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Research Paper

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

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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

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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 Endofree 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

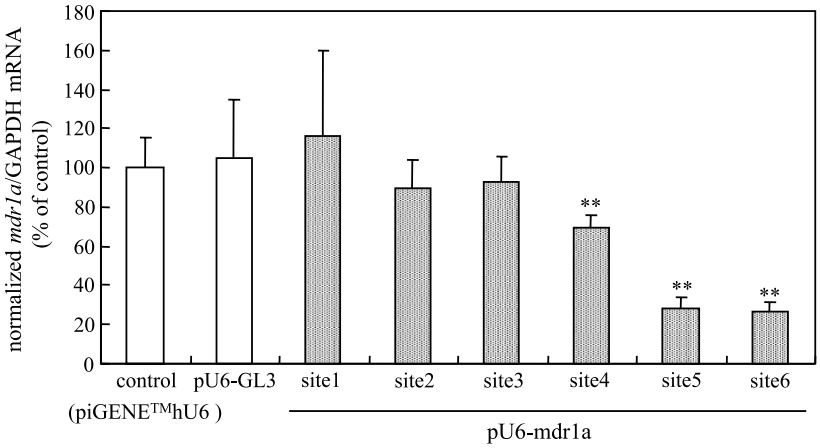

Fig. 1. Target site dependence of the suppressive activity of siRNA-expressing vectors in colon26 cells. Cells were transfected with piGENE™hU6 (control), pU6-GL3 (negative control) or pU6-mdr1a against various targeted sites, and harvested for mRNA isolation at 48 h after transfection. The amount of 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.

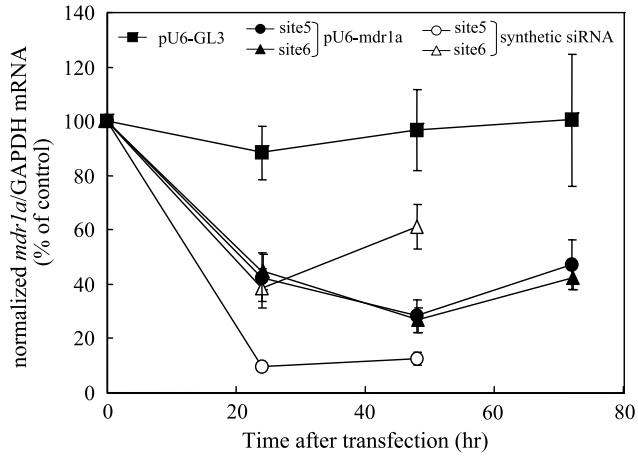

Fig. 2. Duration of siRNA-mediated RNAi against mdr1a in colon26 cells. Cells were transfected with piGENE™hU6 (control), pU6-GL3 (negative control), pU6-mdr1a (sites 5 and 6), or synthetic siRNA (sites 5 and 6), and harvested for mRNA isolation at 24, 48, and 72 h after transfection. The ordinate shows 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, $n = 8$).

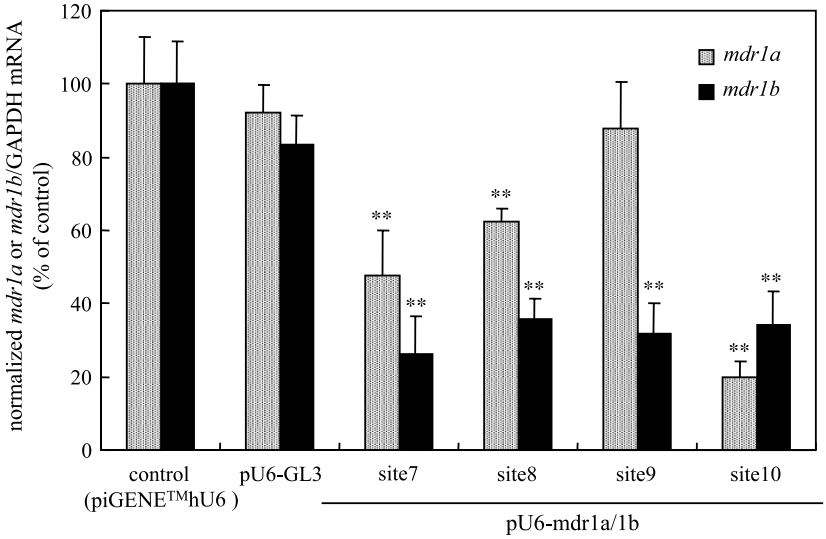

Fig. 3. Simultaneous suppression of *mdr1a*/*1b* by siRNA-expressing pDNAs in colon26 cells. Cells were transfected with piGENE™hU6 (control), pU6-GL3 (negative control), and pU6-*mdr1a*/*1b* (sites 7–10) and harvested for mRNA isolation at 48 h after transfection. The amount of mRNA was determined by 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.

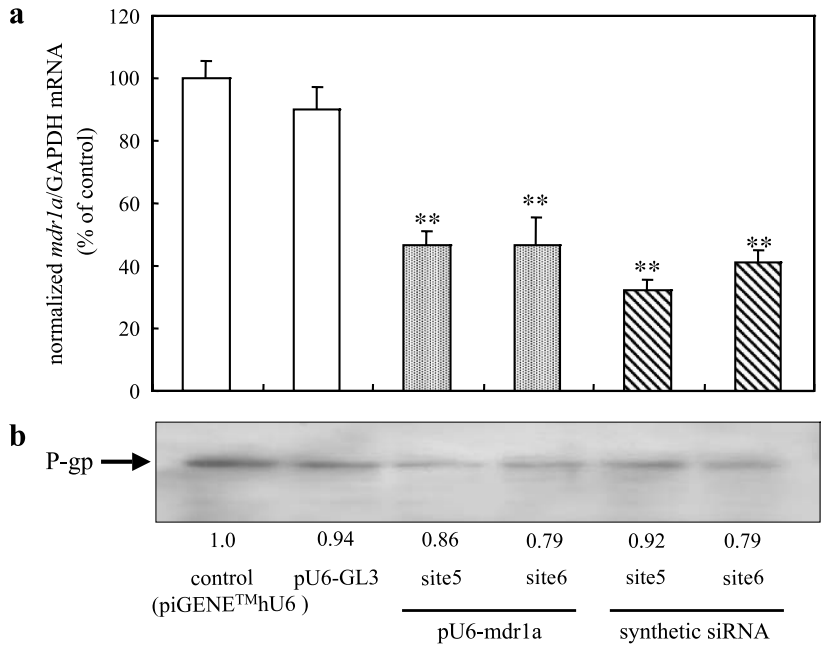

Fig. 4. *In vivo* RNAi targeting endogenous *mdr1a* in the liver by siRNA-expressing pDNAs and synthetic siRNAs. Mice were given two intravenous injections of piGENE™hU6 (negative control), pU6-mdr1a (sites 5 and 6) or synthetic siRNA (sites 5 and 6). Control mice were treated with saline without any 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 ($n = 3$).

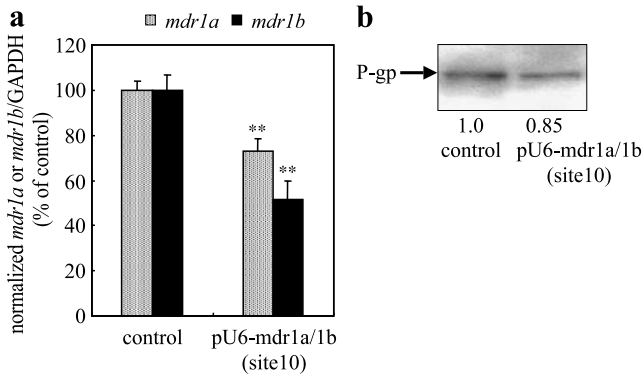

Fig. 5. *In vivo* RNAi targeting endogenous *mdr1a/1b* in the liver by siRNA-expressing pDNAs and synthetic siRNAs. Mice were given two intravenous injections of piGENE™hU6 (control), pU6-*mdr1a/1b* (site 10). Control mice were treated with piGENE™hU6. At 48 h after the first injection, the amount of *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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REFERENCES

1. A. Fire, S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver, and C. C. Mello. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**:806–811 (1998).
2. G. J. Hannon. RNA interference. *Nature* **418**:244–251 (2002).
3. H. Makimura, T. M. Mizuno, J. W. Mastaitis, R. Agami, and C. V. Mobbs. Reducing hypothalamic AGRP by RNA interference increases metabolic rate and decreases body weight without influencing food intake. *BMC Neurosci.* **3**:18 (2002).
4. S. J. Reich, J. Fosnot, A. Kuroki, W. Tang, X. Yang, A. M. Maguire, J. Bennett, and M. J. Tolentino. Small interfering RNA (siRNA) targeting VEGF effectively inhibits ocular neovascularization in a mouse model. *Mol. Vis.* **9**:210–216 (2003).
5. D. R. Sorensen, M. Leirdal, and M. Sioud. Gene silencing by systemic delivery of synthetic siRNAs in adult mice. *J. Mol. Biol.* **327**:761–766 (2003).
6. E. Song, S. K. Lee, J. Wang, N. Ince, N. Ouyang, J. Min, J. Chen, P. Shankar, and J. Lieberman. RNA interference targeting Fas protects mice from fulminant hepatitis. *Nat. Med.* **9**:347–351 (2003).
7. L. Zender, S. Hutker, C. Liedtke, H. L. Tillmann, S. Zender, B. Mundt, M. Waltemathe, T. Gosling, P. Flemming, N. P. Malek, C. Trautwein, M. P. Manns, F. Kuhnel, and S. Kubicka. Caspase 8 small interfering RNA prevents acute liver failure in mice. *Proc. Natl. Acad. Sci. USA* **100**:7797–7802 (2003).
8. J. Yano, K. Hirabayashi, S. Nakagawa, T. Yamaguchi, M. Nogawa, I. Kashimori, H. Naito, H. Kitagawa, K. Ishiyama, T. Ohgi, and T. Irimura. Antitumor activity of small interfering RNA/cationic liposome complex in mouse models of cancer. *Clin. Cancer Res.* **10**:7721–7726 (2004).
9. J. Soutschek, A. Akinc, B. Bramlage, K. Charisse, R. Constien, M. Donoghue, S. Elbashir, A. Geick, P. Hadwiger, J. Harborth, V. J. M. 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, and H. P. Vornlocher. Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature* **432**:173–178 (2004).
10. A. P. McCaffrey, L. Meuse, T. T. Pham, D. S. Conklin, G. J. Hannon, and M. A. Kay. RNA interference in adult mice. *Nature* **418**:38–39 (2002).
11. D. L. Lewis, J. E. Hagstrom, A. G. Loomis, J. A. Wolff, and H. Herweijer. Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. *Nat. Genet.* **32**:107–108 (2002).
12. A. P. McCaffrey, H. Nakai, K. Pandey, Z. Huang, F. H. Salazar, H. Xu, S. F. Wieland, P. L. Marion, and M. A. Kay. Inhibition of hepatitis B virus in mice by RNA interference. *Nat. Biotechnol.* **21**:639–644 (2003).
13. N. Kobayashi, Y. Matsui, A. Kawase, K. Hirata, M. Miyagishi, K. Taira, M. Nishikawa, and Y. Takakura. Vector-based *in vivo* RNA interference: dose- and time-dependent suppression of transgene expression. *J. Pharmacol. Exp. Ther.* **308**:688–693 (2004).
14. M. Miyagishi and K. Taira. U6 promoter-driven siRNAs with four uridine 3′ overhangs efficiently suppress targeted gene expression in mammalian cells. *Nat. Biotechnol.* **20**:497–500 (2002).
15. P. J. Paddison, A. A. Caudy, E. Bernstein, G. J. Hannon, and D. S. Conklin. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev.* **16**:948–958 (2002).
16. T. R. Brummelkamp, R. Bernards, and R. Agami. A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296**:550–553 (2002).
17. L. Zhang, N. Yang, A. Mohamed-Hadley, S. C. Rubin, and G. Coukos. Vector-based RNAi, a novel tool for isoform-specific knock-down of VEGF and anti-angiogenesis gene therapy of cancer. *Biochem. Biophys. Res. Commun.* **303**:1169–1178 (2003).
18. H. Kawasaki and K. Taira. Short hairpin type of dsRNAs that are controlled by tRNA(Val) promoter significantly induce RNAi-mediated gene silencing in the cytoplasm of human cells. *Nucleic Acids Res.* **31**:700–707 (2003).
19. M. T. McManus, C. P. Petersen, B. B. Haines, J. Chen, and P. A. Sharp. Gene silencing using micro-RNA designed hairpins. *RNA* **8**:842–850 (2002).
20. Y. Akazawa, H. Kawaguchi, M. Funahashi, Y. Watanabe, K. Yamaoka, M. Hashida, and Y. Takakura. Effect of interferons on P-glycoprotein-mediated rhodamine-123 efflux in cultured rat hepatocytes. *J. Pharm. Sci.* **91**:2110–2115 (2002).
21. H. Kawaguchi, Y. Matsui, Y. Watanabe, and Y. Takakura. Effect of interferon-gamma on the pharmacokinetics of digoxin, a P-glycoprotein substrate, intravenously injected into the mouse. *J. Pharmacol. Exp. Ther.* **308**:91–96 (2004).
22. A. H. Schinkel, C. A. Mol, E. Wagenaar, L. van Deemter, J. J. Smit, and P. Borst. Multidrug resistance and the role of P-glycoprotein knockout mice. *Eur. J. Cancer* **31A**:1295–1298 (1995).
23. A. H. Schinkel, U. Mayer, E. Wagenaar, C. A. Mol, L. van Deemter, J. J. Smit, M. A. van der Valk, A. C. Voordouw, H. Spits, O. van Tellingen, J. M. Zijlmans, W. E. Fibbe, and P. Borst. Normal viability and altered pharmacokinetics in mice lacking mdr1-type (drug-transporting) P-glycoproteins. *Proc. Natl. Acad. Sci. USA* **94**:4028–4033 (1997).
24. F. Liu, Y. Song, and D. Liu. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther.* **6**:1258–1266 (1999).
25. N. Kobayashi, T. Kuramoto, K. Yamaoka, M. Hashida, and Y. Takakura. Hepatic uptake and gene expression mechanisms following intravenous administration of plasmid DNA by conventional and hydrodynamics-based procedures. *J. Pharmacol. Exp. Ther.* **297**:853–860 (2001).
26. N. Kobayashi, T. Kuramoto, S. Chen, Y. Watanabe, and Y. Takakura. Therapeutic effect of intravenous interferon gene delivery with naked plasmid DNA in murine metastasis models. *Mol. Ther.* **6**:737–744 (2002).

27. H. Xia, Q. Mao, H. L. Paulson, and B. L. Davidson. siRNA-mediated gene silencing *in vitro* and *in vivo*. *Nat. Biotechnol.* **20**:1006–1010 (2002).
28. S. L. Uprichard, B. Boyd, A. Althage, and F. V. Chisari. Clearance of hepatitis B virus from the liver of transgenic mice by short hairpin RNAs. *Proc. Natl. Acad. Sci. USA* **102**:773–778 (2005).
29. X. P. Borst and A. H. Schinkel. Genetic dissection of the function of mammalian P-glycoproteins. *Trends Genet.* **13**:217–222 (1997).
30. T. Holen, M. Amarzguioui, M. T. Wiiger, E. Babaie, and H. Prydz. Positional effects of short interfering RNAs targeting the human coagulation trigger Tissue Factor. *Nucleic Acids Res.* **30**:1757–1766 (2002).
31. H. Kawasaki, E. Suyama, M. Iyo, and K. Taira. siRNAs generated by recombinant human Dicer induce specific and significant but target site-independent gene silencing in human cells. *Nucleic Acids Res.* **31**:981–987 (2003).
32. M. Zuker. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res.* **31**:3406–3415 (2003).
33. H. Wu, W. N. Hait, and J. M. Yang. Small interfering RNA-induced suppression of MDR1 (P-glycoprotein) restores sensitivity to multidrug-resistant cancer cells. *Cancer Res.* **63**:1515–1519 (2003).
34. C. Nieth, A. Priebsch, A. Stege, and H. Lage. Modulation of the classical multidrug resistance (MDR) phenotype by RNA interference (RNAi). *FEBS Lett.* **545**:144–150 (2003).