was significantly ($p < 0.05$) longer than that of the other groups. Furthermore, 2 out of 7 mice survived more than 56 days.

## Discussion

We have already reported that IFN-β or IFN-γ gene delivery by the hydrodynamics-based procedure results in a significant antitumor effect against hepatic metastasis, but a weaker effect against pulmonary metastasis, of mouse colon carcinoma CT-26 cells.[12] The hydrodynamics-based procedure results in transgene expression in various internal organs, but the expression in the lung is much lower than that in the liver.[3] In a previous study, we could detect high IFN activities in the liver after gene transfer by this procedure, but failed to detect significant concentrations of IFNs in the lung. In addition, the plasma concentration of IFNs after gene transfer declined very quickly because of the transient nature of the gene expression driven by the conventional CMV promoter. These characteristics of the hydrodynamic delivery of IFN genes using conventional pDNA with CMV promoter explain the low efficacy of IFN gene transfer against pulmonary metastasis in mice. Consequently, to increase the therapeutic efficacy of IFN cancer gene therapy, we tried to increase and prolong the IFN expression by reducing CpG dinucleotides in pDNA.

As reported with other reporter and therapeutic genes,[25–27,33] the expression of IFN-β and IFN-γ from the pGZB vectors, which was estimated from their serum concentration profiles, was much longer than that from conventional pDNA after intravenous injection by the hydrodynamics-based procedure. A similar prolonged expression with pGZB vector was also obtained with firefly *luciferase* gene (unpublished data). Early studies have claimed that inflammatory cytokines, such as TNF-α produced after pDNA administration, reduce the transgene expression. In the present study, we found that the serum TNF-α level was significantly lower in mice receiving pGZB-Luc than in mice receiving pCMV-Luc (Fig. 1), suggesting that a reduced production of inflammatory cytokines is involved in the prolonged expression of IFNs from the pGZB vector. However, the low and transient nature of the induction of inflammatory cytokines after the injection of naked pDNA by the hydrodynamics-based procedure suggests that sustained gene expression from the pGZB vector is due not only to a reduction of the inflammatory response, but also to other mechanisms that have so far not been investigated.[33]

Transcriptional repression of endogenous genes is often associated with a higher frequency of methylated cytosine residues in the 5′ flanking region of the genes: promoter or enhancer.[34,35] Moreover, CpG methylation has been reported to be associated with the absence of integrated viral gene expression[36] and it has been suggested that *de novo* methylation of foreign DNA represents a cellular defense mechanism against the transcription of a foreign gene.[37] Because all CpG dinucleotides, except for those in the replication origin region, were converted to TpG dinucleotides in the pGZB vector, there was no substrate for DNA methyltransferase in the regions that were relevant to transgene expression. Therefore, sustained gene expression from CpG-reduced pDNA might be closely related to CpG methylation in addition to the reduced inflammatory response.

It has already been reported that the expression of reporter genes, such as chloramphenicol acetyltransferase or a secreted form of human placental alkaline phosphatase, is prolonged by the removal of CpG dinucleotides from the vectors.[25–27] In the present study, we also obtained a sustained transgene expression of either mouse IFN-β or IFN-γ by using CpG-reduced pDNA. However, the expression of IFN-β was not as persistent as that of IFN-γ and other proteins in the literature, suggesting that some of the pharmacological activities of IFN-β shut off the expression. Recently, Sellins *et al.*[38] reported that type I IFNs, such as IFN-α and IFN-β, play a key role in suppressing transgene expression following systemic *in vivo* gene transfer. To examine the effects of IFN-β as well as IFN-γ on transgene expression, the transgene expression in the liver was examined in mice using the pDNA encoding firefly *luciferase* gene (pGZB-Luc). Preadministration of pGZB-Muβ or pGZB-Muγ 48 hr prior to the hydrodynamic delivery of pGZB-Luc reduced the luciferase activity in the liver, but the reduction was significantly greater with pGZB-Muβ (data not shown). Therefore, it is probable that a moderate persistence of IFN-β activity after injection of pGZB-Muβ is a consequence of the ability of IFN-β to affect transcription of the transgene.

The increase in the IFN-β activity, which was quantitatively evaluated by a pharmacokinetic analysis to be a 14-fold increase in the AUC, led to a drastic reduction in the number of metastatic colonies in the lung. However, there was no significant difference in the survival time of the tumor-bearing mice between pCMV-Muβ-treated and pGZB-Muβ-treated groups. On the other hand, the use of pGZB-Muγ produced a significantly greater increase in the AUC (60-fold) and MRT (4-fold), resulting in a significant reduction in the number of tumor colonies as well as a significant increase in the survival time. Taken together, these results demonstrate that long-term expression of either IFN can be achieved by CpG-reduced pDNA and that sustained IFN gene expression results in enhanced therapeutic effects against lung metastasis of tumor cells. IFN-γ gene transfer was much more effective in increasing the survival of CT-26-bearing mice than IFN-β gene transfer, probably reflecting the greater increase in the serum IFN concentrations following the use of pGZB vector. Although multiple injections of conventional pDNA, such as pCMV-Muγ, could be effective in increasing the anti-cancer effect of IFNs, high and sustained serum concentrations of IFN-γ achieved by the pGZB-Muγ will not be obtained by increasing the frequency of administration of pCMV-Muγ. However, too high levels of IFN could induce toxic effects as observed in mice receiving 10 μg of pGZB-Muγ (data not shown). Therefore, the administration dose of pDNA should be carefully adjusted for future clinical applications.

Transfection of CT-26 cells with the IFN genes may occur by the hydrodynamic delivery of pDNA, which could be responsible for the antitumor effects of IFN gene transfer as reported in previous studies.[39,40] However, the level of transgene expression in the liver is far greater than those in other organs,[3] including the lung, the organ where metastatic tumor cells colonize in the present study. We also demonstrated a similar organ expression spectrum of the transgene following injection of pCMV-Muβ and pCMV-Muγ.[12] Therefore, direct transfection of tumor cells with the IFN genes, if it occurs, could have little contribution to the antitumor effects of hydrodynamic IFN gene transfer.

In conclusion, the CpG depletion from pDNA has been proved to be a useful approach for IFN cancer gene therapy. Because the sustained expression from pGZB vector may not be specific for its intravenous injection by the hydrodynamics-based procedure, less invasive methods of administration may be possible for future applications of IFN gene transfer to patients.

## Acknowledgements

The authors thank Dr Seng H. Cheng and Dr Nelson S. Yew (Genzyme Corporation) for providing the pGZB vector.

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# Design of PCR-Amplified DNA Fragments for *In Vivo* Gene Delivery: Size-Dependency on Stability and Transgene Expression

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*Received 18 August 2006; revised 16 November 2006; accepted 13 December 2006*

*Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jps.20879*

**ABSTRACT:** PCR-amplified DNA fragments can be more efficient and safer vectors than conventional plasmid DNA because of their smaller size and fewer numbers of immunostimulatory cytosine-phosphate-guanine (CpG) motifs. In the present study, the expression unit of plasmid DNA encoding farnesylated enhanced green fluorescent protein (EGFPF; pEGFP-F) or firefly luciferase (pLuc) was amplified by polymerase chain reaction (PCR) to obtain DNA fragments (EGFPF-mini, Luc-mini). EGFPF-mini was as effective as pEGFP-F on the basis of the number of EGFPF-expressing cells after intravenous injection into mice by the hydrodynamics-based procedure. Then, the effects of the length of DNA fragments on transgene expression were examined using luciferase-expressing DNA preparations. Luc-mini preparations showed high levels of luciferase activity in cultured cells as well as in mouse liver, even although the levels did not exceed that of pLuc. An elongation of the DNA fragment on either side of the minimal expression unit was effective in increasing the transgene expression and the stability against nucleases. PCR-amplified DNA fragments showed a sustained luciferase activity in mouse liver compared with pLuc, indicating that they are effective in achieving a prolonged expression. Their stabilization against nucleases will further increase the potential of such short, structure-controlled and synthetic DNA fragments for *in vivo* gene delivery. © 2007 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 96:2251–2261, 2007

**Keywords:** hydrodynamics-based procedure; plasmid DNA; PCR-amplified fragment; gene transfer

## INTRODUCTION

For an effective *in vivo* gene therapy, therapeutic genes should be efficiently delivered to target cells within the body. Of various gene delivery methods developed thus far,[1] the use of naked plasmid DNA without any lipids or polymers is the simplest and safest method. The hydrodynamic delivery of naked plasmid DNA, first reported by Liu et al.[2] and Zhang et al.,[3] which involves a large-volume and high-speed intravenous injection of naked plasmid DNA, gives a significantly high level of transgene expression in the liver and other major internal organs.[2–4] A large-volume injection of naked plasmid DNA solution has also been applied to gene transfer to specified organs, including the liver,[5] kidney,[6] and skeletal muscle.[7] In addition, *in vivo* electroporation increased transgene expression by naked plasmid DNA after its injection into the blood circulation[8,9] or tissues.[10] Hydrodynamic pressure or electric fields will allow plasmid DNA to enter cells, probably through pores created on cell membranes.[9,11–13]

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Journal of Pharmaceutical Sciences, Vol. 96, 2251–2261 (2007)

About 75% of cytosine-phosphate-guanine (CpG) dinucleotides in mammalian DNA are methylated at the cytosine position, whereas CpGs are largely unmethylated in bacterial DNA.[14] Because of the presence of unmethylated CpG dinucleotides, or CpG motifs, bacterial and plasmid DNAs stimulate immune responses in mammals.[15] It has been reported that systemically produced TNF-α reduces transgene expression in the lung after intravenous injection of plasmid DNA/cationic liposome complex.[16] Therefore, methylation of CpG motif is a useful method to reduce such immune responses against plasmid DNA, but it generally inhibits the transcriptional activity of most promoters.[17] Reducing the number of CpG motifs from plasmid DNA has been challenged by replacing cytosine of CpG to thimidine[18] or by recombination of plasmid backbone to give minicircles.[19–21] Another concern about the use of plasmid DNA is contamination of lipopolysaccharide (LPS) in DNA preparations, which would also reduce transgene expression.[22] Therefore, it would be useful to develop an alternative method that constructs therapeutic gene-coding DNA preparations without amplification in bacteria.

A unique property of DNA is its reproductive nature, and its amplification by polymerase chain reaction (PCR) has extensively been used in a variety of experimental settings. Because of the convenient nature of PCR, the applications of PCR-amplified DNA fragments to *in vivo* gene transfer have been attempted. Li et al.[23] used PCR-amplified DNA fragments as a model for double-stranded synthetic genes in gene therapy and demonstrated that the DNA fragments could be efficiently transferred into 293-T7 cells. Hofman et al.[24] also examined the transgene expression of such DNA preparations in the liver after intravenous injection into mice by the hydrodynamics-based procedure. These previous studies have revealed that the PCR-based amplification of an expression unit from plasmid DNA gives a therapeutic gene-expressing DNA preparation, which would have a reduced number of CpG motifs and less LPS. Another possible advantage of this approach is that the length of DNA fragments can be easily controlled by selecting appropriate primers for amplification. Because the tissue distribution, cellular entry, and nuclear transport of macromolecular compounds are molecular size-dependent processes,[25,26] smaller ones generally have an advantage in these processes that are involved in plasmid DNA-based *in vivo* gene transfer.

Although the length of DNA fragments may affect their stability, transfection efficiency, and delivery, its importance on gene transfer has hardly been explored. In the present study, we prepared PCR-amplified DNA fragments with varying lengths and examined their transgene expression in cultured cells as well as in mouse liver after intravenous injection by the hydrodynamics-based procedure.

## MATERIALS AND METHODS

### Plasmid DNA

Plasmid DNA encoding firefly luciferase (pLuc) was constructed by subcloning the Hind III/Xba I firefly luciferase cDNA fragment from pGL3-control vector (Promega, Madison, WI) into the polylinker of the pcDNA3 vector (Invitrogen, Carlsbad, CA) as reported previously.[27] pLuc amplified in the DH5α strain of *E. coli* was extracted and purified by a QIAGEN Endofree™ Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). The purity was checked by 1% agarose gel electrophoresis followed by ethidium bromide staining. DNA concentration was measured by UV absorption at 260 nm. pEFGP-F encoding farnesylated enhanced green fluorescent protein (EGFPF) was purchased from BD Biosciences Clontech (Palo Alto, CA). pRL-CMV, a plasmid vector encoding renilla luciferase, was purchased from Promega.

### Primers and PCR Amplification

The sequences of primers used for PCR amplification are as follows: EGFPF-mini (sense primer, CCGTATTACCGCCATGCATT; antisense primer, CCACAACTAGAATGCAGTG); Luc-mini2.6 (sense primer, TTCGCGATGTACGGGCCAGA; antisense primer, CATCCCCAGCATGCCTGC-TA); Luc-mini2.8 (sense primer, GGAGGTCGCTGAGTAGTGCG; antisense primer, TAGCGGTCACGCTGCGCGTA); Luc-mini3.5 (sense primer, GGGAATAAGGGCGACACGGA; antisense primer, CCACACCCTAACTGACACAC); Luc-mini4.5 (sense primer, AGCGGTTAGCTCCTTCGGTC; antisense primer, TGCGCTGACAGCCGGAACAC). Phosphorothioated primers for the Luc-mini2.6, in which 4 linkages at the 3′-terminal were phosphorothioated, were also used to obtain Luc-mini2.6PS. Except for the Luc-mini3.5R and Luc-mini3.5L, the both sides of


DOI 10.1002/jps

the expression unit were extended to obtain DNA fragments of 2.8 kb (Luc-mini2.8) or greater. Only the 3′- or 5′-side of the expression unit was extended for Luc-mini3.5R and Luc-mini3.5L, respectively, using the primers for Luc-mini2.6 and Luc-mini4.5. Figure 1 shows the schematic presentation of the PCR-amplified DNA fragments developed in this study. For PCR, 10 ng of plasmid template, 50 pmol of each primer, and 2.5 U of DNA polymerase (Takara Ex Taq™ or Pyrobest DNA polymerase, Takara Bio Inc., Otsu, Japan), 0.2 mM dNTP mixture were used. The PCR products were extracted with GenElute™ PCR Clean-Up Kit (Sigma, St. Louis, MO).

### *In Vitro* Transfection

Murine melanoma cell line B16-BL6[28] was cultured in Dulbecco's modified Eagle's minimum essential medium supplemented with 10% fetal bovine serum and penicillin/streptomycin/L-glutamine at 37°C and 5% $CO_2$. B16-BL6 cells were plated on 24-well culture plates (at a density of $1 \times 10^5$ cells/well). After an overnight incubation, pLuc or Luc-mini preparation (0.2 pmol/well) was mixed with Lipofectamine2000 (Invitrogen) in Opti-MEM (Invitrogen) and the mixture was incubated for more than 20 min at room temperature. Then, resulting complexes were added to cells. In a separate set of experiments, each DNA preparation (0.2 pmol/well) was added to B16-BL6 cells on 6-well culture plates ($5 \times 10^5$ cells/well) as the naked form and electric pulses (100 V, 4 ms, 10 Hz, 12 pulses) were applied using a rectangular direct current generator (CUY-21, Nepagene, Chiba, Japan). In both cases, cells were collected at 24 h after transfection and lysed in a lysis buffer (0.1 M Tris, 0.05% Triton X-100, 2 mM EDTA, pH 7.8), and the luciferase activity was determined.

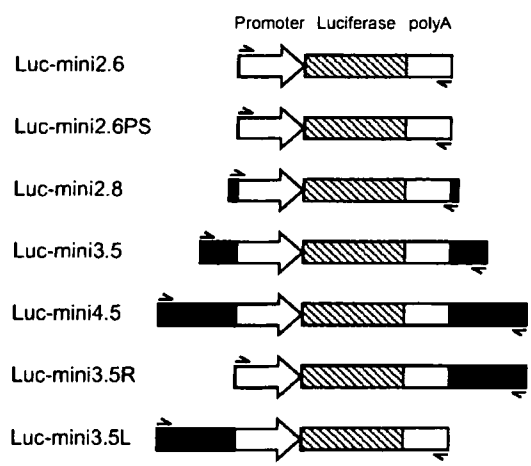


**Figure 1.** Schematic presentation of DNA fragments amplified from plasmid DNA expressing firefly luciferase. DNA fragments, including the minimal expression unit, were amplified by PCR using specific primers (arrows). For Luc-mini2.6PS, primers, in which 4 linkages at the 3′-terminal were phosphorothioated, were used.

### Mice and *In Vivo* Gene Transfer to the Liver by the Hydrodynamics-Based Procedure

Four-week-old female ddY mice (approximately 20 g body weight) were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). The protocols for animal experiments were approved by the Animal Experimentation Committee of Graduate School of Pharmaceutical Sciences of Kyoto University. Mice under light anesthesia by inhalation of diethyl ether received an injection of naked DNA preparations dissolved in 1.6 mL of saline into the tail vein over less than 5 s using a 26-gauge needle.[2] The doses of DNA preparations were determined based on the sensitivity of the assay systems: 0.23 pmol/mouse for luciferase-expressing DNA preparations (1 μg/mouse for pLuc) and 8.4 pmol/mouse for EGFPF-expressing DNA preparations (25 μg/mouse for pEGFP-F). pRL-CMV (0.1 μg/mouse), which encodes renilla luciferase cDNA, was simultaneously delivered with firefly luciferase-expressing pLuc or Luc-mini preparations.

### Confocal Microscopic Observation of Mouse Liver Sections

Mice were euthanized at 6 h after injection of EGFPF-expressing DNA preparations. 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 until use. Frozen liver sections of 8 μm in thickness were made using a cryostat (Jung Frigocut 2800E; Leica Microsystems AG, Wetzlar, Germany) by a routine procedure. The liver sections were fixed with Mildform 20 N (8% paraformaldehyde, Wako, Osaka, Japan) for 4 min at 4°C followed by confocal microscopic observation (MRC-1024, BioRad, Hercules, CA).

### Luciferase Assay

Mice were euthanized at 6 h after injection of luciferase-expressing DNA preparations unless otherwise indicated. The liver was excised and homogenized in the lysis buffer. The homogenate was subjected to three cycles of freezing (−190°C) and thawing (37°C) and centrifuged at 13000*g* for 10 min at 4°C. Appropriately diluted supernatant of the lysate of B16-BL6 cells or liver homogenate was mixed with luciferase assay buffer (Picagene-Dual, Toyo Ink, Tokyo, Japan), and the chemiluminescence produced was measured in a luminometer (Lumat LB 9507; EG and G Berthold, Bad Wildbad, Germany). For liver samples, the ratio of the activity of firefly luciferase (Pp-Luc) to renilla luciferase (Rr-Luc) was calculated to correct for differences in transfection efficiency among mice,[29] and the results were expressed as relative Pp-Luc RLU/s/mg protein, which represents Pp-Luc activity normalized to Rr-Luc activity of the individual animals.

### Stability of DNA Fragments

Bal31 and DNase I were purchased from Takara Bio Inc. Each luciferase-expressing DNA preparation was incubated with Bal31 (1 U/μg DNA) or DNase I (0.005 U/μg DNA) at 37°C. Aliquots (15 μl) were removed at each sampling point and added with EDTA (a final concentration of 50 mM) to stop the degradation of DNA. Each aliquot was mixed with a loading buffer and loaded on a 1.3% agarose gel and electrophoresed in TBE buffer (89 mM Tris-Borate, 25 mM EDTA). After electrophoresis, the gel was stained with ethidium bromide and DNA bands were visualized by fluorescence in an ultraviolet transilluminator system. To determine whether the minimal expression unit remains after the incubation with Bal31, the nuclease-treated samples of Luc-mini2.6 and Luc-mini3.5 were amplified by PCR using the primers for Luc-mini2.6. Then, the PCR product was examined on an agarose gel described above.

### Statistical Analysis

Differences were statistically evaluated by one-way ANOVA followed by the Student-Newmann-Keuls multiple comparison test, and the level of statistical significance was $p < 0.05$.

## RESULTS

### Distribution of EGFPF Expressing Cells in the Liver after Hydrodynamic Delivery of Plasmid DNA and a DNA Fragment

Figure 2 shows the liver sections of mice receiving an intravenous injection of pEGFP-F or EGFPF-mini by the hydrodynamics-based procedure. The dose was set at 8.4 pmol/mouse, that is, 25 and 8.9 μg/mouse for pEGFP-F and EGFPF-mini, respectively. No positive cells were found in

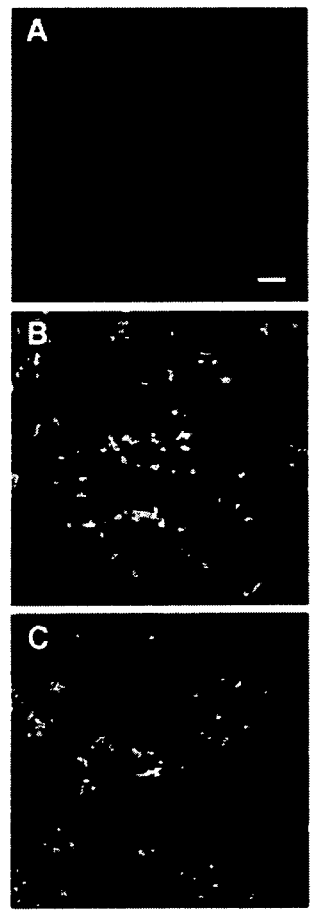


**Figure 2.** Confocal microscopic images of the liver sections following intravenous injection of pEGFP-F or EGFPF-mini in mice by the hydrodynamics-based procedure. Mice were euthanized at 6 h after injection and the liver sections were made. The images shown are typical of those observed in several visual fields of three mice per group. (A) pEGFP-F (0.02 μg/mouse, equal to the amount of template plasmid DNA included in the EGFPF-mini preparation); (B) pEGFP-F (25 μg or 8.4 pmol/mouse); (C) EGFPF-mini (8.9 μg or 8.4 pmol/mouse).


DOI 10.1002/jps

the liver section of mice receiving 0.02 μg pEGFP-F, an equal amount of pEGFP-F used as a template for EGFPF-mini preparation (Fig. 2A). On the other hand, significant numbers of EGFPF-expressing cells were detected in the liver sections of mice receiving pEGFP-F (Fig. 2B) and EGFPF-mini (Fig. 2C). There were no marked differences in the number and distribution of EGFPF-expressing cells in the liver sections. These results suggest that the hydrodynamic delivery of these DNA preparations is hardly affected by their shape and size, although they were quite different from each other, that is, circular 4.8 kb-plasmid DNA and linear 1.7 kb-DNA fragment.

### Transgene Expression by Luciferase-Expressing DNA Preparations in Cultured Cells

Figure 3A shows the luciferase activity in B16-BL6 cells after transfection of pLuc or Luc-mini complexed with Lipofectamine2000. All Luc-mini preparations gave significant levels of transgene expression, although the expression levels were about 100-fold less than that of pLuc. No apparent relationship was observed between the level of transgene expression and the size of the PCR-amplified DNA fragments. Figure 3B shows the luciferase activity in B16-BL6 cells after electric transfer of pLuc or Luc-mini. Again, all Luc-mini preparations gave high levels of luciferase activity, but the levels were significantly smaller than that of pLuc. However, in the case of electric gene transfer, the difference in the luciferase activity between pLuc and Luc-mini preparations was much smaller than that for cationic liposome-mediated transfection (Fig. 3B). In both experiments, Luc-mini2.6PS showed almost identical levels of luciferase activity to Luc-mini2.6, suggesting that the PS substitution have little effects on transgene expression at least under the conditions examined.

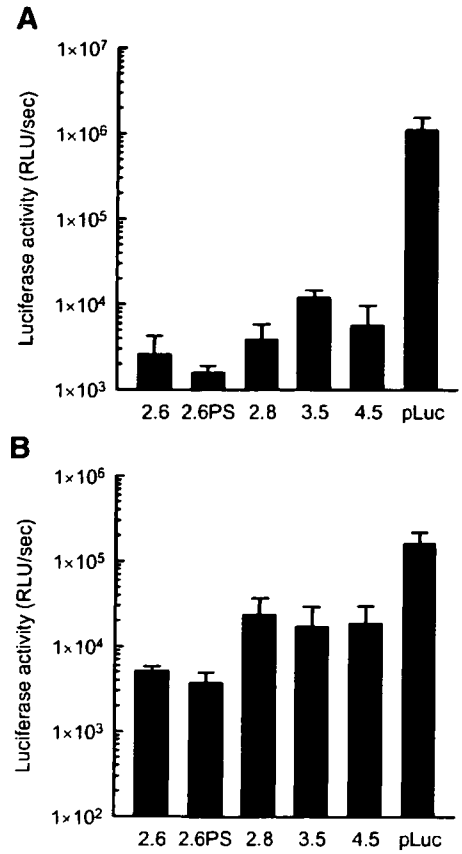


**Figure 3.** Luciferase activity in B16-BL6 cells after transfection *in vitro*. (A) pLuc or Luc-mini preparation (0.2 pmol/well) was mixed with Lipofectamine2000 and resulting complexes were added to cells. (B) pLuc or Luc-mini preparation (0.2 pmol/well) was added to B16-BL6 cells as the naked form and electric pulses (100 V, 4 ms, 10 Hz, 12 pulses) were applied. Cells were collected at 24 h after transfection and lysed in a lysis buffer, and the luciferase activity in the supernatant was measured in a luminometer. PS, phosphorothioated primers were used to obtain PCR-amplified DNA fragments. Results are expressed as mean ± SD of three determinations.

### Transgene Expression in Mouse Liver Following Hydrodynamic Delivery of Luciferase-Expressing DNA Preparations

Figure 4 shows the luciferase activity in the liver 6 h after intravenous injection of each luciferase-expressing DNA preparation into mice by the hydrodynamics-based procedure at a dose of 0.23 pmol/mouse, together with 0.1 μg of pRL-CMV. The relative firefly luciferase activity (Pp-Luc) increased as the length of the DNA fragments became longer. The expression level of Luc-mini4.5 was not significantly different from that of pLuc, but the levels of DNA fragments with the length of 3.5 kb or smaller were significantly ($p < 0.05$) lower than those of Luc-mini4.5 and pLuc. Again, there were no significant effects of the PS substitution in the level of luciferase activity. Pyrobest, a high-fidelity DNA polymerase, was used to prepare a 3.5 kb-DNA fragment, Luc-mini3.5Pyro, but no significant difference in transgene expression was

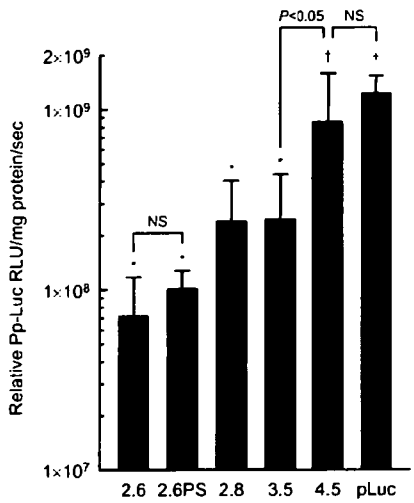


**Figure 4.** Relative luciferase activity in mouse liver after intravenous injection of pLuc or Luc-mini preparation by the hydrodynamics-based procedure. Mice received pLuc or Luc-mini (0.23 pmol/mouse) along with pRL-CMV (0.1 μg/mouse). At 6 h after injection, luciferase activities in the liver were determined. The ratio of the activity of firefly luciferase (Pp-Luc) to renilla luciferase (Rr-Luc) was calculated to correct for differences in transfection efficiency among mice. The results are expressed as relative Pp-Luc RLU/s/mg protein, which represents Pp-Luc activity normalized to Rr-Luc activity of the individual animals. PS, phosphorothioated primers were used to obtain PCR-amplified DNA fragments. Results are expressed as mean ± SD of at least three mice. $^{*}p < 0.05$ versus pLuc; $^{\dagger}p < 0.05$ versus Luc-mini2.6.

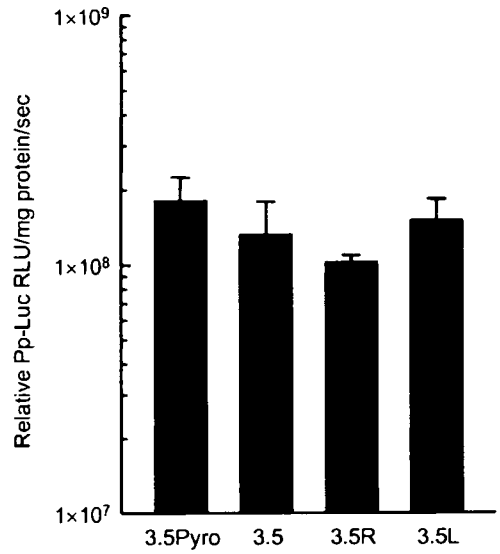


**Figure 5.** Effect of polymerases and the extension of 5′- and 3′-side of the expression unit in DNA fragment on transgene expression. Mice received Luc-mini (0.23 pmol/mouse) along with pRL-CMV (0.1 μg/mouse). At 6 h after injection, luciferase activities in the liver were determined. The results are expressed as relative Pp-Luc RLU/s/mg protein, which represents Pp-Luc activity normalized to Rr-Luc activity of the individual animals. Pyro, a high-fidelity DNA polymerase Pyrobest, which was used to prepare a 3.5 kb-DNA fragment; L, the 5′-side (left-hand side) of the expression unit was extended; R, the 3′-side (right-hand side) of the expression unit was extended. Results are expressed as mean ± SD of at least three mice.

observed between Luc-mini3.5Pyro and Luc-mini3.5 after intravenous injection in mice by the hydrodynamics-based procedure (Fig. 5).

### Effect of the Extension of 5′- and 3′-Side of the Expression Unit in DNA Fragment on Transgene Expression

It was found that the elongation of DNA fragments is effective in increasing the level of transgene expression after *in vivo* gene transfer. Then, we examined the effects of extension of 5′- or 3′-side of the minimal expression unit on transgene expression after hydrodynamic delivery to mouse liver. For Luc-mini3.5, both sides of the expression unit were extended. Only the 5′- or 3′-side of the unit was extended for Luc-mini3.5L and Luc-mini3.5R, respectively. Figure 5 shows the relative firefly luciferase activity of these DNA fragments with an identical length of 3.5 kb. The expression of these three DNA fragments with the same length of 3.5 kb showed similar transgene expression, although Luc-mini3.5R, which has no additional nucleotides on the outside of the promoter, was a little lower than that of the other two preparations, Luc-mini3.5 and Luc-mini3.5L. These results suggest that the length of DNA fragments is a primary factor for the level of transgene expression, and that the extension of the 5′-side of expression units is slightly favorable to some extent over the extension of the other side.

### Time Course of Transgene Expression of Luciferase-Expressing DNA Preparations

Figure 6 shows the time courses of firefly luciferase activity in mouse liver after hydrodynamic delivery of pLuc, Luc-mini2.6, and Luc-mini3.5. The expression levels of Luc-mini2.6 and


DOI 10.1002/jps

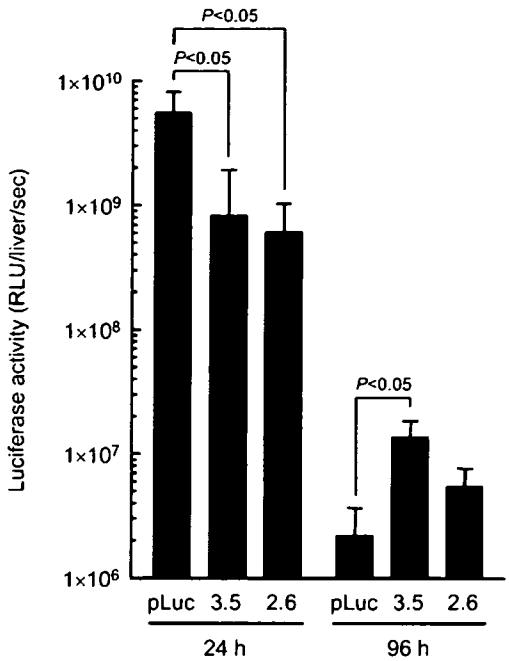


**Figure 6.** Luciferase activity in mouse liver after intravenous injection of pLuc, Luc-mini2.6, and Luc-mini3.5 by the hydrodynamics-based procedure. Mice were injected with pLuc or Luc-mini (0.23 pmol/mouse), and luciferase activities in the liver were determined at 24 and 96 h after injection. Results are expressed as the mean ± SD of three mice.

Luc-mini3.5 at 24 h were lower than that of pLuc as well as at 6 h (Fig. 4). However, the level of Luc-mini3.5 was significantly ($p < 0.05$) higher than that of pLuc at 96 h after intravenous injection by the hydrodynamics-based procedure. These results suggest that the PCR-amplified DNA fragments are effective in achieving prolonged transgene expression than conventional plasmid DNA.

### Stability of PCR-Amplified DNA Fragments

To examine the possibility whether the differences in the transgene expression is due to the stability of DNA preparations, we examined the degradation of Luc-mini preparations by an exo- (Fig. 7) or endonuclease. All DNA preparations were degraded with time in the presence of an exonuclease, Bal31. The disappearance of DNA bands was the slowest with pLuc. All DNA fragments showed a reduction in the DNA size with time, suggesting that they are gradually degraded by the exonuclease. In the case of pLuc

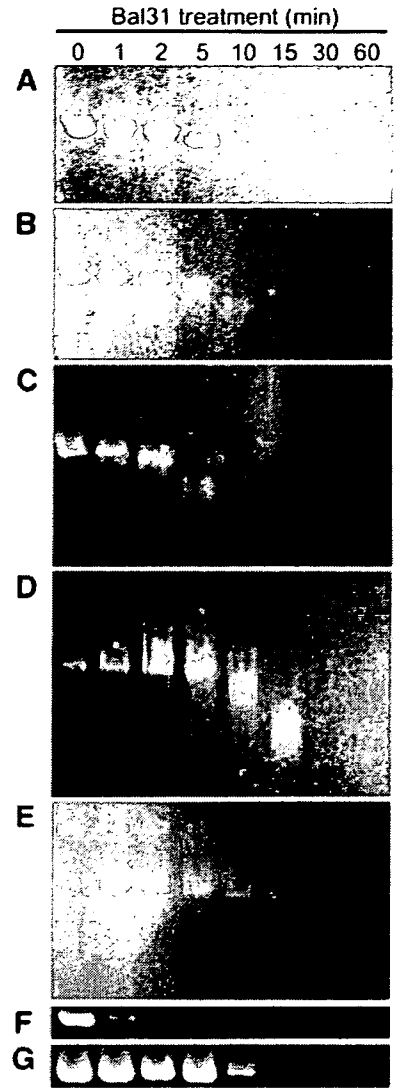


**Figure 7.** Gel electrophoresis of pLuc and Luc-mini after incubation with an exonuclease Bal31. (A–E) Each luciferase-expressing DNA preparation was incubated with Bal31 (1 U/μg DNA) at 37°C. Aliquots were removed at each sampling point and added with EDTA to stop the degradation of DNA. Each aliquot was mixed with a loading buffer and loaded on an agarose gel. (A) Luc-mini2.6; (B) Luc-mini2.6PS; (C) Luc-mini2.8; (D) Luc-mini3.5; (E) pLuc. (F,G) Bal31-treated Luc-mini2.6 and Luc-mini3.5 were amplified by PCR using the primers for Luc-mini2.6, and the PCR products were loaded on an agarose gel. (F) Luc-mini2.6; (G) Luc-mini3.5.

(Fig. 7E), two to three bands were detected, suggesting a time-dependent conversion of plasmid DNA from its supercoiled form to other forms. To confirm whether the minimal

expression unit in DNA fragments remains during the incubation with the nuclease, the unit of Luc-mni2.6 and Luc-mini3.5 was amplified by PCR using the primers used for the preparation of Luc-mini2.6 (Fig. 7F, G). The PCR product from Luc-mini2.6 was detectable only at 1 min after incubation, whereas that from Luc-mini3.5 was able to be detected at least for the first 10 min of incubation. Digestion with DNase I, an endonuclease, also gave similar results to those obtained with Bal31 (data not shown).

## DISCUSSION

Effective *in vivo* gene therapy relies on the controlled expression of transgenes after *in vivo* gene transfer. In spite of the safety of plasmid DNA as a nonviral vector,[1] its clinical applications has significantly been obstructed by the low and transient nature of its transgene expression. Of numbers of problems on plasmid DNA-based gene transfer, inefficient delivery of the DNA inside target cells is the most important one. This is mainly attributed to the large size of plasmid DNA,[30] which is in the range of several millions in molecular weight. One approach to reducing the size is the use of PCR-amplified DNA fragments, which would also have advantages over plasmid DNA, such as small numbers of immunostimulatory CpG motifs and little contamination of LPS. Although errors in PCR amplification could be an inherent problem of this approach for large scale synthesis, previous studies have shown that PCR-amplified DNA fragments are potent vectors to achieve significant transgene expression both *in vitro* and *in vivo*,[23,24] but it has hardly been examined whether the size of such DNA fragments affects the transgene expression. In the present study, we prepared various PCR-amplified DNA fragments and examined their efficiency on transgene expression. Transgene expression from a vector like plasmid DNA would be determined by the accessibility of the vector to the nucleus of target cells, the rate of transcription, and the stability of the vector. In addition to these parameters, promoter attenuation[31] and integration into genome would also determine the duration of transgene expression. First, we used cationic liposome-mediated delivery to cultured cells. The expression of any DNA fragment was almost one hundredth of that of plasmid DNA (Fig. 3A), indicating that the DNA fragments are not as effective for transgene expression as plasmid DNA. It was reported that DNA fragments larger than 1 kb remained in the cytoplasm,[32] suggesting that DNA fragments have little advantages over plasmid DNA as far as the size-dependent nuclear transport is considered. In nondividing cells like hepatocytes, such large DNAs are transported into the nucleus through the nuclear pore complex.[33] Dean et al.[34,35] reported that the process of nuclear transport of DNA is sequence specific and SV40 enhancer is all that is necessary for this DNA nuclear import. Therefore, pLuc might be more efficiently transported into the nucleus than DNA fragments via sequences outside the fragments. In addition, the stability of the DNA and rate of transcription can also be the reasons for the inefficient transgene expression of DNA fragments when delivered as cationic complexes.

Electroporation creates pores on cell membranes, and charged molecules, such as DNA, can be delivered inside cells by electrophoresis.[11] The hydrodynamic delivery method greatly increases the membrane permeability of macromolecules including plasmid DNA in the liver, probably through the creation of membrane pores.[12,13] Because the passage through such pores is a function of molecular size of solutes, the efficiency of cellular entry of DNA preparations could be a function of molecular size. We found that the DNA fragments prepared showed less transgene expression than plasmid DNA, when delivered to cells by electroporation (Fig. 3B). However, the difference between plasmid DNA and DNA fragments was much smaller when they were delivered by electroporation than by cationic liposomes (Fig. 3A). These results suggest that the limited delivery of plasmid DNA through membrane pores is somewhat improved by the reduction in size by PCR amplification of the expression unit of plasmid DNA. This improvement in the delivery of DNA inside cells seemed to be more pronounced in mouse liver after intravenous injection by the hydrodynamics-based procedure. In addition to this hypothesis, the hydrodynamic or electroporative delivery of DNA will take much shorter time than lipofection as far as the delivery of DNA into target cells is concerned. Therefore, a reduced nuclease degradation of DNA may also contribute to the improved gene expression from the PCR-amplified DNA fragments by hydrodynamic delivery and electroporation. No significant differences were observed in the luciferase assay between pLuc


DOI 10.1002/jps

and Luc-mini4.5 (Fig. 4). Similar results were obtained with EGFPF-expressing DNA preparations (Fig. 2). These results indicate that the reduction in size of DNA preparations would be promising for an enhanced delivery, although the level of transgene expression may be low because of the other factors than delivery.

The size of the DNA fragments significantly affected transgene expression in mouse liver after intravenous injection by the hydrodynamics-based procedure. The longer the fragment was injected the higher transgene expression was. The incubation of DNA preparations with nucleases clearly demonstrated that the expression unit in Luc-mini3.5 remained much longer than that in Luc-mini2.6 (Fig. 7), suggesting that the presence of additional nucleotides retards the degradation of the expression unit and the following transgene expression. Because PCR-amplification is highly versatile, there would be least limitations on selecting the size and location for amplification. Loss of some nucleotides in the promoter region would largely reduce the transcriptional activity of DNA preparations. We found that the extension of 5′-side of the expression unit, that is, the promoter side, could be a little better in increasing transgene expression (Fig. 5). However, the effects of the location of the expression unit in DNA fragments were much smaller than those of their length. A possible concern about using PCR amplification is that errors occur during the amplification. We used two polymerases with different fidelities: Taq and Pyrobest polymerases, but observed no significant differences in transgene expression between Luc-mini3.5 and Luc-mini3.5Pyro (Fig. 5). These results do not exclude the possibility of errors in PCR amplification of DNA fragments, but the frequency could be very low.

In spite of the lower expression by DNA fragments at 6 h after injection, Luc-mini2.6 and Luc-mini3.5 showed significantly greater expression at 96 h after injection than pLuc (Fig. 6). Duration of transgene expression may be affected by several factors, including the stability of vectors, promoter attenuation, and integration into genome.[31,36] CMV promoter, the one used in the present study, has been reported to be attenuated in various experimental settings.[37,38] The methylation of CpG dinucleotides in the promoter would be involved in this attenuation.[39] In addition, the presence of CpG motifs triggers the induction of inflammatory cytokines upon administration to animals,[16] because mammalian TLR-9 recognizes unmethylated CpG dinucleotides and induce such cytokines.[15] Although this feature of plasmid DNA can be a drawback for sustained transgene expression, the induction of the cytokines or the attenuation of CMV promoter by itself may not entirely account for the difference in the duration of transgene expression between pLuc and Luc-minis. Another possibility is the difference in the integration into genome of DNA preparations, as reported by Nakai et al.[40] which may cause toxic side effects. However, Chen et al.[41] reported that linear DNA-treated mice showed a 10-fold drop in transgene expression after partial hepatectomy, which suggests that transcriptionally active liner DNAs remained predominantly extrachromosomal in the liver. Similar results were also reported by Kameda et al.[42] using PCR-amplified DNA fragments expressing erythropoietin. These previous reports would support an idea that PCR-amplified DNA fragments are hardly integrated into the genome. Further studies are needed to understand the reason for the prolonged transgene expression by PCR-amplified DNA fragments.

In conclusion, we demonstrated that PCR-amplified DNA fragments are effective vectors to achieve significant transgene expression both *in vitro* and *in vivo*. They do not require bacteria for production, and they can be manipulated without the possible contamination of LPS once template DNA is prepared. Fragmentation tended to reduce the stability and transcriptional activity of expression units, but a relatively prolonged transgene expression can be obtained with DNA fragments. An extension of DNA fragments would retard their degradation, resulted in high level of transgene expression. This effect seems to be enhanced in the present study, because the dose was set at low levels. The stabilization of DNA fragments against nucleases will further increase the potential of such short, structure-controlled, and synthetic DNA fragments for *in vivo* gene delivery.

## ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan, by grants form the Ministry of Health, Labour and Welfare, Japan, by a grant from Uehara Memorial Foundation.

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YAKUGAKU ZASSHI 127(10) 1525—1531 (2007) © 2007 The Pharmaceutical Society of Japan 

—Reviews—

# がん細胞へのsiRNAデリバリーとがん遺伝子治療への適用

高橋有己,* 西川元也, 高倉喜信

# *In vivo* siRNA Delivery to Tumor Cells and Its Application to Cancer Gene Therapy

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(Received June 14, 2007)

RNA interference (RNAi) is a posttranscriptional gene-silencing event in which short double-stranded RNA (siRNA) degrades target mRNA. Because of its potent and highly specific gene-silencing effect, RNAi is expected to be used in the treatment of various diseases. Cancer is one of the major targets of RNAi-based therapy, because silencing oncogenes or other genes contributing to tumor progression can be target genes for RNAi. The delivery of RNAi effector to target cells is one of the key factors determining therapeutic efficacy, because gene silencing is limited to cells reached by RNAi effectors. Tumor cell lines stably expressing reporter genes were confirmed to be effective in sensitively and quantitatively evaluating RNAi effects in tumor cells *in vitro* and *in vivo*. Quantitative analyses of the gene-silencing effect revealed that short-hairpin RNA expressing plasmid DNA (pshRNA) has more durable effects than siRNA. Intratumoral injection of RNAi effectors was effective in suppressing target gene expression in tumor cells, and silencing of $\beta$-catenin or hypoxia-inducible factor-1$\alpha$ (HIF-1$\alpha$) significantly inhibited tumor growth. RNAi effectors were successfully delivered to tumor cells colonizing the liver through the vascular route. We found that tumor-bearing liver showed elevated HIF-1$\alpha$ expression in the cells, and the silencing of the expression in normal liver cells is also effective in inhibiting metastatic tumor growth. These results indicate the possibility of RNAi-based cancer therapy.

**Key words**——RNA interference; short hairpin RNA; hydrodynamic delivery; electroporation; tumor growth

## 1. はじめに

RNA干渉（RNA interference）は二本鎖RNAにより配列特異的にmRNAが分解される現象である.[1] 2001年にTuschlらにより，この現象が活性本体である21-23塩基対の2本鎖RNA（siRNA）を用いることで哺乳動物にも適用可能であることが明らかとされ，それ以来，合成されたsiRNA，あるいは細胞内でプロセシングを受けることによりsiRNAとなるショートヘアピン型RNA（shRNA）を用いることでRNA干渉が誘導可能であることが*in vitro*・*in vivo*の実験系において証明されてきた.[2,3] RNA干渉は，標的遺伝子の発現を簡便かつ特異的に抑制可能であることから，現在では特定遺伝子の機能を評価するための実験手技として幅広く活用されている．また，がんやウイルス感染などに代表される病原タンパク質の発現亢進が原因となる疾患に対しては，その発現を特異的に抑制することによる分子標的治療法としての応用が期待されている.[4,5] その一方で，RNA干渉による遺伝子発現抑制効果はsiRNAが存在する細胞に限局されるため，RNA干渉の治療手段としての適用にはsiRNAの標的細胞へのデリバリーが必要条件となる.[6] しかしながら十分に治療可能なsiRNAのデリバリー方法はいまだほとんど開発されておらず，より効率的なデリバリー方法の開発が必要である．そこで本稿ではわれわれの検討結果を中心に，siRNAをデリバリーすることで発がん遺伝子やがん細胞の増殖・転移等に関係した遺伝子の発現を抑制することによるがん治療の可能性について解説する．

## 2. *In vivo*におけるがん細胞へのsiRNAのデリバリー

RNA干渉の誘導には，siRNAが標的細胞に活性を保持した状態で到達することが必要であるが，

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本総説は，日本薬学会第127年会シンポジウムSD1で発表したものを中心に記述したものである．

siRNA は核酸であることから生体内における安定性が低い．したがって，化学的安定性の改善を目的とした化学修飾 siRNA の有効性も報告されている．[7] また，細胞での転写により shRNA を発現するベクターも有効であり，ウイルスベクター，非ウイルスベクターが利用可能である．[8,9] ウイルスベクターは，アデノウイルスベクター投与による死亡事例やレトロウイルスベクターを用いた遺伝子治療における白血病の発症など，安全性の面で解決しなければならない課題が多い．[10-12] これに対し，プラスミド DNA（pDNA）に代表される非ウイルスベクターは，安全性・生産性の面で優れており，また問題とされてきた導入効率についても近年大幅に改善されてきている．ウイルスを使わない方法で RNA 干渉を誘導し疾患治療を実現するためには，siRNA あるいは shRNA 発現 pDNA（pshRNA）を標的細胞内へデリバリーすることが必須である．しかしながら，siRNA や pshRNA などは，水溶性高分子であるため細胞膜透過性が著しく低く，静脈内投与のような一般的な投与方法ではほとんど細胞内には到達しない．細胞内で作用するこれら化合物による疾患治療の実現には，これらの化合物を効率よく標的細胞内にデリバリーする技術の開発が必要である．膜透過性が制限される水溶性高分子化合物の細胞内デリバリーに関しては，これまでにアンチセンスオリゴヌクレオチドやリボザイム，pDNA などを対象に検討されてきた．[13] 中でも非ウイルスベクターによる遺伝子導入に関する検討においては，pDNA 単独の投与方法の最適化に始まり，電気パルスや超音波の利用，各種高分子・微粒子ベクターとの複合体化による遺伝子発現の増大など，多岐に渡る技術が集積されている．siRNA や shRNA 発現ベクターの利用に際しても，これまでに開発されてきた核酸デリバリー技術が応用可能である．siRNA あるいは pshRNA を生体レベルで標的細胞，すなわちがん細胞へデリバリーすることで標的遺伝子の発現を抑制することが可能となれば，新規分子標的がん治療法になり得るものと考えられる（Fig. 1）．

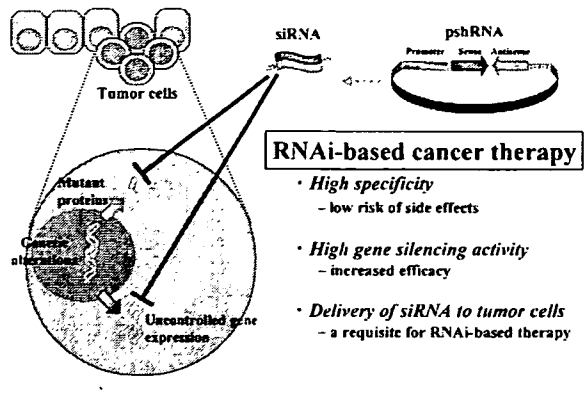


Fig. 1. Schematic Image of RNAi Pathway and Its Application to Cancer Therapy

Because of its highly potent and specific gene silencing effect, RNAi is expected to be applied to cancer therapy. To perform RNAi-based therapy, siRNA or pshRNA should be delivered to the target cells because the RNAi effect is limited in the cells that received RNAi effectors.

**2-1. がん細胞における遺伝子発現抑制効果の定量的解析**　がん細胞での RNA 干渉誘導の検討には，がん細胞内の遺伝子発現量を定量的に評価可能な実験系の利用が非常に有用である．われわれはマウス黒色細胞腫 B16 細胞をモデルのがん細胞とし，ホタル及びウミシイタケの 2 種類のルシフェラーゼを安定に発現する細胞株 B16/dual Luc を構築することによりがん細胞における遺伝子発現抑制効果について定量的に評価可能であることを報告した．[14]

この系では，B16/dual Luc にホタルルシフェラーゼに対する siRNA をトランスフェクションし，一定時間後に測定した両ルシフェラーゼ活性の比を算出することで簡便かつ定量的に RNA 干渉効果を評価可能である．われわれの検討においては，種々の濃度の siRNA をトランスフェクションしたのちのルシフェラーゼ活性を経時的に評価し，遺伝子発現抑制効果が siRNA の濃度依存的であることが示されている（Fig. 2）．[15] RNA 干渉による遺伝子発現抑制効果は，ある時点での最大抑制率を指標に評価されることが多いが，「効果」の観点からは抑制強度だけでなく抑制時間も重要である．われわれは薬物速度論解析において用いられるモーメント解析を，ルシフェラーゼ活性の経時変化データに当てはめることで，遺伝子発現抑制効果の強度及び持続時間の指標として，$AUC_{IE}$ 及び $MRT_{IE}$ を RNA 干渉効果の新たな指標として提唱した（Table 1）．[15] これにより初期濃度（$C_0$）1，10，100 nM の siRNA による遺伝子発現抑制効果について評価を行ったところ，その $AUC_{IE}$ 及び $MRT_{IE}$ と $C_0$ とは薬物用量－反応を表す式 $C_0/AUC_{IE}$ or $C_0/MRT_{IE}=C_0/a+b/a$ (Eq. (1); $a$, $b$ は定数）において線形関係であることを見い出した（Fig. 3）．また，siRNA 及び pshR-

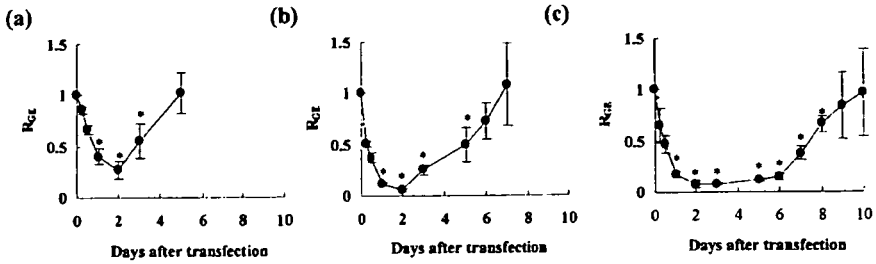


Fig. 2. Time-courses of Gene Expression ($R_{GE}$) following Tansfection of siRNA
B16-BL6/dual Luc cells were transfected with siRNA targeting firefly luciferase at concentrations of 1 (A), 10 (B) and 100 nM (C). Luciferase activities were determined at the indicated times after transfection. The results are expressed as mean ± S.D. ($n$=3). $^*p<0.05$ for Student's $t$-test versus control group. Cited from Ref. 15).

Table 1. $AUC_{IE}$ (day) and $MRT_{IE}$ (day) *versus* siRNA Initial Concentrations ($C_0$; nM)

| $C_0$ | $AUC_{IE}$ | $MRT_{IE}$ |
|---|---|---|
| 1 | 2.00 | 1.98 |
| 10 | 4.00 | 2.72 |
| 100 | 6.59 | 4.21 |

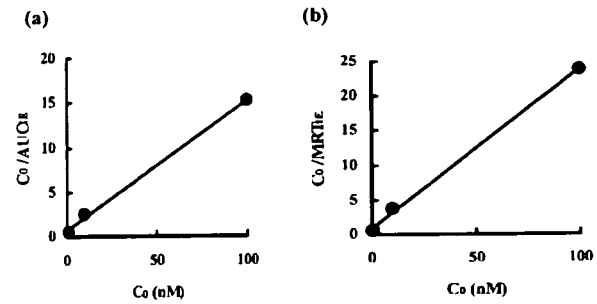


Fig. 3. Linear Plots of $C_0/AUC_{IE}$ and $C_0/MRT_{IE}$ *versus* $C_0$
(a) Linear plots of $C_0/AUC_{IE}$ versus $C_0$. Symbols represent $C_0/AUC_{IE}$ calculated, with lines fitted using Eq. (1). $a$ and $b$, are parameters described in Eq. (1), and were calculated to be 6.90 and 4.72, respectively. r, is the correlation coefficient of the linear plots, and was calculated to be 0.999. (b) Linear plots of $C_0/MRT_{IE}$ versus $C_0$. Symbols represent $C_0/MRT_{IE}$ calculated, with lines fitted using Eq. (1). $a$ and $b$, are parameters described in Eq. (1), and were calculated to be 4.35 and 3.47, respectively. r, is the correlation coefficient of linear plots, and was calculated to be 0.999. Cited from Ref. 15).

NA による遺伝子発現抑制効果についての定量的比較を行う際にも B16/dual Luc 及びモーメント解析の方法論は有用であり，1 分子当たりの遺伝子発現抑制効果は pshRNA の方が約 50 倍強力であることを示した．また，遺伝子発現抑制効果の持続について $MRT_{IE}$ を用いて評価を行い，濃度により多少のばらつきは認められるものの pshRNA の方が 2-3 倍有利であることを明らかにしている．

B16/dual Luc は 2 種類のルシフェラーゼ遺伝子を安定に発現するため，ウミシイタケルシフェラーゼ活性に対するホタルルシフェラーゼの活性の比を取ることにより生体内のがん細胞数のばらつきを補正し，標的遺伝子の発現抑制効果について *in vivo* においてもがん細胞特異的に定量的に評価可能である．

**2-2. 局所投与によるデリバリー**　局所への遺伝子導入を目的としたベクターの投与に関しては既に種々の検討がなされている．腫瘍局所での RNA 干渉の誘導によるがん治療を試みる際には，これら既存の投与方法が利用可能と考えられる．しかしながら遺伝子発現強度が重要な遺伝子導入とは異なり，RNA 干渉の誘導によるがん治療の際には標的細胞群のどれぐらいの割合に siRNA を導入できたか，という標的（がん）細胞へのデリバリー効率が治療効果を左右する重要なパラメーターになるものと考えられる．

局所投与された pDNA のデリバリー効率を改善する手法として各種物理的刺激の適用が試みられてきた．代表的なものとしては，電気パルスを投与部位に加えるエレクトロポレーションが挙げられる．一般にエレクトロポレーションを行うことにより遺伝子発現が増大することが報告されているが，われわれはこのときの発現の増加は導入細胞数の増大も伴うものであることを観察している．[16]

われわれはマウス皮下に B16/dual Luc を移植することにより作製した原発性腫瘍モデルにおいて，ホタルルシフェラーゼを標的とする siRNA 又は pshRNA 水溶液を腫瘍組織内に注入したのちエレクトロポレーションを行うことで投与 24 時間後のルシフェラーゼ活性は対照群の約 30% にまで抑制可能であることを報告している．[14] 培養条件下での遺伝子発現抑制が約 90% 程度であることを考慮すると，本投与方法を用いることで腫瘍組織中の少なくとも 70% 以上のがん細胞において RNA 干渉を

誘導可能であることが推察できる (Fig. 4).[14]

**2-3. 経血管投与によるデリバリー** siRNA 又は pshRNA の経血管投与は血流を介してこれら化合物が分布することから，投与された化合物の分布が投与部位近傍に限局される局所投与と比較して，より多くの細胞へと siRNA・pshRNA を導入可能な投与方法である．これら化合物を血管より投与した検討としては，2002 年 7 月の Kay らのグループの報告がある.[17] 彼らは大容量のプラスミド DNA 水溶液を急速に血管内投与することにより，種々の臓器，特に肝臓において高い遺伝子発現が得られる方法（ハイドロダイナミクス法）を用いた検討を行い，ルシフェラーゼを発現するプラスミド DNA をマウスに投与し，同時投与した siRNA による RNA 干渉効果をルシフェラーゼ活性を指標に評価した．その結果，ルシフェラーゼ遺伝子に相補的な配列を持つ siRNA を投与することにより，約 80%の遺伝子発現抑制を得ている．また，siRNA に加え，pshRNA を投与した場合も siRNA と同等の抑制効果が得られることも報告している．

がん治療を目的としたハイドロダイナミクス法による RNA 干渉の *in vivo* への適用には，標的細胞へのデリバリー効率も効果を左右する重要な因子となる．siRNA と pshRNA とでは，分子サイズに大きな違いが存在するため，デリバリー効率に影響を与える可能性が考えられる．われわれは，分子サイズがハイドロダイナミクス法による遺伝子導入効率に及ぼす影響を明らかにするために，PCR を利用することでサイズの異なる遺伝子導入ベクターを用いた検討を行った.[18] しかしながら，4.8 kbp の GFP 発現プラスミド DNA，あるいは発現に必要な部分のみ（1.7 kbp）を PCR で増幅した DNA 断片をハイドロダイナミクス法でマウスに遺伝子導入したときの肝臓では，ほぼ同数の細胞で遺伝子発現が認められた (Fig. 5).[18] したがって，少なくともハイドロダイナミクス法による肝臓へのデリバリーに関しては分子サイズの影響はあまり認められないものと考えられる．一方，分子量 4000 の PEG を用いた検討では，十分な肝臓での集積がみられなかったことから，デリバリーの観点からはサイズが小さいことがかならずしも有利とは限らないことも推察される.[19]

既述の通りハイドロダイナミクス法によるデリバリーは血流を介して行われることから，肝臓構成細胞のみならず血管に沿って分納する細胞がそのデリバリーの対象となると考えられる．われわれはハイドロダイナミクス法を利用することにより肝臓構成細胞のみならず，肝臓に転移したがん細胞にも血流を介して siRNA・pshRNA をデリバリー可能ではないかと考え，ルシフェラーゼ遺伝子で標識した B16-BL6 細胞を門脈内に移植することで作成した実験的肝転移モデルマウスを用いた検討を行っ

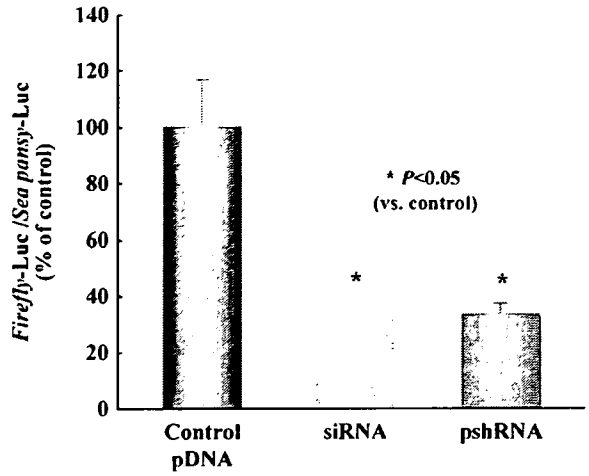


Fig. 4. RNAi in Subcutaneous B16-BL6/dual Luc following Intratumoral Injection of RNAi Inducer Followed by Electroporation

Mice received an intratumoral injection of control pDNA, siRNA targeting firefly luciferase (10 $\mu$g) or pshRNA targeting firefly luciferase (30 $\mu$g) 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. The results are expressed as the mean±S.E. ($n$=8). *$p$<0.05 for Dunnet's test versus control group. Cited from Ref. 14).

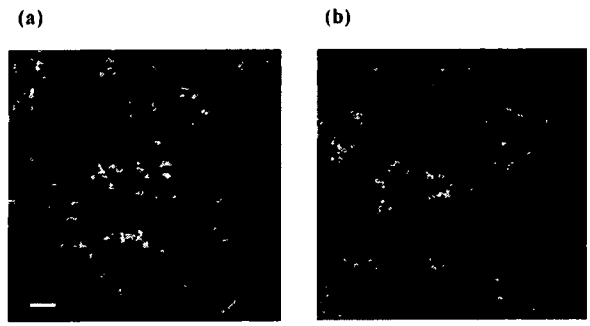


Fig. 5. Confocal Microscopic Images of the Liver Sections following Intravenous Injection of pEGFP-F or EGFPF-mini in Mice by the Hydrodynamics-based Procedure

Mice were euthanized at 6 h after injection and the liver sections were made. The images shown are typical of those observed in several visual fields of three mice per group. (a): EGFP-F-expressing pDNA (25 $\mu$g or 8.4 pmol/mouse), (b): PCR-amplified EGFPF-expressing cassette (8.9 $\mu$g or 8.4 pmol/mouse). Cited from Ref. 18).

た.[14] その結果 siRNA 又は pshRNA のハイドロダイナミクス法による投与により肝臓中がん細胞での標的遺伝子発現を有意に対照群の 40–50%程度にまで抑制可能であることを見い出している（Fig. 6）.[14] ハイドロダイナミクス法による遺伝子導入細胞数は肝臓の約 40%であることが報告されており，それより高い導入効率を得ることができたがこれは転移したがん細胞は血管に沿って分布することを反映した結果と推察される．しかしながら，その遺伝子発現抑制効果は，局所投与の場合と比較して低く，複数回投与による遺伝子発現抑制効率の改善はほとんど認められなかったため，より効率的な遺伝子発現抑制を実現するためにはほかのアプローチの併用等も検討する必要があると考えられる．

**3. RNA 干渉を利用したがん遺伝子治療法の開発**

前述の通り，生体内のがん細胞に対して siRNA 又は pshRNA をデリバリーすることができれば，がん細胞における遺伝子発現を抑制することが可能であり，その遺伝子発現抑制効果を利用したがん治療の試みについても報告されている．既に報告されているものとしては，がん細胞の増殖・生存・転移・血管新生・免疫回避といった性質に関係した遺伝子を標的とした RNA 干渉による検討が報告されている．

**3-1. 局所投与によるがん細胞増殖抑制**　局所に作製した腫瘍に対して siRNA 又は pshRNA の腫瘍内投与とエレクトロポレーションを併用することでがん細胞の遺伝子発現を効率よく抑制可能である

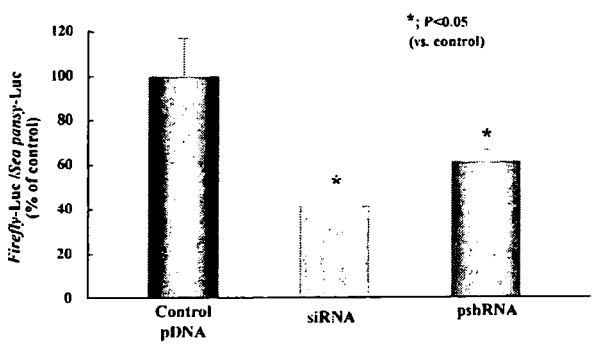


Fig. 6. RNAi in Metastatic Tumors in the Liver following Injection of siGL3 or pU6-siGL3 by the Hydrodynamics-based Procedure

Mice received an intravenous injection of control pDNA, siRNA targeting firefly luciferase or pshRNA targeting firefly luciferase (50 $\mu$g) 13 days after tumor inoculation via the portal vein. The luciferases activities in the liver were measured 24 h after the injection. The results are expressed as the mean±S.E. ($n \geq 4$). *$p<0.05$ for Dunnet's test versus control group. Cited from Ref. 14).

が，われわれはこの方法を用いてがん細胞の増殖等に関与した遺伝子である $\beta$-catenin, hypoxia inducible factor-1$\alpha$ (HIF-1$\alpha$) の発現を抑制することでがん細胞の増殖が抑制可能であることを報告している.[20] pshRNA の腫瘍内注射とエレクトロポレーションにより各内因性の標的遺伝子の mRNA 発現レベルを対照群の 25–35%程度に抑制可能であり，これらがんの増殖や転移に関与する遺伝子の発現を抑制することで有意ながん増殖の抑制が得られることを明らかとしている．このとき，一部マウスではがんのほぼ完全な退縮が認められた（Fig. 7）．こうした pshRNA のデリバリーによる腫瘍増殖の抑制効果は腫瘍サイズ依存的であり，腫瘍サイズの増大とともに抑制効果が減弱することも示されている．これは，腫瘍の増大に伴い pshRNA がデリバリーされるがん細胞数の割合が減少することによるものと考えられる．したがって，大きな腫瘍を標的とする場合にはデリバリー効率を改善することが重要と考えられる．

局所投与により RNA 干渉を誘導する方法としては，エレクトロポレーションの利用のほかにカチオン性物質/siRNA コンプレックスの腫瘍内投与が報告されている．Kim らは VEGF を標的とする siRNA を，マウス皮下に作製した腫瘍組織内に Cholesteryl oligo-D-arginine (Chol-R9)/siRNA コンプレックスの形で単回投与することにより，がん細胞の増殖を対照群と比較して有意に抑制可能であることを報告している.[21] また，sphingosine 1-phosphate receptor-1 (S1P1) を標的とする siRNA とカチオン性リポソームのコンプレックスをマウス皮下に作製した腫瘍組織内に 3 日毎に注入することで腫瘍組織の増殖を有意に抑制可能であることが Chae らにより報告されている.[22] しかしながら，われわれの検討も含めたいずれの報告においても完全な腫瘍の拒絶には至っておらず，更なる効率の改善又はほかの方法を併用することによる治療効果の増強が必要であると考えられる．

**3-2. 経血管投与を利用したがん細胞増殖抑制**

経血管投与は導入細胞数の増大という観点から有用な方法であると考えられ，がん細胞が散在する転移性腫瘍に対してはその適用が特に望ましいと考えられる．ハイドロダイナミクス法を用いることで肝転移性腫瘍に対して RNA 干渉を誘導可能であるの

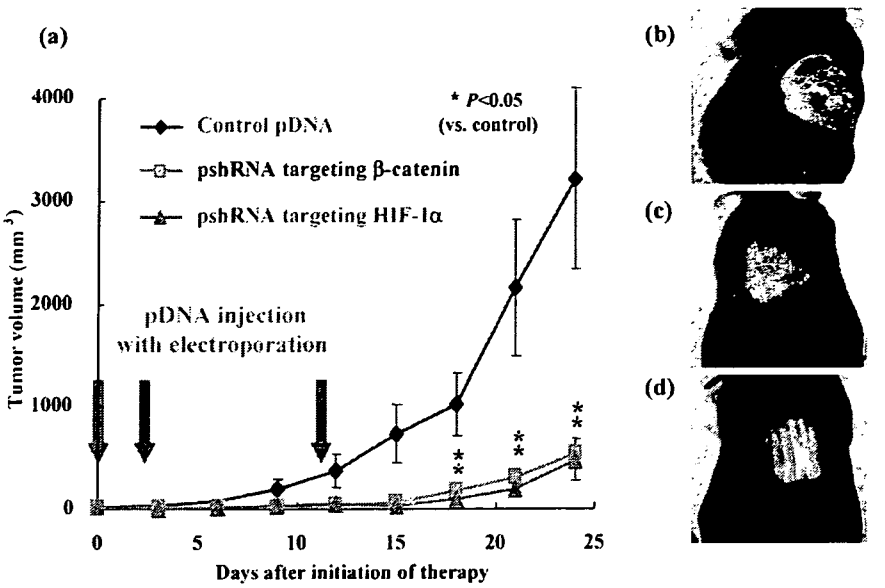


Fig. 7. Effects of Intratumoral Delivery of pshRNAs on the Growth of Primary Tumor Tissue

(a) Mice received an intratumoral injection of 30 $\mu$g control pDNA or pshRNAs followed by electroporation. pDNAs were administrated at day 0, 3 and 12 after the initiation of the therapeutic treatment. The results are expressed as the mean±S.E. ($n$=4). *$p$<0.05 for Student's $t$-test versus control group. (b-d) Photographic image of tumor tissue of mice who received an intratumoral injection of control pDNA (b), pshRNA targeting $\beta$-catenin (c) or pshRNA targeting HIF1$\alpha$ (d) at 18 days after initiation of therapeutic treatment. Cited from Ref. 20).

で，その遺伝子発現抑制効果を利用したがん治療の可能性について評価した．すなわち局所腫瘍モデルと同様に，マウス結腸がん細胞株 Colon26 細胞を門脈より移植することで作製した肝転移腫瘍モデルにおける $\beta$-catenin 又は HIF-1$\alpha$ を標的とする pshRNA のがん細胞増殖抑制効果について調べた．培養 Colon26 細胞を用いた *in vitro* の系においては $\beta$-catenin を標的とする pshRNA の方が HIF-1$\alpha$ を標的とする pshRNA より強力ながん細胞増殖抑制効果を示している．両 pshRNA の物性はほぼ同じであるため肝臓中のがん細胞へのデリバリー効率は同程度であると考えられることから，$\beta$-catenin を標的とする pshRNA の方が肝転移性腫瘍モデルにおけるがん細胞増殖抑制効果は高いと考えられる．しかしながら $\beta$-catenin を標的とする pshRNA の投与ではがん細胞増殖抑制効果はほとんど認められなかった一方で，HIF-1$\alpha$ を標的とする pshRNA を投与したところがん細胞の増殖を効率よく抑制可能であった．このことから，肝臓中のがん細胞内の遺伝子発現抑制以外の要因が肝臓における RNA 干渉の誘導によるがん細胞増殖抑制効果に関与していると推察される．その一因として，ハイドロダイナミクス法を用いることで肝臓中のがん細胞にデリバリー可能ではあるが，投与された pshRNA は肝臓中の正常細胞へもデリバリーされることから，がん細胞のみならず正常細胞における遺伝子発現の抑制ががん細胞の増殖抑制につながったのではないかという仮定が考えられる．詳細については現在検討中である．

全身投与によりがん細胞において RNA 干渉を誘導することによりがん細胞の増殖抑制を試みた検討としては，siRNA 単独投与又はカチオン性物質/siRNA コンプレックスの投与によるものが報告されている．Mook らは，large subunit of RNA polymerase II を標的とする naked siRNA を尾静脈より投与することで，マウス皮下に作製した腫瘍の増殖を有意に抑制可能であることを報告している．[23] また，EphA2 を標的とする naked siRNA を用いた同様の検討結果も報告されている．[24] Pal らは Raf を標的とする siRNA をカチオン性リポソーム/siRNA コンプレックスの形で静脈内投与することにより，マウス皮下に移植したがん細胞における Raf の遺伝子発現及び腫瘍組織の増殖を抑制可能であることを報告している．[25]

また，がん細胞以外の細胞も RNA 干渉の標的細胞とした検討が Santel らにより報告されている．[26] 彼らは，腫瘍組織内の血管内皮細胞における CD31 の発現をカチオン性リポソーム/siRNA を用いて抑

制することにより，腫瘍組織における血管新生並びに腫瘍組織の増殖を抑制可能であることを示している．一般にがん細胞は遺伝子の変異が起こり易いため，RNA 干渉による遺伝子発現抑制に対する耐性を獲得する可能性が考えられるが，それと比較して血管内皮細胞のような正常細胞は遺伝子発現の変異は起こり難いため，彼らの結果は正常細胞を標的とすることによる耐性を生じ難い新しい治療法の可能性を示すと考えられる．

## 4. おわりに

がん細胞特異的に発現している特定の分子を標的とした分子標的治療の実現が望まれており，特定の mRNA を特異的に分解する RNA 干渉は最も有望視される方法の 1 つである．しかしながら，その実現には siRNA あるいは shRNA 発現ベクターを標的細胞（主にがん細胞）に効率よくデリバリーする方法論の開発が不可欠である．一般にがん治療を目的とした場合にはがん細胞に対する RNA 干渉誘導効率が治療効果を左右する大きな要因となることが多いため，研究開発が進むにつれて，RNA 干渉によるがん治療の可能性だけでなく，その限界も明らかとなってきた．標的細胞群の一部において RNA 干渉を誘導することで治療効果が得られる場合，RNA 干渉を利用するシステムは非常に優れた治療法になる可能性がある．今後は治療遺伝子の投与や，DNA ワクチンによる抗原デリバリーなどと組み合わせることで，RNA 干渉により治療効果が増大できるものと期待する．

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