Electronic Supplementary Information (ESI)

Inhibition of cancer-cell migration by tetraspanin CD9-binding peptide

Thanawat Suwatthanarak,[a] Masayoshi Tanaka,[a] Yoshitaka Miyamoto,[b] Kenji Miyado,[b] and Mina Okochi[a]*

[a] Department of Chemical Science and Engineering, Tokyo Institute of Technology, 2-12-1, O-okayama, Meguro-ku, Tokyo 152-8552, Japan

[b] Department of Reproductive Biology, National Research Institute for Child Health and Development, Tokyo 157-8535, Japan.

*E-mail: okochi.m.aa@m.titech.ac.jp

Alanine scanning and truncation of CD9-BP

A peptide library was generated by mutating each amino acid in CD9-BP (RSHRLRLH) into alanine for alanine scanning and another library was obtained by deleting amino acids at N- or C-terminal portion of CD9-BP for truncation. Next, peptide array was synthesized using fluorenylmethoxycarbonyl (Fmoc)-based chemistry (Watanabe Chemical, Japan) and automated peptide spotter (MultiPep RSi; Intavis, Germany). Synthesis cycle was started with deprotecting Fmoc group at the N-terminus by 20% piperidine in N,N-dimethylformamide (DMF). Fmoc L-form amino acid at 0.5 M was then activated by 1.1 M Hydroxybenzotriazole (HOBt) and 1.1 M N,N'-Diisopropylcarbodiimide, and spotted on a cellulose membrane (grade 542; Whatman, UK), activated with β-alanine as the N-terminal basal spacer. The unreacted amine group was capped by 4% acetic anhydride in DMF before the membrane was washed with DMF and ethanol, respectively, at the end of cycle. Peptide spot was 4 mm in diameter and synthesis scale was 100 nmol/cm^2. The synthesis was conducted according to manufacturer's instructions with some modifications. Prior to use, the last Fmoc group was manually deprotected by 20% piperidine in DMF, followed by serial washing the membrane with DMF and ethanol. Side chain-protecting groups were later removed using a mixture of ultrapure water, triisopropylsilane (TIPS) and trifluoroacetic acid (TFA) (2/3/95, v/v/v %), followed by sequential washing the membrane with dichloromethane (DCM), DMF, ethanol and phosphate-buffered saline (PBS, pH 7.4). The prepared peptide array was blocked with 1% bovine serum albumin in PBS for 30 min and then used in sandwich binding assay with CD9 (0.3 μg/mL in PBS, 1 h, ab152262; Abcam, UK), Anti-CD9 antibody (1/1000 in PBS, 1 h, ab92726; Abcam), and Alexa Fluor® 568 secondary antibody (1/1000 in PBS, 1 h, ab175471; Abcam), respectively, at room temperature together with slow agitation. Between each step, the membrane was thoroughly washed with PBS. Fluorescence scanning and imaging of the membrane were done by a biomolecular imager (Typhoon FLA 9500; GE Healthcare, Sweden) and spot intensity was quantified by ImageQuant software (GE Healthcare).

Binding site evaluation of CD9-BP on CD9

Firstly, D-form CD9-BP was synthesized in solid phase using H-Rink-Aminde-ChemMatrix® resin and an automated microwave peptide synthesizer (Initiator+; Biotage, Sweden). In each elongation, Fmoc group at the N-terminus was deprotected by 20% piperidine in DMF before washing the resin with DMF. The carboxyl group of Fmoc-amino acid at 0.5 M was activated by 0.5 M HOBt, 0.5 M O-(1H-benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate and 0.5 M N,N- diisopropylethylamine, and next coupled to the N-terminus, followed by washing the resin with DMF. Synthesis was conducted according to manufacturer's instructions with some modifications: the scale was 0.1 mmol. After the final elongation, Fmoc group at the N-terminus was deprotected by 20% piperidine in DMF before washing the resin with DMF and DCM, respectively. Side chain-protecting groups were

removed and the peptide was cleaved from the resin by a mixture of ultrapure water, TIPS, and TFA (2.5/5/92.5, v/v/v %). The peptide was precipitated and washed by chilled diethyl ether. The peptide pellet, obtained from centrifugation at 4°C and 5,000 rpm for 10 min, was dissolved in 30% acetonitrile and freeze-dried. The peptide was purified and confirmed by high-performance liquid chromatography (HPLC) and mass spectrometry (MS) to ensure >90% purity. For the peptide labeling, CD9-BP and Fluorescein-5-Isothiocyanate (FITC 'Isomer I'; Thermo Fisher Scientific, USA) were incubated together with a mole ratio of 1:2 in sodium bicarbonate buffer (pH 8.7) for overnight at room temperature. Then, the FITC-conjugated CD9-BP was purified and confirmed using HPLC and MS. For the binding assay, a library of twelve-mer peptides was derived from the sequence of CD9's LEL (aa112-aa195) by six residue-frame shifted epitope mapping and synthesized on the membrane as described above. The peptide array was soaked into 10 μM FITC-conjugated CD9-BP in PBS for 1 h at room temperature, followed by washing the membrane with PBS. Finally, the membrane was fluorescently scanned and the spot intensity was quantified.

SPR experiment

For the competitive binding analysis of CD9-BP to interaction between EWI-2 and CD9, EWI-2 protein (13435-H08H; Sino Biological, China) was immobilized as a ligand on CM5 sensor chip using amine coupling kit in Biacore X100 Plus Package SPR system (GE Healthcare, Sweden). CD9 was incubated with CD9-BP to obtain 100 nM of CD9 and 0, 10, 100, 500, 100, 2500 or 5000 nM of CD9-BP in PBS for 1 h before the mixture was applied over the EWI-2-immobilized SPR sensor for 120 s. After each binding analysis, the sensor surface was regenerated with 50 mM sodium hydroxide for 60 s.

CD9 expression in MDA-MB-231 and NHDF cell lines

MDA-MB-231 human breast cancer or normal human dermal fibroblast (NHDF) cell line (American Type Culture Collection, USA) was cultured in a 10 cm dish using Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, and 100 units/mL of penicillin-streptomycin (Thermo Fisher Scientific) under 37°C, 5% CO_2, and a humidified atmosphere. At around 80% confluence, the cells were twice washed with PBS and then trypsinized (0.025% trypsin, 3 min, 37°C). The trypsinization was stopped by adding the culture medium and the cell suspension was centrifuged (1,000 rpm, 37°C, 5 min). The cell pellet was collected, washed with PBS, again centrifuged, and resuspended in PBS. The cell suspension was sonicated on ice (Duty cycle: 30%, Output control: 3, 3 min, Branson sonifier 250 advanced; Branson Ultrasonics Corp., USA) to disrupt the cells. The sonicated cell suspension was centrifuged (21,000 × g, 12 min) before the obtained supernatant was ultra-centrifuged (150,000 × g, 4°C, 45 min) to obtain the membrane fraction pellet. The membrane fraction pellet was resuspended in PBS. A bicinchoninic acid assay (TaKaRa Bio Inc., Japan) was performed to determine a protein concentration in the membrane suspension. The membrane suspension (100 μl) diluted to 0.1 μg/mL was subjected to a human CD9 enzyme-linked immunosorbent assay (LifeSpan BioSciences Inc., USA) to determine a CD9 concentration.

Effect of CD9-BP on cell viability of MDA-MB-231 and NHDF cell lines

MDA-MB-231 or NHDF cell line was cultured in a 96-well dish using the same condition as described above. At around 50% confluence, the cultured medium was removed and replaced with the new medium, containing CD9-BP or AAAA peptide (50, 100, 250, 500 or 1,000 nM). AAAA peptide was synthesized in solid phase as mentioned above. After the 24-h additional culture, a cell viability assay was performed using Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Japan).

Antimigratory effect of CD9-BP to MDA-MB-231 and NHDF cell lines

For the wound healing assay, MDA-MB-231 or NHDF cell line was cultured in a 24-well dish using the same condition as described above. At almost 100% confluence, the cells were scratched by pipet tips to obtain 1 mm wound before the cultured medium was removed and replaced with the new medium, containing CD9-BP, AAAA or Apamin peptide (50 or 100 nM). Apamin peptide was synthesized in solid phase as mentioned above. A wound gap was observed at 0 h and 24 h after the peptide introduction under a microscope (DMi8; Leica, Germany). For the single cell-tracking migration assay, MDA-MB-231 or NHDF cell line was cultured in a 24-well dish using the same condition as described above. At around 20%, the new medium, containing CD9-BP, AAAA peptide, or apamin at 100 nM, was introduced. During further-12 h incubation, microscopy image of cells was taken every 20 min by a microscope. Analysis of the single cell-tracking migration was done by Fiji, an image processing package based on ImageJ program. For the Boyden chamber assay, CytoSelect™ 96-well Cell Migration Assay with 8 μm-pore size (Fluorometric Format, CBA-106; Cell Biolabs, Inc., USA) was employed. Serum-free cell suspension (100 μL, 0.3×10^6 cells/mL) of MDA-MB-231 or NHDF cells was placed in the upper chamber, and 10% FBS-added medium (150 μL) together with a chemoattractant at 100 nM (CD9-BP, AAAA peptide, Apamin peptide, or Anti-CD9 antibody) was placed in the lower well. Epithelial growth factor (AF-100-15; PeproTech, USA) was added to 10% FBS-added medium at 10 nM to increase the cell invasion. After allowing cells to migrate to the other side of membrane under 37°C, 5% CO_2, and a humidified atmosphere for 24 h, the invasive cells were harvested by cell detachment solution, lysed and quantified by a mixture of lysis buffer, CyQuant®GR dye solution. Microplate reader was finally used to measure fluorescence intensity of the solutions at 480/520 nm. The assay was conducted strictly to manufacturer's instructions.

Table S1 Characteristics of alanine-substituted and truncated CD9-BP[a]

Sequence	pI	GRAVY	Charge	Structure (+; helix, -; linear, N/A; not available)	Structural image
RSHRLRLH	12.3	-1.637	3	+++	
ASHRLRLH	12.0	-0.850	2	++-	
RAHRLRLH	12.3	-1.312	3	++-	
RSARLRLH	12.3	-1.012	3	++-	
RSHALRLH	12.0	-0.850	2	++-	
RSHRARLH	12.3	-1.887	3	---	
RSHRLALH	12.0	-0.850	2	++-	
RSHRLRAH	12.3	-1.887	3	---	
RSHRLRLA	12.3	-1.012	3	+++	

Table S1 Characteristics of the alanine-substituted and truncated CD9-BP[a] (Continued).

Sequence	pI	GRAVY	Charge	Structure (+; helix, -; linear, N/A; not available)	Structural image
_SHRLRLH	12.0	-1.229	2	---	
__HRLRLH	12.0	-1.300	2	---	
___RLRLH	12.0	-0.920	2	---	
____LRLH	10.8	-0.025	1	N/A	N/A
RSHRLRL_	12.3	-1.414	3	---	
RSHRLR__	12.3	-2.283	3	---	
RSHRL___	12.0	-1.840	2	---	
RSHR____	12.1	-3.230	2	N/A	N/A
AAAA	3.7	1.800	0	N/A	N/A

[a]Isoelectric point (pI) and charge were obtained from Peptide property calculator (https://pepcalc.com) and grand average of hydropathy (GRAVY) value was obtained from GRAVY calculator (http://www.gravy-calculator.de). Peptide structure was predicted by PEP-FOLD 3 (https://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py#forms::PEP-FOLD3). Structure of some peptides could not be predicted because their sequences are too short.

Table S2 Characteristics of the peptides derived from the LEL of CD9[a]

Sequence	pI	GRAVY	Charge
SHKDEVIKEVQE	4.52	-1.367	-1.9
IKEVQEFYKDTY	4.32	-1.133	-1.0
FYKDTYNKLKTK	10.18	-1.667	3.0
NKLKTKDEPQRE	9.43	-2.683	1.0
DEPQRETLKAIH	5.31	-1.483	-0.9
TLKAIHYALNCC	8.05	0.675	1.0
YALNCCGLAGGV	2.77	1.200	-0.1
GLAGGVEQFISD	0.81	0.383	-2.0
EQFISDICPKKD	4.17	-0.825	-1.1
ICPKKDVLETFT	6.20	0.000	-0.1
VLETFTVKSCPD	3.93	0.233	-1.1
VKSCPDAIKEVF	6.20	0.233	-0.1
AIKEVFDNKFHI	7.83	-0.075	0.1

[a]Isoelectric point (pI) and charge were obtained from Peptide property calculator (https://pepcalc.com) and grand average of hydropathy (GRAVY) value was obtained from GRAVY calculator (http://www.gravy-calculator.de).

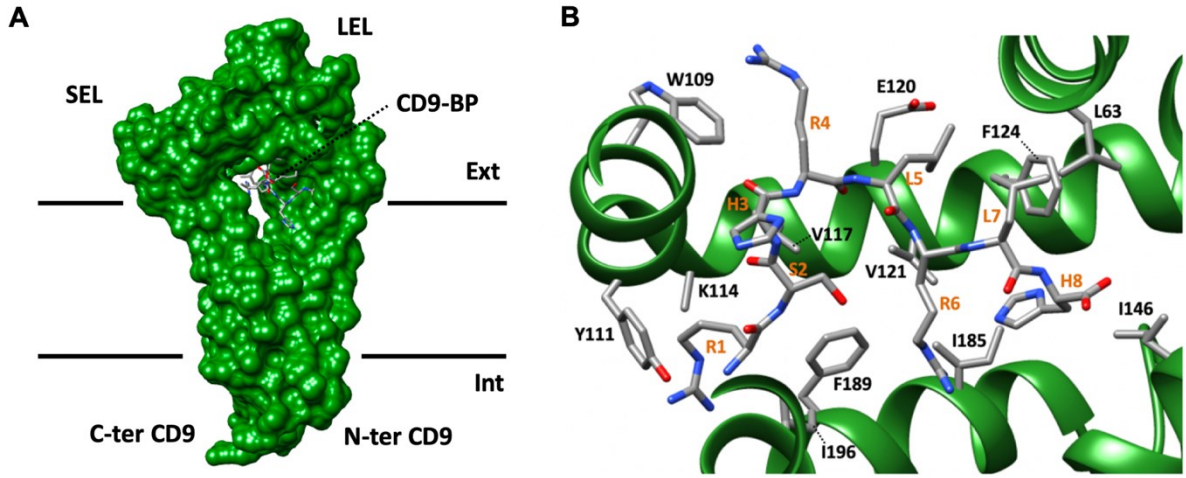

Figure S1. Molecular docking results between CD9 and CD9-BP predicted by MDockPeP, an ab-initio protein-peptide docking server. (A) Surface representation of molecular docking between CD9 and CD9-BP. (B) Zoomed view of molecular docking between CD9 and CD9-BP. LEL, large extracellular loop. SEL, small extracellular loop. Ext, extracellular region. Int, intracellular region.

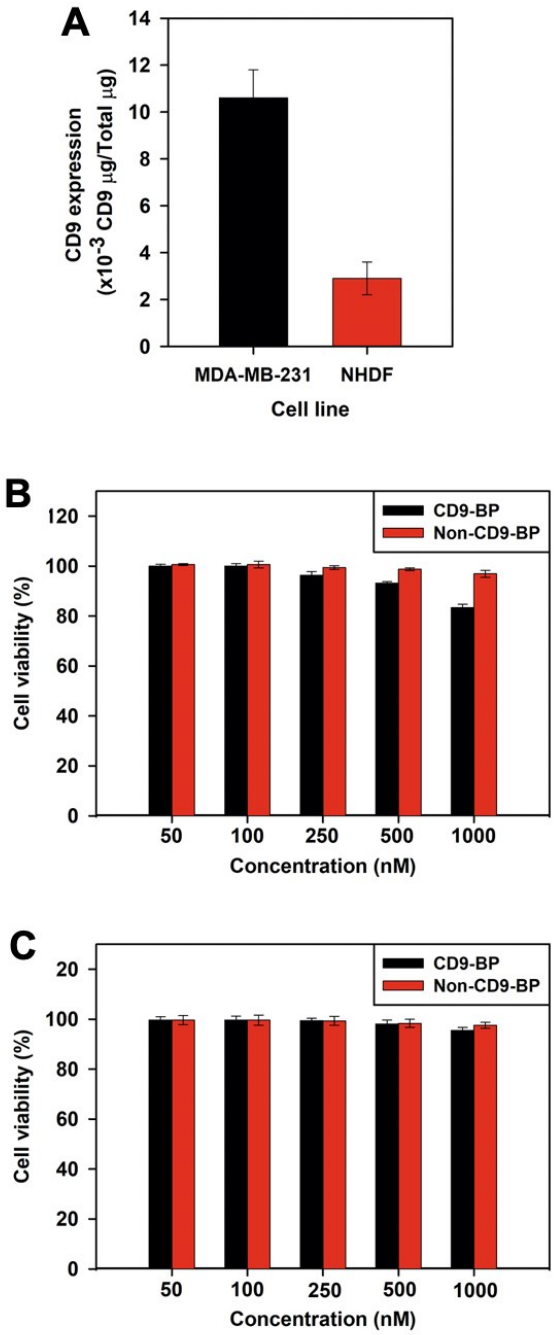

Figure S2. (A) Level of CD9 expression in the membrane fraction of MDA-MB-231 and NHDF cell lines. Effect of CD9-BP on cell viability of (B) MDA-MB-231 and (C) NHDF cell lines. Non-CD9-BP or AAAA peptide was a negative control. Error bar represents a standard deviation from triplicate results.

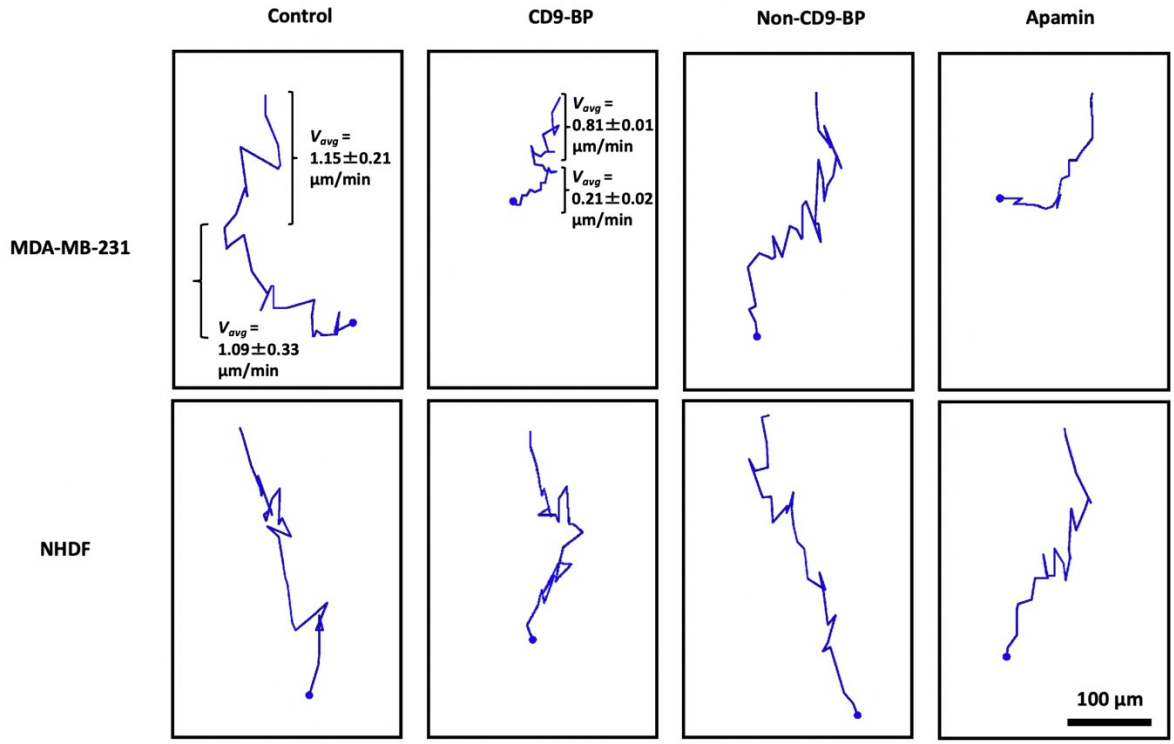

Figure S3. Representative trajectories of MDA-MB-231 and NHDF cells in single cell-tracking migration assay. The dot represents the endpoint. V_{avg}, average velocity.