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Palmitic Acid-Conjugated 21-Nucleotide siRNA Enhances Gene-Silencing Activity

Takanori Kubo,*[,†] Kazuyoshi Yanagihara,[†] Yoshifumi Takei,[‡] Keichiro Mihara,[§] Yasuhiro Morita,[†] and Toshio Seyama[†]

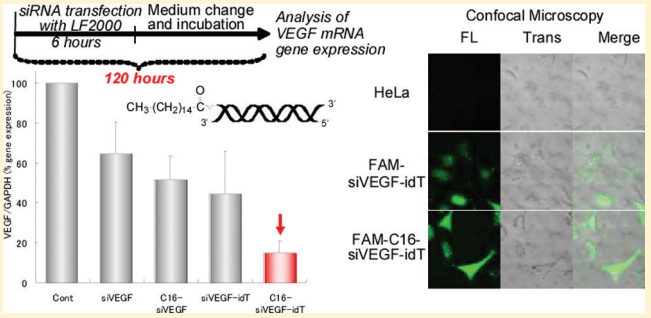
[†]Laboratory of Molecular Cell Biology, Department of Life Science, Faculty of Pharmacy, Yasuda Women's University, Hiroshima, Japan

[‡]Department of Biochemistry, Nagoya University Graduate School of Medicine, Nagoya, Japan

[§]Department of Hematology and Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan

Supporting Information

ABSTRACT: Short interfering RNA (siRNA) technology is a powerful tool for suppressing gene expression in mammalian cells. In this study, we focused on the development of siRNAs conjugated with palmitic acid at the 5′-end of the sense strand (C16-siRNAs) using our novel synthesis strategy in order to improve the potency of siRNA. The C16-siRNAs exhibited enhanced nuclease stability. In addition, they showed potent gene-silencing efficacy against exogenous *Renilla* luciferase in HeLa cells compared with a nonmodified siRNA in the presence of Lipofectamine 2000. The C16-siRNAs also had a more potent inhibitory effect on *Renilla* luciferase activity than the other siRNA conjugated with lipids at the 5′-end and the 3′-end by palmitoyl conjugation. For further improvement, the gene silencing potency of the C16-siRNAs against the endogenous vascular endothelial growth factor (VEGF) gene in HeLa cells was investigated. In this investigation, the siRNAs were prepared not only with the normal RNA sequence but also coupled with an inverted thymidine (idT) at the 3′-ends of both the sense and antisense strands (siRNA-idT), including palmitic acid conjugations at the 5′-end of the sense strand, to improve stability. The C16-siRNA including idT modifications exhibited a significantly greater inhibitory effect on the VEGF gene in the presence of Lipofectamine 2000. It is noteworthy that C16-siRNA-idT demonstrated long-term gene-silencing efficacy of up to 5 days. Interestingly, the C16-siRNAs, including that with idT modifications, exhibited strong RNAi potency in the absence of any transfection reagents, although only at high concentrations. Both the C16-siRNAs and C16-siRNA-idT induced a high level of membrane permeability in HeLa cells. Our developed C16-siRNAs, particularly C16-siRNA-idT, are thus among the promising candidates for a new generation of modified siRNAs that can solve the many problems associated with siRNA technology.



KEYWORDS: *palmitic acid conjugates, intracellular delivery, siRNA, gene silencing, inverted thymidine, long-term RNAi potency*

INTRODUCTION

RNA interference (RNAi) has attracted particular attention as a powerful tool for suppressing gene expression in mammalian cells.[1,2] Fire et al. discovered the phenomenon of the RNAi using a long double-stranded RNA (dsRNA).[3] The dsRNAs are cleaved to 21-nucleotide (nt) short interfering RNAs (siRNAs) by a Dicer enzyme,[4,5] and the siRNAs are bound to a protein complex called an RNA-induced silencing complex (RISC).[6] The RISC cleaves the target mRNA at a sequence-specific position, guided by the antisense strand of the siRNAs. In mammalian cells, however, long dsRNAs induce an interferon response.[7] To prevent interferon activation, Elbashir et al. have used chemically synthesized 21-nt siRNAs.[8]

Although RNAi has many advantages over other genetic drug technologies,[9−11] several problems, such as cellular delivery, instability in the presence of nuclease, and side effects (off-target effects and interferon responses at high concentrations), must be solved before it can be applied in the clinic. To solve the problems associated with RNAi, many chemically modified siRNAs have been developed by improving the properties of siRNAs.[12−17] It has been reported that 21-nt siRNAs with amino acid modifications at the 3′-end are highly stable against nuclease degradation.[12] The 2′-modifications (2′-O-Me and 2′-F) of siRNAs and modifications of a phosphate backbone (e.g., phosphorothioate and boranophosphate) also demonstrate high nuclease resistance.[16,17] Direct conjugation of siRNA to functional molecules, including polymers,[18,19] peptides,[20−22] and lipids,[23−26] has been reported to improve the biological properties of siRNA *in vitro* and

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in vivo. Among these conjugations, covalent bindings of lipids, such as cholesterol, bile acids, and long-chain fatty acids, to siRNAs at the 3′-end of the sense strand have been used to mediate siRNA uptake in cells *in vitro* and *in vivo*.[23,24] These lipophilic siRNAs interact with lipoprotein particles, lipoprotein receptors, and transmembrane proteins, and influence siRNA uptake behaviors. These conjugated siRNAs exhibit RNAi activity without any transfection reagent, but usually only when provided at high concentrations. Although the modifications and conjugations of siRNAs described above can be expected to solve some of the problems associated with RNAi, such as nuclease stability and cell permeability, most of them also weaken the gene-silencing efficacy. There are as yet no reports on the development of functional siRNAs that can solve all the problems of RNAi technology without the weakening or loss of their sequence-specific gene-silencing activity. The other problems with conjugations of siRNA are the complicated synthesis steps, limitation in the conjugating position to siRNA and conjugation molecules, and the high cost of their use in basic research and clinical applications.

In this study, we developed a simple synthesis strategy for conjugated siRNAs, which could be applied to a wide variety of functional molecules conjugated to any siRNA positions. Using this synthesis method, we prepared siRNA conjugated with lipids (palmitic acid, lauric acid, and cholesterol) at either the 5′-end or the 3′-end of the sense strand. The palmitic acid-conjugated siRNA (C16-siRNA) containing inverted thymidine (idT) at the terminal overhang was also synthesized to enhance stability and consequent long-term RNAi potency. The C16-siRNAs, including that coupled with idT, were found to enhance the gene-silencing activity, membrane permeability, and nuclease resistance.

EXPERIMENTAL SECTION

Design and Synthesis of siRNAs Including Amino Modifications. We designed siRNA sequences to target the *Renilla* luciferase, *Firefly* luciferase, and vascular endothelial growth factor (VEGF) genes. The 21-nt single-strand RNAs (ssRNAs; antisense and sense strands) and amino-modified sense-strand 21-nt ssRNAs were purchased from Integrated DNA Technologies (IDT, Coralville, IA). The siRNA sequences targeted to the *Renilla* and *Firefly* luciferase were as follows: *Renilla* sense, 5′-GGCCUUUCACUACUCCUAC-GA-3′; *Renilla* antisense, 5′-GUAGGAGUAGUGAAAGGC-CAG-3′; *Firefly* sense, 5′-CUUACGCUGAGUACUUCGATT-3′; *Firefly* antisense, 5′-UCGAAGUACUCAGCGUAAGTT-3′. The sequences of siRNA targeted to the VEGF gene were as follows: VEGF sense, 5′-UCCUACAGCACAACAAAUGUG-3′; VEGF antisense, 5′-CAUUUGUUGUGCUGUAGGAAG-3′. Amino-modified RNAs at the 5′-end or 3′-end were also purchased from IDT. The molecular weights of all ssRNAs were confirmed by MALDI-TOF mass spectrometry (Ultraflex, Bruker Daltonics, Bremen, Germany) using saturated solutions of 2,4,6-trihydroxyacetophenone (Sigma-Aldrich, St. Louis, MO) in 50 mg/mL diammonium hydrogen citrate in 50% acetonitrile as a matrix.[27] All MALDI-TOF mass spectrometry measurements were carried out in linear-negative mode. The concentrations of all ssRNAs were calculated using their absorbance at 260 nm detected spectrophotometrically (V-550 spectrophotometer, JASCO, Tokyo, Japan). The sense and antisense strands of RNAs were annealed to prepare dsRNAs following the manufacturer's instructions. Briefly, each ssRNA was diluted using sterile RNase-free water to a final concentration of 50 μM, and then 20 μL of each ssRNA (sense and antisense) solution was combined with 10 μL of annealing buffer (5×). The solutions were incubated for 1 min at 90 °C and cooled slowly afterward to room temperature (the final concentration of the siRNA was 20 μM). The dsRNA formation was confirmed by 20% polyacrylamide gel electrophoresis (PAGE; 30 mA, 70 min) and visualized by silver staining (DNA Silver Stain Kit, GE Healthcare, Piscataway, NJ).

Synthesis of Conjugated siRNA. We conjugated 21-nt ssRNAs of the sense strands, which were coded to the target gene (*Renilla* and *Firefly* luciferase, and VEGF gene), with lipids such as palmitic acid, lauric acid, and cholesterol. In the synthesis of palmitic acid-conjugated 21-nt ssRNAs (C16-ssRNAs; 5′-end conjugations: ssRNAs-C16; 3′-end conjugations), the amino-modified ssRNAs at either the 5′-end or the 3′-end (4 nmol in 20 μL of water) were reacted with 40 nmol of palmitic acid *N*-hydroxysuccinimide ester (Sigma-Aldrich) dissolved in 10 μL of *N*,*N*-dimethylformamide (DMF, Sigma-Aldrich) containing 0.7 μL of *N*,*N*-diisopropylethylamine (DIEA, Sigma-Aldrich) in 100 μL of isopropanol/water (1:1) mixture solutions, for 12 h at room temperature. In the synthesis of lauric acid-conjugated 21-nt ssRNA (C12-ssRNA), the ssRNA with amino acid modifications at the 5′-end was reacted with lauric acid *p*-nitrophenyl (Sigma-Aldrich) using the same procedure as described above for C16-ssRNA.

The cholesterol-conjugated 21-nt ssRNA at the 5′-end (Chol-ssRNA) was purchased from Hokkaido System Science (HSS, Hokkaido, Japan).

All 21-nt ssRNAs conjugated with lipids were purified by RP-HPLC using an octadecylsilane (ODS) column (4.6 × 150 mm, 5 μm) under a linear gradient condition of acetonitrile shifting the concentrations from 7% to 70% for 40 min in 20 mM TEAA (pH 7.0). The molecular weights of the conjugates were confirmed by MALDI-TOF mass spectrometry (Ultraflex, Bruker Daltonics) as predicted under the same conditions as above. The yields of the conjugates were spectrophotometrically calculated on the basis of absorbance at 260 nm wavelength.

To prepare siRNA conjugated with lipids, antisense 21-nt ssRNAs, which were complementary to mRNA strands of the target gene and synthesized independently, were annealed with the 21-nt ssRNA conjugated with lipids as in the protocols described above. The quality of the conjugated siRNA was confirmed by 20% PAGE.

Stability against Nuclease Degradation in Serum. Ten microliters of the siRNA (200 pmol), including C16-conjugations, was added to 90 μL of Dulbecco's modified Eagle's medium (DMEM; Wako, Osaka, Japan), containing 10% heat-inactivated FBS (Invitrogen, La Jolla, CA). The samples were incubated for different time intervals (0, 0.5, 1, 2, 4, 6, 8, 12, 24, and 48 h) at 37 °C. Aliquots (10 μL) were taken from each sample. The samples were frozen in liquid nitrogen (to stop the nuclease reaction) and were kept at −20 °C. The RNA products of nuclease degradation were analyzed using 20% PAGE (30 mA, 70 min) and visualized by silver staining (DNA Silver Stain Kit, GE Healthcare). The signals of the RNA products were photographed by LAS4000 (Fujifilm, Tokyo, Japan).

Another approach was attempted to analyze the stability of C16-siRNA in culture medium by RP-HPLC. The RNA samples after incubation with culture medium, obtained in the same way as described above for PAGE analysis, were analyzed by RP-HPLC using an ODS column (4.6 × 150 mm, 5 μm) under a linear condition of acetonitrile shifting the concentrations from 7% to 70% for 40 min in 20 mM TEAA (pH 7.0). The intact RNAs were assessed as a percentage from control (nontreated siRNA and C16-siRNA) using an area of each RNA signal normalized by a constant signal from the medium as an internal control.

The stability of siRNAs, including C16-conjugations, in the 90% FBS was also investigated. Ten microliters of the siRNA (200 pmol), including C16-conjugations, was added to 90 μL of 100% heat-inactivated FBS (Invitrogen). The samples were incubated for different time intervals (0, 5, 10, 20, 30, 45, 60, 90, 120, and 180 min) at 37 °C. The RNA samples after incubation with FBS were analyzed using RP-HPLC by the same procedure as described above.

Cell Culture and Transfection. HeLa cells were cultured in DMEM (Wako) supplemented with 10% heat-inactivated FBS (Invitrogen), 100 U/mL penicillin (Wako), and 100 μg/mL streptomycin (Wako). Cells of the line 44As3Luc, which is a human signet-ring cell gastric cancer cell line that stably expresses the *Firefly* luciferase gene and that was constructed by Yanagihara et al.,[28,29] were cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS (Invitrogen), 100 U/mL penicillin (Wako), and 100 μg/mL streptomycin (Wako).

As for the evaluation of RNAi of siRNA conjugated with lipids targeting the exogenous transiently expressed *Renilla* luciferase gene in either the presence or absence of Lipofectamine 2000 (LF2000; Invitrogen) in HeLa cells, we used psiCHECK-2 Vector (Promega, Madison, WI) as a reporter gene, which contains both the *Firefly* and *Renilla* luciferase genes. *Renilla* luciferase activity is used to estimate RNAi efficacy. The second reporter, *Firefly* luciferase, served as a control. HeLa cells were seeded at 5×10^4 cells/mL in 100 μL of medium in each well of 96-well multiplates and cultured in a 100% humidified atmosphere (5% CO_2, 37 °C). Twelve hours later, 0.02 μg of psiCHECK-2 Vector was first incubated with 0.2 μL of LF2000 in 10 μL of Opti-MEM (Invitrogen) for 30 min according to the manufacturer's protocol, and then 10 μL of the mixtures was added to each well of a 96-well multiplate. Each well contained 90 μL of fresh culture medium without antibiotics at a final concentration of 0.2 μg/mL psiCHECK-2 vector and 2 μL/mL LF2000 per well of 96-well multiplates. To investigate RNAi in the presence of LF2000, the siRNAs against *Renilla* luciferase at different concentrations (10, 5, 2, 1, and 0.5 nM) were preincubated with LF2000 as described for the psiCHECK-2 vector. Four hours after the vectors were transfected, 10 μL of the preincubated mixtures of siRNA with LF2000 was added to each well containing 90 μL of fresh culture medium. After another 8 h incubation, the culture medium was replaced with 100 μL of fresh medium and the cells were cultured for 48 h to assess RNAi. To investigate the RNAi effect against *Renilla* luciferase in the absence of LF2000, first the psiCHECK-2 Vector was transfected using LF2000 by the same procedure as described above, and then the cells were washed three times with culture medium after 4 h incubation. The siRNAs at different concentrations (1000, 800, 600, 400, 200, 100, and 50 nM) without any transfection reagents were added to the cells and incubated for 48 h. The luciferase activity was analyzed 48 h after siRNA transfection. The *Renilla* and *Firefly* luciferase gene expression levels in the cells were measured by the Dual-Glo Luciferase Assay System (Promega). Nontreated cells were used as the control, which was processed according to the same protocols as described above.

As for RNAi analysis of C16-siRNAs against the stably expressed *Firefly* luciferase gene in either the presence or absence of LF2000, 44As3Luc cells were seeded at 5×10^4 cells/mL in 100 μL of medium in each well of a 96-well multiplate and cultured in a 100% humidified atmosphere (5% CO_2, 37 °C). To investigate the RNAi of C16-siRNA against *Firefly* luciferase, the siRNAs were prepared, transfected and cultured as described above for the RNAi against *Renilla* luciferase. The *Firefly* luciferase gene expression levels in the cells were measured by a Bright-Glo Luciferase Assay System (Promega).

As for RNAi analysis of C16-siRNAs against endogenous VEGF gene expression in either the presence or absence of LF2000, the HeLa cells were adjusted to 5×10^4 or 1×10^4 cells in 1 mL of medium for 48 or 120 h, respectively, in each well of 24-well multiplates and were cultured. To investigate RNAi in the presence of LF2000, 2.5 μL of 20 μM siRNAs including C16-conjugations (50 pmol) was preincubated with 2 μL of LF2000 in 100 μL of Opti-MEM for 30 min. Then 100 μL of the mixture (siRNAs and LF2000) was added to each well containing 900 μL of medium dispensed in the 24-well multiplates. After 6 h incubation, the medium was refreshed and the HeLa cells were cultured for 48 or 120 h for RNAi analysis. To investigate the RNAi against VEGF in the absence of LF2000, 100 μL of 10 μM siRNAs including C16-conjugations (1 nmol) was added to each well containing 900 μL of medium dispensed in the 24-well multiplates and cultured for 48 or 120 h. The VEGF mRNA expression in the HeLa cells was analyzed by RT-PCR. Control data were obtained from the nontreated cells.

Gene Silencing of *Renilla* and *Firefly* Luciferase. RNAi toward the transiently expressed *Renilla* luciferase was evaluated by the Dual-Glo Luciferase Assay System (Promega). To detect *Firefly* luciferase activity as an intraplasmid control, 50 μL of Dual-Glo luciferase reagent-1 (beetle luciferin) was added to each well containing 100 μL of culture medium in 96-well multiplates. The plates were incubated in the dark for 10 min at room temperature. Luminescence emitted from the *Firefly* luciferase catalytic reaction was measured for 1 s for each well on the microplate reader (Wallac 1420 ARVO MX, Perkin-Elmer, Waltham, MA).

To measure the *Renilla* luciferase activity and to quench the luminescence from the *Firefly* luciferase catalytic reaction, 50 μL of Dual-Glo Stop and Glo reagent-2 (containing coelenterazine) was added to each well. The multiplates were incubated in the dark for 10 min at room temperature. The luminescence arising from the *Renilla* luciferase catalytic reaction was measured in the same way as described above for *Firefly* luciferase activity, and normalized by the luminescence of *Firefly* luciferase activity in each well of 96-well multiplates. The RNAi of conjugated siRNAs toward the transiently expressed *Renilla* luciferase was assessed as a percentage from the control (siRNA nontreated) sample.

To measure the stably expressed *Firefly* luciferase activity, 50 μL of Bright-Glo reagent was added to each well of 96-well multiplates. The luminescence emitted from the *Firefly* luciferase catalytic reaction was measured in the same way as described above for *Renilla* luciferase activity. The RNAi of conjugated siRNAs toward the stably expressed *Firefly* luciferase was assessed as a percentage of the control value (siRNA nontreated).

Gene Silencing of VEGF. RNAi toward VEGF was evaluated by measuring VEGF mRNA quantitatively using RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was also measured as the intrinsic control. The total RNA was extracted by the RNeasy Plus Mini Kit (Qiagen, Valencia, CA), and the yield was determined by optical density at 260 nm wavelength on a UV spectrometer. RT-PCR analysis was conducted using 100 ng of extracted total RNA. The primers used for the target mRNAs were as follows: VEGF forward primer, 5′-CCCTGATGAGATCGAGTACATCTT-3′; VEGF reverse primer, 5′- ACCGCCTCGGCTTGTCAC-3′; GAPDH forward primer, 5′- GGAAAGCTGTGGCGTGATG-3′; GDPDH reverse primer, 5′- CTGTTGCTGTAGCCGTATTC-3′. RT-PCR was

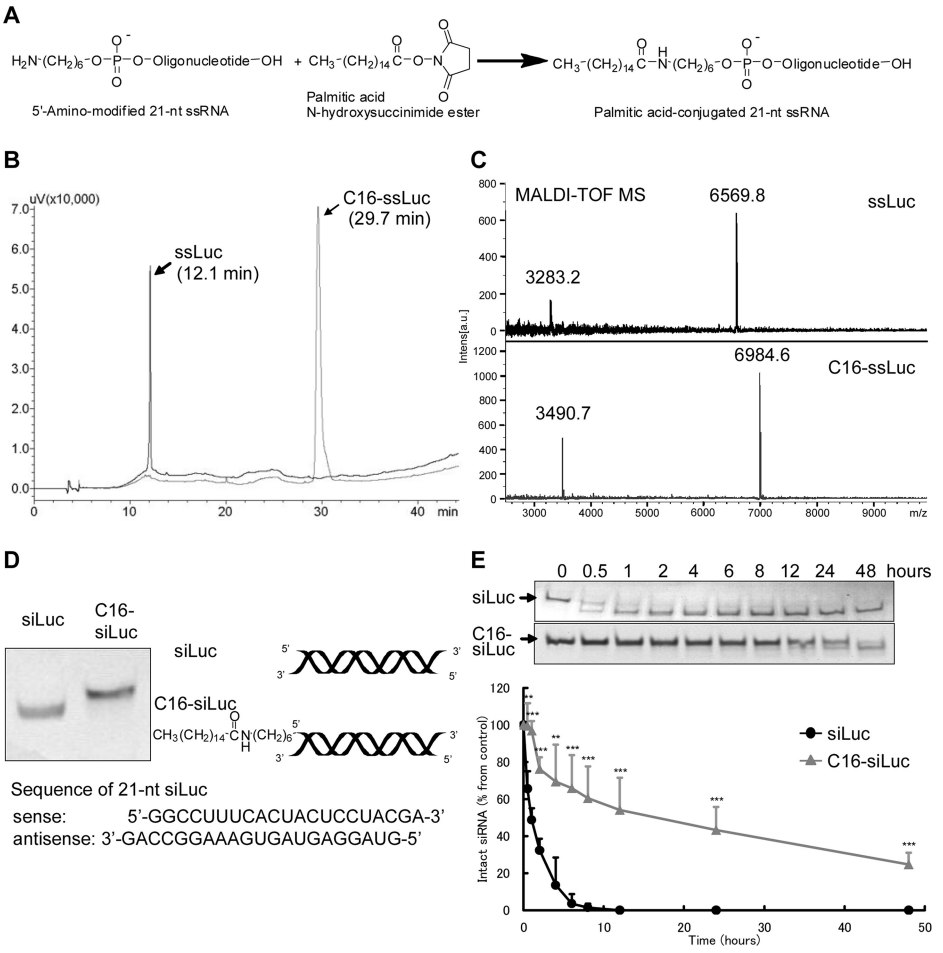

Figure 1. Synthesis and characterization of palmitoyl-siRNA (C16-siLuc). (A) Simple synthesis of C16-siRNA. (B) HPLC profiles of siLuc and C16-siLuc. HPLC was performed using an ODS column (4.6 × 150 mm, 5 μm) under a linear gradient condition of acetonitrile, with the concentrations shifting from 7% to 70% during 40 min in 20 mM TEAA (pH 7.0). (C) Molecular-weight confirmation by MALDI-TOF mass spectrometry. MALDI-TOF mass was carried out in linear-negative mode using a saturated solution of 2,4,6-trihydroxyacetophenone in 50 mg/mL diammonium hydrogen citrate in 50% acetonitrile as a matrix. (D) PAGE analysis of siLuc and C16-siLuc. (E) Serum stability of siLuc and C16-siLuc. Unmodified and palmitic acid-conjugated siLuc was incubated in culture medium containing 10% FBS at 37 °C for 0, 0.5, 1, 2, 4, 6, 8, 12, 24, or 48 h and aliquots were analyzed on 20% PAGE and RP-HPLC. The mean and SD values are from three independent experiments (*P < 0.05, **P < 0.01, ***P < 0.001; t-test vs siLuc treatment).

carried out using the One-Step RT-PCR Kit (Qiagen) according to the manufacturer's protocol. Briefly, the RT step was carried out at 50 °C for 30 min followed by heat denaturation at 94 °C for 10 min. The PCR step was constructed by 25 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s. PCR products were electrophoresed on 2% agarose gel, and the predicted size of amplified molecules was certified with the aid of LAS4000 (Fujifilm).

Intracellular Delivery of C16-siRNAs. To prepare fluorescence (FAM)-labeled siRNA, antisense ssRNA was labeled with 5′-fluorescein phosphoramidite (Glen Research) at the 5′-end. The FAM-labeled antisense 21-nt ssRNA with a normal RNA sequence was prepared for both *Renilla* luciferase and VEGF. The FAM-labeled antisense 21-nt ssRNA consisted of the 19-nt RNA with 2-nt DNA at the overhang, and its idT-modifications at the 3′-termini were prepared for VEGF. The FAM-labeled antisense 21-nt ssRNA was annealed with either palmitic acid-conjugated or unconjugated sense ssRNA including its 2-nt DNA overhang and idT modifications as in the protocols described above.

To deliver the prepared siRNAs intracellularly, 200 pmol of siRNA, including C16-conjugations labeled with FAM, were incubated with 2 μL of LF2000 in 100 μL of Opti-MEM diluted twice for 30 min at room temperature. Then, 100 μL of the mixture was added to 900 μL of culture medium of HeLa cells (5×10^4 cells) and incubated for 6 h in the dark under a humidified atmosphere (5% CO_2, 37 °C). The cells were washed several times with fresh medium, and intracellularly incorporated amounts of 21-nt siRNAs labeled with FAM in cells were examined under a fluorescent confocal microscope (IX70, Olympus, Tokyo, Japan). For quantitative assessment of fluorescence images, the images were analyzed using Scion image software (Scion Corporation, Frederick, MD).

RESULTS

Synthesis and Characterization of C16-siRNA. We initially selected an exogenous *Renilla* luciferase as a target gene because the luciferase reporter assays were among the conventional approaches for RNAi. The ssRNA target to the *Renilla* luciferase gene (ssLuc) modified with amine at the 5′-end was condensed with palmitic acid *N*-hydroxysuccinimide ester in solution (Figure 1A). The crude C16-ssLuc was purified by RP-HPLC (Figure 1B), and the molecular weight

Table 1. Characterizations of ssLuc Conjugated with Bioactive Molecules

name	conjugd molecule	conjugn site	HPLC retention time[a] (min)	MALDI-TOF MS[b] found/calcd	yield[c] (%)
ssLuc	none		12.1	6569.8/6569.9	*d*
C16-ssLuc	palmitic acid	5′-end	29.6	6984.6/6986.4	47.1
Chol-ss21Luc	cholesterol	5′-end	31.9	7324.7/7325.0	*d*
C12-ssLuc	lauric acid	5′-end	24.7	6929.5/6930.3	46.8
ssLuc-C16	palmitic acid	3′-end	30.2	6987.5/6986.4	68.4

[a]A linear gradient condition of CH_3CN shifting the concentrations from 7% to 70% during 40 min in 20 mM TEAA (pH 7.0) using an ODS column. [b]A saturated solution of 2,4,6-trihydroxyacetophenone in 50 mg/ml diammonium hydrogen citrate in 50% acetonitrile was used as a matrix. [c]Overall yields of the products were determined by measuring absorbance at 260 nm after HPLC purification. [d]The purified ssRNAs were purchased.

was confirmed by MALDI-TOF mass spectrometry (Figure 1C). The RP-HPLC retention time of C16-ssLuc was about 17 min slower than that of nonconjugated ssLuc under the purification conditions (see Experimental Section). The purified C16-ssLuc was obtained in 47.1% overall yield by spectrophotometric calculation (Table 1). The C16-ssLuc was annealed with the antisense ssRNA, which was complementary to mRNA strands of the target *Renilla* luciferase gene, in order to prepare palmitoyl-21-nt siRNA (C16-siLuc; Figure 2A). C16-siLuc was

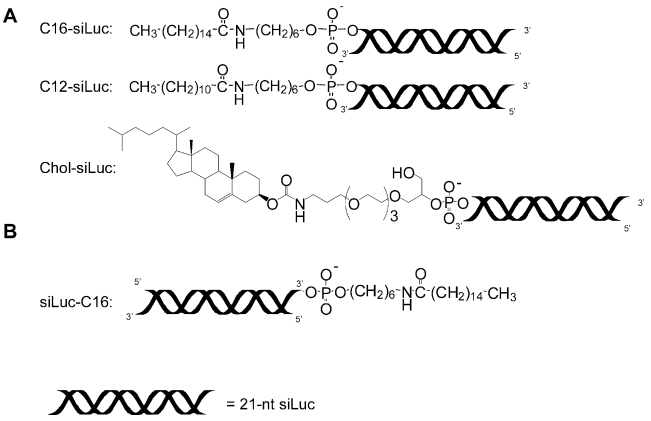

Figure 2. Structures of siLuc conjugated with lipids. (A) Palmitic acid, lauric acid, and cholesterol were conjugated to siLuc at the 5′-end of the sense strand (C16-siLuc, C12-siLuc, and Chol-siLuc). (B) Palmitic acid was conjugated to siLuc at the 3′-end of the sense strand (siLuc-C16).

a confirmed duplex stranded by using 20% PAGE (Figure 1D), and it showed a different mobility shift on PAGE in comparison with the control nonmodified 21-nt siRNA (siLuc). The C16-siLuc was clearly separated and its purity was confirmed for further use.

The nuclease stability of siLuc and that of C16-siLuc in culture medium containing 10% FBS were evaluated at different time intervals (0, 0.5, 1, 2, 4, 6, 8, 12, 24, and 48 h), using PAGE and RP-HPLC analysis (Figure 1E and Figure S1 in the Supporting Information). The siLuc was degraded immediately and completely disappeared after about 5 h of incubation. This was shown by MALDI TOF-MS in our previous report.[30] In contrast, C16-siLuc exhibited greatly increased stability against nuclease; surviving C16-siLuc molecule was found after 48 h incubation. The calculated half-life of the siLuc and C16-siLuc was 1.5 and 16.8 h, respectively. The nuclease resistance of siLuc and that of C16-siLuc in 90% FBS (0, 5, 10, 20, 30, 45, 60, 90, 120, and 180 min) were also evaluated by RP-HPLC (see Figure S2 in the Supporting Information). Despite the 90% FBS, the C16-siLuc exhibited higher resistance compared with siLuc.

RNAi Efficacy of C16-siLuc Targeted to Transiently Expressed *Renilla* Luciferase Gene. We performed a comparative study of the RNAi potency of siLuc, cholesterol-conjugated-siLuc (Chol-siLuc; Figure 2A), and C16-siLuc against the transiently expressed *Renilla* luciferase gene in either the presence or absence of LF2000. In the presence of LF2000, *Renilla* luciferase gene expression was dose-dependently suppressed in all samples with high potency (Figure 3A: +LF2000). The C16-siLuc exhibited stronger gene-silencing

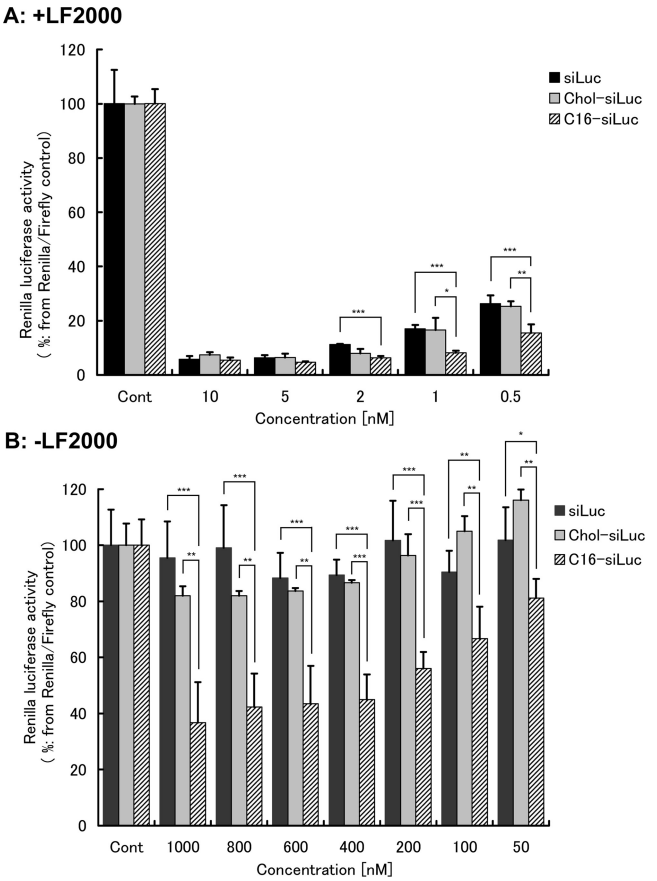

Figure 3. Comparative RNAi efficacy of siLuc, C16-siLuc, and Chol-siLuc to detect the luminescence of *Renilla* luciferase activity in HeLa cells in either the presence (A) or absence (B) of LF2000. The controls were given only PBS(−). The luminescence of *Renilla* luciferase activity was normalized by the luminescence of *Firefly* luciferase activity. The mean and SD values are from three independent experiments (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; *t*-test vs siLuc treatment).

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efficacy than siLuc or Chol-siLuc. A significant difference in the gene-silencing efficacy between siLuc, Chol-siLuc, and C16-siLuc in the absence of LF2000 was verified (Figure 3B: −LF2000). The siLuc did not inhibit *Renilla* luciferase activity at any concentration, even at a concentration as high as 1 μM; in contrast, the *Renilla* luciferase activity was dose-dependently suppressed in both C16-siLuc and Chol-siLuc. The RNAi efficacy of C16-siLuc was especially pronounced.

Comparative Study of C16-siLuc, C12-siLuc and siLuc-C16. We performed a comparative study of conjugated siRNAs between palmitic acid and lauric acid targeted to the *Renilla* luciferase gene. The C12-ssLuc was obtained in a 46.8% overall yield as an isolated yield after RP-HPLC purification (Table 1). The C12-ssLuc was annealed with the antisense ssRNAs to obtain lauroyl-siLuc at the 5′-end of the sense strand (C12-siLuc; Figure 2A). We also conjugated palmitic acid to ssLuc with amino acid modifications at the 3′-end using the same procedure as used for the 5′-end conjugations in order to estimate the influences of siRNA conjugated at the 5′-end and 3′-end on the gene-silencing behavior. The ssLuc-C16 was successfully synthesized (Table 1), and it was annealed with antisense ssRNA to obtain palmitoyl-siLuc at the 3′-end of the sense strand (siLuc-C16; Figure 2B).

The siLuc conjugates with fatty acids were confirmed to be double-stranded forms using 20% PAGE. All conjugations showed different mobility shifts on PAGE than the control siLuc (data not shown). These conjugations were clearly separable, and their purity was confirmed for further use.

All of the siLuc conjugates with fatty acids exhibited dose-dependent suppression of the *Renilla* luciferase activity (Figure 4). The siLuc conjugates with fatty acids manifested strong RNAi

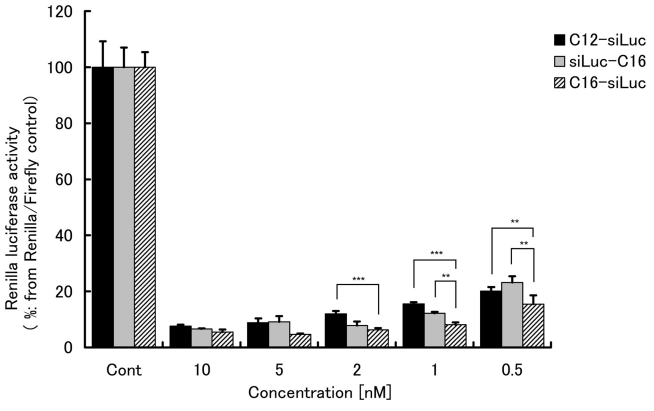

Figure 4. Comparative study of RNAi efficacy of siLuc conjugated with fatty acids. The siLuc conjugated with fatty acids (10, 5, 2, 1, and 0.5 nM) were transfected by LF2000 to HeLa cells, and controls were given only PBS(−). RNAi efficacies of the siLuc conjugated with fatty acids were evaluated to detect the luminescence of *Renilla* luciferase activity, which was normalized by the luminescence of *Firefly* luciferase activity, after 48 h incubation. The mean and SD values are from three independent experiments (*P < 0.05, **P < 0.01, ***P < 0.001; t-test).

efficacy. Among them, the C16-siLuc exhibited higher RNAi efficacy than not only C12-siLuc but also siLuc-C16 in a dose-dependent manner. Therefore, we clarified that the palmitoyl-21-nt siRNA at the 5′-end of the sense strand was the best molecule for enhancing RNAi potency, and we selected this siRNA for use in further studies.

Intracellular Delivery of C16-siLuc in HeLa Cells. The membrane permeability of both siLuc and C16-siLuc was investigated in HeLa cells in the presence of LF2000 using a confocal laser microscope (Figure 5A). No fluorescence signals

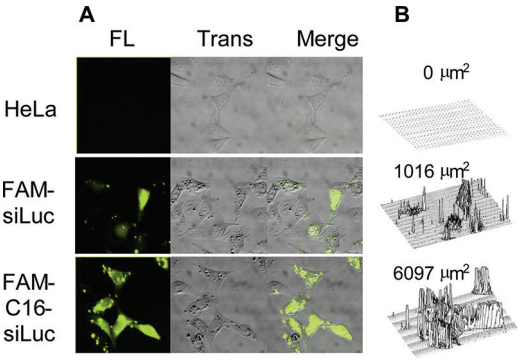

Figure 5. (A) Confocal microscopic images of HeLa cells incubated for 6 h with FAM-siLuc and FAM-C16-siLuc in the presence of LF2000. FL, fluorescent image; Trans, transmission image; Merge, merged image of FL and Trans. (B) The quantitative assessment of fluorescence images was analyzed using Scion image software to obtain area of fluorescence from the image.

were detected in nontreated HeLa cells (control), whereas the cells treated with siLuc and C16-siLuc, both labeled with FAM at the 5′-end of the antisense strand, exhibited bright fluorescence. In particular, C16-siLuc labeled with FAM exhibited an extremely high fluorescence intensity in the cells. We quantitatively analyzed the fluorescent image by Scion image software. The C16-siLuc showed strong fluorescence intensity that was approximately 6-fold higher than that of siLuc (Figure 5B). Accordingly, the C16-siLuc (labeled with FAM) showed enhanced cell membrane permeability in HeLa cells, as predicted by the properties of palmitic acid.

RNAi Efficacy of C16-siRNA Targeted to VEGF Gene. We investigated the RNAi efficacy of siRNA including palmitic acid conjugates against the VEGF gene in either the presence or absence of LF2000 in HeLa cells, in which the gene was activated constantly. VEGF plays a critical role in angiogenesis and has been linked to tumor growth and metastasis.[31−33] Three types of siRNAs, including palmitic acid conjugations, were designed (Table 2). The first consisted of normal 21-nt RNA sequences (siVEGF), the second consisted of 19-nt RNA at the central duplex plus 2-nt DNA at a dangling end at the both 3′-terminus (siVEGF-dT), and the third was a 19-nt RNA at the central duplex plus 2-nt DNA at a dangling end with idT at the both 3′-terminus (siVEGF-idT). The idT molecule is resistant to nuclease degradation, especially for a 3′-exonuclease.[34] The palmitic acid was conjugated to these three siVEGF molecules—named C16-siVEGF, C16-siVEGF-dT, and C16-siVEGF-idT—at the 5′-end of the sense strand via an aminolinker using the same procedure as described above. We also synthesized FAM-labeled siVEGFs including palmitic acid conjugations in order to observe cellular uptake.

We investigated the RNAi efficacy of siVEGFs and C16-siVEGFs in the presence of LF2000 using a HeLa cells. The resultant VEGF mRNA expression was analyzed by RT-PCR assay after 48 h incubation. Although the VEGF mRNA content was reduced to half of the control value by the RNAi of siVEGF, the effect of gene silencing using the C16-siVEGF was

Table 2. RNA Sequence and Molecular-Weight Confirmation of siVEGFs and C16-siVEGFs

name	RNA sequence[a]	TOF MS[b] found/calcd
siVEGF	5′-UCCUACAGCACAACAAAUGUG-3′	6662.8/6663.1
	3′-GAAGGAUGUCGUGUUGUUUAC-5′	6730.5/6731.1
C16-siVEGF	$CH_3(CH_2)_{14}CONH(CH_2)_6$UCCUACAGCACAACAAAUGUG-3′	7080.3/7080.3
	3′-GAAGGAUGUCGUGUUGUUUAC-5′	6730.5/6731.1
siVEGF-dT	5′- UCCUACAGCACAACA AAUGTT-3′	6621.0/6620.1
	3′-TTAGGAUGUCGUGUUGUUUAC-5′	6663.4/6665.0
C16-siVEGF-dT	$CH_3(CH_2)_{14}CONH(CH_2)_6$UCCUAC AGCACAACAAAUGTT-3′	7038.8/7037.3
	3′-TTAGGAUGUCGUGUUGUUUAC-5′	6663.4/6665.0
siVEGF-idT	5′-UCCUACAGCACAACAAAUGTidT-3′	6619.7/6620.1
	3′-idTTAGGAUGUCGUGUUGUUUAC-5′	6664.3/6665.0
C16-siVEGF-idT	$CH_3(CH_2)_{14}CONH(CH_2)_6$UCCUACAG CACAACAAAUGTidT-3′	7037.6/7037.3
	3′-idTTAGGAUGUCGUGUUGUUUAC-5′	6664.3/6665.0

[a]A, G, C, U: bases in RNA. TT: thymidine bases in DNA. idT: inverted thymidine. [b]MALDI-TOF MS carried out in linear-negative mode using a saturated solution of 2,4,6-trihydroxyacetophenone in 50 mg/mL diammonium hydrogen citrate in 50% acetonitrile as a matrix.

intensified to a much greater extent (by less than 30%) than the siVEGF value at a concentration of 50 nM after incubation for 48 h (Figure 6A). The C16-siVEGF-dT and the C16-siVEGF-idT also exhibited strong gene-silencing efficacy the same as C16-siVEGF after 48 h incubation under the same conditions. Interestingly, the RNAi efficacy of the C16-siVEGF-idT potently suppressed VEGF gene expression after 120 h incubation, showing the same RNAi efficacy when it was incubated for 48 h, even though the other siVEGFs that included palmitic acid conjugations showed extremely reduced gene-silencing potency after 120 h (Figure 6B). In the absence of LF2000, the VEGF mRNA expression was not suppressed by siVEGFs even at a concentration as high as 1 μM. In contrast, the C16-siVEGF-dT and C16-siVEGF-idT significantly reduced VEGF mRNA expression at a concentration of 1 μM after incubation for 48 h (Figure 7).

We next investigated the cellular uptake of siVEGFs and their palmitic acid conjugates, all of which were labeled with FAM at the 5′-end of the antisense strand, to HeLa cells in the presence of LF2000 using a confocal microscope (Figure 8A). The area of fluorescence from the image was also quantitatively assessed (Figure 8B). All C16-siVEGFs labeled with FAM showed enhanced membrane permeability to HeLa cells. The quantitative analysis also indicated that the cells treated with C16-siVEGFs labeled with FAM showed a greater fluorescent area than the cells treated with siVEGFs labeled with FAM. There was no remarkable difference in the membrane permeability of C16-siVEGFs affected by the 3′-terminal nucleotide structures. Therefore, the high levels of cellular uptake were attributable to the properties of palmitic acid.

DISCUSSION

Molecules resulting from the direct conjugation of lipids to siRNAs are considered to be promising candidates for enhanced potency of RNAi *in vitro* and *in vivo*. The lipids used in these conjugates, which include cholesterol, bile acids, and long-chain fatty acids, facilitate the cellular penetration of the attached siRNAs through their interaction with the lipid bilayer of the cell membrane, lipoprotein particles, lipoprotein receptors, and transmembrane proteins.[24] In addition, because the lipophilic siRNAs are highly stable against nuclease degradation, the lipoprotein particles help protect their attached siRNAs against the effects of nuclease.[23,24] However, in the literature,[23−26] the direct conjugation of lipids to siRNAs

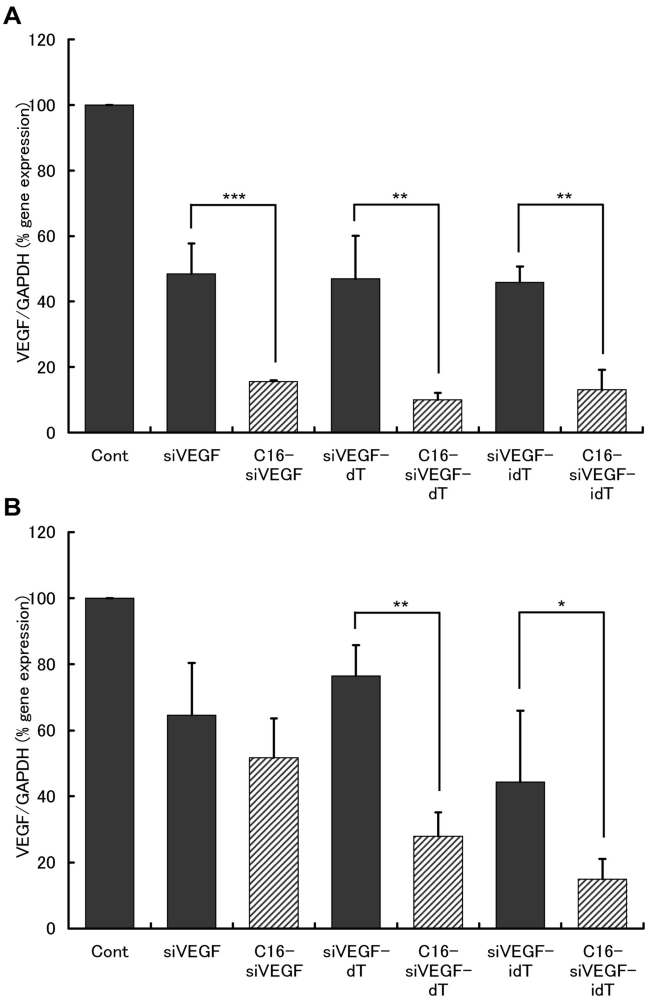

Figure 6. Short-term and long-term RNAi efficacy of siVEGFs, including palmitic acid conjugations, in the presence of LF2000. The siVEGFs used were of three structural types (siVEGF, siVEGF-dT, and siVEGF-idT) and three palmitic acid conjugations (C16-siVEGF, C16-siVEGF-dT, and C16-siVEGF-idT). RNAi efficacies of siVEGFs and C16-siVEGFs were evaluated by measuring VEGF mRNA quantitatively by RT-PCR. GAPDH mRNA was also measured as the intrinsic control. siVEGFs and C16-siVEGFs (50 nM) were transfected into HeLa cells with LF2000 and then incubated for 48 h (A) and 120 h (B). The mean and SD values are from three independent experiments (*P < 0.05, **P < 0.01, ***P < 0.001; t-test).

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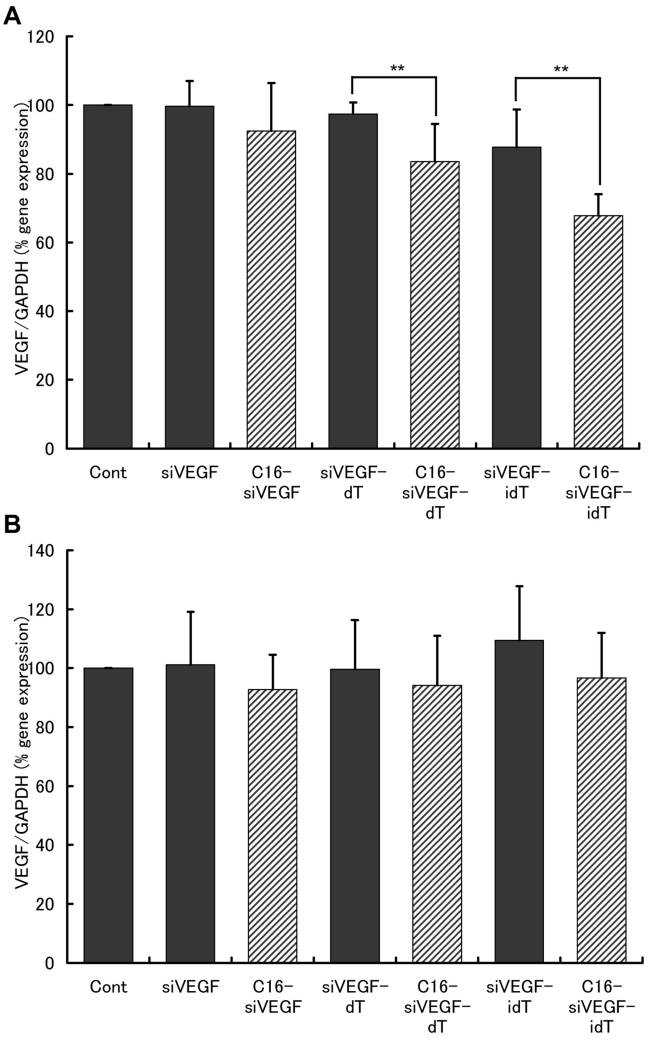

Figure 7. Short-term and long-term RNAi efficacy of siVEGFs, including palmitic acid conjugations, in the absence of LF2000. The siVEGFs and C16-siVEGFs used were of three structural types. RNAi efficacies of siVEGFs and C16-siVEGFs were evaluated by measuring VEGF mRNA quantitatively by RT-PCR. GAPDH mRNA was also measured as the intrinsic control. siVEGFs and C16-siVEGFs (1 μM) were transfected into HeLa cells and then incubated for 48 h (A) and 120 h (B). The mean and SD values are from three independent experiments (**$P < 0.01$; t-test).

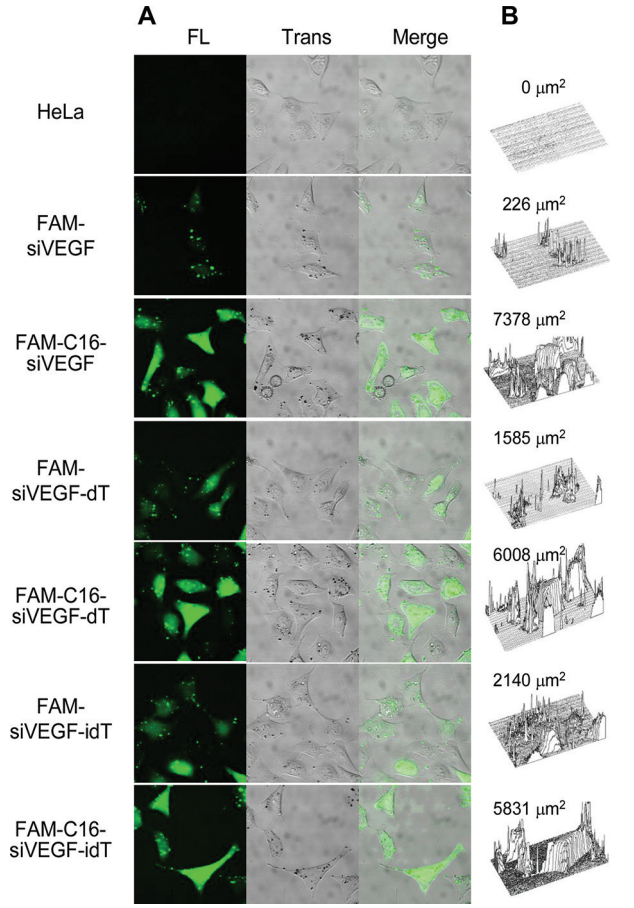

Figure 8. (A) Confocal microscopic images of HeLa cells incubated for 6 h with siVEGFs and C16-siVEGFs in the presence of LF2000. Three types of siVEGF structures were prepared: normal 21-nt RNA sequence (FAM-siVEGF), 19-nt RNA at central plus 2-nt DNA at overhang (FAM-siVEGF-dT), and 19-nt RNA at central plus 2-nt DNA at overhang including idT at the 3′-terminus (FAM-siVEGF-idT). Palmitic acid was conjugated to each siVEGF at the 5′-end of the sense strand (FAM-C16-siVEGF, FAM-C16-siVEGF-dT, FAM-C16-siVEGF-idT). FAM was conjugated to siVEGF at the 5′-end of the antisense strand. FL, fluorescent image; Trans, transmission image; Merge, merged image of FL and Trans. (B) The quantitative assessment of fluorescence images was analyzed using Scion image software to obtain area of fluorescence from the image.

was limited by the conjugating position of the siRNA, which was generally the 3′-end when using lipophile-bearing solid supports[24] or the 5′-end when using phosphoramidite derivatives.[25] Both these conjugations are expensive and involve complicated syntheses.

Our study demonstrated a simple synthesis of covalently conjugated siRNA with lipids in order to enhance the potency of RNAi. Using this synthesis strategy, many kinds of functional molecules (e.g., lipids, PEGs, peptides, and fluorescein) can be attached to any point of the siRNA sequence (e.g., the 5′-end, the 3′-end, or the midpoint). In this study, we synthesized several types of siRNAs conjugated with lipids targeted to the *Renilla* luciferase gene (C16-siLuc, C12-siLuc, Chol-siLuc, and siLuc-C16). Among these, C16-siLuc was determined to have the highest RNAi potential in HeLa cells. C16-siLuc exhibited stronger RNAi activity than Chol-siRNA not only in the presence of LF2000 but also in the absence of LF2000. The data suggested that C16-siLuc is superior to Chol-siLuc as an RNAi molecule. The C12-siLuc also exhibited enhanced RNAi, but C16-siLuc had stronger gene-silencing ability. It seems that the C12-siLuc was also able to interact with the cellular membrane, but palmitic acid, compared to lauric acid, had a more efficient hydrophobic interaction with the lipid bilayer of the cell membrane. In the conjugate of palmitic acid to siRNA, it was demonstrated that palmitic acid at the 5′-end of the sense strand yielded more efficient potency of RNAi compared to that at the 3′-end of the sense strand. Presumably, the RISC more easily recognized the 5′-end conjugations than the 3′-end conjugations in siRNA.[30] This suggested that the potent gene silencing of C16-siLuc was attributable to its potent cellular uptake (Figure 5) and to its strong resistance to nuclease degradation (Figure 1E). In the literature, several studies have reported that the termini-conjugates or modified siRNAs resulted in decreased RNAi potency.[12,35,36] The siRNAs conjugated with peptide at one terminus,[22] or with amine or

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inverted deoxy abasic modifications at all four termini,[12] reduced the RNAi potency for luciferase activity in the presence of LF2000. Our siRNAs conjugated with lipids, particularly C16-siRNA, showed enhanced RNAi potency. We also performed a study of the RNAi potency against a stably expressed luciferase gene using siGL3 and C16-siGL3 in either the presence or absence of LF2000 in 44As3Luc cells to clearly establish the RNAi efficacy of C16-siRNA while avoiding the effect of the residual LF2000 after the washing of the cells. The RNAi potency was found to show the same tendency as the RNAi against the transiently expressed luciferase gene (see Table S1 and Figure S3 in the Supporting Information).

For further improvements, we evaluated the RNAi of C16-siRNAs against the endogenous VEGF gene, because the exogenous gene was much more highly expressed than the endogenous gene. The results of this evaluation showed that the gene-silencing efficacy of siRNA against the exogenous luciferase gene was more potent than that against the endogenous gene. Although the finding that the siRNA silenced the exogenous gene was important, an investigation of the gene-silencing efficacy against the endogenous gene was essential in order to evaluate the applicability of siRNA molecules as therapeutic tools. Accordingly, we investigated the RNAi efficacy of C16-siRNA against the endogenous VEGF gene in HeLa cells. In this study, three types of siRNA molecules were prepared: siVEGF, siVEGF-dT, and siVEGF-idT. These were conjugated with palmitic acid at the 5′-ends of sense strands (C16-siVEGF, C16-siVEGF-dT, and C16-siVEGF-idT). All C16-siVEGFs exhibited strong gene silencing compared with the respective siVEGFs not only after 48 h transfection but also after 120 h transfection in the presence of LF2000. It is noteworthy that C16-siVEGF-idT exhibited strong long-term RNAi efficacy for up to 5 days, at a level that was almost the same as that after 48 h incubation, even though the other siVEGFs including palmitic acid conjugations showed extremely reduced gene-silencing potency after 5 days (Figure 8B). In the absence of LF2000, C16-siVEGF-dT and C16-siVEGF-idT exhibited the suppression of VEGF mRNA gene expression after 48 h of incubation. In particular, C16-siVEGF-idT was remarkably effective for gene silencing in the absence of LF2000. Although the strong RNAi effects of C16-siRNAs in the absence of LF2000 were of interest, the RNAi effects were insufficient and high concentrations of C16-siRNAs were required. We also checked the membrane permeability of C16-siRNA in the absence of any transfection reagent, and the cells treated with FAM-C16-siRNA showed very weak fluorescence signals when the conjugate was administered at high concentration (see Figure S4 in the Supporting Information). These insufficient RNAi effects of C16-siRNAs in the absence of any transfection reagent are one of the problems that remain to be solved before they can be applied in the clinic.

The C16-siRNAs, both C16-siLuc and C16-siVEGF, enhanced cell membrane permeability in HeLa cells in the presence of LF2000. This efficient membrane permeability of C16-siRNAs is attributable to its satisfactory interaction with both the cellular membrane and palmitic acid, as described above. In addition, the membrane permeabilities of the C16-siRNAs were not influenced by the 3′-terminal nucleotide structures such as dT and idT. Several approaches have been used for the efficient delivery of siRNAs for the eventual use of cells, including lipidlike delivery materials,[37] peptide-mediated carriers,[38,39] protein-mediated carriers,[40] polyethylenimine (PEI)-based carriers,[41] and atelocollagen-mediated carriers.[32,42] Some of these were used for *in vivo* application. However, these siRNA delivery materials have also been associated with various problems, such as insufficient delivery, cytotoxicity, and high cost.

In conclusion, the covalent conjugation of palmitic acid to siRNA at the 5′-end of the sense strand using our synthesis procedure can open the way to the development of a new generation of modified siRNAs. By facilitating intracellular delivery of siRNA, preserving RNAi activity, and ensuring long-term gene silencing, C16-siRNA-idT and the other C16-siRNAs examined in this study could solve many of the problems associated with RNAi technology. In addition, the C16-siRNAs presented here might be suitable for *in vivo* application with low toxicity and at low cost.

■ ASSOCIATED CONTENT

Supporting Information

Table S1 displaying characterizations of ssGL3 conjugated with C16. Figures S1−S4. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Laboratory of Molecular Cell Biology, Department of Life Sciences, Yasuda Women's University Faculty of Pharmacy, 6-13-1 Yasuhigashi, Asaminami-ku, Hiroshima 731-0153, Japan. Tel: +81-82-878-9473. Fax: +81-82-878-9540. E-mail: kubo-t@yasuda-u.ac.jp.

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