Journal of Materials Chemistry B

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

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Highly Efficient Gab2 siRNA Delivery to Ovarian Cancer Cells mediated by Chitosan-polyethyleneimine Nanoparticles

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ABSTRACT Malignant bowel obstruction (MBO) is a serious complication which caused high death rate and low quality of life (QOL) for patients diagnosed at the advanced stage of ovarian cancer. RNA interference (RNAi) could be a promising method for treatment of ovarian cancer and decrease the morbidity of MBO. Gab2 gene is overexpressed in ovarian cancer compared with normal ovary tissue, and regulates the migratory behaviors and E-cadherin expression via activation of the PI3K pathway in ovarian cancer cells. Here, chitosan-polyethyleneimine (PEI, Mw 1800) copolymer nanoparticles were synthesized as nanocarrier to deliver Gab2 siRNA into SKOV3 cells, the silencing effects against Gab2 gene and the antitumor effects by chitosan-PEI-Gab2 siRNA nanoparticles (chitosan-PEI-Gab2 NPs) were studied. Results showed that the highly efficient silencing effects against Gab2 expression and its downstream effector AKT protein at more than 90% deregulation were obtained by chitosan-PEI NPs mediated Gab2 siRNA delivery, so as to exhibit obvious antitumor effects against SKOV3 cells with low cytotoxicity, and induce cell apoptosis in early and late stage. The study will provide novel strategies to overcome MBO in ovarian cancer by efficient knockdown of Gab2 expression.

KEYWORDS: Chitosan nanoparticles, Gab2, siRNA, ovarian cancer, MBO.

1. Introduction

Ovarian cancer, one of the most common causes of cancer deaths in women around the world, is often diagnosed at the advanced stage when the cancer cells have already migrated. In these advanced ovarian cancer patients, malignant bowel obstruction (MBO) is a serious complication which causes high death rate and low quality of life (QOL). About 50% of small bowel obstruction and 37% of large bowel obstruction treated in a large gynecological oncology service were founded in ovarian cancer.[1] Cytoreductive surgery combined with platinum-based chemotherapy is the current main treatment for ovarian cancer. However, for the patients with stage III and IV ovarian cancer who are at higher risk for MBO, there is no efficient traditional treatment. Nowadays, more and more researchers pay attention to gene therapy, especially RNA interference (RNAi) triggered by double stranded RNA (dsRNA), a sequence-specific posttranscriptional gene silencing. Grb2- associated binder 2 (Gab2) is a scaffolding potential molecular link between Syk and phosphatidylinositol-3 kinase (PI-3K) pathway. Gab2 mRNA expression is significantly increased in ovarian serious cystadenocarcinoma compared with normal ovary tissue.[2] As is well-known, serious cystadenocarcinoma is the subtype of ovarian cancer.[3] Gab2 is a member of the daughter of sevenless (Dos)/Gab family of scaffolding proteins, and this family also includes mammalians Gab1, Gab2, and Gab3, Drosophila homologue Dos, and Caenorhabditis elegans

homologue suppressor.[4,5] Researchers proved that Gab2 recruits SH2-domain containing signaling proteins such as the regulatory subunit of PI3K p85 and tyrosine phosphatase Shp2, which can activate the Ras-Erk and PI3K-Akt pathways.[6] The phosphatidylinositol 3-kinase (PI3K)/AKT pathway is shown to be closely related to the genesis of ovarian cancer. PI3K is up-regulated in 30% to 45% of ovarian cancers,[7,8] and about 36% of primary tumors show elevated AKT2 activity.[9] AKT has been shown to play an important role in protecting ovarian cancer cells from cytotoxic drug-induced apoptosis.[10,11] In a nutshell, AKT may contribute to tumorigenesis at multiple levels.

All these above-mentioned pathways play a significant role in regulating the growth, survival, differentiation and migration of different cell types.[4,5] The implication of Gab2 in growth factor, cytokine, and multichain immune-recognition receptor signaling have been proved in vitro.[12] Gab2 expression mainly increase the characteristics of epithelial-to-mesenchymal transition (EMT) such as increase in cell migration, invasion and decrease in the expression of E-cadherin (an epithelial cell marker). Gab2 regulates the migratory behaviors and E-cadherin expression via activation of the PI3K pathway in ovarian cancer cells.[13]

Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine

(acetylated unit). Chitosan enhances the transport of polar drugs across epithelial surfaces, and is biocompatible and biodegradable. Chitosan and conjugates such as with hyaluronic acid[14] has a number of commercial and possible biomedical uses, such as nucleic acid drugs' delivery. RNAi has been widely applied in nanomedicine[15] by using of liposome based siRNA nanoparticles,[16] polymeric nanoparticles.[17] FHL2 siRNA formulated within chitosan nanoparticles could knock down FHL2 gene expression about 69.6%, and could also inhibit the growth and proliferation of human colorectal cancer Lovo cells. Folate-chitosan showed higher transfection efficiency and lower toxicity in folate receptor-positive cells, which encourage further study of chitosan as a gene delivery system.[18] Chitosan nanoparticle have been widely used as a good siRNA delivery carrier. However, the silencing efficiency of chitosan nanoparticles mediated siRNA delivery still need to be improved to obtain better antitumor effects.

In order to develop novel highly efficient chitosan based nanocarrier for siRNA delivery with high biocompatibility to treat ovarian cancer and decrease the morbidity of MBO. Here we prepared chitosan-PEI copolymer by conjugating chitosan with polyetherimide (PEI) for Gab2 siRNA delivery, and investigated their silencing effects of prepared chitosan-PEI-Gab2 NPs against Gab2 expression which acts an important role in EMT (epithelial-to-mesenchymal transition), and

its downstream effector protein AKT expression was determined to explain the antitumor mechanism of the chitosan-PEI-Gab2 NPs.

2. Material and methods

2.1. Materials

Carboxylation chitosan with 65% degree of carboxyl substitution was prepared based on the followed protocol: 2 gram of chitosan (Focusgen Co. LTD, Jiaing, China) was added to 100 ml three necked flask, then adding succinic anhydride / ethanol (3 gram succinic anhydride was dissolved in 60 ml of absolute ethanol), reacted under 60 ℃ with magnetic stirring. After the reaction, the products were reduced by pressure filtration, and the solid phase was soaked with ethanol for 8 h. After filtration, the precipitate was washed with ethanol, and then the product was vacuum dried for 48 h under 60 ℃, to get the N-succinate chitosan as starting material. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl: SA = 5:1, mol:mol) was obtained from TCI (Shanghai) Development Co., Ltd. Ethanol (95 wt%) and 20x Phosphate Buffered Saline (PBS) was purchased from Aladdin Chemical Co. (Shanghai, China). Polyethyleneimine (PEI) (Mw 1800) was purchased from Sigma Aldrich (St. Louis, MO). Fetal bovine serum (FBS), ATCC-formulated McCoy's 5a Medium Modified (Catalog No. 30-2007), were obtained from Gibco (Shanghai) Co., Ltd.

SKOV3 was obtained from cell bank of Chinese Academy of Sciences in Shanghai. The fluorescently labeled (5' end FAM modified on the sense strand) Gab2 siRNA (sense strand: 5'GCACCGGCAGCGUUGAUUA, anti-sense strand: 5'UAAUCAACGCUGCCGGUGC), scrambled siRNA (sense strand: 5'UUCUCCGAACGUGUGACGUTT, anti-sense strand: 5'ACGUGACACGUUCGGAGAATT) were purchased from GenePharma (Shanghai, China) Co., Ltd. Primers for PCR and real-time PCR were obtained from Sangon Biotech (Shanghai, China) Co., Ltd. Annexin V-FITC Apoptosis Detection Kit and MTT Cell Proliferation and Cytotoxicity Assay Kit were purchased from KeyGEN BioTHCH (Shanghai, China) Co., Ltd. The quantitative results were plotted on graphs by using the OriginPro 8 software (OriginLab Corp, Northampton, MA).

2.1. Preparation of chitosan-PEI-siRNA nanoparticles

Particle concentration was calculated based on the unit of the chitosan polymer concentration in the chitosan-PEI NPs. At first step, 0.43g PEI (Mw 1800, 10 mmol) was dialyzed with dialysis membrane (MWCO 1000) against 0.01M phosphate buffered saline (PBS) for 48 hours to adjust the pH to 7.0. Chitosan solutions were prepared at unit amount of substance of about 0.01mmol (2.03mg), by dissolving in 3 ml 0.01M PBS with vigorous stirring. 228 μl EDC was added into the chitosan solution with vigorous stirring to activate polymer groups. Then

added dialyzed PEI into the mixture and continued stirring for further 4 hours at room temperature. After 4 hours stirring, the mixture was dialyzed with dialysis membrane (MWCO 7000) against 0.01M PBS for 48 hours to eliminate superfluous PEI and EDC. The final volume of the solution was about 10 ml. The solution was filtered through filtration membrane (diameter: 220nm) to remove bacteria and improve the polydispersion. The concentration of chitosan-PEI was 1mM calculated by the concentration of chitosan unit. Gab2 siRNA (25μM) was mixed with chitosan-PEI (0.01 mM) to form chitosan-PEI-siRNA nanoparticles, at the ratio form 1:40 to 1:800 (mol/mol) at room temperature in RNase-free water for 15～20 minutes, then examined by 0.8% agarose gel electrophoresis to find the most suitable proportion.

2.2. Characterization of chitosan-PEI-siRNA nanoparticles

By Quasi-elastic laser light scattering using a ZetaPALS dynamic light scattering (DLS) detector (NanoZS90, Malvern) at 25 centigrade, nanoparticle size (diameter, nm), polydispersity index, and surface charge (zeta potential, mV) of the chitosan-PEI were determined. The form of chitosan-PEI-siRNA NPs was observed by using transmission electron microscope (TEM). The HNMR spectra of chitosan, PEI, and chitosan-PEI conjugates were determined by DMX 500 (Bruker, Switzerland).

2.3. In vitro Gab2 siRNA delivery by chitosan-PEI nanoparticles

SKOV3 cells were cultured in ATCC-formulated McCoy's 5a Medium Modified (Catalog No. 30-2007) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100 mg/ml streptomycin. Cells was cultured at incubator with 5% CO_2 at 37 °C. SKOV3 cells were seeded in a 24-well plate in 0.5ml complete growth medium per well at a density of 0.5-1.0x10^5 cells/well for 24 hours. Cell density should be 50-80% confluent on the day of transfection. The growth medium from cells was removed and replaced with 0.5 ml of Opti-MEM media. Chitosan-PEI-Gab2 siRNA NPs were diluted in 100 µl of Opti-MEM medium, then added into the Opti-MEM media. The cells were cultured at 5% CO_2 at 37 °C for 18~24 hours post-transfection before assaying for transgene expression. The cells were photographed under confocal microscope 4 hours after transfection. Lipofectamine 2000 was used as the positive control.

2.4. In vitro cytotoxicity assays for chitosan-PEI-Gab2 NPs

3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytetrazoliumromide (MTT) assay was used for evaluating the cytotoxicity of the NPs. SKOV3 cells were seeded in a 96-well plate in 200µl medium per well at a density of 3000~5000 cells per well overnight to allow the cells adhere to the plate. The medium was then replaced with 200µl of medium-containing NPs at different concentrations in quintuplet and

incubated for next 24 hours. Then the supernatant was removed the next day to avoid interference in the assay, 25µl MTT (5 mg/ml) was added to each well. The plate was returned to the incubator for another 4 hours. Then the medium and MTT were aspirate and replaced with 100 µl DMSO. Place the plate adapter on the vortex to shake for 5 minutes to ensure the crystals dissolved. The plate was read in the microplate reader (MK3, Thermo) at 570 nm wavelength. A higher MTT absorbance with background value subtracted indicates higher relative cell viability.

2.5. Cell apoptosis assay

After 18~24 hours post-transfection, the SKOV3 cells can be collected for apoptosis-promoting assay. The cells were digested with the trypsin without EDTA to avoid false-positive. 1~5x10^5 cells were collected by centrifugation. After re-suspend cells in 500 µl of 1x Binding Buffer, 5 µl of Annexin V-FITC and 5 µl of propidium iodide were added. The cells were incubated at room temperature for 5 min in the dark. Analyze Annexin V-FITC binding by flow cytometry (Ex = 488nm; Em = 530nm) using FITC signal detector (usually FL1) and PI staining by the phycoerythrin emission signal detector (usually FL2).

2.6. Real-time PCR and western blot

After incubated for 18~24 hours post-transfection, the chitosan-PEI-Gab2 NPs with Opti-MEM reduced serum media were replaced by complete growth medium and continued incubation for another 24 hours. Then the cells were collected for RNA and protein isolation. Real-time PCR and western-blot were used to determine the expression of RNA and protein. The primer sequences are as following, Gab2-F 5'-GCCGGCACAATACAGAATTCA-3', Gab2-R 5'-GTGAGGCTGCCCTTGGTGT-3', ß-actin-F 5'-TCACCCACACTGTGCCCATCTACGA-3', ß-actin-R 5'-CAGCGGAACCGCTCATTGCCAATGG-3'. For Western blot analyses, SKOV3 cells treated with the chitosan-PEI-Gab2 siRNA, lipo2000-Gab2 siRNA, chitosan-PEI-NC siRNA and PBS were harvested and homogenized in RIPA buffer (Sigma) with a protease inhibitor mix (Roche). Equal amounts of total protein lysate of cells were denatured in Laemmeli buffer at 70 °C for 5 min, separated by SDS/PAGE and transferred onto actived PVDF membranes. The monoclonal antibody against Gab2 (Abcam) was used at a 1:20000 dilution in 5% milk, and the monoclonal antibody against Akt (cell signaling) was at 1:40000 dilution in 5% milk. The membranes were incubated with the primary antibodies at 4°C overnight. Then they were detected with anti-rabbit secondary antibody at 1:40000 dilutions (Abcam) for an hour. GAPDH (Abcam) was used as positive

control at 1:2000 dilution for western blot analysis. The proteins were measured using an ECL detection system according to the manufacturer's protocol.

3. Results and discussion

3.1. Preparation and characterization of chitosan-PEI copolymer

Chitosan is an ideal polymer as gene vector for possessing admirable biocompatibility, biodegradability, and low toxicity.[19,20] While indissolubility under the pH of internal environment is a great shortage for its application, as well as low charge and transfection efficiency.[21] PEI is a widely studied polycationic gene vector for its structure containing abundant amino groups, which resulting in plenty of charge after protonated. PEI has high transfection efficiency with strong nucleic acid condensation capacity and buffering capacity known as proton sponge effects. Nevertheless, PEI exists irreconcilable contradictions as high molecular weight PEI coupled with high transfection efficiency, but high cell toxicity, while low molecular weight PEI possessing low transfection efficiency but low toxicity.[22] To discard the drawbacks and take the advantages of these two polymers, we innovatively construct a chitosan-PEI nanocarrier to achieve a vector with high transfection efficiency and low cell toxicity. As shown in diagrammatic Figure 1, amino groups of PEI were conjugated with the carboxylic groups of chitosan polymer mediated by crosslinker EDC. The chitosan-PEI copolymer can bind and

package electronegative siRNA molecules to improve their stability in serum containing environment.

The conjugation between chitosan and PEI polymer with starting molar ratio at 1:10 was confirmed by HNMR spectra as shown in Figure 2. The change of area ratio of hydrogen spectrum after conjugation was found to match with the starting molar ratio of chitosan and PEI.

3.2. Characterization of chitosan-PEI-siRNA NPs

The prepared chitosan-PEI-siRNA NPs exhibited spherical shape with about 10 nm as observed by TEM (Figure 3A). The particle size and surface charge of freshly prepared chitosan-PEI NPs were determined by dynamic light scattering (DLS), as shown in Figure 3B, the average size of chitosan-PEI NPs were from 5 to 10 nm. The Zeta Potential of the chitosan-PEI NPs was around 10.8 mV (Figure 3C).

Electronegative siRNA is unstable and can be easily degraded by the RNase in the environment. The results of the agarose gel electrophoresis were showed in Figure 3D, Gab2 siRNA (25μM) was mixed with chitosan-PEI NPs (0.01mM) at the ratio 1:20, 1:40, 1:120 and 1:200 (mol/mol) in RNase-free water for 15 minutes. The chitosan-PEI-siRNA NPs stayed in the wells at the ratio of 1:20, 1:40 (mol/mol), the chitosan-PEI-siRNA NPs run to the opposite direction at the ratio of

1:120 and 1:200 (mol/mol). Results showed that the electronegative siRNA and the positively charged chitosan-PEI NPs were combined effectively at 1:12~1:200 molar ratio of siRNA versus chitosan NPs.3.3. In vitro Gab2 siRNA delivery by chitosan-PEI NPs

The siRNA delivery efficiency was evaluated by transferring Gab2 siRNA and negative control (NC) siRNA into SKOV3 cells in vitro. SKOV3 Cells were seeded in a 24-well plate in 0.5 ml complete growth medium per well at a density of 0.5~1.0x10^5 cells/well for 24 hours. Cell density was about 60% confluent when transfection. The chitosan-PEI-siRNA NPs synthesized at the siRNA/chitosan ratio of 1:20, 1:40, 1:120 and 1:200 (mol/mol), and lipofectamine 2000 were used as positive control transfection reagents. For all measurements, the siRNA concentration was fixed at 25 nM. The cells were photographed under confocal laser scanning microscopy after 4 hours' transfection. As shown in Figure 4, chitosan-PEI-Gab2 siRNA NPs exhibited comparable transfection efficiency compared with Lipofectamine group.

In order to determine the quantitative transfection results, flow cytometry was used to determine the intracellular uptake efficiency of FAM labeled siRNA. As shown in Figure 5, compared with other siRNA/chitosan ratio, the Gab2 siRNA had the highest transfection efficiency for about 83.9% at the siRNA/chitosan ratio

of 1:200 (mol/mol). The transfection efficiency of the chitosan-PEI-siRNA NPs depends on the nitrogen atoms (N) in the polymetric/ the phosphorus atoms (P) in RNA (N/P) ratio which was calculated with the siRNA/chitosan volume ratio at which chitosan-PEI-siRNA NPs escapes from the endosomal compartment and transports into the cells. The high transfection efficiency of branched PEI is attributed to the ability to destabilized the endosome membrane.[23,24] Similarly, the chitosan-PEI NPs contains primary, secondary, and tertiary nitrogen atoms, which have proton sponge effects leading to amounts of charged particles absorption into endosomes. This causes increased osmotic pressure allowing plenty of hydrone penetrating into endosomes, thereby giving rise to swelling and burst of endosome membrane. As a result, the chitosan-PEI NPs exhibited high capacity to release siRNA to the cytoplasm after endocytosis. Obviously, the synthesized chitosan-PEI-siRNA NPs could be used as an excellent siRNA vector due to its high transfection efficiency and low cytotoxicity.

3.4. In vitro cytotoxicity and antitumor effects of chitosan-PEI-siRNA NPs

The cytotoxicity of a delivery system is one of the important parameters that should be evaluated when developing a siRNA delivery system. To evaluate the cytotoxicity of chitosan-PEI NPs itself and the antitumor activity of chitosan-PEI-siRNA NPs, we carried out in vitro cytotoxicity experiments using

MTT assay against SKOV3 cancer cell lines. In Figure 6, the relative cell viability of cells after 24 hours incubation with varied concentrations of chitosan, PEI (Mw 1800Da), chitosan-PEI-NC siRNA NPs and chitosan-PEI-Gab2 siRNA NPs. In Figure 6(A), the concentration of chitosan-PEI NPs was set from 0 to 18 μM. The results showed that chitosan-PEI NPs had a significantly lower cytotoxicity at the concentration from 0 to 5 μM compared with PEI (Mw 1800Da) alone. The low cell toxicity of chitosan-PEI NPs derivatives results from the biocompatibility of chitosan and low percentage conjugation of PEI molecules. The poor degradation of the nanomaterial after transfection is the main cause of the cytotoxicity. High-molecular-weight PEI (HMW PEI) (25 kDa) shows a lack of degradable linkages (C-C or C-N bonds) resulting in high toxicity, which is the critical reason we are inclined to choose PEI of Mw 1800 Da.[25] Benefiting from its ester bond hydrolysis reaction in endosome, Chitosan-PEI polymer is degraded into low-molecular weight PEI and Chitosan, both of which are easier to be purged, thereby reducing cytotoxicity. Moreover, the low cell cytotoxicity makes chitosan-PEI NPs a better gene carrier for repeated administrations of nucleic drugs in large doses.

To test the antitumor activity of chitosan-PEI-siRNA NPs, we compared chitosan-PEI-NC siRNA NPs and chitosan-PEI-Gab2 siRNA NPs with the same drug concentration from 0 to 1.1 μM to treat SKOV3 cells. Chitosan-PEI-Gab2

siRNA NPs exhibited much higher antitumor activity compared with chitosan-PEI-NC siRNA NPs (Figure 6B), which proves that the successful Gab2 siRNA delivery can increase the antitumor effects of chitosan-PEI-siRNA NPs.

3.5. Cell apoptosis induced by chitosan-PEI-Gab2 NPs

Cell apoptosis induced by chitosan-PEI-Gab2 NPs was analyzed by flow cytometry (Ex = 488 nm; Em = 530 nm) using FITC signal detector (usually FL1) and PI staining by the phycoerythrin emission signal detector (usually FL2). As shown in Figure 7, 6.84% early apoptosis rate and 24.2% late stage apoptosis rate in SKOV3 cells was induced after treated with chitosan-PEI-Gab2 NPs, higher than the cells treated with chitosan-PEI-NC siRNA NPs (1.2% early apoptotic cells and 14.1% late stage apoptotic SKOV3 cells) and blank control group (0.008% early apoptosis rate and 0.317% late stage apoptotic SKOV3 cells, $p<0.05=$, and similar to the positive control group with Lipofectamine 2000 as the siRNA carrier (8.59% early apoptosis rate and 24.8% late stage apoptotic SKOV3 cells, $p>0.05$). Through the flow cytometry results, Gab2 siRNA molecules were confirmed to be effectively delivered into the SKOV3 cells by the chitosan-PEI NPs, which led to the apoptosis of the ovarian cancer cells obviously.

3.6. Antitumor mechanism of chitosan-PEI-siRNA NPs

The protein was isolated from the SKOV3 cells after different treatments for 48 hours. After treated with chitosan-PEI-Gab2 siRNA NPs, the AKT protein and Gab2 protein of SKOV3 cells were all down-regulated significantly at higher than 90%, superior to down-regulation effects of the Lipo2000-Gab2 siRNA, as showed in Figure 8A. Compared with blank control group, the Gab2 RNA expression of SKOV3 cells treated with chitosan-PEI-Gab2 NPs decreased to 5.928%±0.090, and lower than that of cells treated with Lipo2000-Gab2 siRNA as 6.235%±0.032 (Figure 8B). Down-regulation of the Gab2 RNA and protein all proved that Gab2 siRNAs were successfully transfected into SKOV3 cells and effectively knock down the expression of Gab2 gene. Deregulation of AKT protein was testified as the downstream effector protein of Gab2 for the antitumor mechanism of the chitosan-PEI-Gab2 NPs.

4. Conclusion

Ovarian cancer is one of the most common causes of cancer deaths in women around the world. Overexpression of Gab2 has been proved in ovarian cancer cells, such as SKOV3, OVCAR3, OVCAR5l and others cell lines. Gab2 plays a significant role in Syk and phosphatidylinositol-3 kinase (PI-3K) pathway and epithelial-to-mesenchymal transition (EMT), which plays a significant role in

regulating the growth, survival, differentiation and migration of different cell types.

In this research, we combined the Gab2 siRNA with chitosan-PEI NPs to transfect ovarian cancer cells. The silencing efficiency and antitumor effects of the chitosan-PEI-Gab2 NPs were investigated. The results are extremely encouraging because chitosan-PEI-Gab2 siRNA NPs exhibited highly efficient silencing effects against Gab2 with high biocompatibility. The data indicated that Gab2 gene played a vital role in cell proliferation of ovarian cancer, chitosan-PEI-Gab2 NPs apparently increased cell rate of apoptosis and necrosis in vitro, achieving an excellent tumor inhibition effects. These results pave the way to a new generation of chitosan based siRNA nanocarrier that will allow further preclinical and clinical studies.

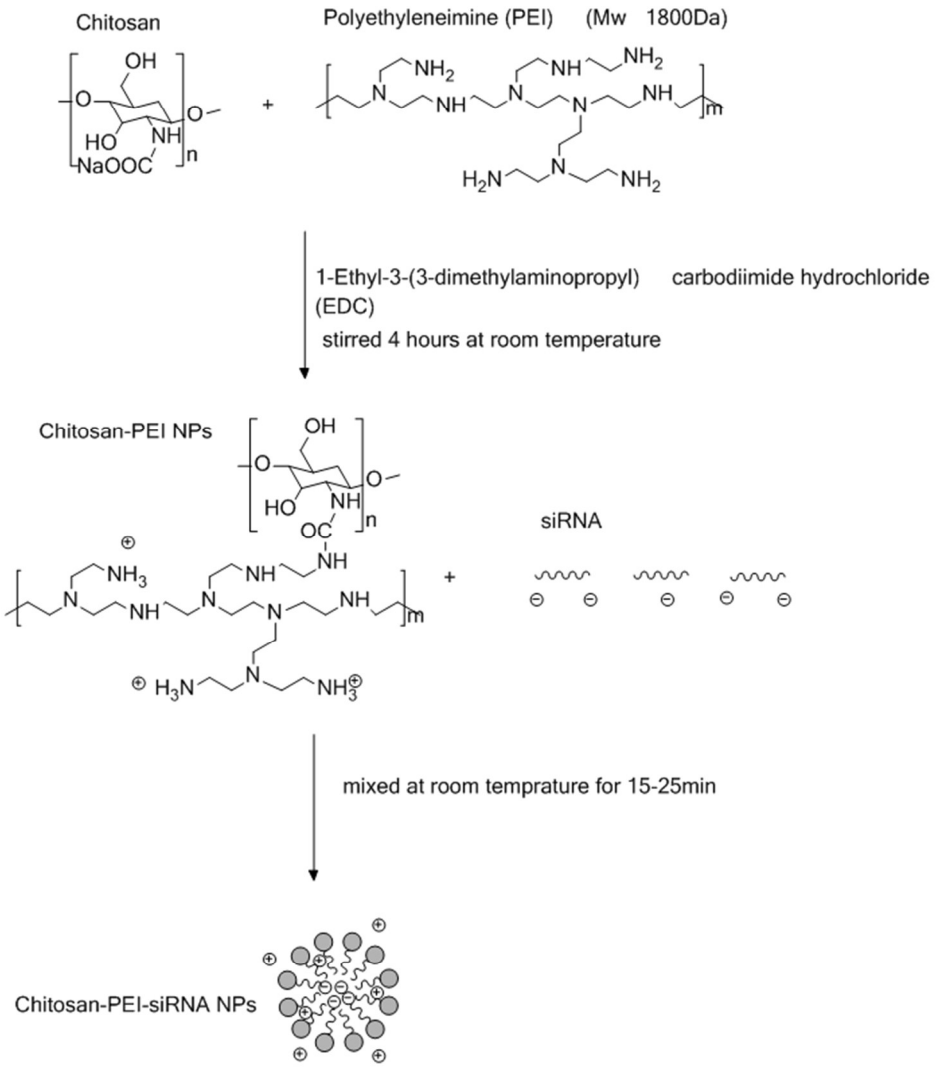

Figure 1. Skemetic diagram of chitosan-PEI copolymer synthesis by chitosan and polyethyleneimine (PEI) (Mw 1800). Chitosan and PEI (Mw 1800Da) are conjugated together by the dehydration between the carboxylic groups of chitosan and amine groups of PEI. And the electropositive chitosan-PEI copolymer bond electronegative siRNA with electro-static adsorption technique.

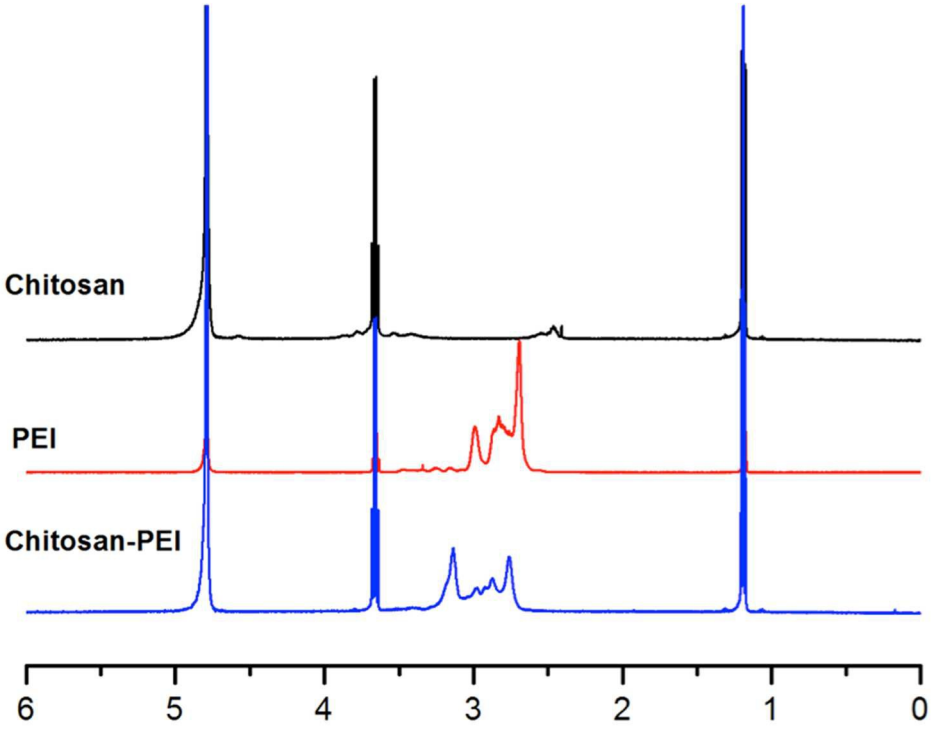

Figure 2. HNMR spectra of chitosan, PEI, and chitosan-PEI copolymer.

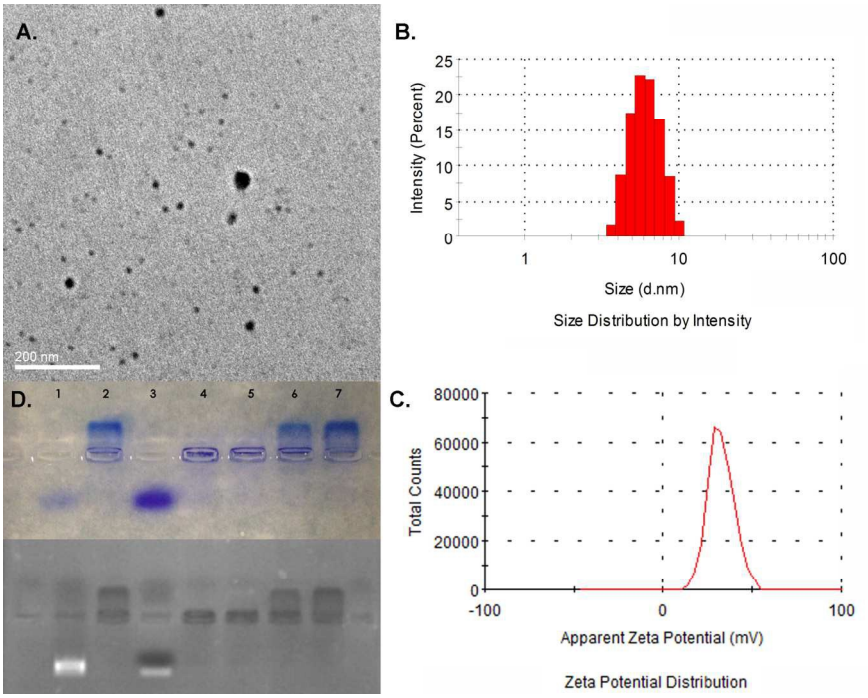

Figure 3. Characterization and siRNA binding efficiency of chitosan-PEI-siRNA NPs. A: Transmission electron microscope (TEM) photograph of the prepared chitosan-PEI-siRNA NPs; B: The DLS of NPs; C: Zeta Potential of chitosan-PEI as about 10.8 mV; D: Photograph of agarose gel electrophoresis of chitosan-PEI-siRNA NPs at different molar ratio of chitosan-PEI copolymer versus siRNA. The number of wells from 1 to 7 represents respectively as ladder, chitosan, siRNA, chitosan-PEI-siRNA NPs (mol/mol=1:20), chitosan-PEI-siRNA NPs (mol/mol=1:40), chitosan-PEI-siRNA NPs (mol/mol=1:120) and chitosan-PEI-siRNA NPs (mol/mol=1:200).

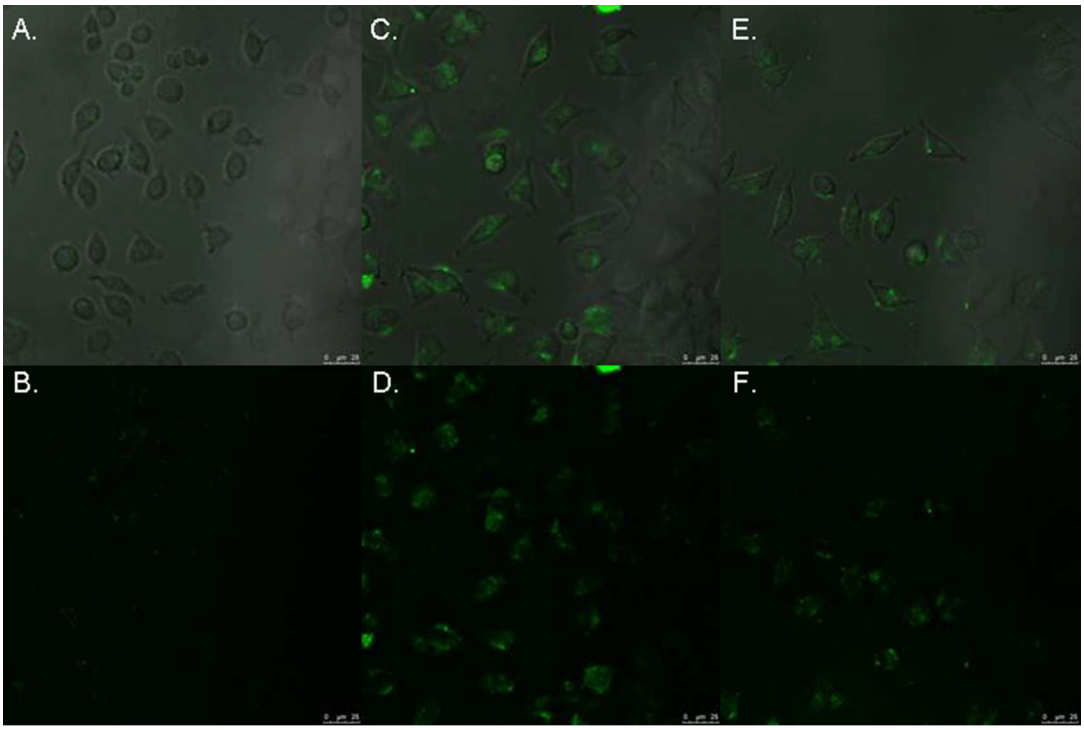

Figure 4. In vitro Gab2 siRNA delivery by chitosan-PEI-Gab2 NPs. A,B: control SKOV3 cells without treatment; C,D: SKOV3 cells treated by chitosan-PEI-Gab2 siRNA NPs, prepared at the ratio 1:200 (mol/mol). E,F: SKOV3 cells treated with Lipo2000-Gab2 siRNA. A,C,E represent the merged fluorescent image and bright field image of SKOV3 cells under different treatment, and B,D,F represent their comparative fluorescent image.

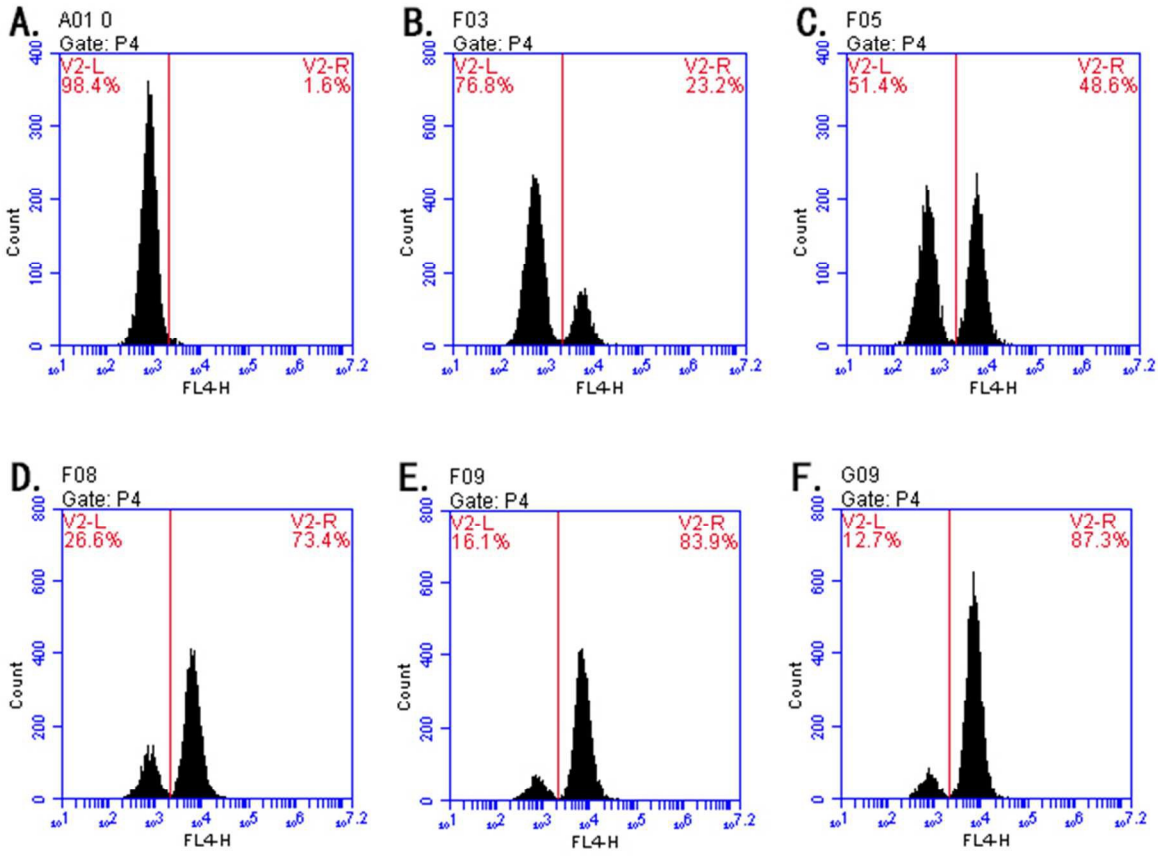

Figure 5. FCM graph for transfection rate of chitosan-PEI-siRNA(FITC) NPs. (A): SKOV3 cells without treatment; (B): SKOV3 cells treated by chitosan-PEI-Gab2 siRNA NPs with siRNA/chitosan ratio of 1:20 (mol/mol); (C): SKOV3 cells treated by chitosan-PEI-Gab2 siRNA NPs with siRNA/chitosan ratio of 1:40 (mol/mol); (D): SKOV3 cells treated by chitosan-PEI-Gab2 siRNA NPs with siRNA/chitosan ratio of 1:120(mol/mol); (E):SKOV3 cells treated by chitosan-PEI-Gab2 siRNA NPs with siRNA/chitosan ratio of 1:200 (mol/mol); (F): SKOV3 cells treated by Lipo2000-Gab2 siRNA group.

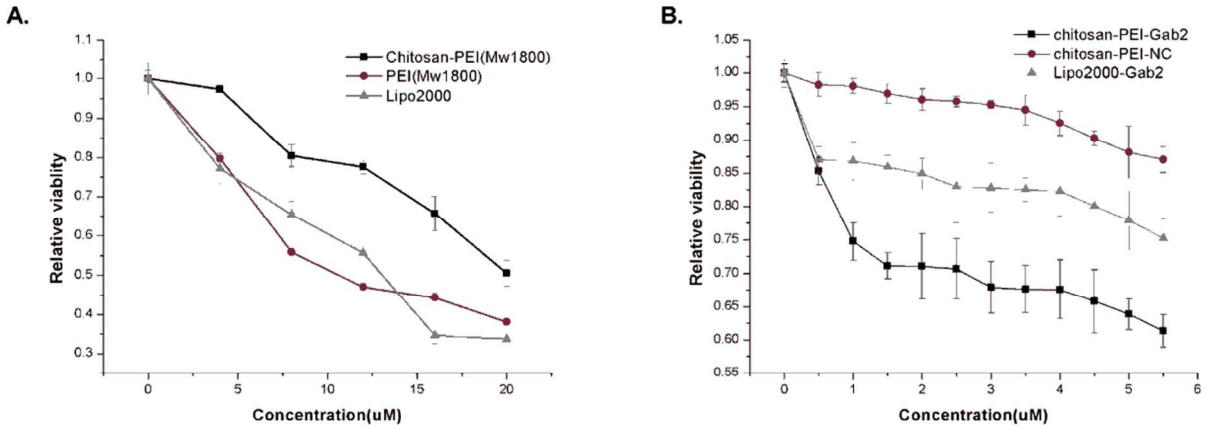

Figure 6. Cytotoxicity of chitosan-PEI copolymer and anti-proliferation effects of chitosan-PEI-Gab2 NPs. A: At the concentration from 0 to 15μM, the chitosan-PEI owns the lowest cytotoxicity compared to PEI (Mw 1800Da) and Lipo2000. At the concentration of 12μM, relative cells viability of the chitosan-PEI NPs group is 77.566%±0.168, while the data of PEI group is 47.069%±0.0022 and the data of Lipo2000 group is 55.660%±0.0338, $p<0.05$. B: At the concentration from 0 to 5.5μM, the chitosan-PEI-Gab2 NPs showed efficient antitumor effects against ovarian cancer cells compared with Lipo2000-Gabs siRNA and chitosan-PEI-NC siRNA NPs.

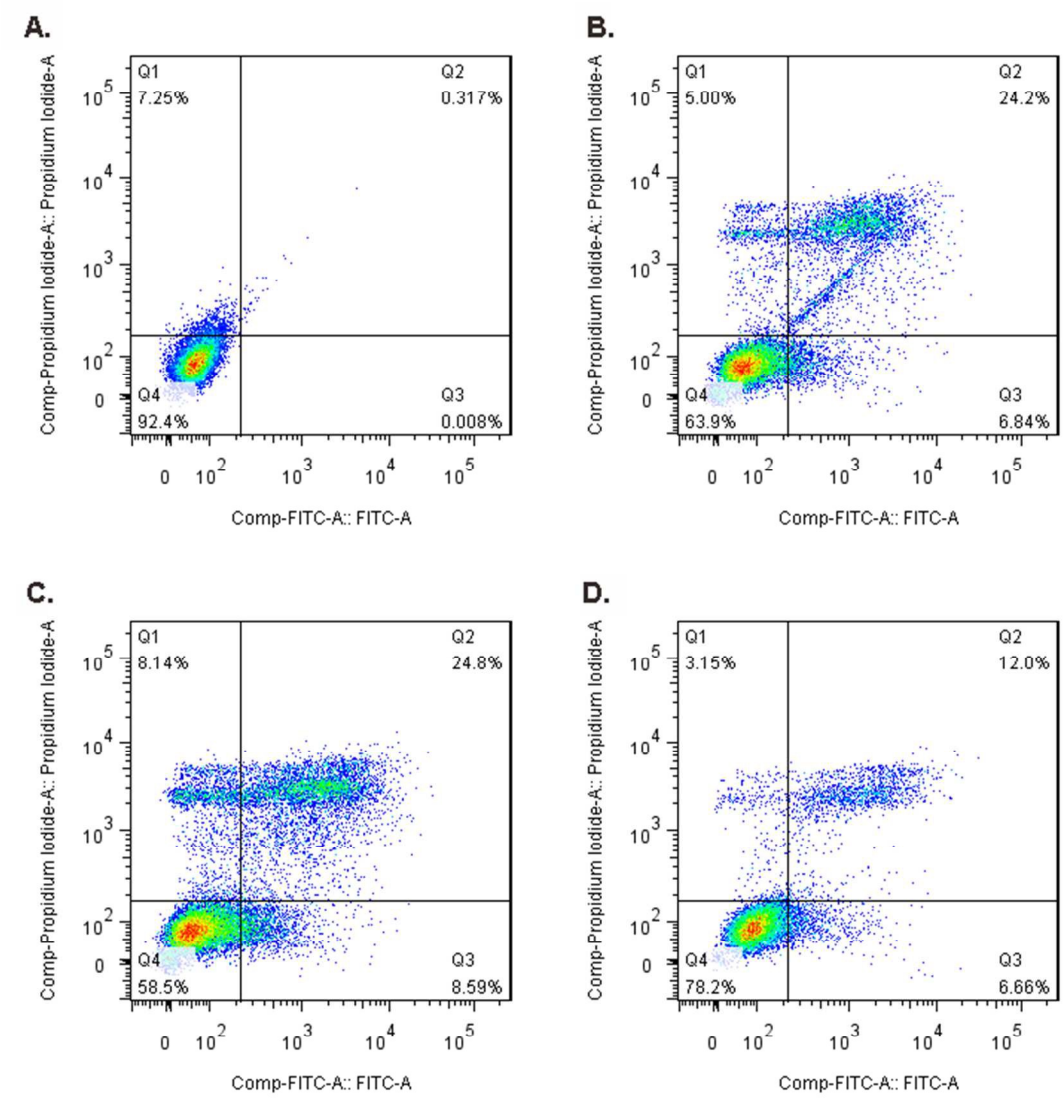

Figure 7. FCM graph for cell apoptosis induced by chitosan-PEI-Gab2 NPs. (A): negative control cells; (B): SKOV3 cells treated by chitosan-PEI-Gab2 NPs, the 24.2% late apoptosis cells and 6.84% early apoptosis cells. (C): Lipo2000-Gab2 siRNA group (24.8% late apoptosis cells and 8.59% early apoptosis cells); (D): Chitosan-PEI-NC siRNA NPs treated cells (12.0% late apoptosis cells and 6.66% early apoptosis cells).

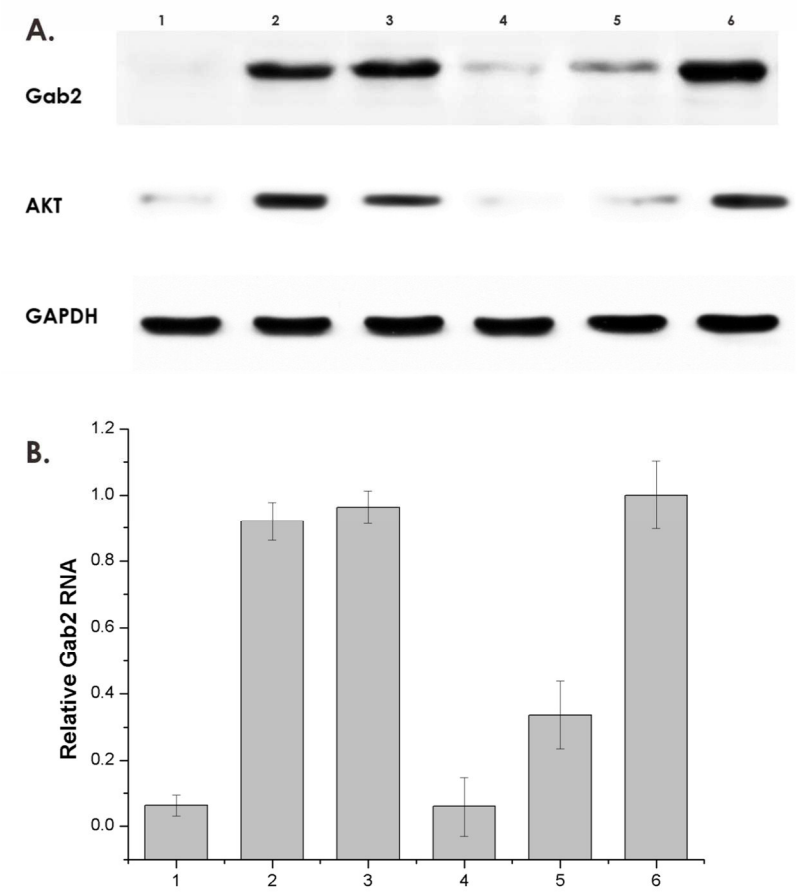

Figure 8. Western blot of Gab2 and AKT protein expression (A) and Gab2 mRNA expression in SKOV3 cells (B). Group 1: Cells treated with lipo2000-Gab2 siRNA; Group 2: Cells treated with lipo2000-NC siRNA; Group 3: Cells treated with chitosan-PEI-NC siRNA NPs; Group 4: Cells treated with chitosan-PEI-Gab2 siRNA NPs at mol/mol=1:200; Group 5: Cells treated with chitosan-PEI-Gab2 siRNA NPs at mol/mol=1:120; Group 6: Cells treated with PBS.

Author Contributions

L.F. Q. conceived and supervised the project, L.W., W.M. Wu, J.S. W., Y. C. performed the experiments, L.W., J.J. W. and L.F. Q. analyzed the data and wrote the manuscript, L.F. Q., J.J. W. were involved in the experiments design. All authors have given approval to the final version of the manuscript.

Acknowledgments

The authors would like to acknowledge the funding support from NSFC (81371682), Shanghai Natural Science Foundation (13ZR1443900). The Foundation for Innovative Research Groups of the National Natural Science Foundation of China (81221001).

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