Supporting Information to

Intracellular Build-up RNAi with Single-Strand Circular RNAs as siRNA Precursors

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Materials and Methods

Preparation of RNA oligonucleotides

RNA oligonucleotides were synthesized on a 0.2 or 1 μmol scale on an H-8 SE DNA synthesizer (Gene World, Tokyo, Japan) or an NR-2A7MX (Nihon Techno Service, Ibaraki, Japan) using 2′-*O*-TOM-protected β-cyanoethyl phosphoramidites. For introducing 5′-terminal phosphate group, 5′-phosphate-ON Reagent (ChemGenes, USA, CLP-1544) was used. To synthesize the RNAs containing a photo-cleavable linker, PC-linker phosphoramidite (Glen Research, USA, 10-4920) was used. To synthesize the RNAs with phosphorothioate at 5′-terminal and alkylthiol at 3′-terminal, 3′-Thiol Modifier C3 S-S CPG (Glen Research, 20-2933) was used and, sulfurizing reagent (ChemGenes, RN-1535) or DDTT (ChemGenes, RN-1588) was used.

After the synthesis on the synthesizer, the CPG were treated with 1:1 mixture of 40% aqueous methylamine and 28% ammonium hydroxide for 30 min at 65 °C and concentrated *in vacuo*. Tetra-*n*-butylammonium fluoride (1 M solution in tetrahydrofuran) was added to the concentrated residue and incubated at 35 °C overnight. The reaction was quenched by the addition of 1 M Tris-HCl (pH 7.2) and desalted with a NAP-25 column (GE Healthcare). Deprotected oligonucleotides were purified by 18% denaturing PAGE (acrylamide : bis-acrylamide = 19:1, 7.0 M urea, 25% formamide, 1 x TBE), and then isolated by the crush and soak method (elution; MQ). After desalted with Amicon-Ultra 3K (Merck) following the manufacture's standard protocols, the RNAs were precipitated with sodium acetate (pH 5.2) and 2-propanol.

For RNAs containing alkylthiol unit, the RNA was incubated in 200 mM DTT, 50 mM tetraborate buffer (pH 8.5) at 30 °C for 30 min. Then, the solution was loaded on NAP-25 column for desalting, and the collected fractions were concentrated *in vacuo.*

For fluorescence labelling, 6-Fluorescein Phosphoramidite (Glen research, 10-1964) or DMT-6-FAM phosphoramidite (ChemGenes, CLP-9780) were used. The RNAs having fluorescein was treated with 28% ammonium hydroxide for 30 min at room temperature and subsequently with 1:1 mixture of 40% aqueous methylamine and 28% ammonium hydroxide for 30 min at 65 °C for deprotection and cleavage from CPG.

Evaluation of immune responses by dsRNA and circular RNAs

RNAs (25 pmol) formulated into liposomes with Opti-MEM (142 μL, Invitrogen) were transfected into T98G cells using Lipofectamine 2000 (Invitrogen), following the manufacturer's method for adherent cell lines. Four hours post-transfection, 125 μL of RPMI (RPMI-1640 with L-Glutamine and Phenol Red, Wako, Japan) supplemented with 30% FBS was added to each well. After 20 h, wells were washed with PBS and total RNA was extracted from T98G cell lysates using ISOGEN (Nippon Gene), following the manufacturer's method. Ten nanograms of each total RNA were

reverse transcribed and amplified in a Thermal Cycler Dice® Real Time System II (Takara Bio) using a One Step SYBR® PrimeScript® PLUS RT-PCR Kit (Perfect Real Time) (Takara Bio) and gene-specific primers. β-actin was used as the internal control. Samples were assayed in triplicate and the data were analyzed according to the comparative threshold cycle (Ct) method, where the amount of target, normalized to an endogenous reference and relative to an experimental control, is given by $2^{-\Delta\Delta Ct}$. Ct indicates the PCR cycle number at which the amount of amplified target reaches a fixed threshold. The ΔCt value is determined by subtracting the mean Ct value of β-actin from the mean Ct value of IFN-β. The ΔΔCt value involves subtraction of the ΔCt experimental control value. Amplification parameters were 42°C for 5 min, 95°C for 10 s, followed by 50 cycles of 95°C for 5 s, and 60°C for 30 s, and terminated by a cycle of 95°C for 15 s, 60°C for 30 s, 95°C for 15 s.
The primer sequences for each gene were as follows;
IFN-β forward: 5'–GTTCGTGTTGTCAACATGACCA–3'
reverse: 5'–TCAATTGCCACAGGAGCTTCT–3'
β-actin forward: 5'–TGGCACCCAGCACAATGAA–3'
reverse: 5'–CTAAGTCATAGTCCGCCTAGAAGCA–3'

Synthesis of PC-Linker RNAs

The reaction was performed in a tube containing solution of 1 μM of linear RNA, 0.01 % of BSA (Takara, 2050A), 0.5 U/μL of T4 RNA ligase (Takara, 2050A), 25% of PEG 6000, 1 x T4 RNA ligase buffer (Takara, 2050A). The mixture was incubated at 15 °C for 18 h. The mixture was mixed with TE-saturated phenol and vortexed, and centrifuged at 20400 x g for 5 min. The aqueous lawyer was collected and mixed with chloroform by vortex, and centrifuged at 20400 x g for 5 min. The aqueous layer was concentrated *in vacuo* and the RNAs were precipitated with sodium acetate (pH 5.2) and 2-propanol. The resulting circular RNAs were separated by 10 ~ 18% denaturing PAGE (acrylamide : bis-acrylamide = 19:1, 7.0 M urea, 25% formamide, 1 x TBE) and isolated by the crash and soak methods (elution; MQ). After desalting with Amicon-Ultra 3K (Merck) following the manufacture's standard protocols, the RNAs were precipitated with sodium acetate (pH 5.2) and 2-propanol. The isolated circular RNAs were analyzed by denaturing PAGE and MALDI-TOF MS. The isolated amount and yields for the circularization reaction (with 4 nmol starting material) are as follows; 21CS) 602 pmol, 15%; 21CA) 313 pmol, 7.8%; 23CS) 505 pmol, 13%; 23CA) 436 pmol, 11%; 27CS) 372 pmol, 9.3%; 27CA) 362 pmol, 9.1%, ApoB-CS) 166 pmol, 4.2%; ApoB-CA) 438 pmol, 11%.

Confirmation of Duplex Formation

Sense and antisense RNA solution was mixed (final concentration; 1 μM) in MQ and the mixture was analyzed by PAGE.

UV irradiation *in vitro*

RNA solution in a tube (0.25 μM in 0.5 x PBS) was irradiated with UV light using a UV lamp (AS ONE, Japan, SLUV-4, 365 nm) with 2 cm distance for 5 ~ 15 min. The mixture was analyzed with 10% dPAGE (staining; SYBR Green II).

Measurements of RNAi activity (UV-irradiation, Lipofection)

HeLa cells were grown at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Wako Pure Chemical Industries) supplemented with 10% FBS (Biological Industries, Kibbutz Beit Haemek, Israel) and Penicillin-Streptomycin Solution (×100) (168-23191, Wako Pure Chemical Industries). Cells were regularly passaged to maintain exponential growth. One day prior to transfection at 70% confluency, cells were plated in 96-well plates (5×10^3 cells/well) for measurement of RNAi activity.

RNA and vectors were transfected into HeLa cells, following the manufacturer's protocols for adherent cell lines. Immediately prior to transfection, the culture medium was replaced with Opti-MEM (Thermo Fischer Scientific, USA), and RNA, pGL3-Control (Promega, USA), and pRL-TK (Promega) formulated in lipofectamine 2000 (Thermo Fischer Scientific, 0.25 μL/well) were added to the medium following the manufacture's standard protocols. (final concentration; RNA 10 nM, pGL3-Control 0.4 ng/μL, pRL-TK 0.4 ng/μL). Four hours after the transfection, the cell medium was exchanged to 10% FBS/DMEM. The cells were irradiated with UV light for 15 min using a UV handy lamp (AS ONE, SLUV-4, 365 nm) placed 10 cm above the 96-well plate. After 20 hours, luciferase expression was monitored with the Dual-Luciferase Reporter Assay System (Promega, Fitchburg, WI) with Wallac 1420 ARVO SX multilabel counter (PerkinElmer, USA) or Mithras LB940 (Berthold). For the experiments in Figure S3, lipofectamine 3000 was used in place of lipofectamine 2000. Scramble dsRNA sequence; 5'-p-CGGAGCUGGUUCGGUCCACUU-3', 5'-p-GUGGACCGAACCAGCUCC GUU-3'.

Measurements of RNAi activity (UV-irradiation, no lipofection)

HeLa cells were grown at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Wako Pure Chemical Industries) supplemented with 10% FBS and Penicillin-Streptomycin Solution (×100) (168-23191, Wako Pure Chemical Industries). Cells were regularly passaged to maintain exponential growth. One day prior to transfection at 80% confluency, cells were plated in 24-well plates (4.2×10^4 cells/well) for measurement of RNAi activity. Before the transfection, cells were washed with PBS twice and Opti-MEM was added. RNA and vectors were transfected into HeLa cells, following the manufacturer's protocols. pGL3-Control and pRL-TK formulated in Lipofectamine 2000 (1.05 μL/well) (final concentration; pGL3-Control; 21 ng/μL, pRL-TK; 21 ng/μL) were transfected to the cells. Four hours after the transfection, the medium was replaced with

10% FBS/DMEM and the cells were cultured for 24 hours. 10 μL of RNA solution in hypertonic solution (2.1 M of sucrose, 7.5% of PEG 2000, 150 mM of HEPES buffer (pH 7.3) in HBSS buffer) was added to the medium (final RNA concentration; 0.5 μM) and the cells were incubated at 37 °C for 10 minutes. Sterile water (52.9 μL) was added and the cells were incubated at 37 °C for 10 minutes. The medium was replaced with 10% FBS/DMEM and the cells were seeded on 96-well plates. The cells were irradiated with UV light for 15 min using a UV handy lamp placed 10 cm above the 96-well plate and were incubated at 37 °C for 24 hours. Luciferase expression was monitored as described above.

Synthesis of disulfide-Linker RNAs

The corresponding disulfide protected RNAs obtained by the deprotection and cleavage from the CPG were used as the stating material. The RNA (final conc. 50 μM) was dissolved in sodium borate buffer (pH 8.5, final conc. 50 mM). To the solution was added DTT solution (final conc. 100 mM) and the mixture was incubated at 30 ºC for 90 min. Then the mixture was desalted with NAP and the collected solution was subjected to the iPrOH precipitation (sodium acetate (pH 5.2) and 2-propanol).The circularization reaction was performed in a tube containing solution of 1 μM of linear RNA, 5 μM of 2,2'-dithiopyridine, 50 mM Tris-HCl pH 7.9, MeOH/Water =1/1. The mixture was incubated at 25 °C for 3-24 h. MeOH was removed under the reduced pressure and the mixture was loaded on NAP 5 column and the fractions containing the RNA were collected and the concentrated. The resulting circular RNAs were separated by 10 ~ 18% denaturing PAGE (acrylamide : bis-acrylamide = 19:1, 7.0 M urea, 25% formamide, 1 x TBE) and isolated by the crash and soak methods (elution; MQ). After desalting with Amicon-Ultra 3K (Merck) following the manufacture's standard protocols, the RNAs were precipitated with sodium acetate (pH 5.2) and 2-propanol. The isolated circular RNAs were analyzed by denaturing PAGE and MALDI-TOF MS. The isolated amount and yields for the circularization reaction are as follows; PS-CS) 2.74 nmol/40 nmol, 6.9%; PS-CA) 4.18 nmol/60 nmol, 7.0%; ApoB-PS-CS) 1.61 nmol/30 nmol, 5.4%; ApoB-PS-CA) 1.66 nmol/30 nmol, 3.8%.

Treatment of disulfide RNA with reductant

RNA samples (0.25 μM) in 1 x PBS containing 10 mM of DTT or GSH were incubated for 30 min at 37 °C. After the NAP 5 treatment and iso-propanol precipitation, the samples were analyzed by dPAGE.

Measurements of RNAi activity (disulfide, Lipofection)

One day prior to transfection at 70% confluency, cells were plated in 96-well plates (1×10^4 cells/well) for measurement of RNAi activity. RNA and vectors were transfected into HeLa cells, following the manufacturer's protocols for adherent cell lines. Immediately prior to transfection, the

culture medium was replaced with Opti-MEM. RNA, pGL3-Control and pRL-TK formulated in lipofectamine 2000 (0.25 μL/well) were added to the medium. (final concentration; RNA 5 nM, pGL3-Control 0.2 ng/μL, pRL-TK 0.2 ng/μL). Four hours after the transfection, the cell medium was exchanged to 10% FBS in DMEM. 20 hours after the medium exchange, luciferase expression was monitored as described above. For the experiments in Figure S3, lipofectamine 3000 was used in place of lipofectamine 2000. Scramble dsRNA sequence; 5'-p-CGGAGCUGGUUCGGUCCACUU-3', 5'-p-GUGGACCGAACCAGCUCCGUU-3'.

Measurements of RNAi activity (disulfide, no Lipofection)

HeLa cells were grown at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Wako Pure Chemical Industries) supplemented with 10% FBS and Penicillin-Streptomycin Solution (×100) (168-23191, Wako Pure Chemical Industries). Cells were regularly passaged to maintain exponential growth. One day prior to transfection at 80% confluency, cells were plated in 24-well plates (4.2×10^4 cells/well) for measurement of RNAi activity. Before the transfection, cells were washed with PBS twice and Opti-MEM was added. RNA and vectors were transfected into HeLa cells, following the manufacturer's protocols. GL3-Control and pRL-TK formulated in Lipofectamine 3000 (0.2 μL/well) (final concentration; pGL3-Control; 15 ng/μL, pRL-TK; 15 ng/μL) were transfected to the cells. Four hours after the transfection, the medium was replaced with 10% FBS/DMEM and the cells were cultured for 24 hours. 10 μL of RNA solution in hypertonic solution (0.5 M of sucrose, 10% of PEG 2000 in 10% FBS DMEM) was added to the medium (final RNA concentration; 0.5 μM) and the cells were incubated at 37 °C for 10 minutes. Sterile water (52.9 μL) was added and the cells were incubated at 37 °C for 10 minutes. The medium was replaced with 10% FBS/DMEM and the cells were seeded on 96-well plates and incubated for another 24h. And then, the cells were rinsed and lysis and the cell lysate were detected by dual luciferase assay as described above.

Measurements of RNAi activity targeting ApoB (UV-irradiation, with lipofection)

Huh-7 cells were grown at 37 °C under 5% CO_2 in RPMI-1640 with L-Glutamine, Phenol Red, HEPES and Sodium Pyruvate (187-02705, Wako Pure Chemical Industries) supplemented with 10% FBS (Biological Industries, Kibbutz Beit Haemek, Israel) and Penicillin-Streptomycin Solution (×100) (168-23191, Wako Pure Chemical Industries). Cells were regularly passaged to maintain exponential growth. One day prior to transfection at 70% confluency, cells were plated in 96-well plates (5×10^3 cells/well) for measurement of RNAi activity.

RNA were transfected into Huh-7 cells, following the manufacturer's protocols for adherent cell lines. Immediately prior to transfection, the culture medium was replaced with OptiMEM (Thermo Fischer Scientific, USA), and RNA, formulated in lipofectamine 3000 (Thermo Fischer Scientific, 0.25 μL/well) were added to the medium following the manufacture's standard protocols. (final

concentration; RNA 1 nM, 10 nM, 25 nM). Four hours after the transfection, the cell medium was exchanged to 10% FBS/DMEM. The cells were irradiated with UV light for 15 min using a UV handy lamp (AS ONE, SLUV-4, 365 nm) placed 10 cm above the 96-well plate. After 20 hours, cell RNA was extracted by NeocleoSpin (Macherey-Nagel, Germany) and reverse transcribed by One Step TB Green™ PrimeScript™ PLUS RT-PCR Kit (Perfect Real Time) (Takara, Japan).

Forward primer (ApoB); 5'-CCTAAAAGCTGGGAAGCTGA-3'

Reverse primer (ApoB); 5'-CCTCCGTTTTGGTGGTAGAG-3'

Forward primer (GAPDH); 5'-GCACCGTCAAGGCTGAGAAC-3'

Reverse primer (GAPDH); 5'-TGGTGAAGACGCCAGTGGA-3'

Evaluation of cellular uptake of dsRNA and circular RNA pairs

HeLa cells were grown at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Wako Pure Chemical Industries) supplemented with 10% FBS and Penicillin-Streptomycin Solution (×100) (168-23191, Wako Pure Chemical Industries). Cells were regularly passaged to maintain exponential growth. One day prior to transfection at 80% confluency, cells were plated in 24-well plates for measurement of cellular uptake. 50 μL of RNA solution in hypertonic solution (0.5 M of sucrose, 10% of PEG 2000 in 10% FBS DMEM) was added to the medium (final RNA concentration; 0.5 μM) and the cells were incubated at 37 °C for 10 minutes. Sterile water (264.5 μL) was added and the cells were incubated at 37 °C for 10 minutes. The medium was replaced with 10% FBS/DMEM and the cells were collected for flow cytometry. Fluorescent signals in cells were detected by laser excitation at 488 nm and emission at 575 nm (at least 5000 events of live cells were collected) on an EC800 flow-cytometer (Sony, Japan). The experiment was performed as three-time independent experiment.

Measurements of RNAi activity targeting ApoB and 5'-RACE experiment (disulfide, Lipofection)

Transfection of "NS*+NA*" and "PS-CS*+ PS-CA*" to HuH-7 were performed following the protocols described above at 100 nM concentration of the samples. Total RNA (1.3 μg) extracted from transfected HuH-7 cells with NucleoSpin RNA (Takara) was ligated to a GeneRacer adaptor (Invitrogen, 5'-CGACUGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAA-3') with T4 RNA ligase (Takara). Ligated RNA was reverse transcribed using a gene-specific primer (5'-GCGGTAGAGTACAGCATTGAAGA-3') with SuperScript III Reverse Transcriptase (Invitrogen). To detect cleavage products, PCR was performed with PrimeSTAR HS polymerase (Takara) using primers that are complementary to the RNA adaptor (5'-CGACTGGAGCACGAGGACACTGA-3') and ApoB mRNA (5'-GGCTTTTGTGGTTGTTGCCA-3'). After that, nested PCR was performed using the following primers; Fw; 5'-GGACACTGACATGGACTGAAGGAGTA-3', Rev; 5'-

CAGAGACAGAGCTGTGGCTAAC-3'. Amplification products were resolved by agarose gel (3% PrimeGel Agarose PCR-Sieve HRS) electrophoresis and visualized by GelRed (Biotium). The corresponding bands were cut out and DNA was isolated by Wizard SV Gel and PCR Clean-Up System (Promega) and the its sequence was analyzed with ABI PRISM 3500xL Genetic Analyzer using reverse primer for 2nd PCR. At the same time, RT-PCR was performed following the protocols described above to confirm the gene silencing effect, using RNA (100 ng) extracted from the same transfected HuH-7 cells.

Measurements of RNAi activity targeting GSK3β (disulfide, Lipofection)

HeLa cells were grown at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Wako Pure Chemical Industries) supplemented with 10% FBS and Penicillin-Streptomycin Solution (×100) (168-23191, Wako Pure Chemical Industries). Cells were regularly passaged to maintain exponential growth. One day prior to transfection at 80% confluency, cells were plated in 24-well plates (2.0×10^4 cells/well) for measurement of RNAi activity. Before the transfection, cells were washed with PBS twice and Opti-MEM was added. RNA and vectors were transfected into HeLa cells, following the manufacturer's protocols. Normal siRNA (GSK3β-S/GSK3β-A) and disulfide-type siRNA (GSK3β-PS-CS/GSK3β-PS-CA) formulated in Lipofectamine 3000 (0.2 μL/well) (final concentration; 50 nM) were transfected to the cells. Four hours after the transfection, the medium was replaced with 10% FBS/DMEM and the cells were cultured for 24 hours. Then, cell RNA was extracted by NeocleoSpin (Macherey-Nagel, Germany) and reverse transcribed by One Step TB Green™ PrimeScript™ PLUS RT-PCR Kit (Perfect Real Time) (Takara, Japan).

Forward primer (GSK3β); 5'- GGTCTATCTTAATCTGGTGCTGG-3'

Reverse primer (GSK3β); 5'- TGGATATAGGCTAAACTTCGGAAC-3'

Forward primer (GAPDH); 5'-GCACCGTCAAGGCTGAGAAC-3'

Reverse primer (GAPDH); 5'-TGGTGAAGACGCCAGTGGA-3'

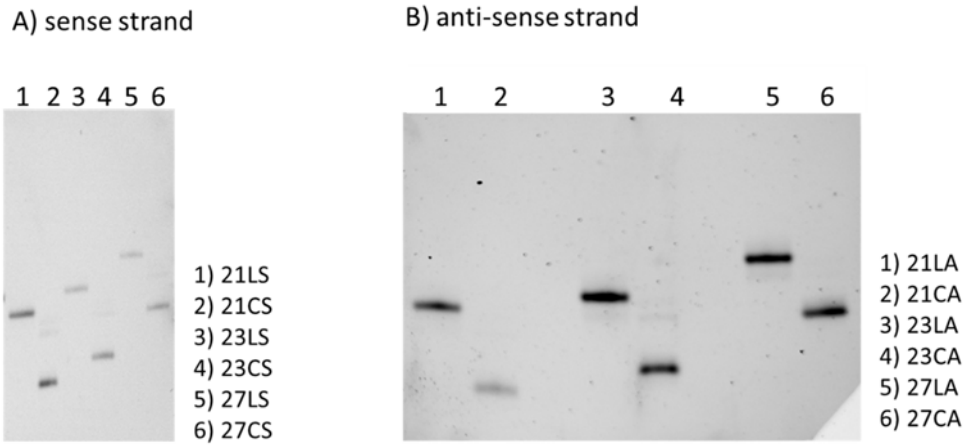


Figure S1 Denaturing PAGE analysis of the PC-linker circular RNAs

The isolated RNAs were analyzed by electrophoresis on 10% denaturing polyacrylamide gel (acrylamide : bis-acrylamide = 19:1, 7.5 M urea, 1 x TBE), stained with SYBR Green II and visualized by scanning on a BioRad Molecular Imager FX (BioRad).

Sample	Calcd. (M+H⁺)	Found	Sample	Calcd. (M+H⁺)	Found
NS	6760.86	6760.29	**27CS**	8859.09	8859.39
NA	6766.91	6767.82	**21LA**	7025.93	7025.47
21LS	7019.89	7021.60	**23LA**	7716.03	7715.40
23LS	7629.97	7630.94	**27LA**	9009.21	9008.43
27LS	8877.10	8878.32	**21CA**	7007.92	7008.66
21CS	7001.88	7001.52	**23CA**	7698.02	7698.47
23CS	7611.96	7612.33	**27CA**	8991.20	8993.18

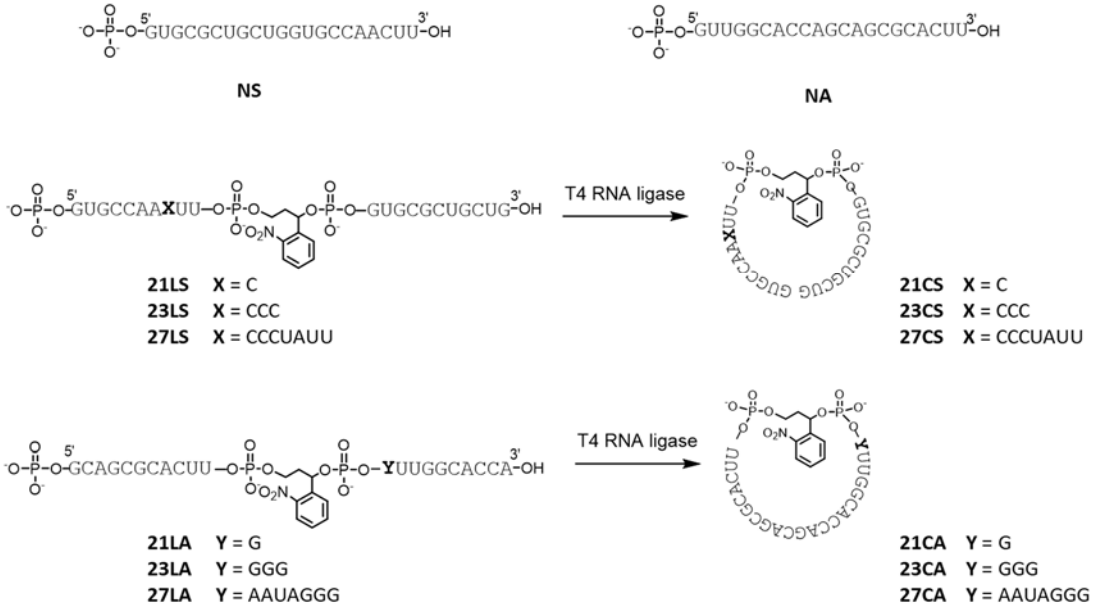


Table S1 Sequences and MALDI-TOF MS analysis of natural siRNAs and PC-linker RNAs

Mass data were obtained using a microflex MALDI-TOF mass spectrometer (Bruker Daltonics) by positive mode using a mixture of 3-hydroxypicolinic acid (HPA) and ammonium citrate as a matrix.

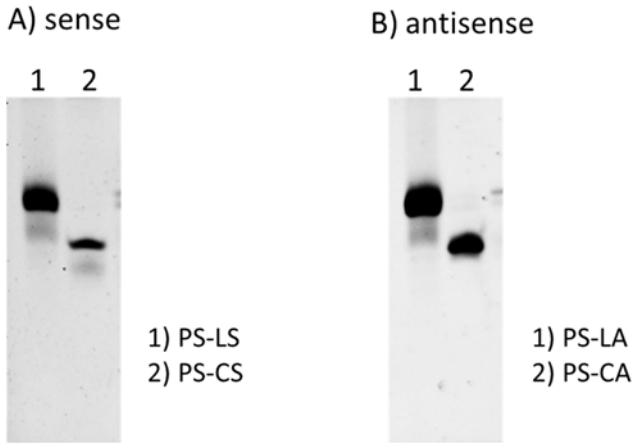


Figure S2 Denaturing PAGE analysis of the disulfide-linker circular RNAs

The isolated RNAs were analyzed by electrophoresis on 10 % denaturing polyacrylamide gel (acrylamide : bis-acrylamide = 19:1, 7.5 M urea, 25% formamide, 1 x TBE), stained with SYBR Green II and visualized by scanning on a BioRad Molecular Imager FX (BioRad).

Sample	Calcd. (M+H$^+$)	Found
PS-CS	6928.81	6931.77
PS-CA	6934.86	6937.81

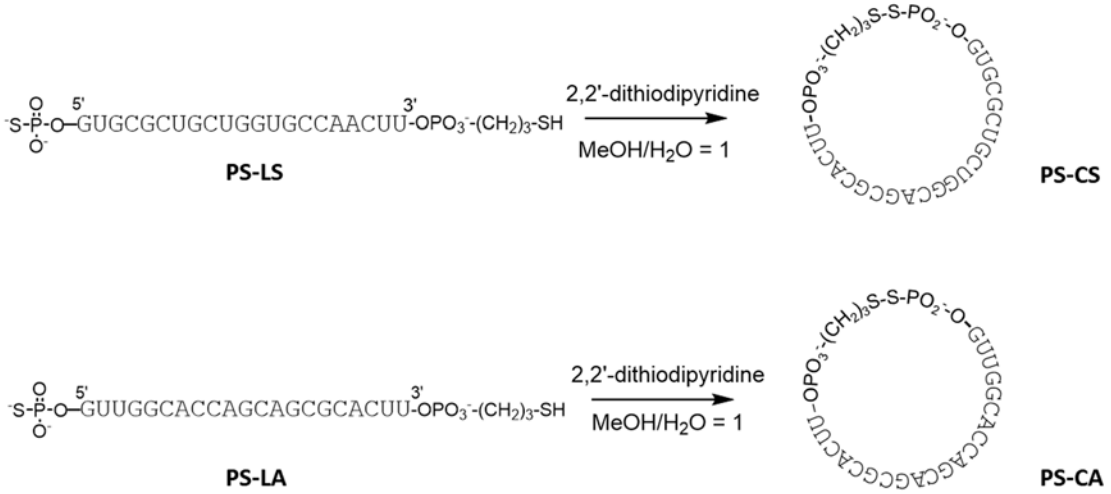


Table S2 Sequences and MALDI-TOF MS analysis of disulfide-liker RNAs

Mass data were obtained using a microflex MALDI-TOF mass spectrometer (Bruker Daltonics) by positive mode using a mixture of 3-hydroxypicolinic acid (HPA) and ammonium citrate as a matrix.

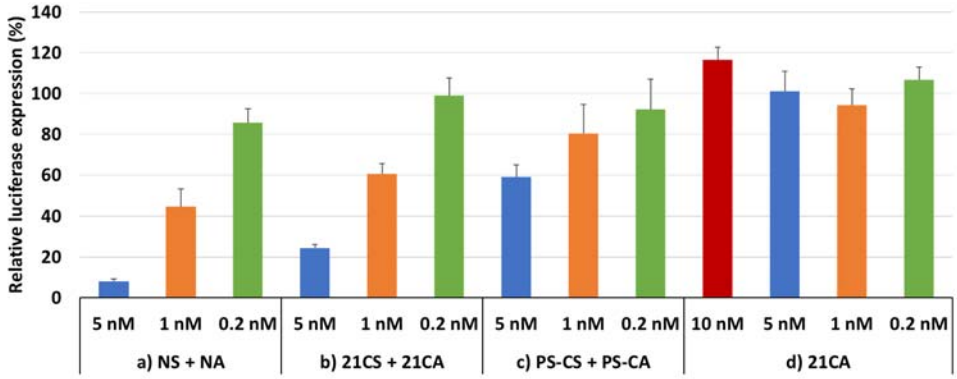


Figure S3 Concentration dependencies of gene silencing effects by siRNA precursors and antisense strand. Relative luciferase expression, Error bars represent the standard deviation of three experiments. The luminescence signals for each sample were normalized to that of the no RNA conditions. a,c); without UV irradiation, b, d); with UV irradiation

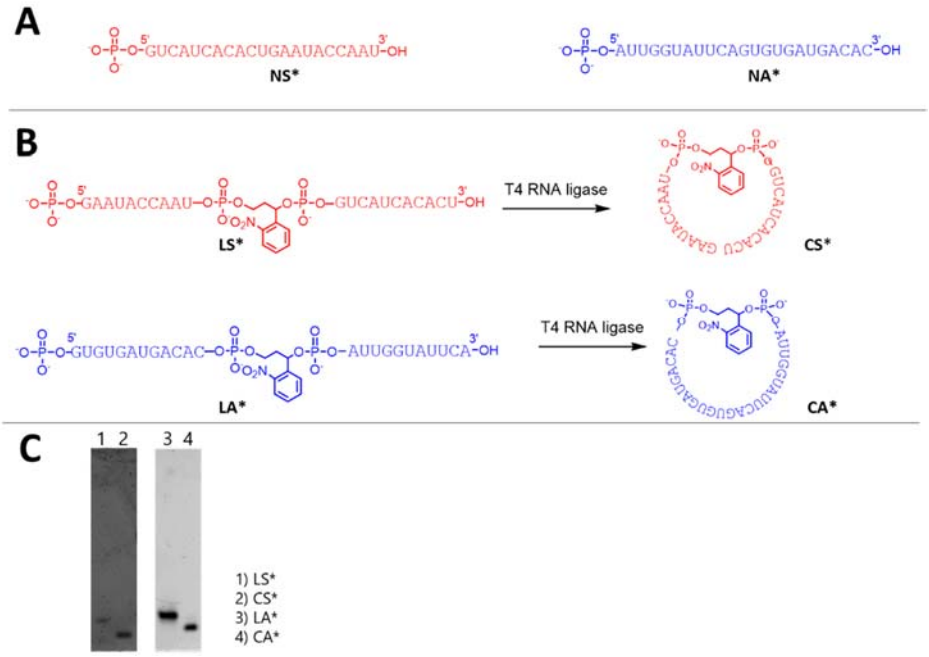


Figure S4. Structure and PAGE analysis of the sample used for the experiments in the supporting information (I). A) Native siRNA targeting ApoB, B) Photo-responsive circular RNAs targeting ApoB, C) dPAGE analysis of the circular RNAs targeting ApoB. 10 % denaturing polyacrylamide gel (acrylamide : bis-acrylamide = 19:1, 7.5 M urea, 25% formamide, 1 x TBE), stained with SYBR Green II and visualized by scanning on a BioRad Molecular Imager FX (BioRad).

Sample	Calcd. ($M+H^+$)	Found
LS*	6962.94	6962.77
CS*	6944.93	6943.95
LA*	7687.98	7687.70
CA*	7669.96	7669.55

Table S3 MALDI-TOF MS analysis of RNA samples for the experiments in the supporting information. Mass data were obtained using a microflex MALDI-TOF mass spectrometer (Bruker Daltonics) by positive mode using a mixture of 3-hydroxypicolinic acid (HPA) and ammonium citrate as a matrix.

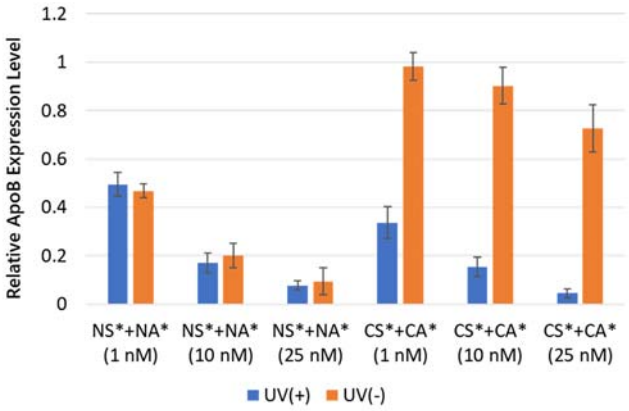


Figure S5 Evaluation of circular RNAs targeting ApoB with the photocleavable linker. A) Gene silencing effects for ApoB by siRNA or circular RNAs with or without photoirradiation. Error bars represent the standard deviation of three experiments.

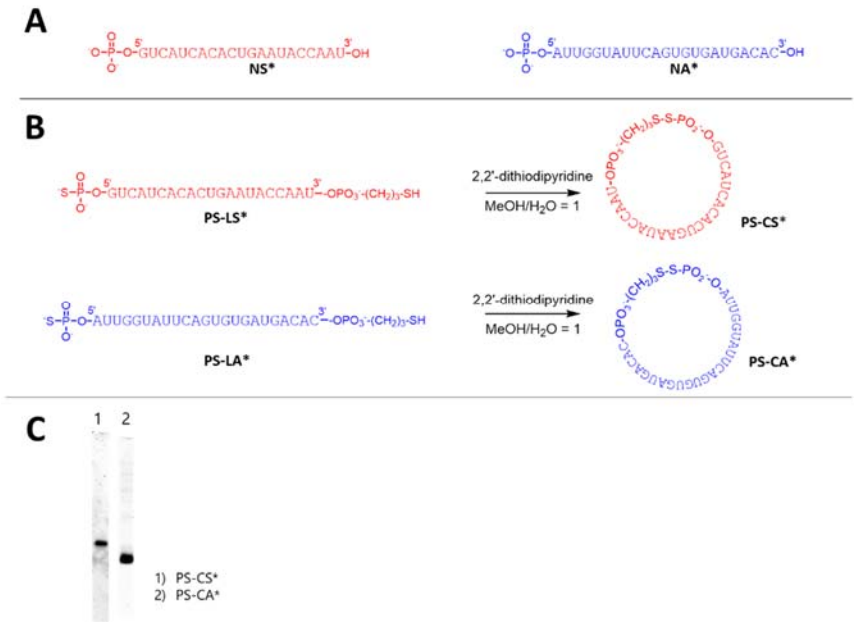


Figure S6 Structure and PAGE analysis of the sample used for the experiments in the supporting information (II). A) Native siRNA targeting ApoB, B) GSH-responsive circular RNAs targeting ApoB, C) dPAGE analysis of the circular RNAs targeting ApoB. 10 % denaturing polyacrylamide gel (acrylamide : bis-acrylamide = 19:1, 7.5 M urea, 25% formamide, 1 x TBE), stained with SYBR Green II and visualized by scanning on a BioRad Molecular Imager FX (BioRad).

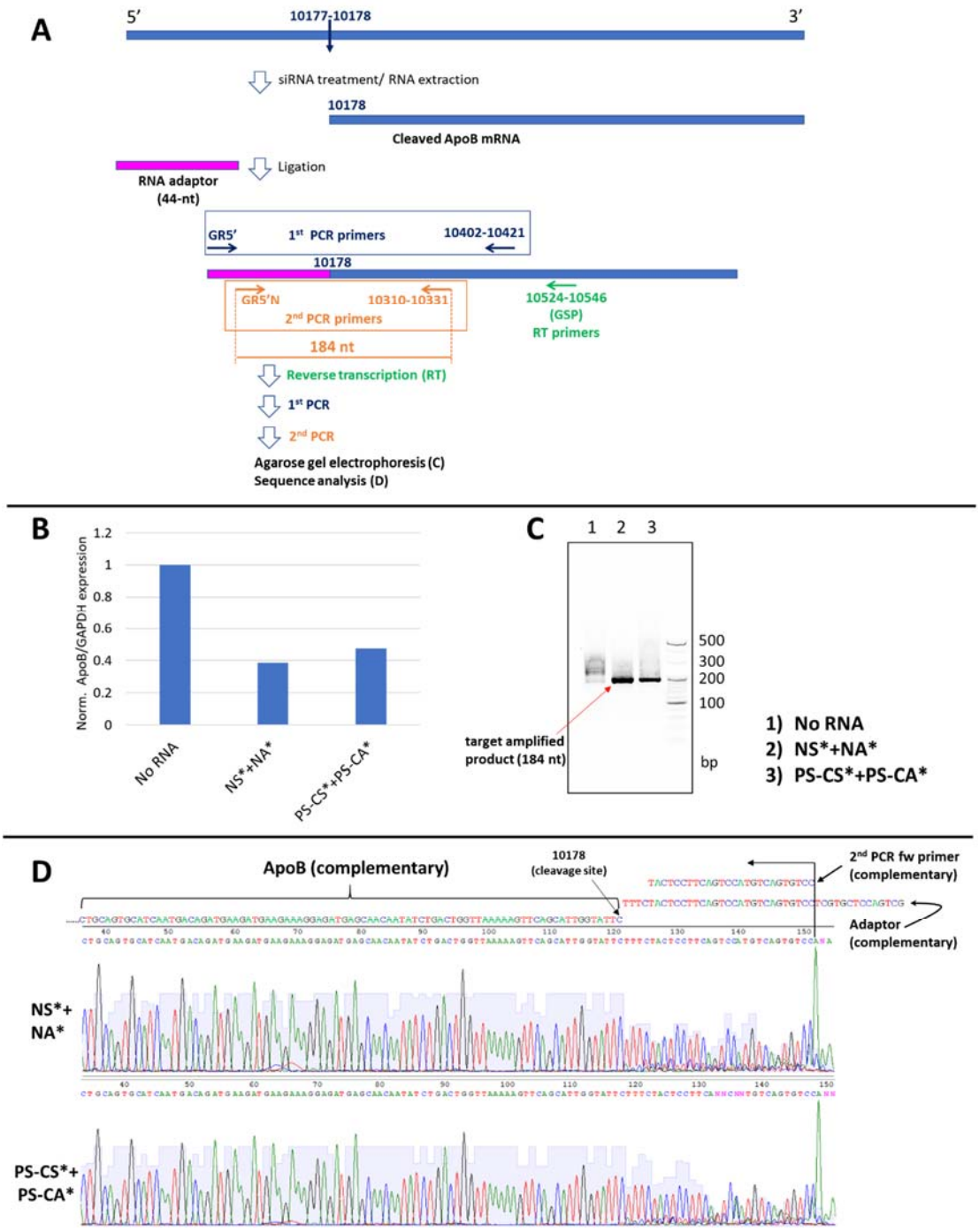
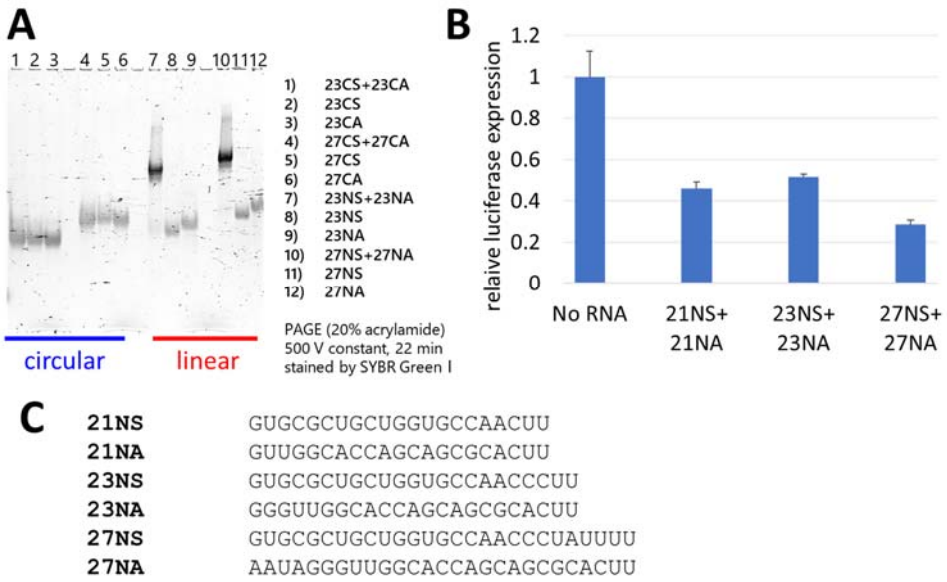


Figure S7 Effects of disulfide-type circular RNAs targeting ApoB. A) Overview of the adaptor and primers for 5'-RACE experiments, B) qPCR evaluation of gene-silencing effect at 100 nM. C) Agarose gel electrophoresis analysis of the amplified DNA products by 5'-RACE experiments. 3% PrimeGel Agarose PCR-Sieve HRS, detected based on GelRed staining. D) Sequence analysis of the amplified products. The sequences of the amplified DNA are fully matched with the predicted sequence based on the expected cleavage site in ApoB mRNA and sequences of the adaptor and 2nd PCR primer.



C

21NS	GUGCGCUGCUGGUGCCAACUU
21NA	GUUGGCACCAGCAGCGCACUU
23NS	GUGCGCUGCUGGUGCCAACCCUU
23NA	GGGUUGGCACCAGCAGCGCACUU
27NS	GUGCGCUGCUGGUGCCAACCCUAUUUU
27NA	AAUAGGGUUGGCACCAGCAGCGCACUU

Figure S8 Property of 23 and 27 mer linear and circular RNAs. A) PAGE analysis of linear and circular RNA pairs of sense and anti-sense strands. Equimolar of sense and anti-sense strands were mixed and analyzed by 20% non-denaturing PAGE. The gels were stained with SYBR Green I. B) Dual luciferase assay of linear 21, 23, 27-mer siRNAs with 25 nM concentration of siRNAs. C) Sequences of the siRNA samples used in A and B. From left to right; 5' to 3' terminus.

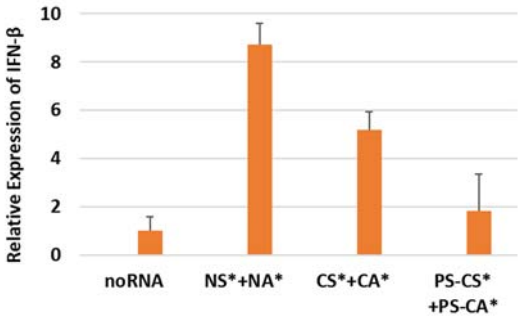


Figure S9 Immunostimulatory effect of normal siRNA (**NS*+NA***) and circular RNAs with stimulus-responsive linker (photocleavable; **CS*+CA***, disulfide; **PS-CS*+PS-CA***). The expression level of IFN-β mRNA was measured by quantitative RT-PCR at 24 h posttransfection of RNA (200 nM) in T98G cells. The expression level of IFN-β mRNA was normalized to that of β-actin. The relative expression of IFN-β mRNA in mock-transfected cells was set as 1. Error bars represent the standard deviation of three experiments. Significant difference between **NS*+NA*** and **CS*+CA*** ($P < 0.05$), and between **NS*+NA*** and **PS-CS*+PS-CA*** ($P < 0.01$) was confirmed by T-test.