Electronic Supplementary Material (ESI) for Chemical Science

Supporting Information

Selective labeling of mature RISC using siRNA carrying fluorophore-quencher pair

Yukiko Kamiya,[a, b] Anna Ito,[a] Hiroshi Ito,[a] Masaaki Urushihara,[a] Junya Takai,[a] Taiga Fujii,[a] Xingguo Liang,[a ,c] Hiromu Kashida[a] and Hiroyuki Asanuma[a] *

Experimental Section

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Materials

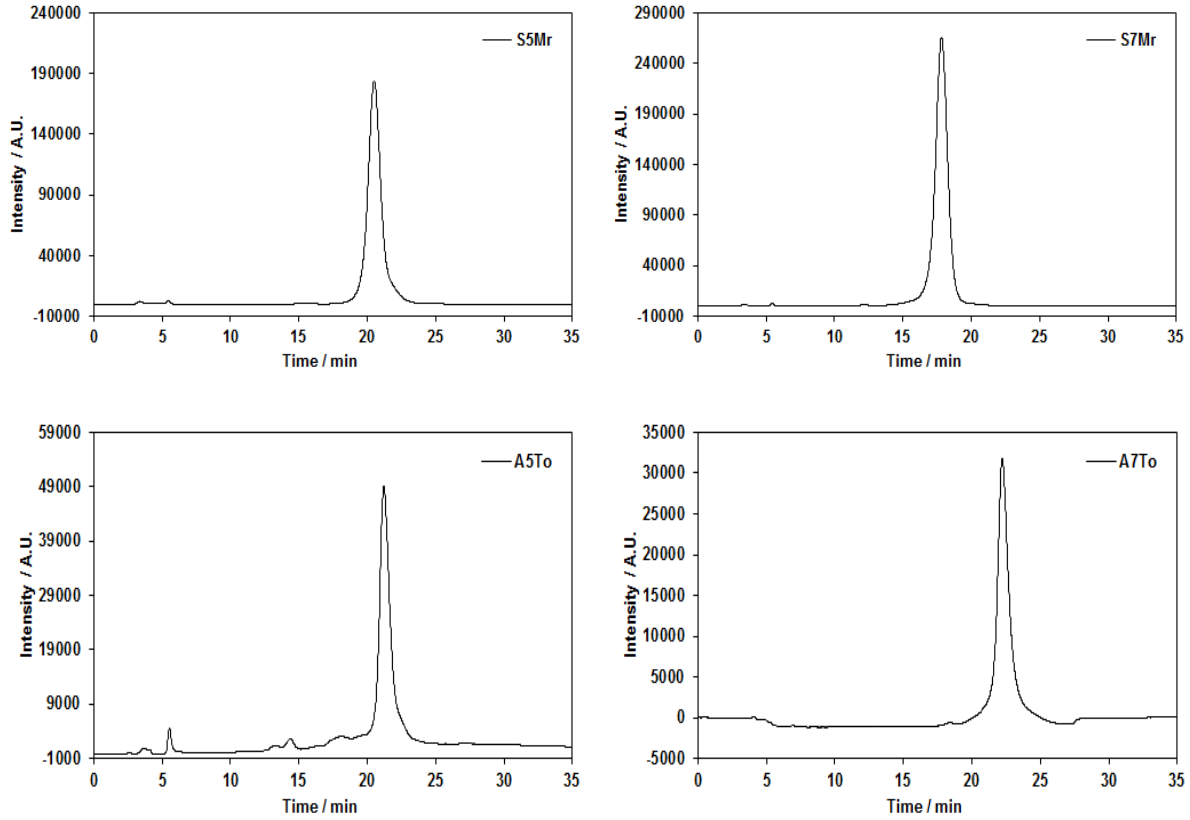
Oligonucleotides containing only natural bases were obtained from Integrated DNA Technologies, Inc.

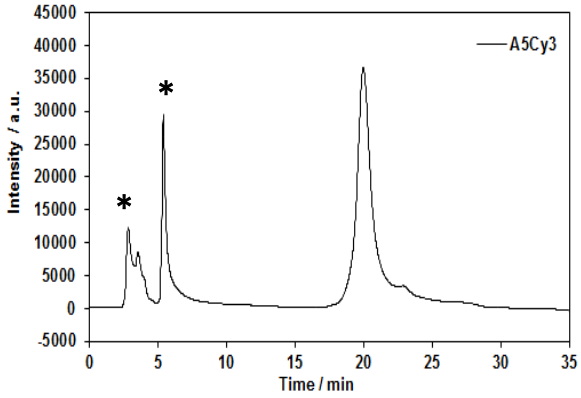
Syntheses of modified RNAs

Phosphoramidite monomers of Methyl red, Thiazole orange, and Cy3 were synthesized according to a previous report with minor modifications [19]. All modified siRNAs were synthesized using an ABI-3400 DNA synthesizer (Applied Biosystems) with conventional and dye-carrying phosphoramidite monomers. After the recommended work-up, the synthesized RNAs were purified by HPLC on a reverse-phase column (Figure S1) and identities were confirmed by MALDI-TOFMS analyses using Autoflex II (Bruker Daltonics).

MALDI-TOF mass: S5Mr: Obsd. m/z 7560.9 (Calcd. for [S5Mr+H^+]: m/z 7563.1); S7Mr: m/z 7559.7 (Calcd. for [S7Mr+H^+]: m/z 7563.1); A5To: m/z 7987.3 (Calcd. for [A5To+H^+]: m/z 7986.4) ; A7To: m/z 7982.7 (Calcd. for [A7To+H^+]: m/z 7563.1) ; A5Cy3: m/z 8078.3 (Calcd. for [A5Cy3+H^+]: m/z 8080.3).

Figure S1 HPLC profiles of synthesized RNA.





Peaks indicated by asterisk contained no detectable oligonucleotides.

Fluorescence spectroscopic measurements

The fluorescence measurements of single-stranded A5To, duplex RNA containing A5To with native sense strand (S) or S5Mr were performed at 20 ^{o}C on a JASCO model FP-6500. Each RNA was dissolved in 10 mM sodium phosphate (pH 7.0) at concentrations of 0.2 μM antisense strand RNA and 0.4 μM sense strand. To conduct the degradation assay, duplex RNA (1 μM A5To or A5Cy3 and 1.2 μM native sense strand or S5Mr) was incubated with HeLa cell lysate in a cuvette at 37 ^{o}C. Fluorescence spectra were monitored at indicated time points after addition of siRNAs into cell lysate. Measurements were begun after 1 min of mixing of the siRNA with cell lysate.

Dual luciferase assay

The dual luciferase assays were performed as described in a previous report [24]. In this assay two types of reporter plasmids, pGL3-Fw and pGL-Rv, were used. The target sequence in pGL3-Fw was 5'-AACCTTACCCACCTCATGTATCT-3' and that for pGL3-Rv was the complementary sequence 5'-AGATACATGAGGTGGGTAAGGT-3'. The target sequences were inserted into the 3'-UTR region of pGL3 which is firefly luciferase expression vector. For this analysis, 293FT cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum, 80 μg/ml penicillin, and 90 μg/ml streptomycin at 37 ^{o}C with 5 % CO_2 in humidified air on a 96-well plate. Co-transfections with siRNA (final concentrations were 0.01, 0.1, 1.0, 5.0, 10, 16, or 20 nM), 100 ng of pGL3, and 0.5 ng of *Renilla* luciferase expression vector were performed by using Lipofectoamine^{TM} 2000 (Invitrogen) according to the manufacturer's instructions. After cultivation at 37 ^{o}C for 48 h, cells were washed with PBS and lysed in 1× Passive Lysis Buffer (Promega). Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions on a Multilabel Plate Reader (EnSpire^{TM}, Perkin Elmer).

Immunofluorescent analyses of siRNA-transfected HeLa cells

HeLa cells were grown in DMEM supplemented with 10 % fetal bovine serum, 80 μg/ml penicillin, and 90 μg/ml streptomycin at 37 ^{o}C with 5 % CO_2 in humidified air. The cells were grown on sterilized cover slips

(Matsunami Glass) placed on the bottom of 12-well plates. The modified siRNA (annealed 48 pmol of sense strand and 40 pmol of antisense strand to avoid incomplete quenching) were mixed with 1 uL of Lipofectoamine™ 2000 (Invitrogene) and incubated for 30 min. The HeLa cells were transfected with the siRNA/Lipofectamine mixture to a final concentration of 0.2 uM siRNA. For the confocal microscopy analyses, HeLa cells transfected with siRNA were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde and then treated with PBS containing 3% BSA and 0.2% Triton-X100 for 10 min. Subsequently cells were stained with anti-AGO2 antibody (Wako) and Alexa Fluor 568-conjugated anti-mouse IgG antibody (Invitrogen) or anti-GW182 antibody (Santa Cruz Biotechnology) and Alexa Fluor 568-conjugated anti-goat IgG antibody (Invitrogen). The stained HeLa cells were visualized by using FD-1000 confocal laser microscopy (Olympus). Images were taken with a 100X oil emission objective lens. The following filter sets were used: excitation DM=DM405/488/543 with SDM490, SDM560, mirror, or none. The 405 nm laser was used to excite DAPI with emission collected using a 461-511 nm band pass filter. The 488 nm laser was used to excite the Thiazole orange with emission collected using a 500-550 nm band pass filter. The 543 nm laser was used to excite the Alexa568 with emission collected using a 560-660 nm band path filter. Fluorescent images were processed with ImageJ.

Time lapse imaging of siRNA-transfected HeLa cells

For time lapse analyses, HeLa cells were cultivated on glass-based dishes (Iwaki). 200 pmol of siRNA (annealed with sense strand antisense strand in 1.2 : 1.0 ratio) were mixed with 500 uL of Lipofectamine 2000 (Invitrogene) and incubated for 30 min. Fluorescent images were obtained after addition of the siRNA / Lipofectamine™ 2000 mixture (a final concentration of 0.4 uM siRNA) onto the HeLa cells in 2 ml of phenol red-free medium using the FD-1000 confocal laser microscopy equipped with cell culture system at 37 ^{o}C and 5 % $CO_{2.}$ Images were taken with a 60X oil emission objective lens. The following filter sets were used: excitation DM=DM405/488 with mirror or none. The 488 nm laser was used to excite the Thiazole orange with emission collected using a 500-600 nm band pass filter. Intracellular fluorescent intensities were evaluated by using FV10-ASW software (Olympus). The fluorescent intensity of each cell at the 24 hr after transfection was set to 1.0 and averaged relative intensity was calculated.

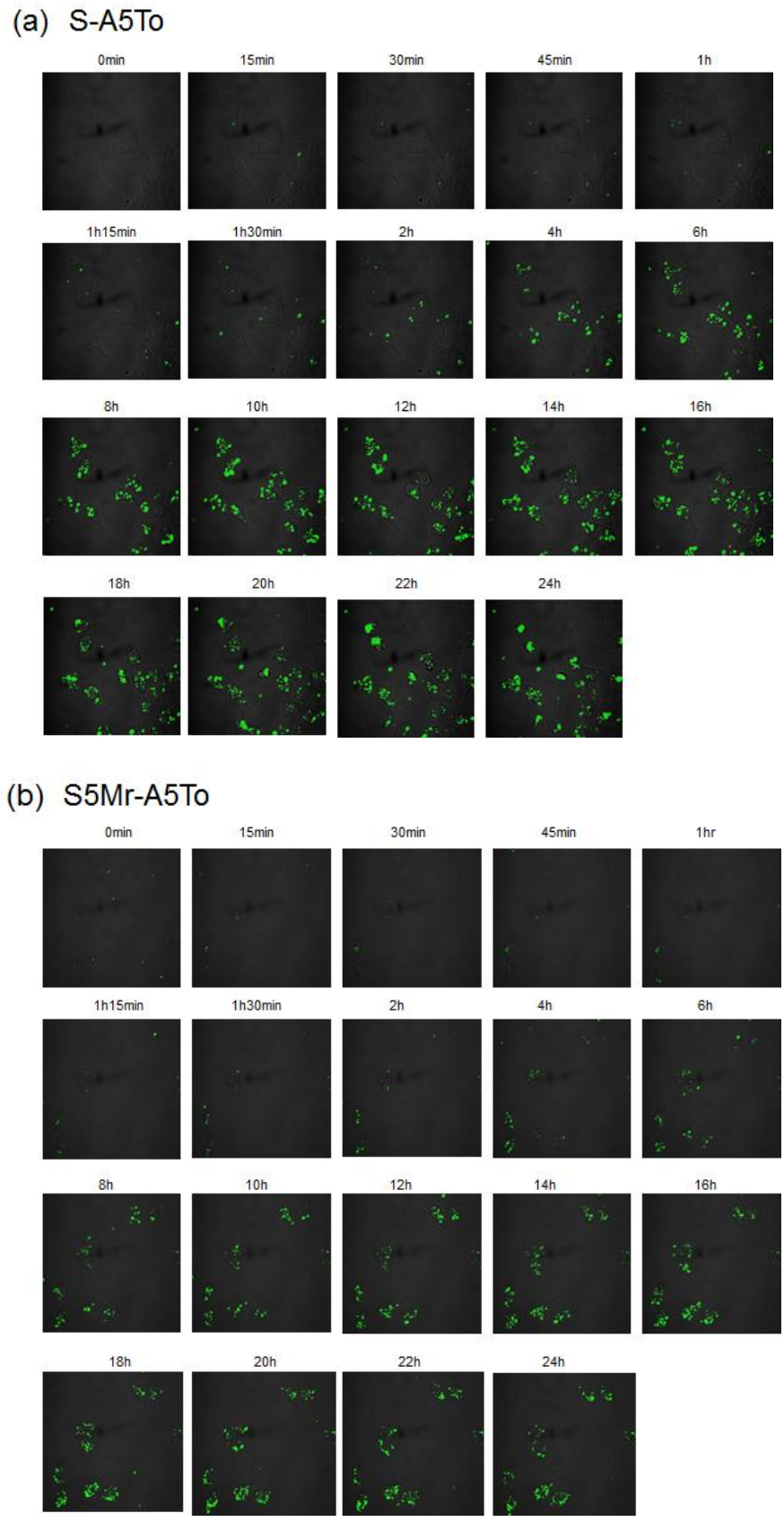


Figure S2. Time lapse images of (a) S-A5To and (b) S5Mr-A5To in living HeLa cells.

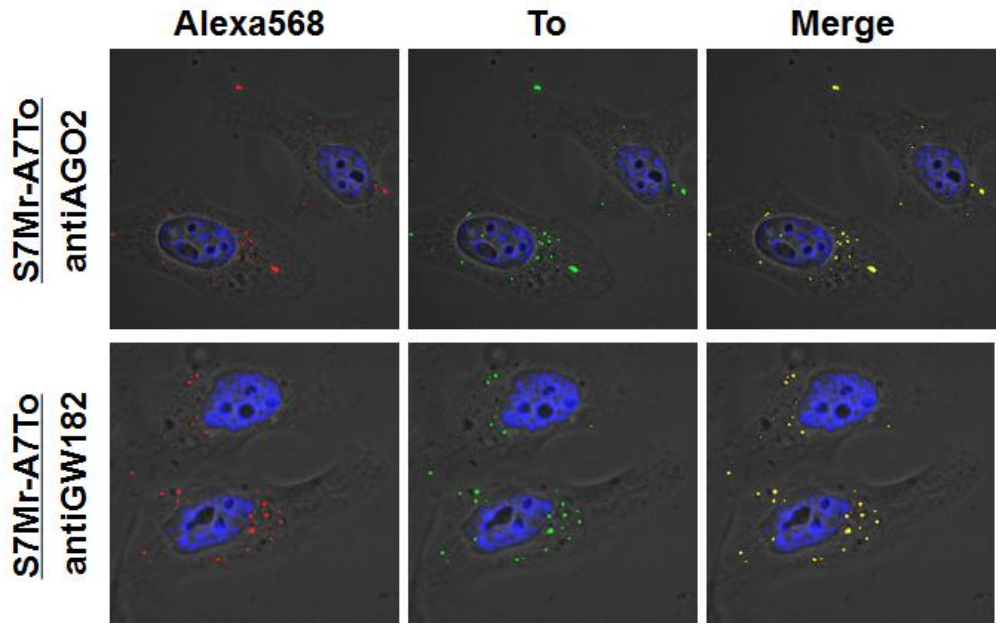


Figure S3. Localization of S7Mr-S7To in cytoplasmic foci in HeLa cells. The fluorescence signals were observed by confocal laser microscopy. Endogenous AGO2 and GW182 proteins were visualized using anti-AGO2 or anti-GW182 antibodies and Alexa568-conjugated secondary antibodies. Green, Thiazole orange-containing siRNAs; blue; DAPI staining; red, Alexa568 staining.