Vector-Based in Vivo RNA Interference: Dose- and Time-Dependent Suppression of Transgene Expression

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ABSTRACT
RNA interference (RNAi) induced by delivery of a small-interfering RNA (siRNA)-expressing vector was characterized in mice. siRNA-expressing plasmid DNA (pDNA) was injected by a hydrodynamics-based procedure along with pDNA encoding an exogenous target luciferase gene. A comparative study showed that stem-loop-type siRNA-expressing pDNA was superior, in terms of the transgene suppressive efficacy, to the tandem-type in the liver following systemic delivery of these pDNAs. Transgene suppression occurred in the liver, kidney, and lung as well as muscle. The degree of suppression was dependent on the dose of siRNA-expressing pDNA and the time at which transgene expression was determined following simultaneous injection of siRNA-expressing and target pDNAs. A reduction in transgene expression became apparent at 1 day after injection, whereas a lower degree of inhibition was obtained before this, as early as 6 h even in mice treated with an excess of siRNA-expressing pDNA. These results suggest that delivery of siRNA-expressing pDNA requires a period of time for induction of RNAi. A study of sequential injections revealed that prior injection of siRNA-expressing pDNA produced a significant suppression for at least 1 day, which disappeared within 4 days. Confocal microscopic studies indicated that the localization of the cells with successful delivery of transgene was different between primary and secondary hydrodynamics-based injections, accounting for the less effective inhibition following the sequential injections. Taken together, these results demonstrate that vector-based in vivo RNAi is a dose- and time-dependent process and offers the possibility of suppressing endogenous targets in a variety of somatic cells.

RNA interference (RNAi) is known as a powerful tool for post-transcriptional gene silencing and expected to be involved in gene therapy strategies (Hannon, 2002; Hutvagner and Zamore, 2002; Dykxhoorn et al., 2003). Small-interfering RNA (siRNA), generated via cleavages of long double-stranded RNA by a member of the RNA III family, Dicer, and typically consisting of two 21- to 23-nucleotide single-stranded RNAs that form a duplex with 2- to 4-nucleotide 3’ overhangs, plays a pivotal role in the RNAi process. Application of RNAi to mammals remained limited due to a sequence-nonspecific gene suppression via the interferon response triggered by long (>30 nucleotides) double-stranded RNA, until it was shown that the use of synthetic siRNA could induce RNAi in mammalian cells without nonspecific inhibition (Caplen et al., 2001; Elbashir et al., 2001). Immediately after the reports of successful induction of RNAi in mammalian cells, we and other groups (Brummelkamp et al., 2002; Lee et al., 2002; Miyagishi and Taira, 2002; Paddison et al., 2002a; Paul et al., 2002; Sui et al., 2002; Yu et al., 2002; Kawasaki and Taira, 2003) developed a vector-based siRNA expression system driven by Pol III promoter such as U6, H1, or transfer RNAval promoter and demonstrated effective induction of vector-based RNAi. Although synthetic siRNA is a functional molecule by itself, which can be incorporated into the RNA-induced silencing complex (RISC) and can guide RISC to the target mRNA of a complementary sequence, direct application of siRNA is accompanied by several disadvantages including an immediate disappearance of the knockdown effect due to the lack of siRNA amplification mechanisms in mammalian cells (Chiu and Rana, 2002; Zamore, 2002; Zeng and Cullen, 2002; Stein et al., 2003).
difficulty in regulating its activities, and the inconvenience and high expense associated with its use. On the contrary, siRNA-expressing vector, which works as a platform to produce a large amount of siRNA for a relatively longer period, can potentially circumvent these problems and is a versatile method of application of RNAi.

For gene function research in animals, RNAi-induced knockdown of genes of interest is attractive for its speed, usefulness, and lower cost, compared with the time-consuming conventional strategies such as gene targeting by homologous recombination. Moreover, introduction of siRNA allows us to achieve simultaneous knockdown of multiple genes or transient knockdown of lethal genes that would otherwise prevent us from investigating their functions in postnatal animals. Therefore, in vivo application of RNAi is likely to prove very popular in terms of functional analysis of unknown genes in addition to therapeutic applications to treat viral infections or tumors. Recently, Song et al. (2003) demonstrated that frequent hydrodynamics-based injections of synthetic siRNA dramatically reduced mRNA and protein levels of the targeted gene-encoded Fas receptor and protected mice from liver failure and fibrosis in experimental hepatitis. Local administrations of synthetic siRNA have been shown to suppress endogenous target genes for agouti-related peptide in the brain (Makimura et al., 2002) and for vascular endothelial growth factor in the eyes (Reich et al., 2003). Intraportal delivery of siRNA/lipid-based transfection reagent complexes resulted in suppression of endogenous β-catenin gene expression in grafted colon cancer cells (Verma et al., 2003) and inhibition of lipopolysaccharide-induced TNF-α gene overexpression (Sorensen et al., 2003). Successful results involving in vivo gene silencing of endogenous targets were achieved predominantly by use of synthetic siRNA. On the other hand, in vivo gene silencing with siRNA-expressing vector has been restricted to topical application (Makimura et al., 2002) or targeting transgenes such as luciferase gene (Lewis et al., 2002; McCaffrey et al., 2002a) and hepatitis B virus mRNA (McCaffrey et al., 2003), apart from a reduction in the endogenous β-glucuronidase mRNA level by adenovirus vector-mediated siRNA delivery (Xia et al., 2002). The delayed success of nonviral vector-based approaches might be attributed in part to the lack of information about vector-based in vivo RNAi. Therefore, in the present study, we characterized the suppression of transgene expression by vector-based RNAi in adult mice, using siRNA-expressing plasmid DNA driven by human U6 promoter.

Materials and Methods

Plasmid DNA (pDNA). siRNA-expressing pDNAs driven by human U6 promoter were constructed from piGENE hU6 vector (iGENE Therapeutics, Tsukuba, Japan) according to the instructions (Miyagishi and Taira, 2003). pU6-tandem19 or pU6-tandem26 transcribe 19- or 26-mer, respectively, of both sense and antisense RNAs that form siRNA with a four nucleotide overhang at each 3′ end, targeted to pGL3 firefly luciferase mRNA (targeted sequence: GTG CGC TGC TGC CAA C). pU6-tandem21 vector, which transcribes nonrelated sequences of RNA 5′-GUG AGC AGG AGU 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. pGL3-control (Promega, Madison, WI) was used as target firefly Photinus pyralis luciferase-expressing pDNA. pRL-SV40 (Promega) encoding sea panay Renilla reniformis luciferase was used as an internal control. pEGFP-N1 encoding enhanced green fluorescent protein (EGFP), pEGFP-F encoding farneylelated EGFP, a modified form of EGFP to bind to the plasma membrane, and pDeRed2-N1 encoding red fluorescent protein DaRed2 were purchased from BD Biosciences Clontech (Palo Alto, CA). We used pEGFP-F for the primary hydrodynamics-based injection to avoid an effusion of the transgene product by the secondary hydrodynamics-based injection, since the unmodified EGFP might diffuse into the circulation following a large-volume injection (Kobayashi et al., 2004). Each pDNA was amplified in the DH5a strain of Escherichia coli and purified using a QIAEX II Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany) or a Geno Pure Plasmid Maxi kit (Roche Diagnostics Corporation, Indianapolis, IN). The purity was checked by agarose gel electrophoresis followed by ethidium bromide staining.

Mice and Intrahepatic Injection. Four-week-old female ddY mice (approximately 20 g body weight), purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan), were used for all experiments. All animal experiments were brought under deliberation and approved for the Ethics Committee for Animal Experiments at the Kyoto University. Mice received an intrahepatic injection or an intramuscular injection of pDNAs. The intranuclear injection was performed by the hydrodynamics-based procedure (Liu et al., 1999) where the described amount of pDNAs dissolved in 1.6 ml of saline (unless otherwise mentioned) were injected into the tail vein over less than 5 s using a 26-gauge needle.

Luciferase Assay. To determine luciferase activities, mice underwent euthanasia at the indicated time and the organs including the liver, kidney, lung, and muscle were excised and homogenized in 5 ml/g (liver and muscle) or 4 ml/g (kidney and lung) lysis buffer (0.1 M Tris, 0.05% Triton X-100, 2 mM EDTA, pH 7.8). The homogenate was subjected to three cycles of freezing (−190°C) and thawing (37°C) and centrifuged at 13,000g for 10 min at 4°C. Then, appropriately diluted supernatant was mixed with luciferase assay buffer (PicageneDual, Toyo Ink, Tokyo, Japan), and the chemiluminescence was measured in a luminometer (Lumat LB 9507; EG and G Berthold, Bad Wildbad, Germany). The ratios were normalized to give percent values relative to those of the corresponding control mice. We set the dose of pGL3-control and pRL-SV40 for the raw values of the luciferase activities to be always at least 10-fold higher than those of the background derived from the liver homogenate of mice without injection.

Confocal Microscopic Study of Liver Sections. Mice were euthanized by cutting the vena cava, and the liver was gently infused with 2 ml of saline through the portal vein to remove the remaining blood. The liver was then embedded 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. Frozen liver sections (8-μm thick) were made using a cryostat (Jung Frigocut 2800E; Leica Microsystems AG, Wetzlar, Germany) by the routine procedure. The sections were directly subjected to confocal microscopy (MRC-1024; Bio-Rad, Hercules, CA) without any fixation, since the fixation step caused massive loss of GFP or DaRed due to...
immediate dissolution in the fixation buffer in our preliminary experiments.

**Results**

Interfering Efficiency of Stem-Loop-Type and Tandem-Type siRNA-Expressing Vectors in Vivo. To determine the preference in the RNAi-inducing efficiency of tandem-type and stem-loop-type siRNA-expressing pDNA in vivo, we first compared the suppressive effect in the liver following simultaneous injection of either type of siRNA-expressing pDNA and target and internal control luciferase-expressing pDNAs by the hydrodynamics-based procedure. As shown in Fig. 1, transgene expression of targeted firefly luciferase $^+$ (Pp-Luc$^+$) was significantly inhibited in mice treated with pU6-tandem26 or pU6-stem21, but not with pU6-tandem(GL2)19, in agreement with the results of in vitro cell culture (data not shown). It was also revealed that pU6-tandem26, which expresses a longer RNA duplex of 26 nucleotides, was superior in inhibitory activity to the pU6-tandem19, which expresses a 19-nucleotide RNA duplex under these experimental conditions (Fig. 1). Since pU6-stem21, which generates the shorter 21-nucleotide RNA duplex, appeared more effective than tandem-type siRNA-expressing pDNAs, we used pU6-stem21 as a model siRNA-expressing vector throughout the following studies.

Vector-Based RNAi in a Variety of Tissues Following Simultaneous Injection of siRNA-Expressing and Target pDNAs. We examined whether transgene suppression was obtained in vivo in a variety of tissues by systemic or local delivery of siRNA-expressing pDNA. Figure 2 shows the inhibitory effect of siRNA-expressing pDNA on transgene expression of the exogenous firefly luciferase gene. In this set of experiments, we used a higher amount of each pDNA to obtain enough luciferase activities for an accurate analysis in the kidney or lung, based on the fact that the level of transgene expression in these organs is approximately 5 to 6 orders of magnitude lower than that in the liver following the hydrodynamics-based procedure (Liu et al., 1999; Kobayashi et al., 2002). As a result, a marked reduction of transgene expression was observed in various organs, predominantly in the liver, following intravenous injection of the pDNAs (Fig. 3).

![Fig. 1. Comparison of interfering efficiency of various siRNA-expressing pDNAs in the liver.](image)

**Fig. 1.** Comparison of interfering efficiency of various siRNA-expressing pDNAs in the liver. Mice received an intravenous injection of different forms of siRNA-expressing pDNA (10 μg) along with pGL3-control (5 μg) and pRL-SV40 (3 μg) by the hydrodynamics-based procedure. Luciferase activities in the liver were determined 3 days after injection. The results are expressed as the mean ± S.D. (n > 4). Statistic significance was analyzed by Dunnett’s test; ***, P < 0.01 versus control.

**Fig. 2.** RNA interference in various organs following siRNA-expressing pDNA injection. Mice received an intravenous injection of piGENE hU6 or pU6-stem21 (80 μg), pGL3-control (10 μg), and pRL-SV40 (10 μg) by the hydrodynamics-based procedure, or an intramuscular injection of piGENE hU6 or pU6-stem21 (20 μg), pGL3-control (1 μg), and pRL-SV40 (1 μg) in a volume of 50 μl. Luciferase activities in the liver, kidney, and lung or treated muscle were determined 1 day after intravenous injection or 3 days after intramuscular injection, respectively. The results are expressed as the mean ± S.D. (n = 4). Significantly different from the corresponding control: *, P < 0.05; **, P < 0.01.

2). RNAi-induced transgene suppression also occurred in the muscle following intramuscular injections (Fig. 2).

Dose- and Time-Dependent Transgene Suppression in Vector-Based in Vivo RNAi. To examine any dose dependence in the suppressive effect in vector-based RNAi, we injected into mice increasing amounts of effector pDNA (pU6-stem21) and a fixed amount of target pDNAs (pGL3-control and pRL-SV40) and determined the degree of transgene suppression after 3 days. As shown in Fig. 3, inhibitory effect was clearly correlated with the dose of effector pDNA injected, with 97% inhibition in mice treated with 100 μg of pU6-stem21. We further investigated the transgene suppression of siRNA-expressing pDNA at different times after simultaneous injection of effector and target pDNAs. A marked
suppression (more than 90%) of transgene expression was obtained from day 1 to day 11 after injection (Fig. 4). However, in the earlier period, only a weak inhibitory effect was seen at 6 h after injection. To examine whether a suppressive effect becomes apparent even at the earlier period following a reduction in the relative amount of target mRNA, we injected into mice a fixed amount of effector pDNA and decreasing amounts of target pDNAs and determined the luciferase activities as early as 6 h after injection. As a result, a dose-dependent decrease in the $P\text{-}\text{Luc}^+/R\text{-Luc}$ value was observed, whereas the suppression was limited to 50% inhibition at most in mice injected with 0.001 μg of target pDNA (Fig. 5).

**Duration of Interfering Activity Following Hydrodynamics-Based Delivery of siRNA-Expressing Vector.** To estimate the duration of suppressing effect of siRNA-expressing pDNA injection, we performed a study of sequential hydrodynamics-based injections of siRNA-expressing pU6-stem21 followed by target pGL3-control and internal control pRL-SV40 at various time intervals. Figure 6 shows the inhibitory effect of siRNA-expressing pDNA injected at various time points before the target pDNA, and in each case, transgene expression was determined at 6 h after the target pDNA injection. The $P\text{-}\text{Luc}^+/R\text{-Luc}$ values were reduced in mice following injection of pU6-stem21 6 h or 1 day before, but not in mice following 4- or 11-day prior injection (Fig. 6).

**Difference in Localization of Transgene-Expressing Cells Following Simultaneous or Sequential Hydrodynamics-Based Delivery of pDNAs.** To examine the intrahepatic localization of the transgene-expressing cells following the hydrodynamics-based procedure, we injected mice with GFP-expressing pDNA and/or DsRed-expressing pDNA. Figure 7 shows the results of confocal microscopic observation of the liver sections. A simultaneous delivery of pEGFP-N1 and pDsRed2-N1 resulted in almost complete overlap of the green and the red signals in the identical cells (Fig. 7A), whereas both GFP and DsRed double-positive cells were very rare following the sequential delivery of pEGFP-F and pDsRed2-N1 (Fig. 7B).

**Discussion**

The efficiency of gene silencing in vector-based RNAi depends on the characteristics of the siRNA-expressing system, in addition to various factors involved in the siRNA itself. Various types of vectors have been designed to generate siRNA (Tavernarakis et al., 2000; Svoboda et al., 2001; Brummelkamp et al., 2002; Lee et al., 2002; McManus et al., 2002; Miyagishi and Taira, 2002; Paddison et al., 2002a, b; Paul et al., 2002; Sui et al., 2002; Yu et al., 2002; Kawasaki and Taira, 2003), and these siRNA-expression systems can be basically divided into two approaches: the sense and the antisense strands of siRNA are expressed from different cassettes aligned in tandem in the same construct (i.e., tandem-type), or the sense and the antisense strands are expressed as a connected RNA with several intermediate bases of in-
It was revealed that a reduction in transgene expression became apparent at day 1 after simultaneous injection and remained thereafter, whereas only a slight inhibition was obtained before this (Fig. 4). In addition, the transgene suppression determined at 6 h was limited to 50% inhibition at most, even in mice treated with an effector:target pDNA ratio of 10,000:1 (w/w) (Fig. 5). On the contrary, an effector:target pDNA ratio of approximately 33:1 or 3:1 (w/w) resulted in a very marked suppression when it was determined 3 days after injection (Fig. 3). These results suggest that it requires a specific period until the suppressive effect becomes apparent following simultaneous injection of effector and target pDNAs. We assumed two possible reasons for the delayed appearance of the transgene suppression. First, for cleavage of target mRNA, an injection of siRNA-expressing pDNA requires many steps, such as cellular uptake and nuclear localization of the injected pDNA, transcription of encoded RNA in the downstream of U6 promoter, transport of the RNA to the cytosol, processing by Dicer to produce functional siRNAs, and incorporation of the siRNAs to RISC, even if the hydrodynamics-based procedure produces rapid intracellular delivery of pDNA through the cellular membrane (Kobayashi et al., 2001, 2004). Second, since we introduced the target mRNA-expressing pDNA exogenously along with the effector pDNA, the expression of target mRNA was transient and the amount of intracellular mRNA varied with time. The promoters used in the present study, a virus-derived cytomegalovirus promoter and a human U6 promoter, were possibly different in their expression profiles, and the target mRNA should reach a maximum level earlier than the effector siRNA due to immediate inactivation of virus-derived promoter (Loser et al., 1998).

The duration of gene suppression is largely dependent on the rate of cell growth and the turnover of the targeted protein in actively dividing cell cultures. Since somatic cells like hepatocytes are not actively dividing, the duration of siRNA-mediated gene silencing in vivo might be governed by the activity of the siRNA-expressing vector and the stability of the functional siRNA as well as the lifespan of the targeted protein. The amount of actively transcribing target pDNA is supposed to decline over time partially in parallel with the amount of available siRNA-expressing pDNA following simultaneous injection of effector and target pDNAs. It might be possible that the observed 11-day persistence of transgene suppression does not represent the actual duration of inhibitory effect but is simply due to a significant inhibition achieved at an earlier time point (Fig. 4). Therefore, to estimate the duration of provision of active siRNA following siRNA-expressing pDNA injection, we performed sequential hydrodynamics-based injections of effector pDNA followed by target pDNAs. Transgene expression was markedly suppressed by 6-h or 1-day prior injection of pU6-stem21, but not by 4- or 11-day prior injection, indicating that the siRNA remained active and sufficient for transgene suppression for at least 1 day after siRNA-expressing pDNA injection under the present experimental conditions (Fig. 6). Since siRNA-expressing pDNA was delivered beforehand and probably had a sufficiently long period for the required processes, including the transcription and processing of targeted siRNA, the suppressive effect could be obtained as early as 6 h after target pDNA injection (Fig. 6; 6 h and 1 day). However, the degree of transgene suppression seemed to be lower compared with the result determined at day 1 after simultaneous injection as shown in Fig. 4.

To address these differences in the efficacy of transgene suppression between the ways of siRNA-expressing pDNA injection, we examined the localization of the transgene-expressing cells following the hydrodynamics-based procedure. It was found that a sequential delivery of the GFP- and DsRed-expressing pDNAs tended to give the green or the red...
signals to separated cells (Fig. 7), indicating that the localization of transgene-expressing cells of the primary and the secondary pDNA injections by the hydrodynamics-based procedure for the case of endogenous targets, simultaneous injection does not affect the transfection efficiency in terms of its suppressive effect since the effector and target pDNAs are supposed to be delivered to identical cells (Figs. 2–5). Moreover, the different hepatic localization of the effector and the target pDNAs, which were injected separately, accounts for the lower inhibitory effect in the sequential injection experiment shown in Fig. 6. In other words, suppression of transgene expression was obtained in only a fraction of the cells where both the siRNA-expressing pDNA and the target pDNAs were delivered, resulting in an apparently lower inhibition. This different localization of transgene positive cells further implies that a study of siRNA-mediated gene therapy in transgene-derived animal models for viral infections, in which hepatitis B, C, or D viral genomic DNA or RNA was introduced by the hydrodynamics-based procedure (Chang et al., 2001; McCaffrey et al., 2002b; Yang et al., 2002), should take the delivery efficiency into account.

In addition to their lower cost of production, vector-based approaches for induction of in vivo RNAi have a number of potential advantages including the possibility of sustained gene silencing and regulation of siRNA expression. The U6 promoter could be controlled with respect to its activity to transcribe RNAs by modification of the promoter to a cyclin-responsive derivative (Ohkawa and Taira, 2000; Taira and Miyagishi, 2001). Xia et al. (2002) recently demonstrated successful RNAi induction via a Pol II, cytomegalovirus promoter-driven siRNA-expression system, indicating the possibility of tissue- or cell-selective induction of siRNA by regulation of the Pol II system. Although further studies are needed to improve the efficacy of siRNA-expressing vector, the present results provide useful information for future strategies for the induction of vector-based in vivo RNAi.

References


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