AT2R Gene Delivered by Condensed Polylysine Complexes Attenuates Lewis Lung Carcinoma after Intravenous Injection or Intratracheal Spray

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Abstract

Transfection efficiency and toxicity concerns remain a challenge for gene therapy. Cell-penetrating peptides (CPP) have been broadly investigated to improve the transfection of genetic material (e.g., pDNA and siRNA). Here, a synthetic CPP (polylysine, K9 peptide) was complexed with angiotensin II type 2 receptor (AT2R) plasmid DNA (pAT2R) and complexes were condensed using calcium chloride. The resulting complexes were small (~150 nm) and showed high levels of gene expression in vitro and in vivo. This simple nonviral formulation approach showed negligible cytotoxicity in four different human cell lines (cervix, breast, kidney, and lung cell lines) and one mouse cell line (a lung cancer cell line). In addition, this K9-pDNA-Ca2+ complex demonstrated cancer-targeted gene delivery when administered via intravenous injection or intratracheal spray. The transfection efficiency was evaluated in Lewis lung carcinoma (LLC) cell lines cultured in vitro and in orthotopic cancer grafts in syngeneic mice. Immunohistochemical analysis confirmed that the complex effectively delivered pAT2R to the cancer cells, where it was expressed mainly in cancer cells along with bronchial epithelial cells. A single administration of these complexes markedly attenuated lung cancer growth, offering preclinical proof-of-concept for a novel nonviral gene delivery method exhibiting effective lung tumor gene therapy via either intravenous or intratracheal administration. Mol Cancer Ther; 15(1); 209–18. ©2015 AACR.

Introduction

Lung cancer is the third most prevalent form of cancer behind prostate and breast cancer; however, treatment options are lagging (1, 2). Even though progress has been made in early detection and prevention, mortality, and morbidity associated with lung cancer is still unacceptably high (3). In 2015, the American Cancer Society reported >430,000 people in the United States are living with lung cancer yielding the largest annual financial burden of any cancer type (1). Although incremental improvements have been made in the palliative care, currently available therapies have had minimal impact in reducing deaths (3). In particular, the need for completely new therapeutics to treat the most aggressive lung cancers is especially great.

Gene therapy is experiencing a renaissance in the United States and around the globe. The European Union has recently approved the first gene therapy drug, Glybera, for the treatment of lipoprotein lipase deficiency (LPLD). Promising late-stage clinical trials suggest the first gene therapy may be approved in the United States in 2016. Most gene therapy clinical trials (64.4%) have aimed at treating cancer, and yet cancer gene therapies have been slow to advance (4, 5). Theoretical, and practical limitations still exist for gene therapy application to lung cancer. Biologic barriers and toxicity continue to confound lung cancer treatment, even though the lungs may be accessed via inhalation or intravenous administration.

Gene vectors (viral and non-viral vectors) have steadily improved over the past 20 years. Viral vectors (e.g., adenoviral) are highly efficient, and facilitate strong transgene expression in different tumor tissues (6, 7). While these vectors are the most effective vectors applied in 70% of clinical trials, they continue to suffer from safety concerns (e.g., immunogenicity and pathogenicity; refs. 8–11), rapid clearance from circulation, and production problems (11–14). For these reasons, nonviral vectors could be more promising gene carriers due to easy synthesis, low cost, and decreased immunogenicity compared with viral vectors (10, 14–18). These attributes suggest nonviral vectors could offer a safer approach for repeated dosing regimens when treating primary as well as recurrent cancers.

Nucleic acids complexed with cationic polymers (polyplexes) or cationic lipids (lipoplexes) are the most commonly used nonviral synthetic gene carriers (16, 19–21). Cationic polymers interact with cell membrane or extracellular components (e.g., glycosaminoglycans) via the positive charge of the amino acid residues (e.g., lysine and arginine; ref. 22). The molecular weight...
and charge of polycations play important roles in complexing nucleic acids for delivering genetic materials (18). High molecular weight polycations often condense the genetic material [e.g., plasmid DNA (pDNA)] into small and stable complexes. On the other hand, low molecular weight polycations often produce large and unstable complexes (18, 23, 24). Unfortunately, low molecular weight polycations have historically exhibited poor transfection efficiency while high molecular weight polycations have been plagued with cytotoxicity (18).

Polylysine was one of the first polycations used for gene delivery (25). Polylysine-pDNA complexes have traditionally required polylysine chains with more than 20 residues to efficiently complex DNA, but yielding modest transfection efficiency and concerns about cytotoxicity (14). Many attempts were made to increase transfection efficiency or reduce cytotoxicity by either chemically modifying polylysine (e.g., PEGylation) or by adding excipients (26). Most research efforts focused on improving tolerability of high molecular weight polylysine (10, 27, 28), but we show that short polylysines with much lower cytotoxicity can indeed condense pDNA into small complexes when calcium is added. Such particles offer an interesting opportunity for repeat dosing to treat lung cancer if efficient transfection can be balanced with low cytotoxicity.

Here, a nine amino acid polylysine (K9) was complexed with pDNA and condensed with calcium chloride. This simple formulation (the K9-pDNA-Ca2+ complex) was explored using four different human cell lines: (i) A549 (a lung cancer cell), (ii) HeLa (a cervix cancer cell), (iii) MDA-MB-231 (a breast cancer cell), (iv) HEK-293 (a virus-immortalized kidney cell) and one mouse cell line, LLC (a lung cancer cell) using a luciferase reported plasmid DNA (pLUC) to assess transfection efficiency. Angiotensin II type 2 receptor (AT2R) is known to stimulate apoptosis and inhibit cell proliferation in different cell lines such as endothelial cells, cardiomyocytes, neuronal cells, prostate cancer cells, and lung cancer cells (5, 16), therefore, AT2R plasmid DNA (pAT2R) was delivered to LLC tumor-bearing mice. These K9-pAT2R complexes were administered via intravenous injection and/or via intratracheal spray to determine lung cancer attenuation in LLC tumor-bearing mice.

Materials and Methods

Materials
pDNA encoding firefly luciferase (pLUC, pGL3) was obtained from Promega. Plasmid DNA (pDNA) encoding human AT2R (pAT2R, pCDNA3.1b) was obtained from the UMR cDNA Resource Center (University of Missouri, Rolla, MO). K9 peptide (KKKKKKKKK; Mw = 1170.65 Da; Purity > 95%) was purchased from Biomatik Corporation. Branched polyethyleneimine (PEI, 25 kDa), mouse serum albumin (MSA) and glucose were from Sigma-Aldrich. Calcium chloride dihydrate (CaCl2·2H2O) was obtained from Fisher Scientific. A549 (CCL-185), Lewis lung carcinoma (LLC; CRL-1642), and HeLa (CCL-2) cell line were obtained from ATCC. MDA-MB-231 and HEK-293 cell line were gifts from Dr. Nikki Cheng (University of Kansas Medical Center, KA).

Preparation of the K9–pDNA–Ca2+ complex
For the in vitro studies, the K9–pLUC–Ca2+ complex solution was prepared by adding 15 μL K9 peptide solution [polymer nitro to pLUC phosphate (N/P) ratio 10] to 10 μL pDNA [0.1 μg/μL in 1× Tris-acetate-EDTA (TAE) buffer], followed by fast pipetting for 20 seconds. Then, 15 μL calcium chloride solution (e.g., 19, 114, and 228 mmol/L) was added and mixed by fast pipetting. The K9–pDNA–Ca2+ complex solution was incubated at 4°C for 20 to 25 minutes and used for each experiment. For the intravenous administration of the K9–pAT2R–Ca2+ complex, 160 μL complex solution was mixed with 40 μL 1% mouse serum albumin (MSA; the final volume of the complex is 200 μL). 4 μg pAT2R, and 53 μg K9 peptide. For the intratracheal administration, 40 μL the K9-pAT2R-Ca2+ complex solution was mixed with 10 μL 10% glucose for the osmolality adjustment (the final volume of the complex is 50 μL; 1 mg pAT2R, and 13 μg K9 peptide).

Preparation of the PEI–pLUC complex
The PEI–pLUC complex solution was prepared by adding 15 μL PEI solution (N/P ratio 10) to 10 μL pLUC (0.1 μg/μL) followed by fast pipetting for 20 seconds. The PEI–pLUC complex solution was incubated at 4°C for 20 to 25 minutes and used for each experiment. The complex solution was prepared immediately before each experiment and used as a control for all in vitro study.

A agarose gel electrophoresis
The K9–pLUC–Ca2+ complex solution was mixed with 4 μL TAE buffer. Then, 4 μL SYBR Green 1 was mixed with the complex solution, followed by incubation at 4°C for 20 to 25 minutes. After adding 7 μL of 6× DNA Loading Dye, the mixture solutions were loaded onto a 1% agarose gel and electrophoresed for 30 minutes at 110 V.

Size and zeta potential
The particle size [effective diameter (nm)] of the K9–pLUC complex with or without calcium chloride was determined by dynamic light scattering (Brookhaven Instruments). The zeta potentials of the complexes were measured by Zeta PALs dynamic light scattering (Brookhaven Instrument). Particle size was measured after dispersing the complexes into nuclease-free water (NFW) or serum-free media (SFM). Zeta potential was measured after dispersing the complexes into 1 mmol/L potassium chloride solution.

Cell culture
A549 cell line were grown in F-12K Nutrient Mixture media (Mediatech, Inc.) supplemented with 10% (v/v) FBS (FBS; Hyclone) and 1% (v/v) penicillin/streptomycin (MB Biomedical, LLC). HeLa, MDA-MB-231, LLC, and HEK-293 cell lines were grown in DMEM invitrogen/Life Technologies supplemented with 10% FBS and 1% penicillin/streptomycin. These cell lines were incubated at 37°C in 5% CO2 humidified the air. Cell line was authenticated by short tandem repeat (STR) DNA profiling. The cells were maintained in low passage (<15) for this study.

Transfection efficiency of the K9–pDNA–Ca2+ complexes to cultured cells
A549, HeLa, MDA-MB-231, LLC, and HEK-293 cell (80,000 cells/well) were cultured in 96-well plates for 24 hours prior to the transfection. The cells were washed once with SFM and 100 μL transfection solution (a mixture of 20 μL of the K9–pLUC–Ca2+ complex and 80 μL of SFM. 0.5 μg pLUC/well) was added to each well. After 5 hours incubation, the transfection solution was replaced with 100 μL fresh growth medium. After 48-hour
incubation, total cellular protein was collected by using BCA Protein Assay Reagent (Thermo Fisher Scientific Inc.). The efficiency of the gene transfection by the complexes was determined by Luciferase Reporter Assay using Luciferase Assay System Freezer Pack (Promega). The Luciferase expression was measured by a microplate reader (SpectraMax; Molecular Devices Corp). The transfection efficiency was expressed as relative light units (RLU) per milligram (mg) of cellular protein.

Cytotoxicity of K9 peptide, PEI, and calcium chloride in vitro

Cytotoxicity of K9 peptide, PEI, and calcium chloride was determined using a CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (MTS) obtained from Promega. A549, HeLa, MDA-MB-231, LLC, and HEK-293 cells were cultured in a 96-well plate in growth media as described previously. Cells were treated with the samples (0–5 mg/mL as indicated in the Fig. 4) for approximately 24 hours. After 24 hours of incubation, the media were replaced with a mixture of 100 μL of fresh serum medium and 20 μL of MTS. Then, the plate was incubated for 3 hours in the incubator. To determine cell viability, the absorbance of each well was measured by a microplate reader (SpectraMax) at 490 nm and normalized to untreated control cells.

Assessment of DNA accessibility to the K9–pLUC complex by SYBR Green assay

The degree of pLUC accessibility in the K9–pLUC complex was assessed by the double stranded DNA binding reagent SYBR Green. Briefly, 10 μL pLUC (0.1 mg pDNA/mL) was mixed with 15 μL of K9 peptide (N/P ratio 10), then 15 μL deionized water or 38 mM/L calcium chloride solution was added. After a 30-minute incubation at room temperature, 120 μL PBS, and 160 μL 10× SYBR Green solutions (Invitrogen) were added. Then 100 μL sample was added to one well of a 96-well cell culture plate. The fluorescence was measured using a fluorescence plate reader (SpectraMax M5, excitation, 250 nm; emission, 520 nm).

Animals

Six-week-old wild-type male C57BL/6 mice were obtained from Charles River Laboratories International, Inc. All mice were housed in a clean facility and held for 10 days to acclimatize. All animal experiments were carried out under strict adherence to the Institutional Animal Care and Use Committee (IACUC) protocols and the Institutional Biosafety Committee set by Kansas State University (Manhattan, KS).

Lung cancer graft in syngeneic mouse and treatment with the K9–pAT2R–Ca2+ complex

Seven-week-old C57BL/6 mice were intravenously injected with 1.2 × 10^6 LLC cells suspended in 200 μL PBS via the tail vein using a 1 mL syringe with a 27G needle. The K9–pAT2R–Ca^2+ complex solution was prepared immediately before injection as described above. For the intravenous administration of the K9–pAT2R–Ca^2+ complex, 160 μL complex solution was mixed with 40 μL 1% MSA. For the intratracheal administration, 40 μL the K9–pAT2R–Ca^2+ complex solution was mixed with 10 μL 10% glucose for the osmolality adjustment. After 7 days of LLC injection, the mice were injected with 200 and 50 μL of the complexes with each composition via intravenous and intratracheal, respectively. PBS and K9–Ca^2+ solution without pAT2R were used as control. In addition, K9–pLUC–Ca^2+ complex prepared as described above was also injected into LLC tumor-bearing mice via intravenous and intratracheal as a nonspecific gene control. Mice were sacrificed by cervical dislocation under deep anesthesia at 14 days after the complex treatment. The lungs were dissected and tumor burden was analyzed.

Immunohistochemical analysis for AT2R, cell proliferation, and apoptosis in LLC allografts

Fixed lung tissues were sectioned at 4 μm and stained with hematoxylin and eosin (H&E) staining for histologic examination. To analyze AT2R expression and cell proliferation by Ki-67 in LLC tumors, sections were deparaffinized and heat-induced epitope unmasking was performed in the citrate buffer followed by incubation with 3% H2O2/methanol for 3 minutes to block endogenous peroxidase activity. Sections were incubated with polyclonal anti-AT2R (1:100 dilution, for 18 hours at 4°C, Abcam) and polyclonal anti-Ki-67 (1:500 dilution, for 18 hours at 4°C, Abcam) antibodies. After the incubation with primary antibodies, sections were incubated with biotin-conjugated anti-rabbit IgG antibody (Vector Laboratories) at a 1:100 dilution for 1 hour at 37°C, followed by reaction with the avidin–biotin–peroxidase complex reagent (Vector Laboratories) for 40 minutes at 37°C. Reactions were developed with 3′,3′-diaminobenzidine tetrahydrochloride (Sigma) and counterstained lightly with Mayer hematoxylin. The cell proliferation...
According to the manufacturer’s instructions with a slight modification using the DeadEnd Colorimetric TUNEL System (Promega), apoptotic cells in LLC tumors were detected by a TUNEL assay by using the DeadEnd Colorimetric TUNEL System (Promega), according to the manufacturer’s instructions with a slight modification (29). The apoptotic index was assessed as the fold change of average number of positive cells in three randomly selected fields in the tumor nodules.

Statistical analysis
Data were analyzed by using GraphPad software. All values were expressed as the mean ± SEM. All experiments were conducted with multiple sample determinations. A statistical evaluation comparing the significance of the difference in gene expression (RLUs/mg protein) between the means of two datasets was performed using a t test. One-way ANOVA, Tukey post test was used to analyze the differences when more than two datasets were compared.

Results
Formation of the K9–pLUC–Ca^{2+} complex
The K9–pDNA–Ca^{2+} and PEI–pDNA complexes were prepared by mixing pLUC or pAT2R with K9 peptide at various N/P ratios as described in the Materials and Methods section. To demonstrate complex formation, agarose gel electrophoresis was performed using 1% agarose gel, and electrophoresed for 30 minutes. Uncomplexed pLUC (naked pLUC) was used as a control. The K9 peptide and pLUC mixture showed ability to form complexes of pLUC and K9 peptide regardless of the presence of 38 mmol/L calcium chloride at N/P ratios 5, 10, and 30. As the net charges of these complexes are positive, the complexes stayed in the loading wells without migrating into the gel and no bands were observed in the electrophoresis (Fig. 1A). Although lower N/P ratios (1–4) also showed no bands in the absence of calcium chloride, the N/P ratios lower than 0.5 showed bands (Fig. 1B). In addition, mixing with calcium chloride and pLUC did not form stable complexes (Fig. 1C).

Physical characterization of the K9–pLUC–Ca^{2+} and the PEI–pLUC complexes
The effect of calcium chloride concentration on the surface charge and particle size of the K9–pLUC complex was investigated. As shown in Fig. 2A, addition of calcium chloride of 37.7 and 113 mmol/L (final concentration) significantly decreased the particle size of the K9–pLUC–Ca^{2+} complex, with relatively narrow polydispersity (~0.1), in both NFW and in SFM. The zeta potential of the K9–pLUC–Ca^{2+} and PEI–pLUC complexes increased significantly with increases in the concentration of calcium chloride (Fig. 2B).

Figure 2. Evaluation of the particle sizes (effective diameters), zeta potentials, and SYBR Green assay of the K9–pLUC and PEI–pLUC complexes with or without calcium chloride. A, the particle size of the K9–pLUC complexes (N/P ratio = 10) were determined by DLS in the presence of various concentrations of calcium chloride (0, 38, and 114 mmol/L) in NFW or SFM. B, zeta potentials of the K9–pLUC and PEI–pLUC complexes (N/P ratio = 10) were determined by Zeta PALS dynamic light scattering in the presence of various concentrations of calcium chloride (0, 38, and 114 mmol/L). C, effect of calcium chloride concentration at 0 and 38 mmol/L on pLUC accessibility in K9–pLUC and PEI–pLUC complexes at an N/P ratio of 10 was evaluated using the SYBR Green assay. The pLUC alone in the solution was used as a control. Results are presented as mean ± SD (n = 3).*** P < 0.0001, one-way ANOVA, Tukey post test comparison to pDNA.
The K9–pLUC–Ca^{2+} complex caused efficient gene transfection with low cytotoxicity in vitro

The in vitro transfection efficiency of the K9–pLUC–Ca^{2+} and the PEI–pLUC complexes was studied using the four different human cell lines and the mouse cell line. Luciferase gene expression was evaluated 48 hours after the transfection using the K9–pLUC–Ca^{2+} and the PEI–pLUC complexes at N/P ratios 5, 10, 20, and 30, and at various calcium chloride concentrations during the complex formulation (Fig. 3A). The K9–pLUC–Ca^{2+} complexes had a high level of gene expression at a calcium chloride concentration of 38 mmol/L (N/P ratio 10) in A549 cells. Luciferase gene expression was decreased when calcium concentration was higher than 114 mmol/L in all N/P ratios tested. This result was further confirmed in other cell lines: A549 cells (Fig. 3B), Hela cells (Fig. 3C), MDA-MB-231 cells (Fig. 3D), HEK-293 cells (Fig. 3E), and LLC cells (Fig. 3F). In all cell lines examined, the transfection efficiency of the K9–pLUC–Ca^{2+} with 38 mmol/L calcium chloride was significantly higher than those with PEI–pLUC and the K9–pLUC–Ca^{2+} with 114 mmol/L calcium chloride (Fig. 3). Significance of including K9 peptide in pDNA transfection was further examined by comparing pLUC expression efficiency in the presence or absence of the peptide in various cell lines (Supplementary Fig. S1). The transfection efficiency of the pLUC–Ca^{2+} complex (without K9 peptide) was significantly lower than that of the K9–pLUC–Ca^{2+} complex at the same calcium chloride concentration.

High transfection efficiency and low cytotoxicity are vital attributes for nonviral gene vectors. To examine whether K9, PEI, and calcium chloride affected the viability of live cells, a membrane translocalization signal (MTS) cytotoxicity assay was conducted using five different cell lines. The five cell lines were individually incubated with up to 5 mg/mL K9, PEI, or calcium chloride for 24 hours and then MTS assay was performed (Fig. 4). The K9 peptide showed no cytotoxicity to 2.5 mg/mL. Only HEK-293 cell viability was decreased at 1 mg/mL levels of K9 peptide. Calcium chloride also did not show strong cytotoxicity until 1 mg/mL. However, PEI induced significant cytotoxicity even at 10 μg/mL in the four cell lines. Although HEK-293 cells were resistant to PEI-induced cytotoxicity, their cell viability was gradually decreased at higher concentrations. This MTS assay strongly suggested that the K9–pDNA–Ca^{2+} complex is a low cytotoxic pDNA transfection vector.

Treatment with the K9–pAT2R–Ca^{2+} complex via intravenous injection or intratracheal spray caused significant growth attenuation of lung tumors

The effect of the K9–pAT2R–Ca^{2+} complex delivering the endogenous apoptosis-inducer gene AT2R was examined using orthotopic LLC allografts in syngeneic C57BL/6 mice. To evaluate the effect of the complex on the lung tumor growth, LLC cells (1.2 × 10^6) were injected via the tail vein. Seven days after cancer cell injection, the mice were treated with a single dose of the K9–pAT2R–Ca^{2+} complexes containing 4 μg pAT2R intravenously or 1 μg pAT2R intratracheally. The tumor growth attenuating the effect of these complexes was observed both macroscopically (Fig. 5A and C) and microscopically (Fig. 5B). Macroscopically, a large number and large size of tumor nodules were detected in PBS- or K9-treated mouse lungs. Average lung weights (mg) of the K9–pAT2R–Ca^{2+} intratracheal (190.6 ± 48.3) and the K9–pAT2R–Ca^{2+} intravenous (201.6 ± 67.0) treated groups were significantly smaller than that of the control PBS group (325.7 ± 69.4, P < 0.05, Fig. 5C and Supplementary Table S1). Histologic examination of tumors in H&E-stained lung sections also displayed only a small number and small size of LLC tumor nodules in mouse lungs treated with the K9–pAT2R–Ca^{2+} complexes (Fig. 5B). Average numbers of tumor nodules in the lungs in the intratracheal (6.0 ± 4.2) and the intravenously (4.6 ± 3.4) groups were significantly smaller than that of the control PBS group (17.8 ± 6.0, P < 0.01, Fig. 5D; Supplementary Table S2). On the other hand, treatment with K9–pLUC–Ca^{2+} complexes did not show any effect on average lung weight (246.8 ± 70.3 in PBS, 213.9 ± 52.3 in K9–pLUC–Ca^{2+} intravenous, and 267.3 ± 6.9 in K9–pLUC–Ca^{2+} intratracheal) and average numbers of tumor nodules (20.4 ± 4.0, 20.2 ± 11.9, and 25.7 ± 1.5 in the same group order as the lung weights described above) in LLC tumor-bearing mouse lung (Supplementary Fig. S2). Both local (i.e., pulmonary) and systemic treatment with the K9–pAT2R–Ca^{2+} complexes were equally effective in attenuating the growth of LLC lung tumor grafts in immunocompetent mice.

Immunohistochemical analysis of AT2R expression, cell proliferation, and apoptosis in LLC grafts

The expression of AT2R gene in the lung was determined immunohistochemically (Fig. 6). As shown in Fig. 6A, AT2R expression was upregulated in the tumor cells in the K9–pAT2R–Ca^{2+} intravenous and intratracheal spray groups but not in other groups. These observations suggested that the K9–pAT2R–Ca^{2+} administration via intravenous and intratracheal significantly attenuated the lung tumor growth by expressing AT2R in the tumor cells. Immunohistochemical analysis of cell proliferation in tumors using anti-Ki-67 antibody did not show any difference (Fig. 6B). Although average number of apoptotic cells detected by TUNEL assay tended to increase in the K9–pAT2R–Ca^{2+} samples. As the analysis was carried out 14 days after treatment with the K9–pAT2R–Ca^{2+} complex, these results may suggest that the treatment with the K9–pAT2R–Ca^{2+} complex attenuated tumor growth during an early stage of LLC tumorigenesis. Nevertheless, these results support that both intravenous and intratracheal administration of the K9–pAT2R–Ca^{2+} offer effective modalities for lung cancer targeted nonviral gene therapy.

Discussion

Development in the field of gene therapy is presently hindered by the lack of robust gene delivery methods. Synthetic, nonviral gene vectors such as polyacationic peptides (e.g., polylysine) are promising vectors (10, 17, 18). The toxicity of these CPPs may be minimized or eliminated by reducing their molecular size. However, the level of transfection efficiency mediated by smaller polypeptides is typically low compared with larger polypeptides (17). CPPs have been used to deliver various anticancer agents (e.g., small molecules, proteins, and nucleic acids) into cells in vivo and have been observed to be effective in inhibiting tumor growth in preclinical tumor models (17, 30, 31). The main goals of this work were to examine the transfection efficiency of the K9–pDNA–Ca^{2+} complex and to determine whether pDNA (pAT2R) can be distributed to lung cancer cells, resulting in therapeutically effective gene expression.

The in vitro transfection efficiency of the K9–pDNA–Ca^{2+} complex was evaluated using luciferase pDNA (pLUC) in the
Figure 3.
The transfection efficiencies of the K9–pLUC complex. A, the transfection efficiencies in various concentrations of calcium chloride (0, 19, 38, 114, and 228 mmol/L) in A549 cells at N/P ratios of 5, 10, 20, and 30. The transfection efficiencies of the K9–pLUC complexes with different concentrations of added calcium chloride (0, 38, and 114 mmol/L) at an N/P ratio 10 in A549 cells (B), HeLa cells (C), MDA-MB-231 cells (D), HEK-293 cells (E), and LLC cells (F). RLUs, relative light units. Results are presented as mean ± SD (n = 4). ***, P < 0.001; **, P < 0.01; one-way ANOVA; Tukey post test.
Ca²⁺ are presented as mean determined by MTS assay. Results cells (E). Cell viability was cells (C), HEK-293 cells (D), and LLC HeLa cells (B), MDA-MB-231 calcium chloride in A549 cells (A), or other type of CPP

Four different human cell lines (kidney, cervix, breast, and lung) and one mouse cancer cell line (lung). Over expression of the AT2R gene in lung cancer cells (32), or other type of CPP–pDNA–Ca²⁺ complex-based intraperitoneal delivery of the AT2R gene was known to reduce lung cancer cell growth (16). Intravenous injection or intratracheal spray of the K9–pAT2R–Ca²⁺ complexes yielded robust gene expression, primarily in lung cancer cells, and significantly attenuated cancer growth. Therefore, this study presents an effective in vitro and in vivo gene delivery system using a low molecular weight cationic CPP (K9 peptide) for lung cancer therapy.

The formation of complexes between K9 and pDNA was observed in both the K9–pLUC–Ca²⁺ and K9–pLUC complexes as observed via agarose gel electrophoresis (Fig. 1) when the N/P ratio is higher than 0.5. By itself, calcium chloride showed negligible ability to complex naked pLUC even at a high concentration (114 mmol/L; Fig. 1C). However, the K9–pLUC complex, without calcium chloride exhibited very low gene expression (Fig. 3A), and the size of this complex was inappropriately large (500–1,500 nm) for gene delivery (Fig. 2A). The addition of calcium chloride in the K9–pLUC complex significantly decreased the complex size in water and culture medium (Fig. 2A) and correspondingly increased gene transfection (Fig. 3A). Therefore, calcium chloride acted as an effective condensing agent to decrease the particle size of the K9–pLUC complex and enhance transfection efficiency. These observations are in good agreement with our previous study in which calcium chloride also decreased particle sizes of CPP–pLUC complexes with other types of CPP (33). In this regard, it is of interest to note that the PEI–pLUC complex did not show a decrease in particle sizes as calcium chloride increased (17). Calcium ion–dependent increase of the total positive charge of the K9–pLUC–Ca²⁺ complex may also play an important role enhancing transfection efficiency by the stronger interaction with the negatively charged plasma membrane (34). In addition, accessibility of SYBR green to the pLUC in the K9–pLUC complex was significantly decreased when calcium chloride (38 mmol/L) was added to the complex solution (Fig. 2C), suggesting the K9–pLUC complex was effectively condensed, and calcium chloride played an essential role in condensation of the complex. The reduction in the particle size of the K9–pDNA–Ca²⁺ complexes likely led to an increase in transfection efficiency.

The effect of calcium chloride on the transfection efficiency of the K9–pDNA complex was first assessed using A549 cells. The best transfection efficiency was achieved at 38 to 76 mmol/L calcium chloride (Fig. 3A). Interestingly, no significant level of gene expression was detected without calcium chloride. As calcium chloride appeared to be an essential component in the condensation of the K9–pLUC complex, yielding small

Figure 4. Cytotoxicity profiles of K9, PEI, and calcium chloride in A549 cells (A), HeLa cells (B), MDA-MB-231 cells (C), HEK-293 cells (D), and LLC cells (E). Cell viability was determined by MTS assay. Results are presented as mean ± SD (n = 3).
compared with the level of PBS control (4). In addition, the low toxicity of the K9 concentrations in all cell lines except for HEK-293 cells (Fig. where PEI exhibited strong cytotoxicity at low micromolar cytotoxicity the host cells. The present study clearly indicated negligible material to the target cells without in (17, 18, 33).

A successful gene vector should be able to deliver genetic material to the target cells without influencing the viability of the host cells. The present study clearly indicated negligible cytotoxicity in vitro up to 2.5 mg/mL for K9 peptide and 1 mg/ mL for calcium chloride after 24 hours for all five cell lines, whereas PEI exhibited strong cytotoxicity at low micromolar concentrations in all cell lines except for HEK-293 cells (Fig. 4). In addition, the low toxicity of the K9–pAT2R–Ca\(^{2+}\) complexes was also observed in the mouse study after intravenous and intratracheal applications, in which all mice receiving K9 alone or the K9–pAT2R–Ca\(^{2+}\) complex survived during the experimental period and did not show acute inflammatory reaction or any histologically detectable abnormality. Therefore, data strongly suggested that the K9–pAT2R–Ca\(^{2+}\) complex represents a safe and efficient gene transfection vector.

Figure 5. Macroscopic analysis of LLC tumors in C57BL/6 mouse lungs (A) and microscopic views (B) of the lung from PBS, K9 alone intratracheal (IT), K9 alone intravenous (IV), K9–pAT2R–Ca\(^{2+}\) intratracheal (complex IT), or K9–pAT2R–Ca\(^{2+}\) intravenous (complex IV)-treated mice (n = 5). Average lung weight (C) and number of tumor nodules (D) in each treatment group were expressed in the bar graphs. Scale bars, 500 μm (B). * P < 0.05; ** P < 0.01 as compared with the level of PBS control (n = 5, one-way ANOVA Tukey post test). K9, nine amino acid polylysine.
The addition of calcium chloride to nascent complexes of polylysine CPP (K9 peptide) and pDNA (pLUC and pAT2R) produced small and stable complexes. These complexes exhibited high gene expression in various human and mouse cancer cells comparable with PEI–pDNA complexes. Single intravenous or intratracheal administrations of the K9–pAT2R–Ca\(^{2+}\) complexes significantly attenuated the growth of LLC cell allografts in mouse lungs, suggesting that K9 peptide–based gene therapy is effective and that the AT2R gene is potentially useful for lung cancer gene therapy. K9 peptide showed negligible cytotoxicity in cell culture, supporting the notion that this CPP is a safe delivery vehicle for genetic materials (e.g., siRNA and pDNA). Although, further studies are required to confirm the in vivo safety of the K9–pAT2R–Ca\(^{2+}\) complexes by formal pharmacokinetics, pharmacodynamics, and multispecies toxicity studies, these data showed that the K9–pDNA–Ca\(^{2+}\) complexes could be an effective and safe nonviral gene transfection tool.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: N.A. Alhakamy, S. Ishiguro, C.J. Berkland, M. Tamura
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