Published in final edited form as:

Methods Mol Biol. 2013 ; 1026: 137–147. doi:10.1007/978-1-62703-468-5_11.

SINGLE-WALLED CARBON NANOTUBE–MEDIATED SMALL INTERFERING RNA DELIVERY AND SILENCING GASTRIN-RELEASING PEPTIDE RECEPTOR IN HUMAN NEUROBLASTOMA CELLS

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Abstract

Small interfering RNA (siRNA) has the potential to influence expression with a high degree of target gene specificity. However, the clinical application of siRNA therapeutics has proven to be less promising as evidenced by poor intracellular uptake, instability *in vivo*, and non-specific immune stimulations. Recently, we have demonstrated that single-walled carbon nanotube (SWNT)-mediated siRNA delivery can enhance the efficiency of siRNA-mediated gastrin-releasing peptide receptor (GRP-R) gene silencing by stabilizing siRNA while selectively targeting tumor tissues. Based on our recent findings, we introduce a novel technique to silence specific gene(s) in human neuroblastoma through SWNT-mediated siRNA delivery *in vitro* and *in vivo*.

Keywords

SWNT; siRNA; GRP-R; Delivery; Image; Neuroblastoma

1. INTRODUCTION

Neuroblastoma is the most common extra-cranial solid tumor in infants and children. Despite recent advances in multimodality therapy, survival rates for all stages of tumors remain a dismal 50%. Novel therapeutic options are, therefore, urgently needed to improve patient outcomes [1]. Gastrin-releasing peptide (GRP), the mammalian equivalent of bombesin, is a neuroendocrine peptide that has been shown to have a growth stimulatory effect on various cancer cell types [2–4]. We have previously demonstrated that GRP and its cell surface receptor, GRP-R, are abundantly expressed in human neuroblastomas [2, 5] and that GRP-R silencing significantly suppresses tumorigenesis and metastatic potential in mouse tumor models of neuroblastoma [6]. To employ siRNA, we relied on the silencing of GRP-R by techniques previously described in the literature [6].

However, current applications of siRNA therapeutics are limited by lack of effective delivery systems. Therefore, an efficient and safe targeted silencing delivery system is highly desired. Recently, single-walled carbon nanotube (SWNT)-mediated delivery systems have garnered significant interest as a novel tool for drug delivery. This is, in large part, due to the ability of SWNTs to cross cell membranes and transport a wide range of biologically active molecules including drugs, proteins, DNA, and RNA into cells [7–9]. The cytotoxicity of SWNTs is mainly dependent on their surface functionalization, where minimal toxic effects were seen in well-functionalized, serum-stable SWNTs. For example, phospholipid-polyethylene glycol (PL-PEG)-coated SWNTs have been shown to be nontoxic and are widely used *in vitro* and *in vivo* [10].

Moreover, SWNTs have emerged as one of the most promising candidates in the field of diagnostic imaging due to their unique optical properties [10–12]. The emission wavelength of SWNTs is in the near-infrared (NIR) range, where most biological tissues and cells are transparent and have low inherent autofluorescence. The fluorescence of SWNTs is extremely stable and can last for months, while fluorescent proteins, which are currently used for imaging living systems, begin to fade within hours. Thus, SWNTs may have significant implications in how we approach fluorescent visualization.

With the capability of penetrating cells and emitting non-bleaching NIR fluorescence, functionalized SWNTs offer the potential to be used as carriers for the delivery of siRNA and diagnostic imaging agents *in vivo*. To address the aforementioned application, we have developed a straightforward protocol for silencing GRP-R in human neuroblastoma through SWNT-mediated siRNA (SWNT-siRNA) delivery *in vitro* and *in vivo*. Western blotting and immunohistochemistry techniques have been used to confirm GPR-R silencing.

2. MATERIALS

2.1 Single-Walled Nanotubes (SWNTs)

1. High-Pressure CO conversion (HiPco) SWNTs.

2.2 Chemicals

1. PL-PEG (1,2-distearoyl- sn -glycero-3-phosphoethanolamine- N –[methoxy (polyethylene glycol)-5000]) (Avanti Polar Lipids Inc).
2. DTT (D,L-dithiothreitol) (Sigma-Aldrich).

2.3 Biological Reagents

1. Antibodies:
 - a. GRP-R primary antibody (Abcam).
 - b. β -actin antibody (Sigma-Aldrich).
 - c. Secondary antibodies against mouse and rabbit IgG (Santa Cruz Biotechnology, Inc.).

- d. Rabbit polyclonal anti-human phospho-histone H3 (Ser10) antibody (Millipore).
 - e. Alexa Fluor 568 Goat Anti-Rabbit IgG (Life Technologies, Grand Island, NY).
2. siRNAs were custom synthesized by Thermo Scientific (Dharmacon):
 - a. Custom siRNA Design Tool, Thermo Scientific siDESIGN Center, <http://www.dharmacon.com/PopUpTemplate.aspx?id=2078>
 - b. The siRNA targeting GRP-R (NM_005314) sequence:
 - sense: 5'-Thiol- UAACGUGUGCUCCAGUGGAdTdT-3'
 - antisense 3'-dTdT AUUGCACACGAGGUCACCU-5'
 - c. Luciferase siRNA (control):
 - sense: 5'-Thiol-CUUACGCUGAGUACUUCGAdTdT-3'
 - antisense: 3'-dTdTGAAUGCGACUCAUGAAGCU-5'
 - d. siRNA solution can be prepared by dissolving siRNA in RNase-free water to reach a siRNA concentration of 100 μ M. Store aliquots of the siRNA solution at -80°C , which can be stable for up to 6 months. However, avoid more than three freeze-thaw cycles.
 3. Cell-Counting Kit-8 (Dojindo Molecular Technologies, Inc.).
 4. Cell lysis buffer (10 $\times$) (Cell Signaling Technology).
 5. Protein inhibitors cocktail (Roche Applied Science).
 6. NuPAGE Novex 4–12% Bis–Tris Gel (Invitrogen).
 7. NuPAGE MOPS Running buffer (20 $\times$) (Invitrogen).
 8. TG (10 $\times$) buffer (Bio-Rad).
 9. Western Lighting Plus-ECL, enhanced Chemiluminescence substrate (PerlinElmer, Inc.).
 10. X-Ray Film, Blue Basic Autorad Film (ISC BioExpress).
 11. Immun-Blot PVDF Membrane (Bio-Rad).
 12. Agarose gels (Cambrex Bio Science Rockland, Inc., Rockland, ME).
 13. DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride, Sigma-Aldrich).
 14. DAKO EnVision + System-HRP (Dako North America, Inc., Carpinteria, CA).

2.4 Cell Culture Reagents

1. The human neuroblastoma cell lines SK-N-SH and BE(2)-C (American Type Culture Collection).
2. RPMI 1640 medium and 0.25% trypsin (Mediatech Inc.).

3. Fetal bovine serum (FBS) (Sigma- Aldrich).
4. Phosphate-buffered saline (PBS), 10×, pH 7.4 (Invitrogen).
5. Penicillin–streptomycin, liquid (10,000 U penicillin; 10,000 µg streptomycin) (Invitrogen, GIBCO).

2.5 Instruments

1. Bath Sonicator, Branson 2510.
2. Cole Palmer CPX-600 cup-horn sonicator.
3. Polyscience X-520 Homogenizer, 750 W.
4. Sorvall Legend X1R Centrifuge (Thermo Scientific); Rotor, FiberLite, F15-8X50C (Piramoon Technologies Inc.).
5. Sorvall WX Ultra Centrifuge with Surespin 630 swing bucket rotor (Thermo Scientific)
6. Dialysis tubing 15 ml, 3,500 MWCO (Fisher Scientific).
7. Amicon centrifugal filter device 4 ml, 100,000 MWCO (Millipore).
8. Microcon Ultracel YM-100 filter device 0.5 ml, 100,000 MWCO (Millipore).
9. Illustra NAP-5 columns, Sephadex G-25 DNA Grade (GE Healthcare).
10. Cary-5000 UV–VIS–NIR Spectrometer (Varian).
11. Cell culture plates and flasks (BD Falcon).
12. Cell culture incubator, Thermo Series II, water Jacketed CO₂ Incubator, HEPA class 100, (Thermo Electron Corporation).
13. FlexStation 3, (Molecular Devices Corp.).
14. Immuno blotting system (Invitrogen).
15. Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad).
16. Nikon Eclipse E600.
17. Leica DMI6000 B.
18. Inverted Microscope IX 71 (Olympus).
19. IR enhanced water immersion objective (Olympus).
20. Liquid N₂ Cooling 2D OMA-V 320 Detector (Princeton Instruments).
21. ProEM 512B CCD (Princeton Instruments).

3. METHODS

3.1 Preparation of Functionalized SWNT

1. Weigh 1 mg of HiPco SWNTs and 5 mg of PL-PEG into a 20 ml glass scintillation vial. Add 5 ml of water. Dissolve PL-PEG completely by shaking.
2. Sonicate the vial in an ice water bath sonicator for 120 min and change the ice water every 30 min to avoid overheating.
3. Centrifuge the SWNT suspension at $122,000 \times g$ and at room temperature for 4 h, and then decant the upper 75 – 80% of the supernatant.
4. Record the UV VIS NIR absorption spectrum of the prepared PL-PEG functionalized SWNT (PEG-SWNT) solution. The final SWNT concentration normally ranges from 20 to 30 mg/l. Store the PEG-SWNT solution at 4 °C.
5. Add 4 ml of the PEG-SWNT solution from the prepared stock in Step 3 into a 4 ml Amicon centrifugal filter device with a molecular weight cutoff (MWCO) of 100 kDa. Centrifuge the device at $4,000 \times g$ and at 4°C for 10 min. The leftover volume in the filter should be <0.5 ml. Fill the filter device with water to 4 ml. Wash five to six times by repeating the centrifuge and water-adding steps in order to completely remove excess PL-PEG in the PEG-SWNT solution. After the final wash, resuspend PEG-SWNTs in PBS and measure the concentration of the PEG-SWNTs solution using a UV VIS spectrometer with a weight extinction coefficient of 0.04651l/mg/cm at 808 nm. Adjust the concentration of the SWNT solution to ~40 mg/l by adding the required amount of water.

3.2 siRNA Conjugation to SWNTs Through Natural $\pi - \pi$ Stacking

1. Dissolve siRNA in RNase-free water to reach a siRNA concentration of 100 μ M. Dilute siRNA in PBS to a concentration of 3.4 μ M.
2. To conjugate siRNA to functionalized SWNT, mix PEG-SWNT solution from subheading 3.1 with the siRNA solution in equal volumes and incubating at 4°C for 24 h. The final SWNT and siRNA concentrations are approximately 20 mg/l and 1.7 μ M, respectively. The SWNT-siRNA solution was ready for cell transfection.

3.3 Cell Culture and Transfection with SWNT-siRNA

1. Culture SK-N-SH and BE(2)-C cells in RPMI 1640 medium supplemented with L-glutamine and 10% FBS. Maintain at 37 °C in a humidified atmosphere of 95% air and 5% CO₂.
2. For the siRNA transfection, plate cells in a 6-well plate at a density of 5×10^5 cells/well in 2 ml medium. Incubate cells overnight under the condition described in **step 1**.
3. To remove aggregates, centrifuge the SWNT-siRNA solution prepared in Subheading 3.2 at $10,000 \times g$ and 4 °C for 10 min. Collect the SWNT-siRNA supernatant containing either SWNT-siGRP-R or SWNT-siLuc conjugates, and add 500 μ l to each well containing cultured cells and 2 ml of medium. The final

SWNT and siRNA concentrations should be approximately ~4 mg/l and ~300 nM, respectively. Incubate the cells at 37 °C, 5% CO₂ overnight.

4. To examine protein expression levels, replat the transfected cells from **step 3** into 6-well plates. Simply remove culture medium and treat the cells with 300 µl of 0.25 % trypsin for 2–5 min. Then add 1 ml of RPMI medium with 10% FBS and spin down the cells at $200 \times g$ for 5 min at 4°C. Resuspend and plate the cells in equal volumes into 35 mm dishes for protein examination at various time points.

3.4 Cell Cytotoxicity Assay

1. Plate cells in 96-well plates at a density of $5\text{--}10 \times 10^3$ cells/well in RPMI 1640 culture medium with 10% FBS and incubate overnight under the condition described in Subheading 3.3, **step 1**.
2. Add PEG-SWNT solution into the well at various concentrations, and culture these cells for up to 3 days after incubation with SWNTs.
3. Assess the cell numbers using Cell-Counting Kit-8 daily. The values, corresponding to the number of viable cells, can be read at OD450 with FlexStation 3. Set up each assay point in triplicate, and repeat the experiment three times for each cell line (Fig.1).

3.5 Cell Preparation for NIR Fluorescence Imaging

1. Trypsinize and wash cells after SWNT–siRNA treatments using culture medium via centrifugation at $200 \times g$ and at room temperature for 5 min.
2. Resuspend cells at the concentration of 2×10^6 cells/ml in RPMI 1640 medium without phenol red.
3. Mix the cell solution with equal volume of 0.8 % agarose gels (warmed at 40 °C) to form a final semisolid cell solution with concentration of 1×10^6 cells/ml in 0.4 % agarose gels.
4. Spread the cell gel solution on the glass slide and cover it with coverslip.

3.6 NIR Fluorescence Imaging of SWNT-siRNA-Treated Cells

A 70 mW/785 nm laser beam (CrystaLaser) is expanded by lenses 1 and 2 and then focused by lens 3 to the back focal plane of an IR-enhanced 60× water immersion objective (Olympus; Fig. 2a). The laser spot in the sample plane is approximately 100 µm in diameter. NIR fluorescence imaging is carried out in epifluorescence mode, and fluorescence images (Fig. 2b) are collected by a liquid nitrogen cooled two-dimensional InGaAs array (2D-OMA V; Princeton Instruments). The excitation light is filtered out using one 1,150 nm long pass filter (Thorlabs). The visible images are collected using an EMCCD (ProEM 512B CCD; Princeton Instruments).

3.7 Protein Western Blotting for GRP-R

1. Prepare whole cell lysate by mixing cells with lysis buffer (1×) containing 1 mM PMSF and protein inhibitor cocktail.

2. Measure the protein concentrations using Bio-Rad Protein Assay kit by reading OD=595 nm with FlexStation 3 and calculate protein concentration. Then prepare a protein sample with a reducing agent (1 mM DTT) and 4× LDS sample buffer, which is then heated at 70 °C for 10 min to denature proteins. Such samples can be stored at −80 °C for several months.
3. Following standard Western blotting technique, electrophorese equivalent amounts of protein (20–30 µg) on NOVEX NuPAGE 4–12 % Bis–Tris gels, and then electro-transfer to polyvinylidene difluoride (PVDF) membranes. Probe with primary antibodies (1:1,000 dilution) overnight at 4 °C followed by incubation with HRP-conjugated secondary antibody (1:5,000 dilution) for 45 min at room temperature.
4. Visualize protein levels by an enhanced chemiluminescent substrate, such as Western Lightning Plus-ECL (Fig. 3).

3.8 SWNT-siRNA Delivery into Subcutaneous Xenograft Tumors in Vivo

All experiments were approved by the Institutional Animal Care and Use Committee in accordance with guidelines issued by the National Institutes of Health.

1. Maintain male athymic nude mice (4–6 weeks old) as described [16].
2. Establish BE(2)-C xenografts by injecting BE(2)-C cells (1×10^6) in 100 µl of HBSS into the bilateral flanks using a 26-gauge needle (n=3–5 per group) as described [6, 16].
3. Perform SWNT–siRNA treatments from day 7 post tumor cell inoculation in mice by injecting 50 µl of the SWNT–siRNA PBS solution with concentrations at 20 mg/l for SWNTs and 1.7 µM for siRNA into each tumor.
4. Harvest tumors at the end point for analysis.

3.9 Immunohistochemistry

1. Perform immunohistochemical staining using DAKO EnVision + System-HRP kit.
2. Fix human neuroblastoma xenografts in formalin overnight and embed it in paraffin wax.
3. Mount tumor sections (5 µm) on glass slides.
4. Deparaffinize sections with xylene.
5. Rehydrate sections with ethanol.
6. Perform antigen retrieval with 10 mM sodium citrate buffer.
7. Block sections with blocking solution for 20 min at room temperature.
8. Incubate slides with GRP-R primary antibodies overnight at 4 °C.
9. Wash slides with PBST buffer three times for 5 min each.
10. Incubate slides with secondary antibodies for 30 min at room temperature.

11. Develop sections with DAB reagent. The reaction was terminated by immersing slides in dH₂O, and sections were counterstained with hematoxylin.
12. Dehydrate slides with ethanol and xylene. Coverslips were mounted on slides.
13. Mount coverslips on the slides and leave them to dry.
14. Take the IHC images under microscope (Leica DMI6000 B) (Fig. 4).
15. For mitosis detection, stain paraffin-embedded sections with rabbit polyclonal anti-human phospho-histone H3 (Ser10) antibody followed by Alexa Fluor 568 Dye (red)-labeled anti-rabbit secondary antibody staining.
16. Stain nucleus with DAPI (blue).
17. Capture images using a fluorescent microscope (Nikon Eclipse E600) (Fig. 5).

ACKNOWLEDGMENTS

The authors would like to thank Nadja Colon and Roel L. Flores for critical review of the manuscript. This work was supported by R01 DK61470 (DHC) from the National Institutes of Health and ECCS-1055852 from National Science Foundation (YQX).

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Fig. 1. PEG–SWNT toxicity assay. Cells treated with PEG–SWNT did not exhibit a significant inhibition on cell proliferation when compared to a control (no SWNT treatment)

Fig. 2.
(a) A schematic diagram of the fluorescence imaging setup; (b) a NIR fluorescence image of SWNT-siRNA-treated cells overlapped with the related optical image. The scale bar is 40 μm

Fig. 3. GRP-R is silenced by SWNT-siRNA transfection. BE(2)-C and SK-N-SH cells were treated with SWNT-siRNA for 72 h. Protein expression was detected by Western blotting. GRP-R expression was significantly knocked down in both cell lines. β -actin levels indicate equal sample loading. Levels of protein expression were quantified by densitometry analysis with Image J (NIH)

Fig. 4. SWNT-siGRP-R silencing in neuroblastoma in vivo. **(a, b)** Representative immunohistochemical staining of GRP-R in tumors treated with SWNT-siLuc or SWNT-siGRP-R. The expression of target GRP-R (*brown staining*) was significantly decreased in SWNT-siGRP-R-treated tumor sections. **(c, d)** Representative H&E-stained tumor sections from mice treated with SWNT-siLuc or SWNT-siGRP-R

Fig. 5. Mitosis detected in paraffin-embedded tumor sections from SWNT–siLuc- and SWNT–siGRP-R-treated tumors, respectively. (**a, d**) Mitotic cells were stained with anti-human phospho-histone H3 (Ser10) primary antibody and Alexa Fluor 568 (*red*)-labeled secondary antibody. (**b, e**) Nuclei were stained with DAPI (*blue*). (**c, f**) Overlapped images. SWNT–siGRP-R treatments resulted in a reduced number of mitotic cells (**d** and **f**), leading to decreased tumor cell proliferation and inhibition of tumor growth