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RESEARCH ARTICLE

Development of a miR-92a delivery system for anti-angiogenesis-based cancer therapy

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Abstract

Background RNA interference has received much attention as a novel therapeutic strategy. MicroRNA (miRNA) appears to be promising as a novel nucleic-acid medicine because it is able to suppress a series of protein expression that relates to a specific event such as angiogenesis. In the present study, we used dicetyl phosphate-tetraethylenepentamine-based polycation liposomes (TEPA-PCL) as a delivery system for miR-92a, one of the miRNAs regulating angiogenesis, and attempted to deliver miR-92a to angiogenic endothelial cells for the development of cancer therapy by anti-angiogenesis.

Methods Cholesterol-grafted miR-92a (miR-92a-C) was bound to TEPA-PCL, and the ratio of nitrogen of TEPA-PCL to phosphorus of miR-92a-C (N/P ratio) was optimized. This complex was transfected into human umbilical vein endothelial cells (HUVECs), and the intracellular localization of miR-92a-C was observed under a confocal laser-scanning microscope by the use of fluorescein isothiocyanate-labeled miR-92a-C. After transfection of HUVECs with miR-92a-C/TEPA-PCL, the expression of miR-92a-target proteins (e.g. integrin α5, mitogen-activated protein kinase kinase 4, sphingosine-1-phosphate receptor 1) was examined by western blotting, and a tube formation assay was performed.

Results The complex of miR-92a-C with TEPA-PCL was formed and miR-92a-C remained stable with TEPA-PCL at the N/P ratio of 10. After transfection of HUVECs with miR-92a-C complex, miR-92a-C spread into the whole cytoplasm of the cells without any change of cellular morphology, and the expression of several proteins encoded by miR-92a-target genes was suppressed. Furthermore, the capability of forming capillary tubes was impaired in complex-treated HUVECs.

Conclusions We have developed a miR-92a delivery system into angiogenic endothelial cells by the use of TEPA-PCL. These results suggest that miR-92a-C/TEPA-PCL is promising for the treatment of tumors via the suppression of angiogenesis. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords angiogenesis; cancer therapy; microRNA; miR-92a; polycation liposome

Introduction

MicroRNAs (miRNAs) are noncoding double-stranded RNAs composed of 16–29 base strands and are involved in endogenous gene silencing. miRNA possesses a sequence complementary to a series of several mRNAs related to important cellular events such as growth and angiogenesis, and it induces knockdown or reduced translation of the target mRNAs [1,2]. miRNAs are

conjugated with RNA-induced silencing complex (RISC), which is a critical protein complex causing RNA interference in the cytosol. Then, this miRNA/RISC conjugate attaches to the 3′-untranslated region of target mRNAs, with the consequence being suppressed protein expression of the target genes by miRNA-induced cleavage or translational repression. Therefore, miRNAs inhibit the protein expression of various target mRNAs collectively, as well as signaling pathways related to these proteins. Recently, the mechanism of action and effect of several miRNAs have been reported, and investigations of miRNAs have been progressing in many fields, especially in fields related to cancer cell progression and its signal transduction [3]. It has been suggested that ras, one of the oncogenes, and bcl-2, a gene related to apoptosis, are controlled by miRNAs [4,5]. Furthermore, recent studies have shown that miRNAs mediate angiogenesis and metastasis by regulating the expression of target genes [6–12]. Therefore, miRNAs have received much attention as a novel therapeutic tool for the treatment of cancer.

In the present study, we have developed a miRNA delivery system for treating cancer by using miR-92a, one of the miRNAs involved in angiogenesis. miR-92a is encoded in the miR-17-92 cluster located in c13orf25 and is composed of two distinct active strands (i.e. miR-92a-1-5p and miR-92a-3p) [13,14]. Furthermore, a recent study indicated that miR-92a suppresses the expression of several proangiogenic proteins, including integrin subunit α5, mitogen-activated protein kinase kinase 4 (MKK4) and sphingosine-1-phosphate receptor 1 (S1P1), and also controls angiogenesis by inhibiting the growth of vascular endothelial cells [15]. These studies suggest that miR-92a could be a valuable therapeutic tool for inhibiting growth of tumor cells through its anti-angiogenesis action.

For the delivery of miRNAs to the desired target site after systemic injection, a delivery system is essential because free miRNAs are unstable in the bloodstream and do not easily permeate cells. We previously developed dicetyl phosphate-tetraethylenepentamine-based polycation liposomes (TEPA-PCL) as a novel nonviral carrier of small interfering RNA (siRNA) [16]. TEPA-PCL is composed of a lipid mixture based on dicetyl phosphate-tetraethylenepentamine (DCP-TEPA) as a polycation lipid derivative and possesses the advantages of both liposomes and polycations for nucleic acid delivery. Therefore, TEPA-PCL has a high transfection efficiency through its proton-sponge effect. We have previously shown that siRNA carried by TEPA-PCL is efficiently taken up into cells and produces a protein-knockdown effect on target genes of siRNAs [17,18]. We also observed that cholesterol-grafted siRNA (siRNA-C) is quite stable in the presence of serum after forming a complex with TEPA-PCL. Moreover, the TEPA-PCL/siRNA-C complex can be decorated with distearoylphosphatidylethanolamine (DSPE)-polyethylene glycol (PEG) conjugate for passive targeting or with ligand-grafted DSPE-PEG for active targeting of the complex [17,19].

In the present study, we prepared the complex of miR-92a with TEPA-PCL and examined the effect of miR-92a delivered to human umbilical vein endothelial cells (HUVECs) with TEPA-PCL as a miRNA-delivery carrier. Furthermore, we also examined the anti-angiogenic effect of miR-92a by monitoring the knockdown of miR-92a-target proteins, including integrin α5, MKK4 and S1P1.

Materials and methods

Preparation of miR-92a-C/TEPA-PCL

Cholesterol-conjugated miR-92a (miR-92a-C) and cholesterol-conjugated control miRNA (miCont-C) were purchased from Hokkaido System Science Co. (Hokkaido, Japan). miR-92a was composed of miR-92a-1-5p and miR-92a-3p (miR-92a-1-5p: 5′-AGGUUGGGAUCGGUUGCAAUGCU-3′; miR-92a-3p: 5′-UAUUGCACUUGUCCCGGCCUGU-3′). DCP-TEPA was synthesized as described previously [16]. Dipalmitoylphosphatidylcholine (DPPC) was a gift from Nippon Fine Chemical Co. (Hyogo, Japan). Cholesterol was purchased from Sigma-Aldrich (St Louis, MO, USA). Dioleoylphosphatidylethanolamine (DOPE) was purchased from NOF Co. (Tokyo, Japan).

For preparation of TEPA-PCL, DPPC, DOPE, cholesterol and DCP-TEPA (3:4:4:1 as a molar ratio) were dissolved in *t*-butyl alcohol and lyophilized. TEPA-PCL was produced by hydration of the lipid mixture with RNase-free water, namely, diethylpyrocarbonate (DEPC)-treated water. These liposomes were sized by extruding them 20 times through a polycarbonate membrane filter with a pore size of 100 nm (Nuclepore, Maidstone, UK). TEPA-PCL and miR-92a-C were mixed in DEPC-treated water and incubated for 20 min at room temperature to form a complex of miR-92a-C/TEPA-PCL. The size and ζ-potential of the liposomes were measured by using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK)

Evaluation of N/P ratio

The ratio of the nitrogen moiety of TEPA-PCL to the phosphorus of miR-92a (N/P ratio) was varied from 1 to 20 equivalents aiming to examine the formulation of miR-92a-C/TEPA-PCL. The complex at each N/P ratio was diluted with TBE-Urea Sample Buffer (Invitrogen, Carlsbad, CA, USA) and subjected to 6% polyacrylamide gel containing 8 M urea. Free miR-92a-C was separated from complexes by electrophoresis and stained with GelRed (Biotium, Hayward, CA, USA) by a 30-min incubation with shaking, and fluorescence was detected by exposure to ultraviloet light using the LAS-3000 mini system (Fuji Film, Tokyo, Japan).

Cytotoxic assay

HUVECs (Lonza, Walkersville, MD, USA) were cultured in endothelial cell growth medium-2 (EGM-2, Lonza) at 37 °C in a humidified atmosphere of 5% CO_2 in air. The cells were seeded onto 96-well plates (Becton-Dickinson,

Franklin Lakes, NJ, USA) at a density of 5.0×10^3 cells and incubated with TEPA-PCL at the same concentration of 100 nM miR-92a-C/TEPA-PCL, or 100 nM miCont-C/TEPA-PCL for 48 h. After having washed the cells with phosphate-buffered saline (PBS) three times, a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan), diluted ten-fold with EGM-2 in accordance with the manufacturer's instructions, was added to each well (100 μl/well). After 1 h of incubation at 37 °C, absorbance was measured with a Tecan Infinite M200 micro plate reader (Tecan, Salzburg, Austria) at a test wavelength of 450 nm and a reference wavelength of 630 nm.

Transfection of HUVECs with miR-92a

HUVECs were seeded onto culture plates and pre-incubated for 24 h. The culture medium was changed to fresh medium without antibiotics, and the cells were incubated with miR-92a-C complexes until the selected period of time, as described for each experimental procedure.

Confocal microscopy

HUVECs seeded onto eight-well chamber slides (Nunc, Rochester, NY, USA) at a density of 1.0×10^4 cells/well were transfected with fluorescein isothiocyanate-labeled miR-92a-C (FITC-miR-92a-C), which was complexed with TEPA-PCL, or mixed with Lipofectamine 2000 (LFA2K; Invitrogen, Rockville, MD, USA) as a control vector (FITC-miR-92a-C concentration was 30 pmol/0.3 ml/well). In the LFA2K group, culturing medium was changed to fresh EGM-2 at 4 h after transfection in accordance with the manufacturer's instructions, whereas the culturing media in the other groups were not changed. Six, 12 or 24 h after transfection with each sample, the cells were washed three times with PBS (pH 7.4) containing heparin (5 units/ml) and three times alone, and fixed with 4% paraformaldehyde. Nuclei were stained with 4′,6-diamino-2-phenylindole (DAPI; 10 μg/ml; Invitrogen) in PBS containing 3% bovine serum albumin (BSA) and 0.1% saponin (Sigma-Aldrich). Next, localization of FITC-labeled miR-92a-C in HUVECs was observed under an LSM510 META confocal laser-scanning microscope (Carl Zeiss, Jena, Germany).

Cellular uptake of miR-92a-C

HUVECs were seeded onto 24-well plates at a density of 2×10^4 cells and transfected with naked FITC-miR-92a-C, FITC-miR-92a-C complexed with TEPA-PCL, or FITC-miR-92a-C mixed with LFA2K at a FITC-miR-92a concentration of 50 pmol/0.5 ml/well, respectively. In the LFA2K group, culturing medium was changed to fresh EGM-2 at 4 h after transfection, whereas the culturing media in the other groups were not changed. Six, 12 or 24 h after transfection, the cells were washed with PBS containing heparin (5 units/ml) three times and PBS only three times, and the cells were lysed by 1% octylglucoside (Dojindo) containing the protease inhibitors aprotinin (50 μg/ml), leupeptin (200 μM), phenylmethylsulfonyl fluoride (PMSF) (2 mM) and pepstatin A (100 μM). The fluorescence intensity of FITC-miR-92a-C in cell lysates was detected and normalized by the total protein of the cells.

Western blotting

Anti-integrin α5 rabbit polyclonal antibody (Upstate Biotechnology, Lake Placid, NY, USA), anti-MKK4 rabbit polyclonal antibody (Cell Signaling, Beverly, MA, USA), anti-S1P1 rabbit polyclonal antibody (Abcam, Cambridge, UK) and anti-β-actin rabbit polyclonal antibody (Sigma-Aldrich) were used for western blotting, diluted in accordamce with the manufacturer's instructions.

HUVECs (1.0×10^5 cells/60-mm dish) were transfected with TEPA-PCL, miR-92a-C/TEPA-PCL, miCont-C/TEPA-PCL as a negative control, or miR-92a-C/LFA2K as a positive control (each miRNA at a concentration of 300 pmol/3.0 ml/dish). In the transfection with miR-92a-C/LFA2K, culturing medium was changed to fresh medium at 4 h after transfection. Forty-eight hours after transfection in each group, the cell extracts were prepared with NDET buffer [composed of 10 mg/ml Nonidet P-40, 4 mg/ml deoxycholic acid sodium salt, 2.5 mg/ml ethylenediaminetetraacetic acid, 50 mM Tris-HCl (pH 7.4), 50 μg/ml aprotinin, 200 μM leupeptin, 2 mM PMSF and 100 μM pepstatin A]. Total protein concentrations were measured using a BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL, USA). Cell extracts were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, separated by electrophoresis, and transferred electrophoretically to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). After having been blocked for 1 h at room temperature with 3% BSA in Tris-HCl-buffered saline containing 0.1% Tween 20 (pH 7.4), the membrane was incubated with a primary antibody (against integrin α5, MKK4, S1P1, or β-actin) overnight at 4 °C. Next, it was incubated with a horseradish peroxidase-conjugated secondary antibody at a dilution of 1:5000 for 1 h at room temperature. Each sample was developed with the use of a chemiluminescent substrate (ECL; GE Healthcare, Little Chalfont, UK), and each protein was detected with the LAS-3000 mini system.

Tube formation assay

Matrigel (BD Biosciences, Bedford, MA, USA) was diluted to 4 mg/ml with EGM-2 without antibiotics, added to each well of a 24-well plate (0.2 ml/well), and allowed to undergo polymerization by a 30-min incubation at 37 °C. HUVECs (1.0×10^5 cells/60-mm dish) were transfected with TEPA-PCL, miR-92a-C/TEPA-PCL at a concentration of 100 or 200 nM, or miCont-C/TEPA-PCL at 37 °C for 48 h (culturing medium in the miR-92a-C/LFA2K

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group was changed to fresh medium at 4 h after transfection). Then, the transfected cells were seeded onto the Matrigel-coated wells (2.0×10^4 cells/well). Cells were incubated at 37 °C for 3.5 h to form capillary tubes, and observed under a microscope (IX71; Olympus, Tokyo, Japan).

Statistical analysis

Differences within a group were evaluated by analysis of variance with Tukey's post-hoc test

Results

Characteristics of miR-92a-C/TEPA-PCL

The particle size and ζ-potential of TEPA-PCL and miR-92a-C/TEPA-PCL complex are shown in Table 1. TEPA-PCL and miR-92a-C/TEPA-PCL had a similar positive charge, although the size of the complex was larger than that of the TEPA-PCL. To assess the stability of miR-92a-C/TEPA-PCL, we performed electrophoresis in 6% polyacrylamide gels. As a result, although free miR-92a-C was detected at an N/P ratio of 1, it was not detected at an N/P ratio of 5, 10, 15 or 20 (Figure 1A). This result suggests that miR-92a-C was released from miR-92a-C/TEPA-PCL complexes with an N/P ratio of 1 but remained stable in the complex when the ratio was 5 or more.

Furthermore, the particle size and ζ-potential of complexes at various N/P ratios did not change significantly above an N/P ratio of 5 (Figure 1B). Especially, the complex at the N/P ratio of 10 was positive-charged stably (approximately 45 mV) among the ratios tested. Therefore, the N/P ratio of 10 was used to form miR-92a-C/TEPA-PCL complexes in subsequent experiments.

The cytotoxicity of TEPA-PCL or miCont-C/TEPA-PCL (100 nM as miCont-C) to HUVECs was determined after 48 h of incubation. As a result, both TEPA-PCL and miCont-C/TEPA-PCL did not show any cytotoxic action.

Table 1. The particle size of TEPA-PCL and miR-92a-C/TEPA-PCL at the N/P ratio of 10, as well as the ζ-potential, were measured using the Zetasizer Nano ZS

	Diameter (nm)	ζ-Potential (mV)
TEPA-PCL	136 ± 13 (PDI: 0.124)	50 ± 4
miR-92a-C/TEPA-PCL	161 ± 10 (PDI: 0.193)	48 ± 13

Data are the mean ± SD.

Uptake of miR-92a-C into HUVECs after transfection with TEPA-PCL

We next examined the intracellular delivery of miR-92a-C into HUVECs by the use of FITC-labeled miR-92a-C. HUVECs were transfected with naked FITC-miR-92a-C, FITC-miR-92a-C/TEPA-PCL or FITC-miR-92a-C mixed with LFA2K in accordance with the manufacturer's instructions. In the miR-92a-C/TEPA-PCL group, miR-92a-C was accumulated in the cells at 6 h after transfection and increased with time up to 12 h, whereas naked miR-92a-C was not taken into the cells even if they had been incubated for 24 h (Figure 2A). In addition, miR-92a-C was taken up into the cells much more by the use of TEPA-PCL than that of LFA2K at each time of incubation. In addition, the intracellular distribution of FITC-miR-92a-C was observed by confocal laser-scanning microscopy. Figure 2B shows the results after a 12-h incubation, although those obtained after a 6- or 24-h incubation were similar. As a result, miR-92a-C carried by TEPA-PCL was observed in the cytoplasm of the cells at 6, 12 and 24 h after transfection, as well as miR-92a-C transfected by the use of LFA2K. The cellular morphology did not change during incubation, except in the case of LFA2K, where degraded cells were observed. This result suggests that TEPA-PCL carried miR-92a-C into the cytoplasm efficiently, with the possibility of a protein-knockdown effect on miR-92a-target genes by RNA interference.

Effect of miR-92a-C transfection with TEPA-PCL on HUVECs

The effect of miR-92a-C/TEPA-PCL transfection on HUVECs was examined by assessing target gene expression and capillary tube formation. Knockdown of miR-92a-target

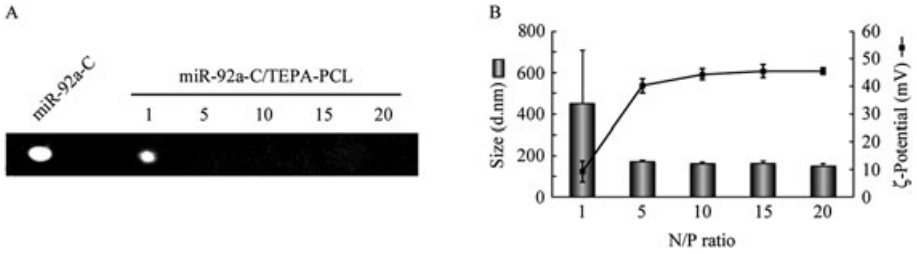

Figure 1. Optimization of the miR-92a-C/TEPA-PCL complex. Cholesterol-grafted miR-92a was mixed with TEPA-PCL for 20 min to form complexes at various N/P ratios. (A) Each complex was subjected to a polyacrylamide gel and examined by electrophoresis. Free miR-92a-C was stained with GelRed. (B) Both the size of complexes at each N/P ratio and the ζ-potential of those were measured using the Zetasizer Nano ZS. The bar and line show the size of particles and the ζ-potential of those, respectively. Data are presented with SD bars ($n = 4$). The means of the polydispersity index (PDI) at each N/P ratio are indicated: 0.367 at the N/P ratio of 1; 0.100 at the N/P ratio of 5; 0.034 at the N/P ratio of 10; 0.078 at the N/P ratio of 15; and 0.075 at the N/P ratio of 20.

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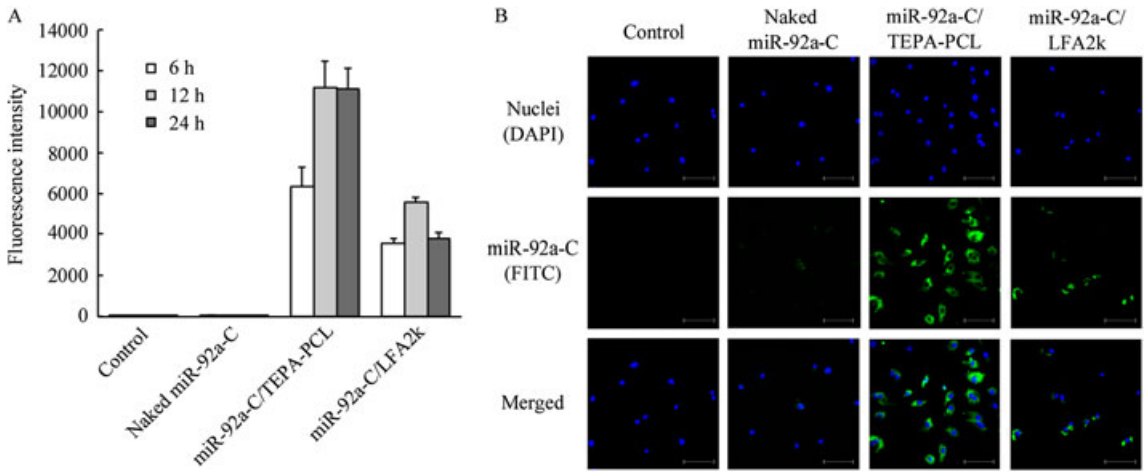

Figure 2. Evaluation of uptake efficiency by transfection of HUVECs with miR-92a-C/TEPA-PCL. (A) HUVECs were transfected with naked FITC-miR-92a-C, FITC-miR-92a-C/TEPA-PCL or FITC-miR-92a-C/LFA2K at a FITC-miR-92a-C concentration of 100 nM. Six, 12 or 24 h after transfection, the cells were washed with PBS containing heparin and PBS alone, and lysed by 1% octylglucoside solution containing protease inhibitors. Then, the fluorescence intensity of FITC-miR-92a-C dissolved in each cell lysate was detected. Data are represented as the fluorescence intensity of FITC (excitation: 485 nm, emission: 525 nm) with SD bars ($n = 4$). (B) HUVECs were transfected with naked FITC-miR-92a-C, FITC-miR-92a-C/TEPA-PCL or FITC-miR-92a-C/LFA2K at a FITC-miR-92a-C concentration of 100 nM. Twelve hours after transfection, the cells were washed with heparin/PBS and PBS alone and fixed with 4% paraformaldehyde. Then, the nuclei were stained with DAPI. The fluorescence intensity of FITC-miR-92a-C taken up into the cells was observed under a confocal laser-scanning microscope. Colors are indicated as green for miR-92a-C and blue for nuclei. Scale bars = 100 μm.

genes was determined by western blotting at 24 and 48 h after transfection with miR-92a-C/TEPA-PCL. As shown in Figure 3A, protein expression of the three target genes examined (i.e. integrin α5, MKK4 and S1P1) was changed little by 24 h of transfection. However, by 48 h post transfection, miR-92a-C complexed with TEPA-PCL suppressed the expression of all of them (Figure 3B). By contrast, the transfection of miCont-C, nonspecific miRNA, by the use of TEPA-PCL did not change the expression of integrin α5, and this expression was repressed by transfection with miR-92a-C/LFA2K (Figure 3C).

For assessment of the anti-angiogenic effect of miR-92a-C/TEPA-PCL, HUVECs incubated with miR-92a-C/TEPA-PCL complexes were seeded onto Matrigel-coated wells, and the ability of the cells to form capillary tubes was examined (Figure 4). In the control and TEPA-PCL groups, a network formed by many tubes was observed. After transfection of the cells with miR-92a-C/TEPA-PCL at a final concentration of 100 nM, tube formation was also observed, and the tube length was not changed compared to that in the control and TEPA-PCL groups. On the other hand, when the concentration was doubled to 200 nM, the capability to form tubes was decreased to a statistically significant extent. Furthermore, the tubes formed by HUVECs transfected with miCont-C/TEPA-PCL at a concentration of 200 nM were not changed compared to those in the control and TEPA-PCL groups. These results demonstrate the expected anti-angiogenic effect of the delivery of miR-92a into anigiogenic-endothelial cells.

Discussion

Recently, nucleic acid medicines have been considered as novel therapeutic drugs for treating intractable and genetic diseases. However, before their clinical use, several problems remain to be solved. For example, nucleic acids are unstable in the bloodstream because of their degradation, and they have a poor efficiency of uptake into target cells. To overcome these problems, we previously developed TEPA-PCL for the delivery of siRNA after systemic

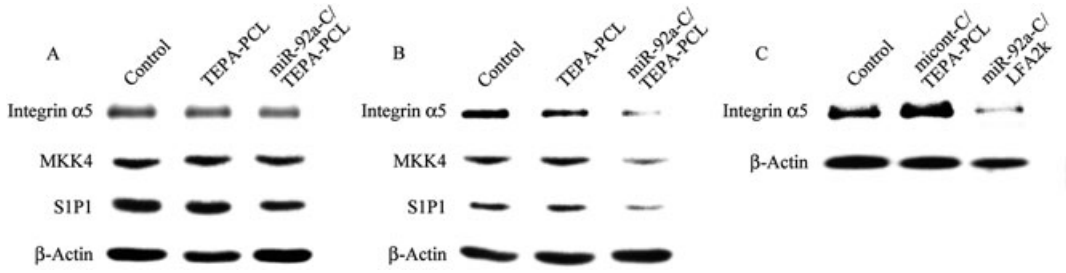

Figure 3. Protein-knockdown effect by transfection of HUVECs with miR-92a-C/TEPA-PCL complexes. HUVECs were incubated with TEPA-PCL or miR-92a-C/TEPA-PCL (miR-92a-C concentration of 100 nM) for 24 h (A) or 48 h (B). The cells were lysed by NDET solution, and the expression of miR-92a-target proteins including integrin α5, MKK4, and S1P1 was examined by western blotting. (C) HUVECs transfected with miCont-C/TEPA-PCL or miR-92a-C/LFA2K at each miRNA concentration of 100 nM. The cells were washed and lysed by NDET buffer. Then, one of miR-92a-target proteins, integrin α5, was examined by western blotting.

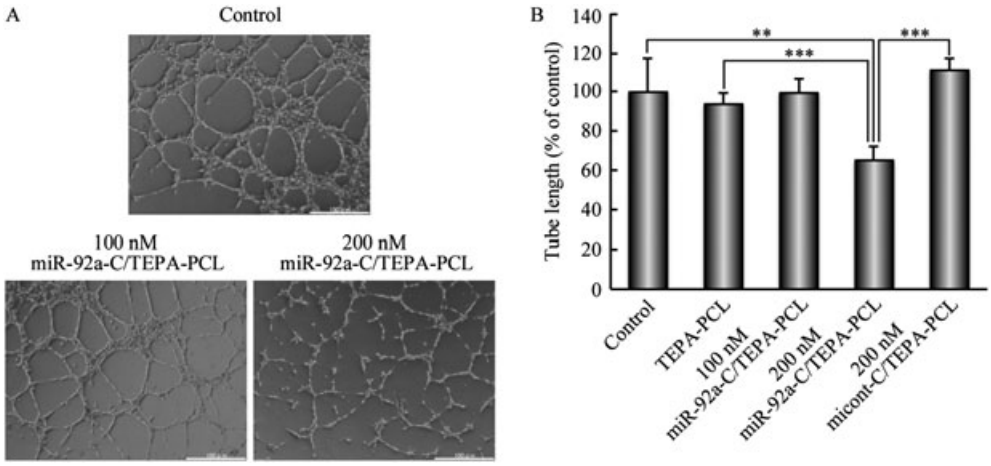

Figure 4. Tube formation assay after transfection of HUVECs with miR-92a-C/TEPA-PCL. HUVECs seeded on 60-mm dishes were incubated with TEPA-PCL, miR-92a-C/TEPA-PCL at a miR-92a-C concentration of 100 or 200 nM, or miCont-C/TEPA-PCL at a miCont-C concentration of 200 nM for 48 h. The transfected cells were collected and seeded on Matrigel-coated wells to form a tube network by incubation for 3.5 h at 37 °C. (A) Photomicrographs show the tube network formed by HUVECs transfected with miR-92a-C/TEPA-PCL at each concentration. Scale bars = 100 μm. (B) The length of tubes was calculated using Multi Gauge software (Fujifilm, Tokyo, Japan). Data are presented as the percentage of tube length versus control with SD bars (n = 4–5). Asterisks indicate a significant difference ($^{**}p < 0.01$, $^{***}p < 0.001$).

injection [16]. The stability of this siRNA bound to TEPA-PCL was achieved by grafting cholesterol to the 3′-end of the sense strand. After forming a complex with TEPA-PCL, siRNA-C does not dissociate from the complex and is not degraded in the presence of serum. TEPA-PCL/siRNA-C complexes may be readily decorated with DSPC-PEG for the purpose of endowing them with the characteristic of long circulation. Moreover, the decoration of the complex with PEG-lipid having a targeting probe attached to the end of the PEG chain enables the delivery of the complex to the desired target cells. When TEPA-PCL/siRNA-C complex is decorated with DSPE-PEG conjugated to the APRPG peptide, which is known to bind to the vascular endothelial growth factor (VEGF) receptor [17], or to an RGD-related peptide, a ligand for integrin αvβ3, these complexes accumulate at the tumor site after systemic injection [16,19]. Additionally, TEPA-PCL efficiently releases siRNA-C into the cytosol after endocytosis of the complex into the target cells [16]. In the present study, which aimed to develop an miRNA delivery system for the treatment of various diseases, we formed a complex of TEPA-PCL with cholesterol-grafted miRNA.

miRNAs have many advantages as a nucleic acid medicine. One such advantage is that miRNAs have high biocompatibility because miRNAs are noncoding RNAs existent in living organisms and regulate various cellular events. Moreover, miRNAs are frequently reported to be useful as a biomarker for diagnosing several diseases [20,21]. These studies suggest that miRNAs have the characteristic of being stable in the bloodstream, although this mechanism remains unknown. The most important characteristic of miRNAs is their ability to repress the translation of various target genes because miRNAs have a partial-complementary sequence against many types of mRNAs related to cellular events such as angiogenesis and cell growth [3,22]. Therefore, miRNAs can suppress certain types of intracellular signaling by inhibiting the expression of proteins involved in such signaling. They also have the potential to be used for the treatment of intractable diseases when an appropriate miRNA is efficiently delivered into the target cells.

miR-92a is reportedly down-regulated in acute leukemia and overexpressed in colorectal cancer in its early phase [23,24]. Additionally, recent research has shown that forced overexpression of miR-92a in endothelial cells blocks angiogenesis both *in vitro* and *in vivo* [15]. Furthermore, transfection of Lipofectamine with miR-92a suppresses the expression of several mRNAs related to angiogenesis (e.g. S1P1, eNOS, MKK4, SIRT1, integrin αv and integrin α5) [15]. Therefore, we hypothesized that the delivery of miR-92a into angiogenic endothelial cells by using polycation liposomes would have an anti-angiogenic effect by translational repression of target mRNAs, thereby suppressing tumor growth by cutting off the oxygen and nutrient supply to the tumor cells.

When the complex of miR-92a and TEPA-PCL was formed, cholesterol was modified on the 3′-terminal end of miR-92a-1-5p, which does not have a sequence complementary to that of the target mRNAs presently examined. Modification by cholesterol improves the ability of miR-92a to bind to TEPA-PCL not only by electrostatic interaction but also by hydrophobic interaction, as in the case of siRNAs [16]. The miR-92a-C/TEPA-PCL complex was optimized, as judged from the results of electrophoresis, and the N/P ratio of 10 was selected for transfection of the miR-92a-C into cells as a result of the stability of miR-92a-C in the complex and the ζ-potential of the complex. The uptake of miR-92a-C/TEPA-PCL into HUVECs was determined by the fluorescence intensity of FITC-miR-92a-C taken into the cells and observed by performing confocal laser-scanning microscopy. As a result, even 6 h after transfection, miR-92a-C was accumulated and observed throughout the cytoplasm when formulated with TEPA-PCL, although miR-92a-C transfected by the use of

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LFA2K was lower than that of TEPA-PCL and taken up only into a localized area of the cytoplasm. These results suggest that TEPA-PCL was more useful for delivering miR-92a into the cytoplasm than LFA2K. Moreover, the TEPA-PCL formulation was less cytotoxic compared to LFA2K. Because miRNAs produce a knockdown effect in the cytoplasm, it was suggested that TEPA-PCL promotes high-knockdown efficiency by the efficient delivery of miR-92a-C into the cytosol. The results of the present study show that translation of target mRNAs, including those of integrin α5, MKK4 and S1P1, was suppressed by 48-h post transfection with miR-92a-C/TEPA-PCL, whereas transfection with miCont-C/TEPA-PCL did not repress the expression of miR-92a-target protein. These results suggest that miR-92a-C taken into cells specifically suppressed the expression of various miR-92a-target proteins.

The ability of HUVECs to form capillary tubes was also impaired by transfection with miR-92a-C/TEPA-PCL complexes at a miR-92a-C concentration of 200 nM. This result indicates that miR-92a has the potential to give an anti-angiogenic effect when delivered to angiogenic endothelial cells: miR-92a-C taken into the cells suppresses the expression of integrin α5, and inhibits tube formation, possibly as a result of the decrease of VEGF signaling. Interestingly, transfection with miR-92a-C/TEPA-PCL at a concentration of 100 nM did not decrease the ability to form a tube network. Because the suppressed expression of integrin does not directly impair VEGF signaling but reduces VEGF binding to VEGF receptors [25,26], the suppressed expression of integrin subunits by miR-92a-C/TEPA-PCL at 100 nM might not be sufficient to decrease VEGF signaling for inducing the inhibition to form a tube network. However, further investigations are required to demonstrate this lag between the miR-92a-C concentration needed to suppress the expression of target-proteins and that needed to impair the ability of the cells to form tubes.

In conclusion, we have developed a miR-92a-C delivery system into angiogenic endothelial cells by the use of TEPA-PCL and show that the expression of various proteins encoded by target genes recognized by miR-92a was inhibited by the transfection. Furthermore, the capability of capillary tube formation was also suppressed by the transfection. These results suggest that the delivery of miR-92a to angiogenic endothelial cells has an anti-angiogenic effect and the potential to suppress the tumor growth.

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