# In vivo gene delivery by cationic tetraamino fullerene

Rui Maeda-Mamiya[a,b], Eisei Noiri[b,1], Hiroyuki Isobe[c], Waka Nakanishi[c], Koji Okamoto[b], Kent Doi[b], Takeshi Sugaya[d], Tetsuro Izumi[e], Tatsuya Homma[a], and Eiichi Nakamura[a,1]

[a]Department of Chemistry and Exploratory Research for Advanced Technology (Japan Science and Technology Agency), University of Tokyo, 7-3-1 Hongo Bunkyo, Tokyo 113-0033, Japan; [b]Department of Hemodialysis and Apheresis, University Hospital, University of Tokyo, 7-3-1 Hongo Bunkyo, Tokyo 113-8655, Japan; [c]Department of Chemistry, Tohoku University, Aoba-ku, Sendai 980-8578, Japan; [d]CMIC Co. Ltd., 7-10-4 Nishi-gotanda Shinagawa, Tokyo 141-0031, Japan; and [e]Laboratory of Molecular Endocrinology and Metabolism, Institute for Molecular and Cellular Regulation, Gunma University, 3-39-15 Showa-machi, Maebashi 371-8512, Japan

Edited by Nicholas J. Turro, Columbia University, New York, NY, and approved January 21, 2010 (received for review August 13, 2009)

**Application of nanotechnology to medical biology has brought remarkable success. Water-soluble fullerenes are molecules with great potential for biological use because they can endow unique characteristics of amphipathic property and form a self-assembled structure by chemical modification. Effective gene delivery in vitro with tetra(piperazino)fullerene epoxide (TPFE) and its superiority to Lipofectin have been described in a previous report. For this study, we evaluated the efficacy of in vivo gene delivery by TPFE. Delivery of enhanced green fluorescent protein gene (EGFP) by TPFE on pregnant female ICR mice showed distinct organ selectivity compared with Lipofectin; moreover, higher gene expression by TPFE was found in liver and spleen, but not in the lung. No acute toxicity of TPFE was found for the liver and kidney, although Lipofectin significantly increased liver enzymes and blood urea nitrogen. In fetal tissues, neither TPFE nor Lipofectin induced EGFP gene expression. Delivery of insulin 2 gene to female C57/BL6 mice increased plasma insulin levels and reduced blood glucose concentrations, indicating the potential of TPFE-based gene delivery for clinical application. In conclusion, this study demonstrated effective gene delivery in vivo for the first time using a water-soluble fullerene.**

carbon nanotube | gene therapy | green fluorescent protein | insulin 2 gene | toxicity

A fullerene carbon nanocluster has unique spherical structures and high hydrophobicity. These unique structures of the carbon clusters lend it several properties including photosensitivity (1), redox property (2), and high chemical reactivity (3). In particular, water-soluble fullerene derivatives, which can be dissolved easily in water by introducing proper hydrophilic residues, have received much attention for their possible biological applications. Recently, introduction of amino, carboxyl, and hydroxyl residues and establishment of chemical modification methods on a fullerene structure enabled us to produce highly biocompatible water-soluble fullerenes (4). Amphipathic fullerenes, when chemically modified with hydrophilic side chains, offer great potential for gene delivery because they can form a complex with DNA effectively.

Gene delivery via nonviral routes has become a powerful and popular research tool for elucidating gene structure, regulation, and function (5). Gene delivery will play a pivotal role in developing new therapeutic approaches (e.g., gene therapy and DNA vaccination), which might have a great impact on the development of clinical medicine. Currently, lipid-based systems are widely used for in vitro and occasionally in vivo experiments because their cationic lipid-DNA complexes can be prepared easily (6, 7). The mechanism of lipid-based DNA delivery systems is to wrap the DNA within a lipid sheath by ionic interaction between the DNA's phosphate anion and the cationic part of the lipid. The lipid sheath is structurally similar to the cell membrane. Therefore, membrane fusion enables the DNA molecules to penetrate into the cell. To date, several reports have described effective in vivo gene delivery using liposomal gene transfer systems (8–12). However, their inefficiency, which is partly attributable to the instability in serum, and cytotoxicity should be addressed (13). Lipofectin is a representative example of widely used lipid-based transfection reagents (14). Among 182,578 hits of transfection-related data in SciFinder database, Lipofectin appears 2,085 times. Application to in vivo gene delivery is also reported (15). In this study, we compared the efficacy of gene delivery between Lipofectin and a water-soluble fullerene derivative described below.

Recently we developed a DNA delivery system using fullerenes that have DNA-binding side chains. Cationic fullerene molecules such as tetraaminofullerene are capable of condensing double-strand DNA into globules smaller than 100 nm. They are therefore penetrable into the cell (16–18). The more hydrophobic nature of fullerenes than alkyl chains in lipids appears to enable fullerenes to form a stable complex with DNA. Tetraaminofullerene is producible in only two steps with fullerene and piperazine derivatives. Moreover, a cationic fullerene can induce gene expression by releasing DNA inside the cell because of its resistance to digestion by nucleases. In an earlier study, we reported the synthesis of tetra(piperazino)fullerene epoxide (TPFE) (Fig. 1*A*) and its binding abilities to DNA, where protection against endonuclease by TPFE was confirmed (17). In vitro gene delivery efficiency was examined by adjusting various conditions including the fullerene/base pair ratio, the transfection time, and the amount of plasmid DNA. In addition, TPFE showed a 4-fold increase of transfection efficiency compared to Lipofectin. Furthermore, TPFE showed reduced cytotoxicity, indicating that TPFE can be used for in vivo transfection (19, 20). To date, no report in the literature has described in vivo gene delivery using carbon nanomaterials. Because TPFE-DNA complex completely differs from Lipofectin-DNA complex in terms of size, charge, and conformational structure, it is expected that gene delivery by TPFE will have different organ affinity and might be able to overcome the problem of being trapped by the lung in the first pass (21).

This study was undertaken to develop an in vivo gene transfer system using a cationic fullurene TPFE. The efficacy of gene delivery to the fetus, organ safety issues, and therapeutic application by TPFE were also evaluated.

## Results

**Size and Stability of TPFE-DNA Complex.** Characterization of the TPFE-DNA complex was performed to confirm the optimal pre-

Author contributions: E. Noiri, H.I., and E. Nakamura designed research; R.M.-M., W.N., K.O., and T.H. performed research; H.I., T.I., and E. Nakamura contributed new reagents/analytic tools; R.M.-M., E. Noiri, K.O., K.D., T.S., and E. Nakamura analyzed data; and R.M.-M., E. Noiri, K.D., and E. Nakamura wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

[1]To whom correspondence may be addressed. E-mail: noiri-tky@umin.ac.jp or nakamura@chem.s.u-tokyo.ac.jp.

This article contains supporting information online at www.pnas.org/cgi/content/full/0909223107/DCSupplemental.

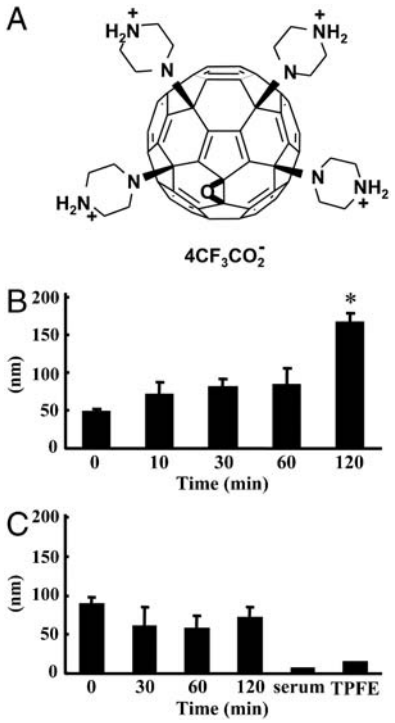


**Fig. 1.** TPFE. (*A*) Structure of TPFE is shown. DLS measurements were done at 0, 10, 30, 60, and 120 min after preparation of TPFE-DNA complexes. (*B*) The size of TPFE-DNA complex was stable for 1 h ($n = 4$). *$p < 0.05$ versus 0 min. (*C*) Adding 10% serum increased the stability of the TPFE-DNA complex ($n = 3$). Error bars are SEM.

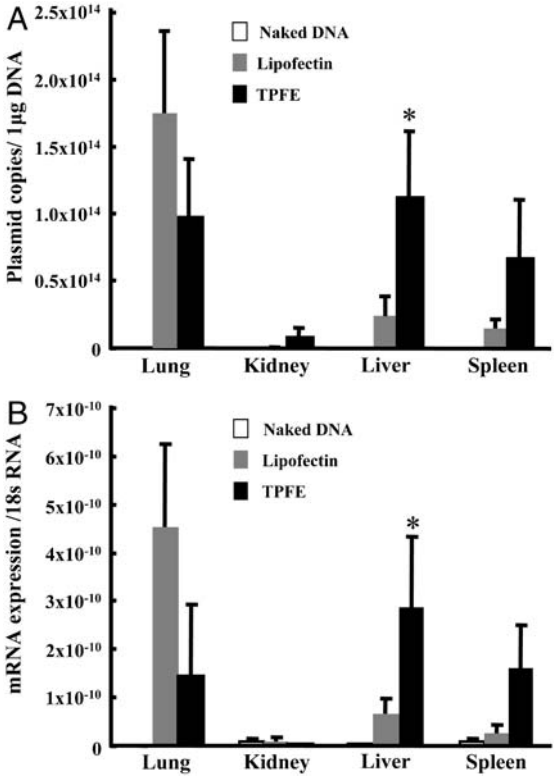


**Fig. 2.** Distribution of injected plasmid DNA and EGFP mRNA expression in each organ. (*A*) After 24 hr injection, plasmid DNA was detected at several organs in the TPFE and the Lipofectin group ($n = 6$ for each group). (*B*) mRNA expression of EGFP gene was analyzed using real-time-PCR at 24 h after injection. mRNA was detected at the lung, liver, and spleen ($n = 6$ for each group). *$p < 0.05$ versus the Lipofectin group. Error bars are SEM.

paration for in vivo injection. Plasmid DNA encoding enhanced green fluorescent protein (EGFP) (720 bp) was used. The size of the TPFE-DNA complex prepared with the reagent-to-base pair ratio (*R*) of 5, by which TPFE showed the highest efficiency on gene transfection in vitro, was measured using dynamic light scattering (DLS) analysis. The average size of TPFE-DNA complex was approximately 50 nm immediately after preparation; it remained stable (less than 100 nm) up to 60 min (Fig. 1*B*). Moreover, the TPFE-DNA complex was able to remain stable as long as 120 min when prepared with 10% bovine serum containing buffer (Fig. 1*C*).

**Gene Delivery Efficiency of TPFE-DNA Complex in Vivo.** The TPFE-DNA complex, Lipofectin-DNA, and naked DNA were injected into the mouse tail vein. The in vivo biodistribution of injected DNA was evaluated by amplifying the EGFP gene encoded in the plasmid DNA. At 24 hr after injection, DNA samples were extracted from the organs of the lung, liver, kidney, and spleen. Comparable localizations of plasmid DNA delivered by TPFE and Lipofectin were found, although it is noteworthy that the amount of DNA in the TPFE group was approximately 2- to 3-fold higher than the Lipofectin group except lung (Fig. 2*A*). Injected plasmid DNA was not detected in the whole blood of any group. The naked DNA group showed virtually no amplification of the EGFP gene in any organ.

Gene expression of the EGFP gene was examined using real-time PCR for evaluating transfection efficacy and confocal microscopy image analysis for detailed localization of gene expression. In the TPFE and the Lipofectin group, mRNA expression was found in the lung, liver, and spleen at 24 hr after injection (Fig. 2*B*). In the TPFE group, the amount of expressed mRNA was higher than that of the Lipofectin group in the liver and spleen, but lower than that of the Lipofectin group in the lung. This tendency resembled that observed in plasmid DNA distribution described above (Fig. 2*A*). In accordance with these results, confocal microscopy image analysis showed EGFP signals in the lung, spleen, and liver of the TPFE and the Lipofectin group. Naked DNA showed no EGFP positive signal (Fig. 3). The similar observation was confirmed by immunohistochemical staining of anti-EGFP antibody (*SI Text* and Fig. S1). The efficacy of gene delivery to the fetus and fetal appendages was also examined. At 24 hr after injection of TPFE-DNA and Lipofectin-DNA to pregnant mice [8 days post coitum (d.p.c.)], fetuses were removed. Plasmid DNA was found in the placenta and yolk sac. The TPFE group showed a higher number of plasmid DNA copies in placenta than the Lipofectin group did (Fig. 4). The mRNA of EGFP was examined using real-time PCR. The mRNA was not consistently detected in fetus or fetal appendage in the TPFE or Lipofectin group (Table 1).

**Evaluation of TPFE Toxicity.** Acute toxicity of the TPFE and the Lipofectin gene transfer systems was evaluated using serum biochemical examinations, which are often used in clinical practice. Blood specimens were collected 24 hr after injections. Liver injury was evaluated by measuring aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Increases of AST and ALT were found only in the Lipofectin group. The Lipofectin group showed modestly but significantly higher blood urea nitrogen (BUN) levels than the TPFE group did, possibly indicating that lipofection caused acute kidney injury (Table 2).

**Delivery of Insulin 2 Gene for Therapeutic Application.** We evaluated the potential application of gene delivery by TPFE with mouse insulin 2 gene (*Ins2*) expressing plasmid. As observed for the experiment of EGFP gene delivery, insulin expression was detected in the lung by immunohistochemistry. Modest insulin expression was also detected in the liver and spleen (Fig. 5*A*). At 12 hr after

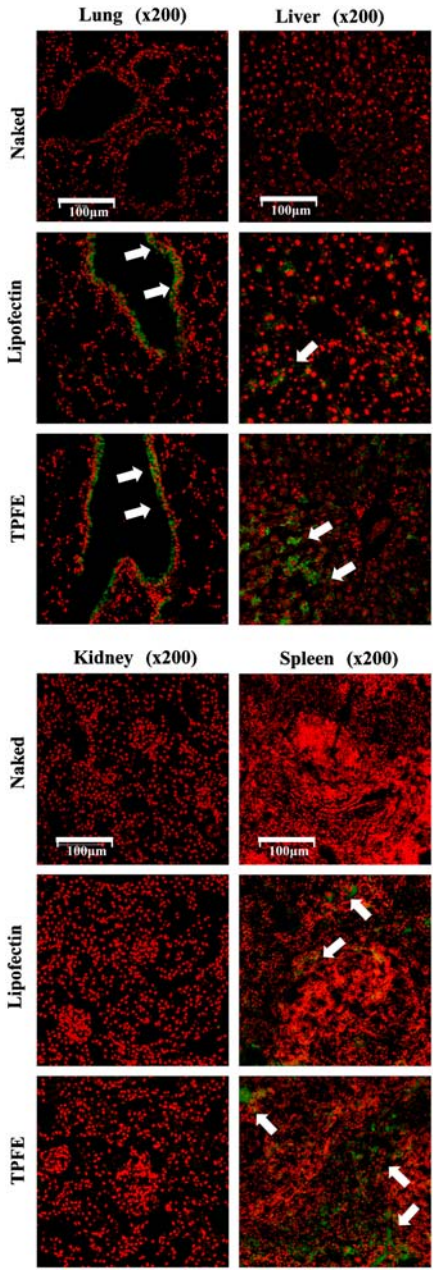


**Fig. 3.** Confocal microscopy images of organ tissue sections. Representative images of EGFP distribution at 24 h after injection. Fluorescence of EGFP (green) and cell nuclei (red) in the lung, liver, kidney, and spleen are shown.

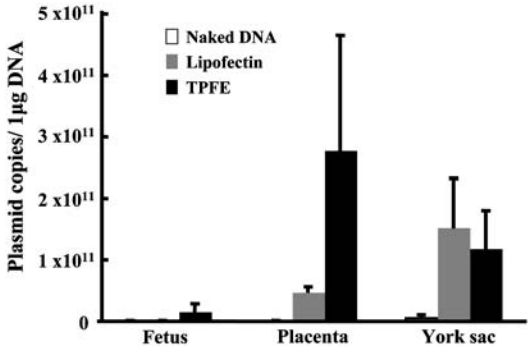


**Fig. 4.** Distribution of injected plasmid DNA in fetus and fetal appendages. Injected plasmid DNA was detected at the fetus and fetal appendage using PCR ($n = 7$–$8$ for each group). Error bars are SEM.

**Table 1. Number of animals showing positive signals of mRNA expression by real-time PCR**

| | Fetus | Placenta | York sac |
|---|---|---|---|
| Naked DNA | 0/7 | 0/7 | 0/7 |
| Lipofectin | 0/7 | 0/7 | 1/7 |
| TPFE | 1/8 | 0/8 | 1/8 |

the injection together with TPFE, plasma insulin levels in the *Ins*2 group were significantly higher than the mock group (Fig. 5*B*). Lower fasting blood glucose levels were observed in the *Ins*2 group until 24 hr after the injection (Fig. 5*C*).

## Discussion

Tetraaminofullerene is a carbon nanomaterial that has an amphiphilic character. In fact, TPFE can form a small aggregate with double-strand DNA, protect nucleic acid from nucleases, and penetrate into the cells. These factors enable us to use this material for gene delivery. In this study, we demonstrated the fact that tetraaminofullerene is applicable as an in vivo gene delivery system. Moreover, we compared the efficiency and toxicity of TPFE with a liposome-based system (Lipofectin). Results show that TPFE can deliver a gene more efficiently in the liver and spleen and showed no acute toxicities to liver or kidney tissues, although Lipofectin induces liver and kidney injury. Finally, we successfully delivered insulin 2 gene by the TPFE system and demonstrated the effect of insulin 2 gene delivery on blood glucose levels.

The specific molecular ratio of DNA to TPFE ($R = 5$) allowed an assembly particle size of 50–100 nm, which is suitably sized for endocytosis. Based on our previous studies, we reported that several amphiphilic compounds showed different optimal $R$ values (18). Results of the present study demonstrated that TPFE can form a more stable complex with DNA under coincubation with serum. In vitro optimization is indispensable to set up effective delivery systems in vivo.

The in vivo biodistribution of TPFE-DNA and Lipofectin complexes showed similar patterns. However, levels of reporter gene expression varied remarkably. The TPFE achieved 5- to 10-fold higher gene expression than Lipofectin in the liver and spleen. Reportedly, lipid-based gene delivery can transfer genes to the lung effectively because cationic liposome can be trapped well at the lung immediately after injection from the tail vein (9, 10, 21). Therefore, many studies have been undertaken to deliver genes to the lung and to treat lung diseases using liposomal gene transfer systems (8, 11, 12). In the TPFE system, the TPFE-DNA complex structure would differ significantly from those of lipid-DNA complex, possibly because of the $\pi$-$\pi$ interaction between the fullerene surface and nucleobases, which might be the origin of the observed organ selectivity. The TPFE will be applicable to other organ diseases including those of the liver and spleen.

Transfection and expression of a foreign gene in embryos and fetuses at desired stages will overcome problems in transgenic animals such as embryonic lethality and unpredictable regulatory effects of transgene integration. In 1995, Tsukamoto et al. reported that cationic liposome efficiently introduces a foreign gene into pregnant mice (22). They showed that a single intravenous injection of foreign gene-cationic liposome complexes into

**Table 2. Acute toxicity of gene delivery carriers**

| | Naked DNA | Lipofectin | TPFE |
|---|---|---|---|
| AST (IU/L) | 21.7 ± 0.8 | 36.5 ± 2.7* | 22.6 ± 1.8 |
| ALT (IU/L) | 3.2 ± 0.3 | 7.3 ± 1.5* | 3.5 ± 0.4 |
| BUN (mg/dL) | 15.2 ± 1.1 | 18.4 ± 0.7* | 15.4 ± 1.0 |

Liver and kidney injuries were evaluated using AST, ALT, and BUN measurements ($n = 6$).
*$p < 0.05$ versus the TPFE group.

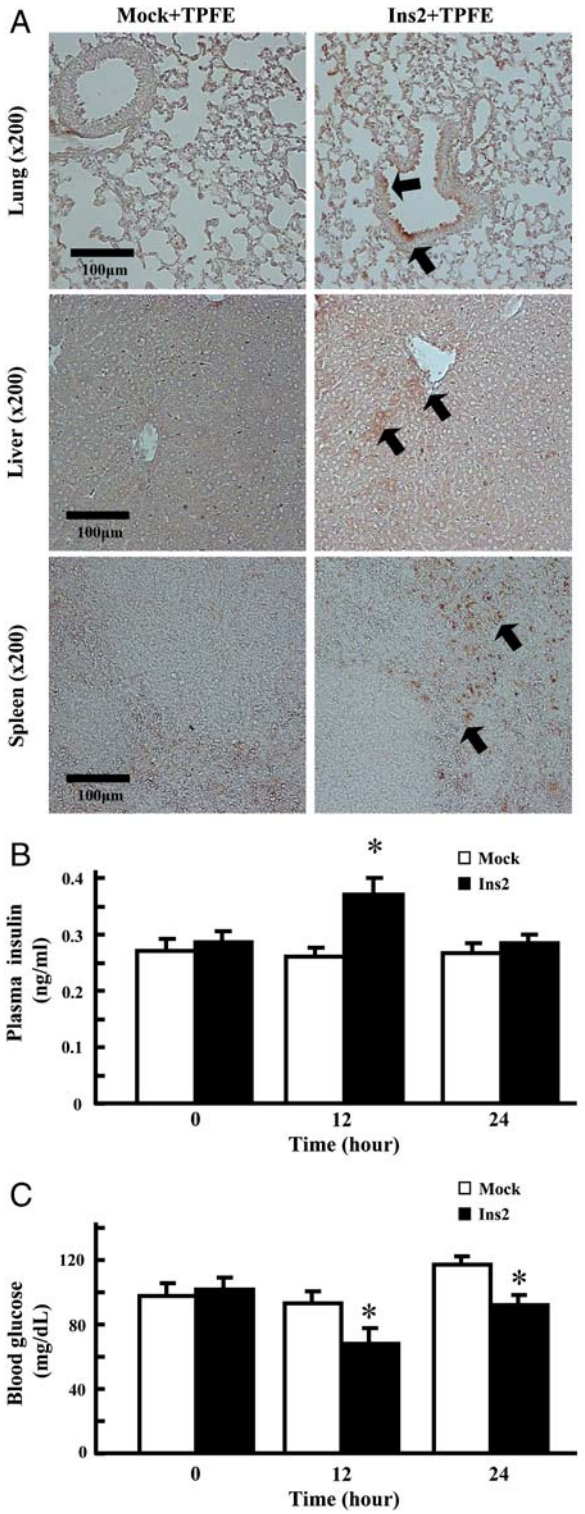


**Fig. 5.** Effect of insulin 2 gene delivery by TPFE. (*A*) Immunohistochemical analysis revealed insulin expression in organ tissue sections as indicated with brown staining (arrows). Hematoxylin eosin was used as a counterstain. Plasma insulin (*B*) and fasting blood glucose levels (*C*) were measured at 0, 12, and 24 hr after injection ($n = 10$ for each group at 0 hr, $n = 5$ for each group at 12 and 24 hr). *$p < 0.05$ versus the mock + TPFE group. Error bars are SEM.

pregnant mice enables the gene to be transferred into embryos and results in gene expression in the fetuses and postpartum progeny. However, the efficiency of gene transfer by this method was not stable (23). Although several reports have described possible gene transfer into pregnant mice and their fetuses, the reported efficiency was insufficient and far from the level necessary for clinical use (24). We evaluated the efficiency of gene delivery to fetuses, the yolk sac, and placenta. The DNA-TPFE complex was trapped at lungs to a lesser degree than Lipofectin-DNA was. Therefore, it was expected that the TPFE system can deliver DNA more efficiently to the fetus. However, virtually no signal was detected in fetuses in either the TPFE or Lipofectin group. The placenta might play a barrier function for these two gene delivery systems.

Our data from this and previous reports indicate several potential advantages of a TPFE-based gene delivery system. First, TPFE can form stable aggregates with DNA, with less than 100-nm diameter with serum containing buffer. In contrast, liposome-based transfection agents are often impeded by plasma proteins (25). Second, TPFE has been demonstrated to have protective ability of DNA against enzymatic degradation (18). Third, TPFE can further penetrate into the nuclear region partly, and the amino-group of TPFE can be removed or neutralized with an amide reaction, by which DNA is released into the nuclear space (18). On the other hand, the mechanisms of cationic liposome penetrating into the cells and nucleus remain unclear.

Cationic liposome-based gene delivery is the most popular technique for in vivo animal experiments. However, the ineffectiveness and toxicity of this method has been described (26). Some reports in the literature describe in vivo gene delivery by Lipofectin, although it induced gene expression found only in lung tissues (27). One report described that expression was under the detection limit in all organs by Lipofectin (20). Song et al. evaluated several different cationic liposomes and found that they needed more than 100 μg of plasmid DNA and a large amount of lipids that were a nearly a lethal dose for mice (19, 20). The present study found virtually the same efficiency of gene delivery with 24 μg of plamid DNA for the TPFE group with no lethal damage to the animals.

Results of this study demonstrated the advantage of fullerene-based transfection systems in terms of toxicity and possible therapeutic application. No acute toxicity was found in the TPFE group, although the Lipofectin group showed acute organ toxicity in the liver and kidney. The two liver enzymes (AST and ALT) are present in hepatocytes and are released rapidly into the bloodstream when hepatocytes are damaged by toxins and/or acute inflammation (28). An increase of BUN indicates dysfunction of the kidney as a filtrating organ. Although these injury markers demonstrate the organ injury and its severity, further investigation is necessary to clarify the mechanism of toxicity by Lipofectin. Several previous reports described no evidence of toxicity by fullerene (29–31). On the other hand, cationic liposome is reported to have toxicity (9, 26, 32). Filion and Phillips showed that cationic liposome can cause remarkable immunological response in vivo (26). Further evaluations of long-term toxicity must be undertaken to apply a fullerene-based gene delivery system to humans. Finally, we demonstrated that insulin gene delivery by TPFE can reduce blood glucose levels. Insulin gene delivery by liposomal gene transfer or hydrodynamics-based system has been reported (33, 34). When TPFE gene delivery system is compared literally with hemagglutinating virus of Japan (HVJ)-liposome-based system, the reduction of blood glucose levels of normal mice is virtually the same degree (20–25 mg/dL). The HVJ-liposome system was also demonstrated to reduce blood glucose in diabetic mice (34). Further evaluation is necessary to clarify the potential application of TPFE gene delivery to animal models of disease. Our data related to safety and therapeutic effects indicate the potential of fullerene-based gene delivery systems for clinical applications.

In conclusion, we demonstrated that a water-soluble fullerene TPFE can deliver genes as efficiently as a cationic liposome-based system in vivo. In fact, TPFE can produce a stable complex

with DNA and show no acute organ toxicity. Moreover, insulin gene delivery by TPFE could reduce blood glucose levels. These data demonstrated successful in vivo gene delivery by carbon nanomaterials.

## Materials and Methods

**Preparation of TPFE-Plasmid DNA Complex.** TPFE transfection reagent was prepared as reported previously (17). Plasmid pCAGGS EGFP was kindly provided by the RIKEN Bioresource Center Cell Bank. This plasmid DNA encoding green fluorescence protein (GFP) was amplified in *Escherichia coli* (DH5; Toyobo Co. Ltd.) and was purified using a commercially available kit (Plasmid GIGA Kit; Qiagen Inc.). For insulin 2 gene (*Ins2*) delivery experiments, we used the pcDNA3 plasmid containing mouse *Ins2* cDNA. Details of this plasmid were described in an earlier report (35).

The TPFE dissolved in 2 mM potassium chloride solution and plasmid DNA dissolved in Tris buffered saline (TBS) were mixed to obtain the reagent-to-base pair ratio (*R*) of 5, which showed the highest transfection efficiency in vitro in our previous report (18). The *R* value was calculated by dividing the nitrogen-to-phosphorus (N/P) ratio by two. The mixture was incubated at room temperature for 5 min before injection.

**DLS Analysis.** The size of TPFE-plasmid DNA complex was measured at 0, 10, 30, 60, and 120 min after complex formation. Dynamic laser light scattering analysis was performed to measure the particle size distribution and average particle sizes using a Zetasizer Nano ZS machine (Malvern Instruments Ltd.).

**Administration of TPFE/EGFP-Expressing Plasmid DNA Complex in Vivo.** Pregnant ICR mice purchased from Saitama Experimental Animals Supply Co. Ltd. were kept at $23 \pm 2$ °C, moisture at $55 \pm 15$% during experiments. Animals were maintained at a 12-hr day and 12-hr night cycle. They ate a regular mouse diet and had free access to water. All animal experiments were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (36).

Pregnant ICR mice (8 d.p.c.) were assigned randomly to one of three groups: the TPFE group, the Lipofectin group, or the naked DNA group. For the TPFE group, TPFE-plasmid DNA complex containing 24 μg plasmid DNA with the *R* value of 5 (described above) was injected intravenously. In the Lipofectin group, the same amount of plasmid DNA encapsulated by Lipofectin was injected according to the manufacturer's protocol (Lipofectin reagent, Invitrogen Corp.). Plasmid DNA dissolved in TBS was injected in the naked DNA group. The injected volume for each group was 300 μL; the total DNA amount was 24 μg for each mouse. At 24 hr after i.v. injection, mice were killed with total body perfusion by cardiac puncture for collecting specimens.

**Delivery of the Insulin 2 Gene and Therapeutic Effects.** Female C57/BL6 mice were obtained from Japan SLC, Inc. Mice were assigned randomly to one of two groups: the *Ins2* + TPFE group and the mock + TPFE group. TPFE-*Ins2* DNA or mock plasmid DNA complex containing 24 μg DNA with the *R* value of 5 was injected intravenously. Plasma insulin and fasting blood glucose levels at 12 and 24 hr after injection were measured with different animal sets because fasting blood samples were obtained after an overnight fast. Glucose concentrations were measured in whole blood using a blood glucose monitor (Terumo Corp.). Plasma insulin was measured using ELISA (Mouse Insulin ELISA kit; Mercodia AB). Measurements were conducted with a 96 well-plate reader (SpectraMax; Molecular Devices Corp.). For immunohistochemistry, mice were killed with total body perfusion by cardiac puncture for collecting specimens at 24 hr after injection.

**DNA Isolation and Semiquantitative PCR Analysis.** At sacrifice, tissue specimens from liver, kidney, lung, and spleen were collected and snap-frozen using liquid nitrogen and kept at −80 °C until analyses. The DNA was isolated from the tissues using a kit (Quick Gene DNA tissue kit S and QuickGene-810; Fujifilm) and quantified with optical density at 260 nm. Then, 0.5 μg of isolated DNA was applied for PCR with AmpliTaq Gold DNA Polymerase (Applied Biosystems) under the following conditions in the Biometra thermal cycler (Biometra biomedizinische Analytik GmbH): denaturation (95 °C for 30 s), annealing (61 °C for 30 s), and extension (72 °C for 30 s) for 50 cycles. The PCR primers (forward, 5′-ACCACATGAAGCAGCACGA-3′; reverse, 5′-TGCTCAGGTAGTGGTTGTCGG-3′) were used for amplification of the EGFP gene sequence (355 bp). Products were analyzed using agarose gel electrophoresis with ethidium bromide staining. Then images were captured using a CCD camera (LAS4000 mini; Fujifilm). Semiquantitative PCR were analyzed using image analysis software (Image Gauge; Fujifilm). To measure the quantity of the delivered plasmid DNA, we used the serially diluted original plasmid DNA as standard (1 μg–0.1 pg). One copy of pCAGGS-EGFP plasmid DNA is equal to $2.960 \times 1 \times 10^{-12}$ μg; the copy number of plasmid DNA was calculated (20).

**RNA Extraction and Real-Time PCR Analysis.** Tissues from the organs were collected at 24 hr after injection and kept in −80 °C before use. Total RNA was extracted using a kit (QuickGene RNA tissue kit SII and QuickGene-810; Fujifilm). To obtain cDNA of transcripts, reverse transcriptase reaction was performed with 1 μg total RNA (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems). Subsequent real-time PCR was performed with the synthesized cDNA and a set of primers for GFP (forward, 5′-CAACTACAACAGCCACAAGGTC-3′; reverse, 5′-GGTGTTCTGCTGGTAGTGGTC-3′) and for internal control (Taqman Eukaryotic 18S rRNA; Applied Biosystems). Subsequently, PCR amplification was performed using a TaqMan universal PCR master mix (Applied Biosystems) under the following conditions (PRISM 7000; Applied Biosystems): denaturation (95 °C for 30 s), annealing (61 °C for 30 s), and extension (72 °C for 30 s) for 40 cycles. The PCR Products were analyzed using software (Sequence Detection version 1.2.3; Applied Biosystems).

**Histological Analysis.** Harvested organs were fixed using 4% paraformaldehyde for 12 hr. Sections were cryoprotected with sucrose 20% and later frozen in optimum cutting temperature. To determine the transfection efficacy, EGFP fluorescence signals were detected using confocal microscopy. Then, 5-μm frozen sections were fixed in 4% paraformaldehyde for 10 min at room temperature, counterstained with TO-PRO3 iodide nuclear stain (1:1000, Invitrogen Corp.) for 60 min, and examined using a confocal microscope (LSM 510 Meta NLO imaging system; Carl Zeiss Inc.).

Insulin immunohistochemical staining of 5-μm paraffin sections was done using indirect biotin avidin technique. The deparaffinized sections were preincubated with ProK solution (DakoCytomation Co. Ltd.), treated with 3% hydrogen peroxide for 15 min, and incubated with a rabbit polyclonal anti-insulin antibody (Santa Cruz Biotechnology Inc.) for 2 hr at 37 °C, followed by incubation with biotin-conjugated anti-rabbit IgG and avidin-conjugated horseradish peroxidase (Vector Laboratories Inc.) and the substrate-chromogen reaction (Nichirei Corp.). Control sections were subjected to secondary antibody only.

**Measurement of Blood Chemistry.** BUN was measured using the urease-indophenol method with Urea NB (Wako Pure Chemical Industries Ltd.). AST and ALT were measured using pyruvate oxidase method with Transaminase CII (Wako Pure Chemical Industries Ltd.).

**Statistical Analysis.** Differences among the experimental groups were detected using Student's *t*-test or Wilcoxon's rank test. Values are expressed as means ±SEM; $p < 0.05$ was considered significant.

**ACKNOWLEDGMENTS.** Part of this study was supported by the Special Coordination Funds for Promoting Science and Technologies of the Ministry of Education, Culture Sports, Science and Technology (MEXT), Japan (Grant 1200015 to E.Noiri); the Global Center of Excellence Program for Chemistry Innovation, MEXT, Japan, Grant-in-Aid for Scientific Research 20108015 and 21685005 (to H.I.); Grant-in-Aid for Scientific Research 21790795, MEXT, Japan (to K.D.); and Grant-in-Aid for Scientific Research 20113005, MEXT, Japan (to T.I.). T.H. thanks the Japan Society for the Promotion of Science for a predoctoral fellowship. The generous supply of [60]fullerene from Frontier Carbon Corporation is acknowledged.

1. Arbogast JW, et al. (1991) Photophysical properties of C60. *J Phys Chem* 95:11–12.
2. Xie Q, Echegoyen L (1992) Electrochemical detection of $C_{60}^{6-}$ and $C_{70}^{6-}$: Enhanced stability of fullerides in solution. *J Am Chem Soc* 114:3978–3980.
3. Hirsch A, Brettreich M (2005) *Fullerenes: Chemistry and Reactions* (Wiley-VCH, Weinheim, Germany), pp 49–344.
4. Nakamura E, Isobe H (2003) Functionalized fullerenes in water. The first ten years of their chemistry, biology and nanoscience. *Acc Chem Res* 36:807–815.
5. Mintzer MA, Simanek EE (2009) Nonviral vectors for gene delivery. *Chem Rev* 109:259–302.
6. Felgner PL, Ringold GM (1989) Cationic liposome-mediated transfection. *Nature* 337:387–388.
7. Felgner PL, et al. (1995) Improved cationic lipid formulations for in vivo gene therapy. *Ann NY Acad Sci* 772:126–139.
8. Barron LG, Gagne L, Szoka FC Jr (1999) Lipoplex-mediated gene delivery to the lung occurs within 60 minutes of intravenous administration. *Hum Gene Ther* 10:1683–1694.
9. Li S, Huang L (1997) In vivo gene transfer via intravenous administration of cationic lipid-protamine-DNA (LPD) complexes. *Gene Ther* 4:891–900.

10. Liu Y, et al. (1997) Factors influencing the efficiency of cationic liposome-mediated intravenous gene delivery. *Nat Biotechnol* 15:167–173.
11. Tsan MF, White JE, Shepard B (1995) Lung-specific direct in vivo gene transfer with recombinant plasmid DNA. *Am J Physiol* 268:L1052–1056.
12. Zou Y, Zong G, Ling YH, Perez-Soler R (2000) Development of cationic liposome formulations for intratracheal gene therapy of early lung cancer. *Cancer Gene Ther* 7:683–696.
13. Filion MC, Phillips NC (1998) Major limitations in the use of cationic liposomes for DNA delivery. *Int J Pharm* 162:159–170.
14. Felgner PL, et al. (1987) Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci USA* 84:7413–7417.
15. Stribling R, Brunette E, Liggitt D, Gaensler K, Debs R (1992) Aerosol gene delivery in vivo. *Proc Natl Acad Sci USA* 89:11277–11281.
16. Nakamura E, et al. (2000) Functionalized fullerene as a new artificial vector for transfection. *Angew Chem Int Ed* 39:4254–4257.
17. Isobe H, et al. (2006) Nonviral gene delivery by tetraamino fullerene. *Mol Pharmaceutics* 3:124–134.
18. Isobe H, et al. (2006) Gene delivery by aminofullerenes: Structural requirements for efficient transfection. *Chem Asian J* 1:167–175.
19. Song YK, Liu F, Chu S, Liu D (1997) Characterization of cationic liposome-mediated gene transfer in vivo by intravenous administration. *Hum Gene Ther* 8:1585–1594.
20. Hofland HE, et al. (1997) In vivo gene transfer by intravenous administration of stable cationic lipid/DNA complex. *Pharm Res* 14:742–749.
21. Thierry AR, et al. (1997) Characterization of liposome-mediated gene delivery: Expression, stability and pharmacokinetics of plasmid DNA. *Gene Ther* 4:226–237.
22. Tsukamoto M, Ochiya T, Yoshida S, Sugimura T, Terada M (1995) Gene transfer and expression in progeny after intravenous DNA injection into pregnant mice. *Nat Genet* 9:243–248.
23. Ochiya T, et al. (1999) Evaluation of cationic liposome suitable for gene transfer into pregnant animals. *Biochem Biophys Res Commun* 258:358–365.
24. Kikuchi N, Nakamura S, Ohtsuka M, Kimura M, Sato M (2002) Possible mechanism of gene transfer into early to mid-gestational mouse fetuses by tail vein injection. *Gene Ther* 9:1529–1541.
25. Yang JP, Huang L (1997) Overcoming the inhibitory effect of serum on lipofection by increasing the charge ratio of cationic liposome to DNA. *Gene Ther* 4:950–960.
26. Filion MC, Phillips NC (1997) Toxicity and immunomodulatory activity of liposomal vectors formulated with cationic lipids toward immune effector cells. *Biochim Biophys Acta* 1329:345–356.
27. Bragonzi A, et al. (1999) Comparison between cationic polymers and lipids in mediating systemic gene delivery to the lungs. *Gene Ther* 6:1995–2004.
28. Sheila S, James SD (1993) *Diseases of the Liver and Biliary System* (Blackwell Scientific, Cambridge, Massachusetts), pp 322–356.
29. Sayes CM, Marchione AA, Reed KL, Warheit DB (2007) Comparative pulmonary toxicity assessments of C60 water suspensions in rats: Few differences in fullerene toxicity in vivo in contrast to in vitro profiles. *Nano Lett* 7:2399–2406.
30. Baker GL, et al. (2008) Inhalation toxicity and lung toxicokinetics of C60 fullerene nanoparticles and microparticles. *Toxicol Sci* 101:122–131.
31. Maeda R, et al. (2008) A water-soluble fullerene vesicle alleviates angiotensin II-induced oxidative stress in human umbilical venous endothelial cells. *Hypertens Res* 31:141–151.
32. Dokka S, Toledo D, Shi X, Castranova V, Rojanasakul Y (2000) Oxygen radical-mediated pulmonary toxicity induced by some cationic liposomes. *Pharm Res* 17:521–525.
33. He CX, et al. (2004) Insulin expression in livers of diabetic mice mediated by hydrodynamics-based administration. *World J Gastroentero* 10:567–572.
34. Morishita R, Gibbons GH, Kaneda Y, Ogihara T, Dzau VJ (2000) Systemic administration of HVJ viral coat-liposome complex containing human insulin vector decreases glucose level in diabetic mouse: A model of gene therapy. *Biochem Biophys Res Commun* 273:666–674.
35. Wang J, et al. (1999) A mutation in the insulin 2 gene induces diabetes with severe pancreatic beta-cell dysfunction in the Mody mouse. *J Clin Invest* 103:27–37.
36. Committee on Care and Use of Laboratory Animals (1985) *Guide for the Care and Use of Laboratory Animals* (Natl Inst Health, Bethesda), DHHS Publ No (NIH) 85-23.