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Self-assemble gene delivery system for molecular targeting using nucleic acid aptamer.

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Abstract

We have developed a novel vector constructed with pDNA, polyethylenimine (PEI), and mucin 1 (MUC1) aptamer for tumor-targeted gene delivery. The MUC1 aptamer and non-specific aptamer were employed to coat the pDNA/PEI complexes electrostatically and stable nanoparticles were formed. The addition of a non-specific aptamer to the pDNA/PEI complex decreased gene expression in the human lung cancer cell line, A549 cells expressing MUC1 regularly. At the same time, the pDNA/PEI/MUC1 aptamer complex showed higher gene expression than pDNA/PEI/non-specific aptamer complex. Furthermore, the pDNA/PEI/MUC1 aptamer complex showed markedly high gene expression in tumor-bearing mice; thus, pDNA/PEI/MUC1 aptamer complexes are useful as a tumor-targeted gene delivery system with high transfection efficiency.

Keywords: aptamer; gene delivery; polyethylenimine; pDNA; MUC1

1. Introduction

In the field of gene delivery, polyethylenimine (PEI) is a common cationic polymer with high transfection efficiency under *in vitro* and *in viv*o conditions among non-viral vectors (Boussif, et al., 1995; Godbey, et al., 1999; Kircheis, et al., 2001; Kichler, et al., 2001; Itaka, et al., 2004). The pDNA/PEI complex, however, binds non-specifically to negatively charged proteoglycans on cell membranes and it is difficult to use the cationic pDNA/PEI complex as the targeted gene delivery system (Lemkine and Demeneix, 2001; Demeneix and Behr, 2005). For cell-specific gene delivery, cell type-specific surface molecules and their antibodies would be useful, and a number of cell-specific gene delivery vectors have been developed (Germershaus, et al., 2006; Chiu, et al., 2004; Liu, et al., 2007). For cancer gene therapy, tumor suppressor genes such as p53 gene and suicide genes such as herpes simplex virus thymidine kinase gene were well studied; and those genes reported to suppress tumor growth by tumor specific gene delivery vector such as transferrin-modified cationic liposome (Nakase, et al., 2005; Neves, 2006).

Aptamers are a family of RNA- or DNA-based oligonucleotides 20-50 nt in length that can specifically bind to selected targets (Guo, et al., 2010). They have often been described as nucleic acid versions of antibodies. However, aptamers unlike antibodies, have yet to elicit immunogenicity *in vivo* and these molecules are readily amenable to chemical synthesis and can be modified easily during the synthesis process, making them more adaptable for

different applications (Eyetech Study Group, 2003; Chan, et al., 2008; Alexander, et al., 2005; Gilbert, et al., 2007; Apte, et al., 2007).

In a previous study, we developed some ternary complexes of pDNA/PEI coated with polynucleotides such as polyadenylic acid (polyA) and polyinosinic-polycytidylic acid (polyIC) (Kurosaki, et al., 2009). It was demonstrated that aptamers could coat pDNA/PEI complexes and the aptamer-coated complex would be taken up by the cells via aptamer-based molecular recognitions. The mucin 1 (MUC1) is unique to tumor cells and overexpressed in most of invasive breast carcinomas, indicating that MUC1 aptamer would be suitable for tumor targeting (Rahn, et al., 2001).

In this experiment, we constructed pDNA/PEI/aptamer complexes with MUC1 aptamer for tumor-targeted gene delivery systems. Then, the pDNA/PEI/MUC1 aptamer complex showed high gene expressions of the plasmid-borne luciferase gene in A549 cells expressing MUC1 regularly under *in vitro* and *in vivo* conditions.

2. Materials and Methods

2.1. Chemicals

PEI (branched form, average molecular weight of 25,000) was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Fetal bovine serum (FBS) was obtained from Biosource International Inc. (Camarillo, CA, USA). RPMI 1640, Opti-MEM I, antibiotics (penicillin 100 U/mL and streptomycin 100 μg/mL), and other culture reagents were obtained from GIBCO BRL (Grand Island, NY, USA). MUC1 aptamer (5'-GAAGTGAAAATGACAGAACACAACA-3') and a non-specific 25 base-long DNA aptamer composed of GATC repeats, according to a previous report (Ferreira, et al., 2009), were purchased from Hokkaido System Science Co., Ltd. (Hokkaido, Japan). All other chemicals were of the highest purity available.

2.2. Preparation of pDNA and Complexes

pCMV-Luc was constructed by subcloning the *Hind* III/*Xba* I firefly luciferase cDNA fragment from the pGL3-control vector (Promega, Madison, WI, USA) into the polylinker of the pcDNA3 vector (Invitrogen, Carlsbad, CA, USA). The pDNA was amplified using an EndoFree® Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). The pDNA was dissolved in 5% dextrose solution (1 mg/mL) and stored at -80 ^{o}C until analysis.

For preparation of the complexes, pDNA solution and PEI solution (pH 7.4 in 5%

dextrose solution) were thoroughly mixed by rapid sequential pipetting and left for 15 min at room temperature, and then each aptamer in 5% dextrose solution was mixed with pDNA/PEI complex by pipetting and left for another 15 min at room temperature. In this study, we constructed various complexes at weight ratios: pDNA: PEI : aptamer = 1:1:0, 1:1:0.25, 1:1:0.5, 1:1:1, 1:1:2, 1:1:4, and 1:1:6.

2.3. Physicochemical Property of the Complexes

The particle sizes and ζ-potentials of the pDNA/PEI/aptamer complexes were measured using a Zetasizer Nano ZS (Malvern Instruments, Ltd., United Kingdom). The number-fractioned mean diameter is shown.

To determine complex formations, 10 μL aliquots of complex solution containing 1 μg pDNA were mixed with 2 μL loading buffer (30% glycerol and 0.2% bromophenol blue) and loaded onto a 0.8% agarose gel containing 0.03% ethidium bromide. For the assessment of complex dissociation, 3 μg of heparin was added to pDNA/PEI complex. Electrophoresis (i-Mupid J®; Cosmo Bio, Tokyo, Japan) was carried out at 35 V in running buffer solution (40 mM Tris/HCl, 40 mM acetic acid, and 1 mM EDTA) for 80 min. The retardation of pDNA was visualized using a FluorChem Imaging Systems (Alpha Innotech, CA, USA).

2.4. In Vitro Transfection Experiments

The human lung cancer cell line, A549 cells, was obtained from the Cell Resource Center

for Biomedical Research (Tohoku University, Japan). A549 cells were maintained in RPMI 1640 supplemented with 10% FBS and antibiotics (culture medium) under a humidified atmosphere of 5% CO_2 in air at 37 ^{o}C. A549 cells were plated on 24-well plates (Becton-Dickinson, Franklin Lakes, NJ, USA) at a density of 2.0×10^4 cells/well and cultivated in 0.5 mL culture medium. In the transfection experiment, after 24 h pre-incubation, the medium was replaced with 0.5 mL Opti-MEM I medium and each complex containing 1 μg pDNA was added to the cells and incubated for 2 h. After transfection, the medium was replaced with culture medium and cells were cultured for a further 22 h at 37 ^{o}C. After 22 h incubation, the cells were washed with PBS and then lysed in 100 μL lysis buffer (pH 7.8 and 0.1 M Tris/HCl buffer containing 0.05% Triton X-100 and 2 mM EDTA). Ten microliters of lysate samples were mixed with 50 μL luciferase assay buffer (Picagene; Toyo Ink, Tokyo, Japan) and the light produced was immediately measured using a luminometer (Lumat LB 9507; EG & G Berthold, Bad Wildbad, Germany). The protein content of the lysate was determined by a Bradford assay using BSA as the standard. Absorbance was measured using a microplate reader at 570 nm. Luciferase activity was indicated as relative light units (RLU) per mg protein.

2.5. In Vivo Transfection Experiments

Animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Nagasaki University with approval from the

Institutional Animal Care and Use Committee. Male KSN mice (5 weeks old) were purchased from Japan SLC (Shizuoka, Japan). After shipping, mice were acclimatized to the environment for at least one week before the experiments. The mice received 1.0×10^6 cells/mouse of A549 cells intradermally. One month after administration when the average tumor weight was approximately 500 mm^3, the mice were injected intratumorally with various complexes containing 20 μg pDNA at a volume of 100 μL per tumor. At 24 h following injection, the mice were sacrificed and the tumors were dissected and homogenized in lysis buffer. The homogenates were centrifuged at 15000 rpm (Kubota 3700; Kubota, Tokyo, Japan) for 5 min. The supernatants were used for luciferase assays, as described above. Luciferase activity was indicated as RLU per gram of tissue.

2.6. Statistical Analysis

Multiple comparisons among groups were made by Sheffe's test. $P < 0.05$ indicated significance.

3. Results

3.1. Physicochemical Characteristics

The size and ζ-potential of the complexes were determined (Fig. 1). The ζ-potential means surface charge of the particles; + means cationic surface charge and – means anionic surface charge. The pDNA/PEI complex was about 52.9 nm particle size with +56 mV ζ-potential. By the addition of aptamers, the ζ-potential was concentration-dependently decreased and reached a plateau at a weight ratio 1:1:6 of pDNA: PEI: aptamer. Addition of aptamers aggregated the pDNA/PEI complex at weight ratio, 1:1:1. Furthermore, MUC1 aptamer increased size of the complex at weight ratio 1:1:2. Conversely, the complexes with other weight ratios had almost 48-75 nm particle size.

Complex formations were examined by a gel retardation assay (Fig. 2). In the lane of naked pDNA, bands of pDNA were detected on agarose gel. On the other hand, in the lanes of pDNA/PEI complex and pDNA/PEI/aptamer complexes, no band was detected. Furthermore, the addition of heparin to pDNA/PEI complex released pDNA from the complex and bands of pDNA were detected clearly.

3.2. In vitro transfection efficiencies

A human lung adenocarcinoma epithelial cell line, A549 cells, regularly expressing MUC1, was transfected with various complexes and gene expression of luciferase carried by

the plasmid vector was evaluated (Fig. 3). The pDNA/PEI complex showed 6.33×10^9 RLU/mg protein in transgene efficiency. In contrast, the high transgene efficiency of pDNA/PEI complexes was significantly decreased in a concentration-dependent manner by the addition of non-specific aptamer ($P < 0.01$). The pDNA/PEI/MUC1 aptamer complexes showed significantly higher gene expressions than pDNA/PEI complex at weight ratios 1:1:0.25, 1:1:0.5, and 1:1:1 ($P < 0.01$) and the gene expressions of pDNA/PEI/MUC1 aptamer complexes were higher than the pDNA/PEI/non-specific aptamer complexes at all weight ratios. The highest gene expression was observed at a weight ratio 1:1:0.5.

3.3. In vivo transfection efficiencies

A549 cells were administrated to mice and tumor-bearing model mice were developed. The pDNA/PEI complex and pDNA/PEI/aptamer complexes at a weight ratio 1:1:0.5 were injected into the tumor. Twenty-four hours after injection, the transgene efficiencies in the tumor were evaluated, as shown in Fig. 4. A high gene expression was observed in mice treated with pDNA/PEI/MUC1 aptamer complexes and was significantly higher than the gene expressions of pDNA/PEI complex and pDNA/PEI/non-specific aptamer complex-treated mice ($P < 0.05$ and $P < 0.01$, respectively).

4. Discussion

MUC1 is a membrane-tethered mucin expressed on the surface of epithelial cells lining mucosal surfaces (Kim and Lillehoj, 2008). Overexpression of MUC1 by most carcinomas and in particular by breast cancers has been reported and correlates with high metastatic potential and poor survival (Gendler, 2001). An underglycosylated form of MUC1 that is unique to tumor cells is overexpressed in virtually all invasive breast carcinomas, making MUC1 a prime candidate for several promising therapeutic vaccine strategies and a potential marker for prognosis (Rahn, et al., 2001).

In previous reports, MUC1 antibody was developed and used in tumor-specific drug delivery systems (Singh and Bandyopadhyay, 2007; Moase, et al., 2011). Recently, MUC1 aptamers were also developed and reported to show high selectivity (Ferreira, et al., 2009; Savla, et al., in press); however, there are few reports about gene delivery systems using MUC1 aptamer. In the previous study, we developed some ternary complexes of pDNA/PEI coated by polynucleic acid, such as polyadenylic acid (polyA) and polyinosinic-polycytidylic acid (polyIC) (Kurosaki, et al., 2009). We also successfully constructed pDNA/PEI/MUC1 aptamer complexes (Japanese Patent Application No. 2010-043186). In this experiment, we investigated the utility of the pDNA/PEI/MUC1 aptamer complex for tumor-targeting gene delivery systems.

The pDNA/PEI/MUC1 aptamer complexes showed high gene expression in MUC1

regularly expressing A549 cells at weight ratios of 1:1:0.25, 1:1:0.5, 1:1:1, and 1:1:2. The high gene expression of the pDNA/PEI/MUC1 aptamer complexes could be explained by the specific molecular recognition of MUC1 aptamer on the complex surface. In fact, the non-specific aptamer inhibited the transgene expressions of pDNA/PEI complex concentration-dependently. A large amount of MUC1 aptamer decreased transgene expression of pDNA/PEI/MUC1 aptamer complex with weight ratios 1:1:4 and 1:1:6. Strong anionic surface charges may repulse the cellular membrane and the rebound might be larger than the binding strength of MUC1 aptamer to MUC1.

Aptamers on the complex surface were also anticipated from the physicochemical properties of the complexes. The addition of aptamer did not disrupt the structure of pDNA/PEI complex, as shown in Fig. 2. However, the aptamer reversed the charge from positive to negative indicating a concentrated distribution of aptamer outside of the particles. MUC1 aptamer increased size of the pDNA/PEI complex at weight ratio, 1:1:2, even if non-specific aptamer did not increase at same weight ratio. It may be caused by the steric structure of MUC1 aptamer.

The pDNA/PEI/MUC1 aptamer complex with a weight ratio of 1:1:0.5, which showed highest gene expressions *in vitro*, was administered to tumor-bearing mice and gene expressions *in vivo* were evaluated. The pDNA/PEI/MUC1 aptamer complexes showed significantly higher gene expressions in the tumor than pDNA/PEI complex and pDNA/PEI/non-specific aptamer complex after intratumoral injection ($P < 0.05$ and $P < 0.01$,

respectively). The results indicate that the pDNA/PEI/MUC1 aptamer complex may be useful for tumor-targeted gene delivery systems.

For the tumor therapy, most current therapies are non-specific, with surgery, radiation, and chemical ablation having the potential to cause damage to surrounding tissue. Similarly non-specific transfection should cause unexpected critical side effects. In this experiment, we constructed pDNA/PEI/MUC1 aptamer complex for tumor specific gene delivery. Furthermore, this pDNA/PEI/aptamer complex could easily change targeting cells by selection of aptamer and could achieve cell-specific gene transfer. The coating and targeting system may be a novel platform technology for targeted gene delivery.

5. Conclusion

In this experiment, we constructed a novel tumor-targeted gene delivery vector, pDNA/PEI/MUC1 aptamer complex. MUC1 aptamer could coat cationic pDNA/PEI complex, electorostatically. The pDNA/PEI/MUC1 aptamer complexes showed higher gene expressions than pDNA/PEI/non-specific aptamer complexes on the A549 cells which are expressing MUC1 regularly in *in vitro* and *in vivo* conditions. The coating system could be applied to other aptamers and may be a novel platform technology for targeted gene delivery.

6. Acknowledgements

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7. References

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8. Figure captions

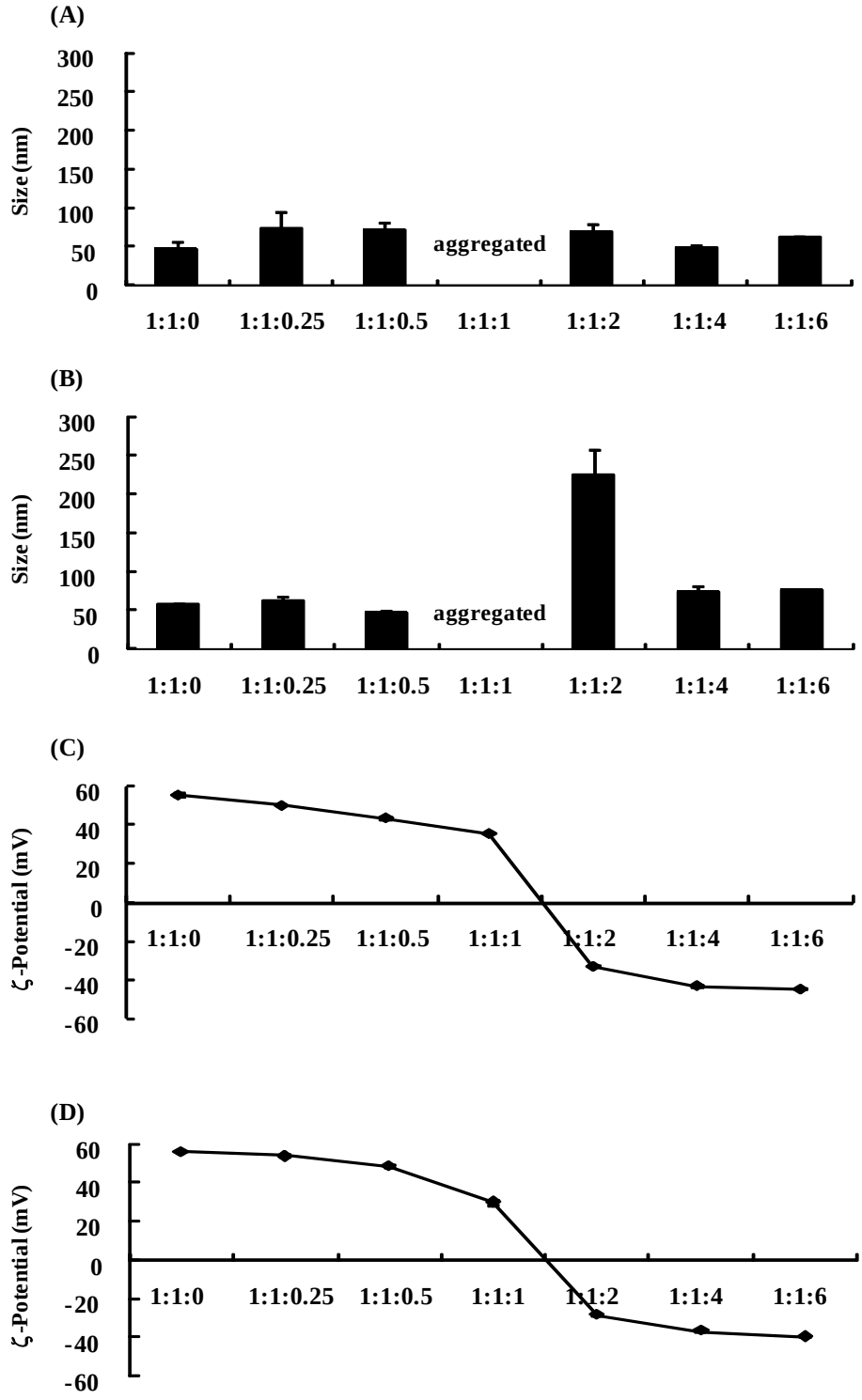

Fig. 1. Sizes (A and B) and ζ-potentials (C and D) of the pDNA/PEI/aptamer complexes. pDNA/PEI/non-specific aptamer (A and C) and pDNA/PEI/MUC1 aptamer (B and D) complexes were constructed with various weight ratios and their size and ζ-potential were evaluated. Each data is the mean ± S.E. of three experiments.

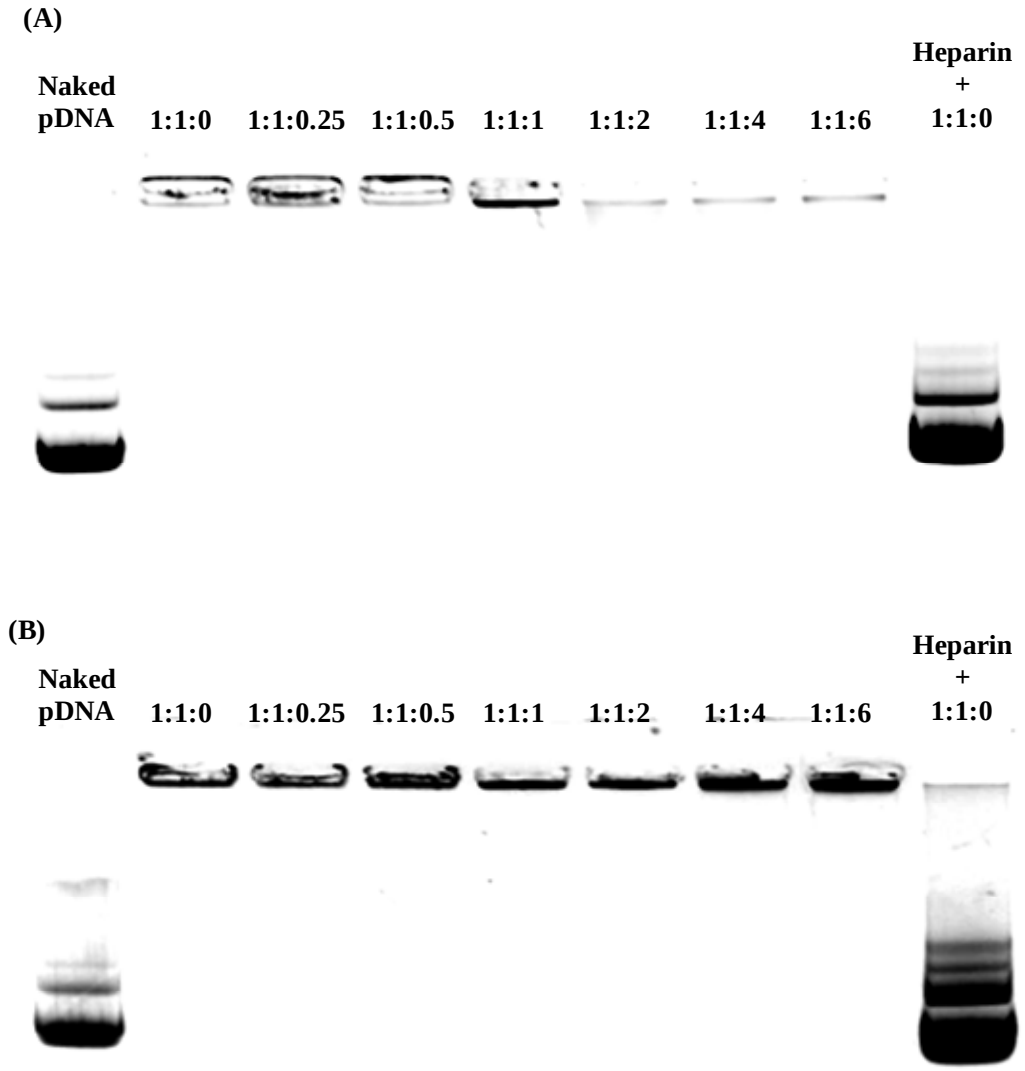

Fig. 2. Effect of non-specific aptamer (A) and MUC1 aptamer (B) on electrophoretic migration of pDNA through an agarose gel.

Each complex was loaded onto agarose gel for electrophoresis. Retardation of pDNA was visualized using ethidium bromide.

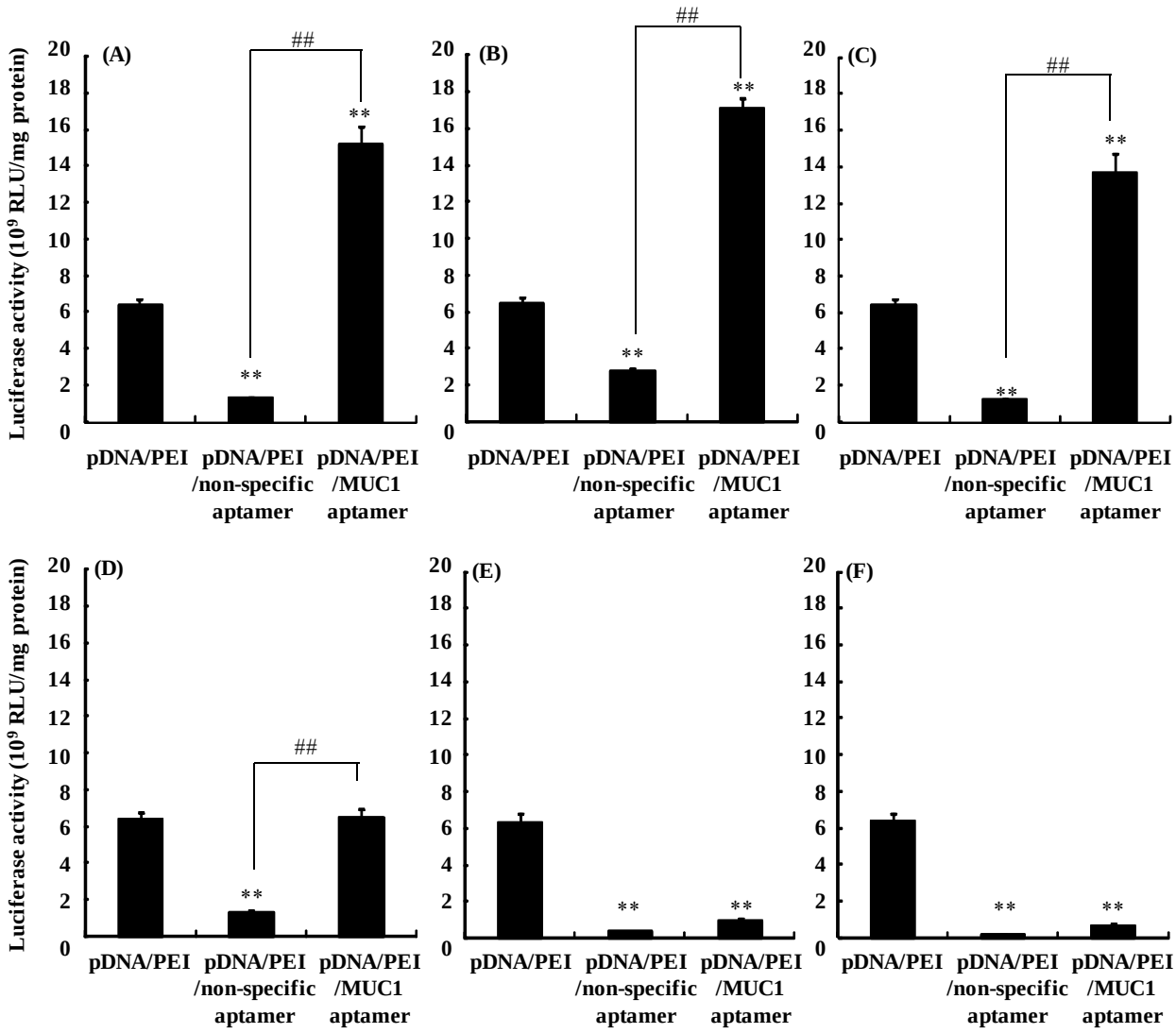

Fig. 3. *In vitro* transgene efficiency of pDNA/PEI/aptamer complexes.

A549 cells were transfected by each pDNA/PEI/aptamer complex with various weight ratios, 1:1:0.25 (A), 1:1:0.5 (B), 1:1:1 (C), 1:1:2 (D), 1:1:4 (E), and 1:1:6 (F). Twenty-four hours after transfection, luciferase activity was evaluated. Each bar represents the mean ± S.E. of three or six experiments. **: $P < 0.01$ vs pDNA/PEI complex, ##: $P < 0.01$.

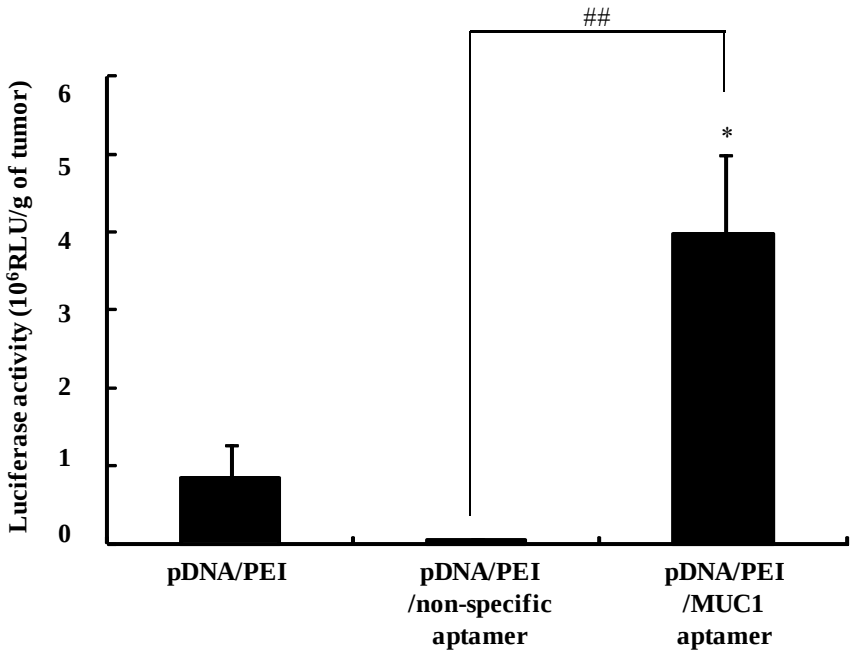

Fig. 4. *In vivo* transgene efficiency of pDNA/PEI/aptamer complexes.

pDNA/PEI complex and pDNA/PEI/aptamer complexes with weight ratio 1:1:0.5 were administrated into the tumors of mice. Twenty-four hours after administration, the tumors were dissected and luciferase activities were evaluated. Each bar represents the mean ± S.E. of three to five experiments. *: $P < 0.05$ vs pDNA/PEI complex, ##: $P < 0.01$.