Chemical Communications

Electronic supplementary information

Title:

Receptor clustering and activation by multivalent interaction through recognition peptides presented on exosomes

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1. Materials and Methods

Peptide synthesis

All peptides were chemically synthesized on a Rink amide resin with a coupling system using 1-hydroxybenzotriazole (HOBt) / 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (Peptide Institute, Osaka, Japan) / N,N-diisopropylethylamine (DIEA) in 9-fluorenylmethyloxycarbonyl (Fmoc) solid-phase peptide synthesis method as previously described.[1,2] The Rink amide resin and the Fmoc-amino acid derivatives were purchased from Shimadzu Biotech (Kyoto, Japan) and Peptide Institute (Osaka, Japan), respectively. For preparation of the stearylated peptide, the N-terminus of the peptide resin was reacted with stearyl acid with diisopropylcarbodiimide in the presence of HOBt as coupling agents, as previously reported.[1] For preparation of the acetylated peptide, the N-terminus of the peptide resin was acetylated using acetic anhydride in the presence of 4-methylmorpholine (NMM) in dimethylformamide (DMF), as previously reported.[3] Deprotection of the protected peptide and cleavage from the resin was performed by treatment with a trifluoroacetic acid (TFA) / ethanedithiol (EDT) mixture (95:5) for 3 h at 20°C, followed by reverse-phase high-performance liquid chromatography (HPLC) for peptide purification. The purity of each peptide was estimated to be >97% on the basis of the analytical HPLC. The structures of the synthesized peptides were confirmed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS) (Microflex, Bruker, Billerica, MA, USA).

Stearyl-K4

($CH_3(CH_2)_{16}$-CO-NH-Lys-Ile-Ala-Ala-Leu-Lys-Glu-Lys-Ile-Ala-Ala-Leu-Lys-Glu-Lys-Ile-Ala-Ala-Leu-Lys-Glu-Lys-Ile-Ala-Ala-Leu-Lys-Glu-Gly-Gly-Cys-amide): MALDI-TOFMS: 3517.0 [calcd. For (M+H)+: 3517.5]. Retention time in HPLC, 20.8 min (column: Cosmosil 5C18-AR-II (4.6 × 150 mm); gradient: 5-95% B in A (A = H_2O containing 0.1% CF_3COOH, B = CH_3CN containing 0.1% CF_3COOH) over 30 min; flow: 1 mL/min; detection: 220 nm). Yield from the starting resin, 18%.

Ac-K4

(CH_3-CO-NH-Lys-Ile-Ala-Ala-Leu-Lys-Glu-Lys-Ile-Ala-Ala-Leu-Lys-Glu-Lys-Ile-Ala-Ala-Leu-Lys-Glu-Lys-Ile-Ala-Ala-Leu-Lys-Glu-Gly-Gly-Cys-amide): MALDI-TOFMS: 3293.9 [calcd. For (M+H)+: 3293.1]. Retention time in HPLC, 10.9 min (column: Cosmosil 5C18-AR-II (4.6 × 150 mm); gradient: 5-95% B in A (A = H_2O containing 0.1% CF_3COOH, B = CH_3CN

containing 0.1% CF_3COOH) over 30 min; flow: 1 mL/min; detection: 220 nm). Yield from the starting resin, 46%.

Fluorescently labeled peptides

Fluorescent labeling with purified peptides of stearyl-K4 or Ac-K4 was performed by treatment with 1.5 equivalents of Alexa Fluor 488 (Alexa488) C5 maleimide sodium salt (Invitrogen, Eugene, OR, USA) in a dimethylformamide / methanol mixture (1:1) for 1.5 h at room temperature followed by HPLC purification, as previously reported.[2]

Stearyl-K4(Alexa488)

($CH_3(CH_2)_{16}$-CO-NH-Lys-Ile-Ala-Ala-Leu-Lys-Glu-Lys-Ile-Ala-Ala-Leu-Lys-Glu-Lys-Ile-Ala-Ala-Leu-Lys-Glu-Lys-Ile-Ala-Ala-Leu-Lys-Glu-Gly-Gly-Cys(Alexa488)-amide):
MALDI-TOFMS: 4215.4 [calcd. For (M+H)+: 4215.2]. Retention time in HPLC, 21.0 min (column: Cosmosil 5C18-AR-II (4.6 × 150 mm); gradient: 5-95% B in A (A = H_2O containing 0.1% CF_3COOH, B = CH_3CN containing 0.1% CF_3COOH) over 30 min; flow: 1 mL/min; detection: 220 nm). Yield from the starting resin, 0.2%.

Ac-K4(Alexa488)

(CH_3-CO-NH-Lys-Ile-Ala-Ala-Leu-Lys-Glu-Lys-Ile-Ala-Ala-Leu-Lys-Glu-Lys-Ile-Ala-Ala-Leu-Lys-Glu-Lys-Ile-Ala-Ala-Leu-Lys-Glu-Gly-Gly-Cys(Alexa488)-amide): MALDI-TOFMS: 3990.3 [calcd. For (M+H)+: 3990.8]. Retention time in HPLC, 16.0 min (column: Cosmosil 5C18-AR-II (4.6 × 150 mm); gradient: 5-95% B in A (A = H_2O containing 0.1% CF_3COOH, B = CH_3CN containing 0.1% CF_3COOH) over 30 min; flow: 1 mL/min; detection: 220 nm). Yield from the starting resin, 9.6%.

Cell cultures

HeLa (human cervical cancer-derived) cells were purchased from the Riken BRC Cell Bank (Ibaraki, Japan). The human breast adenocarcinoma-derived MDA-MB-231 cells were purchased from the European Collection of Cell Cultures (ECACC; Salisbury, UK). Each cell was cultured in α-MEM (Gibco, Life Technologies Corporation, Grand Island, NY, USA) (HeLa cells), minimum essential medium (MEM) (Gibco, Life Technologies Corporation) (MDA-MB-231 cells) containing 10% heat-inactivated FBS (Gibco, Life Technologies Corporation). Each cell was grown on 100-mm dishes and incubated at 37°C under 5% CO_2.

Preparation of HeLa cells stably expressing green fluorescent protein (GFP)-fused CD63

CD63 is a membrane marker tetraspanin protein of exosomes, and we prepared HeLa cells stably expressing GFP-fused CD63 to secrete CD63-GFP-containing exosomes (CD63-GFP-exosomes) as previously reported[5,6]. HeLa cells (1×10^5 cells) were plated on a 24-well microplate (Iwaki, Tokyo, Japan) and incubated for 1 day. They were transfected with CD63-GFP plasmid (pCT-CD63-GFP, pCMV, Cyto-Tracer, System Biosciences, Mountain View, CA) (800 ng) complexed with Lipofectamine LTX reagent (2 μ l) and PLUS reagent (1 μ l) (Invitrogen, Life Technologies Corporation) in α -MEM containing 10% FBS (200 μ l). The cells were also treated with puromycin (3 μ g/ml) (LKT Laboratories, St. Paul, MN) for the antibiotic selection of HeLa cells stably expressing CD63-GFP (CD63-GFP-HeLa).

Plasmid construction for E3-EGFR expression

Preparation of plasmid for E3-EGFR expression was conducted as previously reported.[3] The expression vector for E3-EGFR, E3-EGFR/pDisplayP1 was constructed as follows. pDisplayP1 was created by inserting a DNA fragment including the NheI and XhoI sites into ApaI/XhoI-digested pDisplay (Invitrogen Life Technologies Corporation). The DNA fragment encoding E3 (EIAALEKEIAALEKEIAALEK) was amplified by PCR using a synthetic oligonucleotide as a template, primer 1 (5'-CAAAAAGCTAGCGAAATCGC-3') and primer 2 (5'-GCACACATGGCCGGCGTCCTTCTCAAGAGCTGC-3'). The DNA fragment encoding EGFR (588-1186) was amplified using EGFR cDNA as a template, primer 3 (5'-GGACGCCGGCCATG-3') and primer 4 (5'-GAAAACTCGAGTCATGCTCCAATAAATTCAC-3'). Then the DNA fragment encoding E3-EGFR was amplified using the two PCR products described above as templates, primer 1 and primer 4. The PCR product was cut with NheI and XhoI and inserted between the NheI and XhoI sites of pDisplayP1, yielding E3-EGFR/pDisplayP1.

Preparation of MDA-MB-231 cells stably expressing E3-EGFR

MDA-MB-231 cells (1×10^5 cells) were plated on a 24-well microplate (Iwaki, Tokyo, Japan) and incubated for 1 day. The cells were transfected with E3-EGFR/pDisplayP1 (800 ng) complexed with Lipofectamine LTX reagent (2 μl) and PLUS reagent (1 μl) (Invitrogen, Life Technologies Corporation) in MEM containing 10% FBS (200 μl). The cells were also treated

with geneticin (1 mg/ml) (Gibco, Life Technologies Corporation) for antibiotic selection of MDA-MB-231 cells stably expressing E3-EGFR.

Confocal microscopy (observation for expression of E3-EGFR)

E3-EGFR-expressing or wildtype-MDA-MB-231 cells (each 2×10^5 cells/2 ml) were seeded onto a 35-mm glass dish (Iwaki, Tokyo, Japan) and incubated in MEM containing 10% FBS for 24 h at 37°C under 5% CO_2. The cells were incubated for 15 min at 4 °C. Then, the cells were washed with serum-free F-12 nutrient mixture (Ham's F-12) (Gibco, Life Technologies Corporation), and treated with anti-HA antibody (12CA5, Roche Diagnostics, Indianapolis, IN) (5 μg/ml, 200 μl) (30 min, 4 °C), followed by the treatment with Alexa Fluor 488 goat anti-mouse IgG (4 μg/ml, 200 μl) (30 min, 4 °C) and Hoechst 33342 dye (Invitrogen; 5 μg/ml, 200 μl) (15 min at 4°C). After washing the cells with Ham's F-12 medium, confocal microscopic observation was conducted using a FV1200 confocal laser scanning microscope (Olympus, Tokyo, Japan) equipped with a 40× objective without cell fixation. For detection of E3-EGFR clustering, the cells were treated with each exosome sample (100 μl/well) in MEM containing 10% FBS for 20 min at 37°C, prior to incubation for 15 min at 4 °C and staining with anti-HA antibody.

Flow cytometry (detection for expression of E3-EGFR)

E3-EGFR-expressing or wild-type-MDA-MB-231 cells (each 1.4×10^5 cells/1 ml) were plated onto a 24 well microplate (Iwaki) and incubated in MEM containing 10% FBS for 24 h at 37°C under 5% CO_2. The cells were incubated for 15 min at 4 °C. Then, the cells were washed with serum-free F-12 nutrient mixture (Ham's F-12) (Gibco, Life Technologies Corporation), and treated with anti-HA antibody (12CA5, Roche Diagnostics, Indianapolis, IN) (5 μg/ml, 200 μl) (30 min, 4 °C), followed by the treatment with Alexa Fluor 488 goat anti-mouse IgG (4 μg/ml, 200 μl (30 min, 4 °C). The cells were then treated with 2 mM EDTA (200 μl/well) at 37°C for 10 min, prior to addition of PBS (200 μl) and then centrifuged at 1,500 rpm (200 × g) for 5 min at 4°C. After removal of the supernatant, the cells were washed with PBS (400 μl) and centrifuged at 1,500 rpm for 5 min at 4°C. This washing cycle was repeated, and the cells were suspended in PBS (400 μl) and subjected to fluorescence analysis with a guava easyCyte (Merck Millipore, Billerica, MA, USA) flow cytometer using 488-nm laser excitation and a

525-nm emission filter. Live cells (10,000 cells/sample) for detection of cellular fluorescence intensity were quantified based on forward-scattering and side-scattering analyses.

Isolation of exosomes

HeLa cells or CD63-GFP-HeLa cells (3×10^6 cells) were plated onto 100-mm dishes in 10% exosome-free FBS (EXO-FBS, ATLAS biological, Fort Collins, CO, USA)-contained α-MEM for 3 days at 37°C under 5% CO_2. Secreted exosomes in the cell culture medium were isolated using ultracentrifugation as previously reported.[4] The collected cell culture medium was centrifuged (300 × g) for 10 min at 4° C. The supernatant was centrifuged (2,000 × g) for 10 min at 4°C and again centrifuged (10,000 × g) for 30 min at 4°C to remove cell debris. The supernatant was then centrifuged (100,000 × g) for 70 min at 4°C (Himac CP65β, Hitachi, Tokyo, Japan) in duplicate, and the pellet was collected in PBS. The concentrations of isolated exosomes were showed in terms of their protein concentrations, which were determined using a Pierce BCA protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL, USA).

Western blotting analysis

For detection of exosome marker proteins, isolated exosomes were added to lysis buffer (50 mM Tris-HCl (pH = 7.5), 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate). The boiled samples were separated via 10% SDS-PAGE, transferred onto polyvinylidene fluoride (PVDF) membranes (GE Healthcare, Pittsburgh, PA, USA), and treated with anti-CD9 (EPR2949, Abcam, Cambridge, UK) or anti-CD63 antibody (TS63, Abcam, Cambridge, UK). A secondary antibody labeled with horseradish peroxidase (anti-rabbit IgG HRP-linked whole antibody donkey, GE Healthcare (for anti-CD9) or anti-mouse IgG HRP NA931V (GE Healthcare) (for anti-CD63)) was used, and immunoreactive species were detected using the Enhanced Chemiluminescence (ECL) Plus Western Blotting Detection System (GE Healthcare) with the Amersham Imager 600 (GE Healthcare).

For detection of pTyr1173 of EGFR, MDA-MB-231 cells with or without expressing E3-EGFR (24-well microplate) were washed with MEM without FBS and incubated with the medium for 1 h at 37 °C under 5% CO_2. The cells were treated with each exosome sample in MEM without FBS (200 μl) for 5 min at 37 °C under 5% CO_2. After the treatment of each exosome sample, the cells were scraped in lysis buffer (200 μl). The boiled lysate samples were separated by 10% SDS-PAGE, then transferred to PVDF membranes (GE Healthcare), and

treated with p-EGFR (Tyr1173) antibody (sc-12351, Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibody labeled with horseradish peroxidase (anti-rabbit IgG HRP-linked whole antibody donkey, GE Healthcare) was then used, and immunoreactive species were detected by ECL Plus Western Blotting Detection System (GE Healthcare) with the Amersham Imager 600 (GE Healthcare).

Modification of exosomes with stearyl-K4 peptide

Synthesized stearyl-K4 peptide or Ac-K4 peptide (each 200 μM) diluted with phosphate buffered saline (PBS) was added to a solution of exosomes (4 μg) in PBS (total 25 μl) and incubated for 1 h at 37°C. After the incubation, the solution was added with MEM and FBS to adjust the exosome concentration to final 20 μg/ml in 10% or 0% FBS-containing MEM. To calculate for amount of modified K4 peptides on exosomal membrane, removal of unattached peptide was accomplished by washing with PBS and filtration using Amicon Ultra centrifugal filters (100K device, Merck Millipore). The attachment of stearyl-K4(Alexa488) and Ac-K4(Alexa488) to exosomes was confirmed using a spectrofluometer (FP-6200, JASCO, Tokyo, Japan).

Confocal microscopy (cellular uptake of exosome samples)

E3-EGFR-expressing or wild-type-MDA-MB-231 cells (each 2×10^5 cells/2 ml) were seeded onto a 35-mm glass dish (Iwaki, Tokyo, Japan) and incubated in MEM containing 10% FBS for 24 h at 37°C under 5% CO_2. After complete adhesion, the cells were treated with each exosome sample (100 μl/well). The cells were stained with Hoechst 33342 dye (Invitrogen; 5 μg/ml) for 15 min at 37°C, prior to cell washing. The cells were then washed with fresh cell culture medium and analyzed using a FV1200 confocal laser scanning microscope (Olympus, Tokyo, Japan) equipped with a 40× objective without cell fixation.

Confocal microscopy (phosphorylation of EGFR Tyr1173)

E3-EGFR-expressing MDA-MB-231 cells (2×10^5 cells/2 ml) were seeded onto a 35-mm glass dish (Iwaki, Tokyo, Japan) and incubated in MEM containing 10% FBS for 24 h at 37°C under 5% CO_2. After complete adhesion, the cells were washed with serum-free MEM and treated with serum-free MEM (100 μl/well) for 1 h at 37°C. The cell culture medium was

then removed, and the cells were treated with each exosome sample in serum-free MEM (100 μl/well) for 5 min at 37°C. After removal of the medium, the cells were fixed with 4% paraformaldehyde at room temperature for 30 min and washed with PBS. The cells were then treated with 0.1% Triton X-100 (100 μl/well in PBS) at room temperature for 5 min and again washed with PBS. Phosphorylation of EGFR Tyr1173 was visualized by treatment with p-EGFR (Tyr1173) antibody (sc-12351, Santa Cruz Biotechnology) and secondary antibody labeled with fluorescence (Alexa Fluor 568 goat anti-rabbit IgG (H+L), Invitrogen) was then used (each 30 min at room temperature), prior to analysis using a FV1200 confocal laser scanning microscope (Olympus) equipped with a 40× objective.

Flow cytometry (cellular uptake of exosome samples)

E3-EGFR-expressing or wild-type-MDA-MB-231 cells (each 1.4×10^5 cells/1 ml) were plated onto a 24 well microplate (Iwaki) and incubated in MEM containing 10% FBS for 24 h at 37°C under 5% CO_2. After complete adhesion, the cells were washed with MEM containing 10% FBS and treated with each exosome sample (200 μl/well) prior to washing with PBS (triple washing, 200 μl). The cells were then treated with 0.01% trypsin at 37°C for 10 min, prior to addition of PBS (200 μl) and then centrifuged at 1,500 rpm (200 × g) for 5 min at 4°C. After removal of the supernatant, the cells were washed with PBS (400 μl) and centrifuged at 1,500 rpm for 5 min at 4°C. This washing cycle was repeated, and the cells were suspended in PBS (400 μl) and subjected to fluorescence analysis with a guava easyCyte (Merck Millipore, Billerica, MA, USA) flow cytometer using 488-nm laser excitation and a 525-nm emission filter. Live cells (10,000 cells/sample) for detection of cellular fluorescence intensity were quantified based on forward-scattering and side-scattering analyses.

In macropinocytosis inhibition assay, the cells were pretreated with 5-(N-ethyl-N-isopropyl) amirolide (EIPA, Sigma-Aldrich) in MEM containing 10% FBS (100 μM, 200 μl/well) for 30 min at 37 °C, prior to treatment with each sample (200 μl/well) in MEM containing 10% FBS (200 μl/well) in the presence or absence of EIPA (100 μM) for 1 h at 37 °C. FITC-labeled dextran (molecular weight 70,000) (Sigma-Aldrich) or FITC-labeled transferrin (Sigma-Aldrich) was used as macropinocytosis or clathrin-mediated endocytosis marker, respectively.

Electron microscopy

Suspended exosomes in PBS (30 μg/ml) were dropped onto a carbon-coated grid (400 mesh) and washed with distilled water. Uranil acetate was applied to the grid and left for 10 s at room temperature. Next, the reagent was removed with filter paper and dried, prior to imaging with a transmission electron microscope (TEM) (JEM1200EX, JEOL, Tokyo, Japan).

Zeta-potential and particle size

The zeta-potential and particle size of the exosomes diluted in PBS (20 μg/ml) were determined using a zeta-potential and particle size analyzer ELSZ-DN2 (Otsuka Electronics, Osaka, Japan) according to the manufacturer's instructions.

Confocal microscopy (Cell ruffling assay)

E3-EGFR-expressing or wild-type-MDA-MB-231 cells (each 2×10^5 cells/2 ml) were seeded onto a 35-mm glass dish (Iwaki) and incubated in MEM containing 10% FBS for 24 h at 37°C under 5% CO_2. After complete adhesion, the cells were washed with serum-free MEM and treated with serum-free MEM (100 μl/well) for 1 h at 37°C. The cell culture medium was then removed, and the cells were treated with each exosome sample in serum-free MEM (100 μl/well) for 20 min at 37°C. After removal of the medium, the cells were fixed with 4% paraformaldehyde at room temperature for 30 min and washed with PBS. The cells were then treated with 0.1% Triton X-100 (100 μl/well in PBS) at room temperature for 5 min and again washed with PBS. Cellular F-actin was stained with rhodamine-phalloidin (Molecular Probes) for 20 min at 4°C, and the cells were washed with PBS prior to analysis using a FV1200 confocal laser scanning microscope (Olympus) equipped with a 40× objective.

Preparation of FITC-labeled saporin

For preparation of FITC-labeled saporin, saporin (200 μg, saporin from Saponaria officinalis seeds, Sigma-Aldrich) dissolved in H_2O (100 μl) was reacted with 2 equivalents of FITC (Sigma-Aldrich) dissolved in dimethyl sulfoxide (10 μl) and N,N-diisopropylethylamine (0.5 μl) at 30°C for 2 h, as previously reported.[5,6] To remove the unreacted FITC, gel filtration on a Sephadex G-25 column (PD-10, GE Healthcare) was performed prior to lyophilization. The protein concentration was determined using a Pierce BCA protein assay kit.

Encapsulation of fluorescently labeled dextran and saporin into exosomes

To load fluorescently labeled dextran into exosomes, exosomes (25 μg) were mixed with Texas red-labeled dextran (molecular weight 70,000) (Molecular Probes, Eugene, OR, USA), FITC-labeled dextran (molecular weight 70,000) (Sigma-Aldrich) or saporin (50 μg) in PBS (100 μl). After electroporation (poring pulse: twice pulse (200 V, 5 msec), transfer pulse: five pulse (20 V, 50 msec)) in a 1-cm electroporation cuvette at room temperature using a super electroporater NEPA21 Type II (NEPA Genes, Tokyo, Japan), removal of unencapsulated Texas red-dextran, FITC-dextran or saporin was accomplished by washing and filtration using Amicon Ultra centrifugal filters (100 K device), as previously reported.[5,6] Loading of FITC-dextran and FITC-saporin into exosomes was confirmed using a spectrofluometer (FP-6200, JASCO, Tokyo, Japan). The electroporation method resulted in encapsulation of Texas red-dextran (150 ng/ml) or FITC-dextran (100 ng/ml) in 20 μg/ml of exosomes. The efficiency of dextran encapsulation into exosomes was calculated to be 0.4% (Texas red-dextran) or 0.3% (FITC-dextran). The concentration of saporin encapsulated in 20 μg/ml exosomes was estimated to be approximately 87.0 ng/ml using the FITC-labeled saporin. The efficiency of saporin encapsulation into exosomes was calculated to be 0.2%.

Cell viability (WST-1 assay)

Cell viability was analyzed using the WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) assay, as previously reported.[5,6] E3-EGFR-expressing or wild-type-MDA-MB-231 cells (each 1×10^4 cells/100 μl) were incubated in 96 well microplates in MEM containing 10% FBS for 24 h at 37°C under 5% CO_2. The cells were then treated with each exosome sample (50 μl) at 37°C under 5% CO_2. After the sample treatment, WST-1 reagents (10 μl) were added to each well, and the samples were incubated for 40 min at 37°C. The absorbencies at 450 nm (A450) and 620 nm (A620) were measured, and the value obtained by subtracting A620 from A450 corresponded to the viable cell number.

Statistical analyses

All statistical analyses were performed using GraphPad Prism software (ver. 5.00; GraphPad, San Diego, CA, USA). For comparisons of two groups, unpaired Student's t-test was used after verification of the equal variances with an F-test. Welch's correction was performed

when the variances across groups were assumed to be unequal. For multiple comparison analyses, one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used. Differences were considered significant when the calculated p-value was < 0.05.

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2. Supplementary Figures

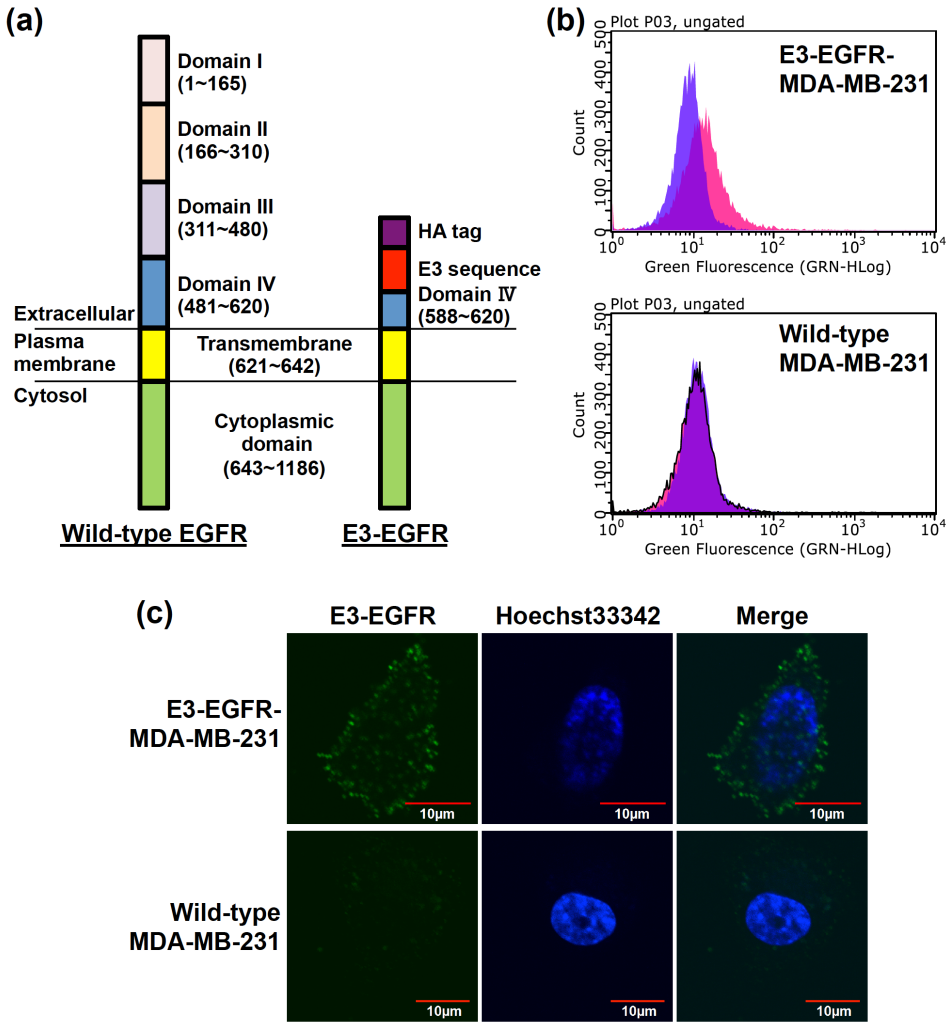


Figure S1. Cellular expression of E3-EGFR on the plasma membrane via gene engineering. (a) Structures of wild-type EGFR and E3-EGFR. Wild-type EGFR is composed of extracellular domains I-IV, the transmembrane domain, and the cytosolic domain. The extracellular domain of E3-EGFR includes the E3 sequence and a partial sequence for domain IV; the domains responsible for dimerization of the receptor (positions 481-587 in domain IV) and for EGF-binding and dimerization of the receptor (domains I-III) were eliminated from the EGFR structure. E3-EGFR carries a hemagglutinin A (HA) epitope tag in the N-terminal region. (b, c) Flow cytometry analysis (b) and confocal microscopic observations (c) of E3-EGFR-expressing or wild-type MDA-MB-231 cells stained with an anti-HA antibody to visualize E3-EGFR expression. Scale bar: 10 μm.

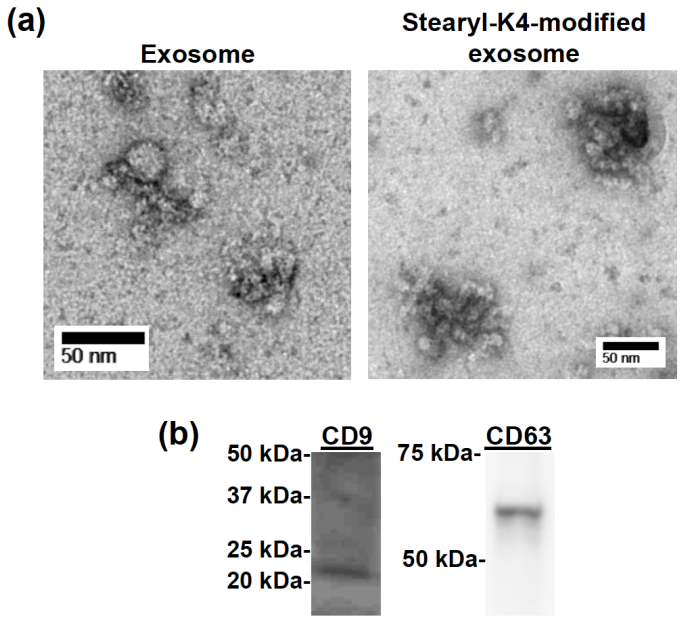
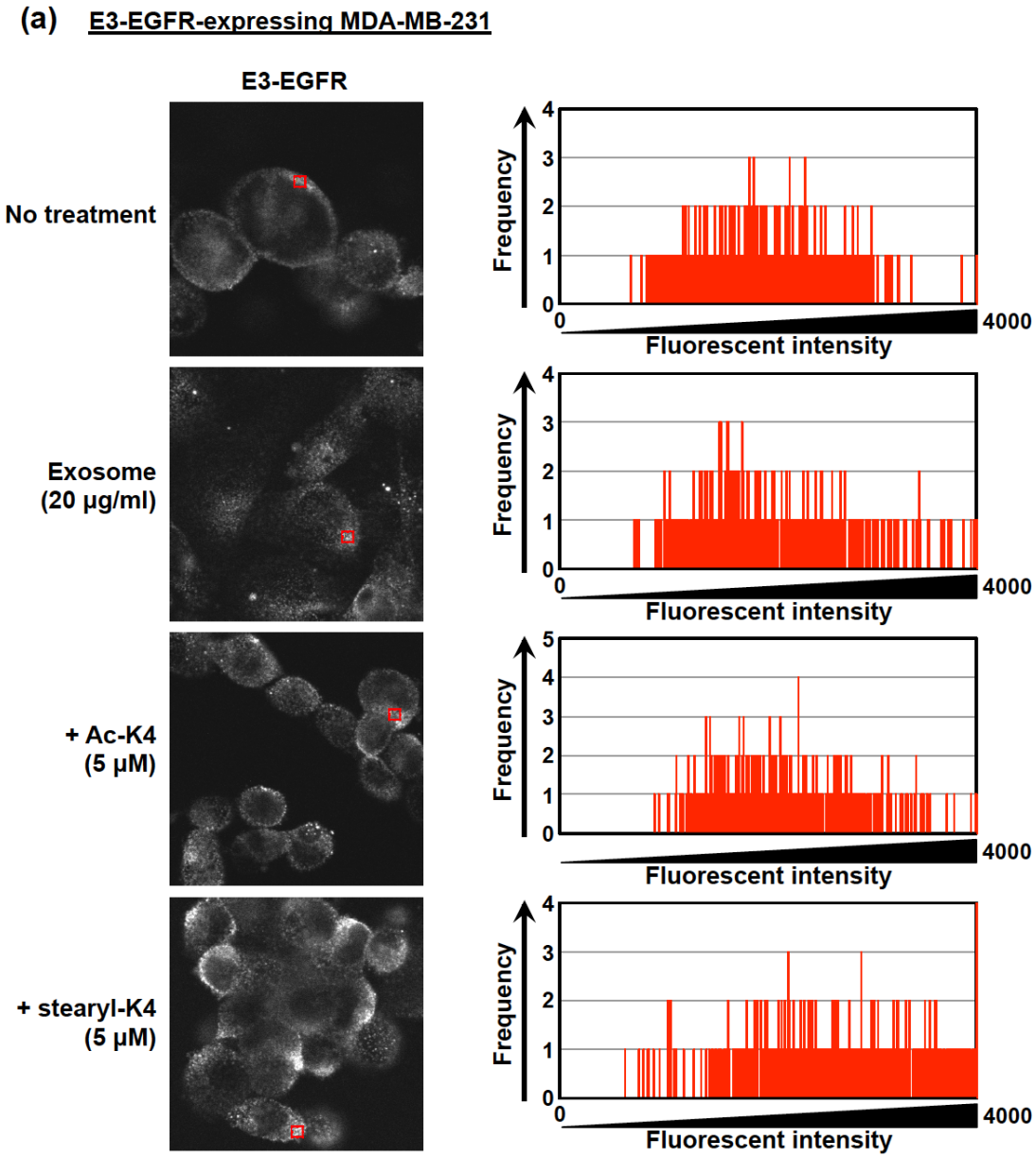


Figure S2. Isolation of exosomes secreted from HeLa cells. (a) TEM observations of isolated exosomes with or without the stearyl-K4 peptide modification. Scale bar: 50 nm. (b) Western blot analyses showing the exosomes secreted from HeLa cells. The CD9 and CD63 exosome marker proteins were detected. The immunoreactive species were detected at positions of approximately 23 kDa (anti-CD9) and 60 kDa (anti-CD63) through SDS-PAGE analysis.


(a)
E3-EGFR-expressing MDA-MB-231
E3-EGFR
No treatment
Exosome
(20 μg/ml)
+ Ac-K4
(5 μM)
+ stearyl-K4
(5 μM)
Frequency
Fluorescent intensity
0
4000

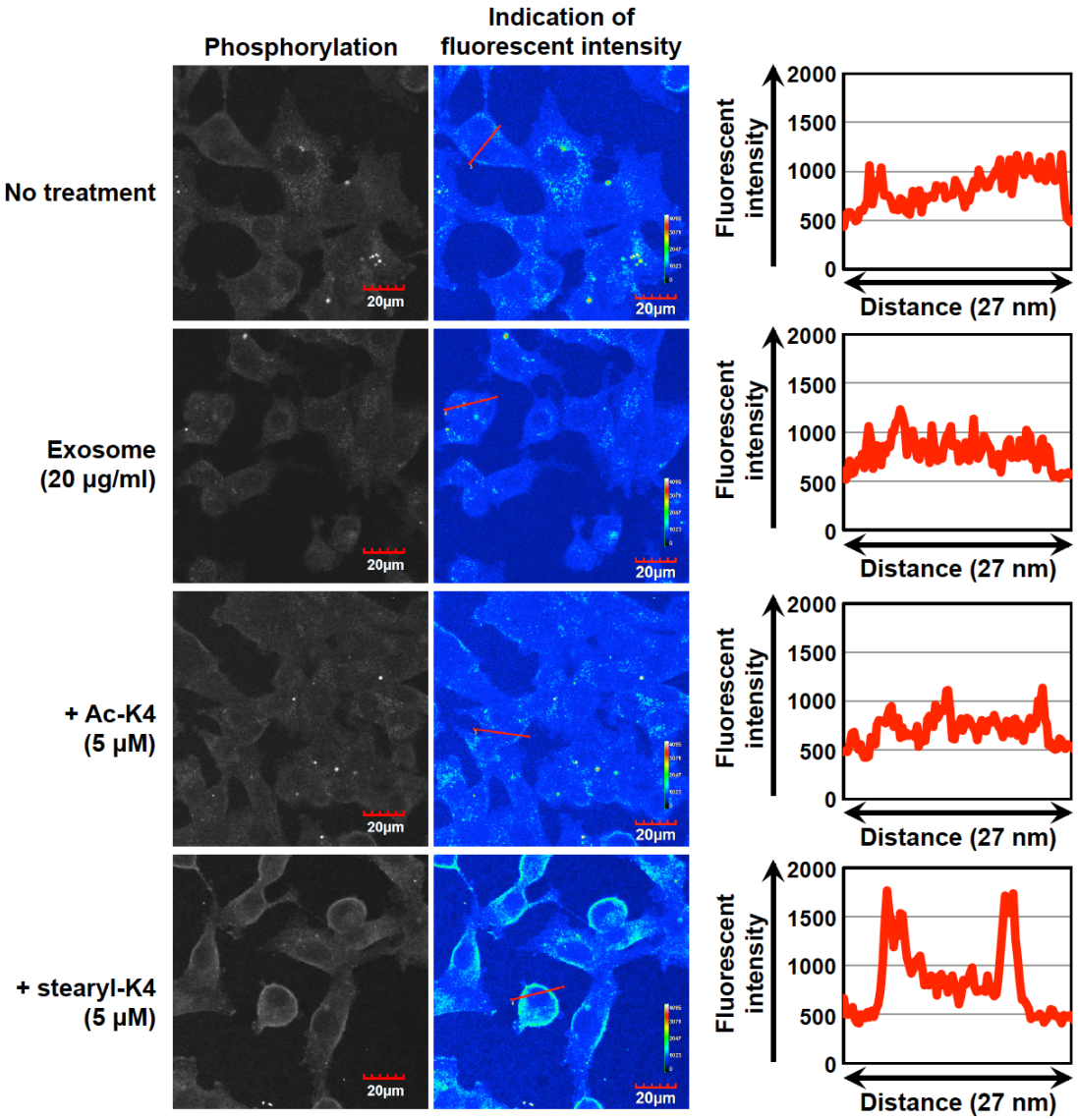


Figure S3. Visualization of E3-EGFR clustering and phosphorylation at Tyr1173 induced by treatment with stearyl-K4-modified exosomes. (a) Confocal microscopic observations of E3-EGFR-expressing MDA-MB-231 cells treated with exosomes (20 μg/ml) with or without the Ac-K4 or stearyl-K4 modification (each 5 μM) for 20 min at 37°C, prior to staining with anti-HA antibody to visualize E3-EGFR on the cellular membrane. All the images were scaled identically and taken with same microscopic settings. Visual appearance of fluorescent intensity of E3-EGFR (red square) was shown. (b) Confocal microscopic observations of E3-EGFR-expressing MDA-MB-231 cells treated with the exosome samples for 5 min at 37°C. Cellular staining with a p-EGFR (Tyr1173) antibody was performed prior to the observations to

visualize EGFR phosphorylation at Tyr1173 (pTyr1173). Scale bar: 20 μm. A line scan (red line) showing the intensity of E3-EGFR across a cell was shown.

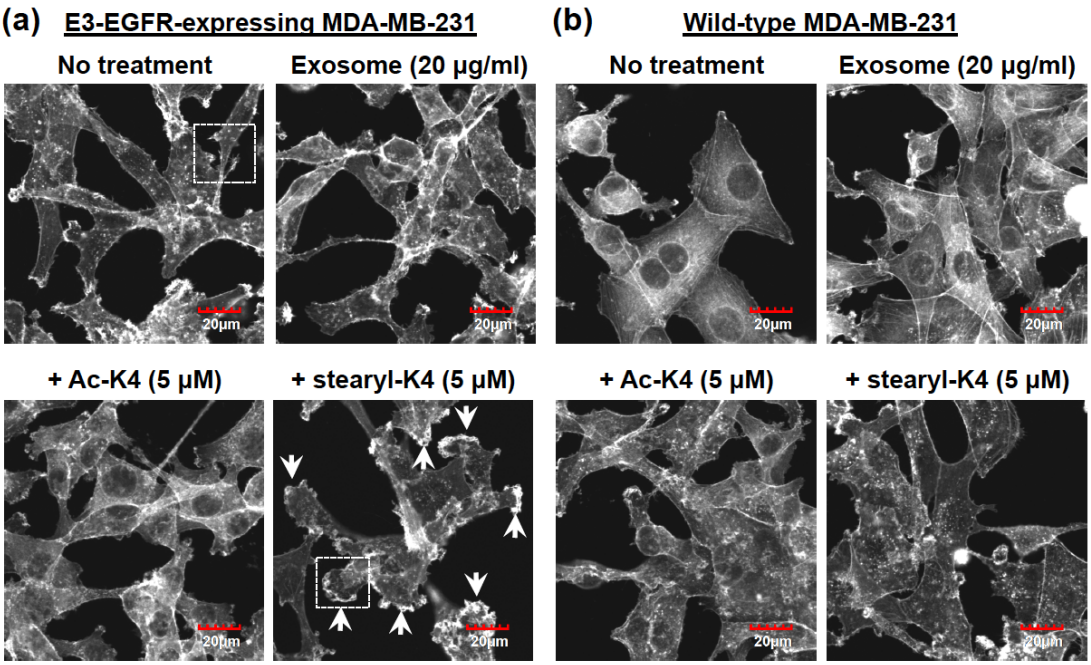


Figure S4. Effects of stearyl-K4-modified exosomes on actin organization in E3-EGFR-expressing cells. (a, b) Confocal microscopic observations of E3-EGFR-expressing (a) or wild-type (b) MDA-MB-231 cells treated with the exosome samples for 20 min at 37°C. Cellular staining with rhodamine-phalloidin was performed prior to the observations to visualize F-actin. Scale bar: 20 μm. The arrows indicate representative enhanced lamellipodia formation. Enlarged images of the areas marked by squares are shown in Fig. 2c.

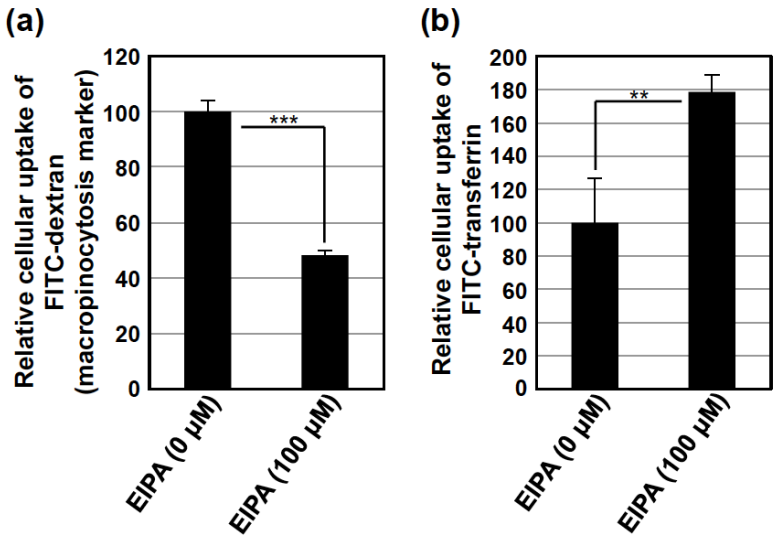


Figure S5. Effects of the macropinocytosis inhibitor EIPA on cellular exosome uptake. (a, b) The relative cellular uptake of FITC-dextran (0.5 mg/ml, molecular weight: 70,000, macropinocytosis marker) (a) and FITC-transferrin (0.4 mg/ml, marker of clathrin-mediated endocytosis) (b) in E3-EGFR-expressing MDA-MB-231 cells, with or without co-treatment with the macropinocytosis inhibitor EIPA (100 μM) for 1 h at 37°C, was analyzed using a flow cytometer. Epidermal growth factor (100 nM) was added to cell culture medium to induce active macropinocytosis in the experimental condition of (a). $^{**}p < 0.01$, $^{***}p < 0.001$.

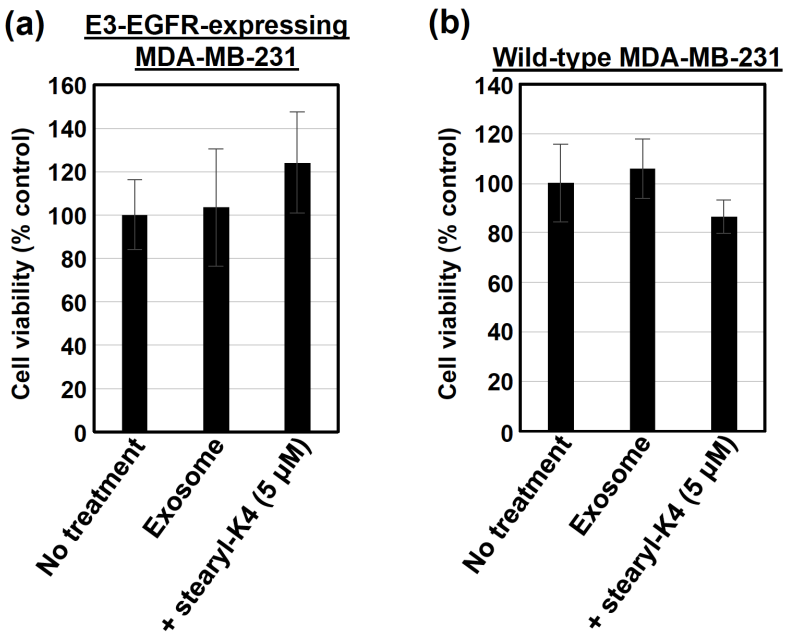


Figure S6. Cell viability. (a, b) E3-EGFR-expressing (a) or wild-type (b) MDA-MB-231 cells were treated with exosomes (20 μg/ml) with or without the stearyl-K4 (5 μM) modification for 24 h at 37°C prior to the WST-1 assay. The data are expressed as the mean (±SD) of four experiments.

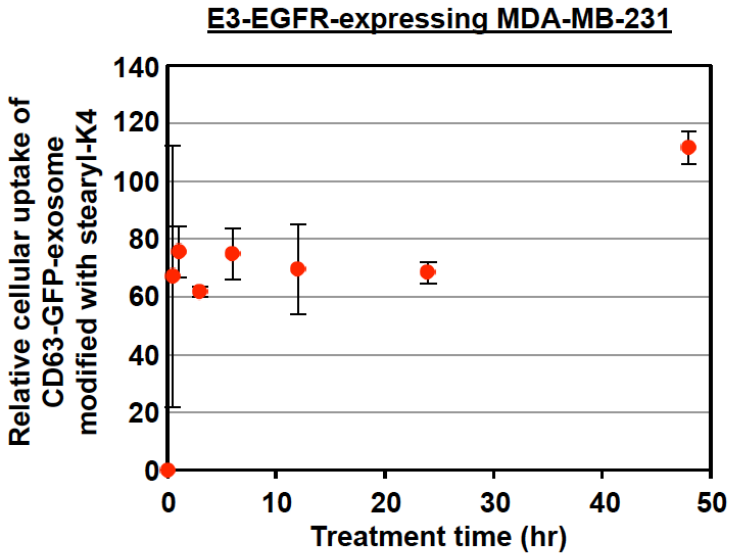


Figure S7. Time-dependent cellular uptake of stearyl-K4-modified exosomes in E3-EGFR-expressing MDA-MB-231 cells. Relative cellular uptake of CD63-GFP-exosomes (20 μg/ml) with the stearyl-K4 (5 μM) modification in E3-EGFR-expressing MDA-MB-231 cells in cell culture medium containing 10% FBS for each period at 37°C was analyzed using a flow cytometer. The data are expressed as the mean (±SD) of three experiments.