated otherwise).

Some mice received an intratumoral injection of pDNA complexed with polyvinylpyrrolydone (PVP, average molecular weight 50 kD, Sigma, St Louis, MO, USA) as described previously [28,29]. In brief, pDNA was dissolved in 5% PVP in 150 mM NaCl. Some groups of mice received electric pulses following intratumoral injection. Twelve electric pulses at the indicated V/cm, of 5 ms duration and 4 Hz were delivered by a pair of 1-cm^2 forceps-type electrodes connected to a rectangular direct current generator (CUY21, Nepagene, Chiba, Japan). Where indicated, elastase (Worthington Biochemical Corporation, Lakewood, NJ, USA), collagenase (type 1-A, Sigma) or hyaluronydase (type VI-S, Sigma) were resuspended in 50 μl saline at the described concentration and injected more than 30 min before electroporation, because the use of these digestive enzymes are reported to increase transgene expression in skeletal muscle by an intramuscular injection of pDNA followed by electroporation [30,31].

2.8. Hepatic metastasis tumor model

Genetically-labeled B16-BL6 cells in the exponential growth phase were harvested by trypsinization and suspended in HBSS. Under ether anesthesia, a midline abdominal incision was made to expose the portal vein, and 1×10^5 B16-BL6/dual Luc cells were injected into the portal vein. Then the opening was sutured and mice were allowed to recover. At indicated times, mice received an intravenous injection of pDNA or siRNA. The intravenous injection was performed by the hydrodynamics-based procedure [32] where pDNA or siRNA dissolved in saline with a volume of 10 % body weight was injected into the tail vein within less than 5 s using a 26-gauge needle. As reported in the literature [32], we confirmed that high transgene expression was achieved in the liver by this procedure.

2.9. Luciferase assay

To determine luciferase activity in vitro, B16-BL6/dual Luc cells were lysed using the cell lysis buffer of an assay kit (PiccageneDual, Toyo Ink, Tokyo, Japan). Then, samples were mixed with the kit luciferase assay buffer, and the chemiluminescence produced was measured in a luminometer (Lumat LB9507, EG and G Berthold, Bad Wildbad, Germany).

To determine luciferase activities in vivo, mice underwent euthanasia at the indicated time and the tumor-bearing liver or the subcutaneous tumor was excised and homogenized in 5 ml/g (liver) or 10 ml/g (tumor) lysis buffer (0.1 M Tris, 0.05% Triton-X-100, 2 mM EDTA, pH 7.8). The homogenate was centrifuged at 13,000 $\times g$ for 10 min at 4 °C. Then the supernatant was mixed with the luciferase assay buffer, and the chemiluminescence produced was measured in a luminometer.

Following subtraction of the background activity using the lysates of B16-BL6 cells, the liver homogenate without tumor inoculation or B16-BL6 tumor homogenate, the ratio of the activity firefly luciferase$^+$ (*Pp*-Luc$^+$) to sea pansy luciferase (*Rr*-Luc) was calculated to correct for differences in the number of tumor cells in each sample. The ratios were normalized to give percentage values relative to those of the corresponding control group.

2.10. Confocal microscopic study of mouse liver sections

Mice were inoculated with B16-BL6/EGFP-F via the portal vein. After 13 days, mice received systemic administration of pDsRed2-N1 by the hydrodynamics-based procedure. Under ether anesthesia, mice were euthanized by cutting the vena cava, and the liver was gently infused with 2 ml saline through the portal vein to remove the remaining blood. The liver was then placed in Tissue-Tek OCT embedding compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan), frozen in liquid nitrogen, and stored in 2-methyl butanol at −80 °C until use. Frozen liver sections (8-μm thick) were obtained by cryostat (Jung CM 3000, Leica Microsystems AG, Wetzlar, Germany) using a routine procedure. The sections were directly subjected to confocal microscopy (MRC-1024; Bio-Rad, Hercules, CA, USA) without any fixation, since the fixation step caused massive loss of DsRed due to immediate dissolution in the fixation buffer as demonstrated in our preliminary experiments.

3. Statistical analysis

Differences were statistically evaluated by Student *t* test or one-way analysis of variance (ANOVA) followed by the Dunnet's test for multiple comparisons. *P*-value of less than 0.05 was considered to be statistically significant.

4. Results

4.1. Construction of tumor cell lines stably expressing model endogenous genes

B16-BL6 cells were transfected with firefly luciferase$^+$ cDNA as a target gene for siRNA-mediated gene silencing, and with sea pansy luciferase cDNA as an internal standard gene that was supposed not to be affected by the treatment. B16-BL6/Luc cells stably expressed the firefly luciferase, and B16-BL6/dual Luc cells expressed both luciferases. The luciferase activities of these cells were found to be stable, and proportional to the number of cells from 10^3 to 10^6 cells/μl. The ratio of firefly luciferase

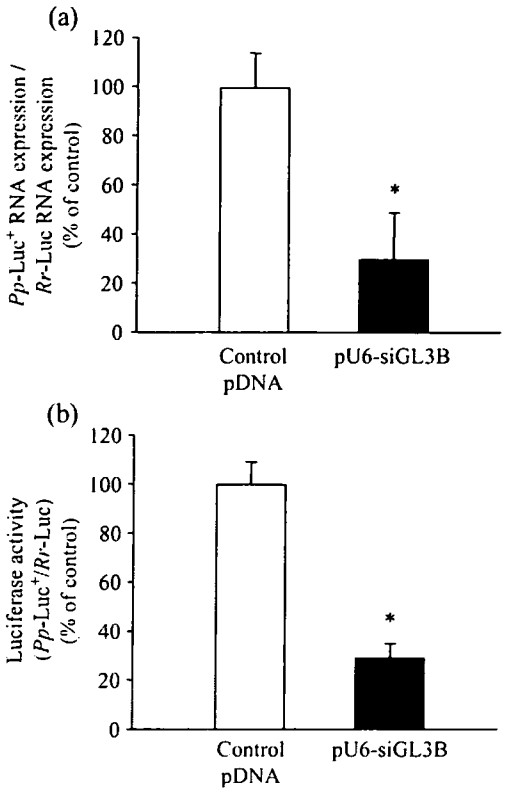

Fig. 1. Reduction of both mRNA expression and luciferase activity in B16-BL6/dual Luc following transfection of siRNA-expressing pDNA. B16-BL6/dual Luc inoculated on 6-well plates was transfected with piGENE hU6 (3 μg) or pU6-siGL3 (3 μg) using Lipofectamine 2000. Amounts of mRNA (a) and luciferase activities (b) were simultaneously measured 24 h after transfection. *Pp*-Luc$^+$ means firefly luciferase$^+$ and *Rr*-Luc means sea pansy luciferase. The results are expressed as the mean ± S.D ($n=5$). $^*P<0.05$ for Dunnet's test versus control group.

activity to sea pansy activity was about 0.3, which was almost constant under any of the conditions examined in this study. These results support the hypothesis that the ratio of the luciferase activities in the tumor cells can be used as an indicator of RNAi and that luciferase cDNAs can be regarded as a model endogenous gene.

4.2. Induction of RNAi in cultured B16-BL6/dual Luc cells

Prior to the in vivo study, we examined whether the siRNA and the siRNA-expressing pDNA were effective in reducing the firefly luciferase expression in cultured B16-BL6/dual Luc cells. Compared with the control vector, pU6-siGL3 reduced the mRNA to 25% of the control, and the luciferase activity was suppressed to about 20% of the control (Fig. 1). These results guarantee the feasibility of using the ratio of the two luciferase activities as an indicator of gene silencing in tumor cells by RNAi.

As the amount of pU6-siGL3 added to the cells increased, the greater the inhibition of firefly luciferase expression (Fig. 2). The luciferase activity was reduced to about 15% of the control by 1 μg pU6-siGL3. Transfection of siGL3, a synthetic siRNA, also reduced the luciferase activity in B16-BL6/dual Luc. pU6-siGL2, which expresses siRNA not targeted to the mRNA of firefly luciferase, had little effect on the luciferase activity.

4.3. Duration of RNAi in B16-BL6/dual Luc cells by siRNA-expressing pDNA

Fig. 3 shows the time-course of the luciferase activity of B16-BL6/dual Luc cells after transfection of pU6-siGL3. A significant reduction in firefly luciferase activity was observed as early as 6 h after transfection. The luciferase activity decreased with time and reached minimal values at 1–2 days after transfection. The suppression by pU6-siGL3 was significant for the

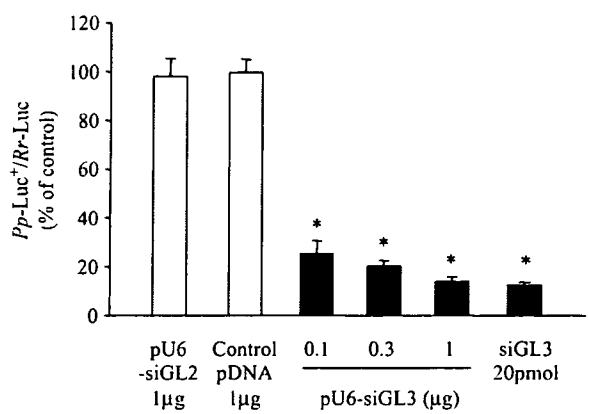

Fig. 2. Reduction of luciferase activity in B16-BL6/dual Luc following transfection of RNAi effectors. B16-BL6/dual Luc inoculated on 24-well plates was transfected with the indicated dose of RNAi effectors using Lipofectamine 2000. Luciferase activities were measured 24 h after transfection. *Pp*-Luc$^+$ means firefly luciferase$^+$ and *Rr*-Luc means sea pansy luciferase. The results are expressed as the mean ± S.D ($n=3$). $^*P<0.05$ for Student *t* test versus control group.

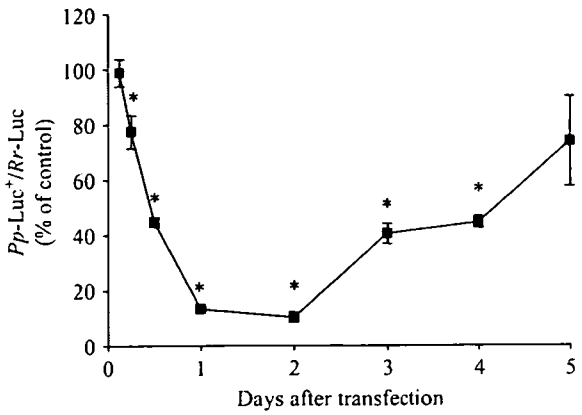

Fig. 3. RNA interference at different times following transfection of siRNA-expressing pDNA. B16-BL6/dual Luc inoculated on 24-well plates was transfected with 1 ig of pU6-siGL3 or piGENE hU6 using Lipofectamine 2000. Luciferase activities were determined at the indicated times after transfection. *Pp*-Luc$^+$ means firefly luciferase$^+$ and *Rr*-Luc means sea pansy luciferase. The results are expressed as the mean ± S.D ($n=3$). $^*P<0.05$ for Student *t* test versus control group.

first 4 days, and then the expression of the firefly luciferase returned to the control level at day 5.

4.4. Delivery of pDNA to subcutaneous tumor tissues following intratumoral injection

To efficiently deliver siRNA or siRNA-expressing pDNA into subcutaneous tumors, several methods reported for gene transfer were examined in terms of their transfection efficiency (Fig. 4). B16-BL6/Luc cells inoculated into the mouse footpad were injected with a fixed dose (10 μg) of pRL-CMV, and the luciferase activities were measured at 24 h after injection. The volume of the solution slightly affected the level of transgene expression with the highest transgene expression at 50 μl. Therefore, 50 μl was used in the following experiments. PVP, which was reported to increase transfection efficiency by naked pDNA after intramuscular injection, reduced the expression. Electroporation increased the transgene expression at the high electric field of 1000 V/cm, but electroporation at 250 V/cm was not effective in increasing the transgene expression. The pre-injection of digestive enzymes also reduced the electroporation-enhanced transgene expression. Therefore, intratumoral injection of naked pDNA followed by electroporation at a field strength of 1000 V/cm without any pretreatment was selected as the method to deliver RNAi effectors to the subcutaneous tumor tissues in the following experiments.

4.5. Induction of RNAi in subcutaneous tumor tissue by intratumoral injection of RNAi effectors followed by electroporation

B16-BL6/dual-Luc cells were inoculated into the foot-pad of mice. When diameter of tumor tissue reached 5 mm (18–20 days after inoculation), siGL3 or pU6-siGL3 was injected into the tumor tissue and electric pulses at a field strength of 1000 V/cm were applied. Fig. 5 shows the firefly luciferase activity at 24 h after injection. The administration of siGL3 or pU6-siGL3 reduced the firefly luciferase expression in the subcutaneous tumors. Both siGL3 and pU6-siGL3 reduced the gene expression to about 60% of the control value. These results indicate that both siRNA and siRNA-expressing pDNA are delivered

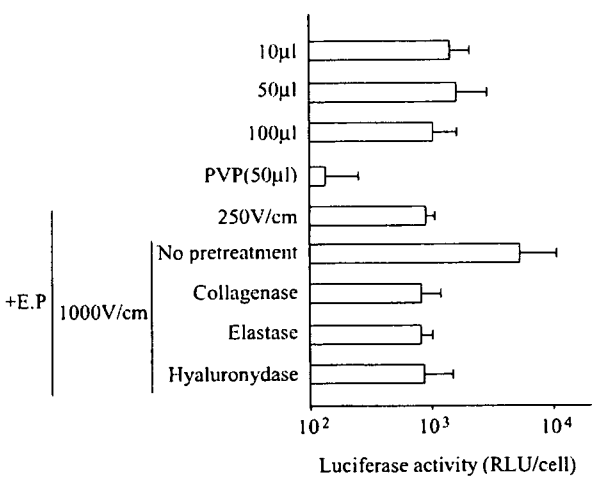

Fig. 4. Transgene expression of luciferase in subcutaneous B16-BL6/Luc following intratumoral injection of pRL-CMV. Mice received an intratumoral injection of pRL-CMV (10 μg) 13 days after subcutaneous B16-BL6/Luc inoculation. PVP groups received an intratumoral injection of pDNA complexed with polyvinylpyrrorydone. Groups of mice received electroporation (E.P) at the indicated electric field strength following intratumoral injection of pDNA. Groups of mice received an intratumoral injection of elastase (10^{-7} U), collagenase (10^{-4} U) or hyaluronydase (36 U) more than 30 min before the electroporation. Twenty-four hours after pDNA administration, luciferase activity in the tumor tissue was measured and the sea pansy luciferase activity per tumor cell was calculated from the firefly luciferase activity which acted as an indicator of the number of tumor cells. The results are expressed as the mean ± S.D ($n=5$). $^*P<0.05$ for Dunnet's test versus control group.

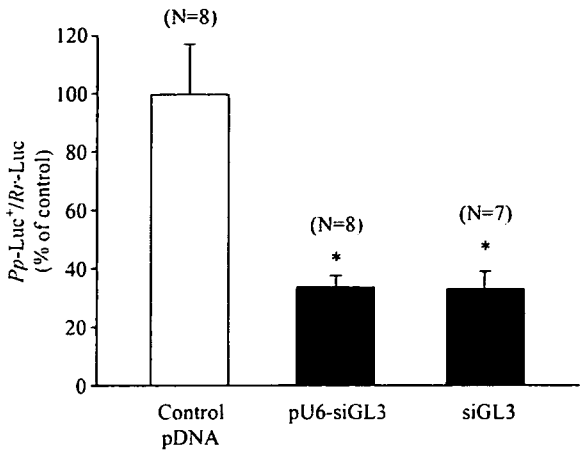

Fig. 5. RNAi in subcutaneous B16-BL6/dual Luc following intratumoral injection of RNAi inducer followed by electroporation. Mice received an intratumoral injection of piGENE hU6 or pU6-siGL3 (30 μg), or siGL3 (10 μg) followed by electroporation at a field strength 1000 V/cm 19 days after subcutaneous B16-BL6/dual Luc inoculation. Luciferase activities in the tumor tissue were determined 24 h after injection. *Pp*-Luc$^+$ means firefly luciferase$^+$ and *Rr*-Luc means sea pansy luciferase. The results are expressed as the mean ± SEM ($n=8$). *$P<0.05$ for Dunnet's test versus control group.

to the subcutaneous tumor with a comparable efficiency under these conditions.

4.6. Hydrodynamic delivery of pDNA to metastatic tumors in the liver

Mice inoculated with B16-BL6/GFP-F cells via the portal vein were intravenously injected with pDsRed2-N1 by the hydrodynamics-based procedure. An efficient transgene expression from the pDNA was observed in normal hepatocytes (Fig. 6a) as well as in the tumor cells localized in the liver (Fig. 6b); the red fluorescence overlapped with the green one, indicating that the dsRed-expressing naked pDNA can be delivered to the tumor cells in the liver.

4.7. Induction of RNAi in metastatic tumors in the liver by hydrodynamic injection of siGL3 or pU6-siGL3

B16-BL6/dual Luc cells inoculated in the liver via the portal vein were targeted by the systemic injection of siGL3 or pU6-siGL3 by the hydrodynamics-based procedure. A single injection of pU6-siGL3 at a dose of 50 μg reduced the firefly luciferase activity to about 40% of the control (Fig. 6c). Three injections of the pU6-siGL3 at 6 h-intervals resulted in about a 60% reduction in the firefly luciferase activity. On the other hand, siGL3 reduced the firefly luciferase expression to about 60% of the control irrespective of the number of injections.

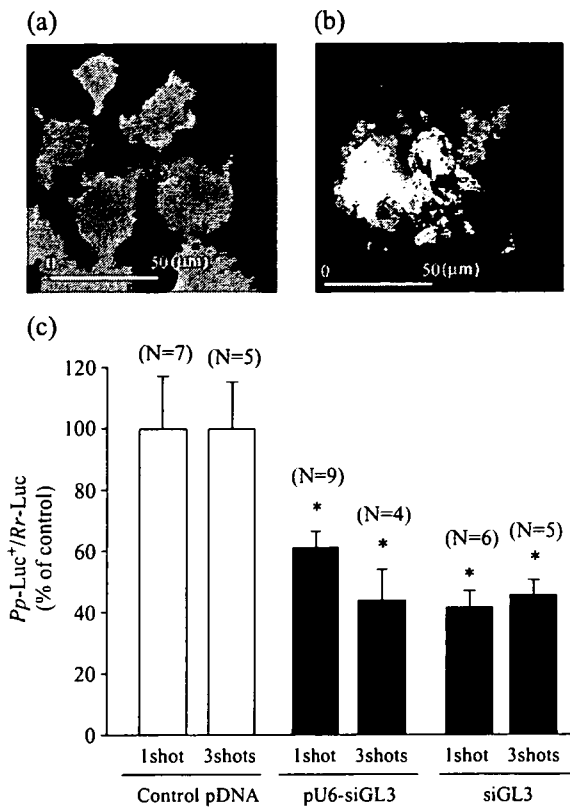

Fig. 6. (a), (b). Confocal microscopic images of the B16-BL6/EGFP-F inoculated liver following intravenous injection of DsRed-expressing pDNA. Mice received an injection of pDsRed2-N1 (15 μg) by the hydrodynamics-based procedure 13 days after tumor inoculation via the portal vein. The mice were euthanized 24 h after the injection, and liver sections were prepared. The images shown are typical of those observed in several visual fields. (c) RNAi in metastatic tumors in the liver following injection of siGL3 or pU6-siGL3 by the hydrodynamics-based procedure. Mice received an intravenous injection of piGENE hU6, or pU6-siGL3 or siGL3 (50 μg) 13 days after tumor inoculation via the portal vein. Mice received either one or three intravenous injections. Mice in the group receiving three injections received a second intravenous injection 6 h after the first injection and the third injection 12 h after the first injection. The luciferases activities in the liver were measured 24 h after the first injection. *Pp*-Luc$^+$ means firefly luciferase$^+$ and *Rr*-Luc means sea pansy luciferase. The results are expressed as the mean ± SEM ($n=4$). *$P<0.05$ for Dunnet's test versus control group.

5. Discussion

RNAi-based cancer therapy is achieved when gene silencing takes place in tumor cells in vivo by siRNA, although it may require a significant reduction in the target gene expression in tumors. The efficiency of gene silencing is determined by two factors: the activity of the siRNA destroying the target mRNA [33] and its delivery to target cells. Regarding the activity, some groups have reported practical approaches to discovering highly-active sequences of siRNA by optimizing its site and sequence [34–36]. However, the delivery issue has hardly been challenged. In the majority of conventional in vivo gene delivery studies, the efficiency of gene transfer has been evaluated by the level and duration of transgene expression, not by the number of cells expressing the gene of interest. This is in direct contrast to the case of in vivo RNAi-based therapy. Considering that mRNA degradation by siRNA occurs only in the cells that are delivered with siRNA or its expressing vector [14,15], the number of target cells receiving RNAi effectors is very important.

An effective delivery method for RNAi effectors cannot be developed without appropriate methods to evaluate the efficiency of gene silencing in target cells in a quantitative manner. Without such methods, it is hard to study the delivery of RNAi effectors in vivo with any degree of accuracy. Therefore, we constructed a system to evaluate the RNAi effect in tumor cells by measuring luciferase activities. This system has the advantage that it can estimate the number of tumor cells that are delivered with RNAi effectors in an easy and sensitive fashion. On the contrary, this system has the disadvantage that tumor cell function is difficult to estimate because the target gene is firefly luciferase.

Transfection of RNAi effectors targeted to firefly luciferase suppressed firefly luciferase expression and had no effects on sea pansy luciferase expression ($8.31 \pm 0.57\ 10^8$ RLU/mg protein with the transfection of control vector, and $8.57 \pm 0.885\ 10^8$ RLU/mg protein with the transfection of pU6-siGL3, $P > 0.05$). The activities and amounts of mRNA of the transfected B16BL6/dual Luc were measured simultaneously, and pU6-siGL3 reduced both the activities and the amounts of mRNA of the targeted luciferase (Fig. 1). These results indicate that the reduction in the luciferase activity reflects the degradation of mRNA, and the luciferase activity can be used as an indicator of tumor gene expression.

Significant reduction in gene expression was sustained at least until 4 days after transfection, but was not observed 5 days after transfection. Based on these results, it appears that the suppressing effect on gene expression in the cultured tumor cells by siRNA-expressing pDNA is only temporary. This transient reduction may be partly explained by the fact that the targeted gene is firefly luciferase$^+$ cDNA that is not related to the survival or growth of tumor cells.

Effective delivery is important for therapeutic treatment with nucleotides, such as pDNA and antisense oligonucleotides, and a number of methods have been developed for their efficient delivery [37,38]. siRNA and siRNA-expressing pDNA are also nucleotides and have similar physicochemical properties to other types of nucleotides, although there are some differences in molecular size, stability and their sites of action within cells. Therefore, the delivery methods developed for various nucleotides can be used for the delivery of RNAi effectors. We believe that the gene transfer methods used for local administration will be useful for delivering RNAi effectors to primary tumors. To achieve an effective delivery, we first investigated transgene expression levels obtained after intratumoral injection of pCMV-RL by these different methods that have been reported to increase transgene expression after intramuscular administration of pDNA [28–31]. Electroporation at a high voltage of 1000 V/cm increased the transgene expression obtained after intratumoral injection of pCMV-RL. Other methods such as the use of PVP and pre-injection of digestive enzymes did not increase the transfection efficiency. The differences in the anatomical and physiological characteristics between skeletal muscle and the subcutaneous tumor tissues may explain the discrepancy in the usefulness of these methods from the literatures as far as the level of transgene expression is concerned. Thus, electroporation at an electric field of 1000 V/cm following intratumoral injection of RNAi effectors was selected to deliver RNAi effectors to primary tumor cells in vivo.

The electroporative delivery of siRNA and siRNA-expressing pDNA was found to be effective in reducing model endogenous gene expression in the primary tumor tissues. This result indicates that intratumoral

injection of RNAi effectors followed by electroporation can inhibit 60% of the gene expression in total tumor tissue. The extent of the inhibition in tumor tissue is determined by two factors. One is the inhibition level per single tumor cell, and the other is the number of tumor cells that received RNAi effectors. Because the in vitro results showed that the maximal inhibitory effect of these RNAi effectors was about 85%, it appears that more than 60% of the tumor cells received RNAi effectors by this administration method. No significant differences were observed in the effects of gene silencing in the subcutaneous tumors between siGL3 and pU6-siGL3.

In the case of treating metastasizing tumor cells, which are distributed mainly via blood and lymph vessels [39], intravascular administration seems to be a promising approach to deliver RNAi effectors to tumor cells. As an intravascular administration method for targeting tumor cells in the liver, we selected the hydrodynamics-based procedure, which was first developed as an efficient method for nonviral gene transfer to hepatocytes [32]. To date, no studies have been performed to see if this procedure can deliver nucleotides to the tumor cells present in the liver. This possibility was investigated qualitatively with fluorescent protein-expressing tumor cells and quantitatively with luciferase-expressing tumor cells. By visualizing tumor cell localization, it was found that the hydrodynamics-based procedure was able to deliver pDNA into the tumor cells inoculated in the liver as well as normal hepatocytes. Based on this observation, we investigated quantitatively the degree of gene silencing in tumor cells inoculated in the liver caused by the administration of RNAi effectors using the hydrodynamics-based procedure. Three administrations of siRNA-expressing pDNA increased the gene silencing effect compared with a single administration, while three administrations of siRNA resulted in almost the same degree of gene silencing effect as that obtained with a single administration. Almost the same degree of gene silencing in tumor cells in the liver can be obtained after the systemic administration of both siRNA and siRNA-expressing pDNA by the hydrodynamics-based procedure. Thus, we have shown for the first time that the injection of pDNA as well as siRNA by the hydrodynamics-based procedure is effective in delivering them to tumor cells metastasizing to the liver. The principle of the hydrodynamics-based procedure is considered to be applicable to treating tumor cells which metastasize to other organs besides the liver. It is obvious that this procedure delivers siRNA and siRNA-expressing pDNA to normal hepatocytes as well as tumor cells in the liver. Therefore, genes mutated in tumor cells can be good targets for siRNA-based therapy to avoid possible side effects.

6. Conclusion

We succeeded in constructing a model system in which the gene expression in tumor cells in vivo can be quantitatively and sensitively evaluated by luciferase activity. With the model constructed in this study, we can investigate the possibility of RNAi induction in primary and metastatic tumor cells. In conclusion, we have shown that gene expression in primary tumor cells can be suppressed to about 60% by optimizing the methods of RNAi effector delivery methods and that, in the case of metastasizing tumor cells, gene expression in tumor cells in the liver can be reduced to about 60% by administration of RNAi effectors by the hydrodynamics-based procedure.

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Research Paper

Sequence-Specific Suppression of *mdr1a/1b* Expression in Mice via RNA Interference

Yumi Matsui,[1] Naoki Kobayashi,[1] Makiya Nishikawa,[1] and Yoshinobu Takakura[1,2]

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Purpose. RNA interference (RNAi) is a powerful tool for silencing gene expression posttranscriptionally. The purpose of this study was to examine whether *in vivo* RNAi can be induced against endogenous *mdr1a/1b* in adult mice and to assess the feasibility of generating P-glycoprotein (P-gp) knockdown mice based on RNAi by a very simple intravenous injection of synthetic small interfering RNA (siRNAs) or siRNA-expressing plasmid DNAs.

Methods. The targeted sequences for silencing *mdr1a* specifically or *mdr1a/1b* simultaneously were examined in an *in vitro* study using a mouse colon carcinoma cell line, colon26 cells, in culture. Mice were repeatedly treated with intravenous synthetic siRNAs or siRNA-expressing plasmid DNAs in naked form administered via a large-volume and high-speed injection, i.e., the hydrodynamics-based procedure. The amount of targeted mRNA and P-gp in the liver were determined by real-time polymerase chain reaction and Western blot analysis, respectively.

Results. Among several targeted sequences, two and one optimized sequences were selected for *mdr1a* and *mdr1a/1b*, respectively, in the *in vitro* study. Following administration of synthetic siRNAs or siRNA-expressing plasmid DNAs directed against *mdr1a*, the mRNA level in the liver was significantly reduced to approximately 50–60% of that in control mice. Furthermore, a slight reduction was observed at the protein level. Similar results were obtained in the experiments using siRNA-expressing pDNA directed against *mdr1a/1b*.

Conclusions. Our results demonstrate that sequence-specific suppression of *mdr1* gene expression is possible at the mRNA level as well as the protein level in mice following intravenous delivery of siRNA effectors.

KEY WORDS: hydrodynamics-based procedure; liver; P-glycoprotein; plasmid DNA; RNA interference.

INTRODUCTION

RNA interference (RNAi) is a process in which double-stranded RNA (dsRNA) induces the sequence-specific degradation of homologous RNA (1,2). RNAi is a powerful tool for silencing mRNA in various organisms and is notable for the application of genetic analysis as well as gene therapy. RNAi-induced suppression of endogenous gene expression is very attractive because of the possibility of obtaining simultaneous knockdown of multiple genes or transient knockdown of fatal genes, which would otherwise prevent us from investigating their functions in postnatal animals. Many studies have already attempted to inhibit the expression of the endogenous genes *in vitro*. Although it is possible to induce RNAi-mediated gene silencing *in vivo*, it is particularly difficult in the case of endogenous genes (3–9).

Previously, it was reported that transgene expression of agents such as luciferase, green fluorescence protein (GFP), and HbsAg derived from hepatitis B virus, could be suppressed in adult mice by synthetic siRNAs and siRNA-expressing vectors (10–12). In a previous study, we have also demonstrated that RNAi-induced transgene suppression against firefly luciferase occurred in a variety of somatic cells *in vivo* (13). On the other hand, few studies have successfully used *in vivo* RNAi against endogenous targets. It has been shown that endogenous gene expression of Fas receptor (6) or caspase 8 (7) is significantly inhibited in the liver following the hydrodynamic delivery of synthetic siRNAs. Several studies have reported RNAi-mediated suppression by local administration of synthetic siRNAs (3–5). Synthetic siRNAs have been used mainly in successful reports involving *in vivo* and *in vitro* studies. Despite their potent knockdown capabilities, synthetic siRNAs have some disadvantages: transient silencing, high cost, and transfection is difficult in some cell types. siRNA-expressing vectors might overcome these problems. In fact, several siRNA-expressing vectors have been

[1] Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan.
[2] To whom correspondence should be addressed. (e-mail: takakura@pharm.kyoto-u.ac.jp

ABBREVIATIONS: pDNA, plasmid DNA; RNAi, RNA interference; siRNA, small interfering RNA.

developed and have been shown to have more efficient suppressive effects (3,14–19).

In the present study, *in vivo* RNAi was attempted by a hydrodynamics-based delivery of siRNA-expressing plasmid pDNA (pDNA), targeting endogenous genes. Murine *mdr1a/1b* P-glycoprotein (P-gp), an efflux transporter for a wide variety of drugs, was selected as a model endogenous target that is expressed in various somatic cells including the liver. This was based on our experiences in earlier studies on the effects of interferons on the P-gp expression and function in primary cultures of rat hepatocytes and mice (20,21). More importantly, Schinkel and colleagues (22,23) have generated mice genetically deficient in *mdr1a*, *mdr1b*, or *mdr1a/1b* and studied the physiological roles of P-gp in these animals. In this study, we assessed the feasibility of generating knockdown mice close to the knockout mice based on RNAi targeting endogenous *mdr1a/1b in vivo* in adult mice following the hydrodynamics-based delivery of synthetic siRNAs or siRNA-expressing pDNAs.

MATERIALS AND METHODS

Plasmid DNA and Synthetic siRNA

siRNA-expressing pDNAs targeting murine *mdr1a/1b* P-gp were constructed from piGENE™hU6 vector (iGENE Therapeutics, Tsukuba, Japan) according to the instructions. pU6-mdr1a (sites 1–6) was designed to produce siRNA targeting to specific sequences in *mdr1a* but not in *mdr1b*, and pU6-*mdr1a/1b* (sites 7–10) was designed to produce siRNA targeting to common sequences in both *mdr1a* and *mdr1b* (Table I). These pDNAs transcribe stem–loop-type RNA with loop sequences of UAG AAU UAC AUC AAG GGA GAU. pU6-GL3, which transcribes unrelated stem–loop-type siRNA targeted to pGL3 firefly luciferase+ mRNA, was used as a negative control pDNA. piGENE™hU6 vector, which transcribes nonrelated sequences of RNA 5′-GUG AGC AGG UGU AAA GCC ACC AUG GAA GAC ACC UGC CAA CUU UU-3′ with partial duplex formation, was used as a control pDNA throughout the present study. Each pDNA was amplified in the DH5α strain of *Escherichia coli* and purified using a Qiagen Endo-free Plasmid Giga kit (Qiagen GmbH, Hilden, Germany) or a Geno Pure Plasmid Maxi Kit (Roche Diagnostics Corporation, Indianapolis, IN, USA).

The synthetic siRNA, annealed duplex of 23-nucleotide RNA targeted to *mdr1a* (site 5 or site 6), was purchased from Qiagen (HPP grade). The sequences of synthetic RNA were as follows: sense 5′-r(AAU GUU GUC UGG ACA AGC ACU) d(TT)-3′ and antisense 5′-r(AGU GCU UGU CCA GAC AAC AUU) d(TT)-3′ for site 5, sense 5′-r(AGA AGG AAC UAG AAG GUU CUG) d(TT)-3′ and antisense 5′-r(CAG AAC CUU CUA GUU CCU UCU) d(TT)-3′ for site 6. Following the dissolution of lyophilized siRNA in suspension buffer (100 mM potassium acetate, 30 mM HEPES–KOH, 2 mM magnesium acetate, pH 7.4), an aliquot of siRNA was heated to 90°C for 1 min and then incubated at 37°C for 1 h to disrupt higher aggregates according to the manufacturer's instructions. The siRNA solution was stored at −20°C until its use.

Cell Culture and Transfection

Colon26 cells, a mouse colon carcinoma cell line, were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum. Cells were seeded to 6-well plates at a density of 5×10^5 cells/well and cultured for 24 h. Transfection was performed in Opti-MEM (Invitrogen, Carlsbad, CA, USA) with pDNA (5 μg/well) or synthetic siRNA (3.3 μg/well) by using LipofectAMINE™ 2000 (10 μg; Invitrogen) for 2 h and the medium was replaced with DMEM. This condition was optimized in our preliminary experiments in terms of the transfection efficiency and the cellular viability. Following incubation for the described time, the cells were washed three times with PBS and total RNA extraction was carried out.

Mice and Intravenous Injection

Five-week-old male BALB/c mice (approximately 20 g body weight) were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). All animal experiments were evaluated and approved by the Ethics Committee for Animal Experiments at the Graduate School of Pharmaceutical Sciences, Kyoto University. For induction of *in vivo* RNAi in mice, we carried out a large-volume and high-speed intravenous injection, the so-called hydrodynamics-based procedure (13,24–26). Mice received two intravenous injections of 1.6 mL saline containing pDNA (50 μg) or synthetic siRNA (50 μg) within 5 s on days 0 and 1. The dose was selected based on the information in the literature (6,7) and in our laboratory (13). On day 2, the mice were euthanized to obtain the total RNAs in the liver.

Measurement of the Amount of mRNA

At the described times after transfection, total RNAs were extracted from the cells using Sepasol-RNA I Super (Nacalai Tesque, Kyoto, Japan). In the *in vivo* experiments,

Table I. Targeted Sequences of siRNA-Expressing pDNA Against *mdr1a* or *mdr1a/1b* P-gp

Sequences against *mdr1a*		Sequences against *mdr1a/1b*	
Site 1	GAA ATG ACC ACG TAC GCC TAC	Site 7	GAC AAG AAC TTC TCA AAG ATG
Site 2	GAA TAC TCT ATT GGA CAA GTG	Site 8	ACA CCC GGC TCA CAG ATG ATG
Site 3	GGG GCT ACA GGG TCT AGG CTT	Site 9	AGA AGG AAC TTG AAA GGT ACA
Site 4	GAT ATT GTC TTC ATT TAC TGA	Site 10	GGC CGC ACC TGC ATT GTG ATC
Site 5	AAT GTT GTC TGG ACA AGC ACT		
Site 6	AGA AGG AAC TAG AAG GTT CTG		

mice were euthanized 1 day after the second injection and total RNAs were extracted from approximately 20 mg of the liver using Sepasol-RNA I Super. Following RNase-free DNase I treatment (Takara, Kyoto, Japan), 2 μg of RNA measured by UV absorption at 260 nm was reverse-transcribed to complementary DNA (cDNA) by SuperScript™ II RNase H⁻ Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions with oligo(dT)$_{12-18}$ primers. The reverse-transcribed product was treated with RNase H (Toyobo, Osaka, Japan). The amount of *mdr1a* and *mdr1b* cDNA was measured by real-time PCR in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster, CA, USA) using Assays-on-Demand™ Gene Expression Products (Applied Biosystems) containing the corresponding specific primers and Taqman probe. PCR amplification was performed in triplicate 50-μL reactions using 1 μL of reverse-transcribed product with TaqMan Universal PCR Master Mix (Applied Biosystems). The amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA in the same sample was also measured with TaqMan Rodent GAPDH Control Reagents (Applied Biosystems) and used for normalization.

Western Blot Analysis

Mice were euthanized 1 day after the second injection and the livers were homogenized individually in PBS containing 250 mM sucrose and 1%(v/v) Sigma protease inhibitor cocktail (Sigma Aldrich) at 4°C. The homogenates were centrifuged at 1500×*g* for 30 min. An aliquot of PBS was added to the resultant pellets and centrifugation was repeated. The protein concentrations of the supernatants obtained after two centrifugations, which were used as protein samples, were determined using a Proteostain Protein Quantification Kit (Dojindo Molecular Technologies, Inc., Tokyo, Japan). Fifty micrograms of proteins was diluted with loading buffer, denatured at 95°C for 3 min, and resolved by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (6.5% polyacrylamide) and transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P; Millipore Corp., Bedford, MA, USA) by semidry blotting with Transblot SD (Bio-Rad, Hercules, CA, USA). To avoid non-specific binding, the membrane was incubated in 5% skimmed milk overnight. Then P-gp protein was detected by primary monoclonal mouse antibody C219 (1:1000) and secondary peroxidase-conjugated rabbit anti-mouse IgG antibody (1:2000). Protein bands were visualized by chemiluminescence on the ECL protein detection system (Amersham Biosciences Inc., Piscataway, NJ, USA) followed by exposure to Hyperfilm ECL (Amersham Biosciences). The densities of the bands obtained on the films were requantitated using ATTO Image Analysis Software (Atto Corp., Tokyo, Japan).

RESULTS

Target Site Dependence of the Suppressive Activity of siRNA-Expressing Vectors in Colon26 Cells

In the present study, murine *mdr1a/1b* was selected as a model endogenous target, which plays an important role in the disposition of drugs in the epithelial cells of various tissues including the liver. The efficiency of siRNA-mediated RNAi is dependent on the mRNA target position. Several putative short interfering RNA target sequences were designed against the murine *mdr1a/1b* gene using an algorithm developed by Miyagishi and Taira (University of Tokyo) (Table I; sites 1 to 6 were specific sequences for *mdr1a* and sites 7–10 were common sequences for *mdr1a* and *mdr1b*), and corresponding siRNA-expressing pDNAs were constructed. First, to investigate target site dependence in *mdr1a*, colon26 cells were transfected with various siRNA-expressing pDNAs against *mdr1a* (pU6-mdr1a-1 to -6), and 48 h later each suppressive effect was compared (Fig. 1). Colon26 cells were used for the *in vitro* study as constitutive

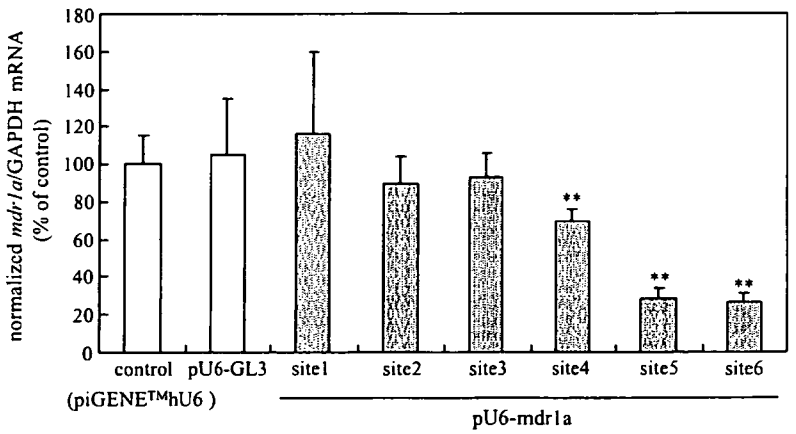

Fig. 1. Target site dependence of the suppressive activity of siRNA-expressing vectors in colon26 cells. Cells were transfected with piGENE™hU6 (control), pU6-GL3 (negative control) or pU6-mdr1a against various targeted sites, and harvested for mRNA isolation at 48 h after transfection. The amount of mRNA was determined by real-time PCR following reverse transcription. Expression levels of *mdr1a* mRNA were normalized to GAPDH and standardized to the cells transfected with piGENE™hU6. The results are expressed as the mean ± SD of at least six determinations. $^{**}p < 0.01$ *vs.* control.

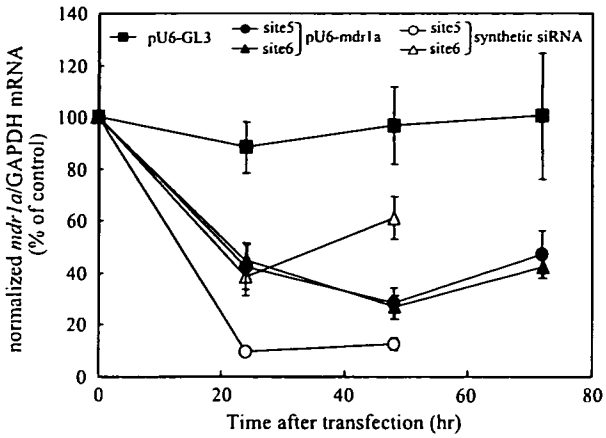

Fig. 2. Duration of siRNA-mediated RNAi against mdr1a in colon26 cells. Cells were transfected with piGENE™hU6 (control), pU6-GL3 (negative control), pU6-mdr1a (sites 5 and 6), or synthetic siRNA (sites 5 and 6), and harvested for mRNA isolation at 24, 48, and 72 h after transfection. The ordinate shows normalized expression levels of *mdr1a* mRNA as a percent of control. The results are expressed as the mean ± SD of at least four determinations.

expression of *mdr1a* and *mdr1b* was confirmed in this mouse cell line (data not shown). As shown in Fig. 1, pU6-mdr1a against different sites on the same target mRNA demonstrated striking differences in silencing efficiency. It was also found that sites 5 and 6 were the most efficient positions for reducing the amount of *mdr1a* mRNA. Therefore these two sites were used in the following studies. It was confirmed that the *mdr1b* expression level was unaffected by pU6-mdr1a (data not shown).

Duration of siRNA-Mediated RNAi Against *mdr1a* in Colon26 Cells

Next, we examined the duration of action of siRNA-mediated RNAi against *mdr1a*. In addition to pU6-mdr1a targeting site 5 and site 6, corresponding synthetic siRNAs were also used in this study. At the described time after transfection, the amount of *mdr1a* mRNA was determined. As expected, both pU6-mdr1a and synthetic siRNAs reduced the amount of *mdr1a* mRNA (Fig. 2). The suppression was confirmed 24 h after transfection and sustained for at least 3 days using siRNA-expressing pDNAs. It was also found that synthetic siRNA could suppress the target mRNA expression more rapidly than siRNA-expressing vectors.

Simultaneous Suppression of *mdr1a/1b* by siRNA-Expressing pDNAs in Colon26 Cells

There is an approximately 80% homology between *mdr1a* and *mdr1b* in mouse mRNA. For the purpose of inducing simultaneous inhibition, four siRNA-expressing pDNAs against common sequences of the two (Table I; sites 7 to 10) were constructed (pU6-*mdr1a/1b*). Figure 3 shows the suppressive effect of pU6-*mdr1a/1b* targeting sites 7 to 10. Whereas any of the pU6-*mdr1a/1b* (pU6-*mdr1a/1b*-7, -8, -9, and -10) could dramatically suppress the amount of *mdr1b* mRNA, the inhibitory effect against *mdr1a* differed among these pDNAs. The amount of *mdr1a* mRNA was markedly reduced in the cells transfected with pU6-*mdr1a/1b*-10, less but significantly with pU6-*mdr1a/1b*-7 and pU6-*mdr1a/1b*-8, and unaffected with pU6-*mdr1a/1b*-9. The different suppression effects on *mdr1a* and *mdr1b* would not be due to the difference in the expression levels because the levels of *mdr1a* and *mdr1b* were similar (*mdr1a*:*mdr1b* = 1.1:1, n = 8).

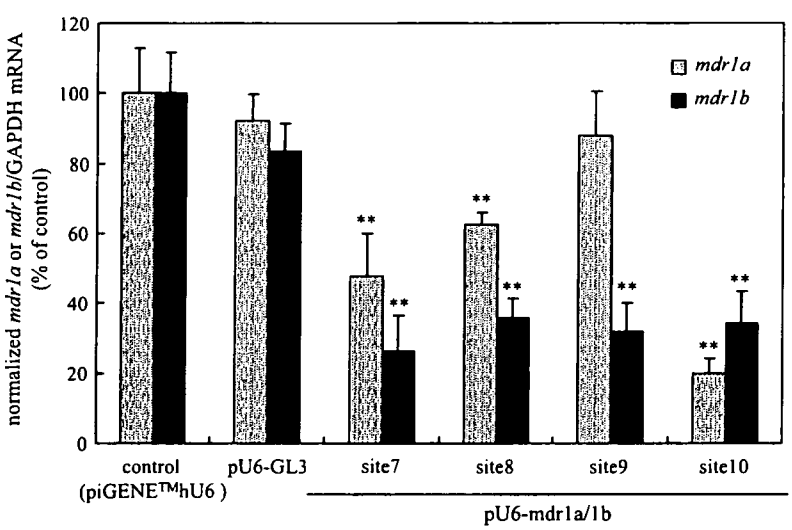

Fig. 3. Simultaneous suppression of *mdr1a/1b* by siRNA-expressing pDNAs in colon26 cells. Cells were transfected with piGENE™hU6 (control), pU6-GL3 (negative control), and pU6-*mdr1a/1b* (sites 7–10) and harvested for mRNA isolation at 48 h after transfection. The amount of mRNA was determined by real-time PCR following reverse transcription. Expression levels of *mdr1a* and *mdr1b* mRNA were normalized to GAPDH and standardized to the cells transfected with piGENE™hU6, respectively. The results are expressed as the mean ± SD (n = 8). $^{**}p < 0.01$ *vs.* each control.

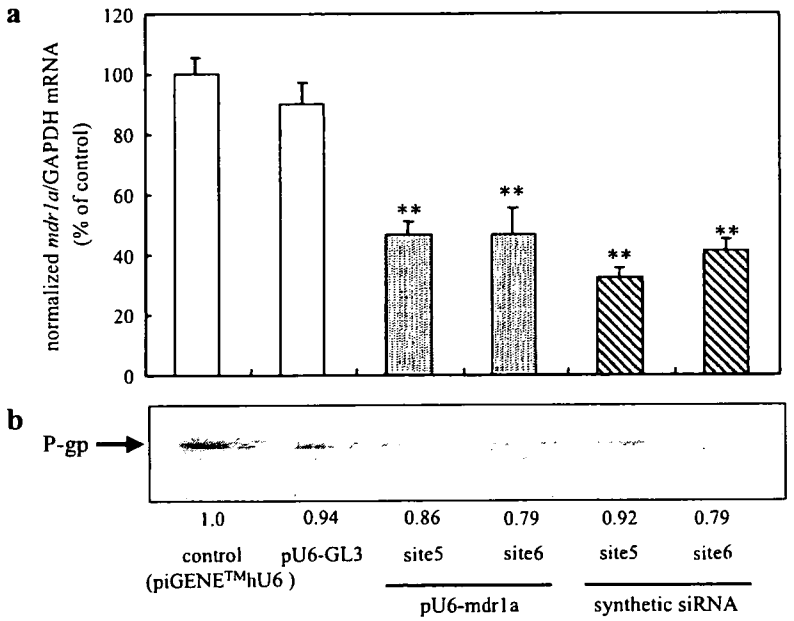

Fig. 4. *In vivo* RNAi targeting endogenous *mdr1a* in the liver by siRNA-expressing pDNAs and synthetic siRNAs. Mice were given two intravenous injections of piGENE™hU6 (negative control), pU6-mdr1a (sites 5 and 6) or synthetic siRNA (sites 5 and 6). Control mice were treated with saline without any pDNA. At 48 h after the first injection, the amount of *mdr1a* mRNA (a) and P-gp (b) in the liver was determined by real-time PCR and Western blot analysis, respectively. (a) Expression levels of *mdr1a* mRNA were normalized to GAPDH and standardized to those of the control group. The results are expressed as the mean ± SE of at least eight determinations. $^{**}p < 0.01$ *vs.* control. (b) Fifty micrograms of proteins was electrophoresed and then visualized. The arrow indicates P-gp bands at 170 kDa. Typical data for P-gp are illustrated together with relative intensity of the protein bands at the bottom ($n = 3$).

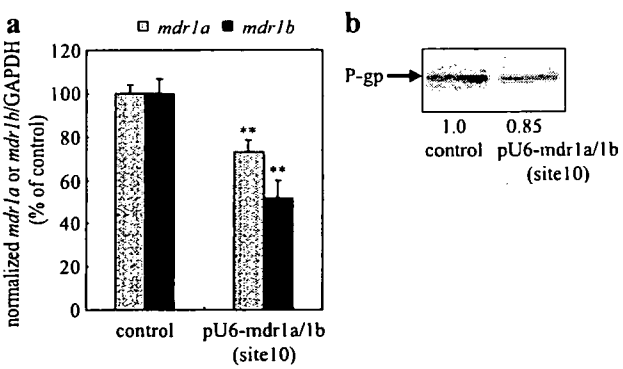

Fig. 5. *In vivo* RNAi targeting endogenous *mdr1a/1b* in the liver by siRNA-expressing pDNAs and synthetic siRNAs. Mice were given two intravenous injections of piGENE™hU6 (control), pU6-*mdr1a/1b* (site 10). Control mice were treated with piGENE™hU6. At 48 h after the first injection, the amount of *mdr1a* mRNA (a) and P-gp (b) in the liver was determined by real-time PCR and Western blot analysis, respectively. (a) Expression levels of *mdr1a* mRNA were normalized to GAPDH and standardized to those of the control group. The results are expressed as the mean ± SD of at least four determinations. $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* each control. (b) Fifty micrograms of proteins was electrophoresed and then visualized. The arrow indicates P-gp bands at 170 kDa. Typical data for P-gp are illustrated together with relative intensity of the protein bands at the bottom ($n = 3$).

In Vivo RNAi Targeting Endogenous *mdr1a/1b* in the Liver by siRNA-Expressing pDNAs and Synthetic siRNAs

To examine the possibility of inducing *in vivo* RNAi targeting endogenous genes, mice were injected intravenously with siRNA-expressing pDNA or synthetic siRNA by the hydrodynamics-based procedure. First, we attempted to induce RNAi in the liver specifically against *mdr1a* by targeting site 5 and site 6, which were the most efficient positions for reducing the amount of *mdr1a* mRNA *in vitro* (Fig. 1). Figure 4a shows the amount of *mdr1a* mRNA in the liver following the hydrodynamics-based delivery of siRNA-expressing pDNA (pU6-mdr1a-5 and pU6-mdr1a-6) or synthetic siRNA against site 5 and site 6 (siRNA-5 and siRNA-6, respectively). The suppressive effect was determined in mice 48 h after the first injection (i.e., 24 h after the second injection). The amount of *mdr1a* mRNA in the liver was significantly reduced in mice receiving the double hydrodynamics-based delivery of both siRNA-expressing pDNAs and synthetic siRNAs (Fig. 4a). Moreover, the expression of P-glycoproteins (P-gp) in the liver was determined by Western Blot analysis (Fig. 4b). P-gp expression levels in the liver of siRNA-treated mice were also slightly reduced. Next, we attempted to induce transgene suppression simultaneously against both *mdr1a* and *mdr1b* by targeting site 10. Because siRNA-expressing pDNAs and synthetic

siRNAs had almost the same potency as far as suppressing the expression of target mRNA was concerned (Fig. 4a, b), we used siRNA-expressing pDNA against site 10, which was the most efficient position for simultaneously suppressing both *mdr1a* and *mdr1b* (Fig. 3). Figure 5a and b shows the amount of *mdr1a* or *mdr1b* mRNA and the amount of P-gp in the liver, respectively, following the hydrodynamics-based delivery of siRNA-expressing pDNA against site 10 (pU6-*mdr1a/1b*-10). The amount of *mdr1a* and *mdr1b* mRNAs was significantly reduced (Fig. 5a). The P-gp expression level in the liver of siRNA-treated mice also seemed to be slightly lower than that of control murine liver (Fig. 5b).

DISCUSSION

RNA interference is a powerful tool for posttranscriptional gene silencing and is expected to be a popular therapeutic approach. It is greatly meaningful to apply RNAi to *in vivo* studies particularly against endogenous genes, and limited reports have been successful so far. Recently, it was demonstrated that endogenous gene expression was significantly inhibited in the liver by hydrodynamics-based delivery of synthetic siRNAs (6,7). Several studies also reported RNAi-mediated suppression by local administration of synthetic siRNAs (3–5). However, there are various strategies to generate siRNAs that silence gene expression including synthetic siRNAs and vector-based procedures. To date, there has been a limited number of reports of RNAi-mediated inhibition against endogenous targets using siRNA-expressing vectors, such as adenovirus-based vectors (27,28).

In the present study, we examined the possibility of siRNA-mediated gene suppression of endogenous *mdr1a/1b* in adult murine liver by the hydrodynamics-based delivery of synthetic siRNA or siRNA-expressing pDNA, because the hydrodynamics-based procedure gives the highest level of transgene expression to this organ (24–26). P-glycoprotein (P-gp), encoded by the multidrug resistance (MDR) gene family (MDR1 in humans; *mdr1a* and *mdr1b* in rodents), is distributed in various normal tissues including liver (29). P-gp is a plasma membrane protein belonging to the superfamily of ATP-binding cassette (ABC) transporters and plays important roles in the pharmacokinetics of xenobiotics. The significance of this transporter was also confirmed by loss-of-function studies using knockout mice (22,23,29).

siRNA-mediated gene silencing is reported to be highly dependent on the target position (30,31). We examined the most potent siRNA-expressing pDNA among various sites chosen against *mdr1a* (sites 1 to 6; Fig. 1) and against *mdr1a/1b* (site 7 to 10; Fig. 3) based on the algorithm, because we thought that the target site was important for obtaining the most efficient gene suppression *in vivo*. In consequence, it was found that in cultured colon26 cells, pU6-mdr1a-5 and pU6-mdr1a-6 effectively inhibited the expression of *mdr1a* (Fig. 1), but not *mdr1b* (data not shown). In addition, in spite of targeting the common sequences, the suppression effects were strikingly different. It was also found that pU6-*mdr1a/1b*-10 could be regarded as a potent siRNA-expressing pDNA simultaneously targeting both *mdr1a* and *mdr1b* and that, in contrast, pU6-*mdr1a/1b*-9 acted as a potent siRNA-expressing pDNA specifically targeting the *mdr1b* gene despite the fact that its targeted sequence was in both *mdr1a* and *mdr1b*. Thus, suitable targeted sequences within murine *mdr1a/1b* were selected by *in vitro* experiments. Although the positional difference might partially depend on the secondary structure of target mRNA, we could not find a clear correlation between the RNAi efficiency at each site and the *mdr1a* or *mdr1b* mRNA structure predicted by the m-fold web server (32). It is possible that other factors, such as mRNA stability and RNA-binding proteins, might be involved. Although various algorithmics to predict the efficiency of targeted sequences have been reported, the present results provide useful information.

In the study to examine the duration of the RNAi effect (Fig. 2), potent suppressive effects of pDNAs were observed for at least 72 h with the highest values at 48 h following transfection, whereas the maximum inhibition by synthetic siRNAs was observed at 24 h and tended to recover 24 h later. The present study supports the previous reports targeting human MDR1 in breast cancer (33), pancreatic carcinoma, and gastric carcinoma cell lines (34) in terms of the duration of suppression of targeted mRNA expression.

Following determination of the most potent candidates of targeted sequences for silencing *mdr1a* specifically or *mdr1a/1b* simultaneously by *in vitro* experiments, we investigated the *in vivo* application of these sequences using synthetic siRNAs and siRNA-expressing pDNAs. The amount of targeted mRNA in the liver of mice, which were repeatedly treated with intravenous synthetic siRNAs or siRNA-expressing pDNAs administered via the hydrodynamics-based procedure, was determined at 48 h after the first injection based on the results of *in vitro* experiments (Fig. 2), although the time course might be different between the *in vitro* and *in vivo* conditions. The mRNA level was significantly reduced to approximately 50–60% of that obtained in the saline-injected group (Figs. 4a and 5a). This may be a reasonable value, because it has been reported that a single intravenous injection of pDNA via the hydrodynamics-based procedure produces detectable transgene expression of β-galactosidase in approximately 40% of liver cells (24). In addition, our previous study also demonstrated that the localization of transgene-expressing cells following the first and second pDNA injections administered via the hydrodynamics-based procedure was apparently different (13). Repeated hydrodynamics-based injections might allow not only synthetic siRNAs (6), but also siRNA-expressing pDNAs, to be taken up by more liver cells. However, all the hepatocytes were not transfected by these RNAi effectors following intravenous delivery.

In this study, it was observed that P-gp expression at the protein level tended to be slightly reduced in mice treated with RNAi effectors against *mdr1a* (Fig. 4b). We also observed an almost similar trend in the P-gp expression levels using immunostaining and employing the same antibody (data not shown). However, the gene silencing effect at the protein level was not so dramatic. One reason could be the characteristic of the antibody C219 used in the experiments: this antibody recognizes P-gp translated from both *mdr1a* and *mdr1b*. However, the effect was still low even if we used RNAi effecters against *mdr1a* and *mdr1b* (Fig. 5b). Therefore, our results suggest that dramatic knockdown at the protein level is difficult because, at least in part, the efficiency of delivery of the RNAi effectors is not very high. Probably

P-gp derived from both *mdr1a* and *mdr1b* is normally present in the liver cells that have escaped the entry synthetic siRNAs or siRNA-expressing vectors. Therefore, it would be difficult to obtain a significant reduction at the protein level using the present method in spite of its simplicity. More efficient *in vivo* transfection with viral vectors, such as adenoviruses, could be an alternative although there are a number of limitations to be overcome.

In conclusion, the present study has demonstrated that siRNA-mediated suppression of endogenous *mdr1a/1b* can be achieved *in vivo* via the hydrodynamics-based delivery of synthetic siRNAs or siRNA-expressing pDNAs, at least in terms of mRNA. The present studies also suggest a limited possibility of generating a knockdown mouse with significantly reduced protein expression and impaired transporter function by this method. Although optimization of the target sequence selection for *in vivo* RNAi induction was achieved in this study, the efficiency of *in vivo* delivery of the RNAi effectors should be improved. The present results provide useful basic information for *in vivo* RNAi directed against endogenous genes including transporters.

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