ELECTRONIC SUPPLEMENTARY INFORMATION

Non-Genetic Cell-Surface Modification with a Self-Assembling Molecular Glue

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Supplementary Contents

Supplementary Methods

1. Chemical synthesis of adh3.0

Synthetic method of adh3.0 was described in our previous research[1]. Briefly, the scheme is shown in scheme S1.

(1) Synthesis of **Aoa-SFFK (alkylchloride-K-) peptide**

The peptide was synthesized with RINK Amide MBHA resin LL by a standard Fmoc-based solid phase peptide synthesis (SPPS). **Alkylchloride-1** was prepared according to our previous report[1]. The resin (0.1 mmol) was swelled in CH_2Cl_2 for 3 hours before use. The resin was treated in succession with Fmoc-Lys(Dde)-OH (Watanabe chemical), Fmoc-Phe-OH (Watanabe chemical), Fmoc-Phe-OH, Fmoc-Ser(*tert*-butyl)-OH (Watanabe chemical), and [(*tert*-butoxycarbonyl)-aminooxy]acetic acid (Boc-Aoa-OH, TCI) by repeating the following A-E steps to obtain **RESIN-1** (scheme S1). The completion of each coupling step was confirmed by the Kaiser test.

A. The deprotection of Fmoc group was performed with 20% (v/v) piperidine in DMF twice (reaction time: 1 min and 10 min).

B. The resin was washed with DMF (2 mL × 5 times).

C. The coupling of the consecutive amino acid was carried out with the corresponding Fmoc-amino acid / Boc-Aoa-OH (0.3 mmol) / alkylchloride-1 (0.3 mmol), *O*-(benzotriazole-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU, 0.3 mmol, Watanabe Chemical), 1-hydroxybenzotriazole (HOBt, 0.3 mmol, TCI), and diisopropylethylamine (DIPEA, 0.6 mmol, Fujifilm-Wako) in the mixture of DMF/NMP = 1/1 for 30 min.

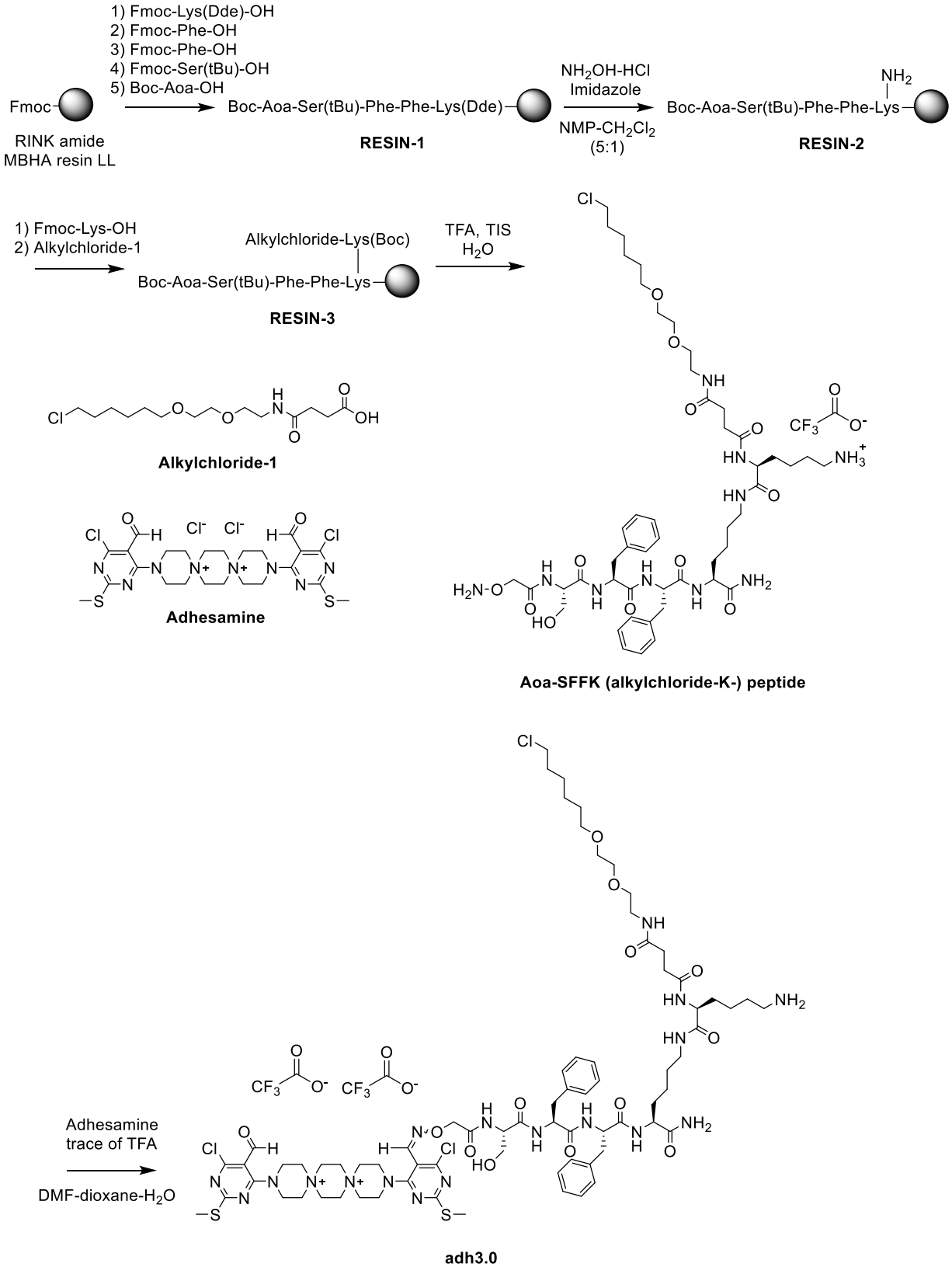
D. The resin was washed with DMF (2 mL × 5 times).

E. The resin was washed with CH_2Cl_2 (2 mL × 3 times) and DMF (2 mL × 3 times).

After the coupling of the consecutive SFFK amino acid, the resin was treated with a solution of $NH_2OH \cdot HCl$ (1.25 g) and imidazole (918 mg) in NMP (5 mL) and CH_2Cl_2 (1 mL) at room temperature for 3 hours for the deprotection of 1-(4,4-dimethyl-2,6-dioxacyclohexylidene)ethyl (Dde) at the Lysine side chain (Scheme S1, **RESIN-2**). The resulting resin and Fmoc-Lys (Boc)-OH was coupled through the B-E steps, followed by the further conjugation with alkylchloride-1 through A-D steps. The resin was washed with methanol (2 mL × 2) and CH_2Cl_2 (2 mL × 2) (Scheme S1, **RESIN-3**). After drying in vacuo, the resin was treated with a mixture of trifluoroacetic acid (TFA) / triisopropylsilane (TIS) / H_2O = 95 / 2.5 / 2.5 for 3 hours. After filtration, cooled diethylether (40 mL × 2) was added to the filtrate. After centrifugation (2,300 g x 5 min), the supernatant was discarded. The precipitate was washed with diethylether (40 mL × 2). The residue was dried in vacuo at room temperature for 3 hours to obtain crude **Aoa-SFFK (alkylchloride-K-) peptide** (91.5 mg) as a white solid. ESI-LRMS, found 1033.40 calcd. 1033.55 ($[C_{49}H_{77}ClN_{10}O_{12} + H]^+$).

(2) Synthesis of adh3.0

Adhesamine (7.94 mg, 11.8 μmol, Nagase Chemical Co.) was added to a solution of **Aoa-SFFK (alkylchloride-K-) peptide** (9.68 mg, 8.44 μmol) in 2 mL solvent (DMF/dioxane/water/TFA = 2/1/2/0.0004). The solution was stirred at 51 °C for 22 hours. After removal of the solvent under a stream of N_2, the residue was purified by HPLC (ODS-A, 30x150 mm, UV detector: 342 nm, flow rate: 15 ml/min, solvent A: water (0.01% (v/v) TFA), solvent B: CH_3CN (0.01% (v/v) TFA), gradient time (min) / B (%) = 0 / 15 → 1 / 15 → 21 / 55 → 25 / 95, retention time 22 min). The collected solution was lyophilized to provide adh3.0 (5.79 mg, 3.14 μmol, 37%) as a white solid. ESI-HRMS: found 806.33881 calcd. 806.33805 ($[C_{73}H_{107}Cl_3N_{18}O_{13}S_2]^{2+}$), purity >95% (calculated in the bellow LCMS analysis). ^{1}H-NMR (600 MHz, DMSO-*d6*) δ 1.22-1.32 (6H, m), 1.35-1.41 (4H, m), 1.46-1.52 (6H, m), 1.65-1.72 (4H, m), 2.29-2.37 (4H, m), 2.52 (3H, s), 2.55 (3H, s), 2.70-2.83 (4H, m), 2.94-3.08 (4H, m), 3.18 (2H, q, *J* = 6.0 Hz), 3.35-3.40 (4H, m), 3.45-3.50 (4H, m), 3.52-3.58 (2H, m), 3.62 (2H, t, *J* = 6.6 Hz), 3.82-4.14 (26H, m), 4.34 (1H, q, *J* = 6.0 Hz), 4.42-4.46 (1H, m), 4.50-4.54 (1H, m), 4.64 (2H, s), 7.05 (1H, br), 7.13-7.27 (12H, m), 7.71 (2H, br), 7.89-7.91 (2H, m), 7.96 (1H, t, *J* = 5.4 Hz), 8.00 (2H, d, *J* = 8.4 Hz), 8.13 (1H, d, *J* = 8.4 Hz), 8.22 (1H, d, *J* = 7.8 Hz), 8.44 (1H, s), 10.1 (1H, s). ^{13}C-NMR (150 MHz, DMSO-*d6*) δ 13.6, 13.8, 22.3, 22.6, 24.8, 26.0, 26.5, 28.7, 28.9, 30.4, 30.5, 31.1, 31.6, 31.9, 36.9, 37.3, 38.4, 38.5, 38.6, 39.9, 40.8, 41.5, 45.3, 51.1, 51.2, 52.3, 52.5, 54.0, 54.7, 61.5, 69.0, 69.3, 69.5, 70.1, 72.3, 103.6, 107.3, 126.1, 126.2, 127.9, 128.0, 129.0, 129.1, 137.5, 137.6, 145.8, 159.6, 160.6, 164.3, 168.5, 169.7, 170.2, 170.6, 170.7, 171.3, 171.5, 171.6, 173.2, 173.3, 186.5.



Scheme S1. Synthesis of adh3.0

2. DNA construction and protein purification

(1) Design of HALO gene encoding plasmid: pET28b-His6-HALO vector

The GGSGGS peptide linker sequence and HALO gene encoding Halo protein was amplified by PCR using forward and reverse primers described in Table S1 from the pET28b-MMP2-HALO-His6 Vector, which we reported previously[1]. The gene was subcloned into the *Eco*RI and *Hind*III site of the pET28b-His6 vector using Infusion® HD Cloning Kit (Takara Bio) to yield a pET28b-His6-HALO vector. The PCR amplified sequence was verified by DNA sequencing.

(2) Design of ANG-2-Halo (ANG2-H) / VEGF-Halo (VEGF-H) / PD-L1-Halo (PDL1-H) fusion proteins

Total RNA from cultured HEK293 cells and HUVECs was prepared using ISOGEN (NIPPON GENE), according to the manufacturer's protocol. Then cDNAs were synthesized using Prime-Script 1st strand cDNA Synthesis Kit (Takara Bio), according to the manufacturer's protocol. The resulting cDNAs were PCR-amplified with forward and reverse primers shown in Table S1. cDNAs derived from HEK293 cells and cDNAs derived from HUVECs were used as templates for amplifying ANGPT2 gene encoding Ang-2 protein, and VEGF-A gene encoding VEGF-A protein, respectively. These genes were further amplified using forward and reverse primers for infusion cloning described in Table S1. Obtained genes were subcloned into the *Nde* I site of pET28b-His6-HALO vector to yield pET28b-His6-ANG-2-HALO and pET28b-His6-VEGF-HALO. The PCR-amplified sequences were verified by DNA sequencing.

The gene encoding human PD-L1 was amplified by PCR using forward and reverse primers described in Table S1 from the pMXx-Neo-hPDL1 vector, which was kindly provided by Prof. Tasuku Honjo, Kyoto University. The PD-L1 gene was further amplified using forward and reverse primers for infusion cloning described in Table S1. The PD-L1 gene was subcloned into the *Nde* I site of pET28b-His6-HALO vector to yield pET28b-His6-PD-L1-HALO. The PCR amplified sequence was verified by DNA sequencing.

(3) Design of secreted PD-L1-Halo (secPDL1-H) fusion protein

The combinatorial gene construct of secPD-L1 and Halo was prepared via following steps. Forward and its complemental sequences (Table S1) of C-terminal region of secPDL-1 were purchased and annealed. PCR product was obtained by using PD-L1 Forward primer for infusion and reverse primer for secPD-L1. In the second step, the annealed C-terminal region of secPD-L1 DNA and the PCR product were used as templates for the following overlap extension PCR under the overlap extension primers (Table S1). Another PCR product was prepared with Forward primer for secPD-L1 and reverse Halo primer for infusion. Finally, these genes were tandemly subcloned in the *Nde I* site of pET28b-His6-HALO vector to yield pET28b-His6-secPD-L1-HALO. The PCR amplified sequence was verified by DNA sequencing.

(4) Design of MMP2-Halo fusion protein (MMP2-H)

Total RNA from cultured HEPA 1-6 cells (Riken source) was prepared using ISOGEN (NIPPON GENE), according to the manufacturer's protocol. Then cDNAs were synthesized using Prime-Script 1st strand cDNA Synthesis Kit (Takara Bio), according to the manufacturer's protocol. cDNAs derived from HEPA 1-6 cells were used as a template for amplifying the gene encoding MMP2 catalytic domain. The gene was amplified by PCR using forward(5'-ATG CAG AAG TTC TTT GGG CTG-3') and reverse(5'- GCA GCC CAG CCA GTC TGA TTT-3') primers described in our previous report[1]. The gene was further amplified for infusion cloning. The resulting gene

was subcloned into the *Nco* I and *EcoR*I site of pET28b-HALO-His6 vector to yield pET28b-MMP2-HALO-His6. The PCR amplified sequence was verified by DNA sequencing.

3. Expression and purification of ANG2-H, VEGF-H, PDL1-H, and secPDL1-H

The expression vectors pET28b-His6-ANG-2/VEGF/PD-L1/secPD-L1-HALO were transformed into BL21-CodonPlus (DE3)-RIPL competent cells (Agilent Technology). These cells were grown in LB (Lennox, Nacalai Tesque) growth media containing kanamycin 50 μg/mL at 37 °C to an optical density (600 nm) of 0.6, followed by the addition of 0.6 mM isopropyl-β-D-galactopyranoside (IPTG, Fujifilm-Wako). After the incubation for 6 hours at 35 °C, the cells were harvested by centrifugation (6,000 rpm, 5 min, 4 °C). The cell-pellet was re-suspended in lysis buffer (20 mM TRIS-HCl (pH 8.0), 10% (v/v) glycerol, 100 mM NaCl, 0.1% (v/v) NP-40, 1x protease inhibitor cocktail) and lysed by sonication. Following centrifugation (16,000 rpm, 30 min, 4 °C) allowed us to remove the soluble fraction. Then, precipitated inclusion body was re-suspended in the 20 mM TRIS-HCl (pH 8.0) buffer with 8 M of urea and centrifuged (16,000 rpm, 30 min, 4 °C) again to remove insoluble fraction. The solution was then enclosed in a dialysis membrane, Size 27 (Fujifilm-Wako) to be dialyzed by gradually titrating 20 mM TRIS buffer (pH 8.0) containing 20 mM NaCl and 0.5 mM dithiothreitol (DTT) for 3 days to refold the fusion proteins. The 6-His-tagged fusion proteins were purified by using Ni SepharoseTM 6 Fast Flow (GE Healthcare). Obtained proteins were concentrated by Amicon-ultra (Merck) 50k for ANG2-H/PDL1-H and 30k for VEGF-H/secPDL1-H. The concentration was determined by the absorbance at 280 nm (molar extinction coefficient, ε values for ANG2-H, VEGF-H, PDL1-H, secPDL1-H were 143,865 $M^{-1}cm^{-1}$, 83,515 $M^{-1}cm^{-1}$, and 91,830 $M^{-1}cm^{-1}$, and 91,705 $M^{-1}cm^{-1}$, respectively).

4. The reaction analysis of Halo-tag fusion proteins with Halo-tag ligands

ANG2-H (7.5 μM) / VEGF-H (7.5 μM) / PDL1-H (7.5 μM) / secPDL1-H (7.5 μM) / MMP2-H (7.5 μM) were reacted with HaloTag® TMR ligand (5 μM, Promega) in the presence or absence of adh3.0 (5 - 250 μM) in PBS at 37 °C with BioShaker M·BR-022UP (TAITEC) 200 r/min. The reaction was stopped by mixing with SDS sample buffer (Nacalai tesque Inc. #09499-14), followed by the electrophoresis with 8% (v/v) poly acrylamide gel. After electrophoresis, proteins were observed with Typhoon FLA 9000 (GE Healthcare) and fluorescence spectra derived from the labelled Halo-tag fusion proteins were taken with λ_{ex} = 532 nm and λ_{em} = 570 nm.

5. Cell culture

NIH3T3 cells were maintained in Dulbecco's modified Eagle medium (DMEM, #11995-065 Gibco®), supplemented with 100 units/mL penicillin (Nacalai Tesque), 100 μg/mL streptomycin sulfate (Nacalai Tesque), and 10% (v/v) fetal bovine serum (FBS, Biowest, S1820-500), at 37 °C and in a humidified 5% CO_2 incubator. Jurkat expressing human PD-1 cells and MDA-MB-231 cells, which were kindly provided by Prof. Tasuku Honjo, Kyoto University, were maintained in RPMI 1640 (Corning, 10040-CV), supplemented with 100 units/mL penicillin (Nacalai Tesque), 100 μg/mL streptomycin sulfate (Nacalai Tesque), and 10% (v/v) FBS at 37 °C and 5% CO_2 in a humidified incubator. HUVECs were maintained in Endothelial Cell Growth Medium Kit (ECM, C-22110, PromoCell) under the full supplement condition at 37 °C in a humidified 5% CO_2 incubator. The medium was changed to the Endothelial Cell Basal Medium (ECM without supplement) 1 hour before the angiogenesis assay.

6. Immunostaining of Halo-tag fusion-protein on cell surface (Fixed cells)

On day 0, NIH3T3 cells were seeded on a Poly-D-lysine coated glass-bottom ViewPlate®-96F microplate (PerkinElmer, Inc.) at 1.5×10^4 cells per well. On day 1, the medium was changed to serum-free DMEM and incubated for additional 1hour. The cells were then incubated for 20 min in the prepared medium which adh3.0 (50 μM) and fusion proteins (7.5 μM) were pre-incubated for 20 min in a serum-free DMEM. Then, the cells were fixed with 4% (w/v) paraformaldehyde solution (Muto Pure Chemicals Co., Ltd) at room temperature for 15 min followed by blocking with 2% (w/v) BSA for 40min. The Cells were then incubated with primary antibody (Anti-HaloTag® Monoclonal Antibody, Promega #G9211) for 1 hour at room temperature. After washing with PBS, cells were exposed to the secondary antibody (AF568 conjugated anti-mouse IgG, Invitrogen) for 1hour at room temperature, followed by the staining with DAPI (4',6-Diamidino-2-phenylindole, dihydrochloride, Dojindo Molecular Technologies, Inc.) solution for 15 min. The cells were observed by Cell Voyager 1000 confocal microscope (CV-1000, Yokogawa Electric Corporation). Cells were washed with phosphate buffer saline (PBS, pH 7.4, 10010-023, Thermo Fisher Scientific) after each step.

7. Immunostaining of cell-surface with ANG2-H, VEGF-H, and adh3.0 (Living cells)

On day 0, NIH3T3 cells were seeded on a Poly-D-lysine coated glass-bottom ViewPlate®-96F microplate (PerkinElmer, Inc.) at 2.0×10^4 cells per well. On day 1, the medium was changed to serum-free DMEM and incubated for additional 1hr. The cells were then incubated for 20 min at 37 °C and 5 % CO_2 in a humidified incubator, in the presence of adh3.0 (50 μM), VEGF-H and ANG2-H proteins (7.5 μM each) cocktail, which were pre-incubated for 20 min in a serum-free DMEM. The cells were proceeded to the blocking with 2% (w/v) BSA for 40 min. The cells were incubated with primary antibody (Anti-VEGFA rabbit monoclonal antibody, abcam, ab52917, abcam and Anti-Ang-2 mouse monoclonal antibody, sc-74403, Santacruz) for 1 hour at room temperature. After washing, cells were exposed to the secondary antibody (AF568 conjugated anti-mouse IgG, #A-11004, Invitrogen, and Goat Anti-rabbit IgG CF®488A, #20012, Biotuim) for 1 hour at room temperature, followed by the staining with Hoechst 33342 (1 μg/mL, Thermo Fisher Scientific) solution for 10 min. The cells were observed by CV-1000. Cells were washed with PBS after each step.

8. IL2-ELISA

The level of human IL-2 cytokine was measured by sandwich enzyme-linked immunoassay (ELISA). Briefly, MDA-MB-231 or NIH3T3 cells (5.0×10^4 /well) were seeded in 96-well plate overnight in the RPMI 1640, containing 10% (v/v) FBS, and incubated for 20 min at 37 °C in a humidified 5% CO_2 incubator. Adh3.0 and secPDL1-H were pre-incubated for 20 min in a RPMI 1640 medium supplemented with 1% (v/v) FBS. The cells were treated with adh3.0 (50 μM) alone , secPDL1-H (7.5 μM) alone, or the combination of both adh3.0 (50 μM) and secPDL1-H (7.5 μM) in the presence or absence of α-human PD-1 antibody (# 329925, 1:1000; BioLegend). After the incubation, cells were washed with RPMI 1640 medium supplemented with 1% (v/v) FBS. Then, Jurkat PD-1 cells (2.5×10^4 /well) were suspended in RPMI 1640 medium supplemented with 1% (v/v) FBS, containing PHA (phytohemagglutinin, #L9017, sigma, 1-3 μg/mL, final) and PMA (phorbpl myristate acetate, #162-23591, Fujifilm-Wako, 2 ng/mL, final) followed by the co-culture. The cells were incubated for 48 hours at 37 °C in a humidified 5% CO_2 incubator. After that, 80 μL of culture supernatant per well were used for an ELISA. The cytokine level was measured using IL-2 ELISA kit from R&D systems (D2050), according to the manufacturer's

protocol. Plates were read at 450 nm using a microplate reader (MTP-880 Lab, CORONA electric Co., Ltd.). Data was analyzed using MS Excel.

9. Wound-healing assay

NIH3T3 cells were seeded on a Poly-D-lysine coated glass-bottom ViewPlate®-96F microplate (PerkinElmer, Inc.) at 2.0×10^4 cells per well in the complete growth medium overnight. When cells reached about 100% confluence, the medium was changed to DMEM, containing 0.5% (v/v) FBS and incubated for an hour at 37 °C in a humidified 5% CO_2 incubator. Then, the cells were scratched with a p-200 pipette tip. Immediately after the wounding, floating cells were removed by washing with DMEM containing 0.5% (v/v) FBS. Followingly, the cells were treated with adh3.0 (50 μM) alone, Halo-tag fusion proteins alone, or the combination of both under the DMEM supplemented with 0.5% (v/v) FBS. After 20 min incubation of the treated cells at 37 °C in a humidified 5% CO_2 incubator, the medium was changed to the fresh DMEM supplemented with 0.5% (v/v) FBS for the purpose of washing the fusion proteins and adh3.0 out. The living cells were observed by CV-1000 microscope (CV-1000, Yokogawa Electric Corporation). Time-lapse images were taken at 20 min or 30 min intervals for up to 14 hours. Each experiment was conducted at least in triplicate. Images were analyzed by NIH ImageJ, using Wound Healing Size Tool (available at https://github.com/AlejandraArnedo/Wound-healing-size-tool/wiki) and MS Excel.

Cell migration activity was calculated as percentage of wound recovery or relative wound recovery;

$$\text{RWA: Recovered wound area (\%)} = (A_0 - A_t) \times 100\ (\%)$$

where, A_0 is the proportion of wound area in the whole image immediately after scratching, and A_t is the proportion of wound area measured 0, 4, and 6 hours after scratching.

$$\text{Relative wound recovery (\%)} = (RWA_x / RWA_{nc}) \times 100\ (\%)$$

where, RWA_x is the RWA of interested sample, and RWA_{nc} is the RWA of its negative control.

10. Angiogenesis assay

Angiogenesis assay was conducted using *In vitro* Angiogenesis Assay Kit (Millipore, ECM625), which consists of laminin, collagen type IV, heparan sulfate proteoglycans, entactin and nidogen. HUVECs were detached from the culture plate with 1 mL Trypsin-EDTA solution and collected in the PCR tube. Cells were centrifuged and washed with the fresh ECM medium containing no supplement. Then, cells were treated with adh3.0 alone, ANG2-H/VEGF-H (7.5 μM) alone, or the combination of both under 20 μL volume of suspended condition for 20 min at 37 °C in a humidified 5% CO_2 incubator. According to the manufacturer's instructions, 96-well Clear Flat-bottom TC-treated Culture Microplate (Falcon®) were coated with ECMatrix™, and HUVECs treated with adh3.0 alone, ANG2-H/VEGF-H (7.5 μM) alone, or the combination of both were seeded on ECMatrixTM (2.0×10^4 cells per well). The capillary tube branch points formed by HUVECs in three random microscopic fields per well were photographed using a microscope. The number of nodes and the number of junctions were quantified from images analyzed by Angiogenesis Analyzer in ImageJ (available at http://imagej.nih.gov/ij/macros/toolsets/Angiogenesis%20Analyzer.txt) as previously reported[2].

Supplementary Figures

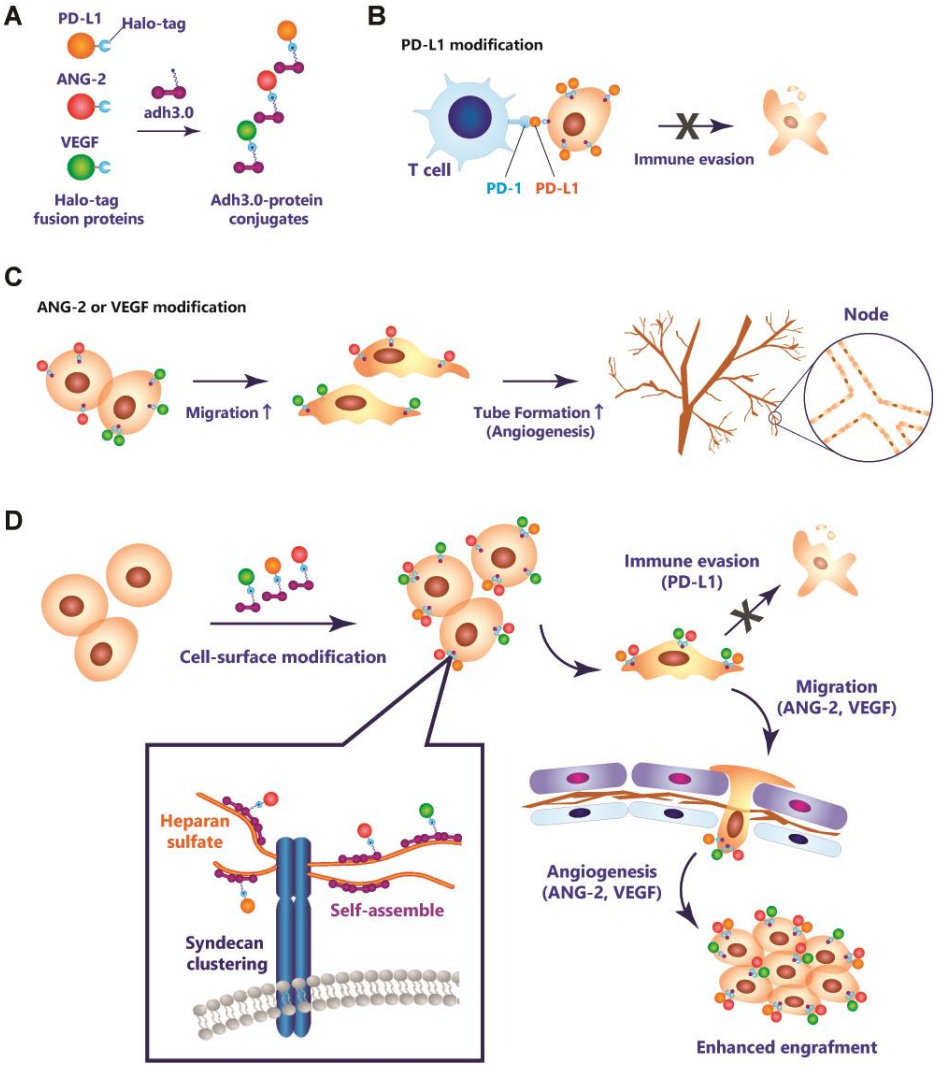


Figure S1. Non-genetic implementation of metastatic cancer properties by adh3.0 conjugates of Halo-tag fusion proteins.
(A) Conjugation of Halo-tag fusion proteins with adh3.0. (B) Adh3.0-mediated presentation of Halo-tag fused PD-L1 on the cell surface is expected to lead to immune evasion through the interaction with PD-1 of T cells. The PD-1-PD-L1 interaction inhibits T cell activation, proliferation, and survival. (C) Adh3.0-mediated presentation of Halo-tag fused Ang-2 and VEGF on the cell surface is expected to enhance cell migration and angiogenic tube formation. (D) The hallmarks of metastatic cancer are cell survival, invasion, angiogenesis, and immune evasion. Non-genetic implementation of these properties into transplanted cells would increase the efficacy of cell engraftment. Toward this end, we employ Halo-tag fusion proteins of PD-L1, Ang-2, and VEGF. Cell-surface modifications with these Halo-tag fusion proteins would enhance immune evasion, cell migration, and angiogenesis.

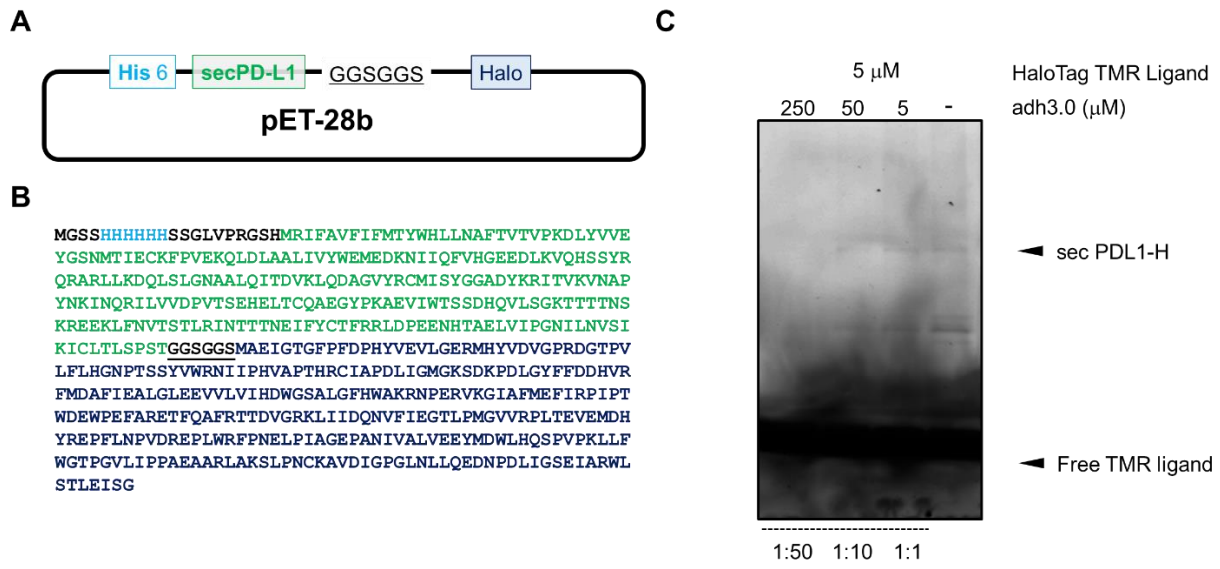


Figure S2. DNA construction and evaluation of recombinant secPDL1-H.
(A) Schematic representation of pET28b vector design for the expression of the secPDL1-H fusion protein. (B) Amino acid sequences of secPDL1-H. 6-His is colored in light blue, GGSGGS linker is underlined, and the Halo-tag region is colored in dark blue. (C) SDS-PAGE analysis of reactions between secPDL1-H and HaloTag® TMR ligand. The conjugation reaction of secPDL1-H (7.5 μM) with HaloTag® TMR ligand (5 μM) was competed through the addition of adh3.0 (5-250 μM). The reaction was completed at 37 °C in 20 min. Reaction products were separated by SDS-PAGE and visualized by in-gel fluorescence scanning.

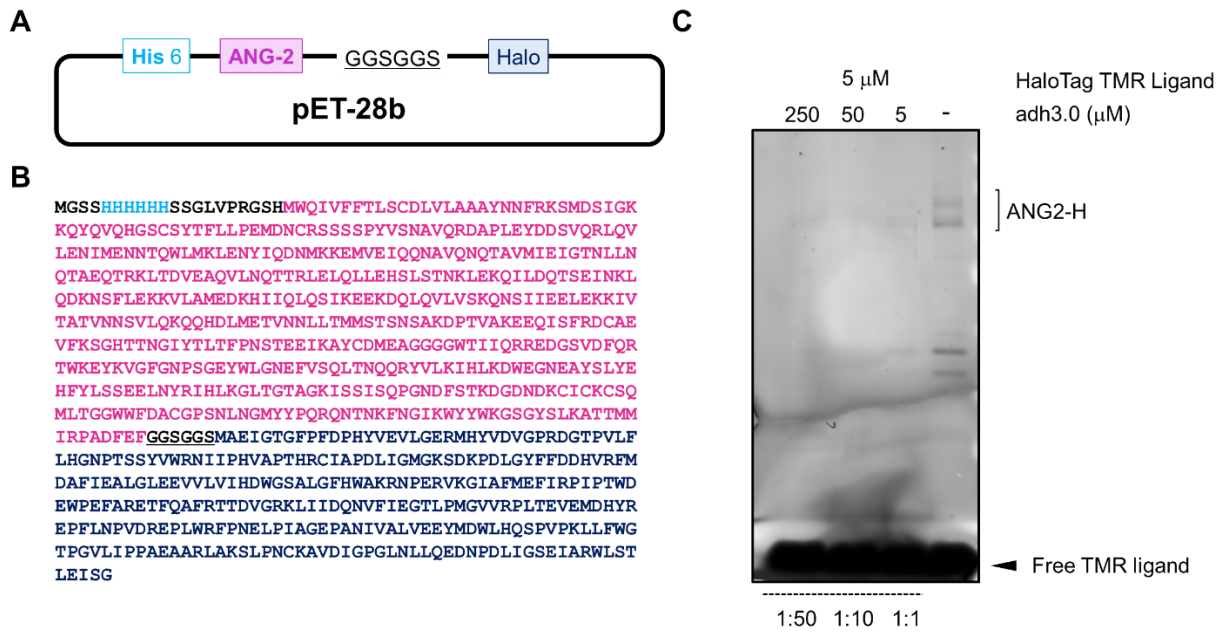


Figure S3. DNA construction and evaluation of recombinant ANG2-H.
(A) Schematic representation of pET28b vector design for the expression of the ANG2-H fusion protein. (B) Amino acid sequences of ANG2-H. 6-His is colored in light blue, GGSGGS linker is underlined, and the Halo-tag region is colored in dark blue. (C) SDS-PAGE analysis of reactions between ANG2-H and HaloTag® TMR ligand. The conjugation reaction of ANG2-H (7.5 μM) with HaloTag® TMR ligand (5 μM) was competed through the addition of adh3.0 (5-250 μM). The reaction was completed at 37 °C in 20 min. Reaction products were separated by SDS-PAGE and visualized by in-gel fluorescence scanning.

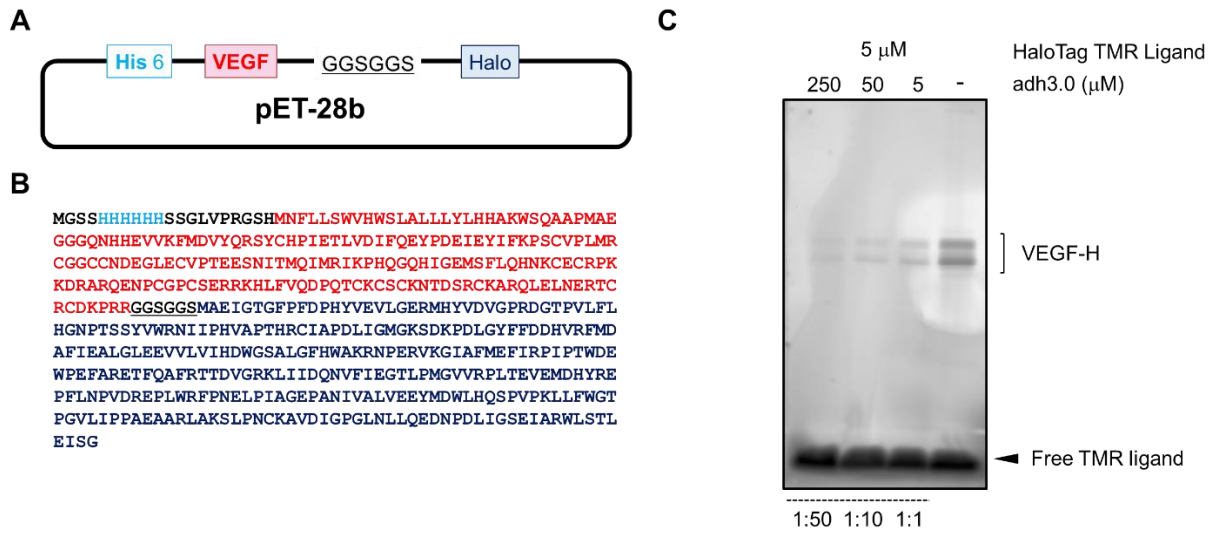


Figure S4. DNA construction and evaluation of recombinant VEGF-H
(A) Schematic representation of pET28b vector design for the expression of the VEGF-H fusion protein. (B) Amino acid sequences of VEGF-H. 6-His is colored in light blue, GGSGGS linker is underlined, and the Halo-tag region is colored in dark blue. (C) SDS-PAGE analysis of reactions between VEGF-H and HaloTag® TMR ligand. The conjugation reaction of VEGF-H (7.5 μM) with HaloTag® TMR ligand (5 μM) was competed through the addition of adh3.0 (5-250 μM). The reaction was completed at 37 °C in 20 min. Reaction products were separated by SDS-PAGE and visualized by in-gel fluorescence scanning.

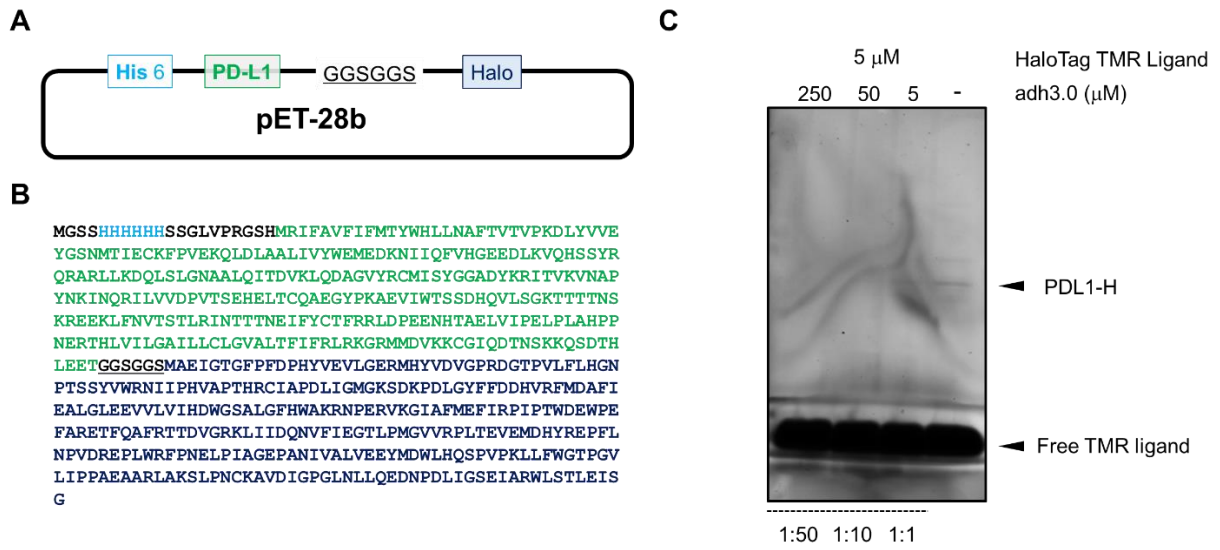


Figure S5. DNA construction and evaluation of recombinant PDL1-H.
(A) Schematic representation of pET28b vector design for the expression of the PDL1-H fusion protein. (B) Amino acid sequences of PDL1-H. 6-His is colored in light blue, GGSGGS linker is underlined, and the Halo-tag region is colored in dark blue. (C) SDS-PAGE analysis of reactions between PDL1-H and HaloTag® TMR ligand. The conjugation reaction of PDL1-H (7.5 μM) with HaloTag® TMR ligand (5 μM) was competed through the addition of adh3.0 (5-250 μM). The reaction was completed at 37 °C in 20 min. Reaction products were separated by SDS-PAGE and visualized by in-gel fluorescence scanning.

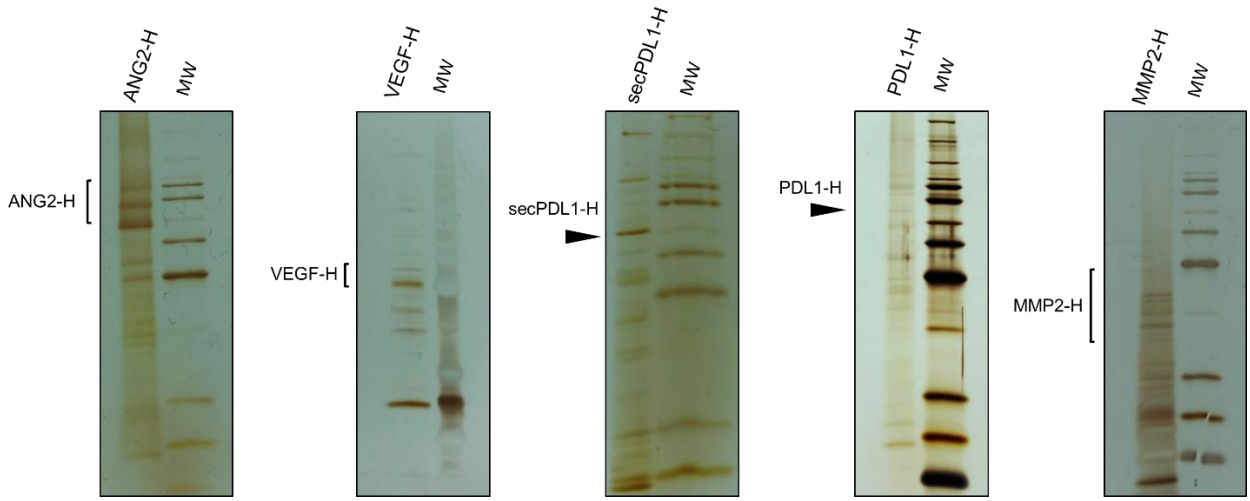


Figure S6. SDS-PAGE analysis of Halo-tag fusion proteins.
Silver staining of Halo-tag fusion proteins indicated the possible degradation or low purity. 10% (v/v) acrylamide gel, MW: molecular weight marker.

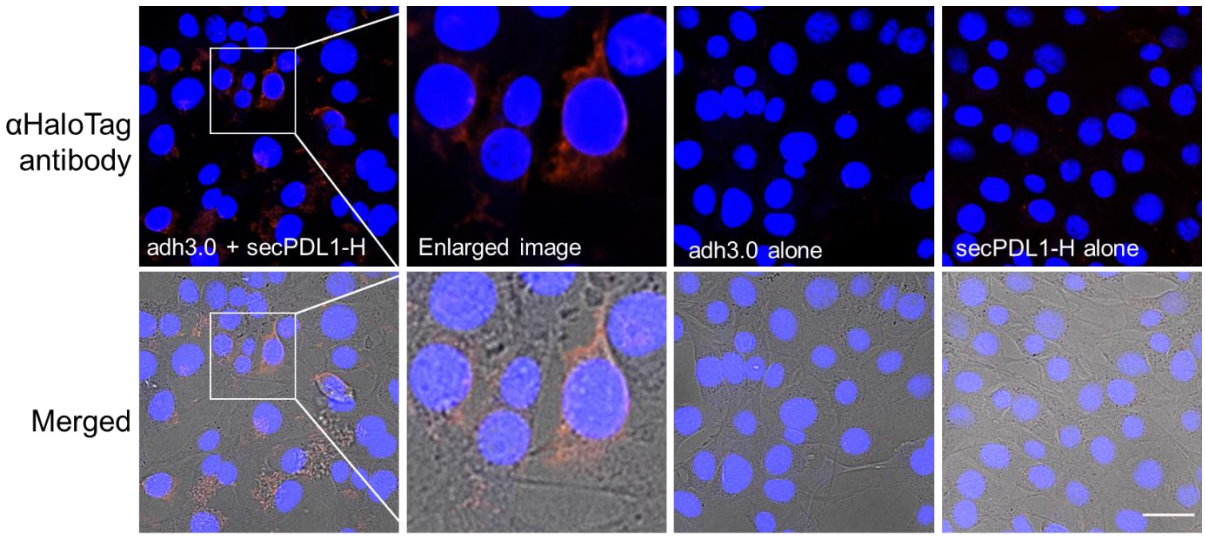


Figure S7. Confocal images of cell surface modified with adh3.0 and secPDL1-H. Cells were treated with adh3.0, secPDL1-H, or the mixture of both in serum-free DMEM ([adh3.0] = 50 μM, [secPDL1-H] = 7.5 μM). Merged images represent the stacked images of bright field, DAPI (blue) and αHaloTag antibody (red). Scale bar represents 33 μm.

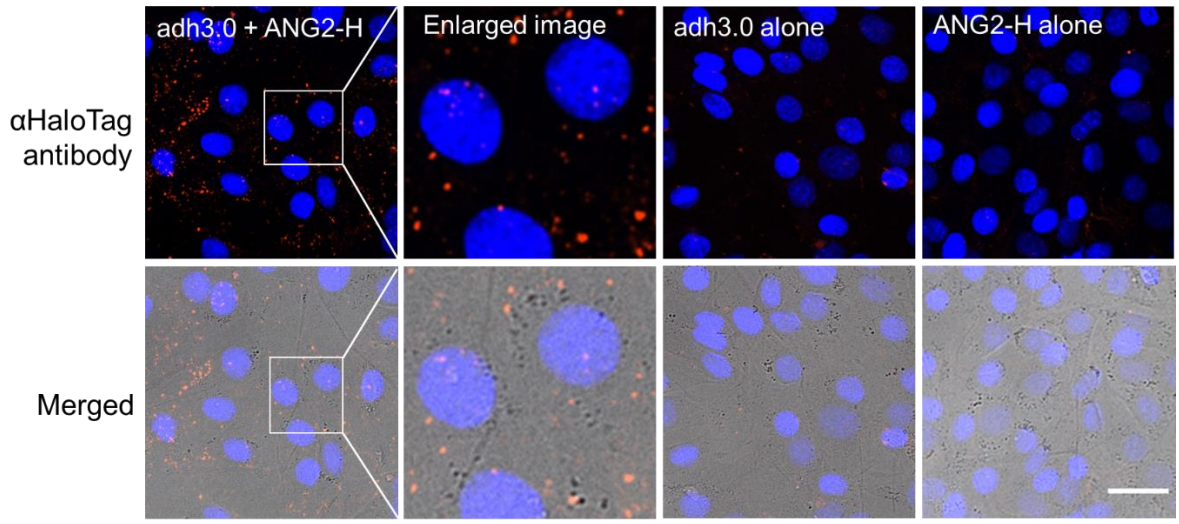


Figure S8. Confocal images of cell surface modified with adh3.0 and ANG2-H.
Cells were treated with adh3.0, ANG2-H, or the mixture of both in serum-free DMEM ([adh3.0] = 50 μM, [ANG2-H] = 7.5 μM). Merged images represent the stacked images of bright field, DAPI (blue) and αHaloTag antibody (red). Scale bar represents 20 μm.

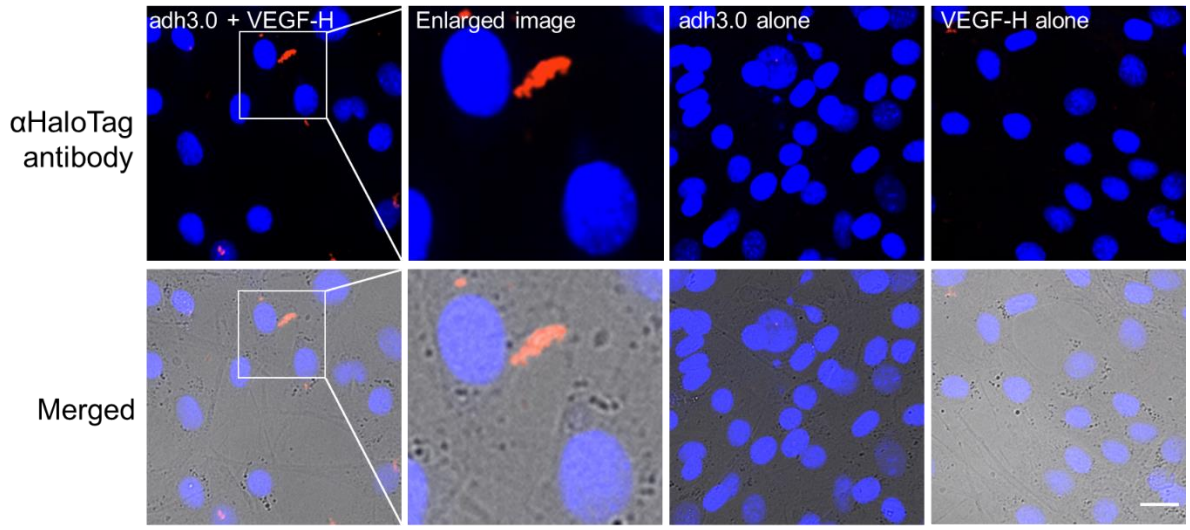


Figure S9. Confocal images of cell surface modified with adh3.0 and VEGF-H.
Cells were treated with adh3.0, VEGF-H, or the mixture of both in serum-free DMEM ([adh3.0] = 50 μM, [VEGF-H] = 7.5 μM). Merged images represent the stacked images of bright field, DAPI (blue) and αHaloTag antibody (red). Scale bar represents 20 μm.

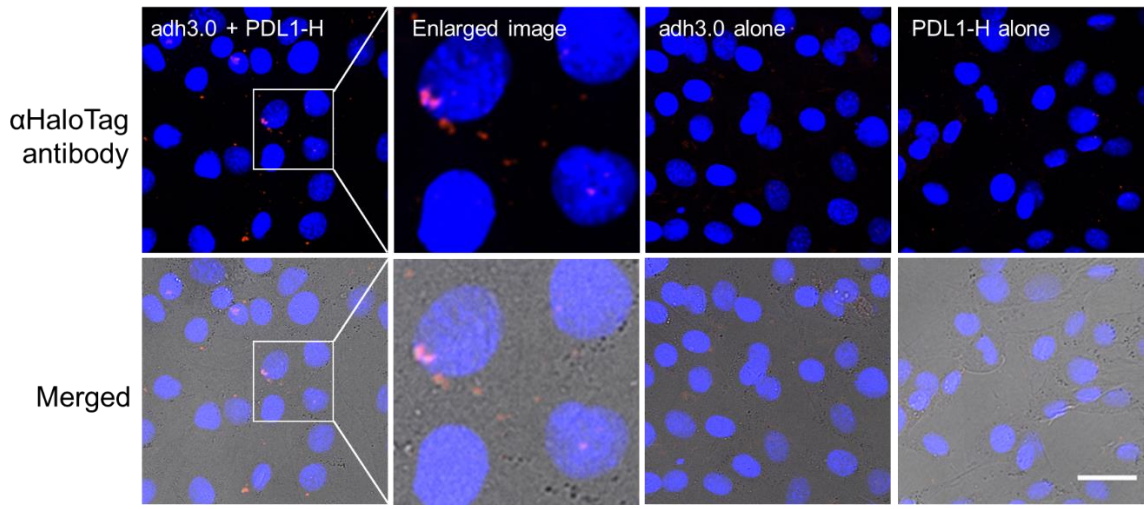


Figure S10. Confocal images of cell surface modified with adh3.0 and PDL1-H.
Cells were treated with adh3.0, PDL1-H, or the mixture of both in serum-free DMEM ([adh3.0] = 50 μM, [PDL1-H] = 7.5 μM). Merged images represent the stacked images of bright field, DAPI (blue) and αHaloTag antibody (red). Scale bar represents 20 μm.

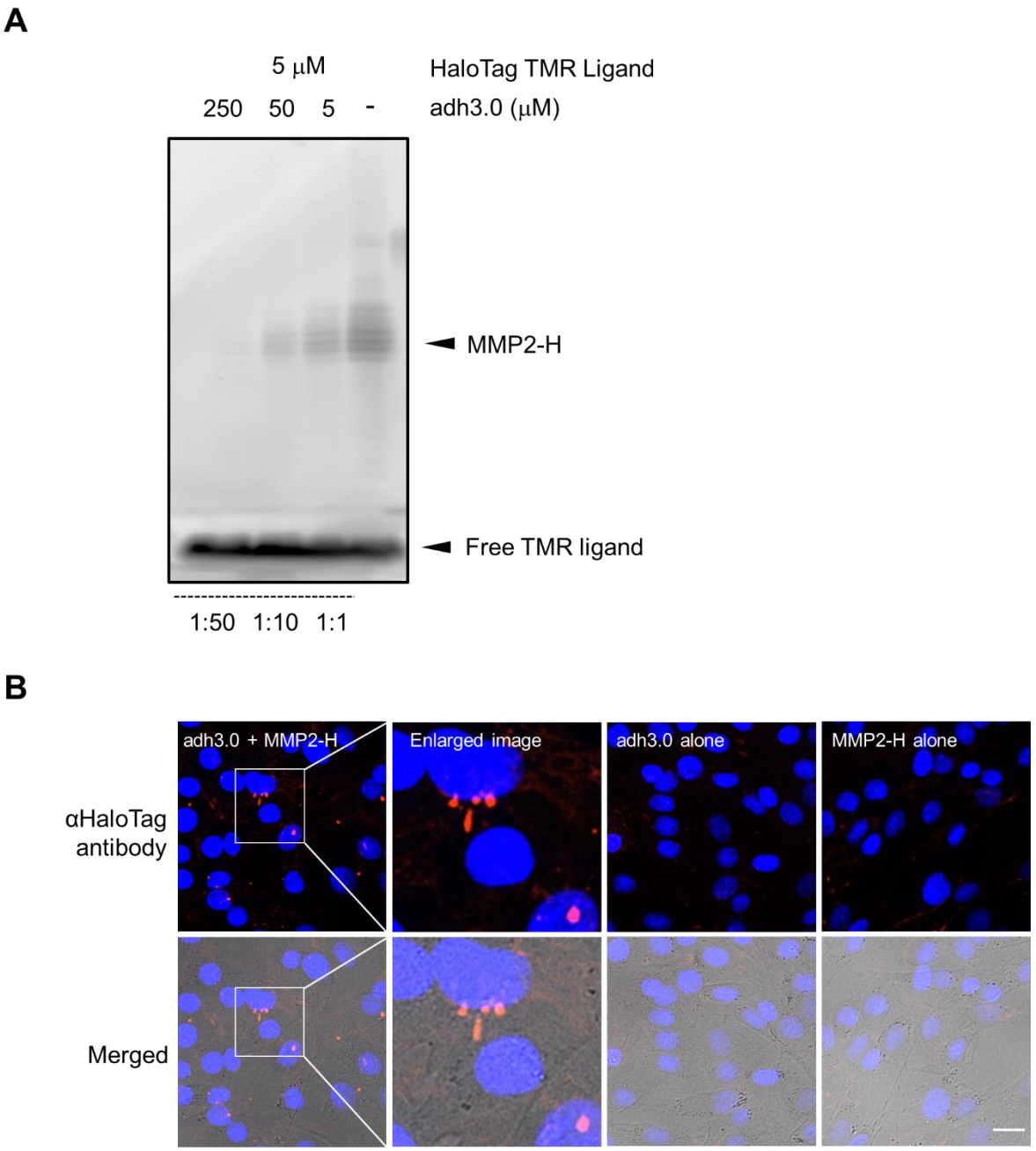


Figure S11. Characterization of the conjugation of MMP2-H with adh3.0 for cell surface modification.

(A) SDS-PAGE analysis of reactions between MMP2-H and HaloTag® TMR ligand. The conjugation reaction of MMP2-H (7.5 μM) with HaloTag® TMR ligand (5 μM) was competed through the addition of adh3.0 (5-250 μM). The reaction was completed at 37 °C in 20 min. Reaction products were separated by SDS-PAGE and visualized by in-gel fluorescence scanning. (B) Confocal images of cell surface modified with adh3.0 and MMP2-H. Cells were treated with adh3.0, MMP2-H, or the mixture both in serum-free DMEM ([adh3.0] = 50 μM, [MMP2-H] = 7.5 μM). Merged images represent the stacked images of bright field, DAPI (blue) and αHaloTag antibody (red). Scale bar represents 20 μm.

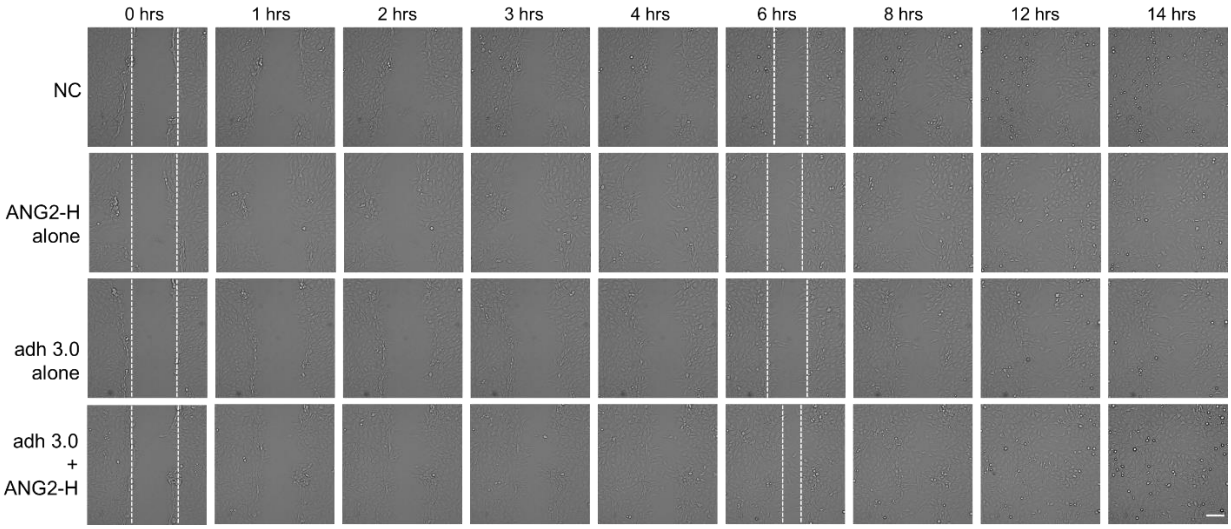


Figure S12. Time-lapse images of NIH3T3 cells modified with adh3.0 and ANG2-H in the wound-healing assay.
Immediately after the scratch was made, NIH3T3 cells were incubated in the condition of DMEM supplemented with 0.5% (v/v) FBS at 37 ºC and observed by CV-1000 microscope over time. The cells were pre-treated with adh3.0 (50 μM) alone, ANG2-H (7.5 μM) alone or the combination of both at 37 ºC for 20 min in a humidified 5% CO_2 incubator before the assay. Scale bar represents 100 μm.

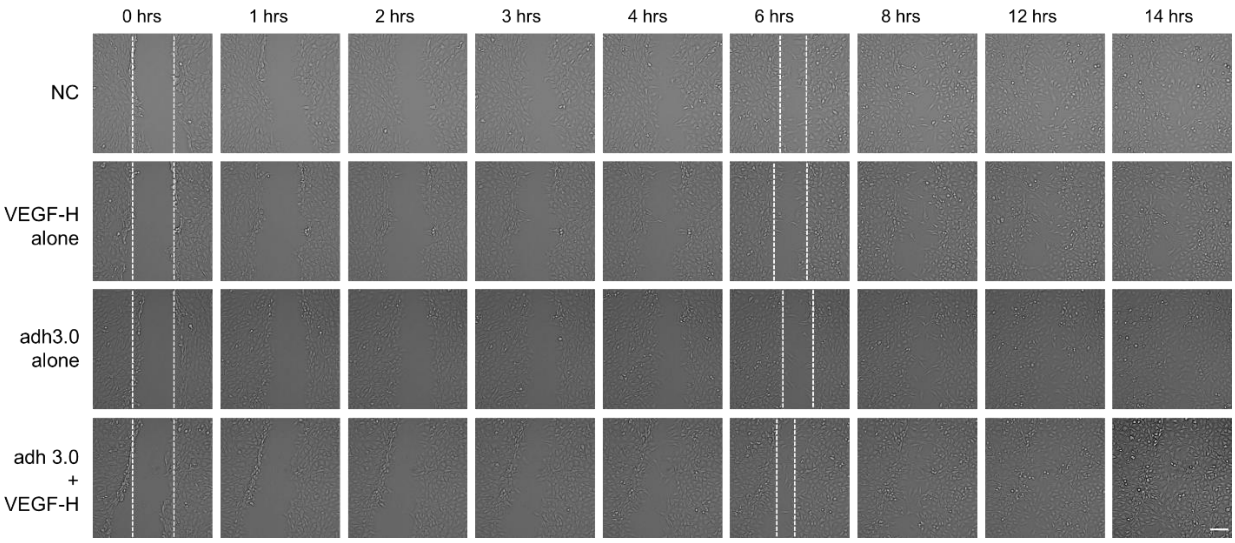
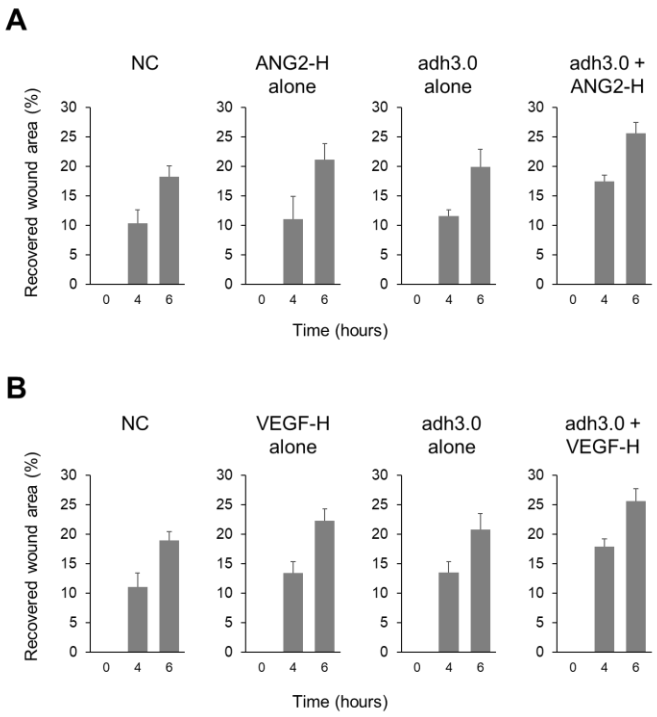


Figure S13. Time-lapse images of NIH3T3 cells modified with adh3.0 and VEGF-H in the wound-healing assay.
Immediately after the scratch was made, NIH3T3 cells were incubated in the condition of DMEM supplemented with 0.5% (v/v) FBS at 37 ºC and observed by CV-1000 microscope over time. The cells were pre-treated with adh3.0 (50 μM) alone, VEGF-H (7.5 μM) alone or the combination of both at 37 ºC for 20 min in a humidified 5% CO_2 incubator before the assay. Scale bar represents 100 μm.



C

Relative Wound Recovery (%)

4hr

	NC	Fusion Protein alone	adh3.0 alone	adh3.0 + Fusion Protein
VEGF-H	100 ± 21	121 ± 18	122 ± 16	162 ± 12
ANG2-H	100 ± 22	107 ± 37	112 ± 28	169 ± 10

6hr

	NC	Fusion Protein alone	adh3.0 alone	adh3.0 + Fusion Protein
VEGF-H	100 ± 8	118 ± 11	110 ± 14	135 ± 11
ANG2-H	100 ± 10	116 ± 15	109 ± 16	140 ± 10

Figure S14. Recovered wound area of NIH3T3 cells modified with adh3.0 and VEGF-H/ANG2-H.

NIH3T3 cell migration was evaluated using scratch wound-healing assay. The cells were pre-treated with adh3.0 alone, ANG2-H alone, or the mixture of both ([adh3.0] = 50 μM, [ANG2-H] = 7.5 μM) were cultured in the conditioned medium for 20 min before the assay. The wound area was recorded over time by CV-1000 microscope. (A) and (B) Recovered wound area (%) after 0, 4, and 6 hours of each treatment. All experiments were replicated at least three times. Data represent mean ± SEM. (C) Relative wound recovery (%) after 4 and 6 hours of the treatment. Data represent mean ± SEM.

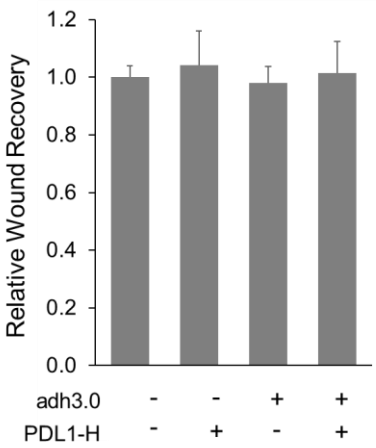


Figure S15. Relative wound recovery of NIH3T3 cells modified with adh3.0 and PDL1-H.

Immediately after the scratch was made, NIH3T3 cells were incubated in the condition of DMEM supplemented with 0.5% (v/v) FBS at 37 ºC and observed by CV1000 microscope after 4 hours of each treatment to measure the wound recovery area. The cells were pre-treated with adh3.0 alone, PDL1-H alone, or the mixture of both ([adh3.0] = 50 μM, [PDL1-H] = 7.5 μM) in the conditioned medium for 20 min before the assay. n = 3. Data represent mean ± SD.

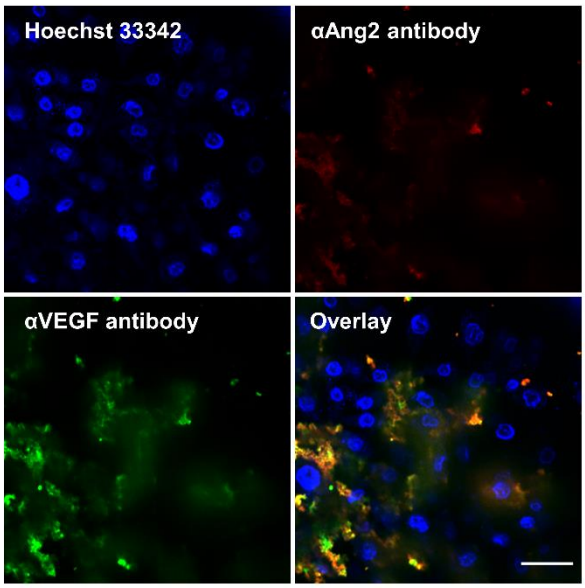


Figure S16. Simultaneous cell-surface modifications with adh3.0-conjugates of ANG2-H and VEGF-H.

Pre-mixtures of adh3.0 (50 μM), ANG2-H (7.5 μM) and VEGF-H (7.5 μM) was prepared and added to the medium of cultured NIH3T3 cells. After incubation for 20 min at 37 °C, the cells were co-stained with αAng2 antibody (red), αVEGF antibody (green) and 20 μM Hoechst 33342 (blue). The cells observed under a confocal microscope. Representative confocal images are shown. ANG2 and VEGF appeared to be co-localized. Hoechst 33342 (blue) is a fluorescent stain for labeling DNA in the nucleus. Scale bar represents 33 μm.

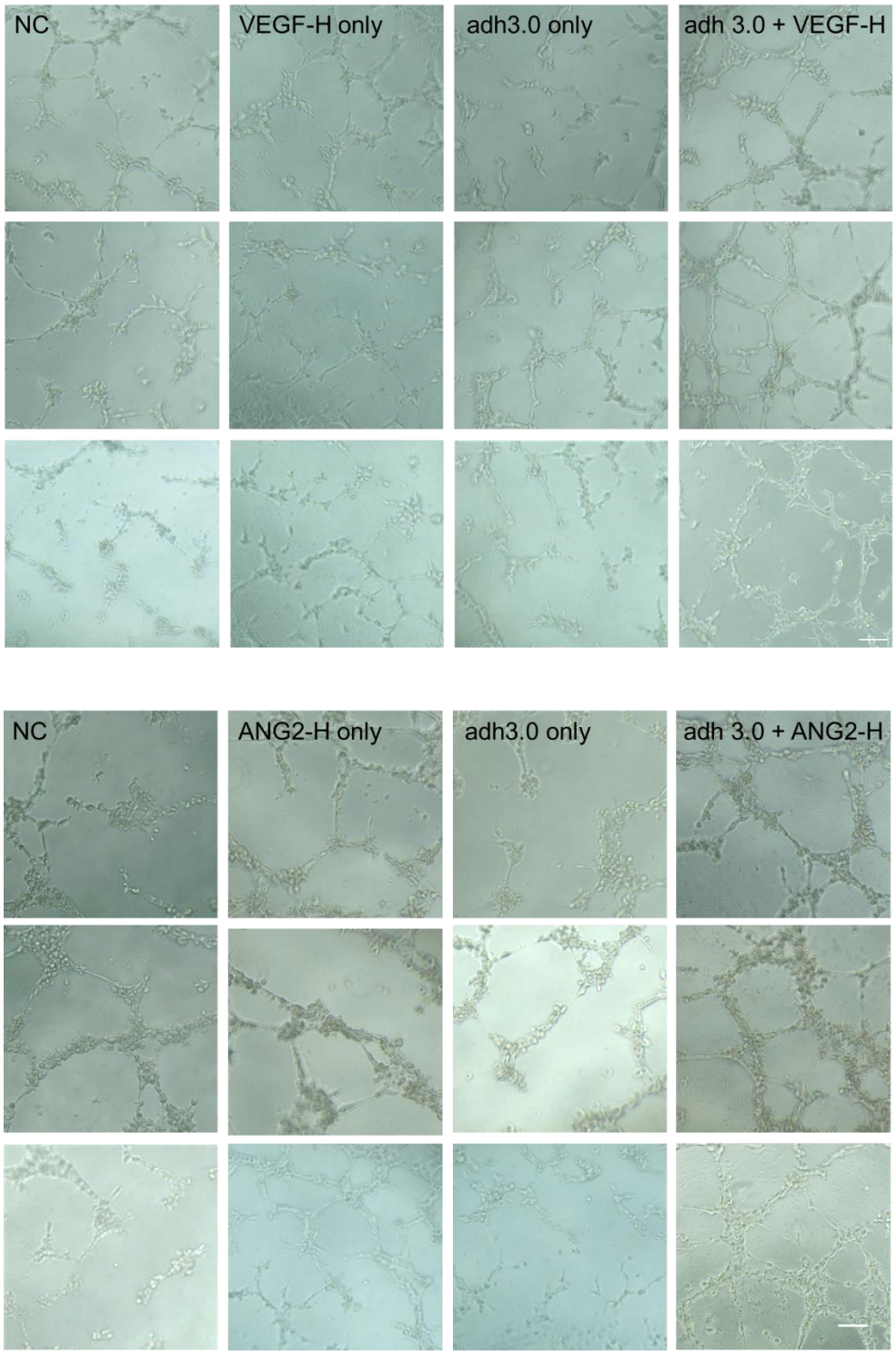


Figure S17. Tube morphology of HUVECs modified with adh3.0 and VEGF-H/ANG2-H.

The bright-field images of the cells of each treatment. The cells were treated with a mixture of adh3.0 (50 μM) and VEGF-H/ANG2-H (7.5 μM). Tube formation was recorded with a microscope after 5-hour incubation. The experiment was replicated three times. Scale bar represents 100 μm.

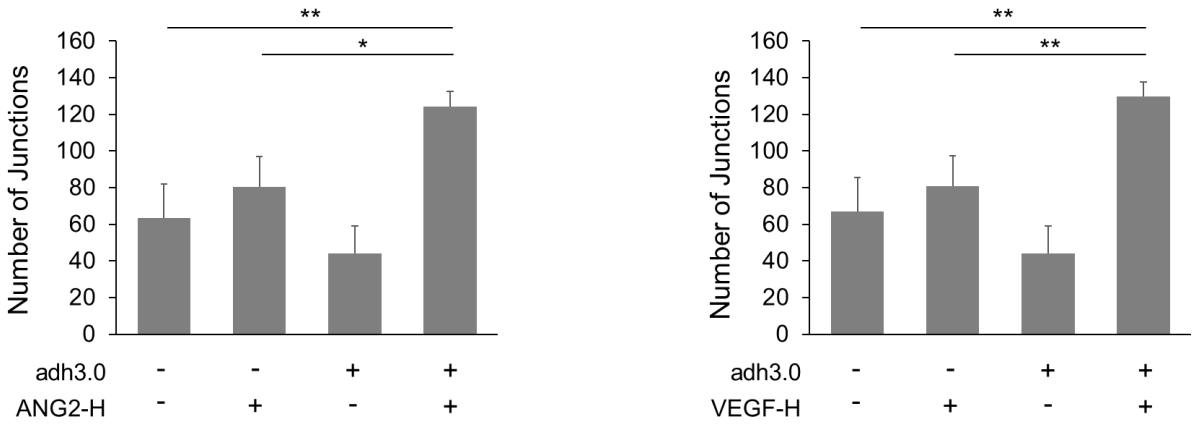


Figure S18. The number of junctions quantified in the angiogenesis assay.
Cell-surface modification with ANG2-H or VEGF-H were evaluated through *in vitro* tube formation assay. HUVECs were treated with adh3.0 (50 μM) alone, ANG2-H / VEGF-H alone (7.5 μM), or the combination of adh3.0 and either Halo-tag fusion protein. Tube formation was recorded with a microscope after 5-hours incubation. The acquired images were analyzed with ImageJ (Angiogenesis Analyzer) for quantification. All experiments were replicated at least three times. n = 3. Data represent mean ± SD. Significance was determined using an unpaired two-tailed Student's t-test. * $p < 0.05$, ** $p < 0.01$.

Supplementary Table

Table S1. DNA oligonucleotide sequences.
Primers used for the construction of MMP2-H is shown in our previous research[1] or briefly mentioned in **supplemental methods**[2].

For Halo gene amplification	
Forward	GGTGGTAGTGGTGGTAGTATGGCAGAAATCG
Reverse	GTTATCGCTCTGAAAGTACAGATCCTCAG
Forward primer for infusion	GTCGGGATCCGAATTCGGTGGTAGTGGTGGTAGTATGGCAGAAATCG
Recerse primer for infusion	GTGCGGCCGCAAGCTTGTTATCGCTCTGAAAGTACAGATCCTCAG
For Ang2-Halo fusion gene	
Forward	ATGTGGCAGATTGTTTTCTTTACTCTGAG
Reverse	GAAATCTGCTGGTCGGATCATCATGGTTGTGG
Forward primer for infusion	GCCGCGCGGCAGCCATATGTGGCAGATTGTTTTCTTTACTCTGAG
Recerse primer for infusion	GTCATGCTAGCCATATCAGCCGGAAATCTCGAGCGTCGACAG
For VEGF-Halo fusion gene	
Forward	ATGACGGACAGACAGACAGACAC
Reverse	CGTACTTGCAGATGTGACAAGC
Forward primer for infusion	GCGGCAGCCATATGAACTTTCTGCTGTCTTGGGTGCATTG
Recerse primer for infusion	GTCATGCTAGCCATATCAGCCGGAAATCTCGAGCGTCGACAG
For PDL1-Halo fusion gene	
Forward	ATGAGGATATTTGCTGTCTTTATATTCATGACCTACTGGCATTTGCTGAACGCATTTACTGTCACGGTTCCC
Reverse	CGTCTCCTCCAAATGTGTATCACTTTGCTTC
Forward primer for infusion	GCCGCGCGGCAGCCATATGAGGATATTTGCTGTCTTTATATTCATG
Recerse primer for infusion	GTCATGCTAGCCATATCAGCCGGAAATCTCGAGCGTCGACAG
For secPDL1-Halo fusion gene	
Cter forward	CTGAATTGGTCATCCCAGGTAATATTCTGAATGTGTCCATTAAAATATGTCTAACACTGTCCCCTAGCACC
Cter reverse	GACTTAACCAGTAGGGTCCATTATAAGACTTACACAGGTAATTTTATACAGATTGTGACAGGGGATCGTGG
Overlap Extension Forward	GCCGCGCGGCAGCCATATGAGGATATTTG
Overlap Extension Reverse	GGTGCTAGGGGACAGTGTTAG
Forward secPDL1-H	GTCTAACACTGTCCCCTAGCACCGGTGGTAGTGGTGGTAGTATG
Reverse secPDL1-H	TGGGATGACCAATTCAGCTGT

Supplementary Videos

Supplementary Video1.
Time-lapse images of cell-migration procedure for live NIH3T3 cells after NC treatment. The area of image is the same as the one shown in Figure S10. Images were captured every 30 min for 14 hours, and playback is at 5 frames per second.

Supplementary Video2.
Time-lapse images of cell-migration procedure for live NIH3T3 cells after VEGF-H (7.5 μM) treatment. The area of image is the same as the one shown in Figure S10. Images were captured every 30 min for 14 hours, and playback is at 5 frames per second.

Supplementary Video3.
Time-lapse images of cell-migration procedure for live NIH3T3 cells after adh3.0 (50 μM) treatment. The area of image is the same as the one shown in Figure S10. Images were captured every 30 min for 14 hours, and playback is at 5 frames per second.

Supplementary Video4.
Time-lapse images of cell-migration procedure for live NIH3T3 cells after adh3.0 (50 μM) + VEGF-H (7.5 μM) treatment. The area of image is the same as the one shown in Figure S10. Images were captured every 30 min for 14 hours, and playback is at 5 frames per second.

Supplementary Video5.
Time-lapse images of cell-migration procedure for live NIH3T3 cells after NC treatment. The area of image is the same as the one shown in Figure S11. Images were captured every 20 min for 14 hours, and playback is at 7 frames per second.

Supplementary Video6.
Time-lapse images of cell-migration procedure for live NIH3T3 cells after ANG2-H (7.5 μM) treatment. The area of image is the same as the one shown in Figure S11. Images were captured every 20 min for 14 hours, and playback is at 7 frames per second.

Supplementary Video7.
Time-lapse images of cell-migration procedure for live NIH3T3 cells after adh3.0 (50 μM) treatment. The area of image is the same as the one shown in Figure S11. Images were captured every 20 min for 14 hours, and playback is at 7 frames per second.

Supplementary Video8.
Time-lapse images of cell-migration procedure for live NIH3T3 cells after adh3.0 (50 μM) + ANG2-H (7.5 μM) treatment. The area of image is the same as the one shown in Figure S11. Images were captured every 20 min for 14 hours, and playback is at 7 frames per second.

Supplementary References

1. I. Takashima, K. Kusamori, H. Hakariya, M. Takashima, T. H. Vu, Y. Mizukami, N. Noda, Y. Takayama, Y. Katsuda and S.-i. Sato, Y, Takakura, M. Nishikawa, and M. Uesugi, *ACS chem. biol.*, 2019, **14**, 775-783.
2. Z. Jia, Y. Zhang, Q. Li, Z. Ye, Y. Liu, C. Fu, X. Cang, M. Wang and M. X. Guan, *Nucleic acids research*, 2019, **47**, 2056-2074.