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GENE DELIVERY

# Target-specific gene silencing by siRNA plasmid DNA complexed with folate-modified poly(ethylenimine)

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## Abstract

A target-specific delivery system of green fluorescent protein (GFP) small interfering RNA (siRNA) plasmid DNA was developed by using folate-modified cationic polyethylenimine (PEI). A GFP siRNA plasmid vector (pSUPER-siGFP), which inhibits the synthesis of GFP, was constructed and used for suppressing GFP expression in folate receptor over-expressing cells (KB cells) in a target-specific manner. A PEI-poly(ethylene glycol)-folate (PEI-PEG-FOL) conjugate was synthesized as a pSUPER-siGFP plasmid gene carrier. KB cells expressing GFP were treated with various formulations of pSUPER-siGFP/PEI-PEG-FOL complexes to inhibit expression of GFP. The formulated complexes were characterized under various conditions. Their GFP gene inhibition and cellular uptake behaviors were explored by confocal microscopy and flow cytometry analysis. pSUPER-siGFP/PEI-PEG-FOL complexes inhibited GFP expression of KB cells more effectively than pSUPER-siGFP/PEI complexes with no folate moieties and showed far reduced extent of inhibition for folate receptor deficient cells (A549 cells). The results indicated that folate receptor-mediated endocytosis was a major pathway in the process of cellular uptake, suggesting that targeted delivery of siRNA vector could be achieved to a specific cell.

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**Keywords:** SiRNA; GFP; PEI-PEG-FOL; Targeted intracellular delivery

## 1. Introduction

RNA interference (RNAi) using small interfering RNA (siRNA), a double-stranded RNA molecule

having 21–23 bp) has recently provided a powerful tool for silencing a target gene in gene therapy. RNAi induces highly sequence-specific degradation of homologous mRNA by double-stranded RNA (dsRNA). The process of RNAi is very useful for genetic analysis and is likely to become a potent therapeutic approach for gene silencing [1,2]. The general mechanism of RNAi involves the cleavage of

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long dsRNA molecules into 21–23 nucleotides small interfering RNAs (siRNAs) by Dicer, an endogenous RNaseIII-like enzyme [3]. The siRNA is incorporated into a ribonuclear protein complex known as the RNA-induced silencing complex (RISC), which contains the proteins necessary for unwinding the double-stranded siRNA, binding, and cleaving the target messenger RNA [4]. In mammalian cells, exposure to dsRNAs with more than 30 bp in length triggers a sequence-nonspecific interferon response that leads to global inhibition of mRNA translation [5,6]. However, introduction of shorter siRNAs into mammalian cells results in mRNA degradation with great sequence specificity without the activation of an interferon response [1]. RNAi by synthetic siRNAs of 21–23 nucleotides depresses endogenous and exogenous gene expression in mammalian cells *in vitro* [7].

Recently, several groups have used siRNA for treating infectious diseases and cancers mediated by variant gene expression [8,9]. For specific gene silencing in a target tissue, a delivery system of siRNA is highly demanded. In the past decade, various kinds of cell-targeting ligands including antibodies, growth factors, peptides, transferrin, and folate have been conjugated to several types of gene carriers, such as polymer conjugates, liposomes, polymer micelles, and nano-particles for target-specific delivery [10–14]. Many bioactive agents including plasmid DNA, anti-sense oligonucleotides, anti-cancer agents, and imaging agents could be delivered site-specifically to target cells and tissues [11]. Among them, folate has been popularly used as a targeting ligand for plasmid DNA, doxorubicin, and anti-sense ODN [15].

In this study, an anti-green fluorescent protein (GFP) siRNA plasmid system (pSUPER-siGFP) was constructed and used to inhibit the expression of exogenous GFP in mammalian cells in a target-specific manner. The anti-GFP siRNA plasmid was complexed with a PEI-based cationic polymer conjugate, poly(ethylenimine)–poly(ethylene glycol)–folate (PEI–PEG–FOL), and the complexes were transfected to folate receptor over-expressing cells that produce exogenous GFP. The pSUPER-siGFP/PEI–PEG–FOL complexes were characterized with dynamic light scattering and gel electrophoresis and their cell-specific gene silencing effect was comparatively and quantitatively examined using folate receptor positive cells (KB cells) and folate receptor negative cells

(A549 cells). The extent of GFP inhibition in KB cells was also analyzed by flow cytometry.

## 2. Materials and methods

### 2.1. Materials

Forward and reverse oligonucleotides for cloning anti-GFP siRNA sequence were synthesized and purified by Bioneer (Daejeon, Republic of Korea). A mammalian siRNA expression vector, pSUPER-RNAi (3176 bp), was purchased from Oligoengine (Seattle, WA). Poly(ethylenimine) (branched PEI, MW 25,000) was supplied from Aldrich (Milwaukee, WI). Poly(ethylene glycol) (COOH–PEG–NH<sub>2</sub>, MW 3400) was obtained from Nektar (Huntsville, AL). Folate and *N*-hydroxylsuccinimide (NHS) were obtained from Sigma (St. Louis, MO). Dicyclohexylcarbodiimide (DCC) was purchased from Fluka Chemie (Buchs, Switzerland). Dulbecco's phosphate buffered saline (PBS), fetal bovine serum (FBS), Roswell Park Memorial Institute 1640 medium (RPMI 1640), and folate-free RPMI 1640 were obtained from Gibco BRL (Grand Island, NY). DNase I was purchased from Promega (Madison, WI). A pEGFP-C1 vector was obtained from BD Bioscience Clontech (Palo Alto, CA). All other chemicals and reagents were of analytical grade.

### 2.2. Construction of anti-GFP siRNA expression vector (pSUPER-siGFP)

A siRNA-expressing sequence for targeting the GFP gene was cloned into a pSUPER RNAi system. As shown in Fig. 1A, forward and reverse target sequences of the anti-GFP siRNA hairpin transcript were GATCCCCGCAAGCTGACCCTGAAGTTC-TTCAAGAGAGAACTTCAGGGTCAGCTTGCTT-TTTGGAAA and AGCTTTTCCAAAAGCAAGC-TGACCCTGAAGTTCTCTCTTGAAGAACTT-CAGGGTCAGCTTGCGGG, respectively [16]. For cloning anti-GFP oligos into pSUPER RNAi vector, the forward and reverse strands of the oligonucleotides (ODN) were annealed to form an anti-GFP ODN duplex. The duplex was then inserted into the *Bgl*III–*Hind*III cleavage site within the pSUPER-RNAi vector pre-linearized with the restric-

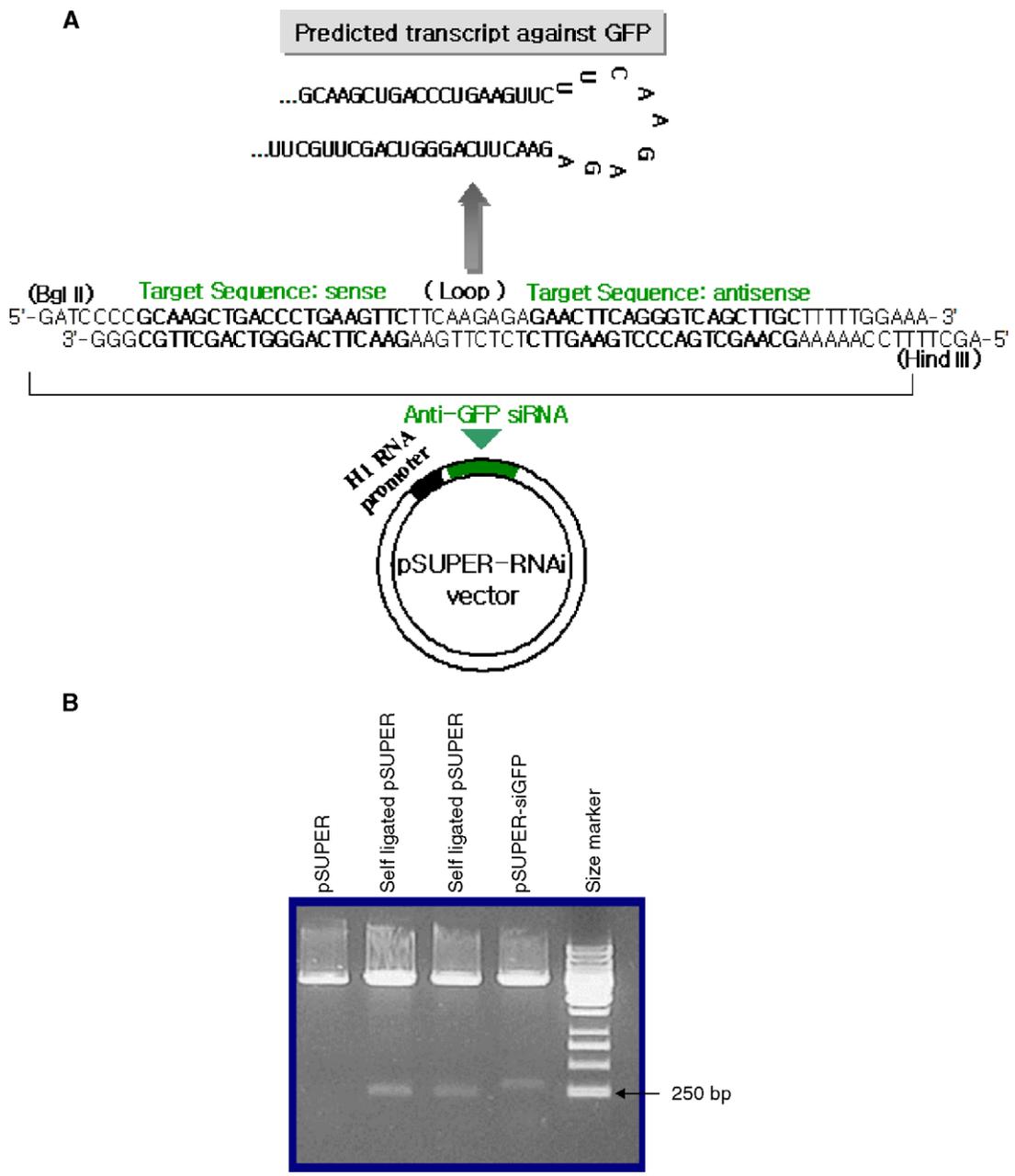


Fig. 1. (A) A schematic diagram of the pSUPER vector expressing anti-GFP siRNA hairpin transcript. (B) Agarose gel electrophoresis analysis of the recombinant pSUPER vectors digested with restriction enzyme. (lane 1: control pSUPER-RNAi vector untreated with enzyme, lane 2 and 3: empty pSUPER vectors, lane 4: pSUPER-siGFP, and lane 5: size marker).

tion enzymes. The recombinant plasmid was transformed into XL1blue *E. coli* strain and the resultant cells were cultured in LB-ampicillin containing medium. Colonies containing empty plasmids or

cloned vectors were recovered. The recombinant plasmids in the selected colonies were prepared, digested with *Hind* III and *Eco*R I, and run on 0.8% agarose gel to isolate the cells containing pSUPER-

siGFP. Fig. 1A shows a schematic diagram of the anti-GFP siRNA expression vector (pSUPER-siGFP), which produces a hairpin type of the siRNA transcript.

### 2.3. Preparation of PEI-PEG-FOL conjugate

The PEI-PEG-FOL conjugate was synthesized according to the previous method with slight modification [13]. Briefly, folate (65 mg, 0.15 mmol), DCC (30 mg, 0.15 mmol), and NHS (17 mg, 0.15 mmol) were dissolved in anhydrous DMSO. The activation reaction proceeded under nitrogen for 1 h at room temperature. The insoluble dicyclohexylurea was removed by filtration (0.45  $\mu\text{m}$  Teflon filter). A hetero-functional PEG derivative (COOH-PEG-NH<sub>2</sub>, 178 mg, 0.05 mmol) dissolved in DMSO was added into the activated folate solution. The PEG-FOL conjugate was dialyzed (MWCO 1000) against deionized water to remove un-reacted substrates and then lyophilized. The terminal carboxylic acid group of the COOH-PEG-FOL conjugate (150 mg, 0.04 mmol) was also activated with DCC/NHS chemistry in DMSO and conjugated to primary amine groups of PEI (320 mg, 12.8  $\mu\text{mol}$ ) dissolved in DMSO. The reaction was carried out at room temperature under nitrogen for 1 h. The reacted conjugate was dialyzed (MWCO 10,000) against deionized water and freeze-dried. Complete removal of PEG-FOL was confirmed by <sup>1</sup>H NMR spectrum, which indicated no change of PEG content in PEI-PEG-FOL before and after dialysis. The stoichiometric molar ratio of conjugation between PEI and PEG-FOL was determined by 400 MHz <sup>1</sup>H NMR spectrometry.

### 2.4. Preparation and characterization of pSUPER-siGFP/PEI-PEG-FOL complex

#### 2.4.1. Preparation of pSUPER-siGFP/PEI-PEG-FOL complex for transfection

For transfection of the pSUPER-siGFP vector, the plasmid was complexed with PEI-PEG-FOL conjugate in an aqueous phase under various conditions. Two micrograms of pSUPER-siGFP in 50  $\mu\text{l}$  of phosphate buffered saline (PBS, pH 7.2) was mixed with PEI-PEG-FOL conjugates in 100  $\mu\text{l}$  of PBS at a desired nitrogen/phosphate (N/P) ratio.

#### 2.4.2. Size and $\zeta$ -potential of pSUPER-siGFP/PEI-PEG-FOL complex

The size and surface charge of pSUPER-siGFP/PEI-PEG-FOL complexes formed at various N/P ratios were estimated by using a dynamic light scattering instrument (Zeta-Plus, Brookhaven, New York). Three milliliters of the complex solution prepared at N/P ratios of 0–16 was analyzed at 25 °C.

#### 2.4.3. Gel retardation assay of pSUPER-siGFP/PEI-PEG-FOL complex

The pSUPER-siGFP/PEI-PEG-FOL complexes prepared at different N/P ratios (0, 1, 2, 4, 8, and 16) were analyzed with gel retardation assay. Electrophoresis was carried out on 0.8% agarose gel with a current of 50 V for 1 h in TAE buffer solution (40 mM Tris-HCl, 1% (v/v) acetic acid, and 1 mM EDTA). The retardation of the complexes was visualized by staining with ethidium bromide.

#### 2.4.4. DNase protection of pSUPER-siGFP/PEI-PEG-FOL complex

Fifty microliters of pSUPER-siGFP vector (20  $\mu\text{g}$ ) in PBS and 100  $\mu\text{l}$  of different amounts of PEI-PEG-FOL or PEI (N/P ratio: 0, 8, 16) dissolved in PBS was mixed with 345  $\mu\text{l}$  of a reaction buffer solution (10 mM Tris-Cl, 150 mM NaCl, pH 7.4). After incubating for 15 min at room temperature to produce pSUPER-siGFP/conjugate complexes, 5 units of DNase I (1 unit/ $\mu\text{l}$ ) were added to the solution. To activate DNase, the sample solution was combined with 50  $\mu\text{l}$  of Mg<sup>2+</sup> solution (50 mM) and gently vortexed. At the start of reaction, absorbance at 260 nm was measured every 10 s for 20 min. The increment value was calculated for each time interval.

### 2.5. Cells and cell culture

KB (human epidermal carcinoma) cells over-expressing folate receptors and A549 (human lung carcinoma) cells deficient of folate receptors were used for receptor-mediated intracellular delivery of pSUPER-siGFP/PEI-PEG-FOL complex. All cell lines were purchased from the Korean Cell Line Bank (Seoul, Republic of Korea). KB and A549 cells were maintained in RPMI 1640 medium with or without folate, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin at

37 °C in a humidified atmosphere of 5% CO<sub>2</sub>. The cells were regularly passaged at sub-confluence and plated 24 h before transfection with pEGFP-C1 vector, an expression vector of green fluorescent protein in mammalian cells.

### 2.6. Preparation of cells expressing green fluorescent protein

The cell lines transiently expressing GFP (GFP-KB cells and GFP-A549 cells) were prepared by transfecting pEGFP-C1 vector into the cells with the use of Lipofectamine™ reagents. The transfected cells were selected by incubation in G418 sulfate (Gibco, Tokyo, Japan) medium as described in previous studies [17,18]. First, the cultured cells were trypsinized and plated 24 h prior to transfection in a cell culture flask (75 cm<sup>2</sup>, BD Falcon) at an initial density of  $1.5 \times 10^7$  cells. pEGFP-C1 vector (6 µg) was mixed with 36 µl of Lipofectamine Plus™ reagent (2 mg/ml, Invitrogen, Carlsbad, CA) in serum-free medium to make a final volume of 600 µl and was incubated for 15 min at room temperature. The solution was combined with 600 µl of dilution medium containing 36 µl of Lipofectamine™ reagent (2 mg/ml, Invitrogen, Carlsbad, CA) and incubated for 15 min at room temperature. Cells were then washed with PBS and the formed complexes were dropped in 4.8 ml of serum-free medium. After 3 h incubation, the reaction medium was replaced with 15 ml of fresh medium containing 10% FBS in the lack of G418 sulfate and cultured for another 24 h. For selection of GFP-expressing cells, the cells were grown in G418 selection medium (550 µg/ml) for 1 day. pEGFP-C1 transfected cells were isolated and used for intracellular delivery of pSUPER-siGFP/PEI-PEG-FOL complexes for suppressing the expression of exogenous GFP gene.

### 2.7. Transfection with pSUPER-siGFP/PEI-PEG-FOL complex

The GFP expressing cells were plated in a 6-well cell culture plate at a density of  $2.5 \times 10^5$  cells/well and incubated for 24 h before transfection. Two micrograms of pSUPER-siGFP was complexed with Lipofectamine, PEI, or PEI-PEG-FOL conjugate at various conditions (Lipofectamine: 12 µg; PEI and PEI-PEG-FOL N/P ratio: 0~24). The complexes were

added to GFP expressing cells in 1ml of serum-free medium and incubated for 3 h at 37 °C. The cells were then cultured in 10% FBS media for 24 h and the level of GFP fluorescence was detected. Transfected cells were harvested by treating with a cell lysis buffer solution (CellLytic M cell lysis reagent, Sigma) followed by centrifugation at 14,000 rpm for 5 min. The supernatant was analyzed using a spectrofluorophotometer (SLM-AMINCO 8100, SLM Instruments Inc., USA) for determining the level of GFP expression in the cells. Excitation and emission wavelengths were 488 and 507 nm, respectively.

### 2.8. Flow cytometry

Fluorescence activated cell sorter (FACS) analysis was performed by seeding GFP transfected and non-transfected KB cells at a density of  $2.5 \times 10^5$  cells/well in a 6-well cell culture plate and treating them as described above. The cells were washed three times with PBS and fixed in 1% para-formaldehyde solution for 30 min at 4 °C. The fixed cells were washed twice with PBS and stored in 0.1% para-formaldehyde. Cells were analyzed by a FACScan instrument (Becton Dickinson) using CELLQUEST software (PharMingen, USA).

## 3. Results and discussion

Instead of synthetic siRNA, GFP siRNA-expression vector system was constructed and used for GFP gene silencing in a cancer cell. Although direct delivery of synthetic siRNA into the cytosol would be more effective for non-dividing cells, the gene silencing effect is transient and non-inducible [19]. In contrast, the siRNA plasmid vector system requiring intranuclear localization can stably express siRNA in an inducible manner for fast dividing mammalian cells. Thus, for rapidly growing cancer cells in vitro, the siRNA plasmid vector system would be more ideal in demonstrating the gene inhibition effect. In this study, GFP was an exogenous target gene and pSUPER-siGFP was a mammalian vector used for anti-GFP siRNA-expression. Forward and reverse ODN sequences were designed according to the siRNA complementary GFP reported by Tiscornia et al. [16]. As described in Fig. 1A, the designed ODN sequence

includes a sense and antisense-GFP sequence, a loop region, a 3' overhanging of T nucleotides, and restriction sites (*Bgl*III site: 5' end, *Hind*III site: 3' end), which produce a hairpin type anti-GFP siRNA transcript. The sense and antisense-GFP sequences, which are composed of nineteen nucleotides, were used for cleavage of the GFP mRNA. The 9-nt spacer that forms a loop is very important in producing a double stranded siRNA after the transcription process [19]. For efficient cleavage for destruction of the target mRNA, the siRNA duplex must have 2 or 3 nt overhanging 3' ends [20]. Successful cloning of target ODN in the recombinant pSUPER vector was confirmed by agarose gel electrophoresis, as shown in Fig. 1B. The self-ligated pSUPER vector with no ODN insert presented a fragment of 227 bp, while the recombinant pSUPER-siGFP vector displayed a fragment approximately 287 bp in length. Colonies containing the cloned vectors were isolated to produce the pSUPER-siGFP plasmids.

To accomplish folate receptor-mediated delivery of the pSUPER-siGFP vector, a cationic polymeric conjugate, PEI-PEG-FOL, was synthesized. It contains a PEI part for condensation of DNA vector, a PEG spacer part for stabilization of polyelectrolyte complex, and a

folate moiety for cancer cell targeting. The terminal  $\gamma$ -carboxylic acid group of folate was activated by NHS/DCC and then conjugated to the terminal amine group of a hetero-functional PEG derivative (COOH-PEG-NH<sub>2</sub>). It is generally known that folate has two  $\alpha$ - and  $\gamma$ -carboxylic acids, but the  $\gamma$ -carboxylic acid is more selectively activated due to its higher reactivity [21,22]. The terminal carboxylic acid of the COOH-PEG-FOL conjugate was subsequently activated by DCC/NHS and reacted with the primary amine group of PEI. The synthesized PEI-PEG-FOL conjugate was corroborated by <sup>1</sup>H NMR spectrum as shown in Fig. 2. From the relative intensity ratio of proton peaks corresponding to the -CH<sub>2</sub>- of PEI (2.8–3.1 ppm) and -CH<sub>2</sub>- of PEG (3.3–3.6 ppm), it was estimated that 4.64 molecules of PEG-FOL were attached to each PEI molecule. The molecular weight calculated was about 43,000. The PEI-PEG-FOL conjugate was complexed with pSUPER-siGFP vector in an aqueous phase at various stoichiometric nitrogen/phosphate (N/P) molar ratios between PEI and the plasmid vector. As shown in Fig. 3A, the size of the resultant pSUPER-siGFP/PEI-PEG-FOL complex decreased sharply with increasing N/P ratio. The size of the complex at the N/P ratio of 4

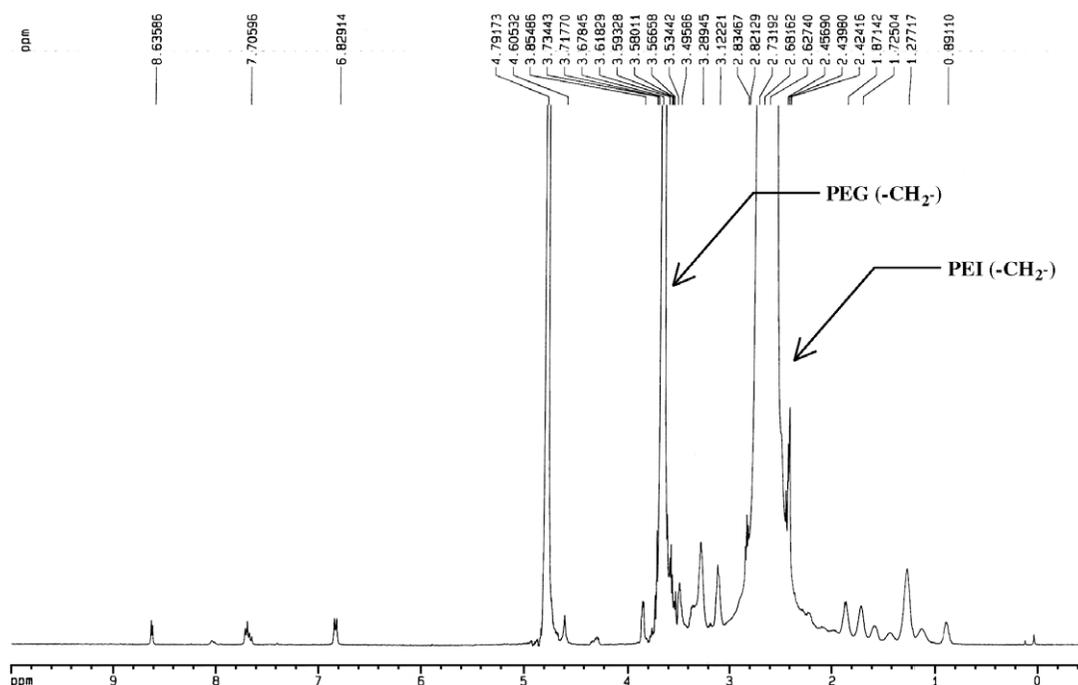


Fig. 2. <sup>1</sup>H NMR spectrum of FOL-PEG-PEI conjugate.

reached  $232.0 \pm 0.4$  nm, where the value of surface zeta-potential became positive at  $10.3 \pm 0.7$  mV. At this N/P ratio, complete gel retardation was observed in 0.8% agarose gel (Fig. 2B). The size and surface charge of the polyelectrolyte complex was stable at the N/P ratio of 16, where the hydrodynamic diameter was  $185.0 \pm 0.3$  nm with a narrow size distribution (polydispersity: 0.055) and the surface zeta-potential value was  $14.9 \pm 0.8$  mV. The PEI-PEG-FOL/DNA complex was stable for more than a week even in serum medium, which was confirmed by 0.8% gel electrophoresis. There was no sign of any decomplexation behavior for PEI-PEG-FOL/DNA complex. The size of pSUPER-siGFP/PEI complexes formed at the same N/P ratio was  $150.5 \pm 1.5$  nm with a surface zeta-potential value of  $28.0 \pm 0.3$  mV. The increase in diameter of about 35 nm and the decrease in surface zeta-potential value upon complexing with the PEI-PEG-FOL conjugate suggest that PEG-FOL segments

formed a surrounding corona around the inner core of pSUPER-siGFP/PEI complexes. The PEG shell was expected to play an important role not only in sterically stabilizing the structure of the complex [23] but also in protecting the siRNA vector from being attacked by nucleases [24–26]. DNase I protection analysis of pSUPER-siGFP vector complexed with PEI or PEI-PEG-FOL under various N/P ratios was depicted in Fig. 4. Naked pSUPER-siGFP vectors and pSUPER-siGFP/PEI complexes formulated at N/P ratios of 8 and 16 exhibited considerable fragmentation of DNA. The complexes with higher N/P ratio showed better protection of the complexed plasmid DNA from enzymatic digestion, suggesting that more condensed polyelectrolyte particles were produced at a higher N/P ratio [27]. pSUPER-siGFP/PEI-PEG-FOL complexes showed no evidence of DNA fragmentation when incubated with DNase I, implying that the inner core composed of PEI and pSUPER-siGFP was additionally

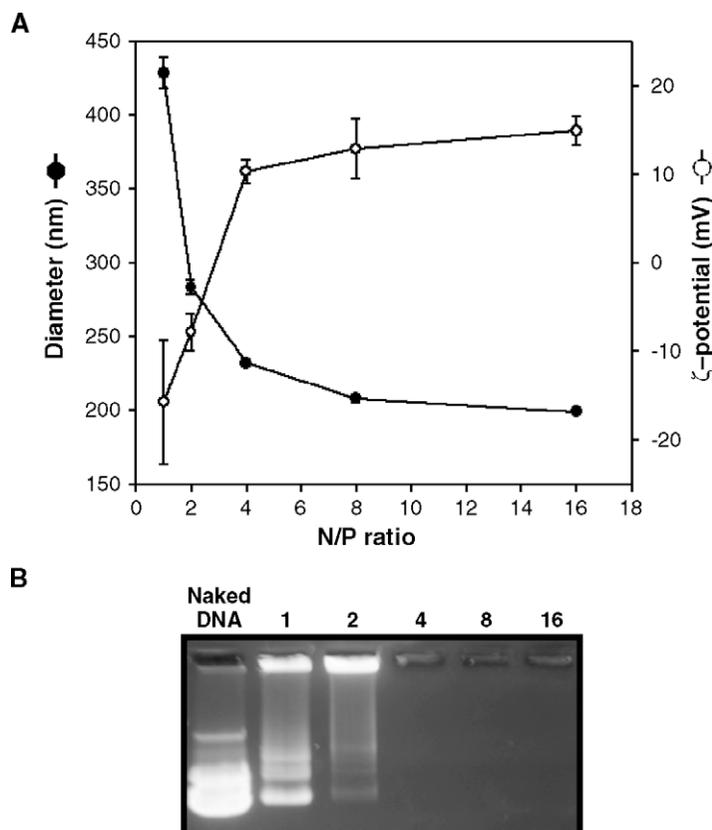


Fig. 3. (A) Effective diameters and surface charges of pSUPER-siGFP/PEI-PEG-FOL complexes as a function of N/P ratio. (B) Gel retardation analysis of the complexes.

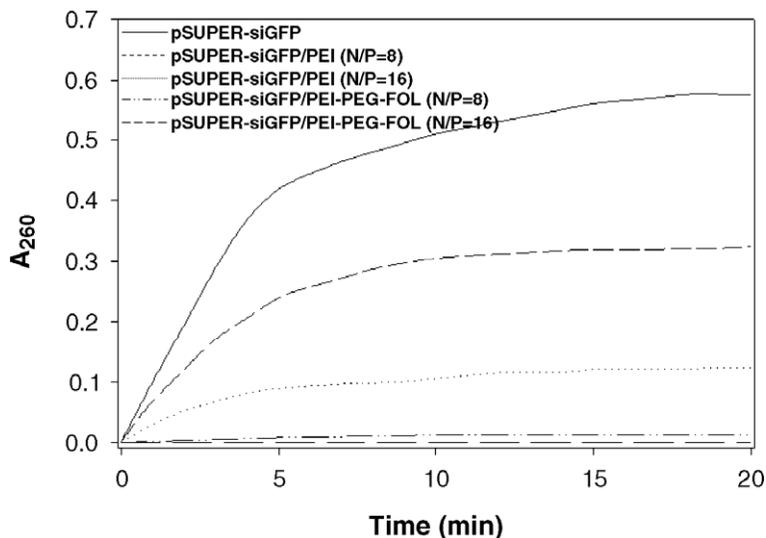


Fig. 4. Enzymatic degradation profiles of variously formulated complexes at N/P ratio of 8 or 16. pSUPER-siGFP vector was complexed with PEI and PEI-PEG-FOL and incubated with 5 units of DNase I for 20 min.

stabilized by PEG chains, as demonstrated in previous studies [28,29].

To investigate the effect of post-transcriptional gene silencing on target cancer cells, the pSUPER-siGFP/PEI and pSUPER-siGFP/PEI-PEG-FOL complexes were transfected to GFP-expressing KB (GFP-KB) cells that were pre-transfected with a mammalian GFP expression vector (pEGFP-C1). Fig. 5 shows inhibition of GFP expression in GFP-KB cells at various N/P ratios of the two complexes. The relative level of GFP expression in cells was determined by normalizing green fluorescence intensity values of extracted proteins from the formulations with different N/P ratios, using non-treated GFP-KB cells as control. The GFP expression level of GFP-KB cells transfected with pSUPER-siGFP/PEI-PEG-FOL complexes was more significantly reduced with increasing N/P ratios than those transfected with pSUPER-siGFP/PEI. The GFP expression level for pSUPER-siGFP/PEI-PEG-FOL complexes reached about  $10.5 \pm 2.5\%$  at the N/P ratio of 24, whereas that for pSUPER-siGFP/PEI complexes showed  $38.6 \pm 1.1\%$  at the same condition. The cytotoxicity of PEI-PEG-FOL was measured with MTT assay over the N/P ratio used for pSUPER-siGFP transfection. There was no detectable cytotoxic effect of PEI-PEG-FOL on KB cells (data not shown). Thus the increasing

extent of gene inhibition with increasing N/P ratio can be attributed to formation of more compact pSUPER-siGFP/PEI inner core that stabilized the complexes. The structural integrity of DNA/PEI complexes was largely dependent on N/P ratio, which directly influenced transfection efficiencies as reported previously [30]. The observed GFP gene

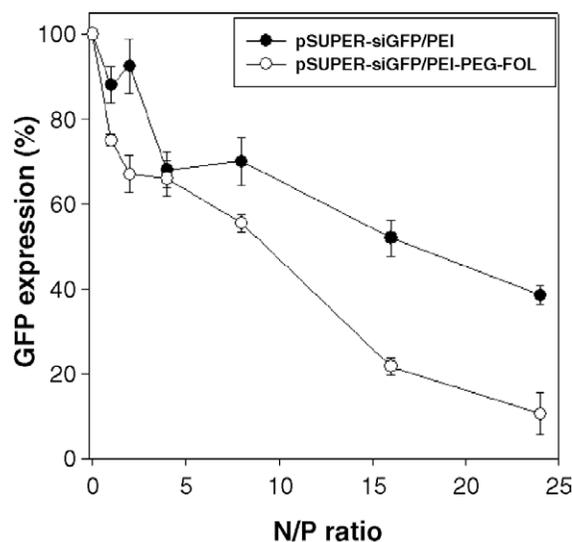


Fig. 5. GFP gene inhibition efficiency of pSUPER-siGFP/PEI and pSUPER-siGFP/PEI-PEG-FOL complexes as a function of N/P ratio against GFP-KB cells.

silencing effect was prolonged for more than 92 h. The enhanced effect in gene silencing for pSUPER-siGFP/PEI-PEG-FOL complexes relative to pSUPER-siGFP/PEI complexes was most likely due to folate receptor-mediated endocytosis that promoted cellular uptake of the complexes. KB cells are human carcinoma cells that particularly over-express folate receptors on their surface [31]. Fig. 6 shows comparative gene silencing effects of pSUPER-siGFP/Lipofectamine, pSUPER-siGFP/PEI, and pSUPER-siGFP/PEI-PEG-FOL complexes for GFP-KB cells and GFP-A549 cells. A549 cells are folate receptor deficient human carcinoma cells. GFP-A549 cells incubated with the three complexes showed GFP inhibition levels from about 56.3% to 63.0%, while GFP-KB cells exhibited those from 10.5% to 38.6%. The different levels of gene silencing for the two cell lines can be attributed to cell-specific transfection efficiencies as reported previously [32]. It can be seen that only pSUPER-siGFP/PEI-PEG-FOL complexes inhibited GFP gene expression markedly in KB cells but not in A549 cells. The enhanced interference of GFP expression in KB cells was caused by increase in cellular uptake of pSUPER-siGFP vector via the process of folate receptor-mediated endocytosis. The results prove that intracellular delivery of pSUPER-siGFP vector occurred in a target-specific manner.

Fig. 7 shows flow cytometric analysis of KB cells and GFP-KB cells transfected with pSUPER-siGFP/

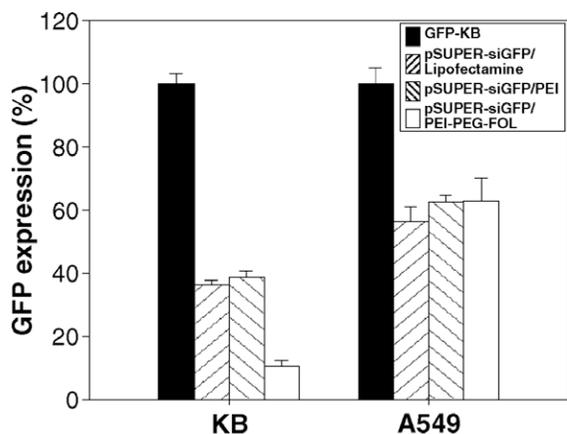


Fig. 6. Comparative inhibition levels of GFP expression by pSUPER-siGFP/Lipofectamine, pSUPER-siGFP/PEI, and pSUPER-siGFP/PEI-PEG-FOL against GFP-KB and GFP-A549 cells (N/P ratio:24).

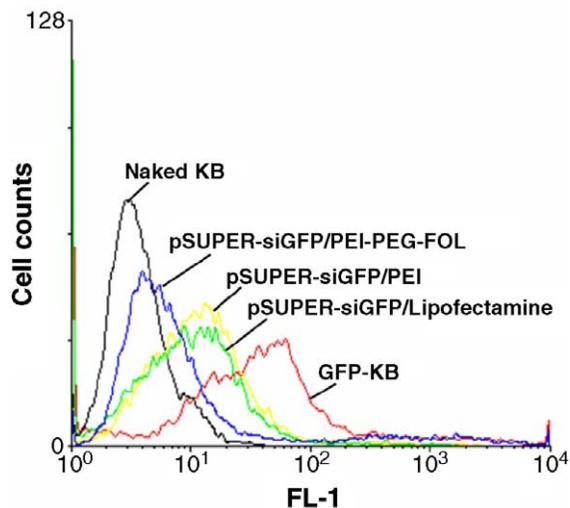


Fig. 7. Flow cytometric analysis of GFP expression interference against GFP-KB cells.

Lipofectamine, pSUPER-siGFP/PEI, or pSUPER-siGFP/PEI-PEG-FOL complexes. The result was consistent with that of the cellular fluorescence intensity assay (Fig. 5). In an arbitrarily selected fluorescence gate region ( $1 < FL-1 < 10$ ), the gene inhibition effect of pSUPER-siGFP/PEI-PEG-FOL in GFP-KB cells was over 90%, while that of complexes without folate moieties was no more than ca. 40–50%. This also demonstrated that a PEI-PEG-FOL conjugate was a useful carrier for cancer cell specific intracellular delivery of anti-GFP siRNA expression vector.

In conclusion, anti-GFP siRNA plasmid DNA was intracellularly delivered to folate receptor positive cells by complexing with a synthetic PEI-PEG-FOL conjugate. The complexes suppressed exogenous GFP expression in a cell-specific manner. Inhibition of the GFP gene expression occurred via a folate receptor-mediated endocytotic process. This formulation strategy can be applied to a wide range of post-transcriptional gene silencing therapeutics including siRNA expression plasmids.

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