Supplemental Data

Effect of PEG anchor in PEGylation of folate-modified cationic liposomes with PEG-derivatives on systemic siRNA delivery into the tumor

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Materials and Methods

Accessibility of siRNA in siRNA lipoplexes

FA-PEG-modified siRNA lipoplexes and FA-PEG- and PEG-modified siRNA lipoplexes were prepared by mixing at charge ratios (+:-) of 1:1~4:1. Amounts of free siRNA in the siRNA lipoplexs were measured using exclusion assays with SYBR® Green I Nucleic Acid Gel Stain (Takara Bio Inc., Shiga, Japan) and calculated based on the standard curves of free siRNA as previously reported [1].

Cellular uptake of FA-PEG-modified siRNA lipoplexes

KB cells were plated into 35-mm culture dishes at a density of 3×10^5 cells 24 h prior to each experiment. LP-0.5F_5, LP-1F_5, LP-2F_5, and LP-3F_5 lipoplexes with 50 pmol Cy5-siRNA were diluted in 1 mL of folate-deficient RPMI-1640 medium, and they were incubated with KB cells for 3 h. After the incubation, the cells were fixed with 4% formaldehyde. The localization of Cy5-siRNA was visualized using an Eclipse TS100-F microscope (Nikon, Tokyo, Japan).

Hemolysis assay

Erythrocytes were collected from blood of female BALB/c mice (8 weeks of age; Sankyo Lab. Service Corp., Tokyo, Japan) at 4°C by centrifugation at 300 g for 3 min and resuspended in PBS as a 2% (v/v) suspension of erythrocytes. siRNA lipoplexes with 2 μg siRNA were added to 100 μl of 2% (v/v) erythrocyte suspension. As a positive control for hemolysis (100% hemolysis), erythrocytes were suspended in hypotonic solution (water). After incubation for 15 min at 37°C, the sample was centrifuged at 200 g for 3 min and hemolysis of erythrocytes was observed. The supernatants were transferred to a 96-well plate and the absorbance of hemoglobin was measured at 570 nm. The hemolysis (%) was calculated as relative to the absorbance of treatment with hypotonic solution.

In vivo antitumor effect

To generate KB tumor xenografts, 1×10^7 KB cells suspended in 50 μL of PBS were inoculated subcutaneously in the flank region of female BALB/c nu/nu mice (8 weeks of age, CLEA Japan, Inc., Tokyo, Japan). The mice were maintained on a folate-deficient rodent diet for the duration of the study. When the average volume of the tumors in each group reached around 100 mm^3 (day 0), LP-0.5P_5/2.5P_2-CS, LP-0.5F_5/2.5P_2-CS, LP-0.5P_5/2.5$P_{1.6}$-CL, and LP-0.5F_5/2.5$P_{1.6}$-CL lipoplexes with 20 μg Cont siRNA or PLK1 siRNA were systemically injected into the mice on days 0, 2, and 4. Tumor volume was measured on days 0, 2, 4, 6, and 8. Tumor volume (%) was calculated as relative to each tumor volume at the day 0.

Reference

[1] Hattori Y, Tamaki K, Sakasai S, et al. Effects of PEG anchors in PEGylated siRNA lipoplexes on in vitro genesilencing effects and siRNA biodistribution in mice. Mol Med Rep. 2020; 22(5):4183-4196.

Results

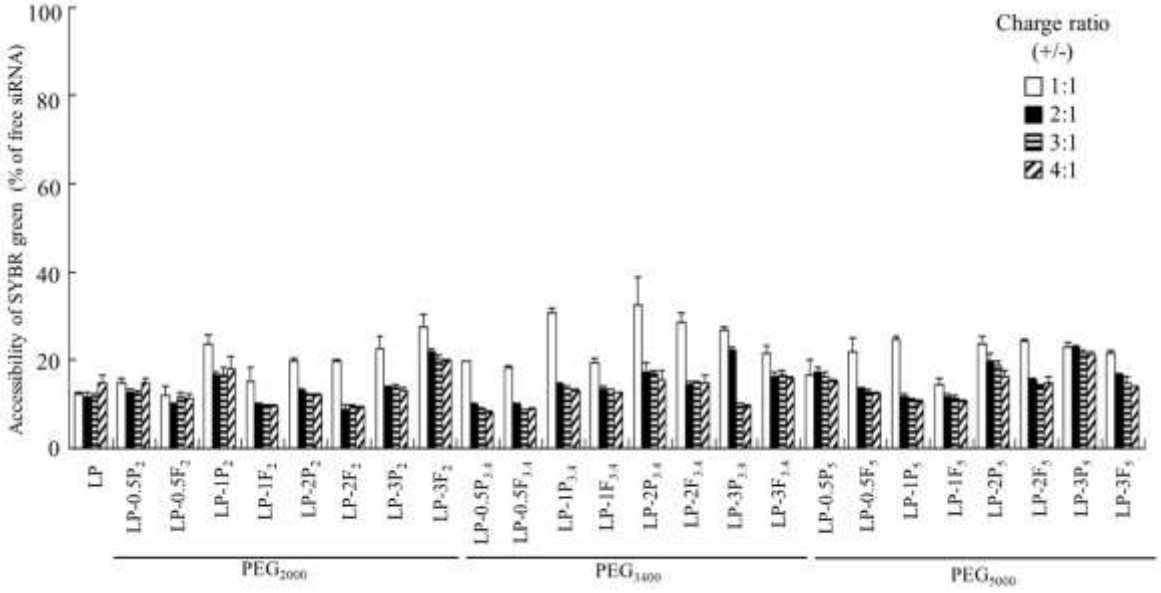

Figure S1 Association of siRNA with 0.5~3 mol% FA-PEG- or PEG-modified cationic liposomes in an exclusion assay using SYBR® Green I Nucleic Acid Gel Stain. FA-PEG- and PEG-modified siRNA lipoplexes were prepared by mixing siRNA with 0.5~3 mol% FA-PEG- and PEG-modified cationic liposomes, respectively, at charge ratios (+:-) of 1:1~4:1. The amount of siRNA available to interact with the SYBR® Green I is expressed as a percentage of the free siRNA unassociated with cationic liposomes. Each column represents the mean+S.D. (n=3).

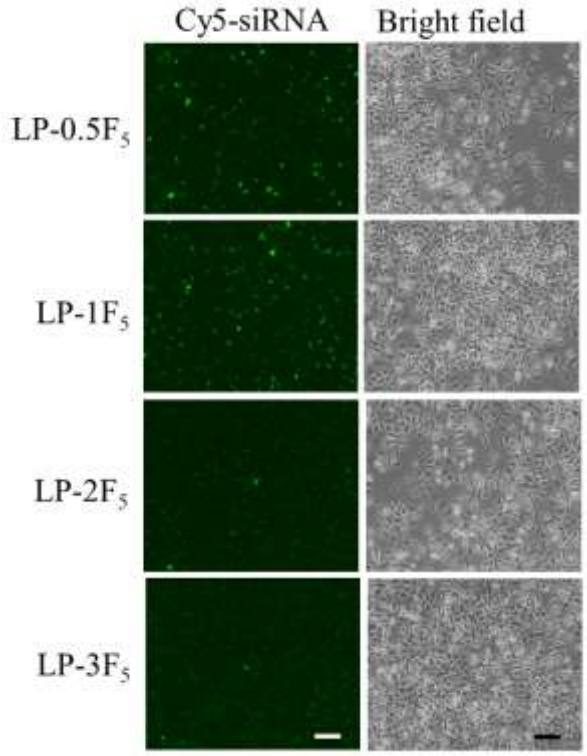

Figure S2 Cellular uptake of FA-PEG-modified siRNA lipoplexes in KB cells after incubation for 3 h. Green signals show localization of Cy5-siRNA. Scale bar = 100 μm.

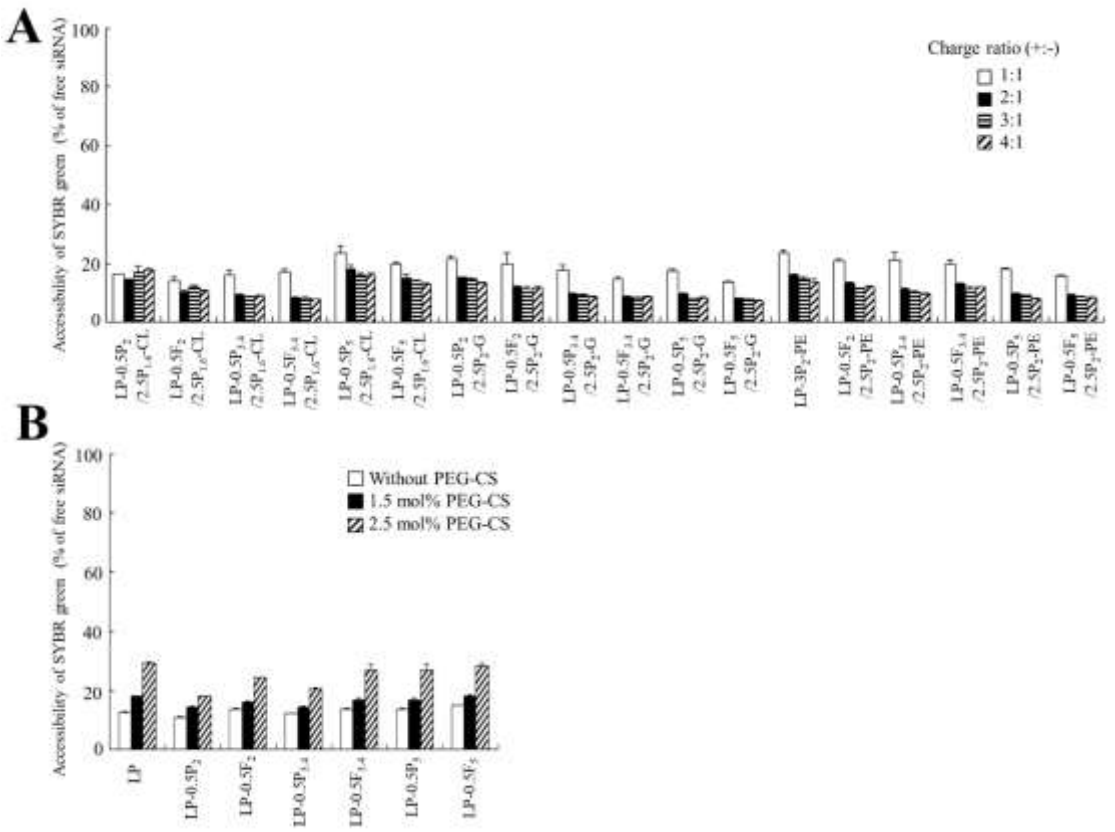

Figure S3 Association of siRNA with FA-PEG- and PEG-modified cationic liposomes in an exclusion assay using SYBR® Green I Nucleic Acid Gel Stain. In A, FA-PEG- and PEG-modified siRNA lipoplexes were prepared by mixing siRNA with 0.5 mol% FA-PEG-DSPE- and 2.5 mol% PEG-derivative-modified cationic liposomes at charge ratios (+:-) of 1:1~4:1. In B, FA-PEG-modified siRNA lipoplexes were prepared by mixing siRNA with 0.5 mol% FA-PEG-DSPE-modified cationic liposomes at a charge ratio (+:-) of 4:1, and PEG_{2000}-CS was then added to the effect of 1.5 or 2.5 mol% PEGylation. The amount of siRNA available to interact with the SYBR® Green I is expressed as a percentage of the free siRNA unassociated with cationic liposomes. Each column represents the mean+S.D. (n=3).

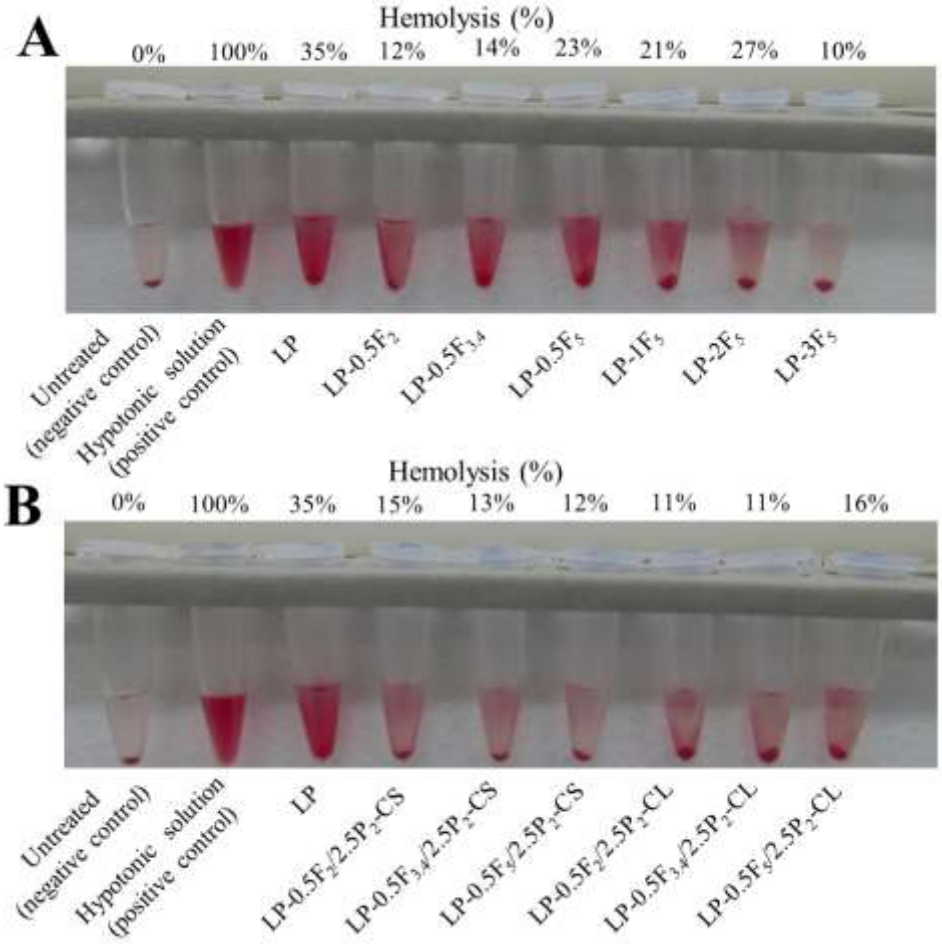

Figure S4 Hemolysis of mouse erythrocytes by addition of siRNA lipoplex. As a positive control for hemolysis (100% hemolysis), erythrocytes were suspended in hypotonic solution (water). The hemolysis (%) was calculated as relative to the absorbance of treatment with hypotonic solution. In A, FA-PEG-modified siRNA lipoplexes, and in B, FA-PEG- and PEG-modified siRNA lipoplexes.

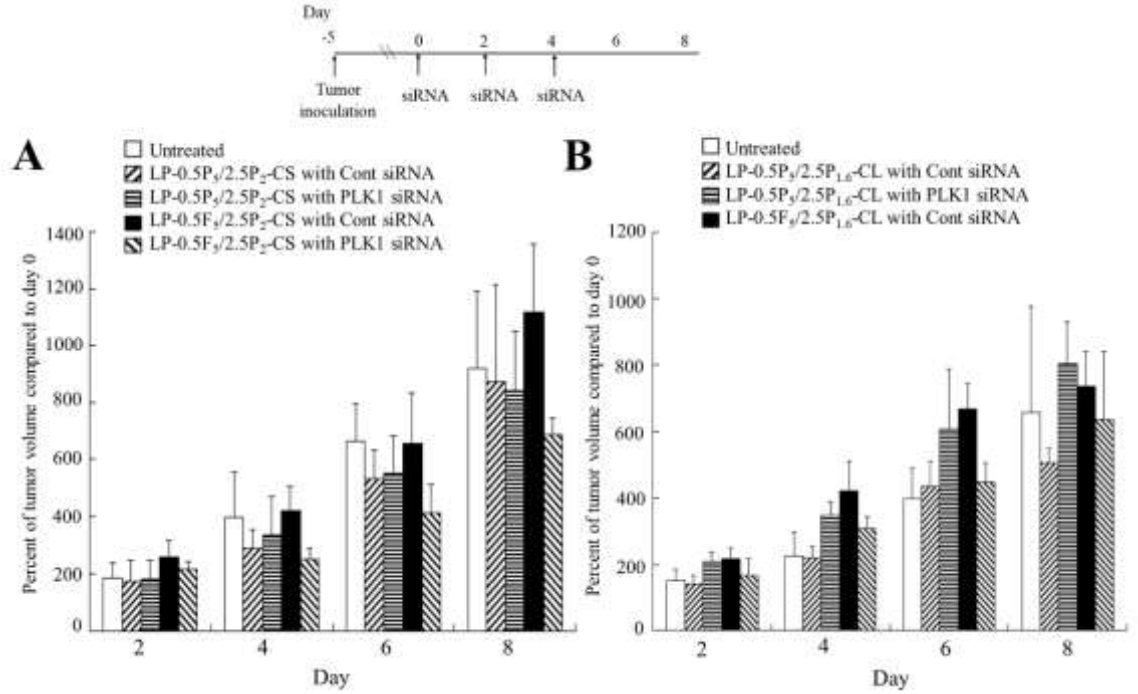

Figure S5 *In vivo* siRNA therapy of KB tumor xenografts using FA-PEG- and PEG-modified lipoplexes with PLK1 siRNA. LP-0.5F_5/2.5P_2-CS and LP-0.5P_5/2.5P_2-CS lipoplexes (A) or LP-0.5F_5/2.5$P_{1.6}$-CL and LP-0.5P_5/2.5$P_{1.6}$-CL lipoplexes (B) with 20 μg of Cont siRNA or PLK1 siRNA, were systemically injected into the mice bearing KB tumor xenografts three times (days 0, 2, and 4). Tumor volume was measured at days 0, 2, 4, 6, and 8. Tumor volume (%) was calculated relative to the volume of each tumor at day 0. No significant differences in tumor volume (%) between Cont siRNA and PLK1 siRNA treatments were observed in the mice after systemic injection with LP-0.5P_5/2.5P_2-CS, LP-0.5F_5/2.5P_2-CS, LP-0.5P_5/2.5P_2-CL, and LP-0.5F_5/2.5P_2-CL lipoplexes, respectively. Each result represents the mean+S.D. (n=3–4 for siRNA-treated groups, and n=5 for untreated groups in A and B).

Table S1 Summary of evaluation for FA-PEG- and PEG-modified cationic liposomes

Liposome	Suppression of cell growth by PLK1 siRNA *via* FR [a)]	Suppression of Luc expression by Luc siRNA *via* FR [b)]	Agglutination with erythrocytes	siRNA accumulation in the lungs
LP-0.5F_2	-	N.D.	+	+
LP-0.5$F_{3.4}$	-	N.D.	+	+
LP-0.5F_5	+	N.D.	+	+
LP-0.5F_2/2.5P_2-CS	-	-	-	-
LP-0.5$F_{3.4}$/2.5P_2-CS	-	-	-	-
LP-0.5F_5/2.5P_2-CS	+	+	-	-
LP-0.5F_2/2.5$P_{1.6}$-CL	-	-	-	-
LP-0.5$F_{3.4}$/2.5$P_{1.6}$-CL	-	-	-	-
LP-0.5F_5/2.5$P_{1.6}$-CL	+	+	-	-
LP-0.5F_2/2.5P_2-G	-	-	-	-
LP-0.5$F_{3.4}$/2.5P_2-G	-	-	-	-
LP-0.5F_5/2.5P_2-G	-	-	-	-
LP-0.5F_2/2.5P_2-PE	-	-	-	-
LP-0.5$F_{3.4}$/2.5P_2-PE	-	-	-	-
LP-0.5F_5/2.5P_2-PE	-	-	-	-

[a)] +: >20% cell growth inhibition ([cell viability (%) after treatment with FA-lipoplexes (Cont siRNA - PLK1 siRNA)] – [cell viability (%) after treatment with non-targeted lipoplexes (Cont siRNA - PLK1 siRNA)]).

[b)] +: >20% Luc knockdown, compared with non-targeted-lipoplexes (Luc activity (%) of non-targeted-lipoplexes - Luc activity (%) of FA-lipoplexes).

N.D., not determined. LP: liposome, P_2: PEG_{2000}, $P_{3.4}$: PEG_{3400}, P_5: PEG_{5000}, F_2: FA-PEG_{2000}, $F_{3.4}$: FA-PEG_{3400}, F_5: FA-PEG_{5000}, CS: chondroitin sulfate, CL: cholesterol, G: DSG, PE: DSPE.