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Antibody-modified lipid nanoparticles for selective delivery of siRNA to tumors expressing membrane-anchored form of HB-EGF

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ABSTRACT

An Fab' antibody against heparin-binding epidermal growth factor-like growth factor (HB-EGF) was applied to achieve advanced tumor-targeted delivery of siRNA. Lipid nanoparticles (LNP) encapsulating siRNA (LNP-siRNA) were prepared, pegylated, and surface modified with Fab' fragments of anti-HB-EGF antibody (αHB-EGF LNP-siRNA). αHB-EGF LNP-siRNA showed high-binding affinity to recombinant human HB-EGF in a Biacore assay. In addition, αHB-EGF LNP-siRNA selectively associated with cells expressing HB-EGF *in vitro*. Confocal microscopic images showed that siRNA formulated in αHB-EGF LNP-siRNA was efficiently internalized into MDA-MB-231 human breast cancer cells, on which HB-EGF is highly expressed. In addition, siRNA encapsulated in αHB-EGF LNP induced obvious suppression of both target mRNA and protein levels in MDA-MB-231 cells. These results indicate that αHB-EGF LNP have excellent potential to deliver siRNA to target cancer cells, resulting in effective gene silencing.

1. Introduction

For clinical application of small interfering RNA (siRNA), many studies have been carried out in both basic and clinical fields [1–3]. Since siRNA is unstable in serum and hardly penetrates the cell membrane, an appropriate siRNA delivery system is necessary for the establishment of siRNA therapies. For this purpose, nanoparticle-mediated delivery of siRNA has been studied to obtain efficient gene silencing [4–6]. Previously, we developed a non-viral vector, named dicetyl phosphate tetraethylenepentamine (DCP-TEPA)-based polycation liposomes (TEPA-PCL), for delivery of double-stranded small RNAs [7]. Our data showed that siRNA or microRNA complexed with TEPA-PCL was highly taken up into cells and had remarkable gene silencing effects [8–10]. Surface modification of TEPA-PCL-based lipoplexes with polyethyleneglycol and peptide ligands, such as Arg-Gly-Asp (RGD)-peptide for targeting integrin $\alpha_v\beta_3$ or Ala-Pro-Arg-Pro-Gly (APRPG)-peptide for targeting vascular endothelial growth factor receptor 1 (VEGFR1), enabled targeted delivery of siRNA and subsequent gene silencing after systemic administration [7,10–12]. On the other hand, we previously designed another type of lipid-based vector for small RNA delivery [13]. We prepared cationic cores carrying siRNA by using palmitoyl RRRRRRGGRRRRG and wrapped them with lipids. lipid nanoparticles (LNP) encapsulating siRNA (LNP-siRNA) thus obtained showed significant gene silencing when they were modified with cell-penetrating peptides on their surface [13].

In the present study, we modified LNP with specific antibody against heparin-binding epidermal growth factor-like growth factor (HB-EGF) to induce gene silencing in target cancer cells in a selective manner. Active targeting of nanoparticles to tumors by antibody conjugation is a promising approach, since tumor cells often express characteristic molecules on their surface that are not found on normal cells [14,15]. HB-EGF is known to be highly expressed on the cell surface of various cancers, such as breast, ovarian, and liver cancers [16,17]. The precursor of HB-EGF is expressed on the cell surface as a membrane-anchored form (proHB-EGF) and then processed to a soluble form (HB-EGF), which mediates the intracellular signaling. Thus, we expected HB-EGF to be a useful target molecule for delivering siRNA to tumors. In fact, our previous study showed that anti-HB-EGF antibody-modified liposomes can efficiently deliver an anticancer agent to cancer cells

Abbreviations: LNP, lipid nanoparticles; LNP-siRNA, LNP encapsulating siRNA; αHB-EGF-LNP, anti-HB-EGF antibody-modified LNP.

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overexpressing HB-EGF both *in vitro* and *in vivo* [18]. Here, we developed LNP-siRNA modified with Fab' fragments of anti-HB-EGF antibody (αHB-EGF LNP-siRNA) and evaluated their potential as a siRNA vector *in vitro*.

2. Materials and methods

2.1. Materials

siRNA was purchased from Hokkaido System Science Co. (Hokkaido, Japan). In this study, siRNA for the luciferase2 gene was used unless otherwise stated. The nucleotide sequences for enhanced luciferase 2 (siLuc2) were 5′-GCUAUGGGCUGAAUACAA-ATT-3′ (sense) and 5′-UUUGUAUUCAGCCCAUAGCTT-3′ (antisense); and for Lamin A/C (siLamin) with a 2-nucleotide overhang (underline) as 5′-GGUGGUGACGAUCUGGGCUTT-3′ (sense) and 5′-AGCCCAGAUCGUCACCACCTT-3′ (antisense). For the use of fluorescein isothiocyanate (FITC)-labeled siRNA, FITC was conjugated to siLuc2 at the 3′ end of the antisense strand.

A palmitoyl derivative of RRRRRRGGRRRRG peptide was purchased from Operon Biotechnologies (Tokyo, Japan). Dioleoylphosphatidylethanolamine (DOPE), dimyristoylphosphoglycerol (DMPG), distearoylphosphatidylethanolamine (DSPE)-polyethyleneglycol (PEG) 5000 (DSPE-PEG), and maleimide-conjugated DSPE-PEG5000 (DSPE-PEG-maleimide) were purchased from NOF Co. (Tokyo, Japan). Cholesterol was kindly provided by Nippon Fine Chemical Co. (Hyogo, Japan). Monoclonal antibody clone 3E9 specific for HB-EGF was obtained by the method described previously [19]. The 3E9 clone recognizes the EGF-like domain of human proHB-EGF, but not that of mouse proHB-EGF. Recombinant human HB-EGF (rhHB-EGF) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2. Preparation of anti-HB-EGF-modified LNP-siRNA

LNP-siRNA were prepared as described previously [13]. siRNA and palmitoyl RRRRRRGGRRRRG (1/16.8 as a molar ratio, containing 1 nmol of siRNA) dissolved in RNase-free water (1 mL, Invitrogen, Rockville, MD) were mixed and incubated for 30 min at room temperature to obtain the cationic cores. On the other hand, DOPE, cholesterol, and DMPG (6/5/2 as a molar ratio, total lipids: 5 μmol) dissolved in chloroform were evaporated under reduced pressure, and stored *in vacuo* for at least 1 h. LNP-siRNA were prepared by hydration of the thin lipid film with 1 mL of the cationic core solution and sized by use of mild sonication for 3 min at room temperature.

Fab' fragments of anti-HB-EGF monoclonal antibody were prepared as described previously [18]. For the modification of LNP-siRNA with Fab' fragments of anti-HB-EGF antibody, 1 mL of the LNP-siRNA solution was incubated with 45 μL of 5 mM DSPE-PEG and 5 μL of 5 mM DSPE-PEG-maleimide dissolved in RNase free water at 37 °C for 2 h, forming PEG/PEG-maleimide-inserted LNP-siRNA (PEG-mal-LNP-siRNA). The coupling reaction of Fab' fragments with the maleimide moiety of PEG-mal-LNP-siRNA was performed according to the method described earlier [20]. Fab' fragments and PEG-mal-LNP-siRNA (1/1 as a molar ratio of Fab' and maleimide moiety) were mixed, and the coupling reaction was carried out at 4 °C for 16 h. Excess Fab' fragments were separated from the Fab'-coupled PEG-mal-LNP-siRNA by gel-filtration chromatography (Sepharose™ 4 Fast Flow column, GE healthcare, Piscataway, NJ), and the LNP-siRNA fractions were collected. After ultracentrifugation (453,000×*g*, 4 °C, 15 min), anti-HB-EGF Fab'-modified LNP (αHB-EGF LNP-siRNA) were resuspended with 1 mL of RNase- free water. Similarly, the surface of LNP-siRNA was decorated with Fab' fragments of control mouse IgG (MGG-0500, MBL, Nagoya, Japan; Control LNP-siRNA). The particle size and ζ-potential of the particles were measured by using a Zetasizer Nano ZS (Malvern, Worcs, UK).

2.3. Transmission electron microscopy (TEM)

Ten microliters of 5 mM αHB-EGF LNP-siRNA was added onto a grid (Nisshin EM, Tokyo, Japan) and dried-out by warm air. After that, the sample was negatively stained with 10 μL of 1 w/v% ammonium molybdate for 1 min, and imaged with an HT7700 TEM System (Hitachi High-Technologies, Tokyo, Japan). TEM images were recorded with a CCD camera at 1024 × 1024 pixels (Advanced Microscopy Techniques, Woburn, MA).

2.4. Assay to detect binding of αHB-EGF LNP to rhHB-EGF

A Biacore sensor chip was activated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/HCl (EDC) and *N*-hydroxysuccinimide (NHS), and then it was coated with rhHB-EGF dissolved in acetate buffer (pH 4.5). Ethanolamine was used as a blank. PEG-mal-LNP-siRNA, Control LNP-siRNA, or αHB-EGF LNP-siRNA were applied to the sensor chip for binding analysis using the Biacore 2000 system (GE healthcare, Tokyo, Japan) (injection time: 10 min, flow rate: 15 μL/min).

2.5. Cell culture

African green monkey kidney-derived Vero cells overexpressing HB-EGF (Vero-H) [21] were cultured in MEM medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, AusGeneX, Oxenford, Australia), 100 units/mL penicillin G (MP Biomedicals, Irvine, CA), 100 μg/mL streptomycin (MP Biomedicals), and 1 μg/mL G418 (SIGMA-Aldrich, St. Louis, MO) in a CO_2 incubator. MDA-MB-231 human breast cancer cells were cultured in RPMI-1640 medium (Life Technologies, Carlsbad, CA) supplemented with 10% FBS, 100 units/mL penicillin G, and 100 μg/mL streptomycin in a CO_2 incubator.

2.6. siRNA Transfection

Cells were seeded onto a culture plate and pre-cultured overnight. Before transfection, the medium was changed to a fresh one containing FBS but not antibiotics. Control LNP-siRNA or αHB-EGF LNP-siRNA was added to the culture medium at a final concentration of 60 nM (as siRNA), and the cells were then incubated for 24 h at 37 °C in a 5% CO_2 incubator. After a medium change, the cells were incubated for the desired time as described for each experimental procedure.

2.7. Association of αHB-EGF LNP-siRNA with cells overexpressing HB-EGF

Vero-H cells (2×10^4 cells/0.5 mL/well) or MDA-MB-231 cells (4×10^4 cells/0.5 mL/well) were seeded onto 24-well plates (BD Bioscience, San Jose, CA). These cells were incubated for 6, 12 or 24 h with FITC-labeled siRNA (60 nM, Hokkaido System Science Co.) formulated in Control LNP or αHB-EGF LNP. Naked FITC-siRNA was also incubated with the cells as a control. The cells were washed 3 times with PBS and lysed with 1 w/v% n-octyl-β-D-glucoside (Dojindo, Kumamoto, Japan) containing protease inhibitors: 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma–Aldrich), 2 μg/mL leupeptin (Sigma–Aldrich), 2 μg/mL aprotinin (Sigma–Aldrich), and 2 μg/mL pepstatin A (Sigma–Aldrich). The fluorescence intensity of FITC was determined with a Tecan Infinite M200 microplate reader (Salzburg, Austria) according to the manufacturer's instructions (ex. 485 nm, em. 535 nm) and corrected by total protein content measured with a BCA Protein Assay Reagent

Table 1
Particle size and ζ-potential of nanoparticles.

	Particle size (nm)	PDI	ζ-Potential (mV)
Cationic cores	98.9 ± 2.76	0.29 ± 0.05	+22.0 ± 2.95
LNP-siRNA	134 ± 21.5	0.27 ± 0.03	−40.5 ± 5.30
αHB-EGF LNP-siRNA	161 ± 24.4	0.27 ± 0.07	−9.50 ± 3.78

Size and ζ-potential of nanoparticles were measured by using the Zetasizer Nano ZS. Data are presented as the mean with SD (PDI, polydispersity index).

Kit (PIERCE Biotechnology, Rockford, IL) according to the manufacturer's instructions.

2.8. Cellular uptake of αHB-EGF LNP into MDA-MB-231 cells

MDA-MB-231 cells were seeded onto 8-well chamber slides (Thermo Fisher Scientific, Roskilde, Denmark) at a density of 1×10^4 cells/well and incubated with FITC-labeled siRNA alone (naked siRNA), Control LNP-siRNA or αHB-EGF LNP-siRNA (60 nM as siRNA) for 24 h. After having been washed with PBS, the cells were fixed with 4% paraformaldehyde for 30 min; and the nuclei were then stained with 4′,6-diamidino-2-phenylindole (DAPI, Life Technologies, Carlsbad, CA, USA). Intracellular localization of siRNA was observed by using confocal laser-scanning microscopy (LSM510 META, Carl Zeiss, Germany).

2.9. Gene silencing effect of siRNA formulated in αHB-EGF LNP

The gene silencing effect of αHB-EGF LNP-siRNA was evaluated by use of siLamin. MDA-MB-231 cells were seeded onto 60-mm dishes at a density of 3×10^5 cells/5 mL/dish and then incubated with Control LNP-siLamin or αHB-EGF LNP-siLamin (60 nM as siRNA) for 24 h. The cells were also treated with RNase-free water as a control. Total RNA of the cells was extracted with an RNeasy Plus Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instruction. Then, cDNA was generated from the total RNA samples (5 μg) by use of a Ready-To-Go T-Primed First-Strand Kit (GE Healthcare). In the presence of either human Lamin A/C primers (Takara Bio, Shiga, Japan) or β-actin ones (Takara Bio) and SYBR Premix Ex Taq (Takara Bio), real-time RT-PCR was performed with a Thermal Cycler Dice Real Time System (Takara Bio). The nucleotide sequences of the primers of Lamin A/C were 5′-GAT GAG GAG GGC AAG TTT GTC-3′ (forward) and 5′-AGG GTG AAC TTT GGT GGG AAC-3′ (reverse); and those of β-Actin, 5′-CAT CCG TAA AGA CCT CTA TGC CAA C-3′ (forward) and 5′-ATG GAG CCA CCG ATC CAC A-3′ (reverse). The conditions for real-time RT-PCR were as follow: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s, 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s.

2.10. Protein-knockdown effect of αHB-EGF LNP-siRNA

Anti-Lamin rabbit polyclonal antibody (Merck Millipore, Billerica, MA), anti-β-actin rabbit polyclonal antibody (Sigma–Aldrich), and horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G (IgG) polyclonal antibody (GE Healthcare) were used at the dilutions recommended in the manufacturer's instructions.

MDA-MB-231 cells cultured on a 6-well plate (1×10^5 cells/5 mL/dish) were transfected with Control LNP-siLamin or αHB-EGF LNP-siLamin (60 nM as siLamin). After 24 h, the medium

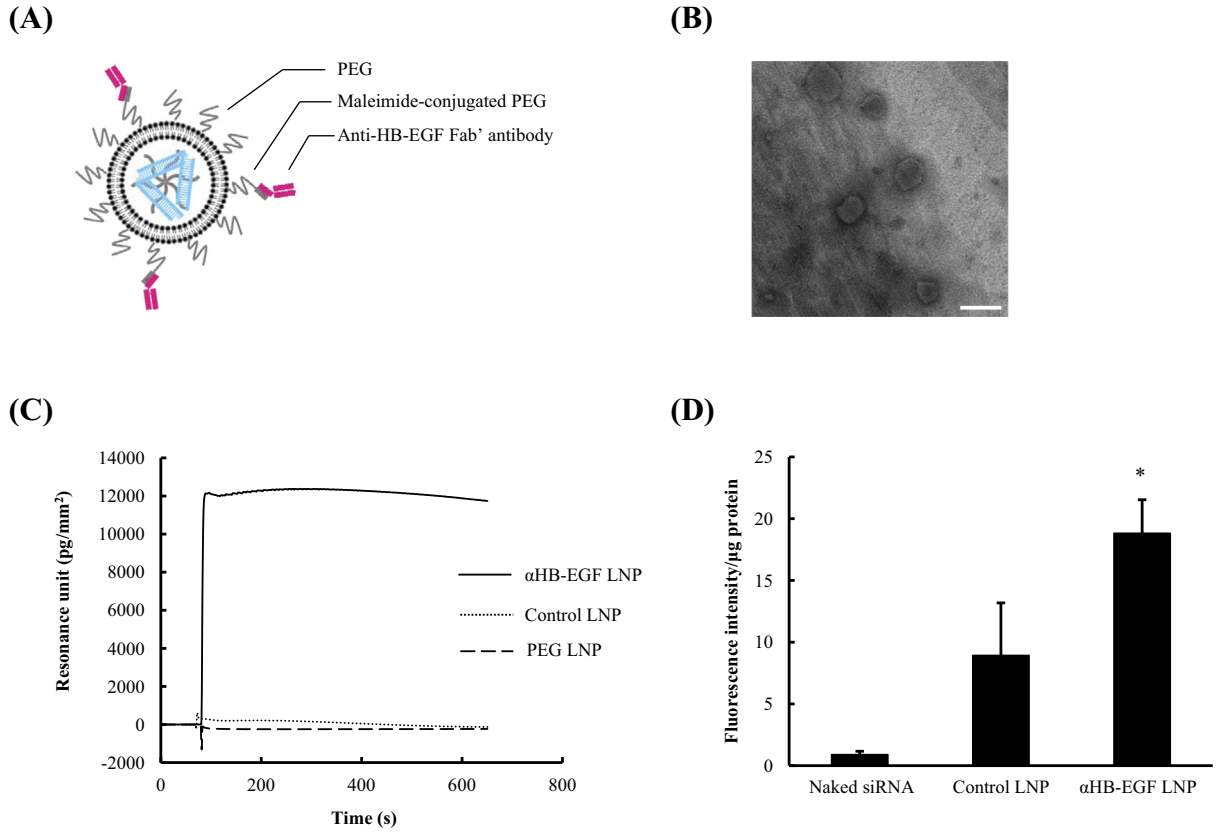

Fig. 1. Characteristics of αHB-EGF LNP-siRNA. (A) Schematic structure of αHB-EGF LNP-siRNA. DSPE-PEG and DSPE-PEG-maleimide were inserted into LNP-siRNA, and the modified nanoparticles were then decorated with anti-HB-EGF Fab' antibody. (B) TEM image of αHB-EGF LNP-siRNA after negative staining with ammonium molybdate. Scale bar indicates 200 nm. (C) Biacore analysis of αHB-EGF LNP-siRNA by use of a sensor chip coated with rhHB-EGF protein. αHB-EGF LNP-siRNA, Control LNP-siRNA or PEG-mal-LNP-siRNA were applied for 10 min to sensitize the sensor chip of the Biacore system. (D) Association of αHB-EGF LNP-siRNA with Vero-H cells. Naked FITC-siRNA (Control) or FITC-siRNA formulated in Control LNP or αHB-EGF LNP was incubated with Vero-H cells for 24 h at 37 °C. After the cells had been lysed, the fluorescence intensity of FITC-siRNA was determined and corrected by the protein contents. An asterisk indicates significant difference (*$P < 0.05$ *vs.* Control LNP-siRNA).

was changed to a fresh one, and then the cells were cultured for an additional 48 h. The cells were subsequently washed with PBS and lysed with lysis buffer composed of 10 mM Tris–HCl (pH 7.5), 0.15 M NaCl, 0.1% sodium dodecyl sulfate (SDS, Wako Pure Chemical Industries, Ltd.), and protease inhibitors (1 mM PMSF, 2 μg/mL aprotinin, 2 μg/mL leupeptin and 2 μg/mL pepstatin A). Total protein content was measured with a BCA Protein Assay Reagent Kit. Cell lysates were subjected to 10% SDS–PAGE and transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA). After having been blocked for 1 h at 37 °C with 5% bovine serum albumin (BSA, Sigma–Aldrich) in Tris–HCl-buffered saline containing 0.1% Tween 20 (TTBS, pH 7.4), the membrane was incubated with a primary antibody against Lamin A/C or β-actin for 24 h at 4 °C, and then with an HRP-conjugated secondary antibody for 1 h at room temperature. Each sample was developed by use of a chemiluminescent substrate (ECL-prime, GE Healthcare), and the chemiluminescence was detected with a LAS-3000 mini system (Fuji Film, Tokyo, Japan).

2.11. Statistical analysis

Differences between groups were evaluated by analysis of variance (ANOVA) with the Tukey *post-hoc* test.

3. Results

3.1. Physicochemical characteristics of αHB-EGF LNP

Particle size and ζ-potential of cationic cores, LNP-siRNA, and αHB-EGF LNP-siRNA are shown in Table 1. Complexes of siRNA and palmitoyl RRRRRRGGRRRRG peptide formed approximately 100-nm cationic cores having a positive charge. LNP-siRNA, i.e., the cores wrapped with lipids containing the anionic phospholipid DMPG, showed an anionic surface charge. The particle size was slightly increased to approximately 130 nm by lipid coating of the cores. After modification of LNP-siRNA with anti-HB-EGF Fab' antibody by using maleimide-PEG-DSPE, the particle size was

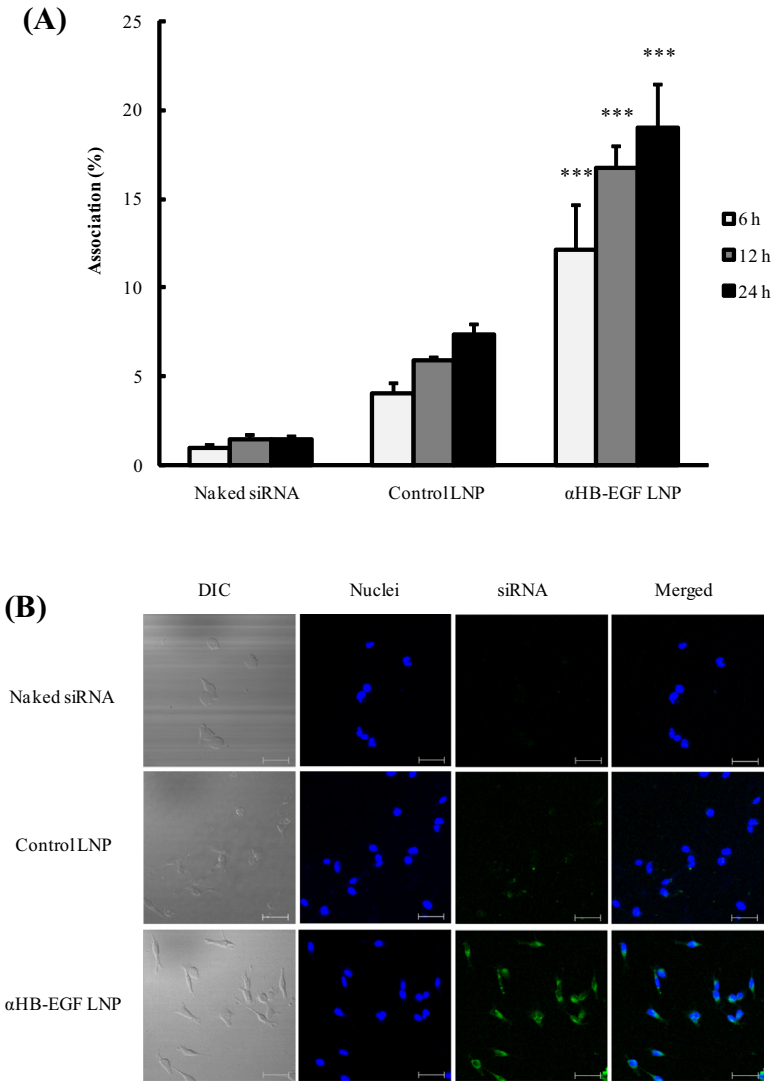

Fig. 2. Uptake of αHB-EGF LNP-siRNA into MDA-MB-231 cells. (A) Association of αHB-EGF LNP-siRNA with MDA-MB-231 cells. Naked FITC-siRNA (Control) or FITC-siRNA formulated in Control LNP or αHB-EGF LNP was incubated with MDA-MB-231 cells for 6, 12 or 24 h at 37 °C. After the cells had been lysed, the fluorescence intensity of the FITC-siRNA was determined. Data are presented as percentages (with SD bars) of siRNA detected in the cell lysate to that in the whole amount added. Asterisks indicate significant differences (***$P < 0.001$ *vs.* Control LNP-siRNA). (B) Intracellular distribution of siRNA in MDA-MB-231 cells that had been transfected with αHB-EGF LNP bearing FITC-labeled siRNA FITC-siRNA (green) taken up into the cells was observed by confocal laser-scanning microscopy. MDA-MB-231 cells were incubated with naked FITC-siRNA, Control LNP-FITC-siRNA, or αHB-EGF LNP-FITC-siRNA for 24 h at 37 °C. The nuclei were stained with DAPI (blue). The scale bars indicate 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

slightly increased further to approximately 160 nm. The ζ-potential of αHB-EGF LNP-siRNA was closer to neutral compared with that of LNP-siRNA. A schematic structure of αHB-EGF LNP-siRNA is shown in Fig. 1A. Anti-HB-EGF Fab' antibody-grafted DSPE-PEG by using DSPE-PEG-maleimide and non-grafted DSPE-PEG were used to modify the surface of LNP-siRNA. The spherical structure of αHB-EGF LNP-siRNA was confirmed by TEM observation (Fig. 1B). The particle size of αHB-EGF LNP-siRNA observed by TEM was about 150 nm. This result was consistent with the laser light-scattering data. The modification efficiency of the Fab' antibody to PEG-mal-LNP-siRNA was determined using high-performance liquid chromatography. When 25 nmol of the Fab' antibody had been used in the reaction mixture, approximately 15 nmol was recovered in the αHB-EGF LNP-siRNA fraction, indicating a modification efficiency of approx. 60%.

3.2. Targetability of αHB-EGF LNP-siRNA

The binding affinity of αHB-EGF LNP-siRNA for rhHB-EGF protein was assessed by use of a Biacore system having the sensor chip coated with this protein. As a result, αHB-EGF LNP-siRNA showed considerably high binding affinity toward human HB-EGF protein, whereas the Control LNP-siRNA and PEG-LNP-siRNA showed no binding at all (Fig. 1C). Next, FITC-labeled siRNA formulated in αHB-EGF LNP was used to transfect Vero-H cells (Vero cells overexpressing HB-EGF) to determine the targetability of αHB-EGF LNP-siRNA *in vitro*. Twenty-four hours after the transfection, αHB-EGF LNP-siRNA were significantly bound to the surface of the cells and/or taken up into the cells compared to the naked siRNA or Control LNP-siRNA (Fig. 1D).

3.3. Uptake of αHB-EGF LNP-siRNA into MDA-MB-231 cells

Overexpression of HB-EGF in MDA-MB-231 triple-negative breast cancer cells was already demonstrated in our previous study [18]. Here, the association of αHB-EGF LNP-siRNA with MDA-MB-231 cells was examined by use of FITC-labeled siRNA. As shown in Fig. 2A, αHB-EGF LNP-siRNA were significantly bound to the surface of the cells and/or taken up into the cells compared to the naked siRNA or Control LNP-siRNA. In addition, the amount of association increased in a time-dependent manner. Then, the intracellular distribution of FITC-siRNA in the transfected MDA-MB-231 cells was observed by confocal laser-scanning microscopy. As a result, FITC-siRNA delivered in the αHB-EGF LNP was homogeneously distributed throughout the cytoplasm of individual cells (Fig. 2B). In contrast, the fluorescence was quite weak or hardly observed when FITC-siRNA was delivered via the Control LNP-siRNA or applied in its naked form, respectively.

3.4. Gene silencing effects of αHB-EGF LNP-siLamin

After MDA-MB-231 cells had been transfected with αHB-EGF LNP-siLamin, knockdown of Lamin A/C mRNA and protein was determined by use of RT-PCR and Western blotting, respectively. αHB-EGF LNP-siLamin showed approximately 80% knockdown of Lamin A/C mRNA, which was significantly different from that obtained with the control (RNase-free water) or Control LNP-siLamin (Fig. 3A). Control LNP-siLamin showed approximately 50% knockdown of Lamin A/C mRNA. Importantly, the αHB-EGF LNP-siLamin clearly suppressed the expression of Lamin A/C protein compared with the control and Control LNP-siLamin (Fig. 3B).

4. Discussion

Specific antibody is expected to have excellent characteristics for use in targeted delivery of siRNA for the following reasons: the specificity and binding affinity are considerably high [22]; internalization occurs via receptor-mediated endocytosis [23]; and practical utility is demonstrated in the clinical setting [24]. For these reasons, we concluded that LNP modified with an antibody is a promising vector for delivering siRNA into the cytoplasm of target cells safely and specifically.

Therefore, in the present study, we conjugated anti-HB-EGF Fab' antibody to the LNP for targeted delivery of siRNA and showed selective gene silencing using αHB-EGF LNP-siRNA. As was shown in Fig. 1C, αHB-EGF LNP-siRNA significantly bound to rhHB-EGF protein attached to the Biacore chip, indicating that the Fab' fragments of the anti-HB-EGF antibody should function adequately as a targeting ligand. In addition, αHB-EGF LNP-siRNA were significantly associated with Vero-H cells compared to the association obtained with Control LNP-siRNA (Fig. 1D). Such selective association of αHB-EGF LNP-siRNA was not observed with control Vero cells, which hardly express HB-EGF (data not shown). These data suggest that αHB-EGF LNP-siRNA were associated with Vero-H cells via the membrane-anchored form of HB-EGF.

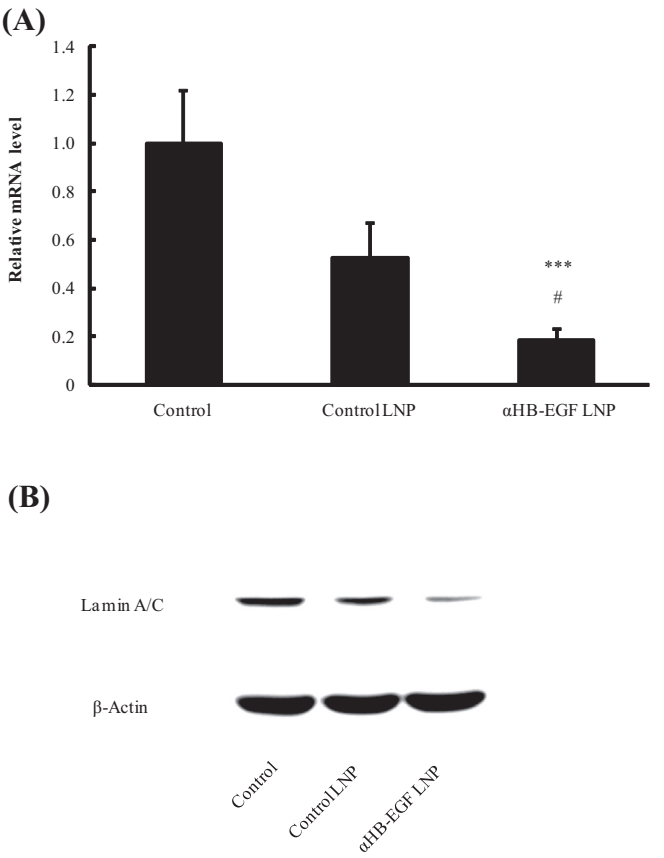

Fig. 3. Gene silencing induced by αHB-EGF LNP-siLamin. (A) Knockdown of Lamin A/C mRNA by αHB-EGF LNP-siLamin. MDA-MB-231 cells were transfected for 24 h with siLamin formulated in Control LNP or αHB-EGF LNP. Other cells were treated with RNase-free water as a control. The expression of Lamin A/C mRNA was determined by real-time RT-PCR. Data are presented as relative expression level of Lamin A/C mRNA to that in the control (RNase-free water) with SD bars. Symbols indicate significant differences ($^{***}P < 0.001$ *vs.* control, $^{\#}P < 0.05$ *vs.* Control LNP). (B) Knockdown of Lamin A/C protein by αHB-EGF LNP-siLamin. MDA-MB-231 cells were incubated for 24 h with siLamin formulated in Control LNP or αHB-EGF LNP. After a medium change, the cells were cultured for an additional 48 h (total of 72 h from the start of transfection). The expression of Lamin A/C and β-actin was determined by Western blotting.

A ligand for the membrane-anchored form of HB-EGF has been reported to be internalized by ligand-mediated receptor endocytosis [25,26]. As expected, αHB-EGF LNP-siRNA were highly taken up into MDA-MB-231 cells which highly express HB-EGF (Fig. 2). Because siRNA was homogeneously distributed throughout the cytoplasm of the cells by delivery in αHB-EGF LNP, the endocytotic pathway via the membrane-anchored form of HB-EGF might be useful for siRNA delivery. The data on gene silencing (Fig. 3) also support efficient and selective siRNA delivery by αHB-EGF LNP.

We previously reported that siRNA encapsulated in LNP is stable even when exposed to 90% FBS for 24 h [13]. In addition, αHB-EGF LNP-siRNA would be expected to have a long half-life in the bloodstream; because the conjugation of Fab' antibody to PEGylated liposomes reportedly does not substantially interfere with their long circulating property [27]. As was shown in Fig. 1B and Table 1, the particle size of αHB-EGF LNP-siRNA appears to be suitable for delivering siRNA to tumors through the gaps in angiogenic vessels. Although *in vivo* studies have not yet been performed, the present findings suggest that αHB-EGF LNP are promising for systemic delivery of siRNA.

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