Electronic Supplementary Information for

A phosphatase-recruiting bispecific antibody-aptamer chimera for enhanced tumor growth suppression

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Contents:

Materials and Methods

Reagents

Dimethyl sulfoxide (DMSO), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide (MTT), BCA protein assay kit, 96-well plates, MDA-MB-231 breast cancer cells, MCF-10A normal mammary epithelial cells, phosphate buffered saline (PBS), parenzyme cell digestion solution, paraformaldehyde (4%), Leibovitz's L-15 medium and Dulbecco modified Eagle medium (DMEM) were purchased from Keygen Biotech (Nanjing, China). Fetal bovine serum (FBS) was purchased from Gibco (Life Technologies, Australia). Transwell insert, 6/12-well plates and confocal imaging cell culture were purchased from NEST Biotech Corp (Wuxi, China). Hepatocyte growth factor (HGF) protein and FITC-labeled anti-Met monoclonal antibody were purchased from Sino Biological Inc. (Beijing, China). Anti-phospho-Met and anti-phospho-Akt monoclonal antibody were obtained from Cell Signaling Technology (Shanghai, China). Anti-GAPDH monoclonal antibody, anti-phospho-Erk1/2 monoclonal antibody and the secondary antibody goat anti-rabbit IgG (H&L)-HRP were purchased from Affinity Biosciences (Changzhou, China). Cy3-labeled secondary antibody goat anti-rabbit IgG (H&L) was obtained from Yifeixue Biotechnology (Nanjing, China). SDS-PAGE gel preparation kit, molecular weight standard, RIPA lysis buffer, protease and phosphatase inhibitor cocktail, QuickBlock™ western solution suit and ECL chemiluminescence kit were purchased from Beyotime Biotechnology (Shanghai, China). Oligonucleotide-conjugation kit and anti-CD148 monoclonal antibody were purchased from Abcam (Shanghai, China). Crystal violet was obtained from Solarbio Life Sciences (Beijing, China). All DNA sequences were obtained from Sangon Biotech (Shanghai, China), and the sequences are listed in Table S1. Water used in all the experiments was purified by a Milli-Q Advantage A10 water purification system (Millipore, Milford, MA, USA).

Instruments

Ultraviolent (UV) spectral analysis was performed with a NanoDrop 2000/2000C spectrophotometer (Thermo Fisher, MA, USA). Microplate reader analysis was carried out on a BioTek Synergy Mx microplate reader (Winooski, VT, USA). Confocal fluorescence imaging of

cells was acquired on a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems CMS GmbH, Mannheim, Germany). The cell migration morphology was observed under a culture microscope (Olympus, Tokyo, Japan), and the time-lapse images were recorded by confocal microscope Nikon Eclipse Ti2 (Eppendorf, Hamburg, Germany). Western blot images were acquired on a Tanon-4600SF chemiluminescence imaging system (Tanon, Shanghai, China).

Preparation of Ab-Ap conjugate

The conjugation of anti-CD148 monoclonal antibody with anti-Met aptamer was accomplished using an oligonucleotide-conjugation kit, according to the manufacturer instructions. Briefly, the antibody and 5’ aminated aptamer were first activated using activation reagent respectively, and followed by desalting steps with separating columns. Then the activated oligonucleotide and antibody was mixed and incubated for 1 h at room temperature to allow for conjugation. After the incubation, the reaction mixture was further purified through the supplied conjugate clean up reagent and centrifugation procedure. Finally, the conjugate concentration was quantified by the BCA protein assay kit, and the quality was confirmed using UV spectra and SDS-PAGE gel electrophoresis analysis. The conjugate was aliquoted and stored at -20 °C until use.

Cell culture

MDA-MB-231 cells were cultured in Leibovitz's L-15 medium. MCF-10A cells were cultured in DMEM medium. Media were supplemented with 10% FBS, and cells were cultured in a humidified incubator at 37 °C under a 5% CO_2 atmosphere.

Confocal laser scanning microscopy (CLSM) imaging

Cells were separately seeded on confocal dishes and cultured overnight before fluorescence imaging analysis. After washing for three times, cells were blocked at 4 °C with 1% FBS-containing PBS for 1 h. Then the cells were incubated with aptamer, antibody or conjugate for 30 min at 4 °C, and washed again. For the fluorescence visualization of anti-CD148 antibody and

conjugate, cells were incubated with Cy3-labeled goat anti-rabbit IgG (H&L) for 30 min at 4 °C and then washed three times with PBS. Finally, 1 mL PBS was supplemented and the obtained cells were imaged under the confocal laser scanning microscope using the 60× oil objective.

Wound healing assay

MDA-MB-231 cells were seeded in a 6-well plate and cultured to whole monolayer. Then the cell monolayer was scratched uniformly using a sterile 20 μL pipette tip. After washing twice to remove floating cells, the wells were supplemented with 1% FBS-containing L-15 medium for cell culture. The initial width of the wound was measured and recorded as t = 0 h under an inverted microscope. Afterwards, the cells were pre-incubated with different formulations (aptamer, antibody or conjugate) for 15 min, and then 30 ng/mL HGF was added to further incubate for 24 h. Cells without any treatment were used as control and cells only stimulated by HGF as contrast. Wound healing was monitored by recording the scratch area every 4 h.

Single-cell tracking assay

MDA-MB-231 cells were seeded on confocal dishes for 24 h and starved in medium supplemented with 1% FBS overnight. After the starvation, the cells were pre-cultured with different formulations for 15 min, and then stimulated with 30 ng/mL HGF. Cells without any treatment were used as control and cells only stimulated by HGF as contrast. The dishes were mounted on the microscope, and time-lapse images were captured every 5 min for 5 h. The migration trajectories of randomly selected cells were constructed by tracking the center position of the individual cell over the observation time using the Image J software. The migration distance during each time-lapse interval is defined as $D_i = \sqrt{|X_i - X_{i-1}|^2 + |Y_i - Y_{i-1}|^2}$, where X_i and Y_i represent the X position and Y position of the cell, respectively. The total migration distance (D_{total}) is given as $D_{total} = \sum_{i=1}^{n} D_i$.

Transwell migration assay

MDA-MB-231 cells were seeded into the upper transwell inserts with an 8-μm pore membrane overnight, and serum-starved for 12 h in L-15 supplemented with 1% FBS. Next, cells were pre-incubated with different formulations for 15 min, and then 30 ng/mL HGF was added. Meanwhile, the lower chambers were filled with 10% FBS supplemented growth medium. Cells without any treatment were used as control and cells only stimulated by HGF as contrast. After further cultivation for 24 h, the cells that migrated through the membranes were fixed with 4% paraformaldehyde and stained with crystal violet solution. Non-migrated cells were removed from the inner side of the upper chamber with cotton swabs. After washing with PBS, migrated cells were visualized through bright field microscope and counted in three random fields.

Western blot assay

MDA-MB-231 cells were cultured in 6-well dishes until they reached to 80% confluence. Subsequently, the cells were starved in medium supplemented with 1% FBS overnight. The cells were pre-incubated with different formulations for 15 min, and then stimulated by HGF for another 30 min. Cells without any treatment were used as control and cells only stimulated by HGF as contrast. After that, cells were washed with precooling PBS twice, and lysed using RIPA lysis buffer supplemented with protease and phosphatase inhibitor cocktail on ice for 10 min. Cells were scraped with clean cell scrapes and the lysate mixture was transferred into centrifugal tubes. The cell lysates were centrifuged at 10,000g for 20 min at 4 °C and the supernatant was collected for further use. Protein concentration was quantified using BCA protein quantitation assay and 30 μg of cell lysates for each sample were loaded for SDS-PAGE electrophoresis. Polyvinylidene fluoride (PVDF) membrane was used to transfer the gels. After blocking, the membrane was incubated with primary antibody at 4 °C overnight and secondary antibody at room temperature for 1 h. Finally, the membrane was washed 3 times to remove unbound antibodies, and then reacted with ECL substrate solution for imaging.

Cytotoxicity of conjugate

Cell viability was determined by the MTT assay. Briefly, MCF-10A cells (around 5000 cells per well) were seeded on 96-well microplates for 24 h prior to the assay. The cells were incubated in culture medium containing Ap-Ab conjugate with different concentrations for another 24 h. Then 50 μL of MTT reagent (1 mg/mL) was added and incubated for 4 h to allow formation of formazan dye. After removal of the supernatant, 150 μL of DMSO was added to each well. After shaking for 10 min on the shaking table, the optical absorption at 570 nm was recorded using a microplate reader. The wells without conjugate were used as control and others were used as test group. And wells without cells were used as background group. Data are reported as the mean of three independent experiments. Cell viability was calculated as following:

$$\text{Cell viability (\%)} = \frac{\text{Abs(test)} - \text{Abs(background)}}{\text{Abs(control)} - \text{Abs(background)}} \times 100\%$$

Cell proliferation inhibition assay

MDA-MB-231 cells (around 2000 cells per well) were seeded on 96-well microplates for 24 h. The cells were respectively treated with different formulations for 15 min, and then 30 ng/mL HGF was added for further incubation 24 h. Then 50 μL of MTT (1 mg/mL) was added to each well and the plates were incubated at 37 °C for another 4 h. The supernatant was discarded, and 150 μL of DMSO was added to each well. After shaking for 10 min on the shaking table, the 570 nm absorption of the solution was monitored on a microplate reader. The group without any treatment were used as control, wells only stimulated by HGF were used as contrast, and wells without cells were used as background group. The calculation of cell viability is the same as above.

Supplementary Data

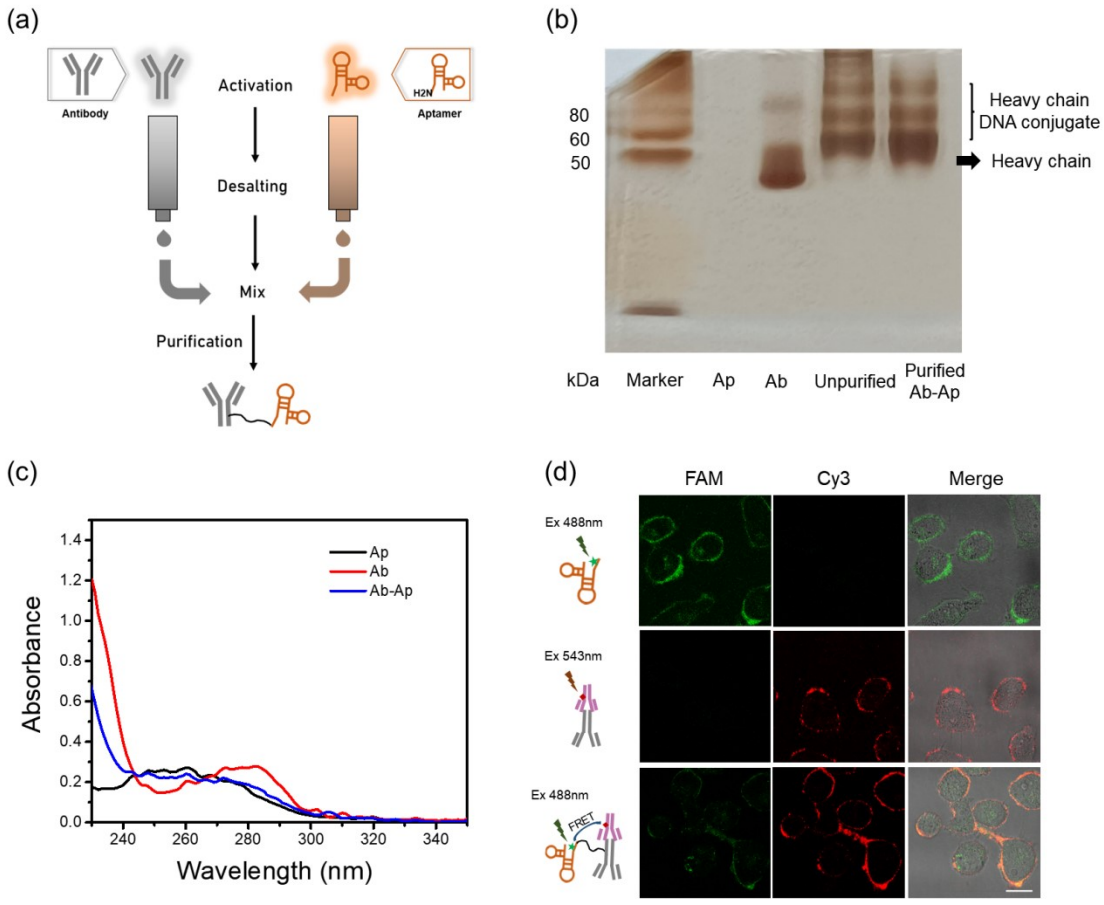

Fig. S1. The characterization of Ab-Ap chimera. (a) The preparation route of Ab-Ap chimera. (b) SDS-PAGE image of Ab and Ab-Ap chimera. (c) UV absorbance spectra of anti-CD148 Ab conjugated with anti-Met Ap. (d) Confocal fluorescence images of MDA-MB-231 cells with Ab-Ap chimera. Green channel: FAM excitation 488 nm/emission 500-550 nm. Red channel: Cy3 excitation 543 nm /emission 560-610 nm. Scale bar: 20 μm.

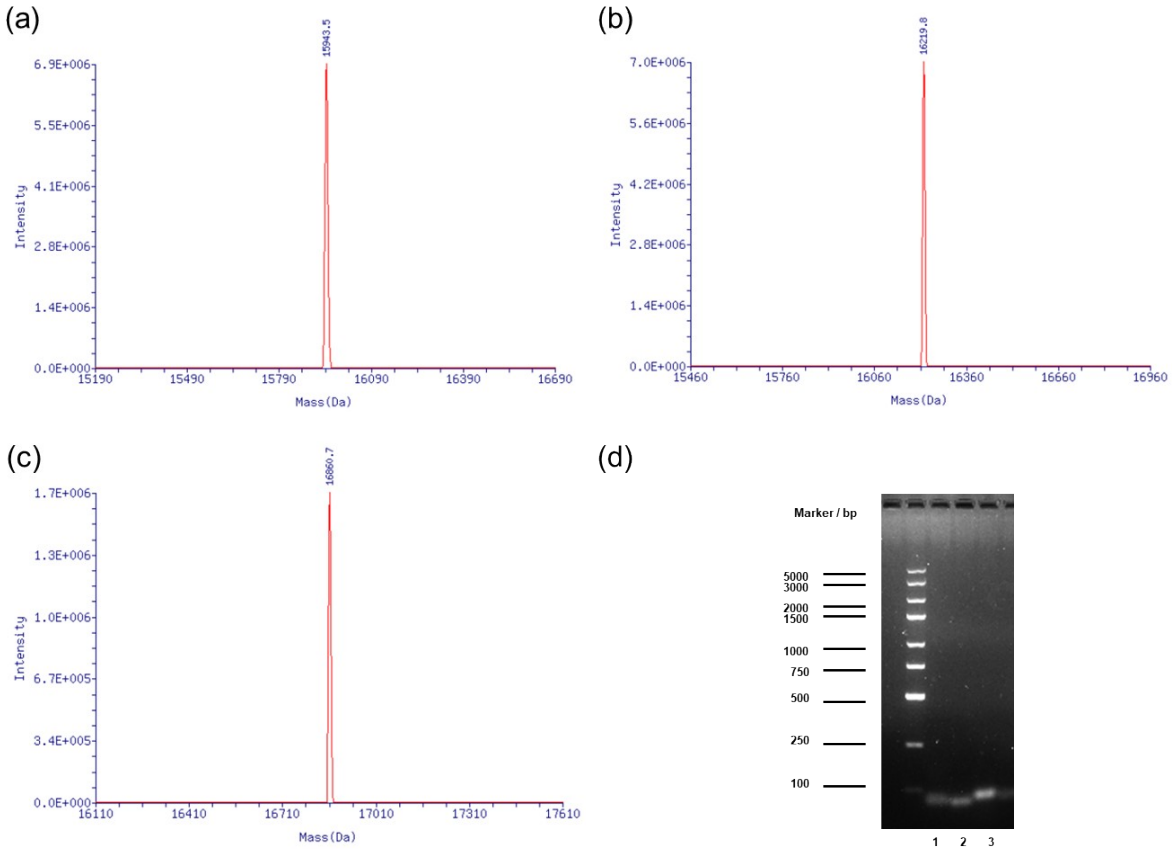

Fig. S2. MS spectra for DNA sequences (a) NH_2-anti-Met aptamer; (b) FAM-anti-Met aptamer; (c) NH_2-/FAM/anti-Met aptamer in Table S1. (d) DNA electrophoresis on 2% agarose gel. Lane details: (1) NH_2-anti-Met aptamer; (2) FAM-anti-Met aptamer; (3) NH_2-/FAM/anti-Met aptamer.

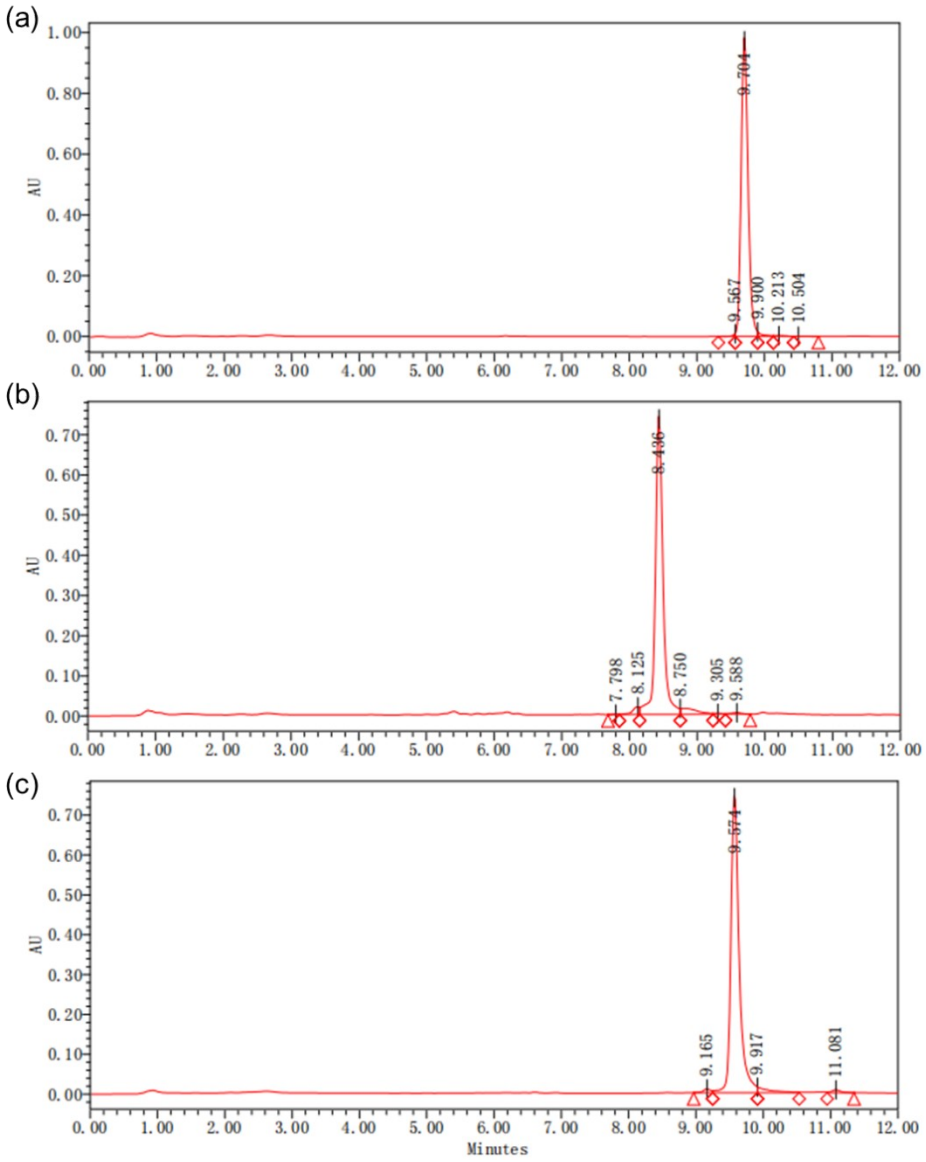

Fig. S3. HPLC detection for DNA sequences (a) NH_2-anti-Met aptamer; (b) FAM-anti-Met aptamer; (c) NH_2-/FAM/anti-Met aptamer in Table S1.

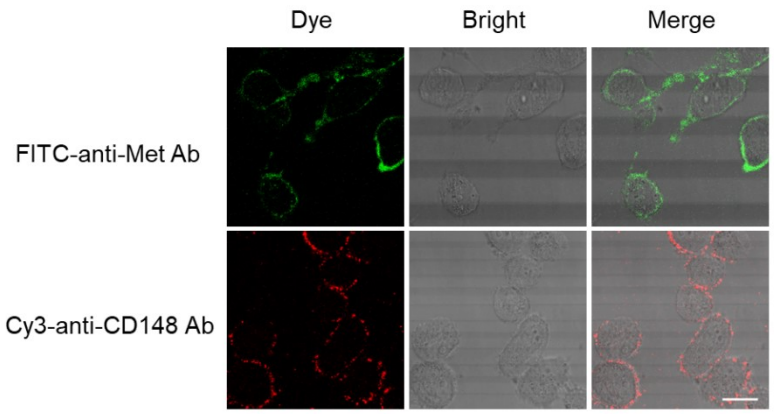

Fig. S4. The verification of Met and CD148 expression through confocal fluorescence images of MDA-MB-231 cells with antibodies. Scale bar: 20 μm.

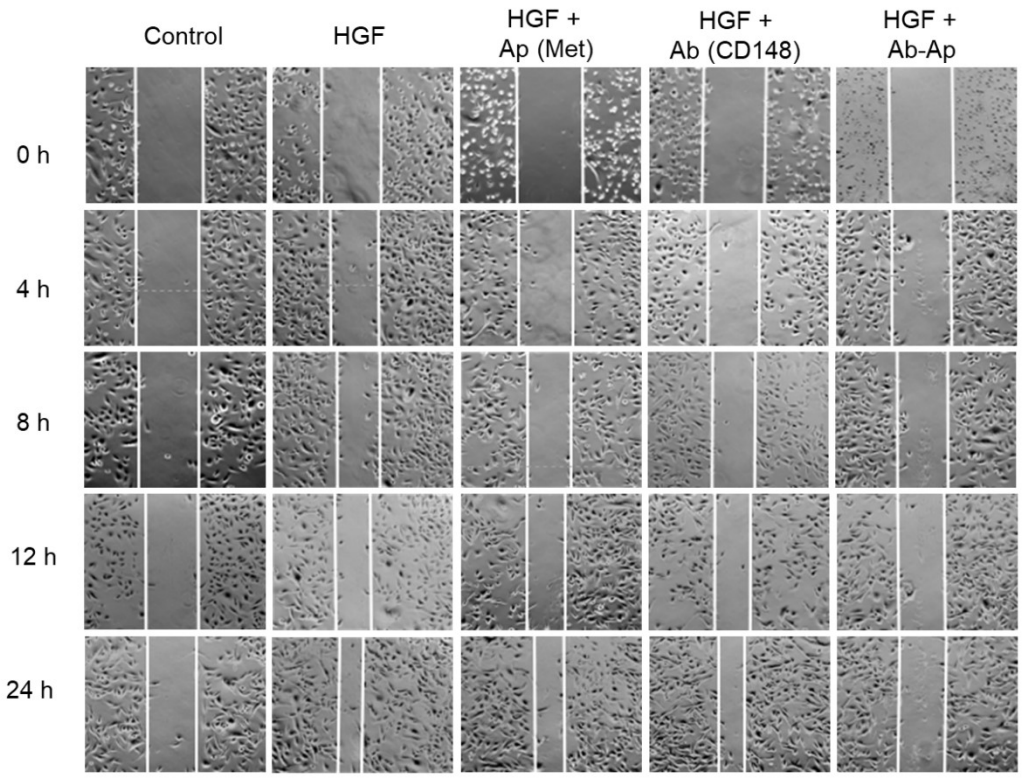

Fig. S5. Monitoring images of wound healing under different conditions.

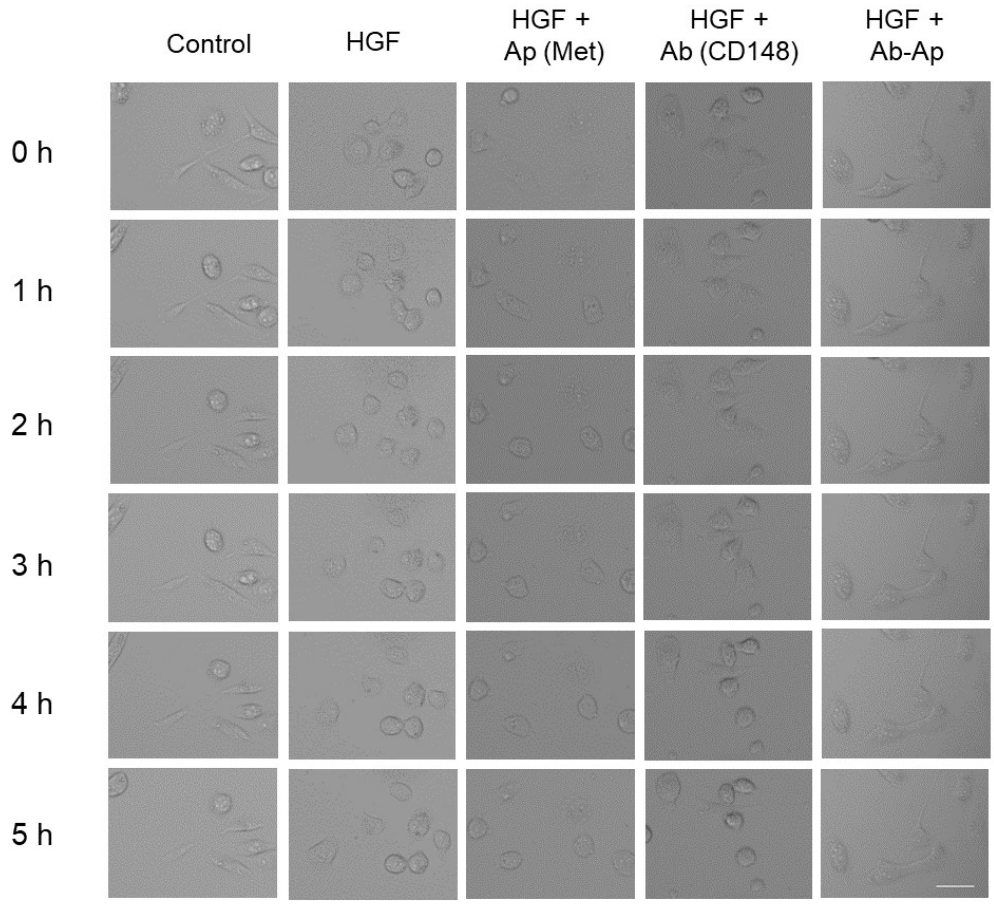

Fig. S6. Images display of cell scattering on the hour under different conditions. Scale bar: 50 μm.

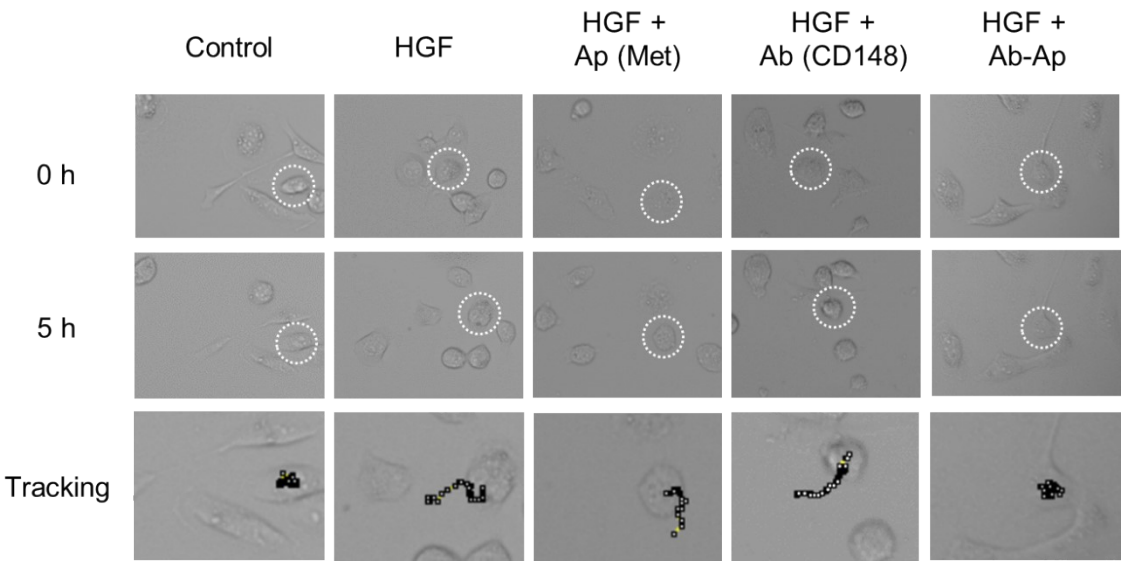

Fig. S7. Single cell movement tracking under different conditions.

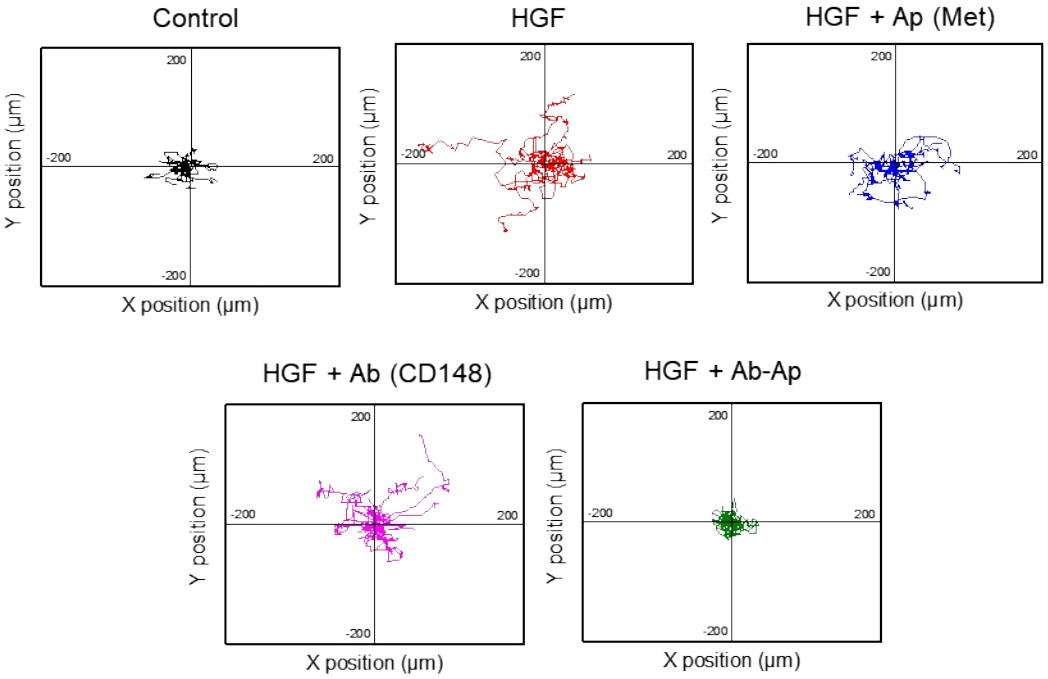

Figure S8. Migration trajectories of randomly selected cells during single cell tracking assay.

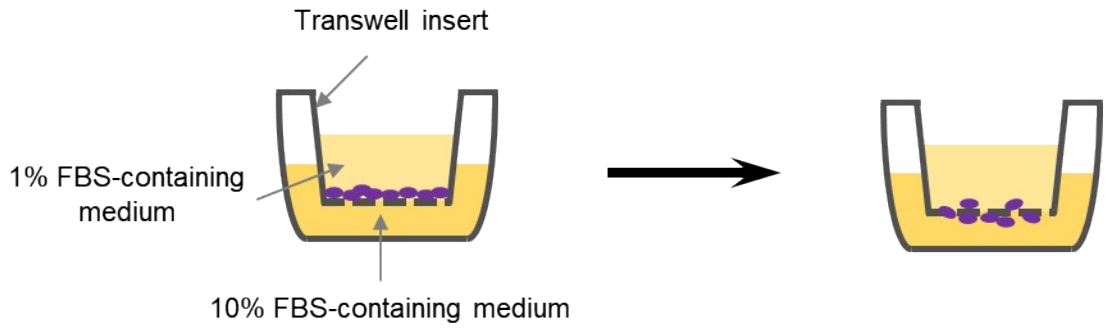

Figure S9. Schematic of the tumor cell migration model from a transwell assay.

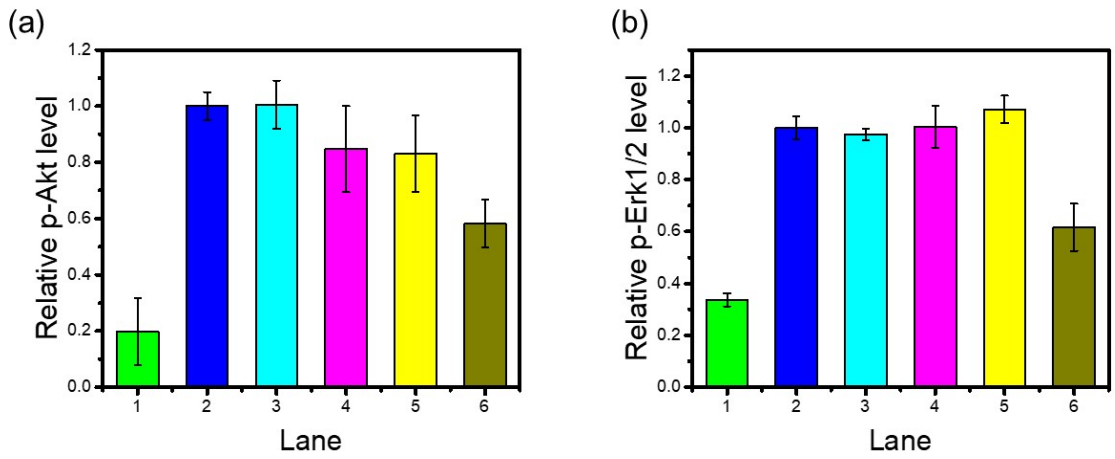

Fig. S10. Quantified western blot analysis of p-Akt (a) and p-Erk1/2 (b) levels in MDA-MB-231 cells incubated with different formulations in Figure 4. Results are presented as means ± standard deviation (SD) (n = 3).

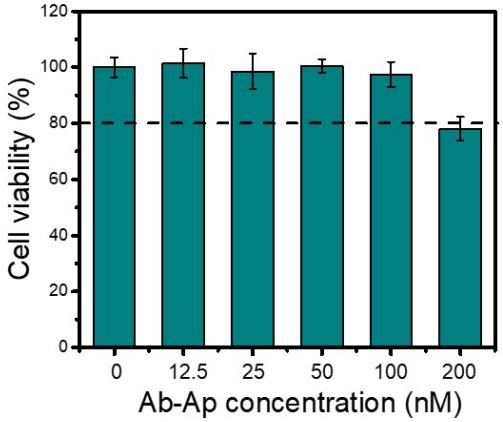

Fig. S11. In vitro cytotoxicity of Ab-Ap chimera toward normal cell MCF-10A. Results are presented as means ± standard deviation (SD) (n = 3).

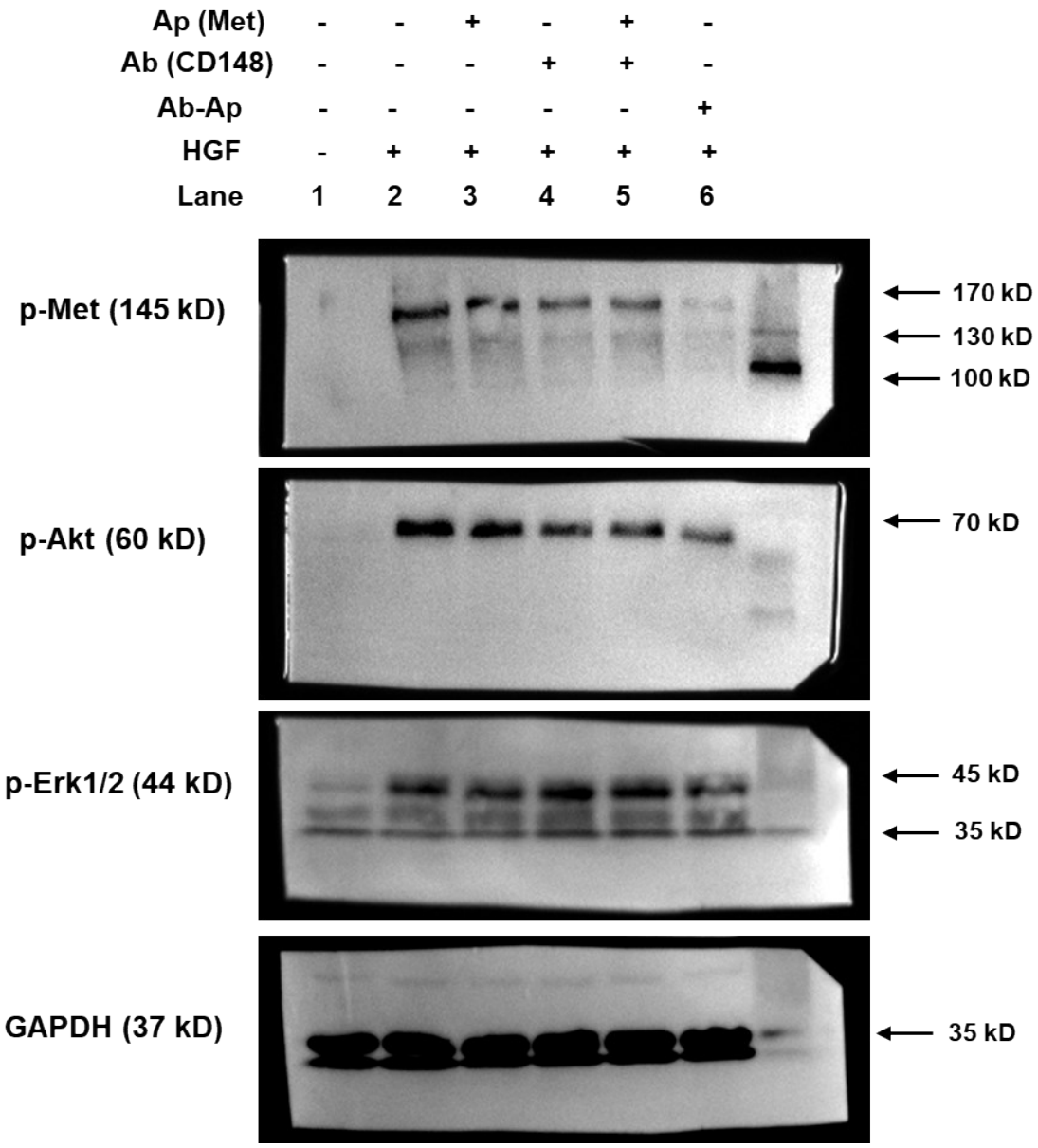

Fig. S12. Full raw images for the western blotting in Fig. 4a. The final western blotting images were obtained by overlaying the chemiluminescence image and the molecular weight markers image. Lane 1-6 represented different treatments and the lane7 were molecular weight markers.

Table S1. The DNA sequences used in this study

Description	Sequence (5’ to 3’)
FAM-anti-Met aptamer	FAM-ATCAGGCTGGATGGTAGCTCGGTCGGGGTGGG TGGGTTGGCAAGTCTGAT
NH_2-anti-Met aptamer	NH_2-C12-ATCAGGCTGGATGGTAGCTCGGTCGGGGT GGGTGGGTTGGCAAGTCTGAT
NH_2-/FAM/anti-Met aptamer	NH_2-C12-/iFAMdT/ATCAGGCTGGATGGTAGCTCGGT CGGGGTGGGTGGGTTGGCAAGTCTGAT