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Hyaluronic acid controls the uptake pathway and intracellular trafficking of an octaarginine-modified gene vector in CD44 positive- and CD44 negative-cells

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Abstract

The cellular uptake pathway for a gene vector is an important factor in transgene expression. We previously constructed an original gene vector, multifunctional envelope-type nano device (MEND). The use of octaarginine (R8), a cell-penetrating peptide dramatically enhanced the transfection activity of the MEND since efficient cellular uptake via macropinocytosis, while the R8 should overcome its poor cell selectivity. Here we prepared an R8-MEND equipped with GALA (a peptide for endosomal escape) (R8/GALA-MEND) coated with hyaluronic acid (HA) (HA-R8/GALA-MEND), a natural ligand for cancer cells overexpressing CD44. We investigated the cellular uptake pathway of the HA-R8/GALA-MEND and the R8/GALA-MEND using HCT116 cells overexpressing CD44. Both carriers were taken up by cells mainly via macropinocytosis, whereas only the HA-R8/GALA-MEND was partially internalized into cells via a CD44-mediated pathway. Investigation of transgene expression showed that the HA-R8/GALA-MEND had a high transfection activity in HCT116 cells via both macropinocytotic and CD44-mediated pathways. On the other hand, the value for the HA-R8/GALA-MEND was significantly decreased compared with the value for the R8/GALA-MEND in NIH3T3 cells (CD44-negative cells). These findings indicate that the HA-coating controls the intracellular pathway for R8-modified

nanocarriers, and that a CD44-mediated pathway is an important route for transgene expression.

1. Introduction

The cellular uptake pathway of a gene vector is a major factor in transgene expression [1-5]. We previously reported that carriers with a high-density of octaarginine (R8), an artificially designed cell penetrating peptide [6, 7], were efficiently internalized by cells primarily via macropinocytosis rather than clathrin-mediated endocytosis, as is the case for cationic liposomes (LPs) [8]. We also developed an R8-modified gene vector, a multifunctional envelope-type nano device (MEND), consisting of a condensed plasmid DNA (pDNA) core and lipid envelopes [9, 10]. The integration of R8 into the MEND (R8-MEND) dramatically enhanced the transfection activity of the MEND, approaching values as high as that for adenovirus [9, 11], while the R8 needs to overcome its poor cell selectivity, since it facilitates the cellular uptake nonspecifically regardless of the cell type.

To date, our group has reported that a dual-ligand liposomal system comprised of a specific ligand and a cell-penetrating peptide (CPP) enhanced both selectivity and cellular uptake efficiency [12, 13]. Takara *et al* prepared dual-ligand PEGylated liposomes by modifying the end of the polyethylene-glycol (PEG) with an NGR (Asn-Gly-Arg) tumor neovasculature-homing motif peptide, which recognizes CD13, a marker for tumor endothelial cells, followed by coating the surface of the liposomes

with CPP [12]. The dual-ligand system stimulated the uptake of the carriers by CD13 positive cells, synergistically [12]. Kibria *et al* also reported that dual-ligand PEGylated R8-modifeid liposomes with the cyclic RGD (Arg-Gly-Asp) peptide, a specific ligand for Integrin $\alpha_v\beta_3$, showed an enhanced cellular uptake as well as a higher transfection efficiency in Integrin $\alpha_v\beta_3$ expressing cells [13]. These reports indicate that a dual-ligand liposomal system can be a useful strategy for achieving cell selective targeting with a high transgene expression.

More recently, we developed a dual-ligand positively charged R8-MEND that was modified with negatively charged hyaluronic acid (HA) via electrostatic interactions but not with PEG [14]. HA is a natural ligand for cancer cells and liver endothelial cells overexpressing CD44, thus it would be expected that HA would be a specific ligand for / targeted to cancer and liver endothelial cells. In that study, we investigated the transgene expression of an R8-MEND modified with HA in liver endothelial cells (liver ECs) (CD44 positive cells), and determined the optimal composition of MEND needed for efficient transgene expression in liver ECs, which possesses an HA-coated lipid envelope modified with the R8 [6, 7] and GALA, a pH-sensitive fusogenic peptide for efficient endosomal escape [15, 16] (HA-R8/GALA-MEND).

The focus of this study was on the mechanism responsible for the cellular uptake pathway and transgene expression of the HA-R8/GALA-MEND. A series of R8/GALA-MENDs coated with various concentrations of HA (600 kDa or 80 kDa) were prepared and the physicochemical properties and the transfection activity of these preparations in HCT116 cells overexpressing CD44 were evaluated. We then investigated the cellular uptake pathway of the HA-R8/GALA-MEND and the R8-MEND using HCT116 cells, and determined the optimal composition of HA-R8/GALA-MEND required for efficient cellular uptake via a CD44-mediated pathway. Moreover, we compared the cellular uptake pathway and transfection activity of HA-R8/GALA-MEND between HCT116 cells and NIH3T3 cells (CD44-negative cells). Based on these results, we analyzed the relationship between the cellular uptake pathway and transgene expression in HCT116 cells and NIH3T3 cells. A model for transgene expression via a CD44-mediated pathway is proposed in an attempt to understand the intracellular fate of HA-coated gene vectors.

2. **Materials and methods**

2.1. Materials

The pcDNA3.1 (+)-luc plasmid was constructed by inserting the firefly luciferase

gene (*Hind*III–*Xba*I fragment) of the pGL3-Control plasmid (Promega, Madison, WI, USA) into the pcDNA 3.1 (+) plasmid containing cytomegalovirus promoter (Invitrogen, Carlsbad, CA, USA) pretreated with the same restriction enzymes. The pDNA was purified using a Qiagen EndoFree Plasmid Mega Kit (Qiagen GmbH, Hilden, Germany). Amiloride and cholesteryl hemisuccinate (CHEMS) was purchased from Sigma (St. Louis, MO, USA). 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) was purchased from AVANTI Polar Lipids Inc. (Alabaster, AL, USA). Stearyl octaarginine (STR-R8) [6, 17] and cholesteryl GALA peptide (Cho-GALA) [15, 16] was obtained from Kurabo Industries, Ltd. (Osaka, Japan). Protamine was purchased from CALBIO CHEM (Darmstadt, Germany). HA (average MW 50-110 kDa, HA (80 kDa); average MW 500-700 kDa, HA (600 kDa)) was obtained from Food Chemifa (Tokyo, Japan). Hoechst 33342 was purchased by DOJINDO Laboratories (Kumamoto, Japan). Fc Receptor Saturation Reagent was purchased from Beckman Coulter, Inc. (Fullerton, CA, USA). Fluorescein isothiocyanate (FITC)-labeled CD44-antibody was purchased from Abcam (Cambridge, United Kingdom). Cy5 labeled pDNA (Cy5-pDNA) was prepared using a *Label* IT® Cy™ 5 Labeling Kit (Takara Bio INC, Shiga, Japan), essentially according to the supplier's instructions. All other chemicals were commercially available, reagent-grade products.

2.2. Cell lines and cell cultures

Human colorectal carcinoma HCT116 cells and mouse embryonic fibroblast NIH3T3 cells were purchased from ATCC (Manassas, VA, USA). HCT116 cells were maintained in McCoy's 5A modified medium (ATCC) with 10% fetal bovine serum (FBS, HyClone; Thermo Fisher Scientific K.K., Waltham, MA, USA), penicillin (100 units/mL) and streptomycin (100 μg). NIH3T3 cells were maintained in Dulbecco's modified Eagle medium (Invitrogen) with 10% FBS, penicillin (100 units/mL) and streptomycin (100 μg). These cells were cultured under an atmosphere of 5% CO_2/air at 37°C. In this experiment, an HCT116 cell line and an NIH3T3 cell line were used as a CD44-positive and CD44-negative cells, respectively.

2.3. Preparation of R8/GALA-MEND and coating the carrier with HA

The R8/GALA-MEND was prepared by the lipid film hydration method, as previously reported [9, 14]. First, pDNA was condensed with polycations to form condensed pDNA particles. The pDNA (0.1 mg/mL) and protamine solutions were mixed in 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4) under vortexing at a nitrogen/phosphate (N/P) ratio of 2.2. Next, lipid films

were produced on the bottom of a glass tube by the evaporation of a chloroform solution containing 138 nmol of lipids (DOPE/CHEMS/Chol-GALA = 9:2:0.2 (molar ratio)). Next, 250 μL of the condensed pDNA particle solution was applied to the lipid film, followed by incubation for 15 min at room temperature to hydrate the lipids. The lipid film was then sonicated for approximately 1 min in a bath-type sonicator (85 W, Aiwa Co., Tokyo, Japan) to produce GALA-MEND. A solution of STR-R8 (10 mol% lipids) was then incubated with the GALA-MEND for 30 min at room temperature to produce R8/GALA-MEND. To coat the R8/GALA-MEND with HA via electrostatic interaction, a solution of HA was added to the R8/GALA-MEND suspensions, and then incubated for 30 min at room temperature (HA-R8/GALA-MEND). To prepare MEND encapsulating Cy5-pDNA, condensed pDNA particles were prepared using Cy5-pDNA as a tracer (3% of total pDNA).

2.4. Preparation of florescent labeled HA-coated R8/GALA-modified liposomes

To observe the intracellular trafficking of the HA-coated nanocarriers, fluorescent labeled LPs were constructed, as described below. LPs containing rhodamine labeled DOPE (AVANTI Polar Lipids Inc.) were prepared by the lipid film hydration method. Lipid films were produced on the bottom of a glass tube by the evaporation of a

chloroform solution containing 138 nmol lipids [DOPE/CHEMS/Cho-GALA/rhodamine labeled DOPE = 9 : 2 : 0.2 : 0.005 (molar ratio)]. Next, 250 μL of 10 mM HEPES buffer (pH 7.4) was applied to the lipid film, followed by incubation for 15 min at room temperature to hydrate the lipids. The lipid film was then sonicated for approximately 1 min in a bath-type sonicator. The resulting suspension was then incubated with a solution of STR-R8 (10 mol% lipids) for 30 min at room temperature to produce R8/GALA-LPs. To coat with HA via electrostatic interactions, a solution of the fluorescein labeled HA (average MW 100-300 kDa; PG Research, Tokyo, Japan) was added to R8/GALA-LPs suspensions (60 g HA / mol lipid), and the resulting mixture was then incubated for 30 min at room temperature (HA-R8/GALA-LP).

2.5. Characterization of prepared carriers

Dynamic light scattering was employed to determine the hydrodynamic diameters and polydispersity index (PDI) of the nanoparticles (Zetasizer Nano ZS; Malvern Instruments, Herrenberg, Germany). The mean diameter was calculated from a cumulants analysis based on the intensity of the scattered light. We also measured the ζ potentials using Laser doppler velocimetry (Zetasizer Nano ZS).

2.6. Transfection and evaluation of transgene expression

Transfection activity was assessed by measurement of luciferase activity described below. Cells (1 x 10^4 cells/well) were seeded on a 96-well plate (greiner bio-one; Frickenhausen, Germany) with complete medium, under an atmosphere of 5% CO_2 / air at 37ºC for 24 hr. After the cells were washed with phosphate-buffered saline (PBS (-)), samples containing pDNA suspended in 70 μL of serum-free medium were added to the cells. After incubation under 5% CO_2 at 37ºC for 3 hr, the cells were washed with PBS (-), and then further incubated for 21 hr in fresh complete medium. The cells were then washed with PBS (-), and luciferase activity (relative light unit (RLU)) was measured using a Luciferase Assay System with a Reporter Lysis Buffer kit (Promega; Madison, WI, USA) by means of a EnSpire™ 2300 Multilabel Reader (PerkinElmer, Inc.; Waltham, MA, USA).

When the CD44-mediated pathway was inhibited, an FITC-labeled CD44-antibody was added to the cells 1 hr before incubation with the carriers. To investigate the effect of CD44-mediated pathway on transgene expression, the relative gene expression as a percent of the transfection activity in the absence of inhibitor was calculated as follows:

Relative gene expression (%) = transfection activity with inhibitor (RLU/well)/

transfection activity without inhibitor (RLU/well) x 100 (1)

2.7. Quantification of the cellular uptake of the carriers by flow cytometry

Cells (1 x 10^5 cells) were seeded on a 12-well plate (BD Falcon; Becton Dickinson, Franklin Lakes, NJ, USA) with complete medium, under an atmosphere of 5% CO_2 / air at 37ºC for 24 hr. The cells were washed with PBS (-) before incubation with the HA-R8/GALA-MEND or R8/GALA-MEND encapsulating Cy5-pDNA (total dose of pDNA, 1 μg). The cells were then atmosphere of 5% CO_2/air at 37ºC. After removing the medium, the cells were washed once with ice-cold PBS (-). The cells were then collected, suspended in complete medium, isolated by centrifugation (1,000 *g*, 4ºC, 5 min) and washed with FACS buffer (0.5% bovine serum albumin and 0.1% NaN_3 in PBS (-)). After resuspension in 0.5 mL of FACS buffer, the cell suspension was filtered through a nylon mesh to remove cell aggregates and dust. The cells were then analyzed by flow cytometry (FACScan; Becton Dickinson). The cells were excited with a 635 nm light from an LD laser for detecting Cy5-pDNA. The fluorescence detection channel was set to the FL4 filter for Cy5-pDNA. The cellular uptake of the carriers was expressed as the mean fluorescence intensity, calculated using the CellQuest software (Becton Dickinson).

These experiments were performed in the presence or absence of an inhibitor. To inhibit CD44-mediated uptake, an FITC-labeled CD44-antibody was added to the cells 1 hr before incubation with the carriers. While, amiloride was used as a macropinocytic uptake inhibitor, and it was added to cells 30 min before with the carriers.

2.8. Visualization of HA coated R8/GALA-modified liposomes in HCT116 cells using confocal laser scanning microscopy

Cells (2 x 10^5 cells) were cultured in 35 mm dishes (IWAKI, Tokyo, Japan) with complete medium under an atmosphere of 5% CO_2 / air at 37ºC for 24 hr. The cells were washed with PBS (-), and the fluorescent labeled HA-R8/GALA-LPs were added to the cells (final lipid concentration, 55 μM). The carrier-envelope was labeled with rhodamine, and the carrier was then coated with fluorescein labeled HA as described above. The cells were then incubated in phenol red-free medium in the absence of serum under an atmosphere of 5% CO_2/air at 37ºC. After a 1-hr incubation, the cells were washed with PBS (-), and then incubated with the in phenol red-free medium containing Hoechst 33342 (final concentration, 5 μg/mL) in the absence of serum to stain the nucleus. After incubation (5% CO_2 / air, 37ºC, 10 min), the cells were washed with PBS (-). The medium was then replaced with fresh phenol red-free complete medium, and

observed by confocal laser scanning microscopy (CLSM) (Nikon A1; Nikon Co. Ltd., Tokyo, Japan). The cells were excited with 405 nm wavelength light from a Diode laser, 488 nm wavelength light from an Ar laser and 561 nm wavelength light from a DPSS laser. A series of images were obtained using a Nikon A1 confocal imaging system equipped with a water immersion objective lens (Plan Apo 60x1.20 PFS WI) and a 1st dichroic mirror (405/488/561/640). The three fluorescence detection channels were set to the following filters: 450/50 (blue color) for nuclei, 525/50 (green color) for fluorescein labeled HA and 595/50 (red color) for a rhodamine labeled carrier-envelope.

2.9. Quantification of expression levels for CD44 in HCT116 and NIH3T3 cells

Cells (2 x 10^5 cells) were collected, suspended in 40 μL of Fc Receptor Saturation Reagent and incubated on ice for 30 min. After the incubation, the cell suspensions were washed twice with FACS buffer, precipitated by centrifugation (1,000 *g*, 4ºC, 5 min). The pellet was suspended in 50 μL of an FITC-labeled CD44-antibody solution (50 μg/mL) and incubated on ice for 30 min. The resulting suspension was washed twice with FACS buffer, precipitated by centrifugation (1,000 *g*, 4ºC, 5 min). After resuspension in 0.5 mL of FACS buffer, the cell suspension was filtered through a nylon mesh to remove cell aggregates and dust. The cells were then analyzed by flow

cytometry (FACScan). The cells were excited with a 488 nm light from an Ar laser. The fluorescence detection channel was set to the FL1 filter for FITC-labeled CD44-antibody. The CD44 expressions were expressed as the mean fluorescence intensity, calculated using the CellQuest software (Becton Dickinson).

2.10. *Observation of CD44 in HCT116 cells using confocal laser scanning microscopy*

Cells (2 x 10^5 cells) were cultured in 35 mm dishes (IWAKI) with complete medium under an atmosphere of 5% CO_2 / air at 37ºC for 24 hr. The cells were washed with PBS (-), and the FITC-labeled CD44-antibody were added to the cells (final concentration, 5 μg/mL). The cells were then incubated in phenol red-free medium in the absence of serum under an atmosphere of 5% CO_2/air at 37ºC. After a 1-hr incubation, the cells were washed with PBS (-), and then incubated in a phenol red-free medium containing Hoechst 33342 (final concentration, 5 μg/mL) in the absence of serum to stain the nucleus. After incubation (5% CO_2 / air, 37ºC, 10 min), the cells were washed with PBS (-). The medium was then replaced with fresh phenol red-free complete medium, and observed by CLSM (Nikon A1). The cells were excited with 405 nm wavelength light from a Diode laser and 488 nm wavelength light from an Ar laser. A series of images were obtained using a Nikon A1 confocal imaging system equipped

with a water immersion objective lens (Plan Apo 60x1.20 PFS WI) and a 1st dichroic mirror (405/488/561/640). The two fluorescence detection channels were set to the following filters: 450/50 (blue color) for nuclei and 525/50 (green color) for FITC-labeled CD44-antibody.

2.11. Statistical analysis

Data are expressed as the mean ± S.D. for the indicated number of experiments. For comparison between two groups, the two tail unpaired student's t-test was used. For multiple comparisons of the cellular uptake of carriers in Result 3.3, one way ANOVA was used. To investigate the effect of the CD44-mediated pathway on transgene expression in Result 3.6, we performed a two-way ANOVA analysis to compare the effect of multiple levels of two factors that are "cell line" and "carrier type". If there was significant interaction between two factors, simple main effect test was also performed. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Preparation of HA-R8/GALA-MEND

A series of R8/GALA-MEND coated with various concentrations of HA (600 kDa or 80 kDa) was prepared as shown in Figure 1. To construct the R8/GALA-MEND, condensed pDNA particles were packaged with a lipid envelope modified with the R8 peptide, a cellular uptake device [7, 8, 11, 17], and GALA, a pH-sensitive fusogenic peptide [15, 16]. In the preparation, HA coats the carrier surface via electrostatic interactions between the positively charged R8/GALA-MEND (~ +50 mV) and the negatively charged HA by simply mixing it with the R8/GALA-MEND. The physicochemical properties of the prepared HA-coated R8/GALA-MENDs (HA-R8/GALA-MENDs) are summarized in Figure 2 and Table 1. The ζ potential of the prepared liposomes was determined as a measure of their surface charge (Fig. 2A (a), 2B (a)). Increasing the concentration of HA caused an initial rapid decrease in ζ potential followed by a charge reversal to a negative charge when the concentration of HA exceeded 18 g HA/ mol lipid, suggesting that HA had fully coated the carrier-surface.

We also observed that the particle-size and the distribution of the carrier changed, depending on the coating ratios of HA. HA-R8/GALA-MENDs were aggregated at 12 g HA / mol lipid, carriers with diameters of ~ 200 nm were prepared at 18 – 60 g HA / mol lipid, and the particle size was increased when the level of HA exceeded 120 g / mol

lipid (Fig. 2A (b), 2B (b)). The PDI of the prepared liposomes was determined to be an indicator of the particle-size distribution (Table 1). HA-R8/GALA-MENDs coated at 18-60 g HA / mol lipid was highly homogeneous (less than 0.25 in PDI value, Table 1). Based on these results, negatively charged homogenous particles could be prepared at 18 - 60 g HA / mol lipid (< 300 nm, < -25 mV, < 0.25 PDI), when the R8/GALA-MEND was simply mixed with HA independent its molecular weight.

3.2. Investigation of the transfection activities of HA-R8/GALA-MEND

We next evaluated the transfection activities of various types of R8/GALA-MENDs coated with HA (600 kDa) or HA (80 kDa) (Fig. 3), to determine which HA-R8/GALA-MEND showed sufficient transfection activity for an analysis of the cellular uptake pathway. The carriers encapsulating 0.04 - 1 μg of pDNA encoding the luciferase reporter gene were transfected to HCT116 cells, which overexpress CD44, which is readily recognized by HA [18], and their transfection activities were then evaluated by measuring the luciferase activity. The transfection activities of all of the HA-R8/GALA-MEND were high and similar to that for the R8/GALA-MEND. Taking the physicochemical properties and transfection activities into consideration, we chose the R8/GALA-MEND coated with HA (600 kDa) and HA (80 kDa) at 60 g HA / mol

lipid, which are the highest coating ratio of HA, among the small sized and homogenous nanoparticles (< 300 nm, < 0.25 PDI).

3.3. Comparison of cellular uptake efficiency between the HA-R8/GALA-MEND and the R8/GALA-MEND using HCT116 cells

We quantitatively compared the cellular uptake of HA (600 kDa)-R8/GALA-MEND, HA (80 kDa)-R8/GALA-MEND and R8/GALA-MEND encapsulating Cy5-pDNA by flow cytometry. As shown in Figure 4A, both HA (600 kDa)-R8/GALA-MEND and HA (80 kDa)-R8/GALA-MEND were effectively internalized into HCT116 cells, similar to the R8/GALA-MEND, even though the HA-R8/GALA-MENDs have a negative charge (Figs. 2A (a), 2B (a)). This interesting tendency for anionic carriers being effectively internalized by cells, which have negatively charged cell membranes, is consistent with previously reported findings [14]. To validate whether the carriers with HA are internalized by cells, we visualized the intracellular trafficking of HA coated carriers using HCT116 cells. R8/GALA-modified carriers labeled with rhodamine (red) coated with fluorescein-labeled HA (green) were incubated with HCT116 cells, and intracellular trafficking was then observed using CLSM (Fig. 4B). We observed several yellow clusters, indicating that HA was localized

with carriers, suggesting that the carriers with HA were internalized into cells.

3.4. Cellular uptake analysis of HA-R8/GALA-MEND in HCT116

To investigate the contribution of the CD44-mediated pathway, we evaluated the cellular uptake of HA-R8/GALA-MEND by HCT116 cells, which overexpress CD44. Carriers with encapsulated Cy5-pDNA were added to the incubation mixture in the presence or absence of an anti-CD44 antibody to specifically inhibit CD44-mediated internalization, and the fluorescent intensity of the carriers taken up by cells were then measured by flow cytometry (Fig. 5A). The anti-CD44 antibody inhibited the uptake of HA (600 kDa)-R8/GALA-MEND and HA (80 kDa)-R8/GALA-MEND, but had no effect on the uptake of the R8/GALA-MEND. We also found that the cellular uptake of the HA (600 kDa)-R8/GALA-MEND was inhibited to a greater extent than that of HA (80 kDa)-R8/GALA-MEND.

A previous study showed that R8-modifeid carriers were efficiently internalized into several types of cells including NIH3T3 cells via macropinocytosis [8]. Thus, the contribution of a macropinocytotic pathway for the carriers in HCT116 cells was investigated using amiloride, which inhibits the Na/H exchange required for macropinocytosis [19, 20] (Fig. 5B). The amiloride strongly inhibited the uptake of the

HA (600 kDa)-R8/GALA-MEND, the HA (80 kDa)-R8/GALA-MEND and the R8/GALA-MEND, indicating that macropinocytosis is the major uptake pathway for all these carriers. Taken together, these results indicate that R8/GALA-MEND, irrespective of whether they are coated with or without HA use macropinocytosis as the major entrance route, whereas only the R8/GALA-MEND coated with HA use the CD44-mediated pathway. The HA(600 kDa)-R8/GALA-MEND was used in further analyses of cellular uptake, because the cellular uptake of HA(600 kDa)-R8/GALA-MEND in HCT116 cells occurred largely via the CD44 pathway compared with HA(80 kDa)-R8/GALA-MEND.

3.5. Determine of the cellular uptake pathway of HA-R8/GALA-MEND in HCT116 cells (CD44 positive) and NIH3T3 cells (CD44 negative)

We compared the cellular uptake pathway of the HA-R8/GALA-MEND between CD44 high and low expression cells. HCT116 and NIH3T3 cells were utilized as CD44-positive and negative cells, and the expression levels of CD44 in these cells was measured using an FITC labeled anti-CD44 antibody. FACS analysis showed that HCT116 cells expressed CD44 at significantly higher levels than NIH3T3 cells (Fig. 6A). Moreover, visualization of CD44 (green signals) in HCT116 cells indicates that the

CD44 is displayed on the cell-surface (Fig. 6B).

The cellular uptakes of HA (600 kDa)-R8/GALA-MEND were investigated in the presence (closed bars) and absence (open bars) of an anti-CD44 antibody or amiloride (Fig. 6C). In the case of HCT116 cells, amiloride strongly inhibited the cellular uptake of the HA (600 kDa)-R8/GALA-MEND, whereas the anti-CD44 antibody showed only a mild inhibition (Fig. 6C (a)), suggesting that the HA (600 kDa)-R8/GALA-MEND was taken up by HCT116 cells via both macropinocytosis and a CD44-mediated pathway. On the other hand, the anti-CD44 antibody had no effect on the cellular uptake of the HA (600 kDa)-R8/GALA-MEND in NIH3T3, although, similar to HCT116, amiloride strongly inhibited cellular uptake (Fig. 6C (b)). These findings suggest that NIH3T3 cells internalize the HA (600 kDa)-R8/GALA-MEND not via a CD 44-mediated pathway but by macropinocytosis.

3.6. Investigation of the transfection activities of HA-R8/GALA-MEND between HCT116 and NIH3T3 cells

To investigate the influence of the cellular uptake pathway on transgene expression, we evaluated the transfection activity of the HA (600 kDa)-R8/GALA-MEND (open bars) and the R8/GALA-MEND (closed bars) using

HCT116 (CD44 positive) and NIH3T3 (CD44 negative) cells (Fig. 7). In the case of HCT116 cells, the HA (600 kDa)-R8/GALA-MEND showed a high transfection activity similar to R8/GALA-MEND (Fig. 7A). On the other hand, the transfection activity of HA (600 kDa)-R8/GALA-MEND was drastically decreased compared with the R8/GALA-MEND in NIH3T3 cells (Fig. 7B).

To investigate the effect of CD44-mediated pathway on transgene expression, we evaluated transgene expression in HCT116 cells and NIH3T3 cells in presence of an anti-CD44 antibody (inhibitor), using the HA (600 kDa)-R8/GALA-MEND (open bars) and the R8/GALA-MEND (closed bars) (Fig. 8). We calculated the relative gene expression as a percent of the transfection activity in the absence of an inhibitor, and also performed two-way ANOVA analyses. When the relative gene expression of the HA (600 kDa)-R8/GALA-MEND and the R8/GALA-MEND for each cell line was compared, in the case of HCT116 cells, the value for the HA (600 kDa)-R8/GALA-MEND was significantly decreased compared to that of the R8/GALA-MEND (*Significant difference ($p < 0.01$), closed bar vs. open bar in HCT116, Fig. 8). On the other hand, in the case of NIH3T3 cells, in which the HA (600 kDa)-R8/GALA-MEND was not internalized via a CD44-mediated pathway as shown in Fig. 6C (b), the use of an anti-CD44 antibody had no effect on transgene expression

(No significant difference ($p > 0.05$), closed bar vs. open bar in NIH3T3, Fig. 8). We next evaluated the relative gene expression among different cell lines, the values for the HA (600 kDa)-R8/GALA-MEND were found to be significantly decreased in HCT116 cells compared to NIH3T3 cells (**Significant difference ($p < 0.01$), open bars in Fig. 8). Based on these results, it can be concluded that the CD44-mediated pathway, one of uptake pathways for the HA (600 kDa)-R8/GALA-MEND, contributes to transgene expression in HCT116 cells, CD44-potiteve cells.

4. Discussion

In this study, we prepared an HA-R8/GALA-MEND, which possesses a lipid envelope modified with R8 and GALA in which the surface is coated with HA. In the preparation, HA coats the carrier surface, when the R8/GALA-MEND and HA are simply mixed with one another. The coating is held in place via electrostatic interactions and does not involve PEGylation, since PEGylation typically results in a decrease in the transfection activity of the carrier, as previously reported [14]. An HA-R8/GALA-MEND prepared using HAs with different molecular weights (600 kDa or 80 kDa) was investigated in terms of the physicochemical properties (Figure 2 and Table 1). We observed that the HA-R8/GALA-MENDs became aggregated when 12 g

HA / mol lipid (Figures 2A(b) and 2B(b)), which would occur when the carriers have an insufficient surface potential for electrostatic stabilization by neutralization (~0 mV in ζ-potentials) as shown in Figures 2A(a) and 2B(a). On the other hand, the increase in carrier-size at higher concentrations than 120 g HA / mol lipid might be contributed by high viscosity due to the high amounts of coated HA. The diameters and ζ potentials of the HA/R8-GALA-MEND could be adjusted by regulating the HA coating weight ratio (g HA /mol lipid), as result, stable HA coated carriers could be prepared at a concentration of 18 – 60 g HA / mol lipid, where the carriers would have sufficient surface potential to permit them to be electrostatically stabilized. We also observed similar a tendency for the physicochemical properties in both types of HA (600 kDa) and HA (80 kDa). These results suggest that similar weights of either type of HA coat the carrier surface at the same coating ratio, although numbers of the coating HA molecules would be different between HA (600 kDa) and HA (80 kDa).

An evaluation of the transfection activities of various types of carriers using HCT116 cells (Figure 3) showed that the transfection activities were increased when the dose of transfected pDNA was increased to 0.4 μg. However, the values were decreased in the case of both the HA-R8/GALA-MEND and the R8/GALA-MEND, when 1 μg of pDNA was transfected to the cell. A previous report showed that excess free cations

released from the gene vectors inhibited the post-transcription process including nuclear mRNA export and the translation process, resulting in low transgene expression [21]. Thus, it was presumed that an excess of cationically charged components in the carriers might affect transfection activity in the case of a high transfection dose.

An investigation of the contribution of a macropinocytotic pathway for the carriers using amiloride (Fig. 5B) indicated that the R8/GALA-MEND coated with or without HA involve macropinocytosis as the major entrance route. The HA coating on the carrier-surface was confirmed based on physicochemical properties such as ζ potentials (Fig. 2), and intracellular observations suggested that the carriers with HA were internalized by cells (Fig. 4B). These results indicate that HA coats the entire surface of the R8/GALA-MEND, but may not be able to inactivate the induction of macropinocytosis. As one possibility, it was presumed that the head group of R8 is displayed on the HA coated carrier-surface, and a part of the R8 could then induce macropinocytosis. In this situation, R8 and HA might function as a ligand for inducing macropinocytosis and the CD44-mediated uptake, respectively. We also concluded that the head group of R8 on the carrier-surface might be an important factor in interactions between the carrires and cell membrane, based on the result showing that the cellular uptake values for the R8/GALA-MEND with or without HA were comparable (Figure

4A).

Both the HA (600 kDa)-R8/GALA-MEND and the R8/GALA-MEND showed similar high transfection activities in the case of HCT116 (CD44 positive) cells (Fig. 7A), while the transfection activity of the HA (600 kDa)-R8/GALA-MEND was drastically decreased in NIH3T3 (CD44 negative) cells compared with that of the R8/GALA-MEND (Fig. 7B). On the other hand, the cellular uptake of both carriers were comparable in each type of cells (Fig. S1). These results suggest that the cellular uptake pathway after the carriers are internalized by cells can have an impact on transgene expression. Based on the analysis for cellular uptake pathway, a model for the intracellular trafficking events of the HA (600 kDa)-R8/GALA-MEND is proposed (Fig. 9). Here we discuss the influence of the cellular uptake pathway on transgene expression in each type of cell.

After being uptaken up via macropinocytosis (about 50%), the HA (600 kDa)-R8/GALA-MEND is able to escape efficiently for transgene expression in both HCT116 cells and NIH3T3 cells, because it is well known that macropinocytosis is an advantageous cellular uptake pathway for endosomal escape [1, 8]. The HA (600 kDa)-R8/GALA-MEND is also internalized by HCT116 cells via a CD44-mediated pathway (about 25%) and another pathway, such as clathrin-mediated endocytosis

leading to lysosomal degradation. As shown in Figure 8, the value for the HA (600 kDa)-R8/GALA-MEND was drastically decreased in the presence of anti-CD44 antibody. This result suggests that CD44-mediated pathway would be largely contributed to the transgene expression in HCT116 cells.

On the other hand, anti-CD44 antibody had no effect on the cellular uptake and transgene expression of the HA (600 kDa)-R8/GALA-MEND in NIH3T3 (Figs. 6C (b), 8), indicating that the HA (600 kDa)-R8/GALA-MEND is internalized by the cells via a different pathway (about 50%) such as clathrin-mediated endocytosis, a disadvantageous pathway for a transgene. As shown in Figure 7, the transfection activities of the HA (600 kDa)-R8/GALA-MEND was drastically decreased compared to the R8/GALA-MEND in NIH3T3 cells. This result may be explained by considering that the hydration layer formed by HA in the carrier surface inhibits membrane fusion between the carriers and the endosomal membrane, resulting in a low endosomal escape and a low transfection activity. This result may be explained by considering that the hydration layer formed by HA in the carrier surface inhibits membrane fusion between the carriers and the endosomal membrane, resulting in a low endosomal escape and a low transfection activity. This HA coating on the carrier surface might have disturbed the endosomal escape activity of the GALA that was used to modify the same carrier-surface, when the

carriers were internalized into cells via a disadvantageous pathway for a transgene such as clathrin-mediated endocytosis. On the other hand, in the case of HCT116 cells, the HA (600 kDa)-R8/GALA-MEND showed a high transfection activity similar to the R8/GALA-MEND (Fig. 7A), suggesting that a CD44-mediated pathway may complement the low endosomal escape caused by the HA-coating in transgene expression in HCT116 cells. A detailed study of this issue will be needed in order to understand the precise mechanism by which carriers are released from endosomes. This issue will be investigated in the future.

In this study, we performed *in vitro* experiments using CD44 positive- and CD44 negative-cells to investigate the cellular uptake pathway and transgene expression of the HA-R8/GALA-MEND. For the therapy targeted to cancer and liver endothelial cells that overexpress CD44, in *in vivo* conditions where a soluble form of CD44 (sCD44) is present in the extracellular space, selective transgene expression in the CD44-positive cells of the target tissue are needed. In a previous study [22], we showed that HA-modified carriers could be targeted to liver endothelial cells (CD44 positive cells) in animal experiments, although gene expression was not investigated. Thus, we expected that the HA-R8/GALA-MEND would distinguish between CD44 positive- and CD44 negative- cells in liver transgene expression, and validation using a model animal

will be conducted in a future study. Further studies should also include an investigation of the difference in affinity between HA-modified carriers for CD44 on the cell surface and sCD44. Studies in this area are currently underway.

5. Conclusion

In this study, we analyzed the cellular uptake pathway involved in the gene expression of an HA coated gene vector (HA-R8/GALA-MEND) using CD44 positive and negative cells. The findings indicate that the HA(600kDa)-R8/GALA-MEND was efficiently taken up by cells and that transfection occurred via macropinocytosis and a CD44-medeiated pathway in HCT116 cells overexpressing CD44. While, in the case of NIH3T3 (CD44 negative) cells, the HA(600kDa)-R8/GALA-MEND was internalized mainly via macropinocytosis and a CD44-mediated pathway was not involved. In addition, the transfection activities of the HA(600kDa)-R8/GALA-MEND were drastically decreased compared with R8/GALA-MEND.

To date, considerable efforts to achieve higher transgene expression in target cells (positive targeting) have been made, in attempts to develop a more efficient gene therapy. On the other hand, our findings indicate that HA decreases the transfection activities of a gene vector in non-target cells (negative targeting). Based on the above

findings, it appears that an HA-coating could be used to control the intracellular pathway of octaarginine-modified nanoparticles, and that an HA coated gene vector achieved selective transgene expression in CD44 positive cell via negative targeting. These results indicates that the cellular uptake pathway involved in such processes would be a major factor in the efficiency of transgene expression in target cells.

Acknowledgements

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Table 1 Polydispersity index (PDI) of R8/GALA-MEND coated with HA (600 kDa) and HA (80kDa) at a series of coating ratios (g HA / mol lipid).

Coating ratio (g HA/mol Lipid)	12	18	24	30	60	120	300
HA (600 kDa)	0.71 ± 0.21	0.20 ± 0.07	0.15 ± 0.02	0.19 ± 0.04	0.24 ± 0.03	0.31 ± 0.04	0.50 ± 0.04
HA (80 kDa)	0.82 ± 0.24	0.10 ± 0.04	0.18 ± 0.11	0.17 ± 0.05	0.21 ± 0.02	0.28 ± 0.06	0.31 ± 0.02

Data are means ± S.D. (3-11).

Figure 1. Schematic image showing the construction of the HA-R8/GALA-MEND.

The construction of the HA-R8/GALA-MEND encapsulating pDNA requires the following three steps: (1) the formation of a condensed pDNA particle with polycation (protamine) via electrostatic interactions, (2) packaging the condensed pDNA particle with a lipid envelope modified with R8 peptide (a cellular uptake device via macropinocytosis) and GALA (a pH-sensitive fusogenic peptide), (3) coating of HA (natural ligand for CD44) to the carrier surface via electrostatic interactions.

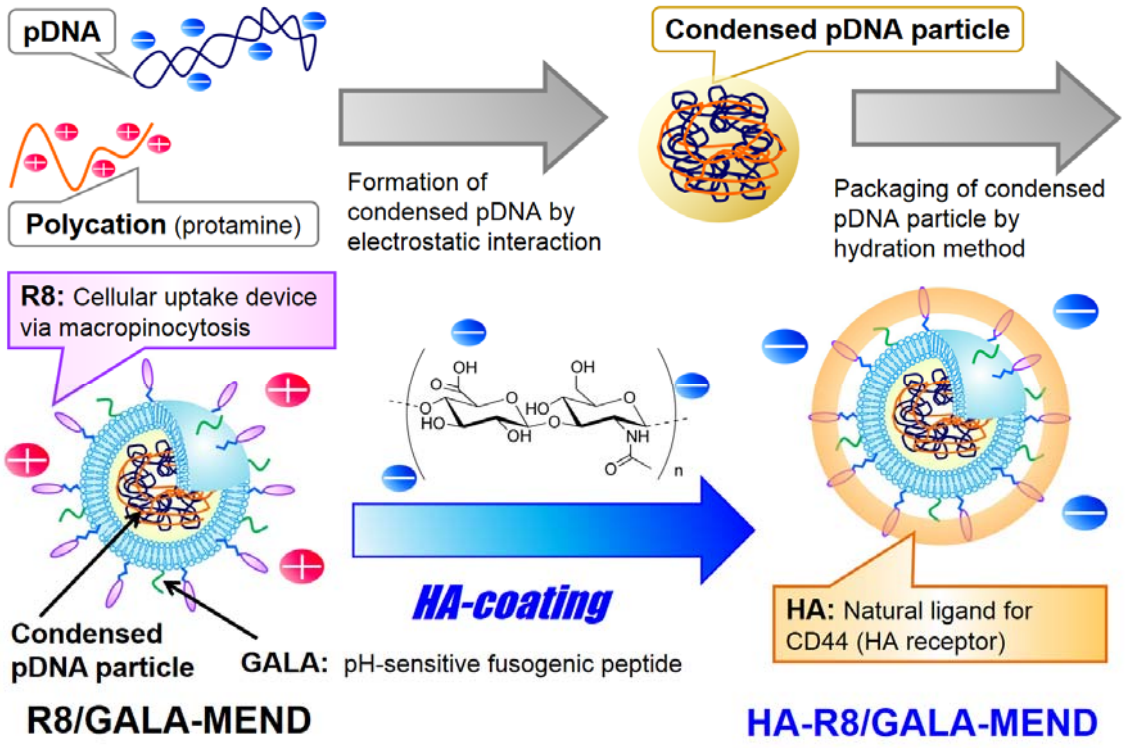


Figure 2. Relationship between the coating ratios of HA and the characteristics of HA (600 kDa)-R8/GALA-MEND or HA (80 kDa)-R8/GALA-MEND.

R8/GALA-MEND was mixed with a solution of hyaluronic acid (HA) at a series of coating ratios (g HA / mol lipid), and then incubated to coat R8/GALA-MEND with HA (600 kDa) (A) and HA (80 kDA) (B). Their ζ-potentials (a) and diameters (b) are summarized. Data are means ± S.D. (n = 3-17).

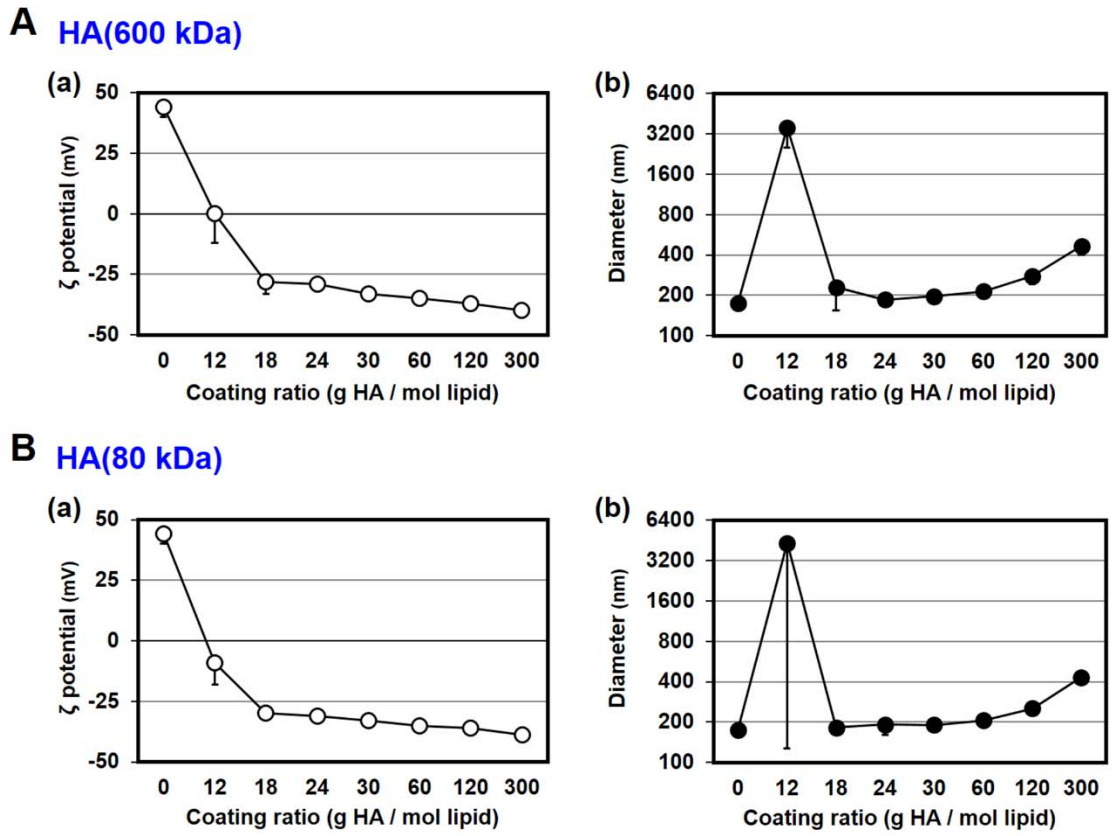


Figure 3. Evaluation of transfection activities of various types of R8/GALA-MEND coated with HA.

Various types of R8/GALA-MENDs coated with HA (600 kDa) or HA (80 kDa) were prepared, and their transfection activities were then evaluated by measuring the luciferase activity (relative light unit (RLU)) when 0.04 - 1 μg of pDNA encoding luciferase protein was transfected to HCT116 cells. Data are means ± S.D. (n = 3-17).

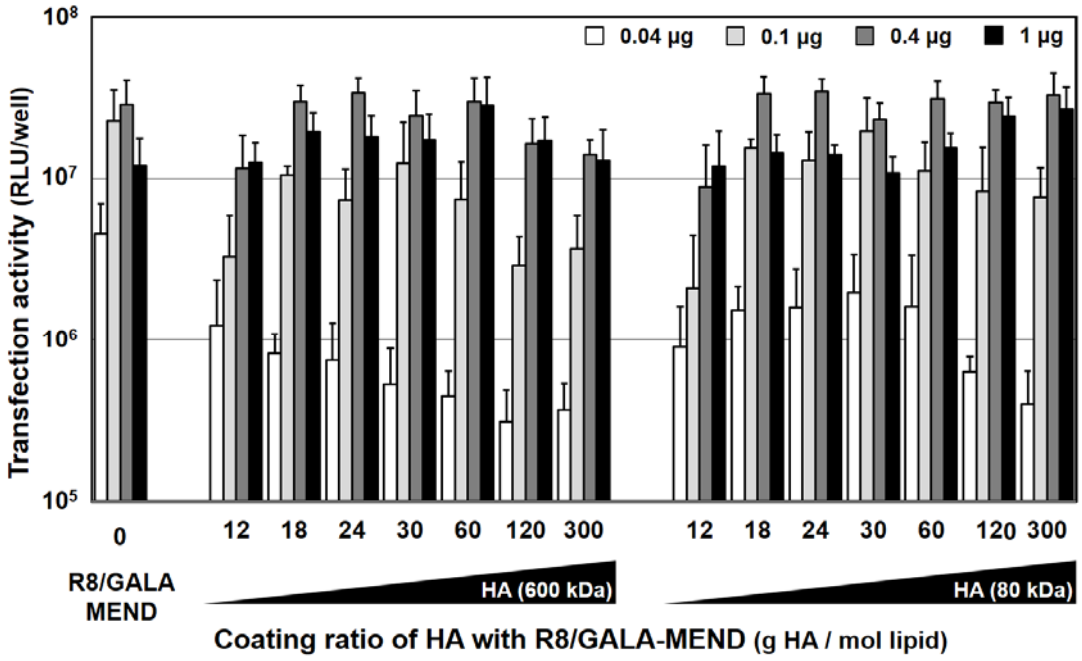


Figure 4. Effect of HA-coating on the cellular uptake of R8/GALA-modified carriers by HCT116 cells.

A. The graph shows the mean fluorescence intensity (MFI) of the R8/GALA-MEND, the HA (600 kDa)-R8/GALA-MEND and the HA (80 kDa)-R8/GALA-MEND encapsulating Cy5-pDNA taken up by HCT116 cells. Data are represented as the mean ± S.D. (n = 3-4). No significant difference was detected ($p > 0.05$ by one-way ANOVA). B. R8/GALA-modified liposomes labeled with rhodamine (red) coated with fluorescein-labeled HA (green) were incubated with HCT116 cells. The nuclei were stained with Hoechst 33342 (blue) prior to intracellular observation: merge image (a), fluorescein-labeled HA (b) and R8/GALA-modified liposomes labeled with rhodamine (c). Scale bar, 50 μm.

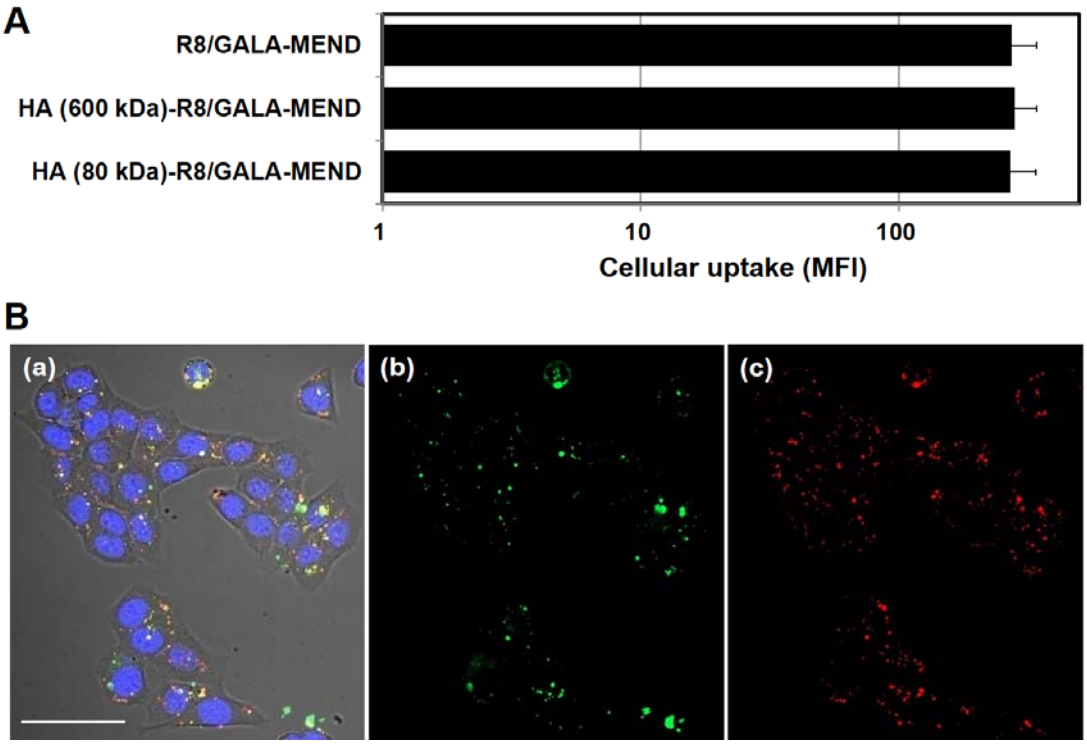


Figure 5. Effect of a CD44-mediated uptake inhibitor and a macropinocytosis inhibitor on cellular uptake by HCT116 cells.

HCT116 cells were incubated with R8/GALA-MEND, HA (600 kDa)-R8/GALA-MEND and HA (80 kDa)-R8/GALA-MEND containing Cy5-pDNA, in the absence (closed bars) and presence (open bars) of the CD44-mediated uptake inhibitor, FITC-labeled CD44-antibody (A), or macropinocytosis inhibitor, amiloride (B). The mean fluorescence intensity of 10,000 cells was measured by flow cytometry, and is expressed as a percent of the fluorescence measured in the absence of inhibitors. Data are represented as the mean ± S.D. (n = 4-5). Significant differences between presence and absence of inhibitors (*p < 0.05, **p < 0.01 by unpaired student's t-test).

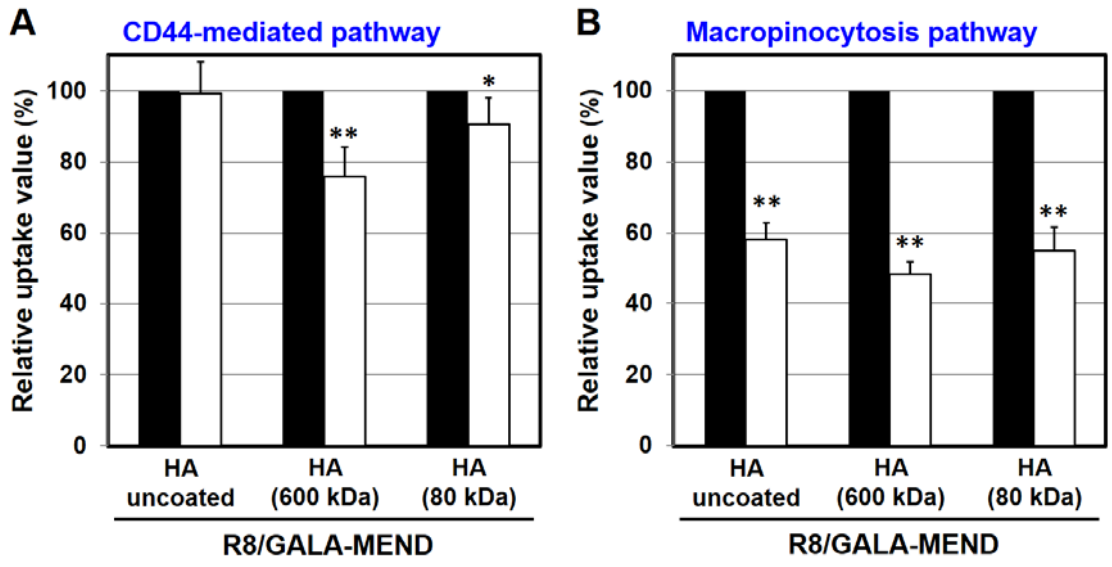


Figure 6. Comparison of the expression levels of CD44 and the cellular uptake pathway between HCT 116 and NIH3T3 cells.

A, The expression levels of CD44 on HCT116 cells and NIH3T3 cells were measured using an FITC-labeled CD44-antibody and flow cytometry analysis (a). Blue and black lines indicate non-treatment and the result with the antibody treatment, respectively. The expression levels of CD44 (MFI) (b) were calculated, using the data shown in (a). MFI, mean fluorescence intensity. Data are represented as the mean ± S.D. (n = 3). Significant difference (**p < 0.01 by unpaired student's t-test).

B, CD44 expressed on HCT116 cells was visualized using FITC-labeled CD44-antibody (green) and CLSM. Nuclei were stained to blue with Hoechst33342 prior to observation. Scale bar, 50 μm. C. HCT116 cells (a) and NIH3T3 cells (b) were incubated with HA (600 kDa)- R8/GALA-MEND containing Cy5-pDNA, in the absence (closed bars) and presence (open bars) of the CD44-mediated uptake inhibitor or macropinocytosis inhibitor. The MFI of 10,000 cells was measured by flow cytometry, and is expressed as a percent of the fluorescence measured in the absence of inhibitors. Data are represented as the mean ± S.D. (n = 3-5). Significant differences between presence and absence of inhibitors (**p < 0.01 by unpaired student's t-test).

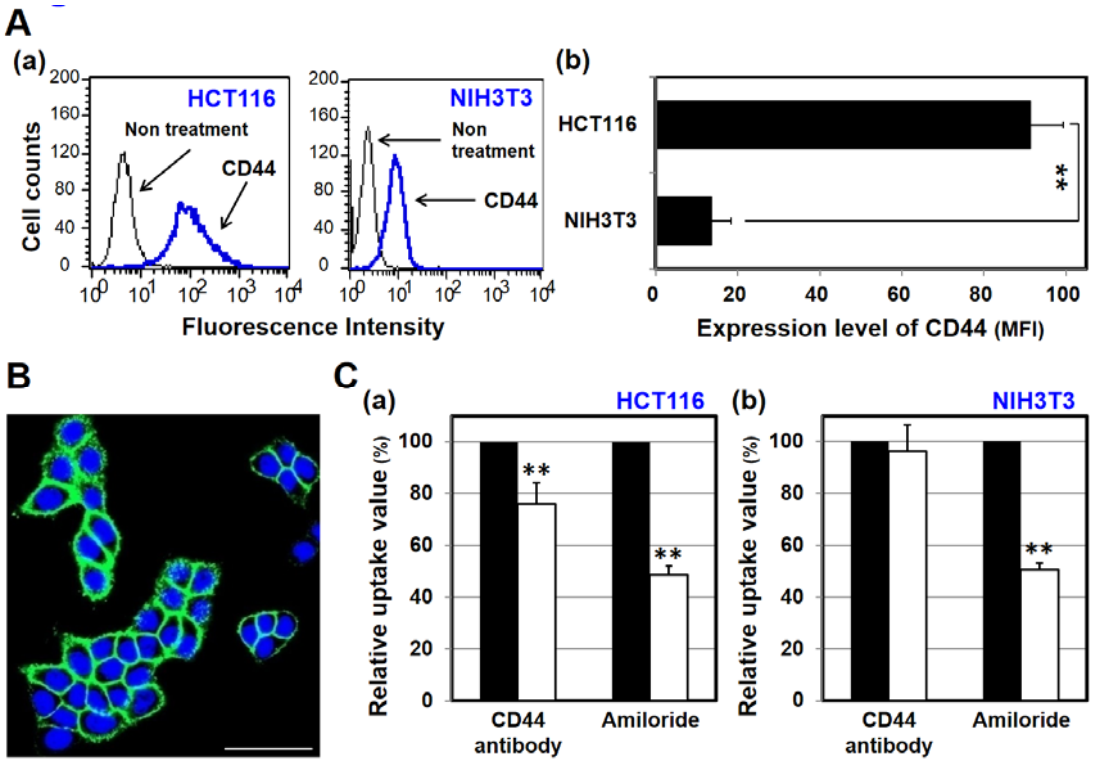


Figure 7. Comparison of transfection activities between the R8/GALA-MEND and the HA (600 kDa)-R8/GALA-MEND in HCT116 cells and NIH3T3 cells.

The R8/GALA-MEND and the HA (600 kDa)-R8/GALA-MEND were prepared, and their transfection activities were then evaluated by means of a luciferase assay when 0.1 and 0.4 μg of pDNA encoding luciferase protein was transfected to HCT116 cells (A) and NIH3T3 cells (B). Closed bars and open bards indicate the transfection activities of R8/GALA-MEND and HA (600 kDa)-R8/GALA-MEND, respectively. Data are means ± S.D. (n = 3-11). Significant difference between R8/GALA-MEND and HA (600 kDa)-R8/GALA-MEND (*p < 0.05 by unpaired student's t-test).

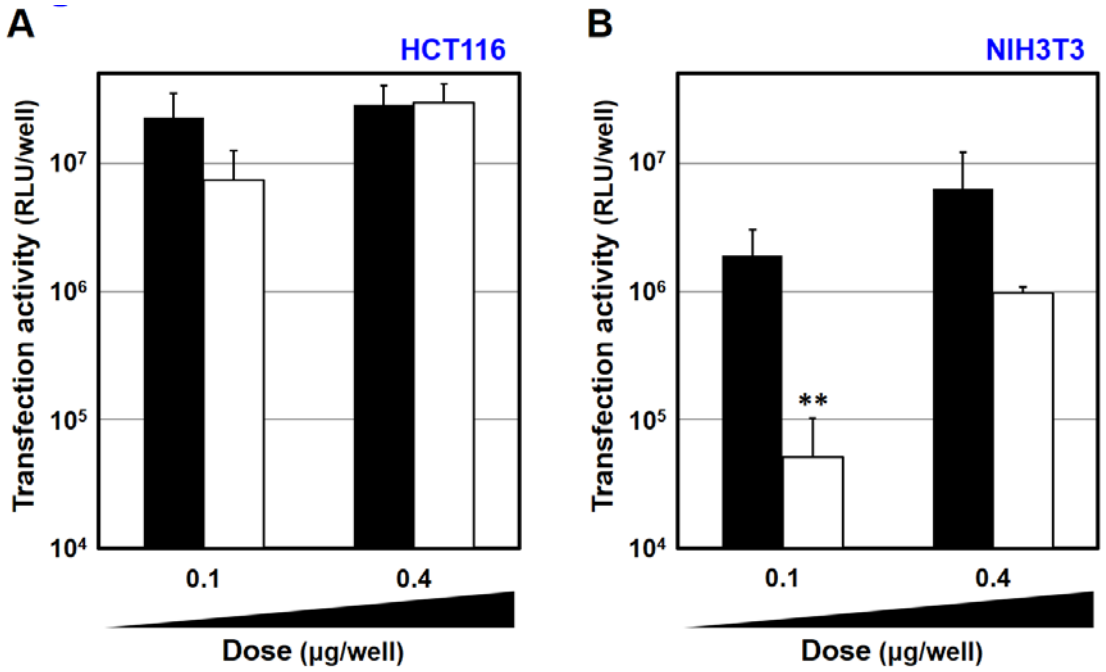
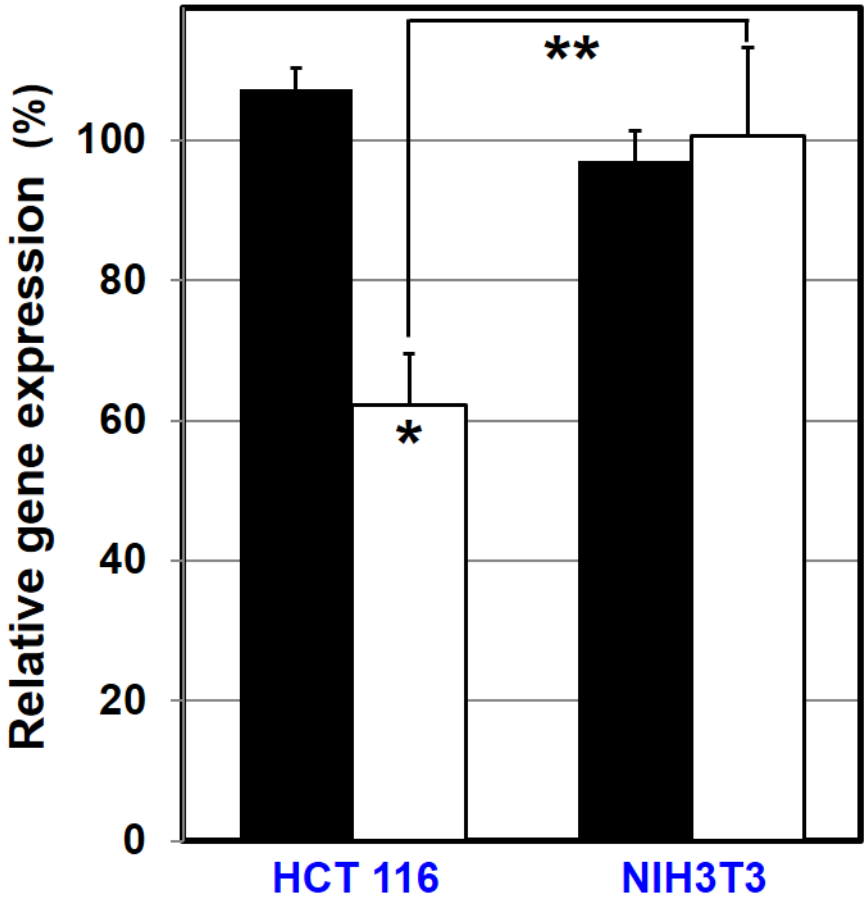


Figure 8. Effect of CD44-mediated uptake inhibitor on transgene expression of R8/GALA-MEND and HA (600 kDa)-R8/GALA-MEND in HCT116 cells and NIH3T3 cells.

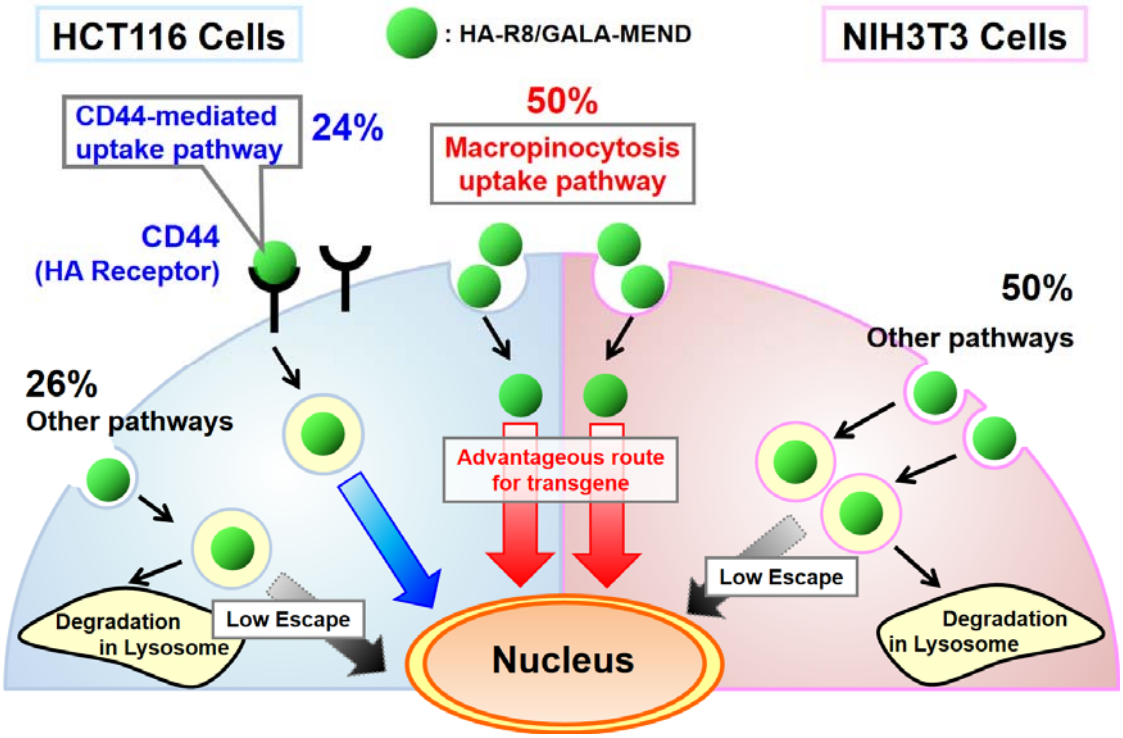
HCT116 cells and NIH3T3 cells were incubated with R8/GALA-MEND and HA (600 kDa)-R8/GALA-MEND containing pDNA encoding luciferase protein, in the absence and presence of the CD44-mediated uptake inhibitor. The transfection activities were evaluated by luciferase assay, and the relative gene expression is expressed as a percent of the transfection activity in the absence of inhibitors (see the Materials and methods section for the detail). Closed bars indicate the transfection activities of R8/GALA-MEND, and open bars indicate the values for HA (600 kDa)-R8/GALA-MEND. Data are represented as the mean ± S.D. ($n = 3$). We performed two-way ANOVA analysis to compare the effect of multiple levels of two factors that are “cell line” and “carrier type”. There were significant differences of different “cell line” ($p < 0.05$) and different “carrier type” ($p < 0.01$). There was also significant interaction between two factors ($p < 0.01$). **Significant differences between HCT116 cells and NIH3T3 cells in each “carrier type” ($p < 0.01$ by simple main effect test, followed by Bonferroni correction). *Significant differences between R8/GALA-MEND and HA (600 kDa)-R8/GALA-MEND in each “cell line” ($p < 0.01$ by simple main effect test, followed by Bonferroni correction).



Two way ANOVA analysis result

Factors	P-value by two way ANOVA
Cell line	0.0133
Carrier-type	0.0017
Interaction between two factors	0.0006

Figure 9. Proposed model of the intracellular trafficking events of HA-R8/GALA-modified nanocarriers. After uptake via macropinocytosis (50%), the HA (600 kDa)-R8/GALA-MEND escapes efficiently for transgene expression in both HCT116 cells and NIH3T3 cells. The HA (600 kDa)-R8/GALA-MEND is also internalized by HCT116 cells via CD44-mediated pathway (26%), and the pathway would have an advantage for transgene expression. While, half of the HA (600 kDa)-R8/GALA-MEND is internalized by NIH3T3 cells via a different pathway, this route would be a disadvantage for transgene expression. These values were quantified based on transfection activity data and cellular uptake analyses.



Supplementary data

1. Materials and Methods

1.1. Preparation of florescent labeled HA-coated R8/GALA-modified liposomes

To observe the intracellular trafficking of the HA-coated nanocarriers, fluorescent labeled LPs were constructed, as described below. LPs containing rhodamine labeled DOPE (AVANTI Polar Lipids Inc.; Alabaster, AL, USA) were prepared by the lipid film hydration method. Lipid films were produced on the bottom of a glass tube by the evaporation of a chloroform solution containing 138 nmol lipids [DOPE/CHEMS/Cho-GALA/rhodamine labeled DOPE = 9 : 2 : 0.2 : 0.005 (molar ratio)]. Next, 250 μL of 10 mM HEPES buffer (pH 7.4) was applied to the lipid film, followed by incubation for 15 min at room temperature to hydrate the lipids. The lipid film was then sonicated for approximately 1 min in a bath-type sonicator (85 W, Aiwa Co., Tokyo, Japan). The resulting suspension was then incubated with a solution of STR-R8 (10 mol% lipids) for 30 min at room temperature to produce R8/GALA-LPs. To coat with HA via electrostatic interactions, a solution of the fluorescein labeled HA (average MW 100-300 kDa; PG Research, Tokyo, Japan) was added to R8/GALA-LPs suspensions (60 g HA / mol lipid), and the resulting mixture was then incubated for 30 min at room temperature (HA-R8/GALA-LP).

1.2. Quantification of expression levels for CD44 in HCT116 and NIH3T3 cells using FITC-labeled CD44-antibody and flow cytometry

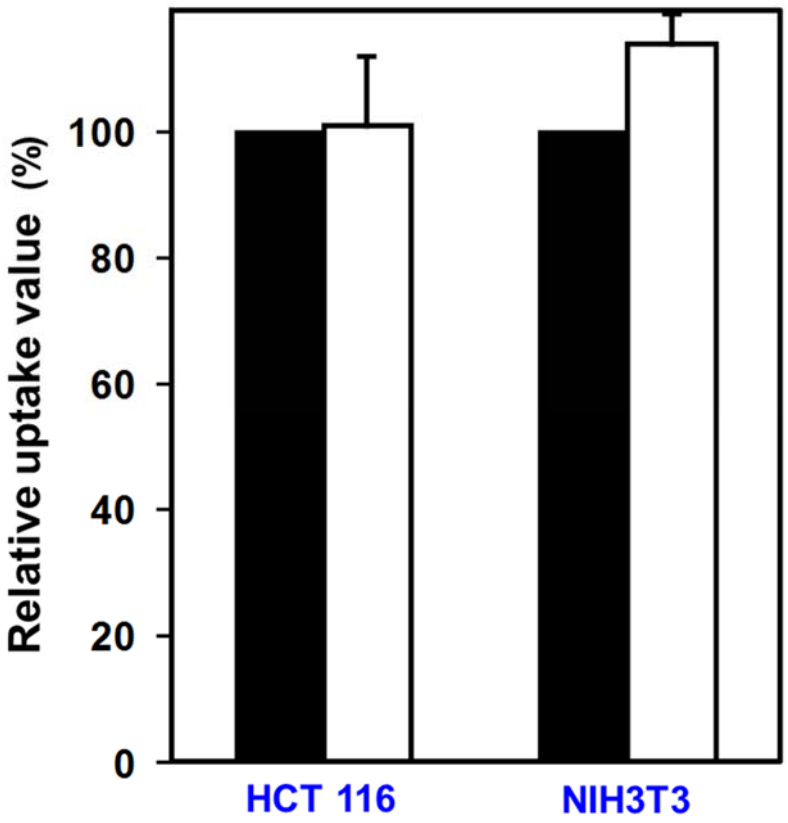
Cells (2 x 10^5 cells) were collected, suspended in 40 μL of Fc Receptor Saturation Reagent and incubated on ice for 30 min. After the incubation, the cell suspensions were washed twice with FACS buffer, precipitated by centrifugation (1,000 *g*, 4°C, 5 min). The pellet was suspended in 50 μL of an FITC-labeled CD44-antibody solution (50 μg/mL) and incubated on ice for 30 min. The resulting suspension was washed twice with FACS buffer, precipitated by centrifugation (1,000 *g*, 4°C, 5 min). After resuspension in 0.5 mL of FACS buffer, the cell suspension was filtered through a nylon mesh to remove cell aggregates and dust. The cells were then analyzed by flow cytometry (FACScan; Dickinson, Franklin Lakes, NJ, USA). The cells were excited with a 488 nm light from an Ar laser. The fluorescence detection channel was set to the FL1 filter for FITC-labeled CD44-antibody. The CD44 expressions were expressed as the mean fluorescence intensity, calculated using the CellQuest software (Becton Dickinson).

1.3. Observation of CD44 in HCT116 cells using confocal laser scanning microscopy

Cells (2 x 10^5 cells) were cultured in 35 mm dishes (IWAKI, Tokyo, Japan) with complete medium under an atmosphere of 5% CO_2 / air at 37ºC for 24 hr. The cells were washed with PBS (-), and the FITC-labeled CD44-antibody were added to the cells (final concentration, 5 μg/mL). The cells were then incubated in phenol red-free medium in the absence of serum under an atmosphere of 5% CO_2/air at 37ºC. After a 1-hr incubation, the cells were washed with PBS (-), and then incubated in a phenol red-free medium containing Hoechst 33342 (final concentration, 5 μg/mL) in the absence of serum to stain the nucleus. After incubation (5% CO_2 / air, 37ºC, 10 min), the cells were washed with PBS (-). The medium was then replaced with fresh phenol red-free complete medium, and observed by CLSM (Nikon A1; Nikon Co. Ltd., Tokyo, Japan). The cells were excited with 405 nm wavelength light from a Diode laser and 488 nm wavelength light from an Ar laser. A series of images were obtained using a Nikon A1 confocal imaging system equipped with a water immersion objective lens (Plan Apo 60x1.20 PFS WI) and a 1st dichroic mirror (405/488/561/640). The two fluorescence detection channels were set to the following filters: 450/50 (blue color) for nuclei and 525/50 (green color) for FITC-labeled CD44-antibody.

2. Supplementary Figure

Figure S1. Comparison of cellular uptake between the HA (600 kDa)-R8/GALA-MEND and the R8-GALA-MEND in HCT116 and NIH3T3 cells.



HCT116 cells and NIH3T3 cells were incubated with the R8/GALA-MEND and the HA (600 kDa)-R8/GALA-MEND containing Cy5-pDNA (1 μg) for 3 hr. The MFI of 10,000 cells was measured by flow cytometry, and is expressed as a percent of the fluorescence measured in the incubation of R8/GALA-MEND. Closed bars indicate the reraltive uptake values of R8/GALA-MEND, and open bars indicate the values for the HA (600 kDa)-R8/GALA-MEND. Data are represented as the mean ± S.D. (n = 3).