Supplementary Information for

The functional activity of E-cadherin controls tumor cell metastasis at multiple steps

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Supplementary Materials and Methods

Generation of activating rabbit mAbs to mouse E-cadherin. Hybridoma cell lines were generated from rabbits immunized with the purified extracellular domain of mouse E-cadherin by Epitomics (Burlingame, CA), which is now owned by AbCam (Cambridge, MA). E-cadherin hybridomas positive for E-cadherin binding in ELISA were then screened in a functional assay in our laboratory as done previously for mouse anti-human E-cadherin, for their ability to activate adhesion in colo205 cells (1). In this case we used colo205 cells expressing mouse E-cadherin after knocking down endogenous human E-cadherin expression with an shRNA. Hybridomas producing activating mAbs 18-5, 56-4 were obtained; also, a hybridoma line producing a neutral antibody 19.1-10 was obtained that binds E-cadherin but does not activate colo205 adhesion.

Generation of recombinant antibodies. Hybridoma cell lines producing mAbs to both mouse anti-human E-cadherin (19A11, 66E8, 46H7) (1) and Rabbit anti-mouse E-cadherin (18-5, 56-4, 19.1-10) were sent to GenScript (Piscataway, NJ) to sequence the variable regions of the heavy and light chains for each mAb. These were all then cloned into the backbone of mouse IgG_1 constant region encoding sequences. The full heavy chain and light chain sequences were then cloned into pcDNA3.4 and expressed in ExpiCHO cells (Invitrogen, Carlsbad, CA) following their protocols. Two weeks post transfection, antibodies were affinity purified from about 350 mLs of media on a 5mL protein G column (HiTrap MabSelect SuRe, GE Healthcare Life Sciences, Pittsburgh, PA), buffer-exchanged to PBS pH7.2 and stored as sterile aliquots at -80 °C until use.

Mouse experiments and *in vivo* treatments with antibodies. FVB MMTV-Polymavirus middle T antigen (MMTV-PyMT) breeders were obtained from The Jackson Laboratory and mated according to the vendor's specifications. All mice were housed and bred under specific pathogen-free conditions at Seattle Children's Research Institute (SCRI) and all animal studies are governed through protocols approved by the Institutional Animal Care and Use Committee (IACUC). Four-week-old transgenic mice were treated twice weekly with neutral mAbs, 19.1-10, or E-cadherin-specific mAb, 56-4 (5 mg/kg

of weight) in saline by intraperitoneal injection. Caliper measurements of primary tumor were done weekly until the end of the experiments. Lung tissues of 14-week-old mouse were isolated and fixed in Bouin's solution and whole blood were harvested.

For 4T1 tumor cell grafting metastatic mouse model study, 4T1 Luc2 expressing hE-cadherin (4T1 Luc-hE) cells suspended in 1x HBSS were injected into the mammary fat pads (1×10^4) of BALB/c mice. To determine the ability of tumor cells to metastasize from bloodstream (or circulation) colonize the lung, 4T1 Luc-hE (3×10^4) were also injected *via* the tail vein (n=8 to 9). No graft control group, no cells but only HBBS buffer was administered (n=3). The generation of 4T1 Luc-hE cell line and animal experiment using 4T1 Luc-hE cells were described previously (2).

Histological analysis, immunofluorescence staining, and detection of apoptosis in tumors. Paraffin section (5 μm) were stained with hematoxylin and eosin (Sigma-Aldrich, St. Louis, MO, USA). For immunofluorescence staining, the tissue sections were hydrated in a series of washes from xylenes to ethanol dilutions to water. Heat-mediated antigen retrieval was conducted with citrate buffer. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Samples were blocked with 5% bovine serum albumin (BSA) (Sigma-Aldrich) and primary antibodies were incubated at 4 °C overnight in 1% BSA in PBS for the following antibodies at indicated concentrations: E-cadherin (BD Biosciences, 610181) 1:1,000; cleaved-caspase 3 (Cell Signaling, 9661) 1:500; pHistone3 (Cell Signaling, 9701) 1:500. Alexa Fluor-conjugated secondary antibodies were incubated on tissues for 1 hour (1:1,000). Hoechst was used to stain the nuclei. For cell culture, 4T1 and MCF10a cells were fixed with 4% paraformaldehyde and incubated with cleaved caspase-3 antibody at 4 °C overnight in 1% BSA in PBS. The coverslips were mounted on glass slides and the slides were imaged using Leica DFC310 FX and Olympus IX71 microscope. For determination of apoptosis in tumor tissues and cell culture, terminal deoxynucleotidyltransferase-mediated dNTP nick end–labeling (TUNEL) assay was performed using the ApopTag® Red *In Situ* Apoptosis Detection kit (S7165, Millipore).

Isolation and detection of circulating tumor cells (CTCs). 900 µl – 1 ml of blood was obtained by cardiac puncture from mouse and processed according to standard separation protocols. After centrifugation, the buffy coat was collected for isolation of CTCs and lysis of any red blood cells collected with the buffy coat is ensured using red blood cell lysis buffer (3). To count the estimated number of circulating cancer cells, we measured mRNA levels of epithelial markers, PyMT, or luciferase expression, which are selectively expressed in PyMT tumor cells or 4T1 cells, respectively. Putative numbers of CTCs were calculated by comparison with the mRNA levels in cultured Py2T or 4T1 Luc2-hE cells. For accurate analysis of apoptosis markers in circulating blood cells, cells expressing epithelial markers were sorted using a BD FACSAria I (BD Biosciences) and the mRNA was measured by qRT-PCR. CTCs were washed and suspended in 500 µL containing ice-cold Hank's Balanced Salt Solution (HBSS) containing 0.5% BSA. Cells were stained with 1 µg/ml 19A11 and fluorescein isothiocyanate-conjugated secondary antibody (1:500) in ice-cold HBSS containing 5% FBS. The antibodies were incubated for 45 minutes and washed to remove the excess of antibodies. The cytometric analysis and cell sorting were carried out using a FACS Aria-II flow cytometer (BD Biosciences, San Jose, CA, USA). Cells were kept in sterile conditions during cell sorting and flow cytometric analysis of membrane E-cadherin protein expression was performed before cell sorting. The Flow Jo software was used for data acquisition and analysis. FACS-purified cell purity was verified by qPCR analysis using the epithelial markers. Total RNA was prepared from FACS-sorted circulating tumor cells or cell lines using TRIzol (Life Technologies). RNA purity was confirmed using a NanoDrop Spectrophotometer (Thermo Fisher Scientific). The RNA samples were reverse-transcribed using a first-strand cDNA synthesis kit (SensiFAST™ cDNA Synthesis Kit) and subjected to qPCR (ΔΔCT) analysis, using KAPA SYBR® FAST qPCR Kits (Kapa Biosystems). cDNA samples contained at least 10 ng/µl. Forward and reverse primer sets were as follows:

hE-cadherin (F: 5′-CCCGCCTTATGATTCTCTGCTCGTGTCC-3′, R: 5′-TCCGTACATGTCAGCCAG CTTCTTGAA-3′), *hBcl-xL* (F: 5′-TCCTTGTCTACGCTTTCCACG-3′, R: 5′-GGTCGCATTGTGGCCT TT-3′), *hBax* (F: 5′-CCCGAGAGGTCTTTTTCCGAG-3′, R: 5′-CCAGCCCATGATGGTTCTGAT-3′), *hki67* (F: 5′-TGACCCTGATGAGAAAGCTCAA-3′, R: 5′-

CCCTGAGCAACACTGTCTTTT-3′), *mE-cadherin* (F: 5′-CGACCCTGCCTCTGAATCC-3′, R: 5′-TACACGCTGGGAAACATGAGC-3′), *mEpCAM* (F: 5′-AGAATACTGTCATTTGCTCCAAACT-3′, R: 5′-GTTCTGGATCGCCCCTTC-3′), *mBcl-xL* (F: 5′-TACCGGAGAGCGTTCAGTGA-3′, R: 5′-CCATCCCGAAAGAGTTCATTCA-3′), *mBax* (F: 5′-TTGCTACAGGGTTTCATCCA-3′, R: 5′-CATATTGCTGTCCAGTTCATCTC-3′), *Luc2* (F: 5′-GCTCAGCAAGGAGGTAGGTG-3′, R: 5′-TCTTACCGGTGTCCAAGTCC-3′), *PyMT* (F: 5′-CTCCAACAGATACACCCGCACATACT-3′, R: 5′-GTATCCAGAAAGCGACCAAGACCAGC-3′

Isolation and 3D culture of primary murine mammary spheroids. Epithelial fragments termed spheroids were isolated from murine mammary tumor. MMTV-PyMT tumors were harvested from mice at 14 wk of age. no. 3 and no. 4 mammary glands were dissected and digested into epithelial fragments by a combination of mechanical disruption and collagenase/trypsin digestion, and DNase treatment to separate epithelial tissue from fat and stromal cells. Briefly, they were minced into small fragments using a sterile razor blade and were incubated (typically in 10 mL of solution in a 15-mL Falcon tube) in collagenase [high-glucose DMEM (D6546; Sigma), 2 mM glutamine (5.1 mL), 200 U/mL penicillin/200 μg/mL streptomycin and 2 mg/mL collagenase I (C2139; Sigma)] with rocking at 37 °C. Successful isolation and culture were achieved with incubation times ranging from 6 h to overnight and in digestion solutions of collagenase alone or collagenase plus trypsin. The epithelial fragments were separated from single cells through differential centrifugation. The final pellet was composed of epithelial fragments, each containing several hundred cells; we term these fragments “organoids”. Spheroids are generated in drops of media that hang from the lid of a tissue culture dish for 72 hr. Next, the drops are pooled, and the spheroids are transferred to a 4 °C mixture of basement membrane materials (1:1 ratio of Matrigel (354230; BD Biosciences) and Collagen, Type I solution from rat tail (C3867-1VL; Sigma-Aldrich)). Following spheroid resuspension, the viscous mixture is pipetted into the wells of a 24 well plate or 8-well Lab-Tek chamber slides (Nalgene-Nunc/Thermofisher Scientific), after which it is given 30 min at 37 °C to solidify into a 3D culture. Warm media containing mAbs is then added to the wells. Spheroid medium: DMEM (Sigma D6546),

2 mM glutamine (ATCC or Invitrogen), 100 U/mL penicillin/100 µg/mL streptomycin, 10 mM Hepes (H3375-250g; Sigma), 0.075% (wt/vol) BSA (A8412; Sigma), 10 ng/mL cholera toxin (C8052; Sigma), 0.47 µg/mL hydrocortisone (H690; Sigma), 5 µg/ mL insulin (I0516; Sigma), and 5 ng/mL EGF (13247-051; Invitrogen). Cell exit from the spheroids is then monitored over time. Image analysis was performed by using Image J software and invasion of spheroids was calculated as a function of the longest invasive distance emanating from the spheroid. (Invasion = longest invasive distance – radius).

3D spheroid formation with Py2T and 4T1 cells. Spheroids are generated in drops of media that hang from the lid of a tissue culture dish for 72 hr. Basement membrane materials were mixed with the cell suspension at a 1:5 ratio and seeded onto a 24-well plate. After 30 min, culture media containing mAbs is then added to the wells. All cell cultures were incubated at 37 °C and 5% CO_2 incubator for 5 days, after which the tumor spheres were observed under an inverted microscope. The diameters of 30 randomly chosen tumor spheres were measured for each group.

Transwell migration and invasion assays. Cells were washed twice with 1x HBSS and harvested after trypsinization. For transwell migration assays, filters (8.0 µm pore size) and 24-well transwell chambers were used. Chambers were rinsed with culture medium without serum 1 hour before the assay. The cells were plated in triplicates in the upper wells at a density of 1×10^5 per well in 0.1 mL of RPMI-0.1% BSA containing mAbs. Chemotaxis was induced using medium with 10% FBS on the bottom side of the chambers. Cells were allowed to migrate for a period of 48 hours at 37°C and 5% CO_2 atmosphere, after which the experiment was stopped by wiping the cells from the upper side of the chamber with cotton swabs and fixed immediately with methanol for 15 minutes and then stained with 0.5% of crystal violet for 15 minutes. A total of ten images were taken for quantification using an inverted microscope. The invasion assay was identical to the above migration assay except that filters were coated with 100 µl of the diluted Matrigel (BD Biosciences). The experiment was stopped after 48 hours as described in migration assay.

Adhesion, activation assay and laminar flow adhesion assay. Cells were seeded in 6-well plates and cultured overnight. The cells were treated with 1 μg/ml control neutral mAb or activating mAb for 24 hr and cell adhesion activation was determined by the extent of morphological change to compact epithelial appearance. The laminar flow cell adhesion assay was conducted as described in a previous report (1). In brief, cells were trypsinized in the presence of 2 mM calcium and washed with 1x HBSS. The cells were pretreated for 2 hr with 3 μg/ml neutral mAb, or activating mAb, and allowed to attach to glass capillary tubes coated with E-cadherin for 10 min, and washed away for 30s at an indicated flow rate. The cells remaining after the wash were counted, and the adhesion percentage was calculated.

Statistics. Statistical analyses were performed using GraphPad Prism software (Graphpad Software, Inc., CA, USA). Statistically significant differences between two groups were determined using the nonparametric Mann-Whitney *U* test, unless otherwise indicated. $P < 0.05$ was considered significantly different. Data presented as images of immunoblots, immunofluorescence staining, H&E, transwell migration and invasion assays, adhesion, activation assay and laminar flow adhesion assay were from a representative experiment, which was qualitatively similar for at least three experiments.

Supplementary Figures

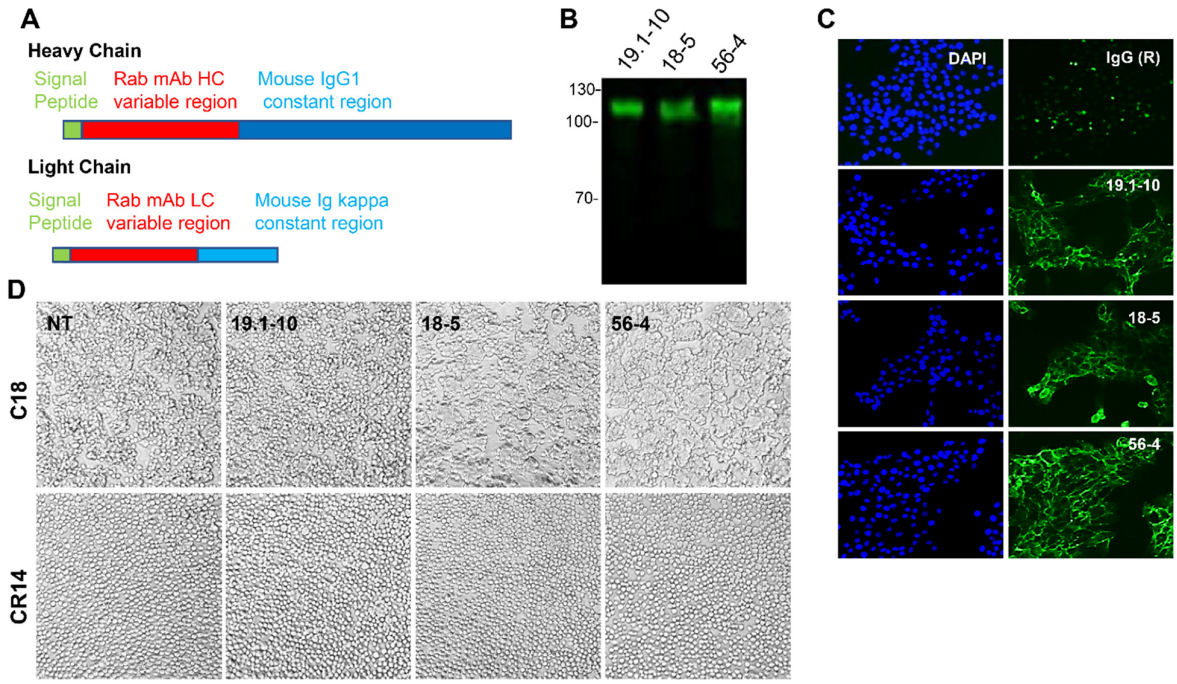

Fig. S1. Recombinant activating mAbs to mouse E-cadherin. (*A*) Schematic of the structure of recombinant heavy and light chain proteins for all mAbs. All the recombinant antibodies contain the mouse IgG1 heavy chain Fc region and the Ig kappa light chain constant region. (*B*) Western Blot analysis of the recombinant antibodies. Neutral (19.1-10) and activating (18-5, 56-4) antibodies at 1 µg/mL were blotted against 20 µg of whole cell lysate from parental 4T1 cells. (*C*) Immunofluorescence staining shows the mAbs bind to endogenous mouse E-cadherin as indicated, respectively. Parental 4T1 cells were incubated with neutral (19.1-10) and activating (18-5, 56-4) antibodies at 1 µg/mL for 7 hrs and then fixed with 4% PFA. (*D*) Activation assay using human colo205 cells expressing mouse E-cadherin (C18) versus colo205 cells in which E-cadherin has been knocked out using CRISPRCas9 (CR14). Shown are cells that were treated with 3 µg/mL of recombinant mAb for 7 hrs.; this assay was also used for the initial hybridoma screen.

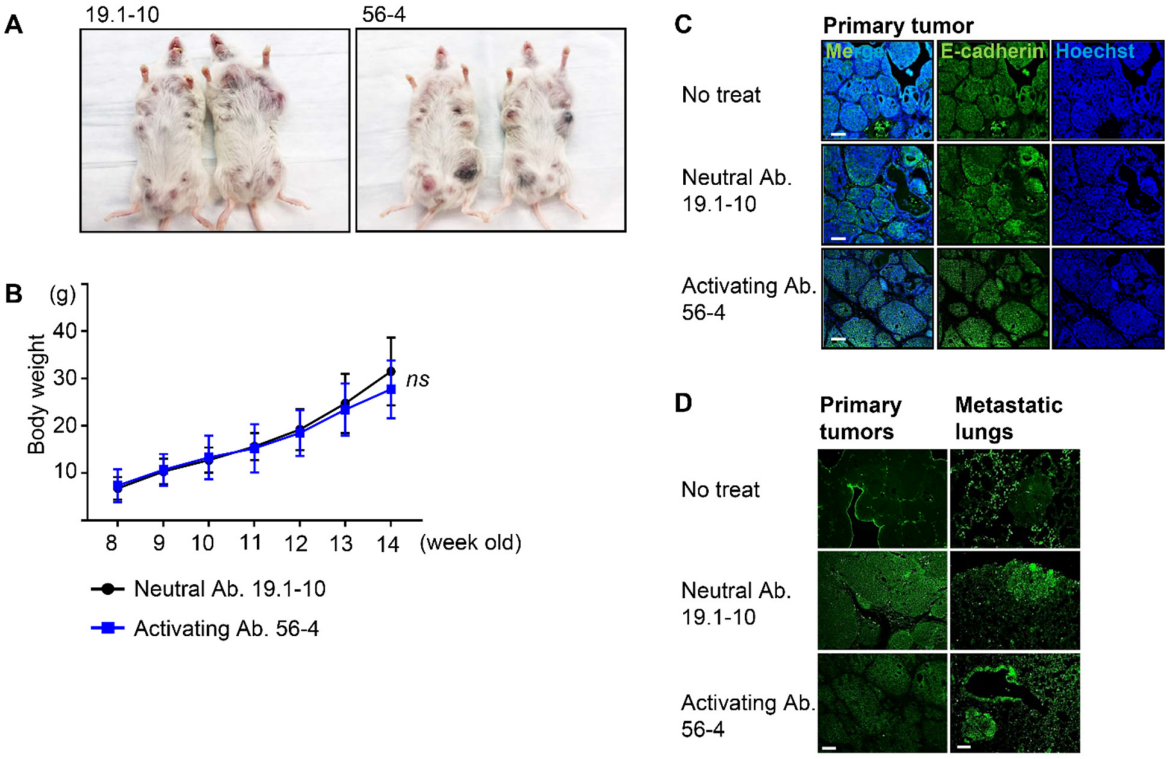

Fig. S2. New mouse E-cadherin activating antibody did not affect in growth in size of primary tumor in MMTV-PyMT mouse model of breast cancer. (*A*) 14-week-old MMTV-PyMT mice developed mammary tumors in similar size. (*B*) Body weight was measured weekly. (n=14 to 16). Representative microscopic images of immunofluorescence staining for E-cadherin (*C*) and injected antibody (*D*) in primary tumors or/ and metastatic lungs of MMTV-PyMT mice. Scale bar, 50 µm. To identify the injected antibodies in tissues, paraffin sections were stained with secondary antibody Alexa Fluor 488 IgG_1 alone without primary antibody staining (*D*).

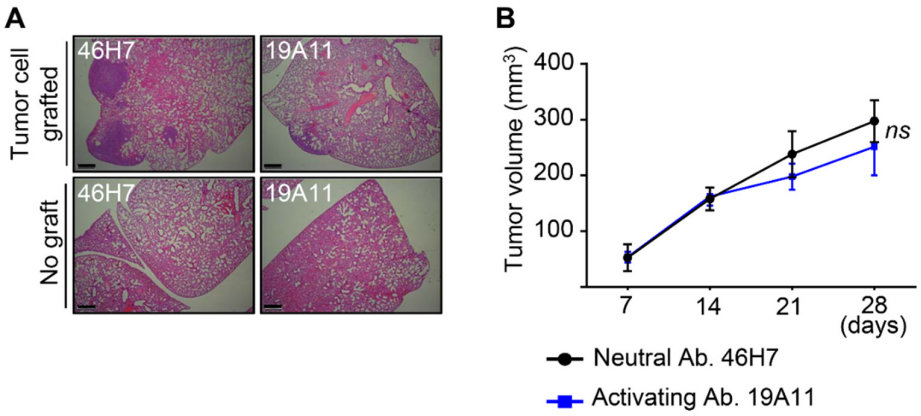

Fig. S3. E-cadherin activating antibody decreases tumor cell dissemination without affecting primary tumor growth. (*A*) Representative microscopic images of H&E staining of the lung from 4T1 Luc2-hE cell injected mice. (*B*) Tumors were measured weekly using external calipers. Scale bar, 500 μm.

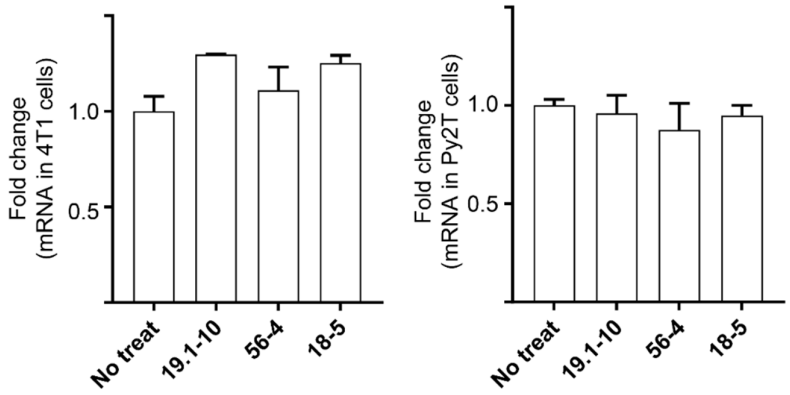

Fig. S4. mRNA levels of endogenous E-cadherin did not change in cell lines treated with E-cadherin activating antibodies. The mRNA expression of E-cadherin was analyzed by qRT-PCR in cultured 4T1 (left) and Py2T (right).

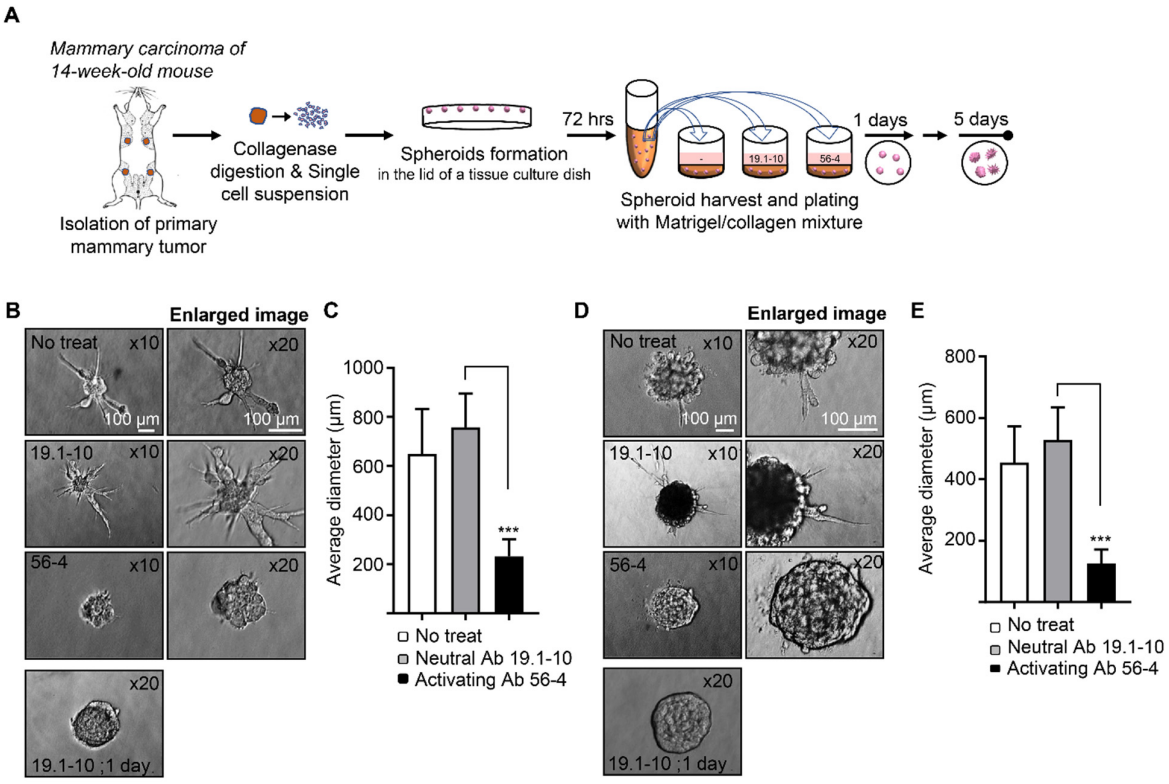

Fig. S5. E-cadherin activating antibody suppresses invasiveness in Py2T and 4T1 spheroids. (*A*) Schema for the preparation of MMTV-PyMT tumor derived spheroids in 3D culture shown in (Fig. 4*A*). (*B-E*) Representative microscopic images of 3D collage-I-embedded organoids treated with neutral mAb or activating mAb in Py2T (*B* and *C*) and parental 4T1 (*D* and *E*) cells. Enlarged views of (*B* and *D*), right of each image. Scale bar, 100 µm. Calculation of invasion as a function of the longest invasive distance emanating from the spheroid. Average of the longest invasive distance (µm) per spheroid. (n=50 organoids per group) ***, $P < 0.001$, statistically significant compared with neutral antibody treatment.

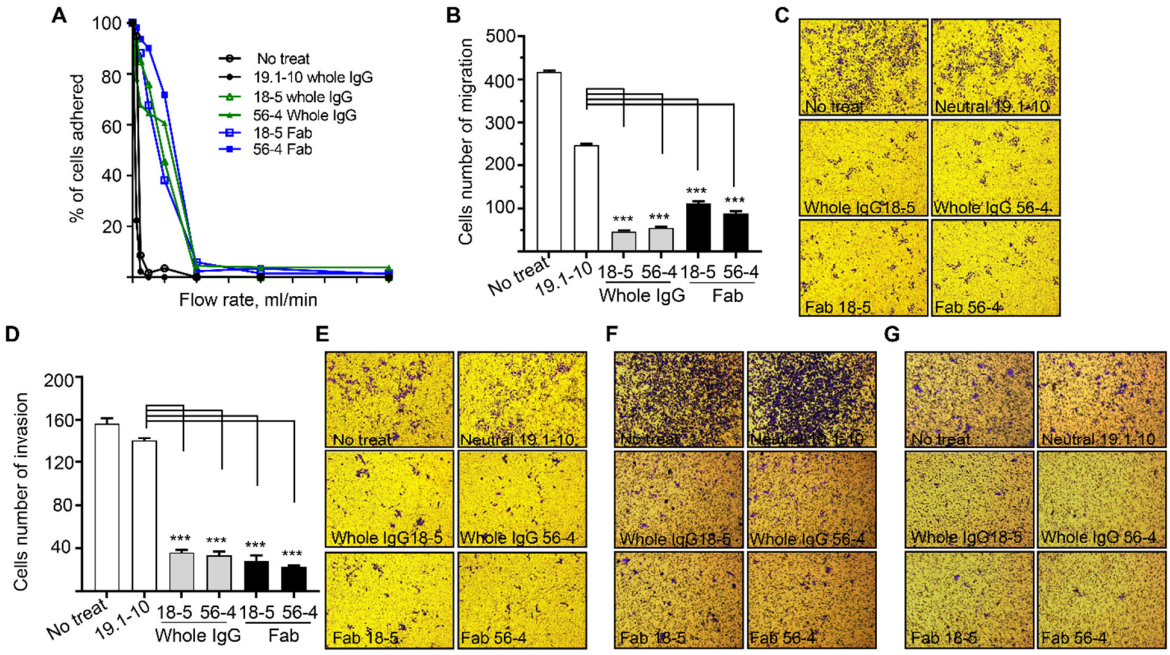

Fig. S6. E-cadherin activating mAbs increase cell adhesion, and inhibit migration and invasion *in vitro*. (*A-E*) Py2T cells. For cell adhesion assay, (*A*) activating mAbs and Fab fragments stimulated adhesion of cells to pure E-cadherin substrate. Py2T cells were untreated, pretreated with 3 μg/ml neutral mAb, 19.1-10 or activating mAbs, 18-5 or 56-4 for 2 hr and cell adhesion strength to E-cadherin-coated capillary tubes was evaluated using increasing laminar flow to determine the force required to detach cells. Quantification (*B*) and representative microscopic images (*C*) of transwell migration assay. Quantification (*D*) and representative microscopic images (*E*) of invasion assay through matrigel. (*F-G*) Parental 4T1 cells. Representative microscopic images of transwell migration (*F*) and invasion through matrigel (*G*) shown in Figs. 4*D* and 4*E*. ***, $P < 0.001$, compared with neutral antibody treatment.

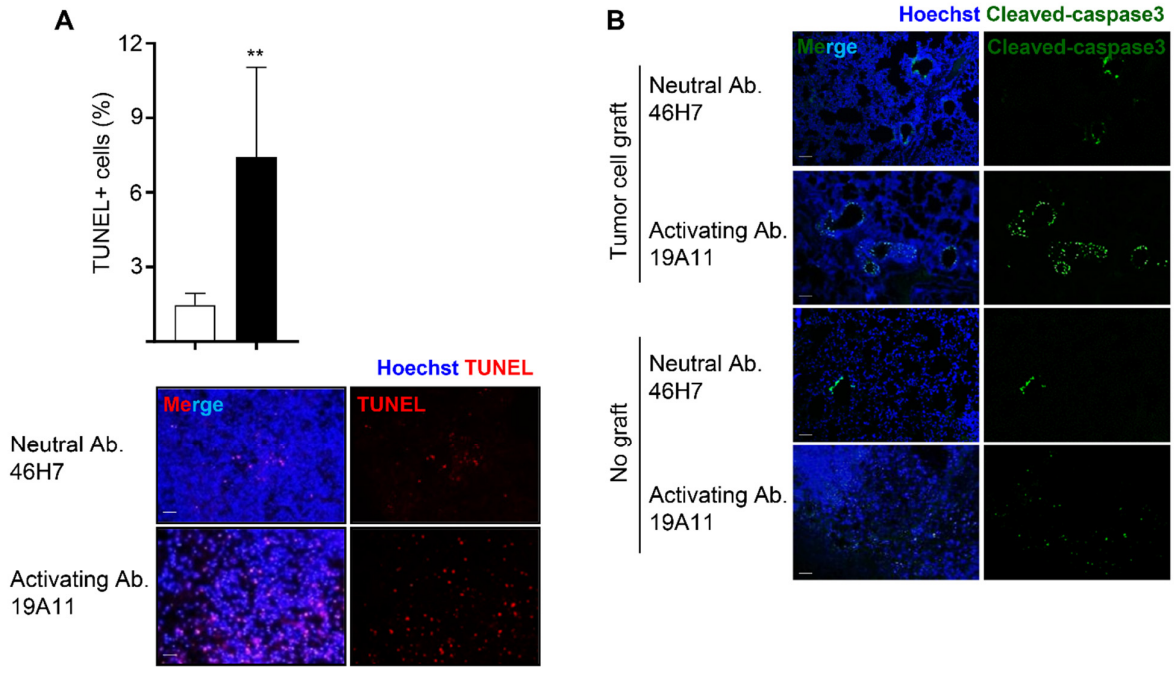

Fig. S7. E-cadherin activating antibody enhanced apoptosis in metastatic lung. Sections of lung from neutral or activating mAb- treated mice described in (Fig. 2*B*) were examined. Quantification (*A*, top) and representative microscopic images (*A*, bottom) of *in situ* apoptosis by TUNEL. Cells were measured in at least 10,000 cells and each percentage represents the average of three randomly chosen fields of 1 sample (×10) (n=4 per group). **, $P < 0.01$, statistically significant compared with neutral antibody treatment. Data are representatives of one out of at least three independent experiments. Representative microscopic images of immunofluorescence staining for Cleaved-caspase-3 (*B*) shown in Fig. 5*A*. Scale bar, 50 μm.

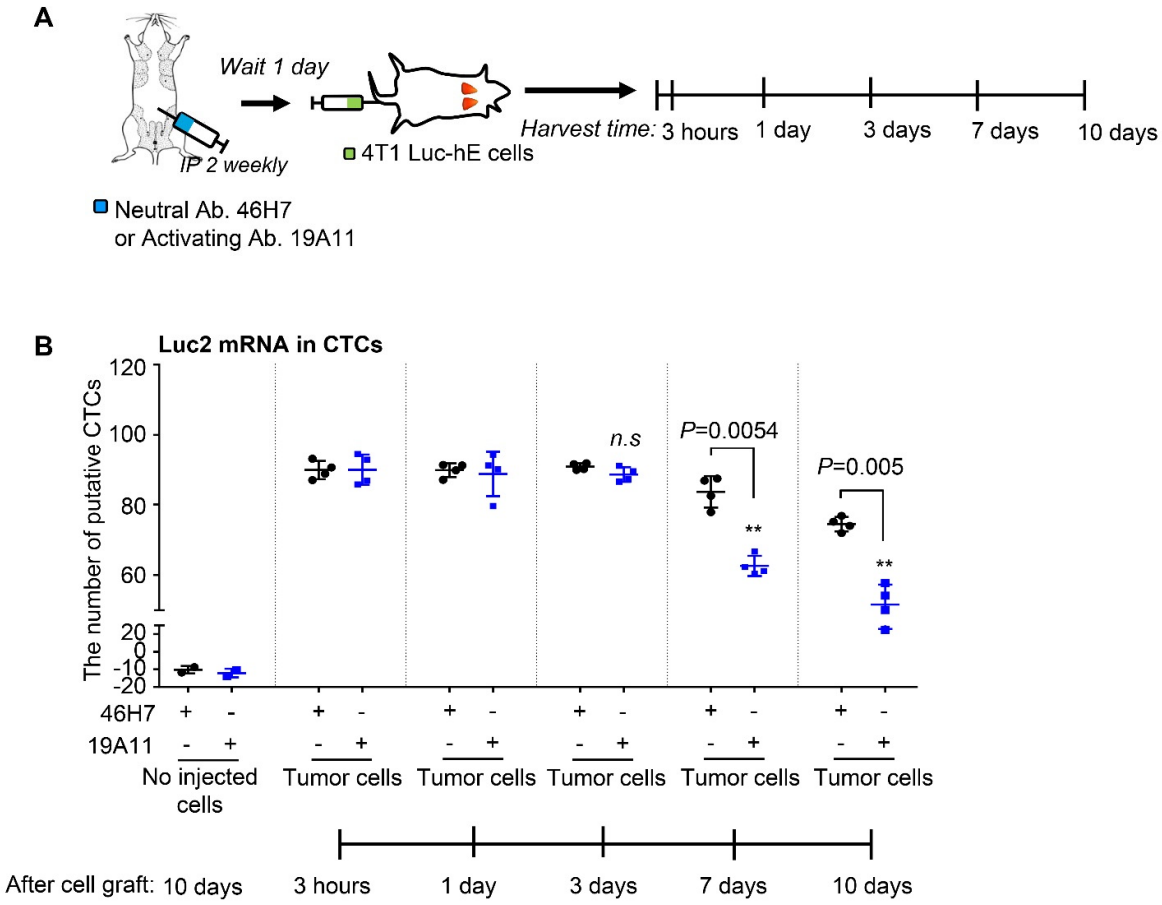

Fig. S8. E-cadherin activating antibody reduces number of circulating tumor cells in the bloodstream. (*A*) Schema of tumor cell graft animal experiment. Mouse was *i.p* injected with either 46H7 neutral mAb or 19A11 activating mAb twice weekly until the end of the experiments. One day after mAb treatment, the mice were *i.v* injected into mouse tail veins with 4T1 Luc-hE cells (3×10^4). The mice were euthanized and then whole blood (900 µl – 1 ml) was collected by cardiac puncture at the indicated time point. (*B*) mRNA level of Luc2 were analyzed by qRT-PCR and the estimated number of circulating tumor cells from equations for level of Luc2 mRNA expression in cultured 4T1 Luc-hE cells was calculated. **, $P < 0.01$, statistically significant compared with neutral antibody treatment at each time point.

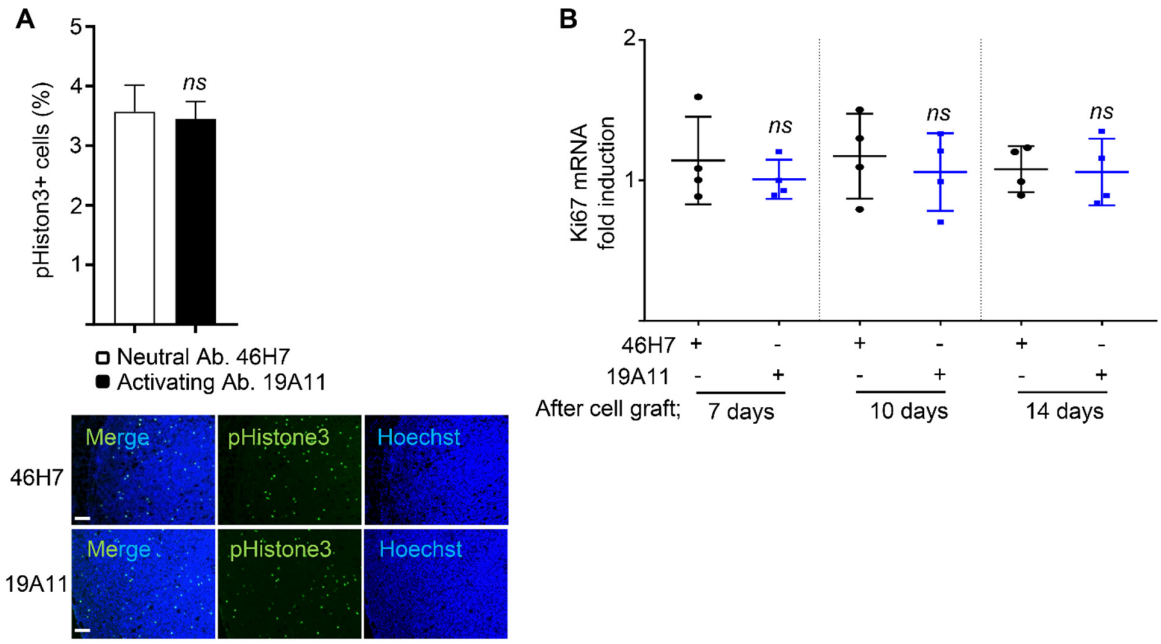

Fig. S9. Cell proliferation was not altered by E-cadherin activating Ab. (*A*) Quantification of immunofluorescence staining for pHistone 3. The same sections as used in (Fig. 5*A*) and Supplementary (Fig. S6) were used. Representative microscopic images (A, bottom). Scale bar, 50 µm. (*B*) Whole blood cells were sorted with hE-cadherin and then Ki67 mRNA level was analyzed by qRT-PCR as described in (Fig. 5 *D-F*).

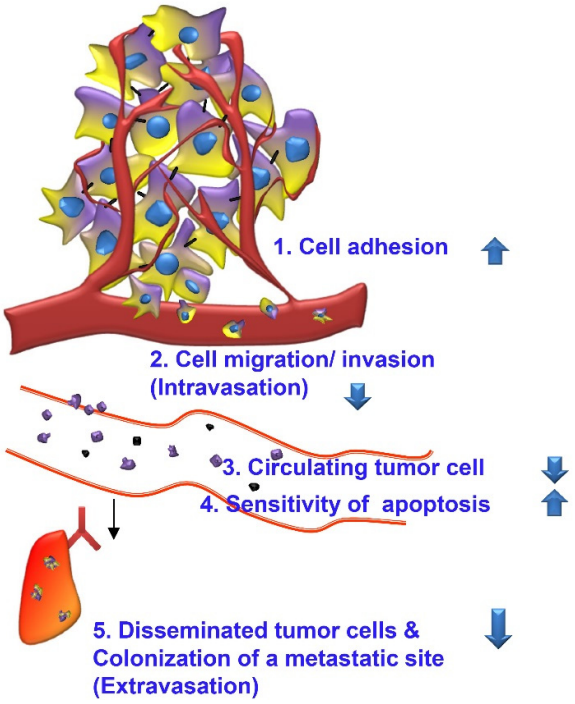

Fig. S10. Summary. Importance of E-cadherin activation in metastatic cascade. The activation of E-cadherin (1) induces the cell adhesion, (2) represses the cell migration/ invasion, (3, 4) suppresses the number of CTCs by increasing the sensitivity of cancer cell apoptosis, and (5) represses colonization of a metastatic site by inhibiting development of invasive protrusions.

Supplementary References

1. Y. I. Petrova, M. M. Spano, B. M. Gumbiner, Conformational epitopes at cadherin calcium-binding sites and p120-catenin phosphorylation regulate cell adhesion. *Mol Biol Cell* **23**, 2092-2108 (2012).
2. Y. I. Petrova, L. Schecterson, B. M. Gumbiner, Roles for E-cadherin cell surface regulation in cancer. *Mol Biol Cell* **27**, 3233-3244 (2016).
3. R.S. Muraoka et al., Blockade of TGF-beta inhibits mammary tumor cell viability, migration, and metastases. *J Clin Invest.* **109**, 1551-1559 (2002).