Supporting Information

Protamine Sulfate-Nanodiamond Hybrid Nanoparticles as Vector for MiR-203 Restoration in Esophageal Carcinoma Cells

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Experimental Section

The formation of protamine functionalized nanodiamonds and the conjugation of miRNA mimics:

The acid-oxidated ND gel (15% w/v in water) was purchased from the NanoCarbon Research Institute Ltd. As-received NDs were dispersed in deionized water and diluted to 1 mg ml^{-1} after overnight ultrasonication. To prepare protamine

sulfate-functionalized nanodiamonds, the ND solution was mixed with protamine sulfate (Sigma Aldrich) at a 1:15 mass ratio of excess protamine and the resulting mixture was agitated at 37 °C for 48h. Unbound protamines were then removed by extensive wash. The protamine@NDs pellet was redispersed in water and the concentration was determined by measuring the lyophilized weight of 1 ml of the solution.

The sequences of microRNA mimics are shown in **Table S2**. Scramble microRNA mimics were designed as negative control microRNA (N.C. miRNA). These mimics were synthesized and labed by Cy3 by RiboBio Co. (Guangzhou, China). MiRNA mimics were incubated with PS@NDs at different mass ratio and incubated for 30 minutes at room temperature before measurement.

Particle size and zeta potential measurements and morphological examination:

Particle size and zeta potential measurements were performed using the Zetasizer Nano ZS (Malvern). PS@NDs complexes with miRNA were prepared in 1mL of pure water at various miRNA:PS@NDs weight ratios ranging from 0 to 1:16. Particle size measurements were performed at 25°C and a 173° scattering angle. The mean hydrodynamic diameter was determined via cumulative analysis. Determination of the zeta potential was based on electrophoretic mobility of the nanoparticles in the aqueous medium, which was performed using folded capillary cells in automatic mode.

NDs and miRNA/PS@NDs (60 μg/ml) diluted in deionized water were deposited onto commercial lacey carbon coated copper TEM grids and air dried for 2 h.

Samples were then imaged by electron microscope (JEOL JEM-2010F, Institute of Microstructure and Properties of Advanced Materials, Beijing University of Technology)

Gel retardation assay:

miRNA/PS@NDs complexes (miRNA : ND-PS w/w ratios of 1:1, 1:2, 1:4, 1:6, 1:8, 1:16) were analyzed by 2% agarose gel electrophoresis. The gels were prepared with 2% agarose in tris-acetate-ethylenediaminetetraacetic acid (EDTA) buffer containing 0.5 μg/ml GelRED^{TM} (Biotium, USA). For gel retardation assay, samples were incubated at room temperature for 15 min, then 10% glycerine was added to each sample. Gel electrophoresis was carried out at 110 V for 10 min and the gel was subsequently photographed using Alpha Innotech gel imager system.

Serum stability assay and the in vitro release of miRNA:

Serum degradation assays were performed as fellows. Briefly, samples of naked miR-203 in aqueous solution or the miR-203/PS@NDs complexes were mixed in a 1:1 ratio with fresh serum (Gibco, USA) to give 50% serum concentration. The mixture were then incubated at 37°C for the indicated times. Aliquots (15μl) from each mixture of the naked miR-203 or the miR-203/PS@NDs with serum at different incubation times were picked up and mixed with 5 μl 2% SDS and 2.5 μl 10% glycerine, then the mixture were loaded onto a 2% agarose gel containing 0.5 μg/ml GelRED^{TM} (Biotium, USA). Gel electrophoresis was carried out at 110 V for 10 min and the gel was subsequently photographed using gel imager system (Alpha Innotech, USA).

To measure the release profile of miRNA, PS@NDs were complexed with Cy3-miRNAs at a weight ratio of 1:4 at either pH 5.5 or pH 7.4 and incubated in a 96-well culture plate at 37°C under moderate shaking. Samples were taken from the plate at scheduled time points and centrifuged at 13,000 rpm for 10 min. The supernatants were moved to another 96-well culture plate, and the concentration of released Cy-3 miRNA was monitored by measuring the fluorescence of Cy-3 using a hybrid multi-mode micro-plate reader (TECAN, Switzerland).

Cytotoxicity analysis:

Ec-109 cells cultured in 96-well cell culture plates (Greiner Bio-one) in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin were incubated with miR-203/PS@NDs conjugates. Cells treated with PEI25K were designed as control. The cell viability was determined at indicated time points using CCK-8 assay according to manufacturer's instructions (Dojindo, Japan).

RNA isolation and real-time quantitative PCR (qRT-PCR):

Total RNA was extracted from cell samples using Trizol reagent (Invitrogen, USA). Reverse-transcription reactions were carried out using an NCode™ VILO™ miRNA cDNA synthesis kit (Invitrogen, USA). The subsequent detections of the expression of miRNA by RT-PCR using EXPRESS SYBR® GreenER™ miRNA qRT- PCR kit (Invitrogen, USA). U6 was used as an endogenous control for normalization. Each sample was analyzed in triplicate. The $2^{-\Delta\Delta Ct}$ method for relative quantification of gene expression was used to determine miRNA expression. The

sequences of primers used for RT-PCR were shown in **Table S3.** All primers were purchased from Sango Co. (Shanghai, China).

Cellular uptake of miRNA/PS@NDs complexes:

Ec-109 cells were incubated with miR-203/PS@NDs, miR-203/NDs, miR-203/PS complexes and naked miR-203, respectively, for 6 h in opti-MEM. The cells were then allowed to grow for an additional 18h in RPIM 1640 containing 10% FBS and fixed with 4% formaldehyde after washing with PBS. MiR-203 was labeled by Cy3. Filamentous actins (F-actin) were stained by green-fluorescent phallodin (Biotium, USA) and nuclei were stained with blue-fluorescent DAPI (Beyotime, China). Fluorescent images were captured with an Olympus FV1000-IX81 confocal microscope imaging system (Olympus, Japan).

Flow cytometry analysis:

2.0×10^5 Ec-109 cells were seeded in 6-well plates and incubated with different formulations at a concentration of 100 nm Cy3-labeled miR-203 for 6 h in opti-MEM at 37℃ in a humidified 5% CO_2 atmosphere. The cells were then harvested and resuspended in PBS at a concentration of 1.0×10^6 cells/ml. The cells were analyzed for fluorescence by flow cytometry (Attune® Acoustic Focusing Cytometer, Life Technologies, USA) after washing three times with PBS.

Plasmid constructs:

3'-UTRs of small GTPase Ran and ΔNp63 were amplified by PCR and cloned in pmirGLO Dual-Luciferase miRNA Target Expression Vector (pmiR-GLO, Promega, USA) into restriction sites *Sac-1*and *Sal-1*. The construct was named pmiR-GLO-Ran

and pmiR-GLO-ΔNp63. Mutant Ran-3'UTR and mutant ΔNp63-3'UTR constructs were formed by site-directed mutagenesis using pmiR-GLO-Ran and pmiR-GLO-ΔNp63 plasmids as template. For the mutated constructs, three nucleotides TCA in miR-203 seed match site were substituted with CGC for both mutants. The accuracy of all cloning was confirmed by sequencing.

Dual-luciferase reporter assay:

Ec-109 cells at about 90% confluence were plated in 6-well plates and transfected with 1 μg of pmirGLO-Ran, pmirGLO-Ran-mut, mutant Ran-3'UTR, mutant ΔNp63-3'UTR constructs and empty vector respectively using Lipofectamine 2000. 1 μg of miR-203 mimics was co-delivered into cells using PS@NDs. Scramble miRNA was used as control and separately co-transfected into cells using PS@NDs. After 24h, Ec-109 cells were collected and analyzed for luciferase activity using the Dual-Glo® Luciferase Assay System (Promegra, USA). Luciferase activity was presented as an average of three independent experiments performed in triplicate.

Western-blot analysis :

Cultured cells were washed with PBS twice and lysed with RIPA lysis buffer (Beyotime, China) for 10 minutes on ice. After centrifugation at 12,000 g for 10 min, the supernatant was collected and the concentrations of protein were measured using Bradford's reagent (Bio-Rad laboratories, USA). The protein samples were loaded onto SDS-PAGE (12%) gel for electrophoresis and transferred onto PVDF membrane (Millipore, USA). The anti-Ran (Novus, USA) and anti-ΔNp63 (Santacruze, USA) antibodies were added into blocking solution and incubated overnight at 4°C. The

membranes were subsequently incubated with the secondary goat anti-mouse antibody conjugated with fluorescent dyes: IRDye 800 CW (KPL, USA). Protein expression was normalized against GAPDH expression (Cell signaling, USA). Blotting images were acquired with the Odyssey infrared imaging system (LI-COR Biosciences, USA) and analyzed by the software provided by the manufacturer.

Cell proliferation assay:

Ec-109 cells were plated into 96-well plates at a density of 5×10^3 cells/well and treated with nanocomplexes. Cell proliferation was evaluated daily using the cell counting kit-8 assay (Dojindo, Japan) in accordance with manufacturer's instructions. Briefly, 10 μl of CCK8 solution was added to the culture medium, and incubated for 3h. The absorbance was determined at 450 nm wavelength with a reference wavelength of 630 nm. The experiments were performed in triplicate and the results were averaged from three replicates.

Transwell assay:

Transwell migration assay was conducted using transwell insert chambers (Corning). 1×10^5 cells were plated into the upper chamber in serum-free medium. Medium containing 20% FBS in the lower chamber served as chemoattractant. After incubation for 24 h at 37 °C in a 5% CO_2 humidified incubator, cells in the upper chambers were removed by wiping with a cotton swab and cells migrated to the lower surface of filter were fixed in 70% ethanol for 30 min and stained with 0.2% crystal violet for 10 min. Cell migration was scored by counting five random fields per

filter under a light microscope. Cell migration rate was calculated by formula as follows: Relative rate of migration (%) = migrating cells with treatment/ migrating cells without treatment ×100. The transwell assay was carried out in triplicate and repeated three times.

Figures

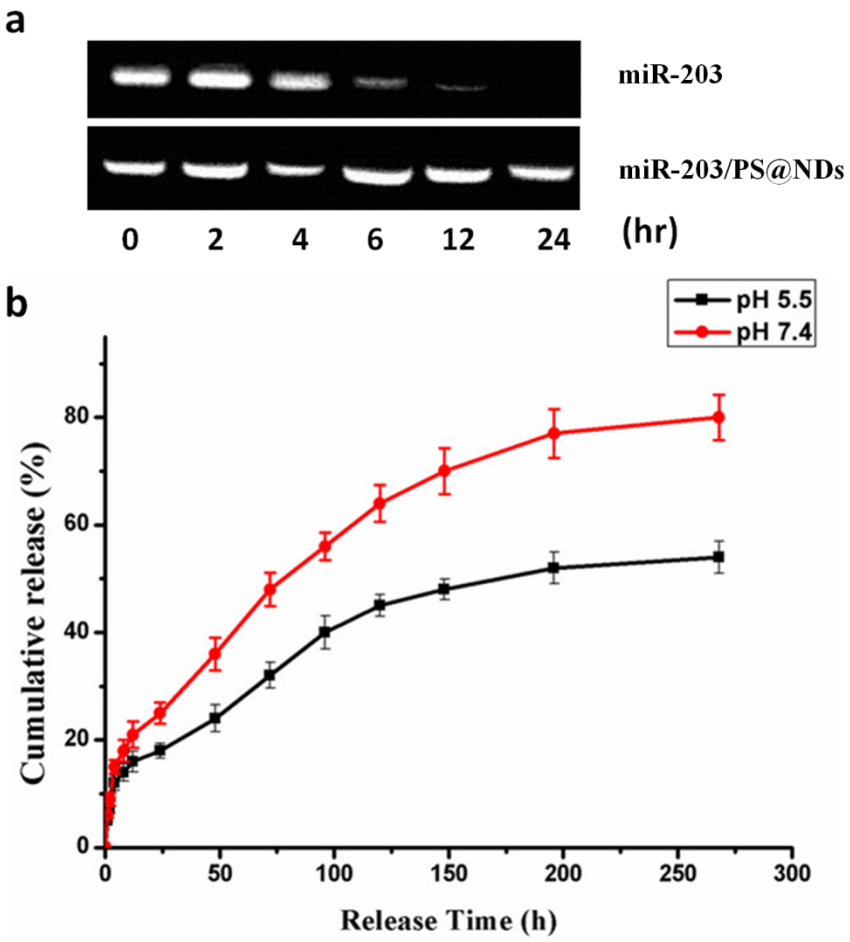

Fig. S1: a: The miRNA stability test under the presence of serum at 37°C. b: Percentage of miRNA released from miRNA/PS@NDs. The results are shown as the mean ± standard deviation (SD) of three independent measurements (n =3).

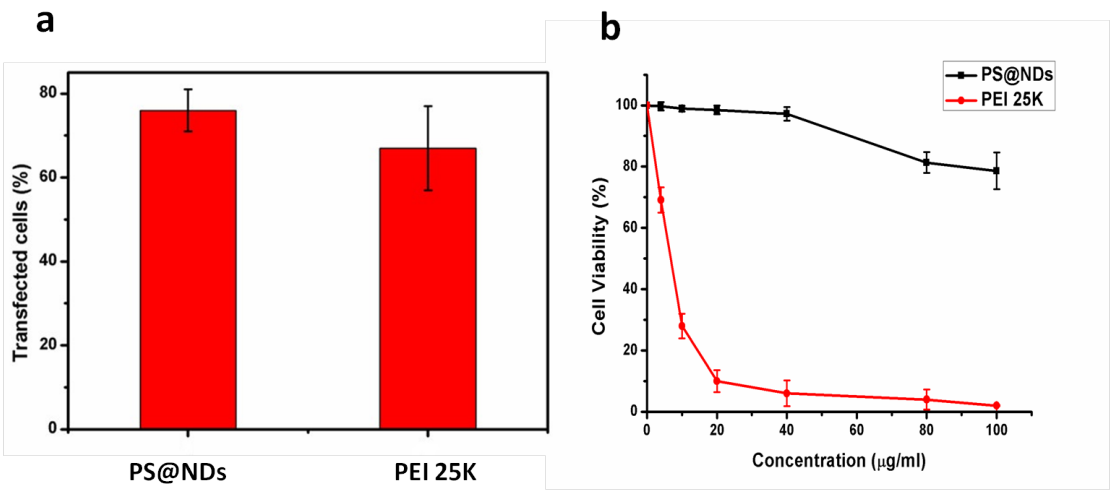

Fig. S2: a: Flow cytometry was used to determine the % transfected cells which treated with miR-203/PS@NDs and miR-203/PEI 25K separately. b: Cytotoxicity assay. Various concentrations of PS@NDs and PEI 25K were incubated with Ec-109 for 48 hours. Cell viability was measured with a cell counting Kit. The results are shown as the mean ± standard deviation (SD) of three independent measurements (n =3).

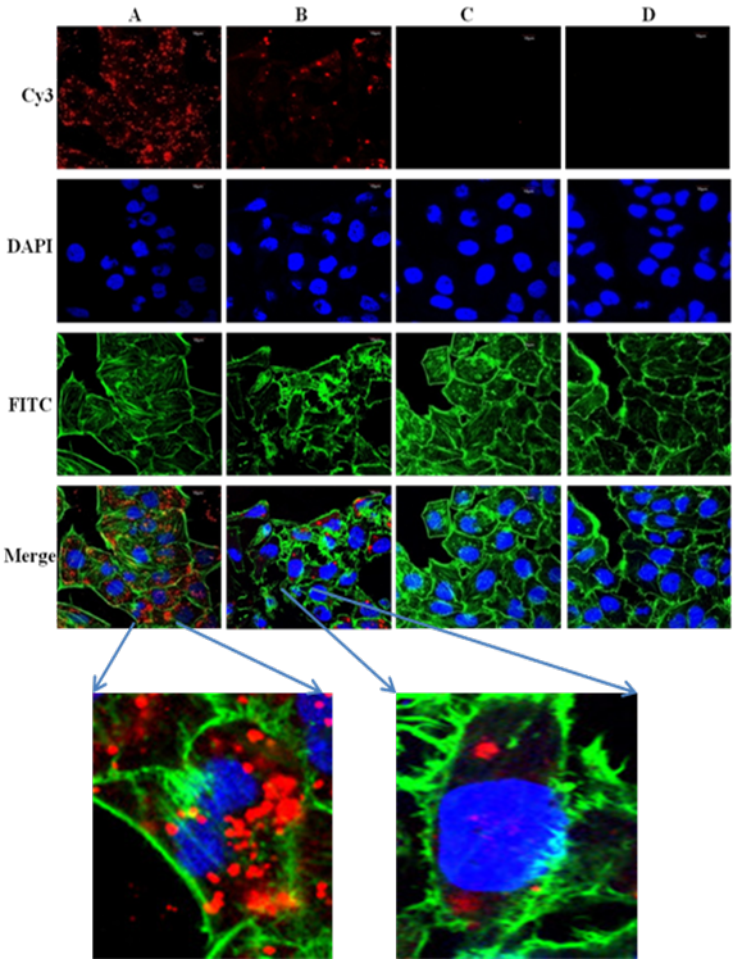

Fig. S3: Confocal microscope images of intracellular uptake of cy3-labeled miR-203 (red) mediated by various types of materials. Ec-109 was incubated with (A) miR-203/PS@NDs, (B) miR-203/PS, (C) miR-203/NDs, (D) miR-203 for 6 h. Cells were allowed to grow in fresh medium for an additional 18 h. F-actins were stained by FITC-labeled phallodin (green) and cellular nuclei were stained with DAPI (blue). Ec-109 cells incubated with miR-203/PS@NDs and miR-203/PS are shown in enlarged views (indicated by arrows).

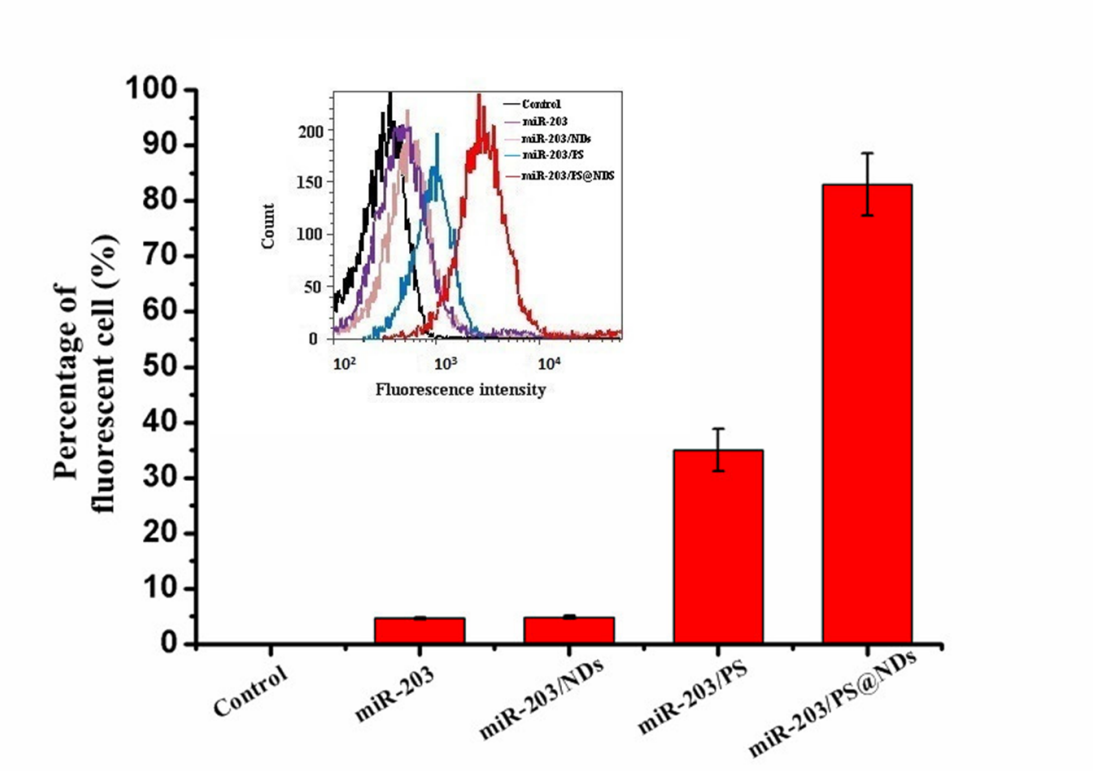

Fig. S4: Flow cytometry analysis of intracellular uptake of cy3-labeled miR-203 mediated by various types of materials. The results are shown as the mean ± standard deviation (SD) of three independent measurements (n =3).

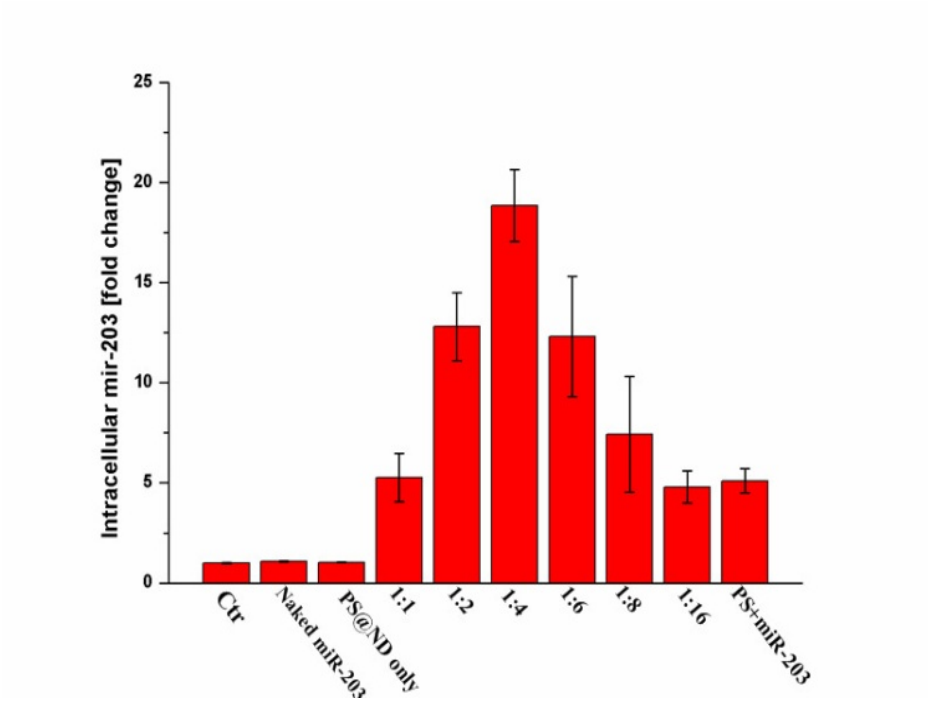

Fig. S5: MiR-203 restoration was measured by quantitative real-time PCR analysis. The results are presented as the mean ± standard deviation (SD) of three independent measurements (n =3).

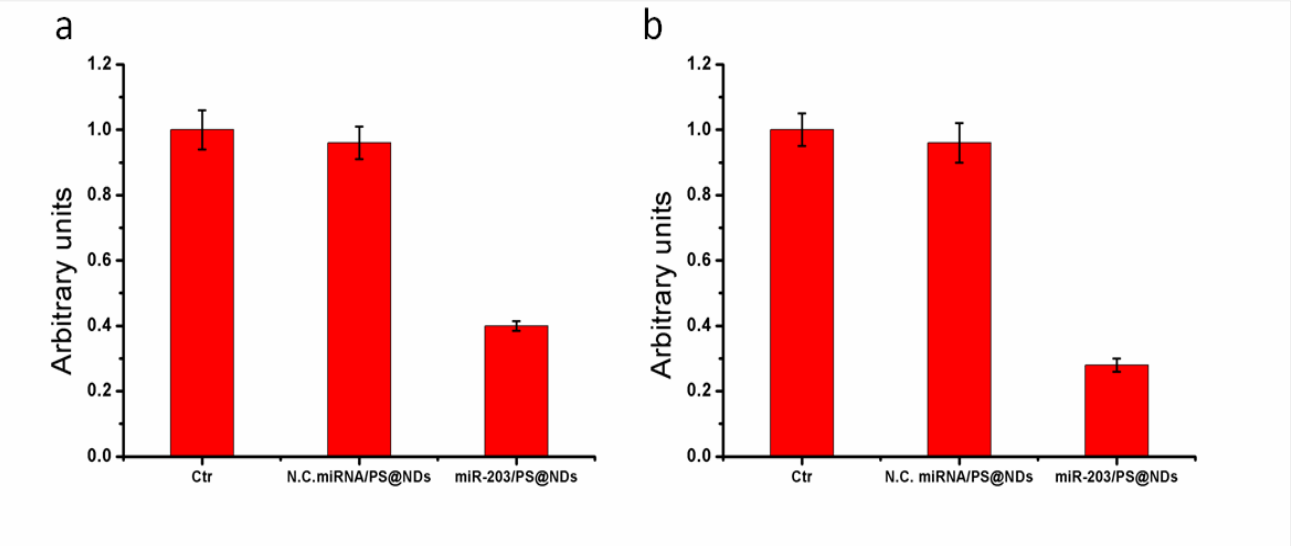

Fig. S6: Quantitative analysis of the knocking down ration of Ran (left) and ΔNp63 (right) detected by Western blot assay. Quantitative data are mean values ± S.D. from at least three independent experiments.

Tables

Table S1 The sizes and zeta potentials of miRNA/PS@ND

The hybrid materials were prepared at different miRNA:PS@NDs ratios. Results are means ± S.D. (n=3).

Different components	Hydrodynamic size (nm)	Zeta potential (mV)
NDs only	52.0±0.3	+14.2±2.0
PS@NDs only (0:1)	117.6±1.2	+34.1±2.2
1:1	132.2±3.3	-33.5±0.6
1:2	147.2±2.3	-26.3±0.6
1:4	186.7±4.2	-20.7±0.8
1:6	3011.7±300	-5.3±1.2
1:8	1024.5±252	+10.6±0.3
1:16	172.3+2.3	+29.4±0.6

Table S2. The sequences of the microRNA mimics used.

Mimic name	Sequence (5'->3')
MiR-203	5'-GUGAAAUGUUUAGGACCACUAG-3'
Scramble microRNA	5'-UCACAACCUCCUAGAAAGAGUAGA-3'

Table S3. The sequences of the primers used in RT-PCR.

Primer name	Sequence (5‘-3‘)
MiR-203	5'- GCGTGAAATGTTTAGGACCACTAG -3'
U6	5'- GCTTCGGCAGCACATATACTAAAAT -3'

Table S4. The sequences of the primers used in plasmid construct.

Primer name	Sequence (5'->3')
Ran 3'-UTR	
Forward primer	5'-CTAAGCGGAACATGTGCTTCATCT-3'
Reverse primer	5'-GATATTTGAAATACAACTTTATTC-3'
Ran 3'-UTR Mutant	
Forward primer	5'- TATAGGCAGCTGTCCTGTGATGTC -3'
Reverse primer	5'-GGCGAATACAACTTTATTCTGATT-3'
ΔNp63 3' –UTR	
Forward primer	5'-GGCCACTAGTGCCTCACCATGTGAGCTCTTC-3'
Reverse primer	5'-GGCCACTAGTGCATGTCCTGGCAAACAAAAAGAG-3'
ΔNp63 3'-UTR Mutant	
Forward primer	5'-GAATGA GTCCTTGATTCGCAAGTTT-3'
Reverse primer	5'-TTTAAGTA CAACAAAACTGCGAATC-3'