Reducible Poly(oligo-D-arginine) for Enhanced Gene Expression in Mouse Lung by Intratracheal Injection

Young-Wook Won[1], Hyun Ah Kim[1], Minhyung Lee[1] and Yong-Hee Kim[1]

[1]*Department of Bioengineering, Institute for Bioengineering and Biopharmaceutical Research, Hanyang University, Seoul, Republic of Korea*

Nonarginine (D-R9) has been reported to be one of the most efficacious protein transduction domains (PTDs) for the intracellular cargo delivery such as DNA, RNA, proteins, and particles. Although oligoarginines are capable of forming polyplex with DNA by electrostatic interaction, the length of oligoarginine can affect the toxicity and gene expression. The reducible poly(oligo-D-arginine) (rPOA) composed of the Cys-(D-R9)-Cys repeating unit forming disulfide bonds between terminal cysteinyl-thiol groups of short peptides was hypothesized to show efficient gene transfection without toxicity. The reducible high molecular weight poly(oligo-D-arginine) may fragment into the Cys-(D-R9)-Cys in cellular environments such as cytosol, cell surface, endosomes, and lysosomes, and enhance DNA transfection efficiency. In the present study, *in vitro* stability, cytotoxicity, and transfection efficiency of DNA/poly(oligo-D-arginine) polyplex were evaluated. In addition, *in vivo* delivery of DNA into the lung was performed by intratracheal injection of DNA/poly(oligo-D-arginine) polyplex. The *in vivo* study with rPOA showed higher level of gene expression than PEI, sustaining for 1 week without toxicity. Reducible high molecular weight poly(oligo-D-arginine) based on R9 PTD is a very promising nonviral gene carrier for lung diseases by efficiently condensing, stabilizing, and transfecting DNA.

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INTRODUCTION

A safe and efficient delivery vector is crucial for the adequate gene expression. Although viral vectors have the high transfection efficiency, they have intrinsic problems such as immunogenicity and genetic recombination.[1,2] Various polymer- and peptide-based gene delivery systems have been developed as alternatives to the viral vector–based gene transfer systems. Various synthetic peptides were developed to facilitate gene delivery in a wide variety of cells *in vitro*; however, toxicity and stability problems remained to be solved for *in vivo* applications. Protein transduction domains (PTDs), also named cell-penetrating peptides, were proposed as a substitute to the polymer- or synthetic peptide–based gene delivery systems because they can enhance cellular uptake and nuclear translocation followed by gene expression.[3]

PTDs have the ability to effectively transduce bioactive cargoes such as drug, protein, gene, and particle through cellular membrane.[4] TAT peptide, Penetratin, transportan, and polyarginine are the most well-known PTDs. Using the TAT peptide, one of the most well-characterized PTDs, the high transduction efficiency of the various cargoes was achieved. Compared to the TAT peptide, L-9-arginines (L-R9) and D-9-arginines (D-R9) have 20~100 times higher transduction efficiency.[5] In addition, TAT and R9 can condense and deliver DNA into cells; however, low molecular weight of the peptides hampers the stability of peptide/DNA complex and reduces transfection efficiency.

Cross-linking of low molecular weight polycations with disulfide bonds increased the complex stability in extracellular condition and reduced cytotoxicity in cytoplasmic space by rapid degradation.[6] It is due to the difference in redox potential (100-fold) between the oxidizing extracellular space and the reducing intracellular space.[7] Reducible poly(L-lysine)[8] and polyrotaxane[9] polymer systems containing disulfide bonds showed enhanced gene delivery efficiency. Various substituted linear poly(amidoamine)s with disulfide bonds were reported to show significantly increased transfection efficiency and cell viability.[10] Recently, disulfide-containing reducible poly(amido ethylenimine)s (SS-PAEIs) were shown to be potential carriers for gene delivery.[1] In addition, reducible cationic peptides with disulfide bonds were reported to show efficient gene expression.[11] This reducible peptide system can be easily constructed from short peptides with terminal cysteine residues by dimethylsulfoxide (DMSO)-mediated oxidation. Small redox molecules or redox enzymes can fragment high molecular weight polypeptides to small peptides in various intracellular environments.[12]

The objective of this study was to develop more effective and less toxic reducible poly(oligo-D-arginine) (rPOA) as a pulmonary gene delivery system. This reducible cationic peptide was considered to stabilize DNA/peptide polyplex in extracellular spaces, facilitate uptake of DNA into cells and release DNA from the polyplex by reduction of disulfides in cytoplasmic space. The transfection efficiency, cytotoxicity, and stability of DNA/rPOA polyplex were evaluated *in vitro*. In addition, *in vivo* delivery of

Correspondence: *Yong-Hee Kim, Department of Bioengineering, Hanyang University, 17, Haendang-dong, Seongdong-gu, Seoul, 133-791, Republic of Korea. E-mail: yongheekim@hanyang.ac.kr*

DNA into the lung was performed using rPOA, representing that rPOA may be a useful gene carrier for lung diseases with high transfection efficiency and low toxicity.

RESULTS

Synthesis of reducible rPOA

The synthesis of rPOA was performed by DMSO-mediated oxidative polymerization of the R9 peptides, Cys-(D-R9)-Cys. The molecular weight distribution of the resulting peptide was measured by the MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) with increasing degree of oxidation in a time-dependent manner until it reaches a plateau. The reaction was continued for 6 days, and low molecular weight impurities were removed by dialysis (MWCO: 10,000 Da). By performing a gel permeation chromatography, the number-average molecular weight (Mn) and mass-average molecular weight (Mw) of the synthesized and purified peptide were determined as 94,757 and 96,204 Da (Mw/Mn = 1.016), respectively (**Figure 1a**). This result represents a significant increase in molecular weight of the resulting peptide over the original peptide molecular weight (1,628 Da).

Characterization of DNA/rPOA polyplex

The DNA condensation efficiency of rPOA was examined by gel retardation and ethidium bromide (EtBr) exclusion assay, and optimum N/P ratios of the cationic peptides to DNA were determined. The binding affinities of anionic DNA with rPOA, D-R9, poly(D-arginine) (PDR), and PEI at various ratios are shown in **Figure 1b**. DNA migration was markedly retarded with increasing amount of cationic peptides and PEI. In comparison with PEI, rPOA and D-R9 showed slightly less efficiency in DNA retardation; however, rPOA and D-R9 have higher DNA-binding affinity than PDR. The complete retardation of DNA with PEI, PDR, D-R9, and rPOA was observed at and above N/P ratios of 3.5, 10.5, 7.0, and 7.0, respectively, demonstrating that the binding and condensation ability of PEI were stronger than those peptides. Quantitative analysis of DNA condensation by cationic peptides and PEI was further examined using the EtBr exclusion assay. As shown in

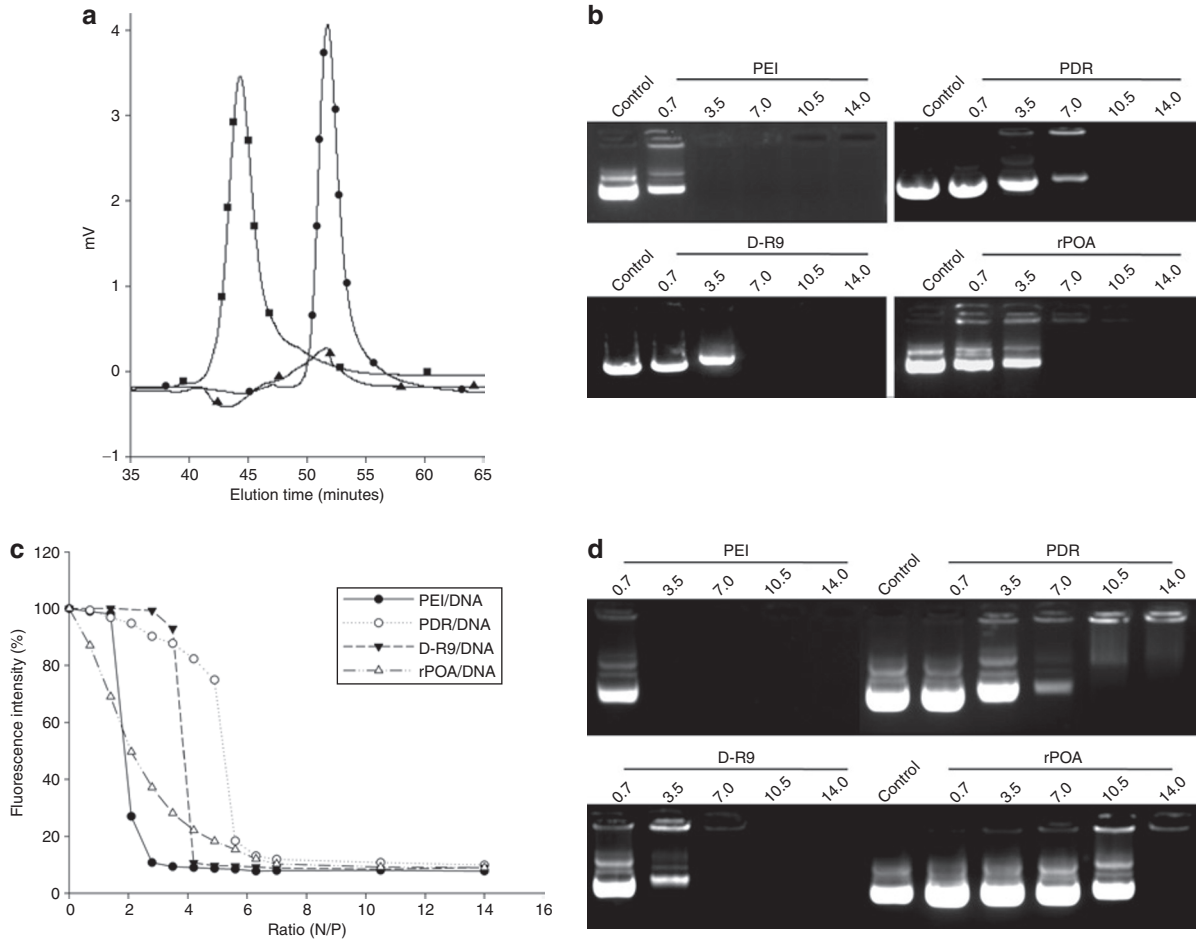

Figure 1 GPC analysis of rPOA, gel retardation, and EtBr exclusion assay. (**a**) Molecular weight of rPOA was measured as ~96 kd by GPC analysis: (closed circles) standard: Mw = 41 kd; (closed squares) standard: Mw = 177 kd; (closed triangles) rPOA. (**b**) DNA migration was retarded by forming polyplexes with PEI, PDR, rPOA, and D-R9 at and above N/P ratios of 3.5, 10.5, 7.0, and 7.0, respectively. One microgram of DNA was condensed for 3 minutes at room temperature with PEI, PDR, rPOA, and D-R9 at various N/P ratios prior to the agarose gel electrophoresis. (**c**) Extension of EtBr exclusion was observed by adding PEI, PDR, rPOA, or D-R9 to the DNA at N/P ratios ranging from 0.7 to 14.0. The fluorescence intensity was expressed as a percentage relative to the initial fluorescence. (**d**) Gel retardation assay was performed in the presence of 40% of β-mercaptoethanol as a reducing agent. GPC, gel permeation chromatography; PDR, poly(D-arginine); rPOA, reducible poly(oligo-D-arginine).

Figure 1c, the fluorescence intensity of the polyplexes with PEI, PDR, D-R9, and rPOA initiated to decrease at N/P ratios of 2.0, 5.0, 4.0, and 0.7, and then reached a steady state at N/P ratios of 3.0, 7.0, 4.0, and 7.0, respectively. Taken together, these observations indicate that both rPOA and D-R9 are capable of forming DNA polyplexes at appropriate N/P ratios. The disulfide bonds of rPOA may be fragmented into the Cys-(D-R9)-Cys in the presence of reducing agents such as dithiothreitol or β-mercaptoethanol. As a result of disulfide bond reduction in the presence of 40% (vol/vol) β-mercaptoethanol, rPOA was not sufficient to condense DNA even high N/P ratios, 7.0 and 10.5, in comparison to the result shown in Figure 1b, whereas PEI, PDR, and D-R9 showed no differences in DNA-condensing ability (Figure 1d).

Chemical properties of cationic peptides, such as the polymerization degree and the type of cationic groups present in the peptides, may influence the biophysical properties of DNA polyplexes, including the size and zeta potential of the DNA polyplex. The mean diameters were not significantly different among polyplexes over a 5-hour period (Figure 2a), suggesting that the condensed polyplex of DNA with the peptides and PEI were stabilized for at least a couple of hours. The average mean diameters of DNA polyplexes with PEI, PDR, rPOA, and D-R9 were approximately 120, 80, 150, and 70 nm, respectively. The surface zeta potential of the polyplexes was measured to compare the difference in zeta potential with the same amount of DNA. At an N/P ratio of 7.0, PDR, D-R9, and rPOA showed negative charge, whereas DNA/PEI polyplex had positive charge (Figure 2b); however, the zeta potential of the peptide/DNA polyplexes rapidly changed to positive at and above N/P ratio of 14.0. The morphological characteristics of the polyplexes and uncondensed DNA are shown in Figure 3. The DNA polyplex was spherical in shape and <200 nm in size, indicating that PEI, rPOA, D-R9, and PDR were capable of condensing DNA into the nano-sized spherical aggregates.

In order to evaluate the effect of rPOA on the protection of DNA against the degradation in serum, naked DNA and the DNA polyplexes prepared at N/P ratios of 7.0 and 14.0 were incubated for 2 hours in phosphate-buffered saline (PBS) in the presence of 50% fetal bovine serum under shaking at 37 °C. Dissociation of DNA from the polyplexes upon exposure to heparin visualized DNA bands in the same position as control and confirmed protective effects of PEI, rPOA, D-R9, and PDR in serum. Significant degradation of naked DNA was observed after 2-hour incubation as shown in Figure 4a. DNA was protected for 2-hour incubation by forming stable polyplexes with PEI, rPOA, D-R9, or PDR, suggesting that those cationic peptides or polymer can protect DNA from degradation in serum under the test conditions. Although all the samples have effective DNA protective ability, rPOA showed the strongest DNA-binding affinity resulting in the weakest DNA intensity in agarose gel electrophoresis as shown in Figure 4a. For the DNA-binding affinity comparison of rPOA with PEI, heparin was added to the polyplexes in a dose-dependent manner. The minimum weight ratio of heparin to PEI for the decomplexation of DNA from PEI polyplex was 5; however, DNA decomplexation from rPOA polyplex was initiated at the heparin to rPOA weight ratio of higher than 50 (Figure 4b).

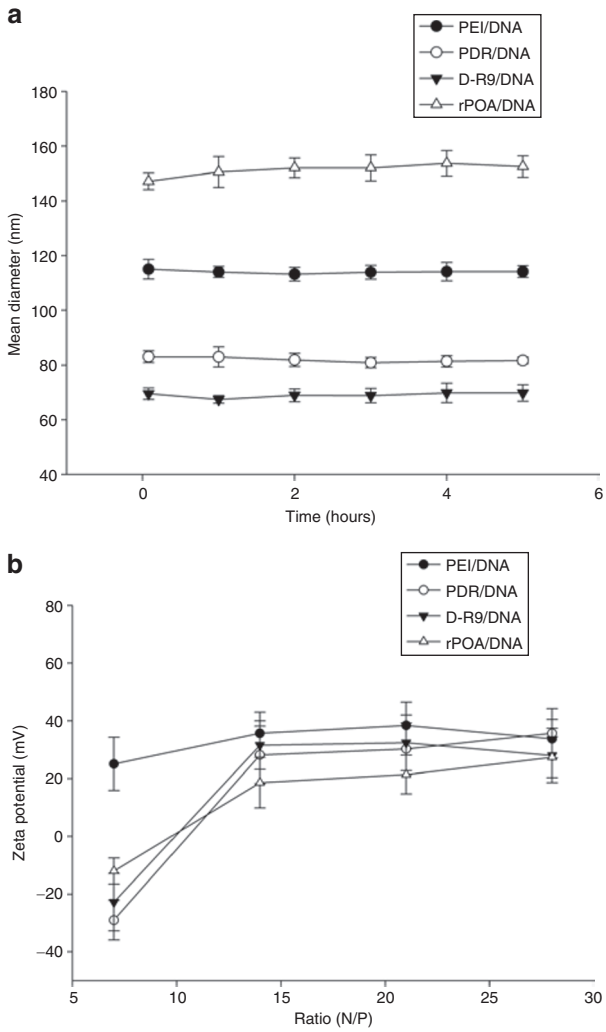

Figure 2 Size and zeta potential of polyplexes. Measurements of (**a**) size and (**b**) zeta potential of polyplexes at various N/P ratios. At N/P ratio of 14.0, the time-dependent variation of the mean diameters of DNA polyplexes with PEI, PDR, rPOA, and D-R9 was observed for 5 hours with 1-hour interval at room temperature. The polyplexes of PEI, PDR, rPOA, and D-R9 were prepared at N/P ratio of 7.0, 14.0, 21.0, and 28.0 for the measurement of the zeta potential. The data represent the average ± SD of three experiments. PDR, poly(D-arginine); rPOA, reducible poly(oligo-D-arginine).

In vitro transfection and cytotoxicity study

The ratio-dependent transfection studies were examined at a constant DNA dose of 2.0 μg per well in 12-well plates at N/P ratios of 7.0 and 14.0 in the presence of serum with three types of cell lines, HEK 293 (human embryonic kidney cell line 293), H9c2, and L2. The transfection efficiency of rPOA was lower than that of PEI in H9c2; however, it showed almost the same level of gene expression in comparison with PEI in HEK 293 and L2 (Figure 5a). The dose-dependent transfection efficiencies in the three cell lines were evaluated at an N/P ratio of 7.0 at DNA doses of 1.0 and 2.0 μg (Figure 5b). The luciferase expression increased as DNA dose increased in all cell lines transfected with both rPOA and PEI. Nevertheless, PDR and D-R9 was sufficient to condense and protect DNA; the gene expression of both peptides was very weak compared to rPOA and PEI polyplexes in all transfection tests.

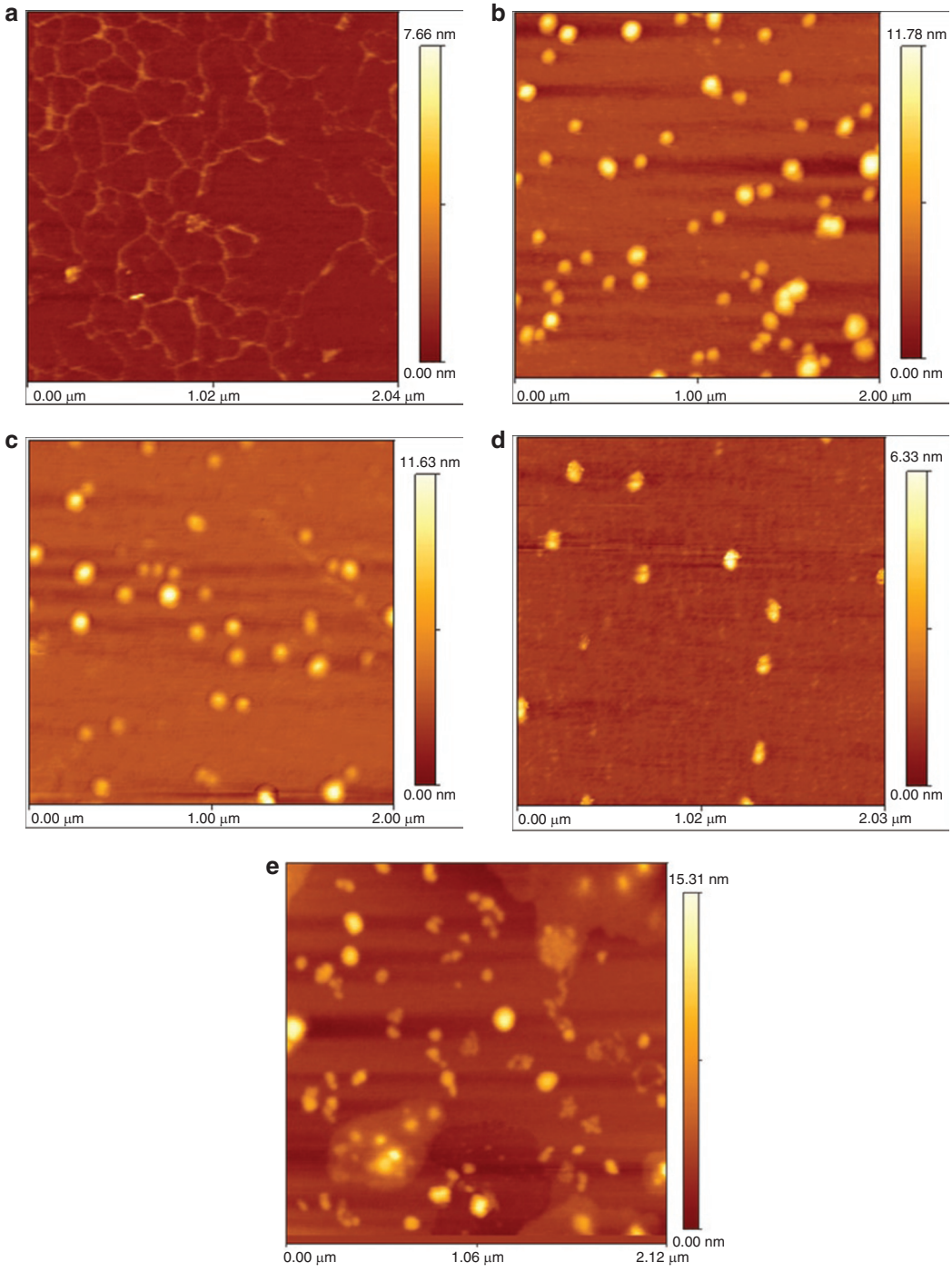

Figure 3 Morphological characteristics of DNA condensed with rPOA, PEI, D-R9, and PDR. Atomic force microscope visualizes (**a**) naked DNA, (**b**) rPOA/DNA polyplex, (**c**) PEI/DNA polyplex, (**d**) D-R9/DNA polyplex, and (**e**) PDR/DNA polyplex. The morphological characteristics of the polyplexes were observed in the close-contact mode in air. Polyplexes of PEI, PDR, rPOA, and D-R9 with DNA were prepared at a 2.5 μg/ml concentration at an N/P ratio of 14.0. PDR, poly(D-arginine); rPOA, reducible poly(oligo-D-arginine).

The cytotoxicity of rPOA, PDR, D-R9, and PEI in L2 cells was measured using CCK-8 (Cell Counting Kit-8) at 48 hours after the transfection of DNA polyplexes with rPOA, PEI, PDR, and D-R9 at an N/P ratio of 14.0 and DNA dose of 2.0 μg. As shown in **Figure 5c**, the viability of L2 cells was >90% in cationic peptide/DNA polyplex treated groups; however, PEI/DNA significantly decreased cell viability to ~60%.

In order to show the influence of the disulfide reduction on the gene expression, cells were incubated in the presence or absence of buthionine-sulfoximine (BSO) known as a blocking agent of glutamyl-S-transferase. Due to the effect of BSO on the inhibition of glutathione (GSH) production, the level of luciferase expression was decreased by ~25 times in the BSO-treated group compared to the control group as shown in **Figure 5d**. The cell viability of

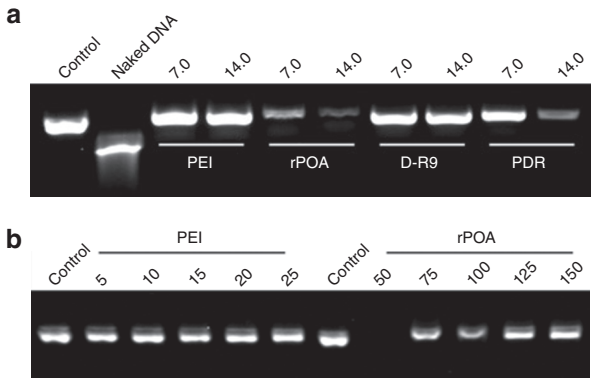

Figure 4 DNA stability in serum and binding affinity of rPOA. (**a**) The polyplexes were prepared at N/P ratios of 7.0 and 14.0, and fetal bovine serum was added to the polyplexes at a final concentration of 50% (vol/vol), and then the samples were incubated for 2 hours at 37 °C. For the decomplexation of DNA from the polyplexes after serum incubation, heparin in phosphate-buffered saline (pH 7.4, 0.5 mol/l NaCl) was added to the samples in the presence of 0.01 mol/l EDTA with additional 1-hour incubation. Protective effects of cationic peptides or PEI against DNA degradation in serum are evident in gel electrophoresis. (**b**) The binding affinity of rPOA to DNA was compared with PEI by adding heparin in a dose-dependent manner. rPOA was maintained in their polyplex formation at the heparin to rPOA weight ratio of higher than 50, whereas PEI was fully decomplexed at the heparin to PEI weight ratio of 5. PDR, poly(D-arginine); rPOA, reducible poly(oligo-D-arginine).

L2 cells in the presence of BSO was evaluated in a dose-dependent manner because BSO is often very toxic in some cells. The percent cell viability of L2 cells was 101 ± 1.09, 103 ± 3.65, 98 ± 1.48, and 99 ± 2.66 under 50, 100, 200, and 400 μmol/l BSO treatment, respectively, suggesting that the reduced gene expression of luciferase in L2 cells in the presence of BSO was not caused from the cytotoxicity of BSO.

In vivo transfection and histology study

The *in vivo* transfection efficiencies were evaluated at various doses of DNA and ratios of rPOA. PEI showed slightly increased level of luciferase expression in comparison to naked DNA; however, the transfection efficiency of rPOA significantly increased under the test conditions (**Figure 6a**). The luciferase expression of rPOA was ~3,000, 7,000, and 5,000 at the dose of 10, 15, and 20 μg, respectively, at an N/P ratio of 7.0. The luciferase activity was slightly decreased at an N/P ratio of 14.0 in the three doses of DNA. The maximum relative light units (RLU) was found at the DNA dose of 15 μg and N/P ratio of 7.0, showing ~20- and ~100-fold increase in luciferase expression compared to the DNA/PEI polyplex and naked DNA, respectively. As shown in **Figure 6b**, the DNA/rPOA polyplex maintained the gene expression for 1 week, and the luciferase activity showed a prolonged transgene expression up to 3 days with high level of gene expression and with a slightly decreasing level up to 7 days. The luciferase activity showed approximately tenfold reduction in the transgene expression at day 7, when the transfection level of rPOA was almost the same as that of PEI shown in **Figure 6a**, compared to 1 day.

Hematoxylin and eosin–stained histological results showing morphological and inflammatory changes revealed no significant differences between the control and the lungs treated with DNA/rPOA polyplex at 1, 3, and 7 days after the intratracheal injection. The histological analysis of airway epithelium and airway perivascular clearly showed that the inflammatory infiltrations were minimally observed near the airway at days 1, 3, and 7 as shown in **Figure 7** and **Table 1**.

DISCUSSION

Short cationic peptides were polymerized into reducible polypeptide by DMSO oxidation of the terminal cysteinyl-thiol groups of the Cys-(D-R9)-Cys repeating units. This reducible high molecular weight peptide can fragment into the Cys-(D-R9)-Cys in the presence of reducing agents such as dithiothreitol or β-mercaptoethanol, both of which reduce disulfide bonds between the two cysteines to free sulfhydryl. In addition, the disulfide bonds can be reduced by various mechanisms in cellular environments such as cytosolic space, cell surface, endosomes, and lysosomes.[13] It has been reported that the redox-triggered destabilization mechanism of the reducible disulfide bonds constitutes a powerful tool to the development of nonviral gene delivery system due to its releasable properties. Although the intracellular delivery mechanism of R9-PTD is unclear, it is assumed that the difference in the GSH content controlling the intracellular redox environment between intracellular and extracellular space could initiate the destabilization of rPOA resulting in DNA release in cytosol and its uptake into the nucleus.[14] The intracellular GSH content in L2 cells decreased up to ~30% compared to control after 6 hours in the presence of 50 μmol/l BSO, an inhibitor of GSH synthesis by blocking glutamyl-S-transferase, which is a key enzyme in the GSH production. A slightly faster decrease in GSH level was observed in the presence of 100 μmol/l BSO, demonstrating that 50–100 μmol/l BSO led to the maximal effect of BSO on the inhibition of GSH activity in L2 cells.[15] In this study, it was hypothesized that rPOA may be able to form a stable polyplex with DNA at predetermined N/P ratio to effectively transfect DNA into the cells and release DNA in cytosol by being fragmented into short peptides resulting in high transfection efficiency and low cytotoxicity. BSO treatment significantly reduced the transfection efficiency of DNA/rPOA polyplex in L2 cells (**Figure 5d**), indicating that the reduction of rPOA facilitates a deformation of DNA/rPOA polyplex, which results in the release of DNA. As a result of the rPOA reduction, the transfection efficiency of DNA/rPOA polyplex in the absence of BSO was higher than that of DNA/rPOA polyplex in the presence of BSO. Reducibility of DNA/polymer polyplex plays an important role for the enhanced and stable transfection efficiency of condensed DNA with minimal cytotoxicity.

It is interesting to note that most PTDs including TAT peptide show similar characteristics containing arginine-rich amino acids.[16] Indeed, oligo-arginine containing ≥6 arginine enters cells more rapidly than peptides of equal length consisted of lysine, histidine, ornithine, or citrulline.[17,18] However, DNA condensed with most short cationic peptides has shown low stability caused by a less cooperative effect of DNA with the peptides, resulting in insufficiency to protect DNA from degradation in serum and/or metabolism *in vivo*.[19] The minimum chain lengths of the cationic peptides for gene delivery are 6–10 amino acids, which are enough to condense DNA into stable nano-sized aggregates.[17] With octaarginine PTD, only 4–5 arginines were considered to involve in forming the complex with the cargo, leaving some

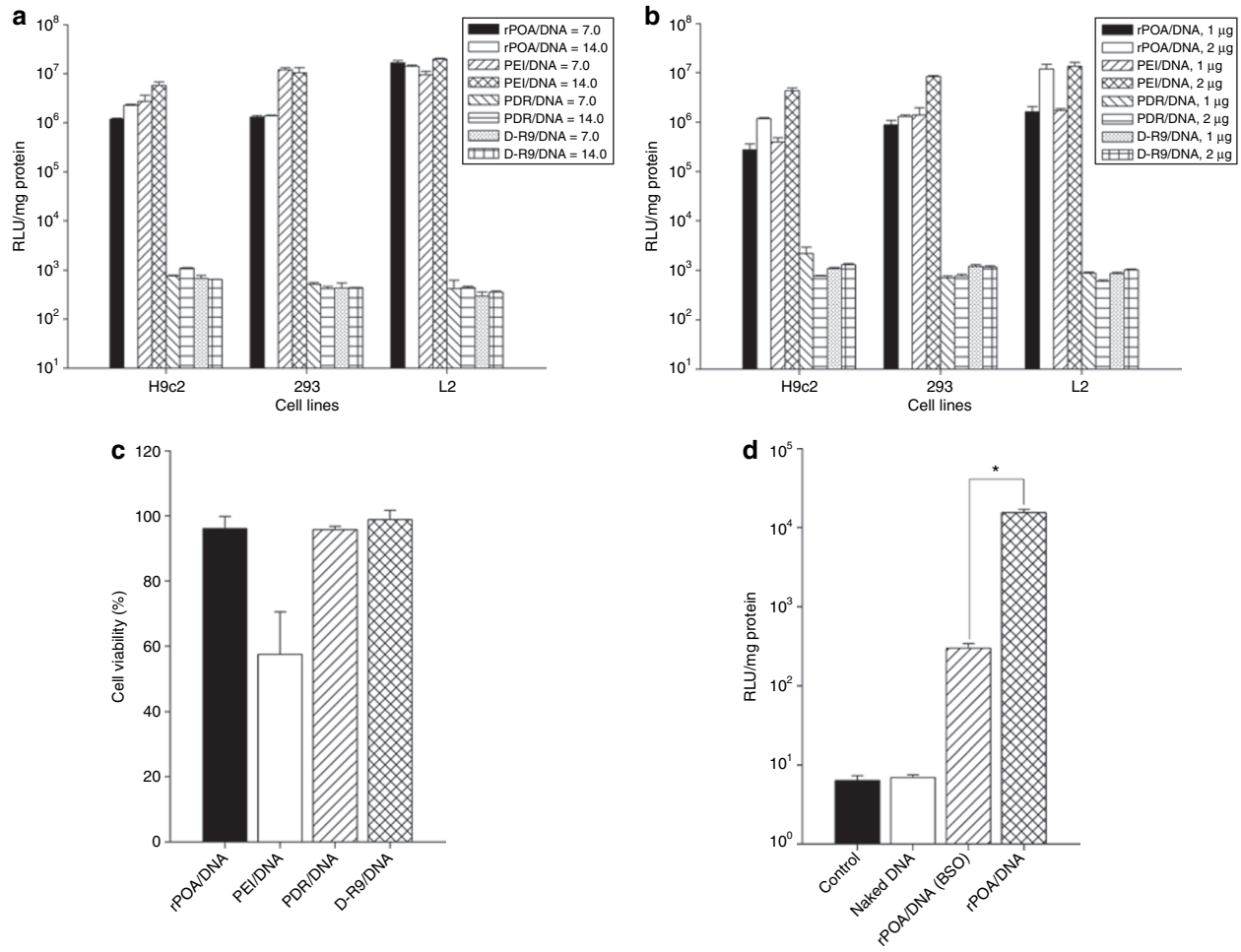

Figure 5 ***In vitro*** **transfection efficiency and cytotoxicity.** (**a**) ratio-dependent and (**b**) dose-dependent transfection efficiency, (**c**) cytotoxicity of rPOA, PEI, PDR, and D-R9, and (**d**) a decrease in luciferase expression in the presence of BSO. The complexes were prepared with a DNA dose of 2 μg at N/P ratios of 7.0 and 14.0, and transfected for 48 hours for the ratio dependency. For the dose-dependent transfection study, 1 and 2 μg of DNA were complexed with the cationic peptides at an N/P ratio of 7.0 followed by transfection. The cell viability was examined in L2 cells with the complexes prepared with 1 μg of DNA at N/P ratio of 7.0 and 14.0. The level of luciferase was significantly decreased in BSO-treated group. The graph represents the average ± SD of three experiments (*P < 0.05). BSO, buthionine-sulfoximine; PDR, poly(D-arginine); RLU, relative light units; rPOA, reducible poly(oligo-D-arginine).

arginines open for interaction with the cell membrane. Cargo/PTD complexes containing 7–9 free arginines exhibited considerably higher uptake than did complexes composed of 1–5 free arginine residues.[20,21] Furthermore, when the cationic peptides bind too strongly with the negatively charged gene, the gene may not be released from the polyplex. The required size of the peptide/DNA polyplex for efficient intracellular delivery of subsequent intracellular processing is <400 nm in size.[22] Taken together, the PTD-based carrier should have >10 cationic amino acids, formed the polyplexes of <400 nm in size, protected DNA from degradation and released DNA for the sufficient gene expression. The DNA/rPOA polyplexes and the molecular weight of rPOA are <400 nm in size and ~96 kd, respectively, suggesting that the rPOA has enough arginines to condense and protect DNA and to bind with cell membrane. These characteristics make rPOA effective to transfect DNA in terms of the polyplex size, the length of the peptide, and the reducibility.

High gene delivery efficiency *in vitro* does not guarantee high transgene expression *in vivo* because cationic vector systems are inactivated in as low as 5–10% serum.[23] In the present study, transfection results performed with 10% serum showed that DNA was well protected and efficiently expressed by the polyplex formation with rPOA in the three cell lines (**Figure 5**). Although the transfection efficiency of rPOA was low or same as that of PEI *in vitro*, rPOA showed significantly high level of transgene expression compared with PEI in mice lung delivered by intratracheal injection (**Figure 6**). *In vivo* transfection result with 10 μg DNA/rPOA polyplex showed approximately tenfold higher level of luciferase expression compared with DNA/PEI polyplex prepared at a DNA dose of 15 μg. This result suggests that rPOA is more potent than PEI to the *in vivo* gene delivery because rPOA showed higher gene expression than PEI at even low dose of DNA. In addition to the high gene expression *in vivo*, rPOA had a potential to prolong gene expression up to 7 days. This result demonstrates that the high transfection ability *in vitro* does not assure the high level of DNA expression *in vivo*, and rPOA is a more effective gene delivery system than that of PEI *in vivo*. PEI is the most frequently used cationic polymer in the pulmonary gene delivery among several gene

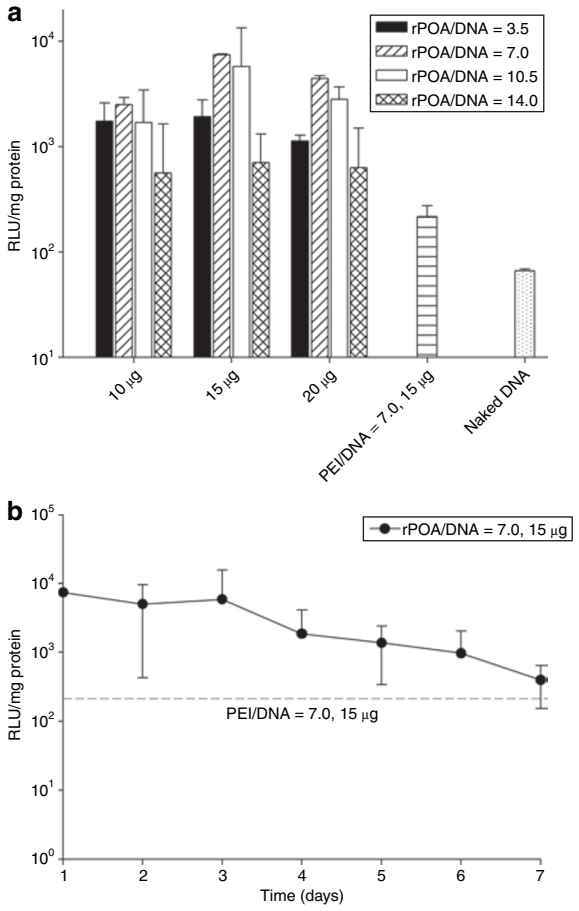

Figure 6 *In vivo* transfection efficiency. (**a**) The ratio- and dose-dependent luciferase expression and (**b**) prolonged expression of luciferase *in vivo*. The intratracheal injection of the complexes prepared with three doses of DNA at four N/P ratios of rPOA to DNA. The luciferase was assayed 1 day after the injection for the ratio and dose dependency, and at each day up to 7 days for the observation of prolonged gene expression prepared with 15 μg of DNA at an N/P ratio of 7.0. The graph represents the average ± SD of six mice. RLU, relative light units; rPOA, reducible poly(oligo-D-arginine).

carriers, in terms of high transfection efficiency both *in vitro* and *in vivo* due to its proton-buffering effect. Considerable increase in gene expression was achieved using various types of commercially available PEI, such as 22 kd linear and 25 kd branched PEI. In particular, studies with 25 kd branched PEI have reported significantly enhanced levels of gene expression in the lung by an aerosol administration with minimal toxicity.[24–28] These observations reveal that PEI-based aerosol administration of gene into the lung is, at least in part, a safe and efficient method for the pulmonary gene delivery. In the present study, the gene transfection efficiency, long-term expression, and toxicity of rPOA were evaluated in the mice lung after the aerosol administration of rPOA/DNA polyplexes as a pulmonary gene carrier by comparing the levels of transgene expression with PEI.

In summary, reducible high molecular weight rPOA based on the R9-PTD was developed and shown to efficiently condense, stabilize, and transfect DNA both *in vitro* and *in vivo*. High molecular weight rPOA showed DNA condensation ability as effective as D-R9, PDR, and PEI, and protected DNA from degradation in serum. Evaluation of the rPOA reducibility using BSO confirmed that the disulfide bonds can be reduced by several mechanisms in various cellular environments, and it facilitates release of gene from the polyplexes and accelerates gene expression. Moreover, rPOA showed high transfection efficiency with minimal toxicity *in vivo*, whereas PEI showed poor gene expression *in vivo*, suggesting that rPOA is appropriate for the *in vivo* applications as a gene carrier. rPOA based on nonarginine is a very promising nonviral gene carrier in terms of intracellular reducibility, low toxicity, protection of DNA against serum, and effective transfection *in vivo*.

MATERIALS AND METHODS

Materials. Peptides C-R9-C (CRRRRRRRRRC, Mw 1628) and D-R9 (RRRRRRRRR, Mw 1422) were purchased from Peptron (Daejeon, Korea). PEI (branch form, average molecular weight 25,000), PDR, and 0.1% trifluoroacetic acid were obtained from Sigma (St Louis, MO). Plasmid DNA (pGL3-promoter, 5,010 bp) and the luciferase assay kit were from Promega (Madison, WI). All other reagents were of analytical grade.

Synthesis of reducible rPOA. Preparation of the rPOA was carried out by the conventional oxidative polycondensation using DMSO as previously described.[29] The condensation was performed at 57 mmol/l Cys-(D-R9)-Cys concentration in PBS (pH 7.4) containing 30% DMSO for 6 days under stirring at 150 rpm and room temperature (RT). During the reaction, 10 μl of samples were taken at 24-hour interval and terminated by adding 5 mmol/l HEPES buffer, and the aliquots were analyzed by MALDI-TOF (Ultraflex; Bruker Daltonics, Bremen, Germany). The average molecular weight of the rPOA was measured by gel permeation chromatography (1515 isocratic pump, 2414 refractive index detector; Waters, Milford, MA) with a calibration using commercially available poly(L-lysine) after no change in molecular weight distribution was confirmed by a MALDI-TOF. The low molecular weight peptides were removed using a dialysis membrane (MWCO: 10,000). Purified peptides were collected and lyophilized with a vacuum-freeze dryer (Freezone 4.5; Labconco, Kansas City, MO). For the gel permeation chromatography analysis, peptides were dissolved in 0.1% trifluoroacetic acid containing 0.2 mol/l NaCl at a concentration of 1 mg/ml. The peptides were eluted with 0.1% trifluoroacetic acid containing 0.2 mol/l NaCl at a flow rate of 0.6 ml/minute.

DNA condensation

Agarose gel electrophoresis assay: Various amounts of the cationic peptides including rPOA, D-R9, PDR, and PEI were added to PBS containing 1 μg of DNA, and then the samples were incubated for 15 minutes at RT and electrophoresed on 0.8% (w/v) agarose gel stained for 30 minutes at 100 V in 0.5% TBE buffer solution. An image of the agarose gel was captured to display the location of the DNA. Ratios were expressed as N/P ratios of the cationic peptides or PEI to DNA for all data. Polyplexes were prepared at various ratios of the cationic peptides or PEI to DNA at a final volume of 6 μl, and then the samples were incubated for 15 minutes at RT. Four microliters of β-mercaptoethanol were added to the mixture for the reduction of the disulfide bond between the cysteines. The reaction was performed under gentle stirring for 1 hour at 37 °C. The samples were electrophoresed as described above.

EtBr exclusion assay: The ability of cationic peptides to condense DNA was tested by a standard EtBr exclusion assay. DNA (15 μg) and EtBr (4 μg) were incubated in 3 ml of PBS for 15 minutes and the resultant fluorescence taken as 100%. Various amounts of the cationic peptides and PEI were incrementally added to the DNA-EtBr mixture at a final volume of 3 ml. The samples were mixed by inversion, covered with foil, and incubated at RT for 3 minutes prior to the measurement of fluorescence. The relative fluorescence intensity of the mixture was expressed as a percentage of the fluorescence intensity relative to that of the DNA-EtBr

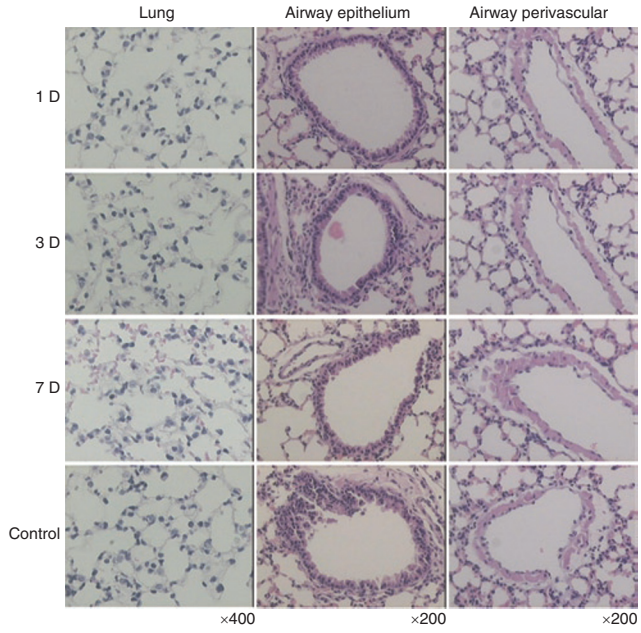

Figure 7 Morphological and inflammatory changes in the lungs, airway epithelium, and airway perivascular after the injection. Lung tissue, airway epithelium, and perivascular were shown with hematoxylin and eosin staining at 1, 3, and 7 days after the transfection of rPOA/DNA polyplexes. No significant differences were observed in all groups. rPOA, reducible poly(oligo-D-arginine).

Table 1 Inflammatory cell infiltration

	Peribronchial	Perivascular	Alveolar septa	Edema[a]
1 Day	None	None	None	None
3 Days	Mild[b]	None	Minimal	Minimal
7 Days	Minimal	Minimal	Minimal	Minimal
Control	Minimal	None	None	None

[a]Edema: widening of the interstitial space surrounding the airway and vasculature; [b]Mild inflammatory cell infiltration: 2~5/ × 1,000.

mixture. The fluorescence was measured using a UV/fluorescence reader (SpectraMax M2[e]; Molecular Devices, Sunnyvale, CA) at excitation and emission wavelengths of 516 and 598 nm, respectively.

Characterization of condensed DNA

Size and zeta potential determination: The mean diameter and surface zeta potential of the polyplexes were measured using a dynamic light scattering using Zetasizer-Nano ZS (Malvern Instruments, Worcestershire, UK). Polyplexes of DNA with the cationic peptides and PEI were prepared in PBS. At an N/P ratio of 14.0, the time-dependent variation of the mean diameters was observed for 5 hours with 1-hour interval. DNA/cationic peptides and DNA/PEI polyplexes were examined at N/P ratios of 7.0, 14.0, 21.0, and 28.0 for the measurement of the zeta potential. The mean diameters and zeta potential of the polyplexes were measured in triplicate.

Atomic force microscopy: The morphological characteristics of the polyplexes were observed by an atomic force microscope (AFM) with Nano-R AFM (Pacific Nanotechnology, Santa Clara, CA) in the close-contact mode in air. Polyplexes of the cationic peptides/DNA and PEI/DNA were prepared at 2.5 μg/ml DNA and ratio of 14.0. To visualize naked DNA, DNA was prepared at 2.5 μg/ml DNA with 3 mmol/l NaCl. After 3 minutes, 10 μl of the polyplexes and naked DNA were vortexed for 10 seconds and placed on freshly cleaved mica. After 3 minutes, the remaining solution was washed with a drop of water and the excessive water was dried with a gentle stream of nitrogen.

DNA stability in serum: For the protection of DNA in serum, cationic peptides/DNA and PEI/DNA polyplexes were prepared at an N/P ratio of 14.0 in PBS and incubated at RT for 15 minutes. After incubation, fetal bovine serum was added to the polyplexes at a final concentration of 50% (vol/vol). The stability of DNA in the serum was tested by incubating for 2 hours under heating at 37 °C and shaking at 150 rpm. After the 2-hour of incubation, heparin was added to the polyplexes at a final heparin to the cationic peptides or PEI weight ratio of 60 in the presence of 0.01 mol/l EDTA. After 1 hour of incubation, the mixtures were electrophoresed under the same conditions described above.

In vitro *transfection study*

Cell culture: HEK 293, rat cardiac cell line H9c2, and rat lung epithelial cell line L2 were obtained from ATCC, Manassas, VA. All three cell lines were cultured in Dulbecco's modified Eagle's medium, 10% fetal bovine serum, and penicillin (100 IU/ml)/streptomycin (100 μg/ml). Cells were incubated to 80% confluency at 37 °C with 5% CO_2.

In vitro *transfection efficiency*: HEK 293, H9c2, and L2 cells were seeded on 12-well plates at a density of 8.0×10^4, 4.0×10^4, and 1.0×10^4 cells/well, respectively. After 1 day of incubation, the culture media were replaced with 1 ml of 10% fetal bovine serum–supplemented Dulbecco's modified Eagle's medium–containing DNA polyplexes with rPOA, D-R9, PDR, and PEI. The polyplexes were prepared by mixing 2 μg of DNA and various amounts of rPOA, D-R9, PDR, and PEI in Dulbecco's modified Eagle's medium. After 48 hours of cell incubation, cells were washed twice with PBS and treated with 150 μl of 1× cell lysis buffer reagent (Promega) for 20 minutes. The cell lysates were scraped, harvested, and transferred to 1.5 ml microtubes, and centrifuged for 3 minutes at 13,000 rpm. Luciferase RLU of the cell lysates were measured on a 96-well plate luminometer (Berthold Detection Systems, Pforzheim, Germany) with 20-second integration, and the results were expressed as RLU/mg of cell protein determined by the DC protein assay kit with bovine serum albumin standard (Bio-Rad Laboratories, Hercules, CA). L2 cells were seeded on 12-well plates at a density of 2.0×10^4 cells/well and incubated in the absence or presence of 200 μmol/l BSO for 24 hours prior to transfection. After 24 hours of incubation, the level of gene expression was measured using luminometer as previously described. In all cases, the transfection efficiency was measured three times with three replicates.

Cytotoxicity: For the measurement of cytotoxicity, L2 cells were seeded on 96-well plates at a density of 1.0×10^4 cells/well. After 24 hours of incubation, all the culture media were replaced with 200 μl of fresh media containing DNA polyplexes with rPOA, D-R9, PDR, and PEI at a predetermined N/P ratio. After 48 hours, 20 μl of the CCK-8 (Dojindo Laboratory, Tokyo, Japan) solution replaced the media. After 2 hours of incubation, the absorbance of each well was measured by a UV/Vis spectrophotometer at 540 nm. The relative cell viability was calculated and expressed as percent cell viability. The viability was measured three times with eight replicates.

In vivo *transfection study.* DNA/rPOA polyplexes were prepared in 100 μl of PBS at DNA doses, 10, 15, and 20 μg, and at N/P ratios, 3.5, 7.0, 10.5, and 14.0. DNA/PEI polyplex was prepared in PBS at DNA dose of 15 μg and an N/P ratio of 7.0 as a positive control, and the 15 μg of naked DNA was used as a negative control. Mice were anesthetized by an injection of ketamine at a dose of 100 mg/kg and of xylazine at a dose of 100 mg/kg intraperitoneally. The trachea of suspended mice by upper teeth at an appropriate angle was illuminated by optical light source. The lower jaw was open and the tongue was displaced in order to view the trachea. The aerosolization of 100 μl of DNA/rPOA and DNA/PEI polyplexes was performed using a MicroSprayer aerosolizer (IA-1C; Penn-Century, Philadelphia, PA). The ratio- and dose-dependent gene expressions were measured 1 day after the injection, and the time-dependent luciferase expression was examined for

1 week with the polyplexes prepared at DNA dose of 15 μg and an N/P ratio of 7.0 in 100 μl of PBS. The mice were killed at a predetermined time. Lungs were homogenized, and the luciferase RLU in the tissue protein was measured using a 96-well plate luminometer as previously described. The harvested lungs 1, 3, and 7 days after the injection were instilled with 4% buffered formaldehyde solution for a histology examination.

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