

Full-length article

Development of efficient RNA interference system using EGF-displaying phagemid particles

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Key words

epidermal growth factor; siRNA; gene delivery; phagemid particles; hydroxycamptothecin

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Abstract

Aim: To develop an efficient RNA interference system using phagemid particles displaying the epidermal growth factor (EGF) ligand. **Methods:** pSilencer1.0-siEGFP and pSilencer4.1-siAkt plasmids were constructed by gene clone technology. The modified helper phage genome (plasmid) M13KO7EGFCT was used to package phagemids, such as pSilencer1.0-siEGFP and pSilencer4.1-siAkt. ELISA was used to quantify the titer of the progeny virus particles. Single-strand DNA was extracted and analyzed by agarose gel electrophoresis to evaluate the percentage of the phagemid particles. The expression level of the reporter gene enhanced green fluorescence protein (EGFP) was determined by transducing phagemid particles packaging pSilencer1.0-siEGFP into cells. The level of Akt gene expression in cells transduced phagemid particles packaging pSilencer4.1-siAkt was examined by Western blotting. Hydroxycamptothecin (HCPT) was used to enhance the gene transduction efficiency. **Results:** RNAi vectors pSilencer1.0-siEGFP and pSilencer4.1-siAkt were successfully constructed. Phagemid-encoding siRNA can be packaged efficiently. After the cells were infected by EGF displaying phagemid particles in the presence of HCPT, the expression of the target gene EGFP or Akt was substantially downregulated. **Conclusion:** Cell-targeted phagemid particles are efficient siRNA delivery vectors in the presence of HCPT.

Introduction

In previous studies, scientists have successfully modified the filamentous bacteriophages, which are capable of delivering genes to mammalian cells using targeting ligands, such as growth factors^[1–5], antibodies^[6], and viral capsid proteins^[7]. To improve the ligand display level on phages, we developed LMP cells (*Escherichia coli* bearing the ligand-pIII-encoding helper phage) to make cell-targeted phagemid particles^[8]. We also demonstrated that it can deliver reporter genes into target cells.

RNAi techniques are a promising way to silence therapeutic target genes. Both non-viral vectors and viral vectors have been used as a vehicle to deliver siRNA *in vitro* and *in vivo*. The major problem of non-viral vectors lies in its low gene delivery efficiency and hard quality control. Animal viruses, such as adenoviruses, have broad natural tropism

which makes them difficult to selectively transfer siRNA genes into target cells. In contrast to these animal viruses, filamentous bacteriophage has no natural tropism to mammalian cells. However, it can highly efficiently infect the targeted cells when the peptide ligand was displayed on the surface. Like non-viral vectors, the major problem of this vector is its low gene delivery efficiency. Fortunately, hydroxycamptothecin (HCPT), a topoisomerase I inhibitor and also anticancer drug, can substantially improve its gene delivery efficiency^[1,13,17,18]. In this study, we tested whether cell-targeted phages can be used as siRNA gene delivery tools or not.

Materials and methods

Reagents Restriction endonucleases were purchased from TaKaRa Biotechnology (Dalian, China). Dulbecco's

modified Eagle's medium (DMEM), Dulbecco's phosphate-buffered saline, fetal bovine serum (FBS), and trypsin/ethylenediamine tetraacetic acid were purchased from Invitrogen (Grand Island, NY, USA).

Cell culture H1299 (large-cell lung carcinoma; ATCC, Manassas, VA, USA) cells were cultured at 37 °C in medium consisting of 90% DMEM and 10% FBS in a humidified atmosphere of 95% air and 5% CO₂.

Plasmid construction All oligonucleotides were synthesized by Sangon (Shanghai, China) and annealed to be double-stranded, as previously described^[10]. The selection of the coding sequences of EGFP and Akt for siRNA was based on 2 different publications, respectively^[9,10]. Detailed information of the oligonucleotides is shown in Table 1.

To generate the pSi1.0-EGFP RNAi plasmid, a 55-nt oligo-DNA duplex (annealed by siEGFP-f and siEGFP-r) corresponding to nucleotides 106–127 of the pEGFP-N1 (BD Biosciences Clontech, Palo Alto, CA, USA)-coding region was directly inserted into the pSilencer1.0 (Ambion, Austin, TX, USA) vector digested with *Apa*I (blunted) and *Hind*III.

The pSilencer4.0-f plasmid was generated as follows: the pGI3-control plasmid (Promega category no.: E1741; Promega, Madison, WI, USA) was digested with *Sca*I, and the 1.08 kb fragment were used to ligate with pSilencer4.0 (Ambion, Austin, TX, USA) which is linearized with *Sca*I. The inserted subclones with the reversed orientation of cytomegalovirus (CMV) promoter are genetically selected with the supplementary of ampicillin resistance. This modified pSilencer4.0 was prepared for the phage package and siRNA expression.

To generate the pSi4.0-Akt plasmid, a 52 nt oligo-DNA duplex (annealed by siAkt-f and siAkt-r) corresponding to nucleotides 1462–1482 of the Akt1 (NM_001014432)

and 1244–1264 of Akt2 (NM_001626)-coding region was directly inserted into the pSilencer4.0 vector digested with *Bam*HI and *Hind*III. The mock Akt sequence is only one nucleotide different from siAkt, as listed in Table 1.

Preparation of phagemid particles The phagemid particles were prepared following previously published protocols^[11] with some modifications. M13KO7EGFCT^[8] was transformed into *Escherichia coli* to make LMP cells. The phagemid carrying the siRNA-encoding sequence was transformed into LMP cells. The cells were then plated on LB agar (containing 70 µg/mL kanamycin and 50 µg/mL ampicillin) and incubated at 37 °C overnight. A cell clone was picked the next day and transferred into 1 L LB containing 70 µg/mL kanamycin and 50 µg/mL ampicillin. After shaking at 37 °C for 14–16 h, the supernatant of the culture was collected and the phagemid particles were purified with polyethylene glycol (PEG)/NaCl precipitation.

Quantification of phagemid particles The phagemid particles were quantified by ELISA, as previously described^[12]. Briefly, serial dilutions of phagemid particles in coating buffer (0.1 mol/L NaHCO₃, pH 9.1) were coated in microtiter plates (Corning; Corning, NY, USA) at 4 °C overnight. After blocking with 1% bovine serum albumin (BSA; Sigma, St Louis, MO, USA) in phosphate-buffered saline (PBS), the bound phages were stained with a mouse anti-M13 antibody conjugated with horseradish peroxidase (HRP; Amersham Biosciences, Piscataway, NJ, USA) diluted at 1:200 in PBST (PBS containing 0.1% Tween-20). The signal was developed using the 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid) substrate and quantitated in an ELISA reader (Bio-Rad Laboratories, Hercules, CA, USA). The M13KO7 helper phage of a known number of pfus was used for standardization.

Table 1. Oligonucleotide sequences used to synthesize siRNA *in vitro* targeting EGFP and Akt (human) genes.

Targets	Target sequences	Synthesized oligonucleotide	
EGFP	siEGFP-f	GGCGATGCCACCTACGGCAAGC	5'-GGCGATGCCACCTACGGCAAGCTCGAGC TTGCCGTAGGTGGCATCGCCCTTTTG-3'
	siEGFP-r		5'-AATTCAAAAAGGGCGATGCCACCTACGGC AAGCTCGAGCTTGCCGTAGGTGGCATCGCC-3'
Akt	siAkt-f	CCTGCCCTTCTACAACCAGGA	5'-GATCCTGCCCTTCTACAACCAGGACTGCA GTCCTGGTTGTAGAAGGGCAGG A-3'
	siAkt-r		5'-AGCTTCCTGCCCTTCTACAACCAGGACTG CAGTCCTGGTTGTAGAAGGGCA G-3'
	siAktm-f	CCTGCCTTCTACAACCAGGA	5'-GATCCTGCCTTCTACAACCAGGACTGCA GTCCTGGTTGTAGAAAAGGCAGG A-3'
	siAktm-r		5'-AGCTT CCTGCCTTCTACAACCAGGA CTGCAG TCCTGGTTGTAGAAAAGGCA G-3'

Purification of single-strand DNA from phagemid particles Single-strand DNA (ssDNA) was extracted from the phagemid particles following the supplied protocol (Ph.D.-12 phage display peptide library kit, New England Biolabs, Beverly, MA, USA). Briefly, the phagemid particles were precipitated by PEG/NaCl. The pellet was then suspended with 100 μ L iodide buffer. In total, 250 mL ethanol was added and incubated for 10 min to allow for ssDNA precipitation. The pellet was collected after centrifugation at 12 000 \times g for 10 min, washed in 70% ethanol, and air dried for 10 min. It was finally dissolved in 30 μ L TE buffer (10 mmol/L Tris-HCl [pH 8.0] and 1 mmol/L EDTA) and analyzed by agarose gel electrophoresis.

In vitro phagemid particle transfection The cells were plated into 24-well plates at densities of 10 000 cells per well, 24 h prior to the phage particle addition. The phages were added at 10¹¹ pfu/mL and incubated with the cells for 48 h at 37 °C in complete media. The cells were visualized under an epifluorescent inverted microscope (IX71; Olympus, Tokyo, Japan). All the transfections were done in triplicate and performed at least twice.

HCPT treatment HCPT treatments were performed 48 h after the addition of the phages in medium containing 10% FBS. The medium was removed at 48 h after the phage addition, and the cells were incubated with HCPT at 2.5 μ mol/L for 6 h at 37 °C. This was followed by replacement with fresh medium and an additional incubation of 18 h at 37 °C.

Western blot analysis In total, 10⁶ H1299 cells transfected transiently with a variety of phagemid particles were lysed after HCPT treatment, as described in the Results. Prior to the lyses, the cells were washed with PBS and collected by scraping. Then, they were lysed in ice-cold Tris buffer (50 mmol/L, pH 7.5) containing 5 mmol/L EDTA, 300 mmol/L NaCl, 0.1% Igepal, 0.5 mmol/L NaF, 0.5 mmol/L Na₃VO₄, 0.5 mmol/L phenylmethylsulfonyl fluoride, and antiprotease mixture (Roche Molecular Biochemicals, Indianapolis, IN, USA), sonicated, and centrifuged at 13 000 \times g for 10 min. The supernatant was used for protein determination by the Bradford procedure (Bio-Rad, USA) and Western blotting. The proteins were resolved on 12% SDS-PAGE, transferred onto nitrocellulose membranes, and incubated with the appropriate antibodies. The following protocols are the same as described earlier. The anti-pSer473-AKT antibody (Cell Signaling Technology, Beverly, MA, USA) was used in 1:1000 dilutions. The goat antimouse immunoglobulin G conjugated with HRP (ImmuClub Labs, Sunnyvale, CA, USA) was diluted at 1:10 000 in 1% BSA in PBST. After 1 h incubation, the filters were washed with PBST, developed using a SuperSignal West Pico kit (Pierce Biotechnologies, Rockford,

IL, USA) for 5 min, and exposed to X-ray film.

Results

Construction of siRNA expression vectors To improve the expression of siRNA against Akt, the pSilencer4.1-CMV vector which carries the CMV promoter was used. pSi4.1CMV-fl was constructed by inserting the F1 origin sequence into pSilencer4.1-CMV. pSilencer4.0-siAKT was obtained by insert shRNA against Akt into pSi4.1CMV-fl.

ssDNA analysis To examine the ratio of phagemids to helper phage genomes packaged in the phagemid particles, we analyzed the ssDNA. The results indicate that almost all of the DNA packaged were phagemids (Figure 1).

In vitro inhibition of EGFP expression by EGF displaying pSil.0-siEGFP phagemid particle infection To test whether phagemid particles carrying siRNA against EGFP can inhibit EGFP expression, we transfected the pEGFP-N1 plasmid into NCI-H1299 cells. At 12 h after transfection, the phagemid particles (10¹¹ pfu/mL) were transduced into the cells. At 24 h after transduction, HCPT was added into the culture at a final concentration of 2.5 μ mol/L; the cells was incubated for another 24 h. The data shown in Figure 2 indicate that pSil.0-siEGFP can significantly inhibit the expression of EGFP.

Phagemid particles of pSi4.1-siAkt can inhibit Akt expression of NCI-H1299 in the presence of HCPT The NCI-H1299 cells was infected with pSi4.1-siAkt phagemid particles packaged by M13KO7EGFCT. HCPT was added to improve the siRNA expression. Western blotting was performed to examine the Akt expression.

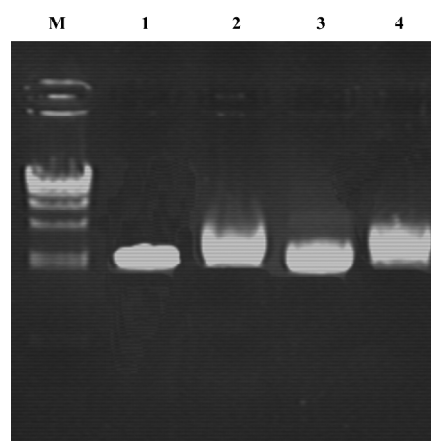


Figure 1. Analysis of the ssDNA released from phagemid particles Lane M: λ DNA/*Hind*III; Lane 1: pSi1.0-siEGFP phagemid particles; Lane 2: pSi4.1-siAkt phagemid particles; Lane 3: pSi1.0-Mock phagemid particles; Lane 4: pSi4.1-Mock phagemid particles

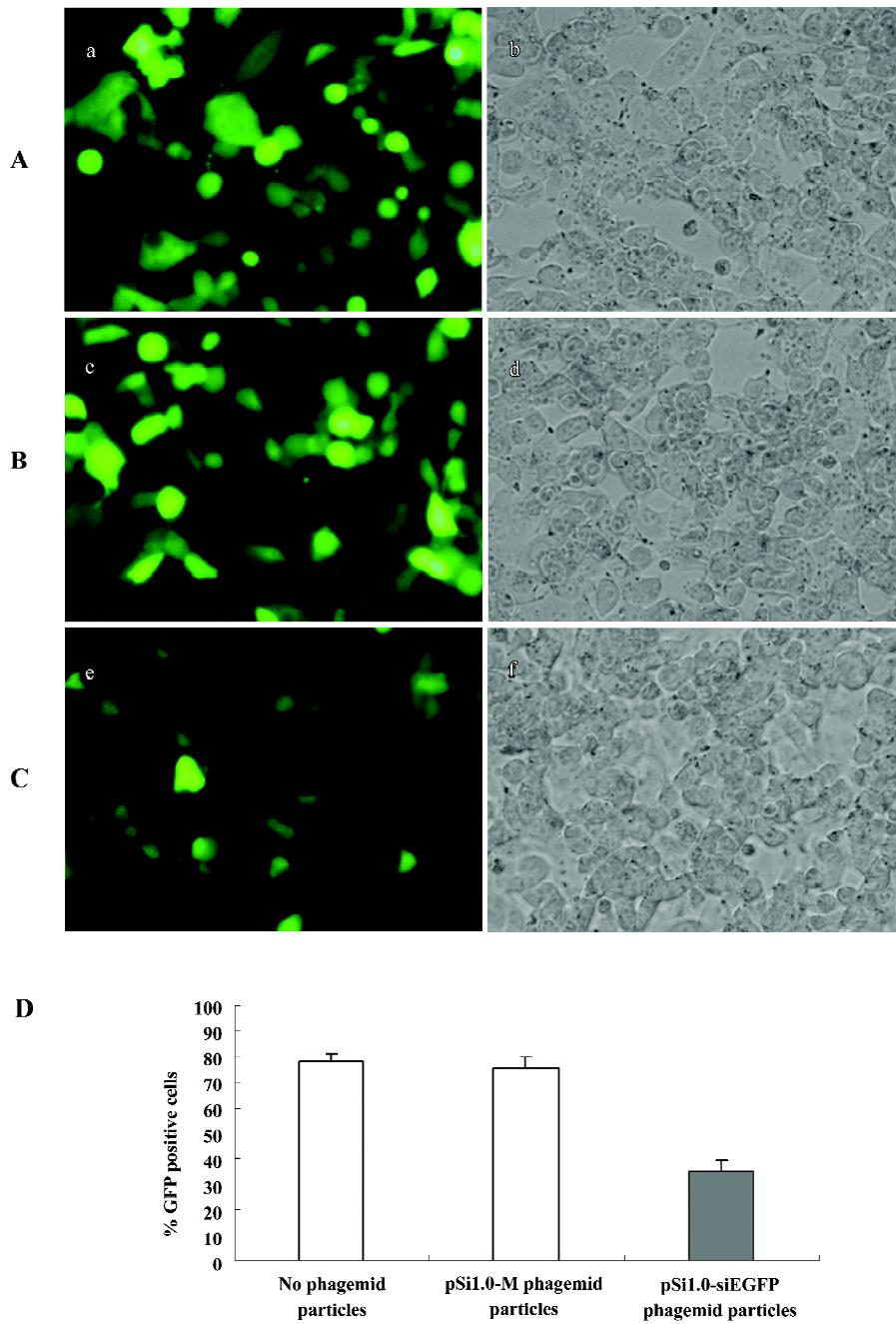


Figure 2 Inhibiting EGFP expression by transduction of pSi1.0- siEGFP phagemid particles into NCI-H1299 cells carrying pEGFP-N1 in the presence of hydroxycamptothecin. (A) No phagemid particles added; (B) pSi1.0-M phagemid particles packaged with M13EGFKO7CT; (C) pSi1.0-siEGFP phagemid particles packaged with M13EGFKO7CT; (D) Quantitation of GFP positive cells transfected by phagemid particles (GFP positive cells from duplicate wells were averaged and experiments were performed in triplicate. In total, 50 000 cells per well were counted each time. Error bars represent the standard deviation of the mean). All the images were taken at the same exposure. (Magnification: 400). a,c,e, represent cells visualized under fluorescence microscope; b, d, f, represent cells visualized under light microscope.

As shown in Figure 3A, the pSi4.1-siAKT plasmid transfected by Lipofectamine can inhibit the Akt expression by approximately 50%. No inhibition was found in mock vector

pSi4.1-M.

As shown in Figure 3B, in the absence of HCPT, the phagemid particles of siAkt can not efficiently inhibit the

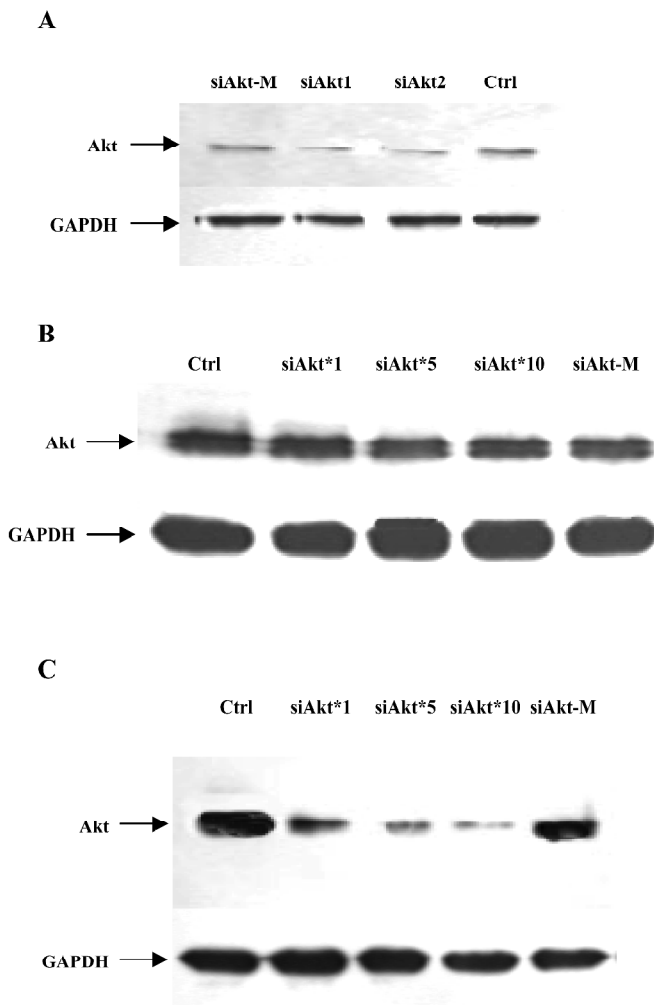


Figure 3 Western-blot analysis of the specific Akt gene silencing by EGF-targeted phagemid particles mediated RNA interference. (A) pSi4.1-siAkt RNAi expression plasmid was transfected into NCI-H1299 cells using Lipofectamine 2000. 72 h later the cells were harvested, and the obtained total cell lysates were subjected to SDS-PAGE and immunoblotting; siAkt1 and siAkt2 are duplicate experiments of the siAkt transfection. (B) NCI-H1299 cells were transfected with different doses of pSi4.1- siAkt phagemid particles in the absence of hydroxycamptothecin; (C) NCI-H1299 cells were transfected with different doses of pSi4.1- siAkt phagemid particles in the presence of 2.5 $\mu\text{mol/L}$ hydroxycamptothecin. siAkt*1, siAkt*5 and siAkt*10 represent 1×10^{10} pfu/mL, 5×10^{10} pfu/mL, 1×10^{11} pfu/mL pSi4.1-siAkt phagemid particles respectively. Ctrl represent no phagemid particles added. siAkt-M represent mock siRNA phagemid particles.

Akt expression. However, in the presence of HCPT (Figure 3C), the siAKT phagemid particles can inhibit the Akt expression in a dose-dependent manner. The highest inhibition was 50%–60% when 1×10^{11} pfu/mL particles were added.

Discussion

In this study, we utilized a novel phagemid system to deliver several siRNA into mammalian cells and estimated their effect *in vitro*. The phagemid vectors have a number of advantages over other vectors. Unlike other viral gene delivery vector, filamentous phages have no natural mammalian cell tropism. Phagemid particles can be easily retargeted by displaying various ligands, such as peptides, scFv and natural ligands (eg EGF, FGF)^[5,8,14–16]. In addition, it is very efficient to prepare the phagemid particles rapidly (about 2–3 d). In previous studies, phages were modified so that they could be used in gene delivery. The ability of cell-targeted

phages to deliver siRNA would be of interest. Since phage genomes for packaging replicates by rolling circles, we examined in this study whether the hairpin structure of the siRNA-encoding sequence will impede its replication and package. The results clearly demonstrate that phagemid particles carrying siRNA can be prepared as efficiently as those carrying other protein-encoding genes.

This study also demonstrates that cell-targeted phagemid particles carrying siRNA can be expressed efficiently in the presence of HCPT, although further studies need to be performed to elucidate the mechanism.

We also tested whether the phagemid particles carrying siRNA against Akt have some effect on cell growth. However, no significant inhibition was obtained. This is not uncommon, because the EGF ligand on the phagemid particles has a cell-growth stimulatory effect that might compensate the cell growth inhibitory effect contributed by the siRNA. Thus, to be a cancer gene-delivery vector, phage vectors should display ligands that do not prompt cell growth

or carry siRNA against other oncogenes, such as PI3K.

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