Supplementary Information

Promotion of cytoplasmic localization of oligonucleotides by connecting cross-linked duplexes

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Contents

Experimental

Preparation of Fluorescence Labelling ASOs

We obtained 5′- and 3′-amino-modified 2′-*O*-methyl RNAs (MeRNAs) containing deoxyuridine from Gene design (Japan). First, 2 nmol of 5′- or 3′-amino-modified MeRNAs was reacted with 100 nmol Alexa-Fluor 546-NHS (Molecular Probe) in 100 μL of 100 mM borate buffer (pH 8.0) with 50% dimethyl sulfoxide at 30°C. After 90 min, the reaction mixture was desalted with a cartridge column (NAP5), and the products and percentage yield were analysed using high-performance liquid chromatography (HPLC) equipped with a reversed-phase column and a photodiode array detector (Waters). Additionally, cross-link reactions of MeRNAs were performed using a bifunctional linker (N^1, N^5-Bis(aminooxyacetyl)-1,5-diaminonaphthalene; aoNao) under the same conditions previously reported [1,2]. All oligonucleotides were purified with HPLC using a reverse-phase column.

Cell Culture and Transfection of ASOs

HeLa cells were cultured in Dulbecco's Modified Essential Medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 units/mL penicillin, and 100 μg/mL streptomycin (Gibco). HeLa cells were seeded at 5×10^4 cells per μ–Dish (Ibidi, Munich, Germany) in 500 μL of DMEM with 10% FBS the day before transfection. After 200 μL of the medium was removed from each dish, the cells were transfected with 30 μL of Opti-MEM containing 0.9 μL of Lipofectamine® 2000 (Invitrogen) and 5 nM ASOs or 20 nM for 12-mer CDs to measure the intracellular localization of ASOs.

Luciferase Assays

A target sequence complementary to mature miR-21 was inserted into the 3′-UTR of the *Renilla* luciferase (hRluc) gene in the psiCHECK-2 vector (Promega) to generate plasmid psiCHECK-2-miR21, which contained both hRluc and *firefly* luciferase (Fluc) genes.[1] Next, HeLa cells were seeded at 1.5×10^4 cells per well in 100 μL of DMEM with 10% FBS in 96-well plates (Nunc) the day before transfection. Cells were transfected in triplicate with 10 μL of Opti-MEM containing 0.3 μL Lipofectamine® 2000 per well, 100 ng of psiCHECK-2-miR21, and 0 to 10 nM fluorescence-labelled ASOs. Luciferase activity was measured 48 h post-transfection using the Dual-Glo Luciferase Assay System (Promega). The ratios of Renilla luciferase to firefly luciferase (hRluc/Fluc) were first calculated by averaging the triplicate well measurements. Ratios were then normalized using the measurement of the psiCHECK-2-transfected cells without ASO.

Fluorescence Microscopy

The subcellular localization of oligonucleotides was measured 6 h post-transfection by fluorescently staining nuclei and whole living cells with Hoechst 33342 (Dojindo, Japan)[3] and calcein acetoxymethyl ester (calcein-AM, Dojindo, Japan)[4], respectively, and imaging with confocal microscopy (Leica TCS SP8). Calcein-AM passively crosses the cell membrane in an electrically neutral form and is converted by an intracellular esterase into a negatively charged form, fluorescent calcein that is retained in the intracellular compartment as long as the plasma membrane is intact. Images were acquired at a resolution of 512 × 512 pixels with a 63 × oil immersion objective and z-stacks of 11 sections at 0.3-μm thickness. The resulting stacks had an X × Y × Z dimension of 185 × 185 × 3 μm or 92.5 × 92.5 × 3 μm and were acquired. For each position, three sequential images (Alexa-Fluor 546 labelled oligonucleotide, calcein-AM, and Hoechst 33342) were acquired. Alexa-Fluor 546 labelled Oligonucleotides were detected in photon counting mode for quantitative analysis. The measurement range (z-axis) in the cell height was set so that the center of most of the cell nuclei in the image was large and visible. Therefore, the quantitative analysis and fluorescence imaging used images that contained the center of most of the nuclei.

Based on the calcein-AM and Hoechst 33342 fluorescence staining data, the outline of a whole cell and the nucleus were segmented using the ImageJ ROI manager, and the fluorescence intensity of Alexa-Fluor 546 in each segment was quantified. Next, the fluorescence intensity of Alexa-Fluor 546 in the cytoplasm was calculated by subtracting the fluorescence intensity in the nucleus from that of the whole cell. Finally, the nuclear and cytoplasmic localization ratios were calculated using total fluorescence intensities in the nucleus and the cytoplasm from the average values of the triplicate images (10–30 cells in 185 × 185 μm). We performed each experiment three or more times independently, and the data were expressed as means ± SD.

Analysis of Cell Extracts

A cytoplasmic fraction was prepared from 5×10^7 HeLa cells 6 h post-transfection using a nuclear/cytosolic fractionation kit (CELL BIOLABS, Inc.). Fluorescently labelled oligonucleotides were extracted with phenol/chloroform from the cytoplasmic fraction and purified by ethanol precipitation. The obtained sample was analysed using a 20% polyacrylamide gel with 8 M urea. The gel was imaged at the excitation wavelength of 532 nm.

References

1 Y. Mie, Y. Hirano, K. Kowata, A. Nakamura, M. Yasunaga, Y. Nakajima and Y. Komatsu, *Mol. Ther. - Nucleic Acids*, 2018, **10**, 64–74.

2 K. Ichikawa, N. Kojima, Y. Hirano, T. Takebayashi, K. Kowata and Y. Komatsu, *Chem. Commun.*

3 M. J. Lydon, K. D. Keeler and D. B. Thomas, *J. Cell. Physiol.*, 1980, **102**, 175–181.

4 N. G. Papadopoulos, G. V. Z. Dedoussis, G. Spanakos, A. D. Gritzapis, C. N. Baxevanis and M. Papamichail, *J. Immunol. Methods*, 1994, **177**, 101–111.

1. Intracellular localization of ASOs.

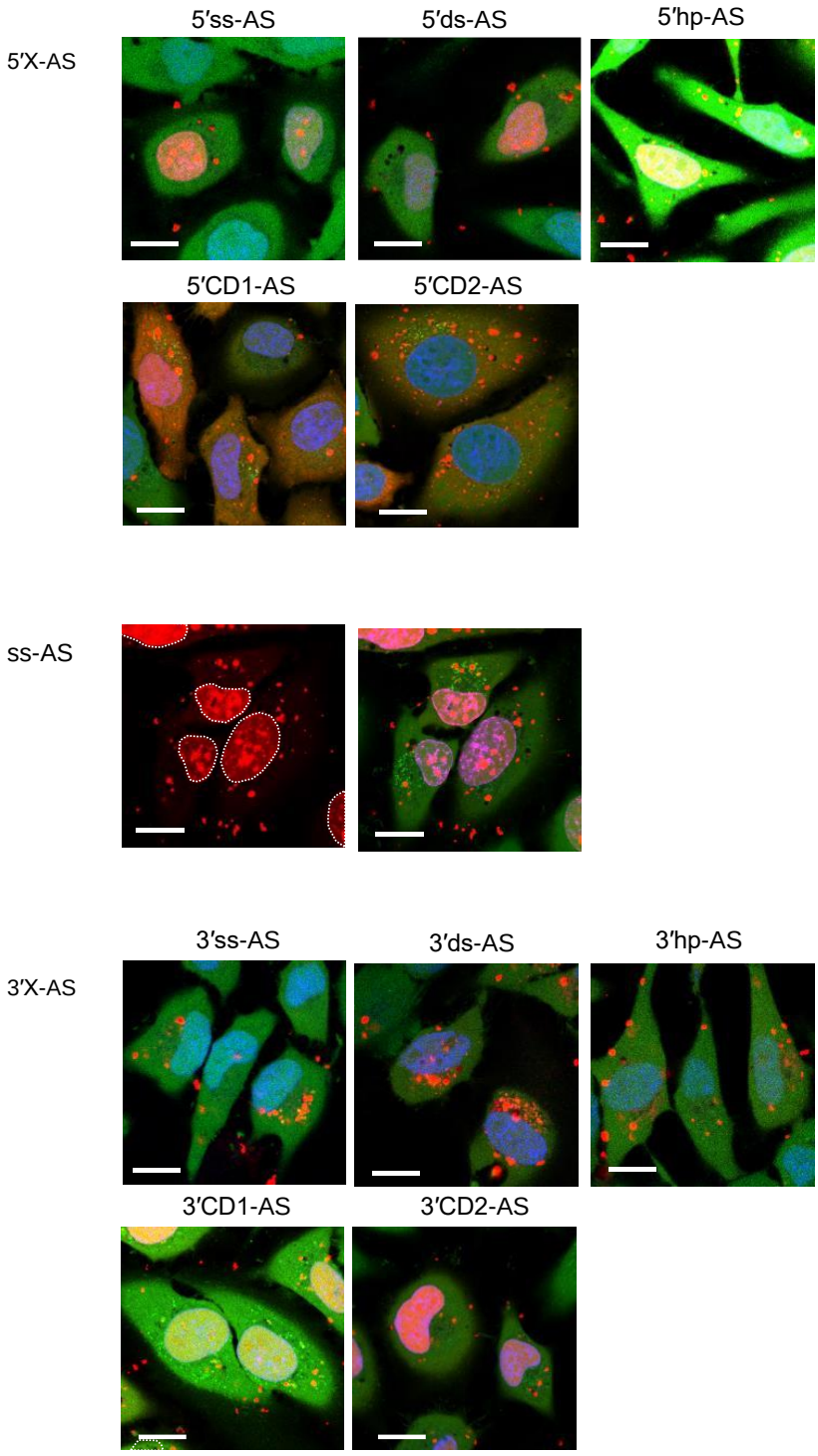


Figure S1. Intracellular localization of ASOs with CD.

Representative fluorescent images of triple-stained living HeLa cells with ASOs (red), calcein-AM (green), and Hoecst 33342 (blue) (lower panel). Scale bar: 20 μm.

2. Sequences of fluorescently labelled ASOs.

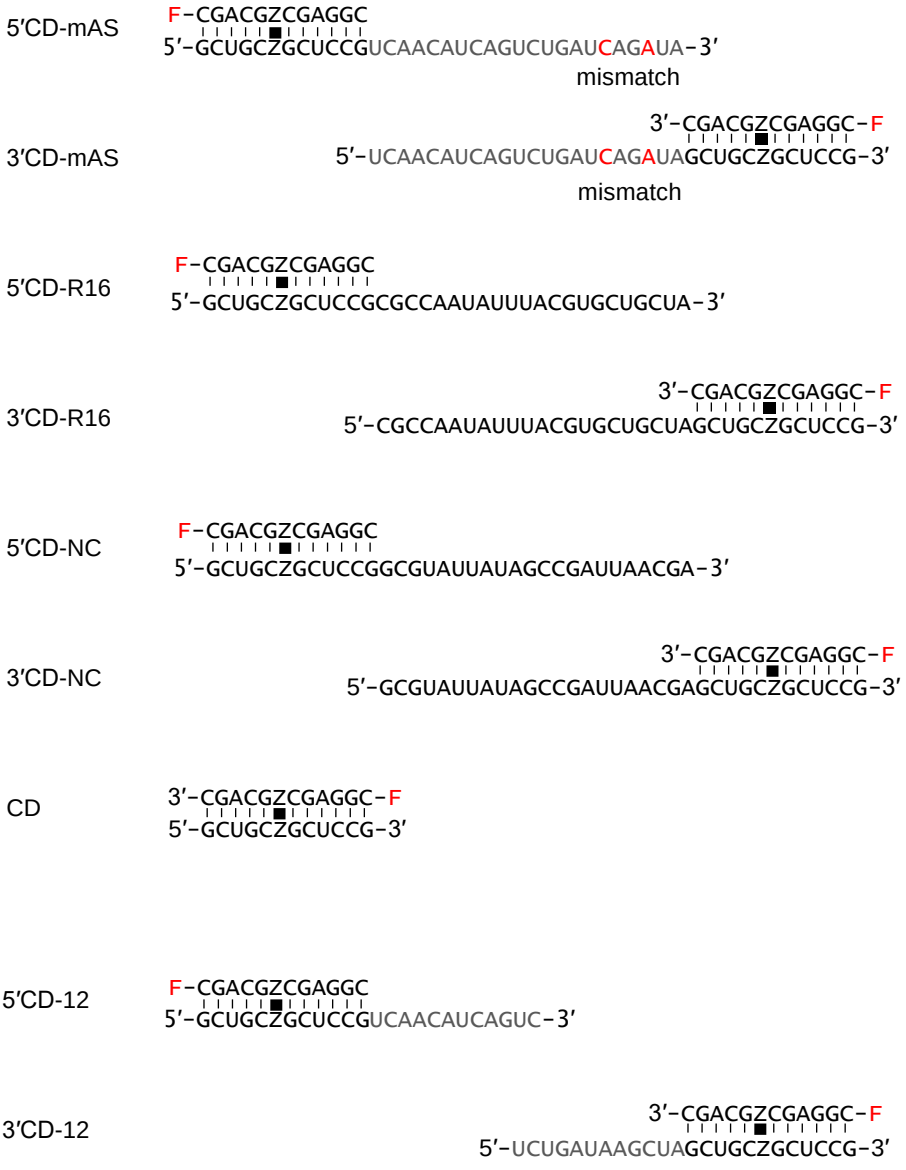


Figure S2. Sequences of fluorescence labelling ASOs.

The complementary sequence to miR-21 is shown in gray. F indicates the Alexa-Fluor 546. Black squares and Z indicate the cross-linkers and cross-linked sites, respectively. Both 5′CD-mAS and 3′CD-mAS contain mismatch (red) sequences. Both 5′CD-R16 and 3′CD-R16 contain complementary sequence of microRNA-16. Both 5′CD-NC and 3′CD-NC contain a scrambled sequence.

3. Intracellular localization of ASOs with CD after 48 h transfection.

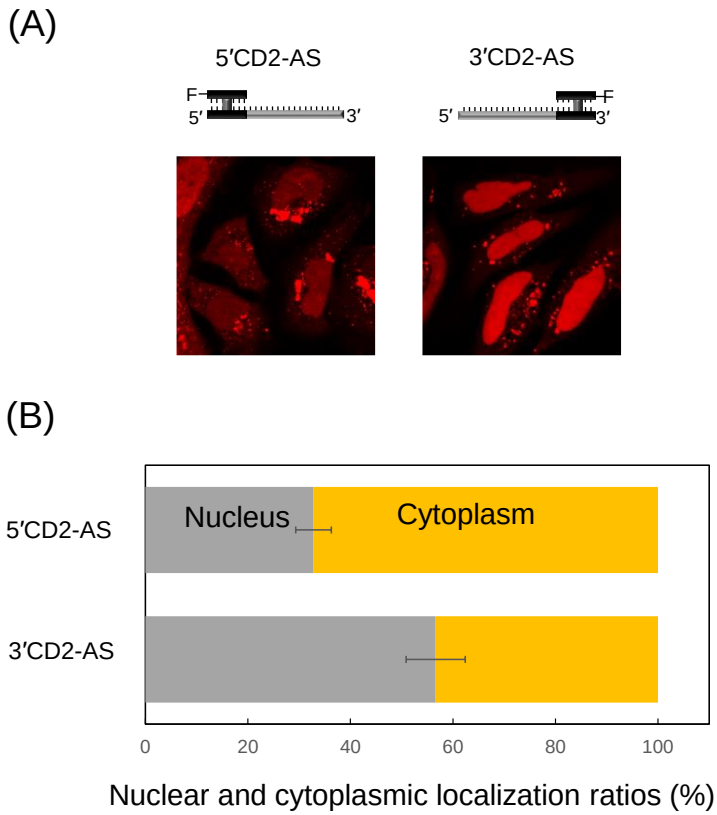


Figure S3 Intracellular localization of ASOs with CD after 48 h transfection. (A) Imaging analysis in HeLa cells. Representative fluorescent images of Alexa-Fluor 546-labelled ASOs. A dotted line indicates the nucleus. Scale bar: 20 μm. (B) The nuclear and cytoplasmic localization ratio of the ASOs CD at the 5′- or 3′-end are indicated by gray and orange bars, respectively. F indicates the Alexa-Fluor 546.

4. Effect of sequences in 5′- or 3′-CD-modified single-strand on intracellular localizations.

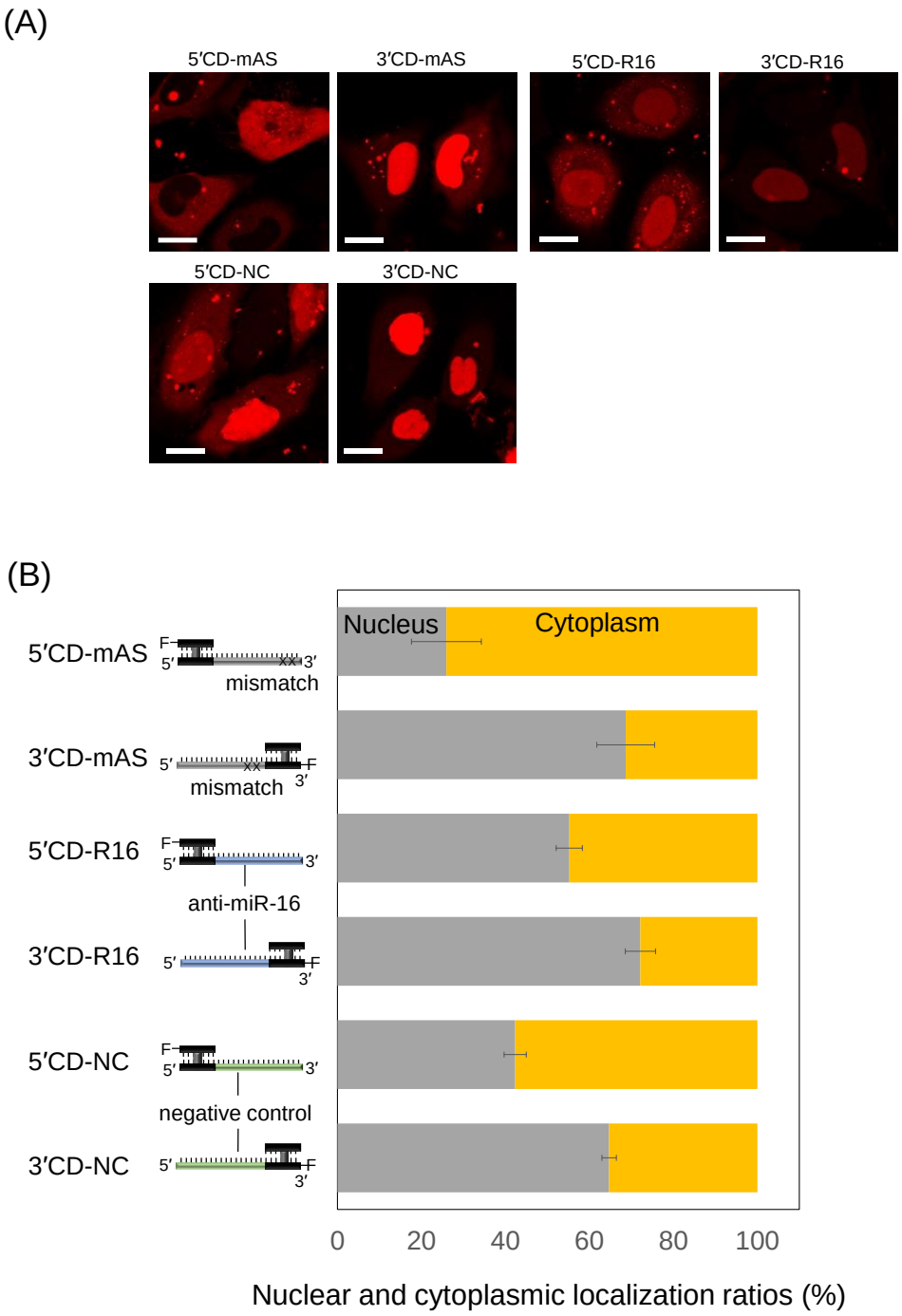


Figure S4. The effect of sequences in 5′- or 3′-CD-modified single-strand on intracellular localizations. The intracellular localization of various ASOs with a single CD. (A) Representative fluorescent images of Alexa-Fluor 546-labelled ASOs. Scale bar: 20 μm. (B) The nuclear and cytoplasmic localization ratios of the ASOs.

5.Uncropped image of denaturing PAGE analysis

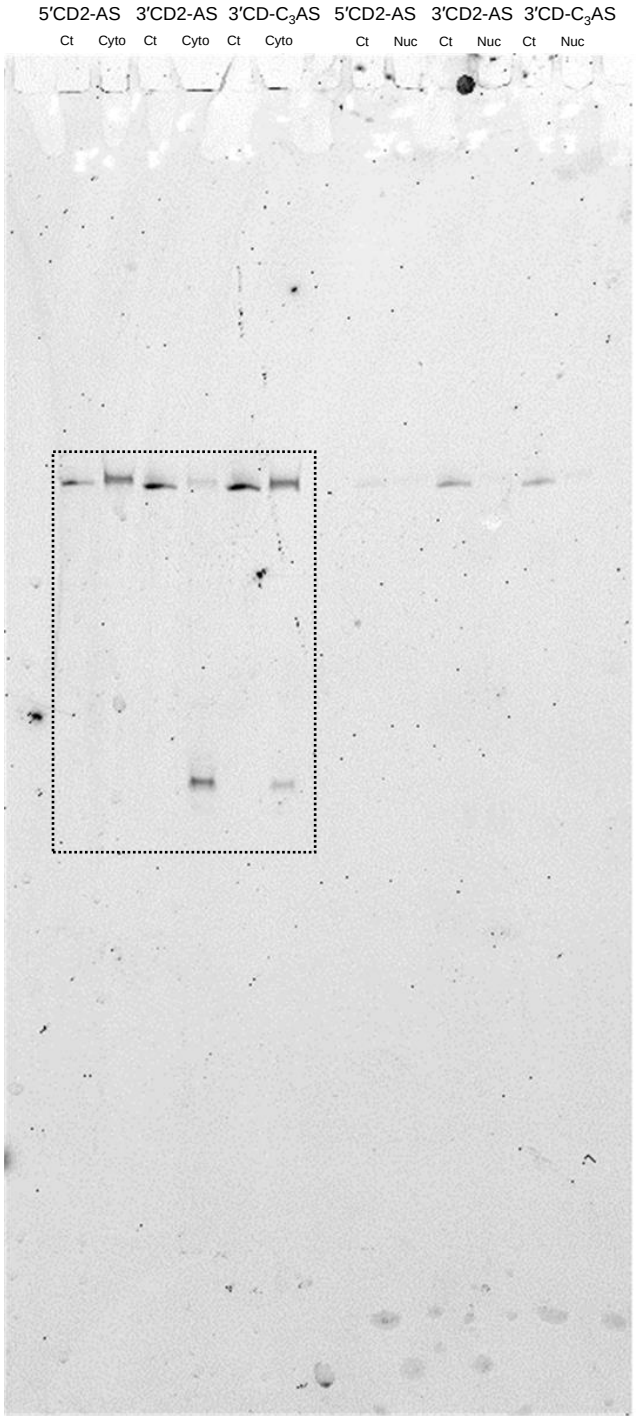
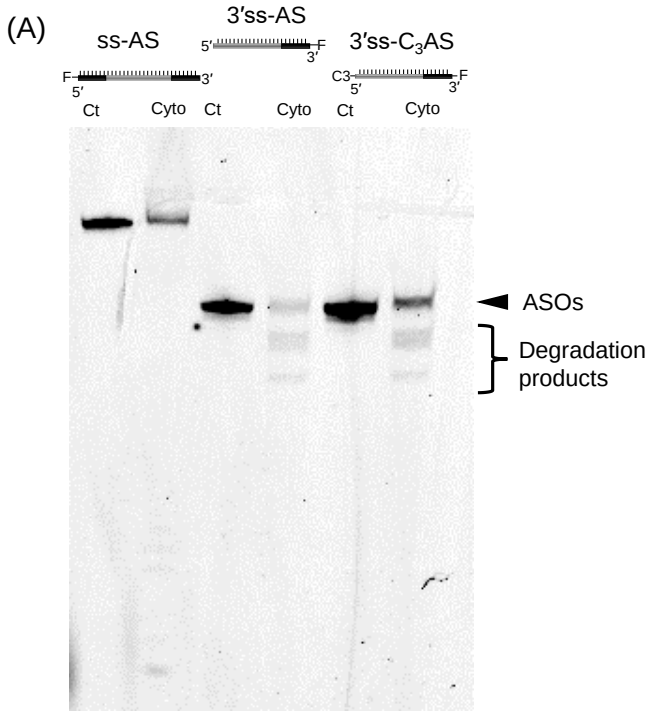


Figure S5. Uncropped image of denaturing PAGE analysis (5′CD2-AS, 3′CD2-AS, and 3′CD-C_3AS) . Ct, Cyto, and Nuc indicate the control, cytoplasmic, and nucleus fraction, respectively. The region of interest in this image used to Figure 5a is shown with the dotted square.

6. Stability of ASOs in the cytoplasm.



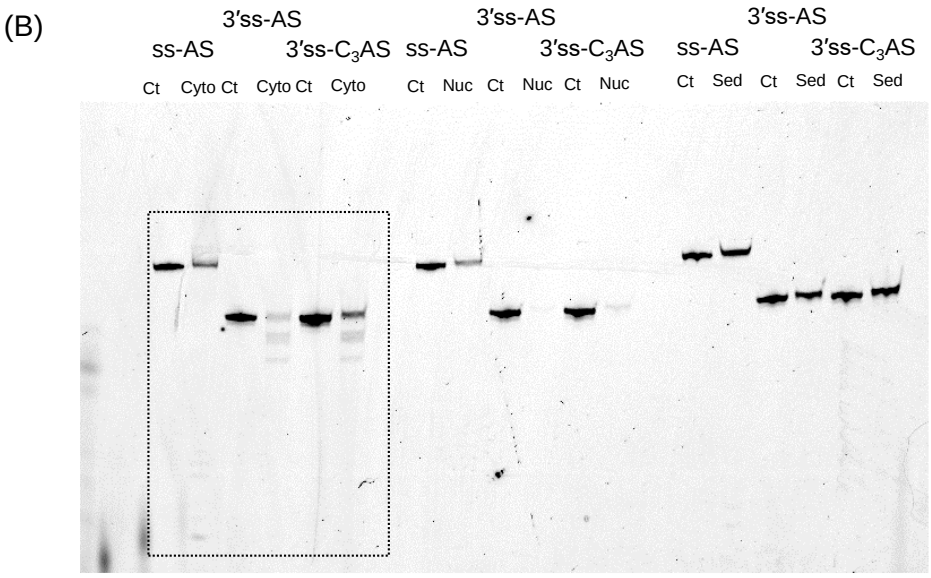


Figure S6. Stability of ASOs in the cytoplasm.

(A) Denaturing PAGE analysis of the cytoplasmic fractions of Hela cells transfected with fluorescently labelled single-stranded ASOs. (B) Uncropped image of denaturing PAGE analysis. The region of interest in this image used to (A) is shown with the dotted square. Untransfected ASOs were loaded as a control. Ct and Cyto indicate the control and cytoplasmic fraction, respectively. Nuc and Sed indicate the nucleus and sedimentation fraction, respectively. The gel image was obtained from scanning the gel at the excitation wavelength of 532 nm.

7.Anti-miRNA activities of ASOs without fluorescence labelling in HeLa cells

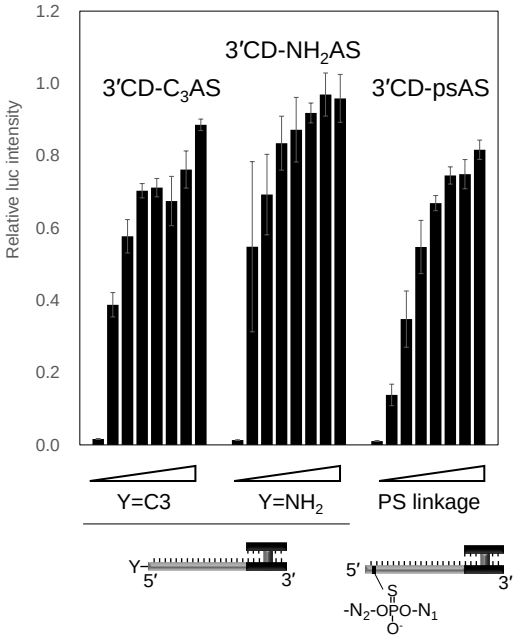


Figure S7. Anti-miRNA activities of ASOs without fluorescence labelling in HeLa cells.

Schematic drawings of the secondary structure of each ASO. Dual-luciferase assays indicate the relative luciferase intensity in the cells with 0, 0.5, 1, 2, 3, 4, 5, and 10 nM of ASO. The normalized intensities are represented as mean ± SD.