Supplementary Materials for

Biocompatible, Chimeric Peptide-Condensed Supramolecular Nanoparticles for Tumor Cell-Specific siRNA Delivery and Gene Silencing

Hangxiang Wang[1‡], Wei Chen[2‡], Haiyang Xie[1], Xuyong Wei, Shengyong Yin[1], Lin Zhou[1], Xiao Xu[1]* & Shusen Zheng[1]*

[1]First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang, PR China.

[2]Zhejiang University School of Medicine, Hangzhou, Zhejiang, PR China.

*Correspondence should be addressed to X. X. (zjxu@zju.edu.cn) or S. Z. shusenzheng@zju.edu.cn

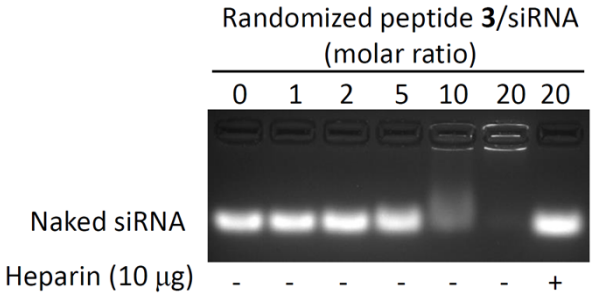

Figure S1 Agarose gel (2%) retardation assay at different molar ratios of randomized peptide **3** to siRNA.

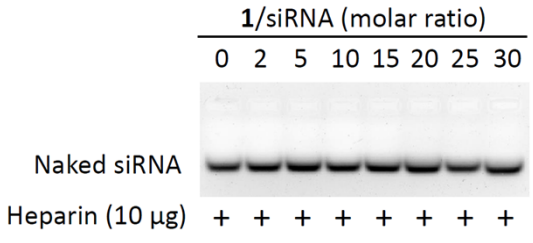

Figure S2 siRNA encapsulated inside the SP94-dR **1** nanoparticle can be released upon addition of heparin (10 μg).

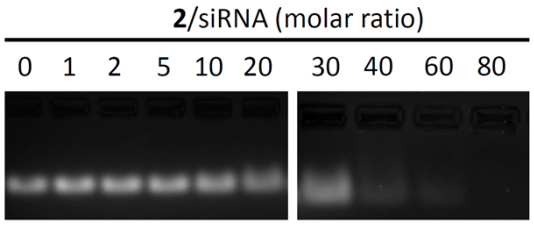

Figure S3 Agarose gel (2%) retardation assay at different molar ratios of peptide **2** to siRNA. The consecutive D-arginine was replaced by L-arginine in peptide **2**.

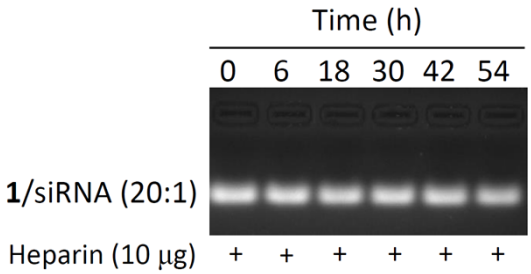

Figure S4 Stability of siRNA complexed with **1** against RNase degradation over the course of the 54 h experimental period. Before the agarose gel assay, heparin (10 μg) was added to release the intact siRNA.

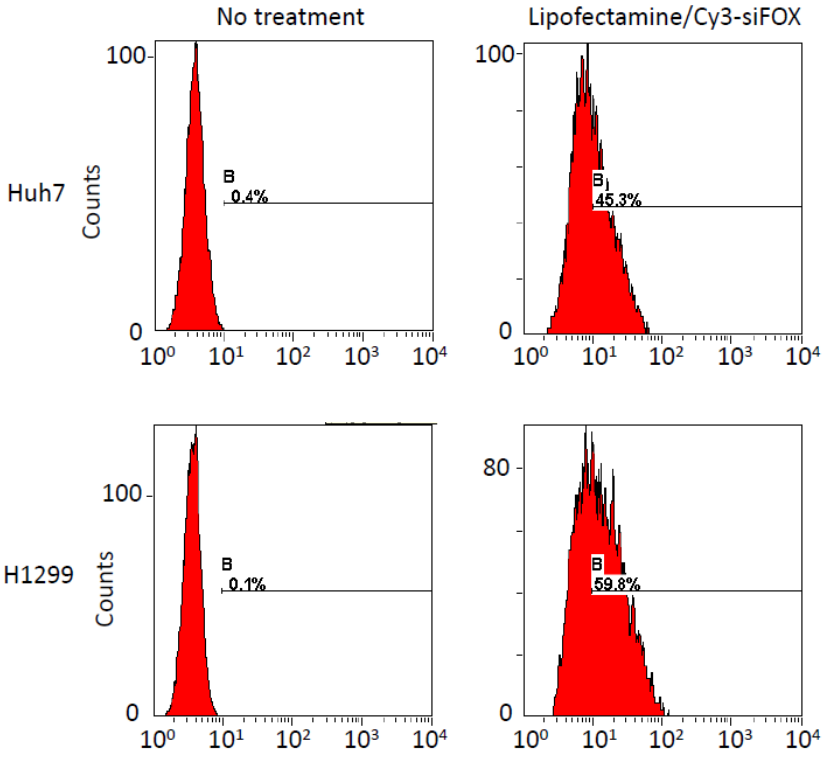

Figure S5 Flow cytometry evaluation of Lipofectamine 2000 (Invitrogen) for intracellular delivery of Cy3-labeled siFOX (50 nM) on Huh7 (upper panel) and H1299 (lower panel) cells. No treatment (left) and Lipofectamine-mediated transfection.

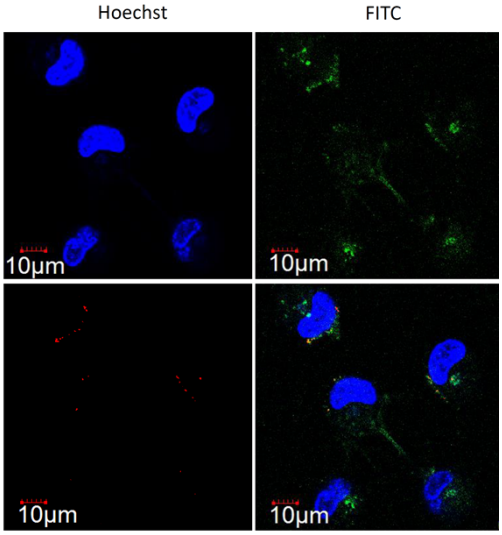

Figure S6 Representative confocal fluorescence microscopy images of H1299 cells that were treated with Cy3-labeled siRNA (50 nM) complexed with the SP94-dR **1** peptide. The nuclei and the endosomes/lysosomes were stained with Hoechst 33342 (blue) and FITC-labeled Dextran (green, endo/lysosome tracker), respectively.

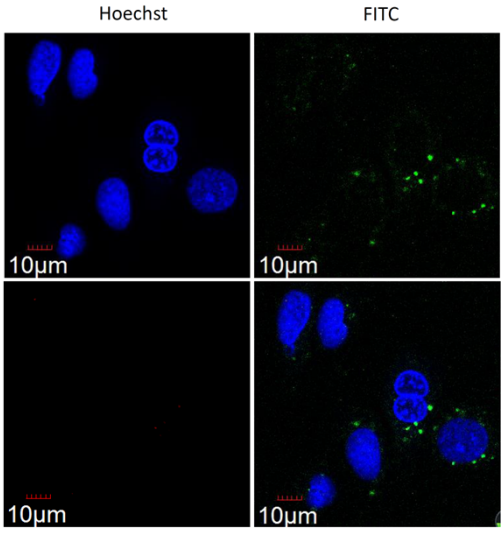

Figure S7 Representative confocal fluorescence microscopy images of Huh-7 cells that were treated with Cy3-labeled siRNA (50 nM) complexed with the scrambled peptide **3**. The nuclei and the endosomes/lysosomes were stained with Hoechst 33342 (blue) and FITC-labeled Dextran (green, endo/lysosome tracker), respectively.

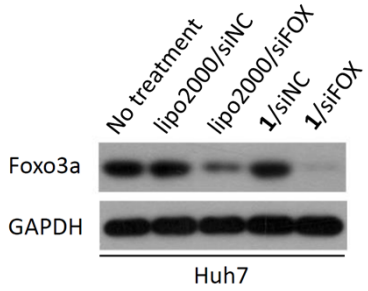

Figure S8 Endogenous foxo3a gene expression was reduced in the Huh7 cells by SP94-dR **1**/siFOX (100 nM) nanoparticle, but the control siRNA (siNC, 100 nM)-encapsulated nanoparticle did not reduce the foxo3a expression by western blotting analysis. As a comparison, cells were treated with Lipofectamine 2000-complexed siFOX (100 nM) and siNC (100 nM).

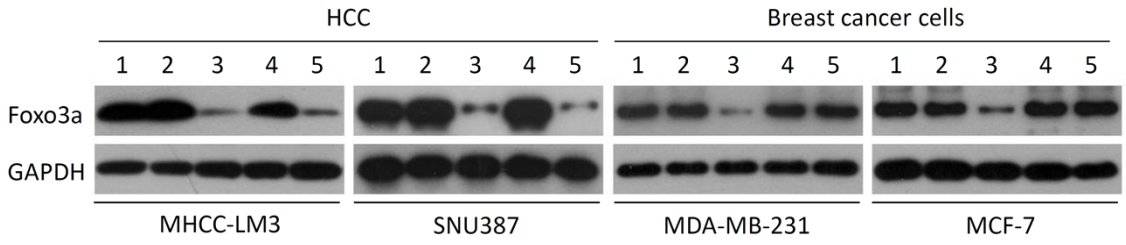

Figure S9 SP94-dR **1**-mediated Foxo3a gene-silencing in HCC and breast cancer cells. As a comparison, cells were treated with Lipofectamine 2000-complexed siFOX (100 nM) and siNC (100 nM). Lane 1: No treatment; Lane 2: lipo2000/siNC ; Lane 3: lipo2000/siFOX; Lane 4: SP94-dR **1**/siNC; Lane 5: SP94-dR **1**/siFOX.

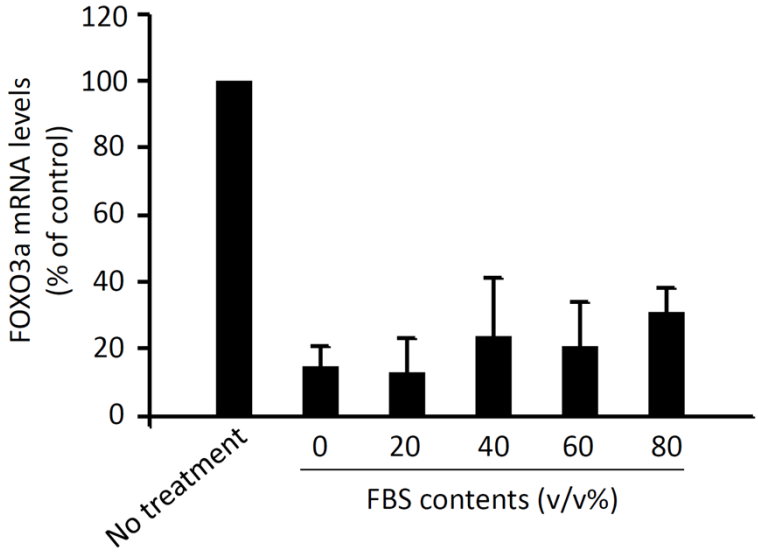

Figure S10 In vitro quantification of Foxo3a mRNA levels in Huh7 cells after transfection of SP94-dR **1**/siFOX (at a 20:1 molar ratio with 50 nM siRNA) nanoparticles in the presence of fetal calf serum (FBS).

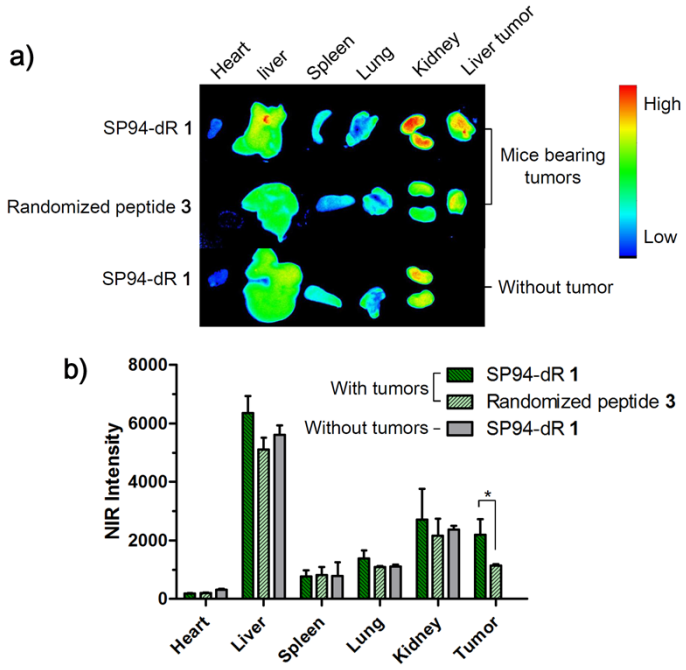

Figure S11 In vivo distribution of peptide-encapsulated Cy5.5-ODN nanoparticles in orthotopic MHCC-97H hepatocellular carcinoma mice model. (a) Representative images of SP94-dR **1**-complexed and randomized peptide **3**-complexed Cy5.5-ODN nanoparticles at 24 h after intravenous injection. Mice without tumors were also injected with SP94-dR **1**/ Cy5.5-ODN nanoparticles for comparison. Ex vivo NIRF images of the major organs. (b) NIR fluorescence intensity in major organs at 24 h after injection of **1** and **3**/ Cy5.5-ODN nanoparticles (n=3). * indicates $p < 0.05$.

Table S1 Formulations of peptides **1-3** with siRNA.

Peptide/siRNA nanoparticle (molar ratio)	Size (nm)	PDI	Zeta-potential (mV)
1/siRNA (20/1)	190	0.137	25.9±2.6
2/siRNA (40/1)	ND	ND	24.6±5.5
3/siRNA (20/1)	326	0.185	15.3±1.7

PDI, polydispersity index; ND, not determined. Values are mean±SD.

Materials and Methods

Peptide synthesis

Peptides were synthesized by solid-phase synthesis and purified by high-performance liquid chromatography.

SP94-dR, **1**, NH_2-SFSIIHTPILPLGGGG*RRRRRRRRR*-COOH

SP94-R, **2**, NH_2-SFSIIHTPILPLGGGGRRRRRRRRR-COOH

Randomized SP94-dR, **3**, NH_2-FLSISTHPPILIGGGG*RRRRRRRRR*-COOH

In **1** and **3**, the C-terminal nine arginine residues are $_D$-arginine.

ESI-MS:

1: calcd for $[M+3H]^{3+}$ = 991.51, obsd 991.69

2: calcd for $[M+3H]^{3+}$ = 991.51, obsd 991.79

3: calcd for $[M+3H]^{3+}$ = 991.51, obsd 991.66

siRNA sequence

siRNAs were synthesized by GenePharma Co. Ltd (Shanghai, China) and the sequences of Foxo3a siRNA (siFOX) are: 5'-GCACAGAGUUGGAUGAAGUTT-3' (sense); 5'-ACUUCAUCCAACUCUGUGCTT-3' (antisense). For some experiments, siRNA with Cy3 label at the 5' end of the sense strand was used. The sequences of negative control siRNA (siNC) are: 5'-UUCUCCGAACGUGUCACGUTT-3' (sense); 5'-ACGUGACACGUUCGGAGAATT-3' (antisense).

Encapsulation of siRNA and agarose gel electrophoresis

The siRNA (20 μM) in RNase-free distilled water was complexed with various amounts peptides, at different molar ratios from 1 to 40 (peptide/siRNA). After incubation at room temperature for 10 min, gel retardation assay (2% agarose with ethidium bromide) was used to determine the complex formation. Electrophoresis was performed at 30 V for 30 min in TAE buffer (20 mM Tris-HCl, 10mM glacial acetic, 0.5mM EDTA, pH 8.0), and the siRNA bands on the gel was visualized under a UV transilluminator at a wavelength of 365 nm using gel imaging system (Alphalmager HP).

Characterizing of nanoparticles

The hydrodynamic diameters and zeta-potential of the peptide/siRNA nanoparticles in water were analyzed at 25 ºC.

TECNAL 10 (Philips) was used to obtain transmission electron microscopy (TEM) images, operating at an acceleration voltage of 80 kV. The sample solution at a concentration with 5 μM siRNA (peptide **1**/siRNA=20:1) was placed onto a 300-mesh copper grid coated with carbon. Approximately 2 min after deposition, the surface water was removed with filter paper and then air-dried. Positive staining was performed using a 4 wt % aqueous uranyl acetate solution.

siRNA stability test against RNase A degradation and serum stability

For stability against RNase A degradation, siRNA (20 μM, 15 μL) was mixed with SP94-dR **1** at a molar ratio of 20:1 and incubated at room temperature for 10 min. After adding 1 μL of RNase A (diluted to 0.001 μg/μL, Takara, Japan) and incubated for a predetermined period at 37 ºC, 3 μL of the sample was frozen with liquid N_2 and stocked in -80 ºC. Before loading to 2% agarose gel (ethidium bromide staining), the samples were treated with heparin (10 μg) to release intact siRNA. As a control, naked siRNA without peptide **1** was treated with RNase A under identical conditions and subjected to agarose gel analysis.

For serum stability assay, siRNA (20 μM, 15 μL) was mixed with SP94-dR **1** at a molar ratio of 20:1 and incubated at room temperature for 10 min. After adding mouse serum at a final 50% (v/v) concentration, the samples were incubated for a predetermined period at 37 ºC and then performed the agarose gel analysis.

Cell culture

The human hepatocellular carcinoma cell Huh7 was maintained in DMEM medium. HCC cells LM3, SNU387 and lung cancer cell H1299 were maintained in RPMI-1640 media. Breast cancer cells, MDA-MB-231 and MCF-7 were maintained in L-15 and MEM media, respectively. All media were supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were incubated at 37 ºC in a 5% CO_2 atmosphere.

Cellular uptake studies

For microscopic observation, Huh7 or H1299 cells (2×10^5 cells) were seeded on glass coverslips and incubated for overnight. SP94-dR **1** or randomized peptide **3** carrying

Cy3-siFOX were added and incubated with the cells. The final concentration of Cy3-siFOX in the Opti-MEM was 50 nM. After 6 h of incubation, cells were cultured in freshly replaced DMEM growth media for further 20 h. The nuclei and the endosomes/lysosomes were stained with Hoechst 33342 (Invitrogen) and FITC-labeled Dextran (Sigma-Aldrich), respectively. The cells were imaged by CLSM (Olympus, IX81-FV1000) to determine the localization of siRNA inside the cells.

For flow cytometric analysis, Huh7 or H1299 cells were seeded into 6-well plates at 3×10^5 cells per well for overnight. After 6 h transfection with Cy3-siFOX-formulated nanoparticles (50 nM final concentration in Opti-MEM media), the DMEM media were freshly replaced and cultured at 37 ºC in a 5% CO2 humidified atmosphere for 12 h. the cells were rinsed twice with cold PBS, trypsinized and washed with cold PBS. After filtration through 40-μm cell strainer, the cells were analyzed using a Beckman Coulter (FC500MCL).

In vitro gene silencing

To assess the cell-specific gene silencing capability of nanoparticles, cells were seeded into 6-well plates (2×10^5 per well) and incubated overnight. The cells were transfected with SP94-dR **1**/siFOX (molar ratio with 20:1) formulations at different siRNA doses (0-200 nM) in Opti-MEM or in the presence of FBS. Lipofectamine 2000 carrying 50 or 100 nM of siFOX or siNC in Opti-MEM was used as the positive control. After 6 h incubation, the transfection solution was changed to fresh growth media and incubated for further 24 h at 37 ºC in a 5% CO_2 atmosphere. The cellular levels of Foxo3a mRNA and protein were assessed using quantitative real time PCR (qRT-PCR) and western blot, respectively.

For qRT-PCR analysis, the cells were collected and total RNA was extracted using the TriZol reagent (Invitrogen) and Prime Script reagent RT Kit (Takara, Dalian, China) following the manufacturer's protocol. The primers for Foxo3a and β-actin and procedure for qRT-PCR analysis was reported in the previous publication.[S1]

For Western blot analysis, transfected cells were first washed twice with cold PBS, then resuspended in 50 μL of RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1% SDS) freshly supplemented with Roche's Complete Protease Inhibitor Cocktail Tablets. The cell lysates were incubated on ice for 1 h. The lysates were then clarified by centrifugation for 15 min at 14000 g. The protein concentration was

determined using the BCA Protein Assay Kit (Thermo Fisher Scientific Inc.; Rockford, IL, USA). The samples were mixed with 2×sample buffer (pH 6.8, 125 mM Tris•HCl, 20% Glycerol, 4% SDS, 0.01% Bromophenol blue, 100 mM DTT). The samples then were resolved by 10% SDS-PAGE and electrotransfered onto an Immun-Blot PVDF membrane (Bio-Rad). The immunodetection of Foxo3a was performed with anti-Foxo3a antibody (Cell Signaling) and anti-rabbit IgG-HRP conjugate (Santa Cruz Biotechnology).

Biodistribution of nanoparticles *in vivo*

For the investigation of biodistribution of nucleic acid-loaded nanoparticles *in vivo*, MHCC-97H tumor was inoculated to liver of six-week-old Balb/c nude mice. After one months, tumor-bearing mice were injected via the tail vein with Cy5.5-labeled ODN (20 μg per mouse, sequence: 5'-TCT CCC AGC GTG CGC CAT-3' was custom synthesized by Alpha DNA, Inc (Montreal, Canada)) formulated with SP94-dR **1** or non-targetable peptide **3**. After 4 h or 24 h, the NIR fluorescence signal was monitored using animal imaging system (Carestream MS FX PRO). The excised tissue' fluorescence including livers, lungs, spleens, kidneys, and hearts, as well as the tumors, were imaged. All animal procedures were performed according to national regulations and approved by the local animal experiments ethical committee.

Reference

S1) Liang, C.; Chen, Wei.; Zhi, X.; Ma, T.; Xia, X.; Liu, H.; Zhang, Q.; Hu, Q.; Zhang, Y.; Bai, X.; Liang, T. *Mol. Cancer* **2013**, 12:14.