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Targeted Delivery with Imaging Assessment of siRNA Expressing Nanocassettes into Cancer

Wei Chen^{1,2} and Lily Yang¹

Lily Yang: Lyang02@emory.edu

¹Department of Surgery, Emory University School of Medicine, Clinic C, Room C-4088, 1365 C Clifton Road, NE, Atlanta, GA, 30322 USA

²Department of Gastrointestinal Surgery, First Affiliated Hospital of Sun Yat-Sen University, Guangzhou, China

Abstract

Molecular therapy using small interfering RNA (siRNA) shows great promise in the development of novel therapeutics for cancer. Although various approaches have been developed for in vivo delivery of siRNAs into tumors, stability of siRNA in blood circulation, and low efficiency of siRNA delivery into tumor cells are the major obstacles for further translation into cancer therapeutics. In this protocol, we describe methods of the production of shRNA expressing DNA nanocassettes by PCR amplification of double-stranded DNA fragments containing a U6 promoter and a shRNA gene. Those DNA nanocassettes can be conjugated to the polymer coating of nanoparticles that are targeted to cellular receptors highly expressed in tumor cells, such as urokinase plasminogen activator receptor (uPAR), for targeted delivery and receptor mediated internalization of shRNA expressing DNA nanocassettes. Methods for in vitro and in vivo evaluation of target specificity and gene-knockdown effect are also provided.

Keywords

Targeted nanoparticle; In vivo siRNA delivery; Imaging; Cancer therapy; shRNA expressing nanoparticle

Introduction

Human cancer cells are highly heterogeneous with dysfunctional signal pathways and intrinsic resistant mechanisms to stress and therapeutic agent induced cell death. To effectively treat drug resistant tumor cells, inhibition of key molecules that play the major roles in drug resistance by molecular targeted therapy using small interfering RNA (siRNA) is a promising approach in sensitizing tumor cells to therapeutic agents [1]. Although various approaches have been developed for delivering siRNAs into tumor cells, it has been very challenging to efficiently deliver sufficient amounts of siRNA into tumor cells in vivo following systemic delivery. The development of targeted siRNA delivery approaches that

enable effective delivery of siRNA or siRNA producing vectors into tumor cells in vivo is significant for translating this molecular therapy into clinical applications [2, 3].

siRNA is a double-stranded RNA molecule with 19–23 base pairs in length and interacts with mRNA to inhibit the level of a specific gene expression. Short hairpin RNA (shRNA) is a hairpin RNA molecule that is processed by endoribonuclease (Dicer) to siRNA. shRNA can be chemically synthesized or expressed from a gene expression vector containing a U6 or H1 promoter and an shRNA gene [4]. Advantages of delivery a DNA-based shRNA expressing vector are its in vivo stability and the ability of expression of a high level of shRNA inside cells to prolong the effect of the gene knocking-down. However, the whole length shRNA plasmid or viral vector is very large (>4000 base pairs), with a large proportion of sequence supporting vector propagation and selection. Thus, to enable efficient delivery of shRNA into cells, we developed an shRNA expressing DNA cassette containing only core targeting sequence of shRNA and a U6 promoter to support its transcription inside eukaryotic cells [5].

We started by cloning the shRNA gene sequence into an shRNA expressing plasmid backbone, such as pSilencer. shRNA expressing DNA nanocassettes were then amplified by Polymerase Chain Reaction (PCR) using a pair of primers covering a short 5'-flank region of U6 promoter and a short 3'-terminal flank region following the shRNA core targeting sequence. This shRNA expressing double-stranded DNA nanocassette is around 500 base pairs (bps). It has equivalent inhibitory efficiency as the full length plasmid [5].

To deliver the shRNA cassette into targeted cells, we used quantum dots (QDs) or magnetic iron oxide nanoparticles (IONPs) to carry the shRNA cassette and serve as imaging probes for monitoring the delivery of the DNA nanocassettes. QDs produces fluorescent signal and can be used to track localization of the nanocassettes in vitro by cell imaging and in vivo by optical imaging. However, potential clinical application in humans may be limited due to a low sensitivity of optical imaging to detect signals in deep tissues and long-term toxicity concerns [6]. IONP is biocompatible and biodegradable, and produce MRI contrast for noninvasive tumor imaging [7]. The surface of the above nanoparticles has been functionalized with carboxyl residuals, which can be used for conjugation with amine containing molecules [7]. To conjugate shRNA nanocassettes to IONP, the reverse PCR primer is modified with an amine at the 5' end, so that the PCR products can conjugate to the carboxyl group of the polymer coating by an amide bond mediated by EDAC [8].

Another advantage of these nanoparticles is that they can be decorated with many molecules simultaneously to enable multifunction, such as dyes for optical imaging, targeting ligand for tumor specific delivery, and chemotherapeutic drugs to enable therapeutic effect [9]. In this protocol, we use amino terminal fragment (hATF) of human urokinase plasminogen activator (uPA) as a targeting ligand, which targets its receptor uPAR in tumors [10, 11]. The design of this nanoparticle is illustrated in Fig. 1. Using a luciferase shRNA as a model system, we show the methods of conjugation of shRNA expressing DNA nanocassettes to nanoparticles and evaluation of the effect of the nanoparticle shRNA delivery carriers in vitro in cancer cell lines and in vivo in animal tumor models. Results of our studies showed that uPAR targeted nanoparticles could efficiently deliver luciferase shRNA cassettes into

cancer cells in vitro and inhibit target gene expression. In vivo administration of nanoparticles in human cancer xenograft models led to the inhibition of the level of luciferase gene expression in tumors. This protocol provides a framework for the development of targeted delivery of siRNA using shRNA expressing DNA cassettes both in vitro and in vivo, as well as method for monitoring delivery of nanoparticle-shRNA delivery carriers by noninvasive optical imaging.

Materials

shRNA Cloning

1. An appropriate shRNA expressing plasmid, such as pSilencer™ 2.1-U6 neo that can be purchased from Applied Biosystems (Life Technologies, Carlsbad, CA).
2. Synthesizing shRNA template sequences. The inserted shRNA fragments are chemically synthesized (Integrated DNA Technology, Coralville, Iowa) containing the following structure: 5'-GATCC (BamHI)-19–23 nt shRNA sense sequences-TTCAAGAGA (Loop sequence)-19–23 nt shRNA antisense sequences-TTTTTTGGAAA (Terminate sequence)-A (HindIII). The shRNA sense sequences are listed in Table 1.
3. Restriction endonucleases BamHI and HindIII, T4 DNA ligase (Sigma-Aldrich, St. Louis, MO).
4. Synthesizing PCR primers for amplification of shRNA cassettes (*see* Note 1). The sequences of PCR primers are listed in Table 1.
5. OneTaq® DNA Polymerase PCR kit for PCR reaction (M0480, New England Biolabs, Ipswich, MA).
6. Spectrophotometer for DNA quantification (Bio-Rad Laboratories, Inc., Hercules, CA)

Nanoparticles and Bioconjugation

1. Amphiphilic polymer coated quantum dots (QDs, emission wavelength of 620 nm) or iron oxide nanoparticles (IONPs) with 10 nm core size are provided by the Ocean Nanotech (Ocean Nanotech, San Diego, CA).
2. 6.5 mM (1 mg/mL) EDAC (Pierce, Rockford, IL) dissolved in borate buffer (pH 5.0). EDAC solution should be prepared before conjugation and used immediately.
3. 4.6 mM (1 mg/mL) sulfo-NHS (Pierce, Rockford, IL) dissolved in borate buffer (pH 5.0). sulfo-NHS solution should be prepared before conjugation and used immediately.

¹Use sterile water, pipette tips, and tubes in the experiment. Apply aseptic techniques as strictly as possible. This is extremely important when the conjugated nanoparticles will be used in animals.

4. 50 mM borate buffer prepared by diluting the 20× stock solution (Pierce, Rockford, IL) with water. Adjust to pH 5.0 or pH 8.6 (*see* Note 2).
5. Nanosep 100 k column (Pall Corp, Ann Arbor, MI).
6. Targeting ligands can be antibodies, antibody fragments, peptides, and recombinant natural ligands. For the uPAR targeting ligand, we use the 135 amino acids of recombinant receptor binding domain of uPA. cDNA of the recombinant amino terminal fragment of human uPA (hATF) is cloned into pET20a plasmid (Life Technologies, Carlsbad, CA), and expressed in *E. coli* BL21 bacterial expression system [10, 11].
7. Ni-NTA agarose beads (Qiagen, Valencia, CA).
8. Bradford protein assay (Bio-Rad Laboratories, Inc., Hercules, CA).
9. Zetasizer Nano (Malvern Instruments Inc., Southborough, MA).
10. Syngene G:BOX imaging system (Syngene, Frederick, MD).
11. Centrifuge 5430 R (Eppendorf, Hamburg, Germany).

Cell Lines and Animals

1. Cancer cell lines that stably express a firefly luciferase gene can be used to establish human tumor xenograft model. We use both the MCF-10DCIS human breast cancer cell line (Asterand US, Detroit, MI) [12] and the MIAPaCa-2 human pancreatic cancer cell line (MIAPaCa-2-luc, kindly provided by Dr. Rosa Hwang, MD Anderson Cancer Center, Houston, TX) [13]. MCF-10DCIS cells are cultured in the DMEM/F12 medium supplemented with 5 % horse serum. MCF-10DCIS cell line was transfected with a lentiviral vector, LV-pUB-Fluc-eGFP that provided dual firefly luciferase and enhanced green fluorescence protein (eGFP) expression (hereafter MCF-10DCIS-luc). MIAPaCa-2-luc cells are cultured in the DMEM medium supplemented with 10 % fetal bovine serum.
2. Athymic nude mice (6–8 weeks old) for establishment of human tumor xenograft models can be purchased from qualified vendors.
3. D-Luciferin substrate (Caliper Life Sciences, Hopkinton, MA) is prepared into a fresh stock solution at 15 mg/ml in DPBS.
4. Xenogen IVIS Spectrum system (Caliper Life Sciences, Hopkinton, MA).
5. Olympus OV-100 imaging system (Olympus America Inc., Central Valley, PA) or other in vivo optical imaging systems.
6. Lipofectamine 2000 (Life Technologies, Carlsbad, CA).

²To protect the DNA cassettes from digestion by deoxyribonucleases after injected into mice, the PCR primers can be modified at the 5' end with a phosphorothioate linkage [14].

Methods

Cloning Plasmid

1. Anneal the shRNA templates by heating the templates followed by gradually cooling.
2. Linearize pSilencer™ 2.1-U6 neo plasmid with restriction endonucleases BamHI and HindIII.
3. Ligate the annealed shRNA template sequences with the linearized plasmid using T4 DNA ligase.
4. Transform competent cells, such as DH5 alpha, with the ligation products. Plate the transformed cells on LB plates containing 100 µg/ml ampicillin and grow overnight at 37 °C.
5. Pick clones, isolate plasmid DNA, and validate the plasmid by DNA sequencing. For detailed information of cloning procedure, please check the protocol provided by the manufacturer of the plasmid.

Amplification of shRNA Expressing DNA Cassettes

1. Set up PCR reaction with the following components: 10 µl 5× OneTaq Standard Reaction Buffer, 1 µl of 10 mM dNTPs, 10 µM of forward and reverse primers each, 0.25 µl of OneTaq DNA Polymerase, and 1 ng of plasmid DNA. Add water to make the final volume to 50 µl.
2. Run the PCR using the following thermocycling condition: initial denaturation at 94 °C for 5 min; then 94 °C for 30 s, 58 °C for 30 s, 68 °C for 50 s for a total of 32 cycles; final extension at 68 °C for 5 min (see Notes 3 and 4).
3. Purify the PCR products by ethanol precipitation and resuspend the purified DNA in water.
4. Quantify the DNA with a spectrophotometer.
5. Test DNA by 2 % agarose electrophoresis.

Preparing hATF Protein

Recombinant hATF protein is expressed in *E. coli* BL21 and purified from bacterial extracts under native conditions using Ni-NTA agarose beads. The purified protein is then examined by 12 % SDS-PAGE, and followed by Coomassie Blue staining. The concentration of protein is quantified by Bradford protein assay.

³PCR conditions may need to be optimized according to different shRNA, PCR kit used in order to get the highest yield of PCR reaction.

⁴For in vivo study, a large amount of shRNA cassettes will be needed. To generate enough shRNA cassettes, simply set up large scales of PCR reactions and run the PCR in parallel. In our experiment, about 5 µg of product can be generated after a standard PCR run with 50 µl volume.

Production of Targeted Nanoparticles

Conjugation of Targeting Ligand to Amphiphilic Polymer-Coated Nanoparticles, Such as QDs and IONPs

1. Spin down the buffer that is used to store nanoparticles with a Nanosep 100 k column at $2000 \times g$ for 3 min.
2. Resuspend nanoparticles with 100 μ l of borate buffer (pH 5.0).
3. Add fresh made EDAC and sulfo-NHS solution into nanoparticles at a molar ratio of 1:400:200 for nanoparticles:EDAC:sulfo-NHS. Mix thoroughly by vortexing. Rotate the tube at room temperature for 10 min.
4. Stop the reaction by spinning the solution in a Nanosep 100 k column at $2000 \times g$ for 3 min. Thus, the total reaction time (rotation plus centrifuging) should be less than 15 min.
5. Resuspend the activated nanoparticles by adding 200 μ l of borate buffer (pH 8.6). Carefully add small drops of hATF into the solution and mix by vortexing. Repeat the process until all hATF is added. The molar ratio of nanoparticles:hATF is 1:10. Rotate the solution at room temperature for an additional 4 h.
6. Transfer the solution into a 100 K Nanosep column and centrifuge at $2000 \times g$ for 3 min until all the media is filtered through (longer time of centrifuging may be needed).

Assembling Nanoparticle-hATF and shRNA

1. Resuspend nanoparticle-hATF conjugates from the previous step with 100 μ l of borate buffer (pH 5.0).
2. Conjugate nanoparticle-hATF with shRNA using the EDAC method as described in Subheading 3.4.1. The molar ratio of nanoparticles:shRNA can be adjusted based on the applications, typically in a range of 1:1 to 1:10.
3. Wash nanoparticles with sterilized water for two times, and spin down the media in a 100 K Nanosep column at $2000 \times g$ for 3 min until all the media is filtered through (longer time of centrifuging may be needed).
4. Resuspend nanoparticles with desired amount of water based on nanoparticle concentrations required for specific applications and collect the products.

Characterizing Nanoparticles

1. Determine concentration of nanoparticles. For IONPs, read the OD at 500 nm after 50–100 times dilution of nanoparticle in water. Multiply the absorbance value with the dilution factor then divide by a factor of 4.3 to get the iron concentration in mg/ml. For QDs, read the fluorescence at emission wavelength of 620 nm. Use standard sample (like the stock QDs) to create a reference curve, and calculate the concentration of QD products based on the curve.

2. Examine the particle size distribution using Zetasizer Nano.
3. Examine the nanoparticles using electrophoresis in 2 % agarose gel at 100v for 30 min. Stain the gel with DNA dye, then examine the gel using an optical imaging system (QDs) or UV-transilluminator (IONPs) (*see* Note 6) (Fig. 2).

Testing the Nanoparticle In Vitro

1. A luciferase gene stably transfected cell line, such as MIAPaCa-2-luc, is added into a 96-well tissue culture plate for 24 h to grow to 50 % confluence.
2. Dilute shRNA DNA cassettes or hATF/shRNA conjugated nanoparticles containing equivalent of shRNA in RPMI-1640 media and then add 100 μ l of prepared media containing 20 pmol of shRNA DNA cassettes to each well, and treat cells for 4 h.
3. Replace the media with full culture media containing 10 % FBS, and keep for 44 h.
4. To serve as a positive control, transfect the same amount of shRNA cassettes using a transfection reagent, such as Lipofectamine 2000 using the manufacturer's protocol.
5. Two days after treatment, perform optical cell imaging by adding 150 μ g/ml D-Luciferin substrate to the culture media, and detecting luciferase activity using Xenogen IVIS Spectrum system as shown in Fig. 3 (*see* Note 5).

Testing the Nanoparticle In Vivo

Establish Human Tumor Xenograft Mode—The MCF-10DCIS-luc human breast cancer cells at 80 % confluence are trypsinized and washed with PBS. Orthotopic human breast cancer xenograft model is established by injecting 1×10^6 cells into the mammary fat pad of the nude mice.

Administrate Nanoparticles into Tumor Bearing Mice—One week after tumor inoculation when tumor is about 5–8 mm in diameter, Inject 200–300 picomolar of nanoparticles, such as QDs, carrying hATF and luciferase siRNA-expressing DNA cassettes (about 2 nmol of DNA cassettes) into the tail vein of mice.

In Vivo Bioluminescence Imaging (BLI)

1. Before and at different time points following the nanoparticle administration, perform the bioluminescence imaging to assess luciferase activity of tumors. Firstly, anesthetize the mice by injecting a mixture of 95 mg/kg ketamine hydrochloride and 5 mg/kg xylazine in sterile saline intraperitoneally (i.p.).

⁶For examination of IONP conjugates, nanoparticles and DNA cassettes are loaded onto agarose gel and run the electrophoresis at 100 v for 30 min. Stain the gel with DNA dye. Then the gel can be visualized using a gel imaging system, for example Syngene G:BOX imaging system. Apply the UV filter to see the fluorescence from DNA or IONP-hATF/shRNA conjugates. Switch off the UV filter to visualize IONP (brown color).

⁵Various methods can be used to assess the delivery efficacy of nanoparticles in vitro, for example RT-PCR, western blot or bioluminescence imaging for detecting target the level of gene expression.

2. Inject D-Luciferin substrate 150 mg/kg body weight i.p. into the nude mice 5 min before each bioluminescence imaging procedure.
3. Perform the bioluminescence imaging using Xenogen IVIS Spectrum system by following the instructions of the manufacturer (Fig. 4).

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Fig. 1. Schematic illustration of the design of siRNA expression nanoparticle. siRNA expressing DNA cassette is cloned from a plasmid by PCR amplification. The PCR products encompass a U6 promoter and shRNA gene sequence, and are labeled with an amine group at one end. The shRNA cassettes and targeting ligands are then conjugated to nanoparticles using carboxyl/amine reaction. (Reprinted with permission from John Wiley & Sons, Inc. [5])

Fig. 2.

Agarose gel electrophoresis of QD-hATF/shRNA nanoparticles. The *left panel* shows QDs in *red* the *middle panel* shows DNA in green fluorescence; the *right panel* shows the overlay of QDs with DNA. QDs that conjugated with DNA nanocassettes show *orange color*. (Reprinted with permission from John Wiley & Sons, Inc. [5])

Fig. 3.

Testing gene knockdown efficacy of luciferase shRNA nanoparticle carriers in vitro. MIAPaCa-2-luc cells cultured in 96-well plates are incubated with 20 pmol of luciferase shRNA cassette (shRNA-luciferase) or IONP-hATF-luc siRNA expressing cassettes containing equivalent molar of DNA. To serve as a positive control, shRNA-luciferase cassette is also delivered into cells with a classic transfection reagent Lipofectamine 2000. Luciferase activity of cells is measured 48 h following the incubation with D-Luciferin substrate using the Xenogen IVIS system. The result shows that IONP-hATF-luc has better inhibitory efficacy of luciferase activity than the other groups. (Reprinted with permission from John Wiley & Sons, Inc. [5])

Fig. 4.

Evaluation of targeted delivery and efficiency of inhibition of gene expression using uPAR targeted shRNA delivery nanoparticles in vivo in a human tumor xenograft model. QD-hATF-luc or control scrambled shRNA nanocassette are injected into nude mice bearing human breast cancer via the tail vein. Bioluminescence images are taken at different time points following the injection. Numbers in the figure show changes in the percentages of luciferase activity compared with the level in the tumor before the treatment. QD-hATF-luc nanoparticle shows substantial inhibitory effects during a 6-day observation post treatment. (Reprinted with permission from John Wiley & Sons, Inc. [5])

Table 1

Oligonucleotides for shRNA plasmid cloning and PCR

	Name	Sequence
Cloning primers	Random control	5'AAGAGGCTTGCAACAGTGCA3'
	Firefly luciferase	5'CGGATTACCAGGGATTCA3'
PCR primers	Forward	5'GATGTGCTGCAAGGCGATTA3'
	Reverse	*5'GGAAACAGCTATGACCATGA3'

* The reverse primer is modified at the 5' end with an amine group for conjugation to nanoparticles