Supporting information

Enhanced intercellular delivery of cRGD-siRNA conjugates by an additional oligospermine modification

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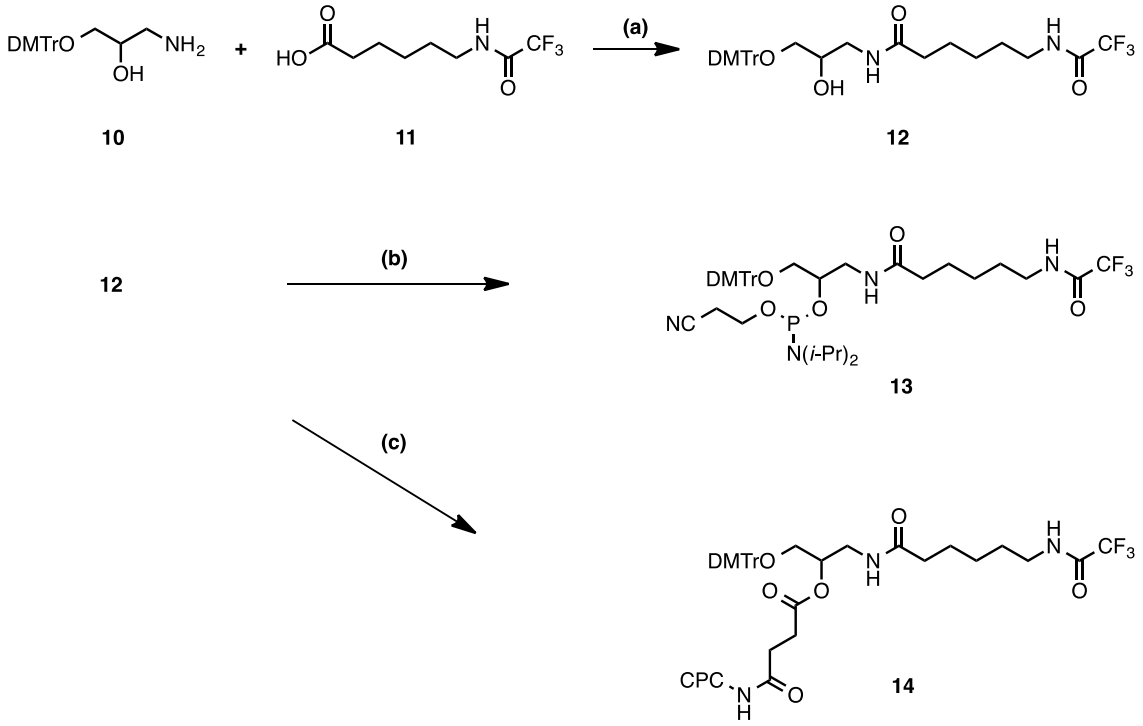
Contents

General remarks

^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded at 600, 500 and 400 MHz (^{1}H), at 150 and 125 MHz (^{13}C), and at 162 MHz (^{31}P). $CDCl_3$ (CIL) or DMSO-d_6 (CIL) was used as a solvent for obtaining NMR spectra. Chemical shifts (δ) are given in parts per million (ppm) downfield from $(CH_3)_4Si$ (δ 0.00 for ^{1}H NMR in $CDCl_3$), a solvent (δ 39.5 for ^{13}C NMR and δ 2.49 for ^{1}H NMR in DMSO-d_6), or H_3PO_4 (δ 0.00 for ^{31}P NMR in $CDCl_3$) as an internal reference with coupling constants (*J*) in hertz. The abbreviations s, d, t, q and m signify singlet, doublet, triplet, quartet and multiplet respectively. Mass spectra were obtained by electrospray ionization (ESI) time-of-flight (TOF) mass spectrometry. DNA and RNA syntheses were carried out with a DNA/RNA synthesizer. Silica gel chromatography was done with Kanto silica gel 60 N.

Synthesis of amino modified phosphoramidite and CPG. Amino modified phosphoramidite (**13**) and CPG (**14**) were synthesized as shown in Scheme S1. (*S*)-3-amino-1-*O*-(4,4'-dimethoxy)trytil-2-propandiol (**10**) and 6-(trifluoroacetamido)-hexanoic acid (**11**) were synthesized according to previous reports[1,2].



Scheme S1. Synthesis of amino modified phosphoramidite and CPG. (a) DMAP, NEt_3, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, DMF, room temperature, overnight; (b) *N*,*N*-diisopropylethylamine, chloro(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphine, CH_2Cl_2, room temperature, 1 hr. (c) (1) succinic anhydride, DMAP, pyridine, room temperature, overnight. (2) aminopropyl controlled pore glass, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, DMF, room temperature, 3 days.

(*S*)-3-(6-Trifluoroacetylamino)hexanecamido-1-*O*-(4-4'-dimethoxy)trityl-2-propanol (12). To a solution of **10** (0.50 g, 1.27 mmol) in DMF (10 mL) was added **11** (0.35g, 1.52 mmol), DMAP (0.19 g, 1.52 mmol), NEt_3 (0.21 mL, 1.52 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.29 g, 1.52 mmol) at room temperature. The mixture was stirred at room temperature for overnight. The solvent was

evaporated in vacuo, and the resulting residue was purified by column chromatography (SiO_2, 3% MeOH in $CHCl_3$) to give **12** (0.44 g, 0.73 mmol, 57%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42-7.39 (m, 2H), 7.31-7.27 (m, 6H), 7.24-7.20 (m, 1H), 6.83 (d, 4H, *J* = 9.2), 6.71 (s, 1H), 5.70-5.69 (m, 1H), 3.88 (s, 1H), 3.79 (s, 1H), 3.57-3.52 (m, 1H), 3.36 (q, 2H, *J* = 6.4), 3.26-3.12 (m, 3H), 3.05 (d, 1H, *J* = 4.4), 2.11 (t, 2H, *J* = 6.8), 1.64-1.53 (m, 4H), 1.36-1.29 (m, 2H); ^{13}C NMR (151 MHz, DMSO-d_6) δ 172.2, 158.0, 145.1, 135.8, 129.7, 127.7, 127.7, 126.5, 113.1, 85.1, 68.8, 65.7, 55.0, 42.6, 40.0, 35.1, 28.0, 25.8, 24.9; HRMS (ESI-TOF) *m/z* $[M + Na]^+$ Calcd for $C_{32}H_{37}F_3N_2Na_1O_6$ 625.2501; Found 625.2494.

(*S*)-3-(6-Trifluoroacetylamino)hexanecamido-1-*O*-(4-4'-dimethoxy)trityl-2-*O*-[(2-cyanoethoxy)(*N*,*N*-diisopropyamino)]phosphanyl-propane (13). A mixture of **12** (0.64 g, 1.06 mmol), *N*,*N*-diisopropylethylamine (0.74 mL, 4.24 mmol), and chloro(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphine (0.5 mL, 2.12 mmol) in CH_2Cl_2 (6.4 mL) was stirred at room temperature for 1 hr. The mixture was partitioned between $CHCl_3$ and aqueous $NaHCO_3$ (saturated). The organic layer was washed with brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (a neutralized SiO_2, 66% EtOAc in hexane) to give **26** (0.43 g, 0.54 mmol, 51%): ^{31}P NMR (242 MHz, $CDCl_3$) δ 149.7, 149.0; HRMS (ESI-TOF) *m/z* $[M + Na]^+$ Calcd for $C_{41}H_{54}F_3N_4Na_1O_7P_1$ 825.3578; Found 825.3570.

Solid support synthesis. A mixture of **12** (0.10g, 0.17 mmol), succinic anhydride (0.10 g, 1.00 mmol), and DMAP (61 mg, 0.50 mmol) in pyridine (2 mL) was sstirred at room temperature for overnight. The mixture was partitioned between EtOAc and aqueous $NaHCO_3$ (saturated). The organic layer was washed with brine, dried (Na_2SO_4), and

concentrated to give the corresponding succinate. A mixture of the succinate, aminopropyl controlled pore glass (0.26 g, 40 μmol), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (32 mg, 0.17 mmol) in DMF (1.7 mL) was shaken at room temperature for 3 days. After the resin was washed with pyridine, a capping solution (15 mL, 0.1 M DMAP in pyridine:Ac_2O = 9:1, v/v) was added and the whole mixture was shaken at room temperature for overnight. The resine was washed with pyridine, EtOH, and MeCN, and dried *in vacuo*. The amount of the compound **12** loaded on the solid support **13** was 26 μmol/g from the calculation based on absorbance of dimethoxytrityl cation released by treating with a solution of 70% $HClO_4$:EtOH (3:2, v/v).

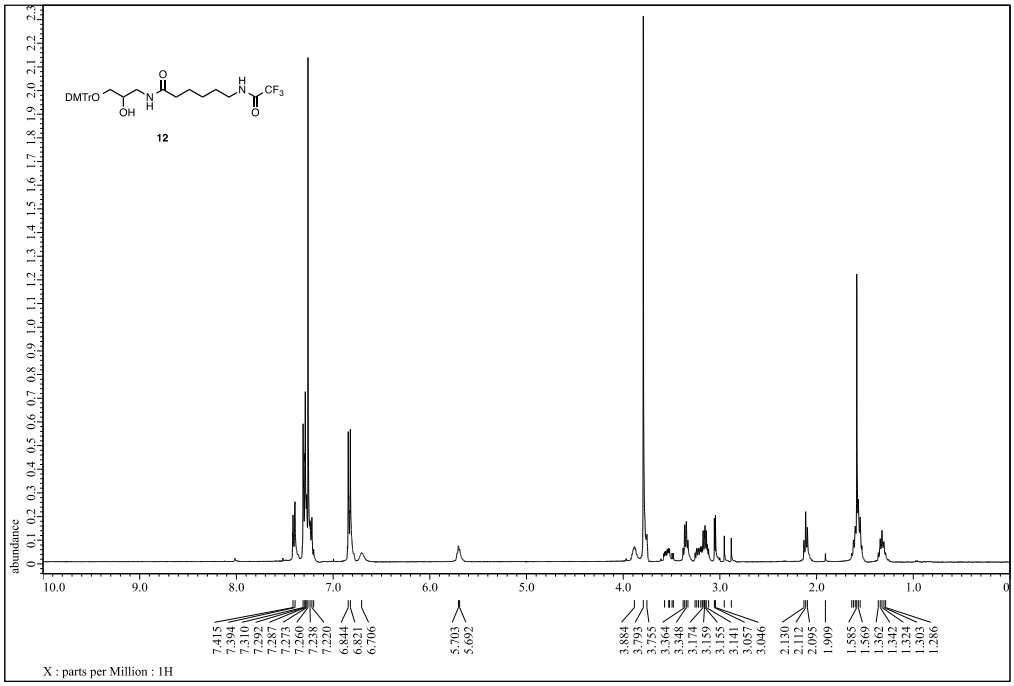


Figure S1. ^{1}H NMR spectra of compound **12** in $CDCl_3$

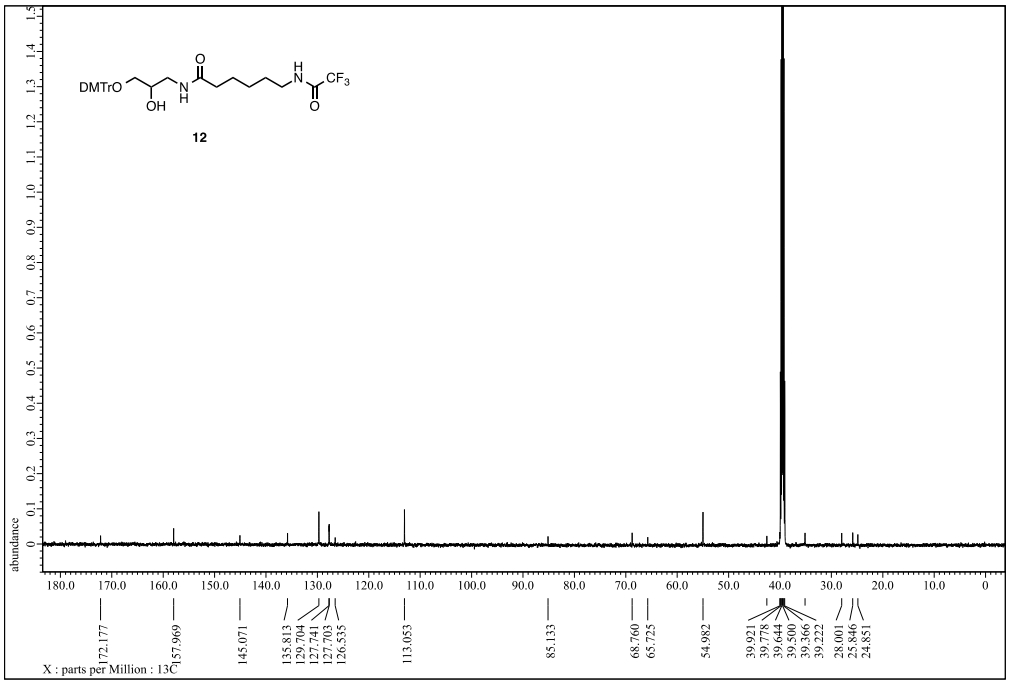


Figure S2. ^{13}C NMR spectra of compound **12** in DMSO-d_6

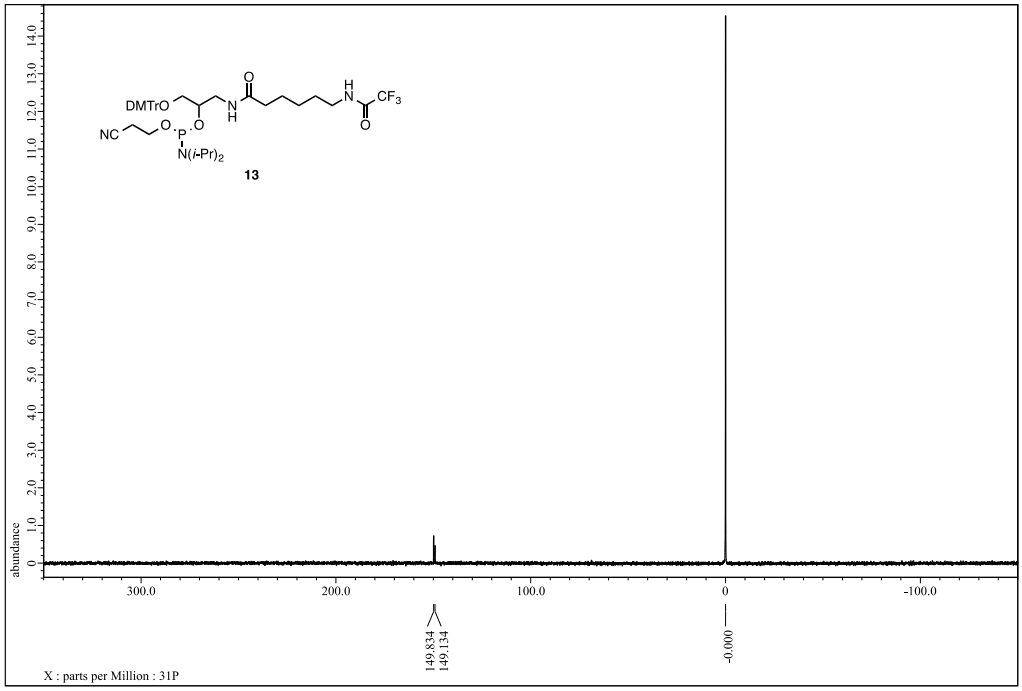
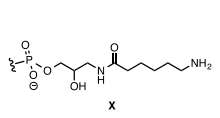
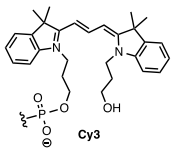


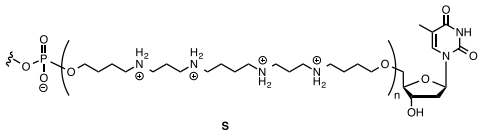
Figure S3. ^{31}P NMR spectra of compound **13** in $CDCl_3$

Table S1. Sequences of ssRNAs.

No	Sequence (5'-3')[a]	calcd. mass	observed mass
6	AAGCUGAAGUGGuCuGAAcdTdT	6775.2	6775.6 ([M-H]$^-$)
7	AAGCUGAAGUGGuCuGAAcdTdT-X	7041.4	7039.6 ([M-H]$^-$)
14	AAGCUGAAGUGGuCuGAAcdTdT-X-Cy3	7549.0	7548.5 ([M-H]$^-$)
15	GuucAGACCACuucAGcuudTdT	6693.2	6692.1 ([M-H]$^-$)
16	GuucAGACCACuucAGcuudTdT-S_5-dT	9040.0	9038.9([M-H]$^-$)
17	GuucAGACCACuucAGcuudTdT-S_{10}-dT	11082.6	11080.7([M-H]$^-$)
18	GuucAGACCACuucAGcuudTdT-S_{15}-dT	13125.19	6773.7([M-2H]$^{2-}$)
19	GGCCUUUCACUACUUCUACUU-S_{15}-dT	12938.85	6468.52([M-2H]$^{2-}$)
20	GUAGGAGUAGUGAAAGGCCUU-X	7083.3	7081.2 ([M-H]$^-$)

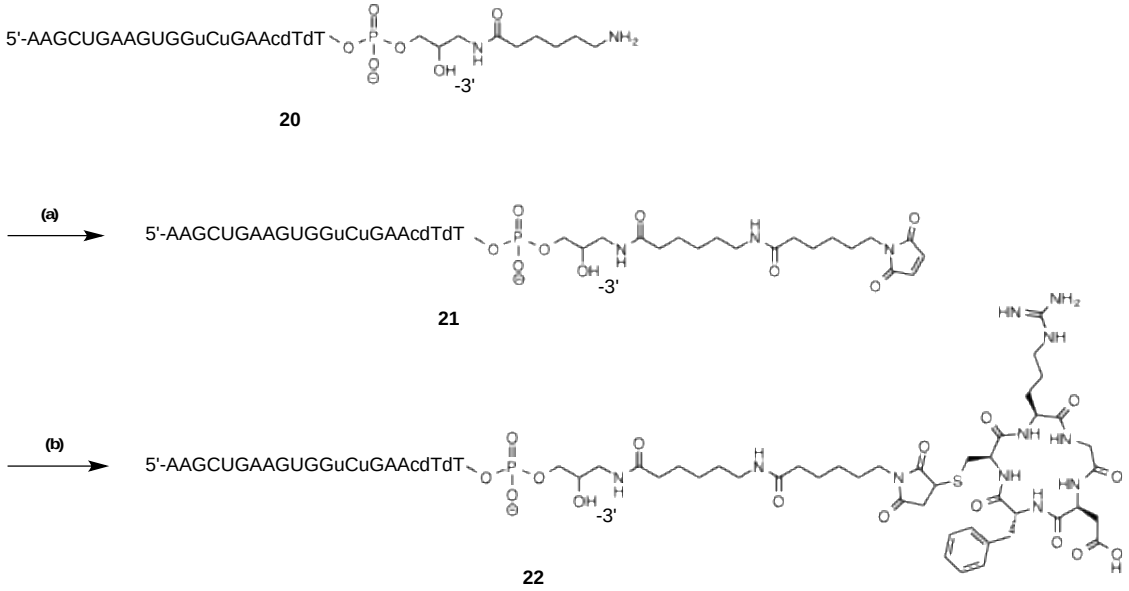






[a] Small letters indicate 2'-*O*-methyl RNA, dT indicates 2'-deoxythymidine.

Synthesis of cRGD conjugates 22. To a solution of **20** (15 nmol) in 10 mM NaCl, 100 mM phosphate buffer (pH 7.4) (100 μL) and DMSO (30 μL) was added 0.2 M EMCS in DMSO (15 μL) at room temperature. The mixture was incubation at 37 ºC overnight and maleimide-modified **21** was collected by RP-HPLC. Subsequently, 50 mM c(RGDfC) peptide in DMSO (3 μL) was directly added to the collected solution containing **21**. The mixture was incubation at room temperature overnight and cRGD conjugated RNA **22** was purified by RP-HPLC (1.47 nmol, 10%).



Scheme S2. Synthesis of cRGD conjugate **22**. (a) *N*-(6-maleimidocaproyloxy)succinimide (EMCS), Phosphate buffer (pH 7.4), room temperature, overnight; (b) c(RGDfC), 0.1 M TEAA, 50% MeCN aq., room temperature, overnight.

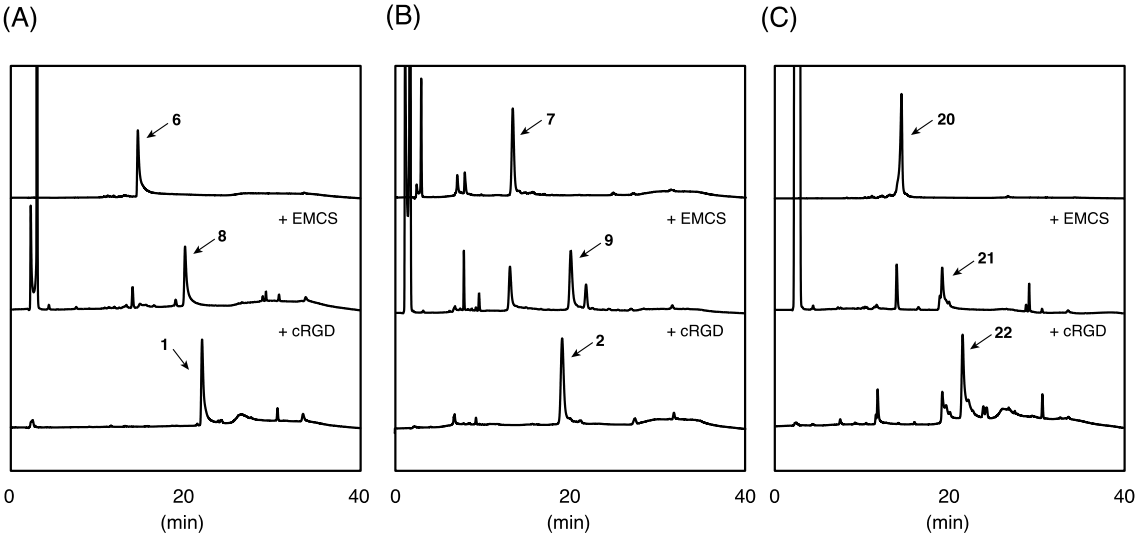


Figure S4. RP-HPLC profiles of the reaction mixtures of cRGD conjugates. (A) cRGD-antisense strand conjugate of *RecQL1* siRNA (**1**). (B) Cy3-labeled cRGD-antisense strand conjugate of *RecQL1* siRNA (**2**). (C) cRGD-antisense strand conjugate of *Renilla*

luciferase siRNA (**22**).

Table S2. Sequences of cRGD conjugated antisense strands.

No	Sequence (5'-3')[a]	calcd. mass	observed mass
1	AAGCUGAAGUGGuCuGAAcdTdT-RGD	7813.3	7812.6 ([M-H]$^-$)
2	AAGCUGAAGUGGuCuGAAcdTdT-RGD-Cy3	8320.9	8319.0 ([M-H]$^-$)
22	GUAGGAGUAGUGAAAGGCCUU-RGD	7854.2	7853.1 ([M-H]$^-$)

[a] Small letters indicate 2'-*O*-methyl RNA, dT indicates 2'-deoxythymidine.

Table S3. Sequence of *Renilla* luciferase siRNA, N/P ratio.

Name	S/AS[a]	Sequence (5'-3')[b]	N/P
siLuc[S15/RGD]	S	GGCCUUUCACUACUUCUACUU-S_{15}-dT	1.05
	AS	GUAGGAGUAGUGAAAGGCCUU-RGD	

[a] S denotes sense strand, AS denotes antisense strand.

[b] dT indicates 2'-deoxythymidine.

Table S4. Primers used for determination of target mRNA levels.

Abbreviation of primer[a]	Sequence (5'-3')
RecQL1-F	TGATTCTTCACCTGCCGCTT
RecQL1-R	TGACGAGTGTAAAACCATCTGA
β-actin-F	GAGCACAGAGCCTCGCCTTT
β-actin-R	CCTCGTCGCCCACATAGGAA

[a]Fs denote forward primers. Rs denote revers primes.

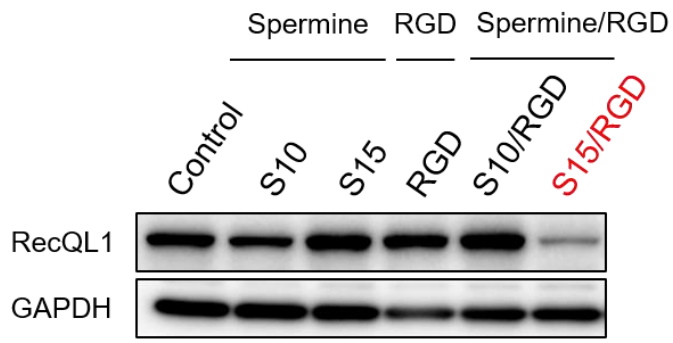


Figure S5. Gene silencing activities of the synthesized siRNA conjugates. Each siRNA was transfected into A2058 cells at 250 nM. Whole cell protein samples were collected from A2058 cells, separated by 12.5% SDS-PAGE, blotted onto a PVDF membrane and then, visualized by chemiluminescence.

Western blotting. 24 h before transfection, A2058 cells (0.4×10^5/mL) were transferred to a 6-well plate (1 mL/well). Cells were transfected with siRNA conjugates in serum free OPTI-MEM medium at 250 nM final concentration. After 4 hours transfection, FBS was added to each well at 2% final concentration and further incubation for 92 hours. Whole cells were homogenized in chilled lysis buffer (10 mM Tris-HCl (pH 7.4), 1% NP-40, 0.1% deoxycholic acid, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, and 1% Protease Inhibitor Cocktail) and stood for 20 min on ice. After centrifugation at 13,000 rpm for 20 min at 4 °C, the supernatants were collected as whole cell protein samples. Protein contents were measured with a DC Protein assay kit (Biorad, Hercules). 10 μg of lysate protein was separated by 12.5% SDS–PAGE, and blotted onto a PVDF membrane (PerkinElmer Life Sciences). After blockage of nonspecific binding sites, the membrane was incubated overnight at 4 °C with primary antibody followed by incubation with HRP-

conjugated secondary antibody (Cell Signaling Technology) at room temperature. After washed the membrane, the immunoblots were visualized by use of Ammersham ECL Plus Western Blotting Detection Reagents (GE Healthcare) according to the manufacture's protocol.

References

S1. Azhayev, A. V.; Antopolsky, M. L. Amide group assisted 3′-dephosphorylation of oligonucleotides synthesized on universal A-supports. *Tetrahedron* **2001**, *57*, 4977–4986.

S2. Chen, Q.; Chu, T. A two-step strategy to radiolabel choline phospholipids with ^{99m}Tc in S180 cell membranes via strain-promoted cyclooctyne–azide cycloaddition reaction. *Bioorg. Med. Chem. Lett.* **2016**, *26*, 5472–5475.

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