Title

Failure of active targeting by a cholesterol-anchored ligand and improvement by altering the lipid composition to prevent ligand desorption.

Shoshiro Yamamoto[†], Yu Sakurai[†], Hideyoshi Harashima*

Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan

[†]: These two authors equally contributed to this manuscript

*Corresponding author

Correspondence should be addressed to Hideyoshi Harashima (harasima@pharm.hokudai.ac.jp)

Kita-12, Nishi-6, Kita-ku, Sapporo 060-0812, Japan.

E-mail: harasima@pharm.hokudai.ac.jp, TEL: +81-11-706-3919, FAX: +81-11-706-4879

Abstract

Although anti-angiogenic therapy is predicted to be an effective therapy for treating cancer, selectively targeting tumor endothelial cells (TECs), and not normal endothelial cells, remains a major obstacle. Modifying a drug carrier with a targeting ligand is a popular strategy for developing an active-targeting type drug delivery system (DDS). We previously reported that a cyclo(Arg-Gly-Asp-D-Phe-Lys) (cRGD)-equipped liposome that contains encapsulated siRNA (RGD-MEND) achieved an efficient therapeutic outcome in a murine cancer model. To develop a more efficient TEC-targeting DDS, we examined the effect of the length of the polyethylene glycol (PEG) that is used as a peptide-linker on the cholesterol-scaffold, and liposomal composition on the efficiency of delivery of siRNA to cRGD receptor $\alpha_V\beta_3$ integrin positive cells. An RGD-MEND modified with shorter linker/no-linker, PEG350 or no-PEG, showed a higher cellular uptake *in vitro*. However, a shorter or no-linker RGD-cholesterol-modified MEND showed no silencing effect despite its high, *in vitro* silencing efficiency. To examine the possibility that the cholesterol-scaffold ligand was removed from the surface of the RGD-MEND by interactions with serum proteins, the RGD-MEND was incubated in the presence of a 50% serum solution. The cellular uptake of the cholesterol-scaffold ligand was drastically reduced by the incubation in serum. Increasing the cholesterol ratio in the lipid envelope and adding a helper lipid improved the *in vivo* knockdown efficiency, probably due to an enhanced ligand retention, even in in *vivo* conditions. The findings

reported herein suggest that the lipid composition and the ligand scaffold of the MEND are major factors in successfully developing an efficient active-targeting DDS.

Keywords: nanoparticle, liposome, active targeting, siRNA, cancer; cyclic RGD, anti-angiogenic therapy

1. Introduction

Given the fact that small interfering RNA (siRNA) causes a specific cleavage of mRNA in a sequence-specific manner (Elbashir et al., 2001), an efficient method for delivering siRNA could result in the suppressed expression of selected genes in cells. Therefore, siRNA is generally thought to be a potentially useful solution for treating diseases that are caused by a known gene. However, siRNA cannot passively pass across the plasma membrane of cells due to its high molecular weight (~13 kDa) and negative charge, and is degraded by ribonuclease enzymes in the circulation, which results in a low silencing efficiency (Butler et al., 2016; Judge et al., 2005). Additionally, siRNA must be delivered to cells that are responsible for the disease. To protect the siRNA molecule from degradation and deliver it to a cell of interest, a active-targeting–type drug delivery system (DDS) will be needed.

Anti-angiogenic therapy is one of the most powerful treatments for suppressing tumor growth and metastasis (Liu et al., 2015b). Moreover, anti-angiogenic therapy enhances the therapeutic effect of anti-cancer drugs and radiation therapy via remodeling of the tumor microenvironment (Chen and Xu, 2015; Yoshizawa et al., 2012). Many different molecules are involved in excessive angiogenesis, including fibroblast growth factor (FGF), transforming growth factor (TGF)-α, TGF-β, hepatocyte growth factor (HGF), angiopoietin-1 (Ang-1), Ang-2 and vascular endothelial growth factor (VEGF) (Ferrara et al., 2003). Inhibiting the VEGF signaling pathway has been well researched for

anti-angiogenic therapy over the past decade, since VEGF was first identified in 1989 (de Vries et al., 1992; Keck et al., 1989). On the other hand, tumor growth suppression and remodeling of the tumor microenvironment by blocking the VEGF pathway is a temporary measure, since some types of tumor tissue have the ability to develop resistance against VEGF inhibition (Bergers and Hanahan, 2008; Huang et al., 2012). This resistance against VEGF blocking might be due to the expression of other pro-angiogenic factors, and the recruitment of endothelial progenitor cells and monocytes (Grunewald et al., 2006; Winkler et al., 2004). That is to say, anti-cancer therapy in which multiple factors are targeted will likely be necessary in cases of long-term drastic treatments. In fact, inhibiting both Ang-2 and VEGF receptors has been reported to prolong survival over each therapy alone in murine glioblastoma models (Peterson et al., 2016). To achieve this therapy, siRNA will likely be one of these treatments because siRNA DDS can simultaneously inhibit the expression of multiple genes by encapsulating several siRNA molecules in DDS (Love et al., 2010).

We previously developed a multifunctional envelope-type nano device (MEND) intended for delivering siRNA to target cells (Hatakeyama et al., 2011; Sato et al., 2016a). The MEND is composed of a pH-sensitive cationic lipid, YSK05. YSK05 has the ability to fuse with endosomal membranes under conditions of a high pH, because of its unique head group and unsaturated fatty acid. To improve selectivity to a target cell, the MEND surface can be easily modified with various targeting ligands such as transferrin (Hatakeyama et al., 2004), a sugar (Masuda et al., 2008), an

antibody (Hatakeyama et al., 2007), and a peptide (Hatakeyama et al., 2007; Mudhakir et al., 2008; Takara et al., 2010) via the lipophilic groups of two stearic acids as an anchor.

The preparation of active targeting carriers modified with the cRGD-peptide has been previously reported (Kim et al., 2014; Liu et al., 2015a; Sakurai et al., 2013). Basically, the cRGD-peptide is incorporated into liposomes or micelles as a ligand-linker-anchor conjugate scaffold. Most carriers were prepared with PEG2k as cRGD-peptide linkers, which are used to conjugate the targeting-ligand (Torchilin and Lukyanov, 2003). Regarding other targeting ligands, other groups have reported that the length of the PEG linker is a determinant of the binding affinity between ligands and receptors (Perche and Torchilin, 2013; Stefanick et al., 2013b). For example, when folic acid was incorporated into the surface of liposomes, the optimized PEG molecular weight was 3,400, not 2,000 or 5,000 (Gabizon et al., 1999). The effect of linker length on the targeting ability, however, was not examined in that study. Moreover, although it has been reported that the leakiness of a cargo encapsulated in a liposome is dependent on the composition of the liposome, only limited information concerning the interaction between linker-scaffold and the lipid composition of the carrier is available (Pereira et al., 2016; Stefanick et al., 2013b).

In this study, we examined the influence of these factors, i.e., the length of linker, linker scaffold, and lipid composition of the carrier, on cellular uptake and the localization of the RGD-MEND with *in vivo* murine colorectal cancer model. This is because colorectal cancer is known to be sensitive to

anti-angiogenic therapy and anti-VEGF antibody was approved for colorectal cancer patients (Hurwitz et al., 2004). We previously examined the effect of a longer PEG-linker length (over 2,000) on *in vivo* knockdown efficiency. However, no drastic change was observed among $PEG_{2,000}$, $PEG_{3,400}$ and $PEG_{5,000}$ linkers (Hada et al., 2015). It is generally known that long PEG chain hinders the cellular uptake and the endosomal escape of nanocarrier due to the ability to stabilize the membrane of liposomes, so called “PEG-dilemma” (Hatakeyama et al., 2011). We inferred that shorter PEG-linker would enhance cyclic RGD-medicated targeting. Based on this presumption, the RGD-MEND was modified with a shorter PEG-linker, PEG350, PEG1k, PEG2k, or the absence of PEG. Serum proteins greatly influence the morphology and leakage of inclusions and the liposomal component and size are factors in this process (Guo et al., 1980). Therefore, the interaction between the linker-scaffold and MEND composition were characterized in the presence of 50% (v/v) fetal bovine serum (FBS). The findings indicate that the affinity between ligand scaffold and liposome surface was directly correlated with *in vivo* localization and gene silencing ability in the TECs.

2. Materials & methods

2.1. Materials

YSK05 was synthesized as previously reported (Sato et al., 2012). Cholesterol was purchased from Avanti Polar Lipid (Alabaster, AL, USA). 1,2-dimyristoyl-s*n*-glycerol, methoxy poly(ethyleneglycol) 2,000 ether (PEG-DMG), cholesterol-poly (ethyleneglycol) 1,000 (PEG1k-chol), PEG2k-chol and *N*-hydroxy-succinimide poly (ethyleneglycol) 2000 (NHS-PEG2k-DSPE) were purchased from NOF Corporation (Tokyo, Japan). Cyclo(Arg-Gly-Asn-*D*-Phe-Lys) peptide (cRGD) was purchased from Peptides International, Inc. 1,1'-dioctadecyl-3,3,3',3'- tetramethylindodicarbocyanine (DiD) and 3,3'-dioctadecyloxacarbocyanine (DiO), Quant-iT^{TM} RiboGreen RNA and High Capacity RNA-to-cDNA kit were purchased from ThermoFisher Scientific (Waltham, MA). Roswell Park Memorial Institute medium-1640 (RPMI-1640), Fetal Bovine Serum (FBS), TRI Reagent and Hoechst33342 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primers were purchased from Hokkaido System Science (Sapporo, Japan). OS-RC-2 (human renal cell carcinoma cells) was a generous gift from K Hida (Hokkaido University, Sapporo, Hokkaido, Japan). C26 (murine colon carcinoma cells) was purchased from the ATCC. THUNDERBIRD SYBR qPCR Mix was purchased from TOYOBO LIFE SCIENCE (Osaka, Japan). Amicon Ultra-15 Centrifugal Units was purchased from MERCK MILLIPORE (Darmstadt, Germany). Phosphate buffered saline without Ca^{2+} and

Mg^{2+} (PBS) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Anti-mouse CD31 Antibody (#102423) was purchased from BioLegend (San Diego, CA, USA). ICR mice (female, 4-week-old) were purchased from Japan SLC (Shizuoka, Japan). Balb/c mice were purchased from Japan CLEA (Shizuoka, Japan). All other chemicals used in this study were commercially available.

2.2. Cell Culture and preparation of C26 tumor bearing mice

OS-RC-2 and C26 cells were cultured in RPMI-1640 medium supplemented with FBS (10% v/v), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37 ℃ in a humidified 5% CO_2 air. The cells were trypsinized and transferred to new dishes when the cells were 70%-80% confluent.

2.3. Synthesis of RGD-PEG-chol

PEG2k-chol (100 mg, 40 μmol) was mixed overnight with succinic anhydride (10 mg, 0.1 mmol) under an Ar atmosphere. The mixture was then dialyzed with SpectraPor 6 (MWCO 1,000) to remove excess succinic anhydride. The resulting preparation was freeze-dried to obtain 78.0 mg of COOH-PEG2k-chol as a white powder (yield 78%). The COOH-PEG2k-chol (2.0 mg) was then incubated in N,N-anhydrous dimethylformamide (DMF) with 2 *eq.* N,N'-dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide (NHS) for 12 hours at room temperature. After the incubation, the mixture was filtered through a syringe filter (0.2 μm) to remove the insoluble compounds. The

resulting preparation was then mixed overnight with 1.2 mg of cRGD and 1 μL of trimethylamine (TEA) at room temperature. cRGD conjugated chol-PEG2k (RGD-PEG2k-chol) was purified by dialysis with SpectraPor6 (MWCO 1000) against DDW. cRGD conjugated to PEG1k-chol was synthesized using the same methodology. Concerning the synthesis of cRGD directly conjugated to chol, the carboxyl group of cholesterol hemi succinate (CHEMS, Sigma-Aldrich) was activated and conjugated to cRGD via an amide bond by the same methodology as described above. PEG350-chol was synthesized by conjugating CHEMS and COOH-hexaethylene glycol-NH_2 (MW 353.4) by EDC, NHS and TEA in DMF. The obtained PEG350-chol was similarly conjugated to the cRGD peptide. RGD-PEG2k-DSPE was synthesized as previously described (Sakurai et al., 2016). The structures of all of the products were confirmed by electrospray ionization or matrix-assisted laser desorption ionization-mass spectrometry.

2.4. Preparation of MENDs modified RGD-PEG-lipid

The MENDs were prepared with YSK05, cholesterol, PEG-DMG, and DSPC at the indicated ratios using the *t*-BuOH dilution method (Sato et al., 2012). Briefly, a 40-160 μg siRNA solution (pH 4.0) was added into a *t*-BuOH solution containing the lipids. This solution was then added stepwise to 2 mL of citric buffer (pH 4.0) under vigorous stirring. The mixture was diluted with PBS, and the *t*-BuOH was removed with an Amicon Ultra-15 (MWCO 100 kDa). The MEND was modified with

each of the RGD-PEG-lipids (3 mol % of total lipid) and EtOH (7.5 v/v%) solution (pH 5.5) and then incubated for 30 minutes (60 ℃, 1100 rpm) aspreviously reported (Hada et al., 2015). EtOH was removed with an Amicon Ultra-15 (MWCO 100 kDa). The encapsulation efficiency (EE) and recovery rate (RR) of siRNA were measured with a RNA quantification kit, RiboGreen, as previously reported (Sakurai et al., 2014). When MENDs were labeled with DiO, 0.5 mol% to total lipid of DiO in ethanol solution was added to the lipid mixture prior to mixing the lipids with siRNA.

2.5. Cellular uptake experiments

Since it is difficult to culture an endothelial cell line, we used a human renal cell carcinoma OS-RC-2, which expresses cRGD receptor $\alpha v\beta_3$ integrin, similar to endothelial cells. Previously, we confirmed that the silencing efficiency of RGD-modified MEND in OS-RC-2 cells was modestly correlated to the *in vivo* silencing efficiency (Hada et al., 2015). OS-RC-2 cells were seeded at a density of 1×10^5 cells/well 24 hours before transfection. For transfection, the DiO-labeled RGD-MENDs were applied to the wells (20 nM). Two hours later, the cells were collected and washed twice with FACS buffer (0.5 w/v% bovine serum albumin, 0.01w/v% sodium azide in PBS) to remove cell debris and unincorporated MENDs. The cell pellet was suspended in 500 μL FACS buffer. The fluorescence of the RGD-MEND within the cells was determined with a FACSCalibur

instrument (Becton Dickinson, Franklin Lakes, NJ, USA). Data were analyzed using Cell Quest (Becton Dickinson).

2.6. In vitro gene silencing experiment

OS-RC-2 cells were seeded at a density of 1×10^5 cells/well 24 hours before transfection. To evaluate the knockdown activity of the RGD-MENDs, they were added to the cell cultures at concentrations of 0.03 nM-0.3 nM. At 24 hours after transfection, the medium was removed and 500 μL of TRI Reagent was added to the wells. RNA was extracted from cell lysates according to the manufacture's protocol. The RNA was subjected to reverse transcription with a High Capacity RNA-to-cDNA kit. To quantify the expression of mRNA, cDNA was subjected to qRT-PCR with a Fast SYBR Green Master Mix using LightCycler-480 (Roche Diagnostics, Germany) according to the manufacture's protocol. All primers were checked for amplification efficiency.

2.7. Animal experiment

Balb/c mice (male, four week-old) were subcutaneously injected with C26 cells (1.0×10^6 cells/mouse) to prepare C26 bearing mice. When the tumor volume reached 100 mm^3 about 10 days after the inoculation, the mice were used in the experiments described below. For *in vivo* knockdown experiments, mice were intravenously administered with the RGD-MENDs at a dose of 1.0 mg/kg.

The mice were sacrificed 24 hours after administration and tumor tissue was harvested. TRI Reagent (500 μL), was then added to the tumor tissue and resulting mixture was vigorously homogenized with PreCellys 24 (Bertin Technologies, Montigny-Le-Bretonneux, France). The procedure used to measure mRNA expression is the same as that used in the in vitro experiment. These animal experimental procedures were reviewed and approved by the Hokkaido University Animal Care Committee in accordance with the Guide for the Care and Use of Laboratory Animals.

2.8. Observation of RGD-MENDs in vivo

The tumor tissues that were harvested from C26 bearing mice were cut at a thickness of 400 μm with a microslicer DTK-1000 (Dosaka-em, Kyoto, Japan). The tumor tissue slices were subjected to immunostaining with Anti-mouse CD31 Antibody (#102423) to visualize blood vessels. Blood vessels and fluorescence of DiD-labeled RGD-MEND were observed with Con-Focal Laser Scanning Microscopy (CLSM) using an A1R confocal imaging system (Nikon, Tokyo, Japan).

2.9. Incubation of RGD-MENDs in FBS

To confirm that the RGD-PEG-lipid was desorbed from the MEND, RGD-MENDs were incubated in 50 v/v% FBS at 37℃ for 3 hours. After dilution with RPMI-1640 to adjust the fluorescent intensity, the MEND solutions were added to each cells at the same intensity. The cells

were then analyzed with a FACSCalibur instrument, as above described.

2.11. PEG-MENDs PK in vivo

ICR mice (male, four week-old) were treated with 200 μL DiD-labeled PEG-MEND by intravenous injection. A 12 μL volume of mouse blood was collected from the tail vein at 0.017, 0.50, 1.0, 3.0 hours. The blood was diluted with 228 μL 1 w/v% SDS solution. The fluorescence of the mixture was measured with an Infinite M200 (Tecan, Männedorf, Switzerland). The calculated area under the curve (AUC) was determined by calculating the total area of the trapezoid under the concentration curve.

3. Results and discussion

3.1 Effect of PEG-length and lipid structure of the PEG scaffold on delivery

3.1.1 Influence of PEG-length in the RGD-MEND on cellular uptake and knockdown efficiency.

We previously reported that an RGD-MEND formulation that was modified with RGD-PEG2k-DSPE efficiently delivered siRNA to tumor endothelial cells in xenografts (Hada et al., 2015; Sakurai et al., 2014). We first assessed the effect of length of a PEG-linker that was shorter than the “conventional” (RGD-PEG2k-DSPE). The characterization of RGD-MEND with shorter linker was summarized in Table 1. To evaluate the cellular uptake and gene silencing potencies of the RGD-MEND, the surfaces of the RGD-MENDs with encapsulated siRNA were modified with lipophilic fluorescent DiO molecules with different molecular weight (MW) PEG linkers, 350, 1k, 2k, or no-PEG linker. The lipid scaffold DSPE was altered with cholesterol because of the ease of synthesis of derivatives. The cellular uptake of each MEND was determined by FCM with an OS-RC-2 cell line that expressed a receptor for $\alpha v\beta_3$ integrin **(Figs. 1A, B**). Modification with RGD-PEG350-cholesterol or RGD-cholesterol effectively facilitated the cellular uptake of the MEND in comparison with that of RGD-PEG1k-cholesterol or RGD-PEG2k-cholesterol. Similarly, the silencing efficiency of the MEND was enhanced more effectively by modification with RGD-PEG350-cholesterol or RGD-cholesterol (**Fig. 1C**). We conclude that an RGD-MEND modified with a shorter PEG-linker would be suitable for binding between the cyclic cRGD-peptide

and $\alpha v\beta_3$ integrin. Similar experimental results have been reported by other groups researching the modification of nanoparticles with targeting ligands. For example, the cellular uptake of liposomes and micelles was significantly enhanced when the VLA-4 and Her2 targeted-peptide was tethered by a shorter ligand-linker (Stefanick et al., 2013a; Stefanick et al., 2013b). On the other hand, modification of the PEG5k-linker to a liposome with folate resulted in a higher uptake by the human oral carcinoma KB cells than folate with a PEG2k-linker or a PEG3.4k-linker (Kawano and Maitani, 2011). The exact reason for this difference is currently unclear. It is possible that relationship between linker length and binding affinity might be determined by whether the ligand was sufficiently flexible or not.

3.1.2 Comparison of in vivo knockdown activity among RGD-PEG350-cholesterol-MEND, RGD-cholesterol-MEND, and RGD-PEG2k-DSPE-MEND.

To evaluate gene silencing activity *in vivo*, we conducted animal experiments with mice with murine colorectal cancer C26. We prepared a RGD-PEG350-cholesterol-modified MEND (RGD350C), a RGD-cholesterol-modified MEND (RGDC), and a RGD-PEG2k-DSPE MEND (Conventional) encapsulating siRNA targeting the *Pecam* (also known as *Cd31*) gene, a marker of endothelial cells. The mice were administered with the liposomes via the tail vein at a dose of 1.5 mg/kg respectively. At 24 hours after the injection, *Pecam* mRNA expression was measured by

qRT-PCR. The conventional RGD-MEND resulted in a gene silencing of approximately 50%-60% in both experiments (**Fig. 2**). However, RGD350C and RGDC failed to induce gene silencing, although both preparations showed high gene silencing potencies *in vitro*. We assumed that this difference in gene silencing between *in vitro* and *in vivo* conditions can be attributed to the action of serum proteins. It was possible that recognition by more abundant serum proteins in the blood could decrease the TECs targeting ability of the RGD-peptide in some way, compared to the *in vitro* situation (10% serum).

3.2. Desorption of RGD-PEG-cholesterol from liposome and the solution

3.2.1 Incubation of RGD-MENDs in the FBS

We then explored the reason for the reduced knockdown potencies of RGD350C and RGDC in the *in vivo* study. To verify whether serum proteins in the blood were involved in this low gene silencing activity, we simulated the environment in the blood. RGD-MENDs were incubated in FBS at 37℃ for 3 hours. After this, they were added to culture of OS-RC-2 cells at a dose of 20 nM. After 2 hours, we evaluated cellular uptake using FCM. The conventional liposome, whose anchor of the RGD-conjugate was DSPE, showed no difference between before and after incubation, while RGDC, whose anchor of the RGD-conjugate was cholesterol, showed a drastically impaired potency for cellular uptake after incubation (Fig. 3). From examining these findings, we conclude that

RGD-cholesterol incorporated into the surface of MEND impaired the TECs targeting ability in the blood due to the high concentration of serum proteins. Therefore, the silencing ability of RGD350C and RGDC was decreased considerably, because the anchor moiety was composed of cholesterol.

From previous research, it was shown that a lipid with a long acyl chain, such as DSG, was held tightly by particles, while a lipid with a short acyl chain, such as DMG, was removed from particles in the blood (Ambegia et al., 2005; Mui et al., 2013). These findings indicate that interactions between the linker-scaffold and LNPs should be carefully considered when a ligand-liposome is administered *in vivo*. The logP value denotes the Partition coefficient of a compound. The larger the logP is, the higher is the hydrophobicity. The logP of DSPE is 12.23, and that of cholesterol is 7.02 (Sun, 2004). Considering these logP values, the cholesterol scaffold would have a lower interaction for the MEND surface than the DSPE scaffold. From these results and previously reported studies, these findings indicate that the RGD-cholesterol diffused from the MEND surface in the blood stream because of its weak association with the particle. This hypothesis is consistent with the results showing that the PEG2k-DSPE-modified liposome remained in the circulation longer than the PEG2k-cholesterol-modified liposome (**Supplemental Figure 1**).

3.2.2 Change of lipid composition for MEND

In liposome preparations, it is known the insertion affinity and stability of the anchor molecule linker to the liposomal bilayer is highly influenced by the liposomal component (Hwang et al., 2010). When a liposome is exposed to a temperature above the phase transition temperature (Tm), it shifts to a liquid crystal phase which causes serious issues such as drug leakage (Belsito et al., 2001). Because YSK05 is predicted to have a low Tm, YSK05 destabilizes the liposomal membrane (Sato et al., 2016b). To prevent this expected desorption of RGD-cholesterol, we changed the MEND composition from YSK05/cholesterol/PEG-DMG 70/30/3 (RGDC) to YSK05/cholesterol/PEG-DMG 50/50/3 (RGDC_50) or YSK/cholesterol/DSPC/PEG-DMG 60/30/10/3 (RGDC_DSPC), all of which were modified with RGD-cholesterol. Their physical properties were summarized in Table 2. Cellular uptake was evaluated after the new RGD-cholesterol-modified MENDs were incubated in FBS. If the MENDs were not incubated with FBS, there was no significant difference among new and old MENDs. However, and interestingly, MENDs with the new lipid composition (RGDC_50 and RGDC_DSPC) remained stable, even after being incubated in FBS. These results suggest that serum proteins play a key role in the reduced cellular uptake of RGD-cholesterol-modified MENDs, and that this reduction could be suppressed by preparing lipid nanoparticles with a more stable lipid composition.

It is known that cholesterol contributes to the stabilization of liposomes or phospholipid biomembranes because the formation of phase separated lamellae is completely inhibited

(Bedu-Addo et al., 1996; Cao et al., 2012; Drolle et al., 2013). Moreover, the addition of cholesterol to a liposome can also alter membrane permeability (Ridgway et al., 1999). The hydrophilic region of phosphatidylcholine (PC) occupies a relatively large volume and, therefore, stabilizes the lamella membrane. Further, DSPC has high Tm value. Accordingly, cholesterol and DSPC in a liposomal lipid results in the stabilization of the liposome membrane and prevents leakage of an encapsulated drug. However, in this instance, we discovered that the stability of the membrane conferred by cholesterol or DSPC would affect not only the retention of the cargo but also the targeting of the modified ligand-lipid to the liposome membrane. In the case of stable RGD-cholesterol-modified MENDs (RGDC_50, RGDC_DSPC), lowering the liposomal fluidity by increasing the ratio of cholesterol or the addition of DSPC suppressed the diffusion of RGD-cholesterol from the surface of the MEND. Accordingly, we conclude that the desorption of RGD-cholesterol from a MEND with a traditional lipid composition (YSK05/cholesterol, 70/30) resulted in the loss of TECs targeting ability in the blood.

3.2.3 in vivo experiments of the new RGD-cholesterol-MEND

We observed the localization of the MEND in tumors by CLSM. DiD-labeled RGD-MENDs were prepared and injected into mice at a dose of 1.5 mg/kg. As shown in Fig. 4, the fluorescence of DiD labeled RGDC had diffused into tumor tissue. This indicates that the cRGD peptide was not retained

on the surface of MEND, and hence RGDC failed to target TECs. On the other hand, the fluorescence of the RGD-cholesterol-modified MENDs with a stable lipid composition (RGDC_50, RGDS) or RGD-PEG2k-DSPE-MEND was co-localized with tumor vessels. Considering these results, stable RGDC_50 and RGDC_DSPC were able to effectively bind to TECs via the cRGD peptide because RGD-cholesterol was retained in the MEND and did not diffuse into the blood. We then examined the gene expression of *Pecam*. As previously indicated, RGDC failed to reduce the expression of mRNA. Nevertheless, the MEND with a new composition, RGDC_50 and RGDC_DSPC, caused a significant reduction in the *Pecam* mRNA expression to the same extent as that for the RGD-PEG2k-DSPE-MEND. These findings indicate that changing the lipid composition permitted the RGD-cholesterol-MEND to regain its gene silencing ability.

On the other hand, the gene silencing activity of the stable RGDC_50 and RGDC_DSPC was not superior to that of the conventional RGD-PEG2k-DSPE-modified MEND *in vivo*, although the cellular uptake *in vitro* of RGDC_50 and RGDC_DSPC were better than that of RGD-PEG2k-DSPE-MEND. It is possible that the RGD-cholesterol that was retained on the MEND with rigid lipid envelop was inferior to RGD-PEG2k-DSPE, therefore RGDC_50 and RGDC_DSPC failed to show gene silencing ability. Further changes in lipid composition, for example, increasing the ratio of cholesterol or DSPC might further improve the retention of RGD-cholesterol by the MEND membrane. However, further stabilization of the liposome membrane might block effective

membrane fusion (Bailey and Cullis, 1997), or a decrease in the ratio of YSK05 would reduce the efficiency of escaping from endosomes due to the loss of cationic properties. Hence, to further improve gene silencing efficiency, this dilemma needs to be addressed and resolved. Further, the cholesterol-anchored ligand might tend to detach from the surface of lipid nanoparticle (LNP) in case of fusogenic lipid composition. Chen *et al.* reported that stabilization of limit-size LNP surface (<30 nm) by addition of PEG-lipid with carbon 18 acyl chain suppressed a removal of lipid component such as cationic lipid and helper lipid (Chen et al., 2016). Likewise, the detachment of ligand-lipid conjugate would more frequently occur when lipid composition is unstable, fusiogenic. As for colloidal stability in both *in vitro* and *in vivo* situation, diameter and polydispersity index was measured after an incubation in 10% of fetal bovine serum. There is no difference among these MENDs even after the incubating MENDs in serum (**Supplemental Table 1**). This result supports our conclusion that not colloidal stability but ligand detachment would be attributed to a reduced targeting ability.

We also prepared a RGD-PEG350-cholesterol-modified MEND and a RGD-PEG2k-DSPE-modified MEND, and evaluated cellular uptake *in vitro* (data not shown). However, no effective cellular uptake was detected, although the reason for this is not clear. In any event, the findings reported here clearly show that changing the liposomal composition is an

effective strategy for preparing liposomes in which the targeting ligand is retained on the liposome surface.

Conclusion

We explored the effect of anchor and linker structure in a cyclic RGD peptide (cRGD) ligand scaffold on targeting. The findings show that, concerning a linker for a ligand with a cholesterol anchor, the shorter the poly(ethylene glycol) was, the more efficiently were the cRGD-modified MENDs taken up in the *in vitro* experiment. On the other hand, in the case of an *in vivo* study, MENDs modified with cRGD with a cholesterol-anchor failed to induce a gene silencing effect in tumor endothelial cells (TECs). Pre-incubation of the MEND with the cholesterol-anchor in 50% serum resulted in a significant impairment in the cellular uptake of cRGD-modified MENDs. This result suggests that the failure in *in vivo* gene silencing can be attributed to the removal of cRGD from the surface of the MEND. To prevent cRGD derivatives from being removed from the surface, the lipid composition was altered to a more stable one by increasing the ratio of cholesterol instead of cationic lipid or introducing a stable helper lipid, DSPC. As a result, these stable MENDs that were modified with a cholesterol-anchor caused *in vivo* gene silencing. Our results reveal that the retention of a specific ligand in the bloodstream should be taken into the account for developing active targeting-type drug delivery systems.

Acknowledgement

The authors wish to thank Dr. Milton S Feather for appropriately modifying the manuscript. This study was supported, in part, by research grants (Research on Development of New Drugs, Health and Labour Sciences Research Grant, and Initiative for Accelerating Regulatory Science in Innovative Drug, Medical Device, and Regenerative Medicine) from the Japan Ministry of Health, Labour and Welfare (MHLW) Grant Number PH44280004.

Figure legends

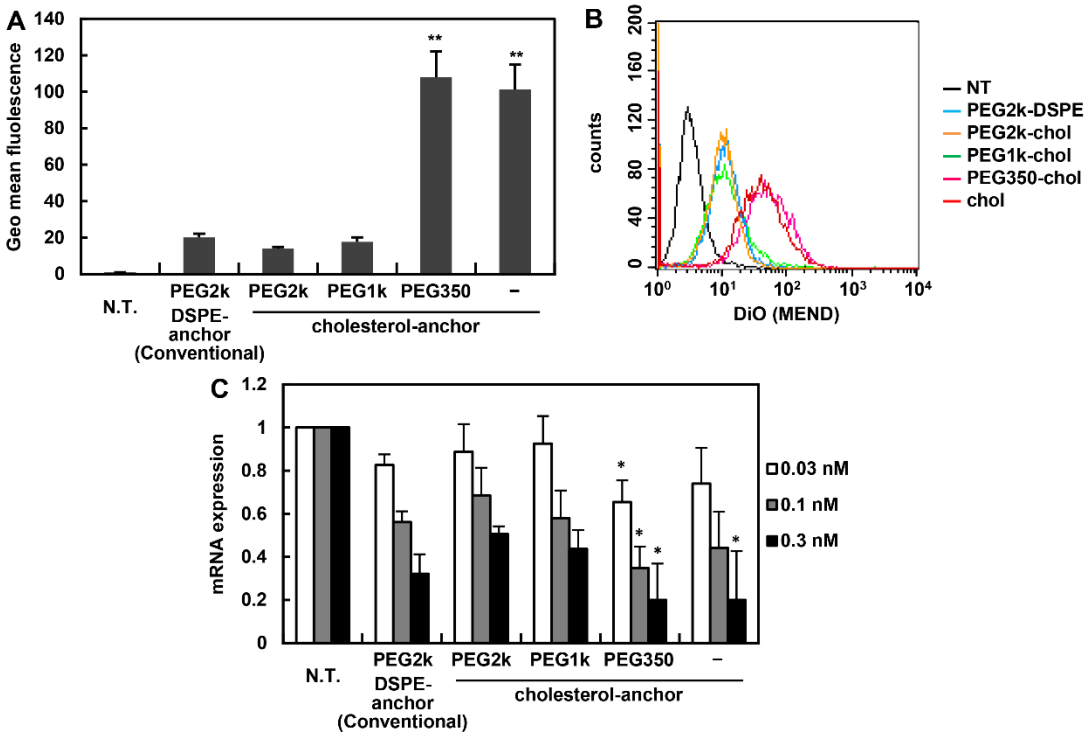

Figure 1. *in vitro* Cellular uptake and gene silencing efficiency of RGD-MENDs A) Effect of PEG length on the cellular uptake of MENDs targeting $\alpha_V\beta_3$ integrin. Cells were transfected with DiO-labeled MENDs for 2 hours, and the fluorescence intensity of cells was measured by flow cytometry. The mean of 3 independent experiments are shown. B) A representative histogram of cellular uptake. C) Effect of PEG length on gene silencing efficiency. Cells were transfected with MENDs at the indicated concentrations. The expression of mRNA was measured by qRT-PCR 24 hours after the transfection. The data are represented as the mean ± S.D. (n=3) **, $p < 0.01$ and *, $p < 0.05$ one-way ANOVA followed by Bonferroni test (*vs.* RGD-PEG2k-cholesterol on each concentration).

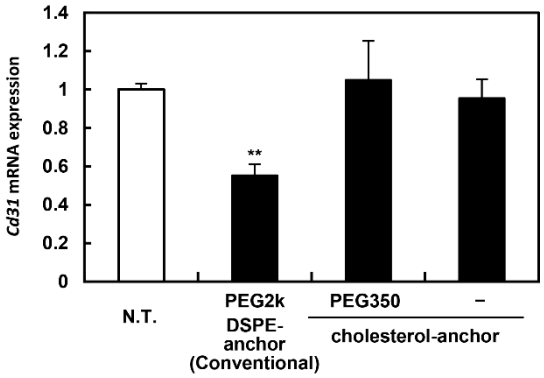

Figure 2. Gene silencing activity of RGD-MENDs *in vivo*. mRNA primarily expressed in TECs (*Pecam*) was quantified by qRT-PCR at 24 hours after the administration of 1.5 mg siRNA/kg body weight of RGD-MENDs. The data are represented as the mean ± SD (n = 3). $^{**}P < 0.01$ ANOVA followed by SNK test (*vs*. N.T.).

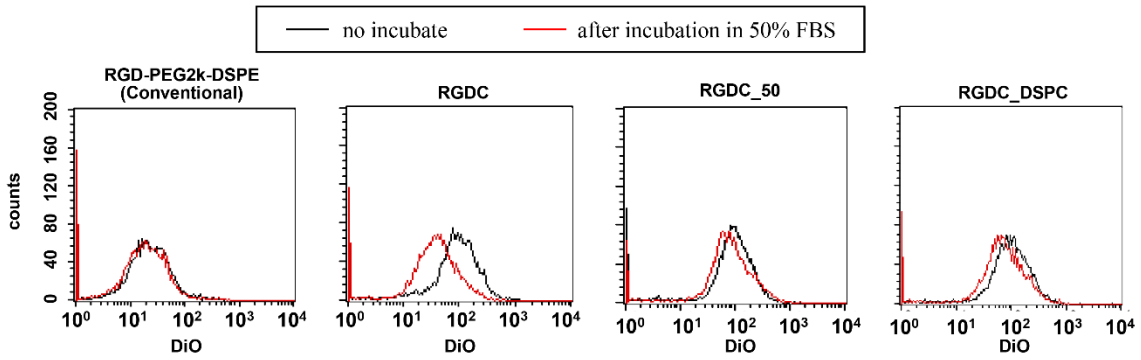

Figure 3. Cellular uptake of MENDs after incubation in serum. A) Composition of the MENDs. B) Cellular uptake of the MENDs before and after incubation in serum. MENDs were incubated in the serum for 3 hours and applied into the cell cultures. Black and red lines denote the cellular uptake of each RGD-MEND before incubation and after incubation, respectively.

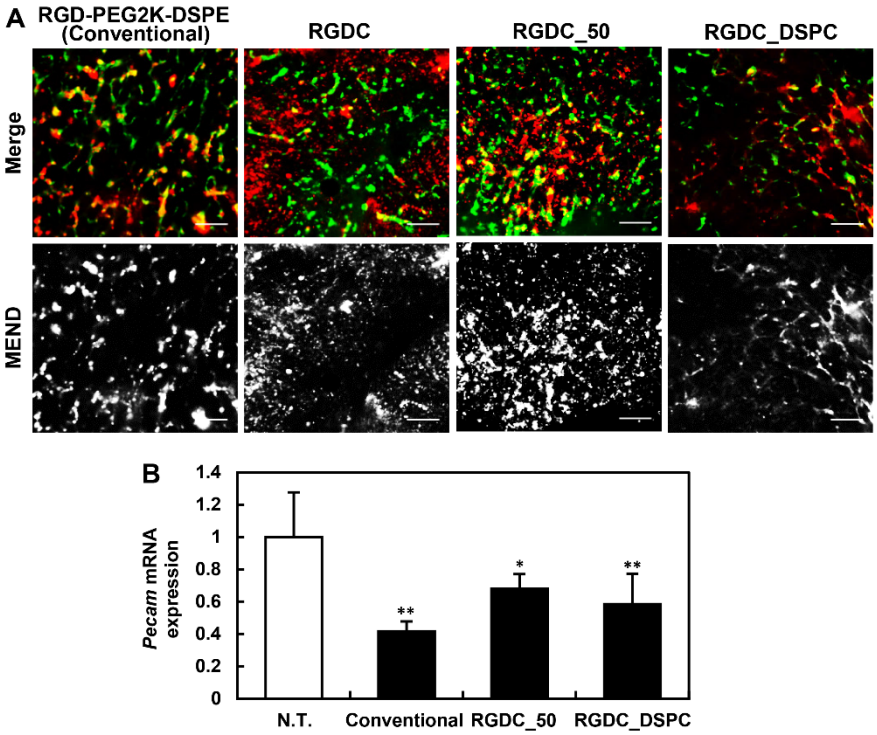

Figure 4. Intratumoral localization of MENDs 24 hours after injection and knockdown activity *in vivo*. A) Distributions of MENDs in tumors 24 hours after injection. Green, tumor blood vessels, red DiD-labeled MENDs, and observed with CLSM with a 20× objective lens. Scale bar = 100 μm. B) *Pecam* mRNA knockdown activity in TECs of MENDs. mRNA levels were measured with qRT-PCR after mice bearing C26 tumors were injected with the MENDs at a dose of 1.5 mg siRNA/kg body weight. The data are represented as the mean ± SD ($n = 3$). $^{**}P < 0.01$ ANOVA followed by SNK test (*vs*. N.T.).

References

Ambegia, E., Ansell, S., Cullis, P., Heyes, J., Palmer, L., MacLachlan, I., 2005. Stabilized plasmid-lipid particles containing PEG-diacylglycerols exhibit extended circulation lifetimes and tumor selective gene expression. Biochim Biophys Acta 1669, 155-163.

Bailey, A.L., Cullis, P.R., 1997. Membrane fusion with cationic liposomes: effects of target membrane lipid composition. Biochemistry 36, 1628-1634.

Bedu-Addo, F.K., Tang, P., Xu, Y., Huang, L., 1996. Interaction of polyethyleneglycol-phospholipid conjugates with cholesterol-phosphatidylcholine mixtures: sterically stabilized liposome formulations. Pharm Res 13, 718-724.

Belsito, S., Bartucci, R., Sportelli, L., 2001. Lipid chain length effect on the phase behaviour of PCs/PEG:2000-PEs mixtures. A spin label electron spin resonance and spectrophotometric study. Biophys Chem 93, 11-22.

Bergers, G., Hanahan, D., 2008. Modes of resistance to anti-angiogenic therapy. Nat Rev Cancer 8, 592-603.

Butler, J.S., Chan, A., Costelha, S., Fishman, S., Willoughby, J.L., Borland, T.D., Milstein, S., Foster, D.J., Goncalves, P., Chen, Q., Qin, J., Bettencourt, B.R., Sah, D.W., Alvarez, R., Rajeev, K.G., Manoharan, M., Fitzgerald, K., Meyers, R.E., Nochur, S.V., Saraiva, M.J., Zimmermann, T.S., 2016. Preclinical evaluation of RNAi as a treatment for transthyretin-mediated amyloidosis. Amyloid 23, 109-118.

Cao, Z., Zhang, L., Jiang, S., 2012. Superhydrophilic zwitterionic polymers stabilize liposomes.

Langmuir 28, 11625-11632.

Chen, S., Tam, Y.Y.C., Lin, P.J.C., Sung, M.M.H., Tam, Y.K., Cullis, P.R., 2016. Influence of particle size on the in vivo potency of lipid nanoparticle formulations of siRNA. J Control Release 235, 236-244.

Chen, Z., Xu, X.H., 2015. Combining antiangiogenic therapy and radiation in nasopharyngeal carcinoma. Saudi Med J 36, 659-664.

de Vries, C., Escobedo, J.A., Ueno, H., Houck, K., Ferrara, N., Williams, L.T., 1992. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science 255, 989-991.

Drolle, E., Kucerka, N., Hoopes, M.I., Choi, Y., Katsaras, J., Karttunen, M., Leonenko, Z., 2013. Effect of melatonin and cholesterol on the structure of DOPC and DPPC membranes. Biochim Biophys Acta 1828, 2247-2254.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., Tuschl, T., 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411, 494-498.

Ferrara, N., Gerber, H.P., LeCouter, J., 2003. The biology of VEGF and its receptors. Nat Med 9, 669-676.

Gabizon, A., Horowitz, A.T., Goren, D., Tzemach, D., Mandelbaum-Shavit, F., Qazen, M.M., Zalipsky, S., 1999. Targeting folate receptor with folate linked to extremities of poly(ethylene glycol)-grafted liposomes: in vitro studies. Bioconjug Chem 10, 289-298.

Grunewald, M., Avraham, I., Dor, Y., Bachar-Lustig, E., Itin, A., Jung, S., Chimenti, S., Landsman, L.,

Abramovitch, R., Keshet, E., 2006. VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells. Cell 124, 175-189.

Guo, L.S., Hamilton, R.L., Goerke, J., Weinstein, J.N., Havel, R.J., 1980. Interaction of unilamellar liposomes with serum lipoproteins and apolipoproteins. J Lipid Res 21, 993-1003.

Hada, T., Sakurai, Y., Harashima, H., 2015. Optimization of a siRNA Carrier Modified with a pH-Sensitive Cationic Lipid and a Cyclic RGD Peptide for Efficiently Targeting Tumor Endothelial Cells. Pharmaceutics 7, 320-333.

Hatakeyama, H., Akita, H., Harashima, H., 2011. A multifunctional envelope type nano device (MEND) for gene delivery to tumours based on the EPR effect: a strategy for overcoming the PEG dilemma. Adv Drug Deliv Rev 63, 152-160.

Hatakeyama, H., Akita, H., Ishida, E., Hashimoto, K., Kobayashi, H., Aoki, T., Yasuda, J., Obata, K., Kikuchi, H., Ishida, T., Kiwada, H., Harashima, H., 2007. Tumor targeting of doxorubicin by anti-MT1-MMP antibody-modified PEG liposomes. Int J Pharm 342, 194-200.

Hatakeyama, H., Akita, H., Maruyama, K., Suhara, T., Harashima, H., 2004. Factors governing the in vivo tissue uptake of transferrin-coupled polyethylene glycol liposomes in vivo. Int J Pharm 281, 25-33.

Huang, Y., Yuan, J., Righi, E., Kamoun, W.S., Ancukiewicz, M., Nezivar, J., Santosuosso, M., Martin, J.D., Martin, M.R., Vianello, F., Leblanc, P., Munn, L.L., Huang, P., Duda, D.G., Fukumura, D., Jain, R.K., Poznansky, M.C., 2012. Vascular normalizing doses of antiangiogenic treatment reprogram the

immunosuppressive tumor microenvironment and enhance immunotherapy. Proc Natl Acad Sci U S A 109, 17561-17566.

Hurwitz, H., Fehrenbacher, L., Novotny, W., Cartwright, T., Hainsworth, J., Heim, W., Berlin, J., Baron, A., Griffing, S., Holmgren, E., Ferrara, N., Fyfe, G., Rogers, B., Ross, R., Kabbinavar, F., 2004. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 350, 2335-2342.

Hwang, S.Y., Cho, D.Y., Kim, H.K., Cho, S.H., Choo, J., Yoon, W.J., Lee, E.K., 2010. Preparation of targeting proteoliposome by postinsertion of a linker molecule conjugated with recombinant human epidermal growth factor. Bioconjug Chem 21, 345-351.

Judge, A.D., Sood, V., Shaw, J.R., Fang, D., McClintock, K., MacLachlan, I., 2005. Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. Nat Biotechnol 23, 457-462.

Kawano, K., Maitani, Y., 2011. Effects of polyethylene glycol spacer length and ligand density on folate receptor targeting of liposomal Doxorubicin in vitro. J Drug Deliv 2011, 160967.

Keck, P.J., Hauser, S.D., Krivi, G., Sanzo, K., Warren, T., Feder, J., Connolly, D.T., 1989. Vascular permeability factor, an endothelial cell mitogen related to PDGF. Science 246, 1309-1312.

Kim, M.S., Lee, D.W., Park, K., Park, S.J., Choi, E.J., Park, E.S., Kim, H.R., 2014. Temperature-triggered tumor-specific delivery of anticancer agents by cRGD-conjugated thermosensitive liposomes. Colloids Surf B Biointerfaces 116, 17-25.

Liu, J., Deng, H., Liu, Q., Chu, L., Zhang, Y., Yang, C., Zhao, X., Huang, P., Deng, L., Dong, A., Liu, J., 2015a. Integrin-targeted pH-responsive micelles for enhanced efficiency of anticancer treatment in vitro and in vivo. Nanoscale 7, 4451-4460.

Liu, Y., Xia, X., Zhou, M., Liu, X., 2015b. Avastin(R) in combination with gemcitabine and cisplatin significantly inhibits tumor angiogenesis and increases the survival rate of human A549 tumor-bearing mice. Exp Ther Med 9, 2180-2184.

Love, K.T., Mahon, K.P., Levins, C.G., Whitehead, K.A., Querbes, W., Dorkin, J.R., Qin, J., Cantley, W., Qin, L.L., Racie, T., Frank-Kamenetsky, M., Yip, K.N., Alvarez, R., Sah, D.W., de Fougerolles, A., Fitzgerald, K., Koteliansky, V., Akinc, A., Langer, R., Anderson, D.G., 2010. Lipid-like materials for low-dose, in vivo gene silencing. Proc Natl Acad Sci U S A 107, 1864-1869.

Masuda, T., Akita, H., Nishio, T., Niikura, K., Kogure, K., Ijiro, K., Harashima, H., 2008. Development of lipid particles targeted via sugar-lipid conjugates as novel nuclear gene delivery system. Biomaterials 29, 709-723.

Mudhakir, D., Akita, H., Tan, E., Harashima, H., 2008. A novel IRQ ligand-modified nano-carrier targeted to a unique pathway of caveolar endocytic pathway. J Control Release 125, 164-173.

Mui, B.L., Tam, Y.K., Jayaraman, M., Ansell, S.M., Du, X., Tam, Y.Y., Lin, P.J., Chen, S., Narayanannair, J.K., Rajeev, K.G., Manoharan, M., Akinc, A., Maier, M.A., Cullis, P., Madden, T.D., Hope, M.J., 2013. Influence of Polyethylene Glycol Lipid Desorption Rates on Pharmacokinetics and Pharmacodynamics of

siRNA Lipid Nanoparticles. Mol Ther Nucleic Acids 2, e139.

Perche, F., Torchilin, V.P., 2013. Recent trends in multifunctional liposomal nanocarriers for enhanced tumor targeting. J Drug Deliv 2013, 705265.

Pereira, S., Egbu, R., Jannati, G., Al-Jamal, W.T., 2016. Docetaxel-loaded liposomes: The effect of lipid composition and purification on drug encapsulation and in vitro toxicity. Int J Pharm 514, 150-159.

Peterson, T.E., Kirkpatrick, N.D., Huang, Y., Farrar, C.T., Marijt, K.A., Kloepper, J., Datta, M., Amoozgar, Z., Seano, G., Jung, K., Kamoun, W.S., Vardam, T., Snuderl, M., Goveia, J., Chatterjee, S., Batista, A., Muzikansky, A., Leow, C.C., Xu, L., Batchelor, T.T., Duda, D.G., Fukumura, D., Jain, R.K., 2016. Dual inhibition of Ang-2 and VEGF receptors normalizes tumor vasculature and prolongs survival in glioblastoma by altering macrophages. Proc Natl Acad Sci U S A 113, 4470-4475.

Ridgway, N.D., Byers, D.M., Cook, H.W., Storey, M.K., 1999. Integration of phospholipid and sterol metabolism in mammalian cells. Prog Lipid Res 38, 337-360.

Sakurai, Y., Hada, T., Harashima, H., 2016. Preparation of a Cyclic RGD: Modified Liposomal SiRNA Formulation for Use in Active Targeting to Tumor and Tumor Endothelial Cells. Methods Mol Biol 1364, 63-69.

Sakurai, Y., Hatakeyama, H., Sato, Y., Hyodo, M., Akita, H., Harashima, H., 2013. Gene silencing via RNAi and siRNA quantification in tumor tissue using MEND, a liposomal siRNA delivery system. Mol Ther 21, 1195-1203.

Sakurai, Y., Hatakeyama, H., Sato, Y., Hyodo, M., Akita, H., Ohga, N., Hida, K., Harashima, H., 2014. RNAi-mediated gene knockdown and anti-angiogenic therapy of RCCs using a cyclic RGD-modified liposomal-siRNA system. J Control Release 173, 110-118.

Sato, Y., Harashima, H., Kohara, M., 2016a. A Multifunctional Envelope-Type Nano Device Containing a pH-Sensitive Cationic Lipid for Efficient Delivery of Short Interfering RNA to Hepatocytes In Vivo. Methods Mol Biol 1364, 71-78.

Sato, Y., Hatakeyama, H., Hyodo, M., Harashima, H., 2016b. Relationship Between the Physicochemical Properties of Lipid Nanoparticles and the Quality of siRNA Delivery to Liver Cells. Mol Ther 24, 788-795.

Sato, Y., Hatakeyama, H., Sakurai, Y., Hyodo, M., Akita, H., Harashima, H., 2012. A pH-sensitive cationic lipid facilitates the delivery of liposomal siRNA and gene silencing activity in vitro and in vivo. J Control Release 163, 267-276.

Stefanick, J.F., Ashley, J.D., Bilgicer, B., 2013a. Enhanced cellular uptake of peptide-targeted nanoparticles through increased peptide hydrophilicity and optimized ethylene glycol peptide-linker length. ACS Nano 7, 8115-8127.

Stefanick, J.F., Ashley, J.D., Kiziltepe, T., Bilgicer, B., 2013b. A systematic analysis of peptide linker length and liposomal polyethylene glycol coating on cellular uptake of peptide-targeted liposomes. ACS Nano 7, 2935-2947.

Sun, H., 2004. A universal molecular descriptor system for prediction of logP, logS, logBB, and absorption. J Chem Inf Comput Sci 44, 748-757.

Takara, K., Hatakeyama, H., Ohga, N., Hida, K., Harashima, H., 2010. Design of a dual-ligand system using a specific ligand and cell penetrating peptide, resulting in a synergistic effect on selectivity and cellular uptake. Int J Pharm 396, 143-148.

Torchilin, V.P., Lukyanov, A.N., 2003. Peptide and protein drug delivery to and into tumors: challenges and solutions. Drug Discov Today 8, 259-266.

Winkler, F., Kozin, S.V., Tong, R.T., Chae, S.S., Booth, M.F., Garkavtsev, I., Xu, L., Hicklin, D.J., Fukumura, D., di Tomaso, E., Munn, L.L., Jain, R.K., 2004. Kinetics of vascular normalization by VEGFR2 blockade governs brain tumor response to radiation: role of oxygenation, angiopoietin-1, and matrix metalloproteinases. Cancer Cell 6, 553-563.

Yoshizawa, Y., Ogawara, K., Fushimi, A., Abe, S., Ishikawa, K., Araki, T., Molema, G., Kimura, T., Higaki, K., 2012. Deeper penetration into tumor tissues and enhanced in vivo antitumor activity of liposomal paclitaxel by pretreatment with angiogenesis inhibitor SU5416. Mol Pharm 9, 3486-3494.