Supporting Information

Veiseh et al. 10.1073/pnas.1402383111

SI Materials and Methods

Two-Dimensional Cell Culture. Human breast cell lines were grown to 50–70% confluence on 75 cm^2 tissue culture plastic (VWR) and were maintained in the following manner: Hs578T, MCF-7, and MDA-MB-361 in DME-H21 (University of California, San Francisco); MDA-MB-231 in DMEM (Gibco); and SKBR-3 in McCoy's (Gibco), all in the presence of 1%Pen/Strep plus 10% (vol/vol) FBS (Sigma). HMT-3522 S1 and HMT-3522 T4-2 mammary epithelial cells were cultured as described in the literature (1–3). MCF-10A was propagated in DMEM/F12 (Invitrogen) according to Debnath et al. (4). Media was changed every 3–4 d for all cell lines except for HMT-3522 cells, which required a media change every 2 d. All cell lines were subcultured every 4–5 d except S1 cells, which were subcultured every 8–10 d. Cell viability was assessed with dye exclusion using a Vi-CELL cell analyzer (Beckman Coulter).

Three-Dimensional Cell Culture of FACS-Sorted Cells. FACS-sorted cells were cultured on top of Matrigel, a laminin-rich gel isolated from Engelbreth–Holm–Swarm mouse sarcoma cells, in the same manner as described in the original paper (5) except that cells were suspended in ice-cold medium (20,000 cells per 250 μL per 1.8 cm^2 surface area) and mixed with 10% (vol/vol) solution of ice-cold Matrigel at equal medium volume (250 μL) and then added drop-by-drop to the surface of Matrigel-coated wells. Cells were then incubated, and 500 μL of same culture medium used for 2D was added every 2 d.

Three-Dimensional Culture Reversion Assay. MDA-MB-231 cells were prepared as described above with the following modifications. A function blocking anti-β1 integrin antibody (AIIB2; Aragen Bioscience) was added (10 μg/mL) to the cell suspension in media, and the mixture was seeded using the on-top procedure (5). On the second day of 3D culture, 2 mM LY294002 (Calbiochem), a small molecule inhibitor of PI3K (6), was added to the medium. On day 4, reverted (AIIB2 + LY294002 treated) and nonreverted (untreated control) cells were photographed with a Zeiss Axiovert microscope. Cells were released from their matrices using PBS-EDTA as described below for use in assays.

Extraction of Cells from 3D Cultures. PBS-EDTA solution (containing 5 mM EDTA Invitrogen, 1 mM $NaVO_4$, 1.5 mM NaF, 100× protease inhibitor mixture in 1× PBS) was prepared, filtered, and placed on ice, along with cell culture plates and conical tubes that had been coated with 5% (wt/vol) BSA solution 1× PBS (Sigma-Aldrich) for 15 min at room temperature (RT). Three-dimensional cultures were washed once with one volume culture medium then twice with two volumes of PBS-EDTA solution. Matrigel was detached from bottom of culture wells using polyethylene cell scrapers (Corning, Inc.), and collected into ice-cold 15-mL conical tubes. Conical tubes containing polymerized Matrigel and cells in PBS-EDTA were oriented horizontally on an ice bucket, which was shaken on a Thermoscientific shaker at 4 °C for 1 h to depolymerize and liquefy the Matrigel. Tubes were then centrifuged at 800 rpm (Eppendorf Centrifuge 5804, A-4-44 rotor) for 5 min, the supernatant discarded, and cells resuspended in PBS-EDTA solution. This was repeated two additional times to minimize the amount of Matrigel in the final extract. Cells were counted using a Vi-CELL cell analyzer (Beckman Coulter).

Fluorescent Hyaluronan Probe Synthesis. Soluble hyaluronan (HA) (0.2 mL; 1% HA with molecular weight average 240 kDa) was activated in 1 mL of a 0.0028-g/mL *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDAC) solution (Sigma-Aldrich) in 2-morpholineethanesulfonic acid (MES) (20 mM, pH 4.5; Sigma-Aldrich), and the resulting solution was reacted with 0.3 mL of Alexa Fluor hydrazide, Tris(triethylammonium) salt or Texas Red (TR) hydrazide (1 mg/mL in H_2O; Invitrogen) in the dark at RT. The solution was covered in aluminum foil and placed on a rocker for 12 h at RT. Unconjugated dye was removed by dialysis against 75 mM NaCl in deionized (DI) water (8.8 g NaCl in 2 L DI water) using a Slide-A-Lyzer 10K molecular weight cut off dialysis cassette (3–12 mL capacity; Pierce) in the dark for up to 4 d at 4 °C. NaCl solution was changed four times and the Alexa Fluor 647 (A^{647})-HA or TR-HA probes retained in the cassettes were sterilized by filtering through a 0.2-μm filter (Corning, Inc.). The probes were dissolved in supplemented 1×PBS solution as explained below for binding/uptake assays.

Fluorescent HA Probe Binding in Suspension. In preparation for FACS analysis, the culture medium was aspirated and cells were washed 1× with Hank's balanced salt solution (HBSS) in 20 mM Hepes (Sigma). Cells were detached in nonenzymatic Ca^{2+}-free HBSS cell dissociation solution (Sigma). Harvested cells were suspended in ice-cold sterile 1×PBS supplemented with 1% antibiotic (Gibco), 1% Hepes, 0.1% human recombinant insulin, zinc (Invitrogen), and 0.1% human recombinant transferrin (Invitrogen), and then centrifuged at 1,200 rpm for 3 min in Eppendorf Centrifuge 5804, A-4-44 rotor. Cells were resuspended in 1 mL of the above PBS solution and then counted and assessed for viability using a Vi-CELL cell analyzer (Beckman Coulter); 1×10^6 cells in 1 mL of the supplemented 1×PBS solution were incubated on ice with 1 mL of A^{647}-HA (433 μg HA/mL) and dissolved in the supplemented 1×PBS solution for 45 min in the dark on ice. Unbound A^{647}-HA was removed by centrifugation (2×) in the supplemented 1×PBS solution. Cells were resuspended in the PBS solution, filtered through cell strainers, and analyzed by flow cytometry.

Fluorescent HA Probe Uptake in 2D. Subconfluent cells were grown in DMEM plus 10% (vol/vol) FCS to 50% subconfluence to maximize receptor for HA-mediated motility (RHAMM) surface display. Cells were rinsed in HBSS solution/20 mM Hepes, pH 7.3, detached with nonenzymatic HBSS (Sigma), which preserves cell-surface epitopes, and were exposed to A^{647}-HA and TR-HA at 37 °C as described (7, 8) in 2D. To block the HA-binding function of cluster designation 44 (CD44), 50 μg/mL anti-CD44 antibody (clone KM201; R&D Systems) was added to live BCa cells for 5 min, per the manufacturer's instructions, before the addition of TR-HA (7). Fluorescent HA (F-HA) intensities in the nuclei were quantified by Adobe Illustrator software.

F-HA Probe Uptake in 3D. Cells were cultured on top of Matrigel in the same manner as described in the original paper (5) for up to 5 d and were rinsed in HBSS solution/20 mM Hepes (3×) before exposure to A^{647}-HA at 2.5 mg/mL in sterile 1×PBS supplemented with 1% antibiotic (Gibco), 1% Hepes, 0.1% human recombinant insulin, zinc (Invitrogen), and 0.1% human recombinant transferrin (Invitrogen) for 30 min in the dark at 37 °C. Unbound A^{647}-HA was removed by HBSS solution/20 mM Hepes rinse (3×, 5 min each) and were fixed with 1% cetylpyridinium chloride (Sigma-Aldrich)/4% (wt/vol) paraformaldehyde (Sigma-Aldrich) in PBS for 10–20 min at RT in the dark. Excess

fixative was removed by 1 M buffered glycine solution (3×) before addition of DAPI (1:5,000 dilution in PBS; Sigma). Cells were washed with PBS (2×) and DI water (1×) before imaging with an LSM 710 confocal microscope (Carl Zeiss).

Antibody Preparation and Labeling. Mouse monoclonal anti–HA-mediated motility receptor (anti-HMMR) was prepared against a purified recombinant human HMMR peptide (mouse amino acid 706–767), which includes the HA-binding region (amino acid 719–750), clone 6B7D8 (ProMab). RHAMM antibodies were directly conjugated to Alexa Fluor 488 tetrafluorophenyl ester (Invitrogen) before sample preparation for cell-surface RHAMM detection by single-or multiplexed-color flow cytometry. CD44 monoclonal antibody (rat anti-mouse, clone IM-7; BD Pharmingen) was conjugated to A^{647} succinimidyl ester (Invitrogen) before detection of cell-surface CD44 by single-color flow cytometry, and commercially available CD44-phycoerythrin (PE)-cyanine 5 (Cy5) (rat anti-mouse, clone IM-7; BD Pharmingen) was used for multiplexed-color flow cytometry.

Multicolor Sample Preparation for Flow Cytometry. We selected three fluorochromes for simultaneous detection of HA binding, CD44, and RHAMM displays based on the following criteria: (*i*) minimal fluorescent spectral overlap among fluorochromes, (*ii*) maximal detection brightness for the low-expressing markers (i.e., RHAMM), and (*iii*) far red/near-infrared fluorochrome for HA to extend signal stability. Multicolor staining was performed on cells that were first incubated with A^{647}-HA then with anti-RHAMM and -CD44 antibodies. Before the addition of antibodies, A^{647}-HA–treated cells were blocked in a cold HBSS solution [5% (vol/vol) FCS plus HBSS/20mM Hepes] for 45 min in the dark to avoid fluorescence quenching, and were washed 2× in a PBS/2% (wt/vol) BSA solution (Sigma-Aldrich). Aliquots of 1.25×10^5 cells per 50 μL were incubated simultaneously with anti-RHAMM-6B7B7-Alexa Fluor 488 monoclonal antibody (2.5 μL) and rat anti-mouse CD44-PE Cy5 (0.75 μL of 0.2 mg/mL; BD Pharmingen) on ice for 30 min in the dark. Cells were then washed 2× in the cold 5% FCS/HBSS/Hepes buffer to remove unbound primary antibodies, filtered through cell strainers, reacted with propidium iodide (PI) (1:5,000 dilution from 20 μg/mL stock), and analyzed for multicolor staining.

Flow Cytometry Parameters. ***Single-color analysis.*** Forward scatter (FSC) and side scatter (SSC) lights were collected through a neutral-density filter in the FSC path, and a neutral-density filter at a 90° angle, respectively. The 488-nm lasers excited Alexa Fluor 488 and PE, while the 633 nm laser excited Alexa Fluor 467 and allophycocyanin (APC). Fluorescence emissions were collected although the FITC (533/30 Bandpass), PE (585/42 Bandpass), and APC (660/20 Bandpass) filters in fluorescence channels FL-1, FL-2, and FL-4, respectively. Unstained cells were first run as controls, and 30,000 events were analyzed for each sample. FSC and SSC settings were adjusted until the cells appeared in the middle of the FSC vs. SSC dot plot so that any cell aggregates or debris were excluded. The photomultiplier tube voltage of the FITC, APC, and PE detectors was adjusted until the cells appeared within the lower quadrant of the different dot plot, setting the background or unstained fluorescence levels according to these parameters. Next, single-color samples (A^{647}-HA, fluorescent anti-CD44, or fluorescent anti-RHAMM) were analyzed as needed. To verify antibody specificity, we ran positive controls (cell lines with known expression of antigens) and negative controls (isotype-matched nonimmune IgG) for each type of sample. ***Multiplexed-color analysis.*** With the parameters set for single-color analysis, single-color samples were run to correct the spectral overlaps using digital compensation by subtracting the overlap signal of single colors from the overall signals detected in each channel. Compensation was applied to confirm the positive population was directly horizontal or vertical to the negative population and not detectable in the other detector regions. Compensation was not required for the APC detector. The initial fluorescent levels of control unlabeled or nonimmune IgG-treated cells in the absence of added F-HA was measured for the RHAMM (FL-1) and CD44 (FL-4) channels and compared with the real-time display of RHAMM and CD44 (detected by antibodies) as well as A^{647}-HA binding. Compensation was performed using single-color samples as described above for double- and triple-color samples (RHAMM/CD44 and PI/RHAMM/HA) with spectral overlap. After parameters for defining background/negative signal and compensation values were established, the multiplexed three-color samples (RHAMM/CD44/HA) were analyzed. Data analysis was performed using FlowJo software (www.flowjo.com).

Transmission Electron Microscopy. Subconfluent cells were serum-starved overnight as described above, then exposed to HA-Au nanoparticles and DMEM plus 10% (vol/vol) FCS for 30 min. Cells were fixed in situ in 2.5% (wt/vol) gluteraldehyde (electron microscope grade; Pierce Chemicals) followed by osmium tetroxide for 2 h at 4 °C. Adherent cells were negatively stained with uranyl acetate, dehydrated in an alcohol series, and then infiltrated with epon epoxy resin. Sixty- to 90-nm sections of the polymerized resin embedded cells were cut with a diamond knife onto copper grids. Sections were examined with a Zeiss Libra 120 plus transmission electron microscope.

Scanning Electron Microscopy. Three-dimensional cultured cells, as explained above, were washed gently with HBSS (2×) before fixation with 2.5% (wt/vol) glutaraldehyde in 0.1 M phosphate buffer for 30 min at RT. Excess glutaraldehyde was removed with PBS/glycine treatment for 10 min followed by two washes with distilled DI water at RT. Dehydrated cells were Au-coated (60% Au and 40% palladium) in SPI Module Sputter/Carbon Coaters (SPI Supplies/Structure Probe, Inc.) under a 100-mbar vacuum for 50 s. Images were taken using a Sirion scanning electron microscope (FEI Company) with through-the-lens detection technology.

Isolation of $HA^{-/low}$ and HA^{high} Subsets by FACS. A^{647}-HA–bound cells were sorted using gates set to an 8% threshold on a histogram profile. The threshold was set based on the intensity with which cells bound A^{647}-HA, and cells were sorted into $HA^{-/low}$ and HA^{high} subpopulations based on differential binding of cells to HA (8% maximum and minimum signal selection). Sorted cells were collected in fresh culture medium and cultured under 2D or 3D conditions. Data analysis was performed using FlowJo software.

Cell Morphology and Growth Analyses. Immediately following FACS sorting, $HA^{-/low}$ and HA^{high} subpopulations of MDA-MB-231 were plated at equal densities in triplicates onto four-well glass chamber slides (10,000 cells per well) or onto Matrigel as described above. Cells were photographed on alternating days for 8 d using a Zeiss Axiovert 200 microscope. The surface area occupied by adherent cells was quantified using the particle analyzer module of ImageJ software (http://imagej.nih.gov/ij).

Colony-Forming Assays. ***Two-dimensional culture.*** Immediately following FACS sorting, single cells were seeded into six-well plates (1×10^3 cells per well) in triplicate in DMEM plus 10% (vol/vol) FBS and incubated at 37 °C for 13 d. Cells were stained with 0.2% methylene blue in 50% (vol/vol) ethanol and destained with tap water. Each well was photographed, and the size and number of colonies were calculated.

Three-dimensional culture. Agar (1%) in H_2O was mixed with equal volumes of 2× DMEM plus 20% FCS and 2% (vol/vol) antibiotic at 40 °C. One milliliter of this solution (base agar) was poured

into a 35-mm^2 plate in triplicate and solidified at RT. Agar solution (0.7%) equilibrated to 40 °C was combined with 2× DMEM and the sorted subpopulations at 5,000 cells per mL. The resulting mixture was poured onto the base agar at 1 mL per plate (four plates per condition). After solidification of the top layer of agar and cells, 500 μL of the base agar solution was added to each plate which was then placed in a humidified 37 °C incubator for 1 h. The solidified multilayered agar plates were coated with 500 μL DMEM containing 10% FCS and 1% antibiotic then incubated in a humidified atmosphere containing 5% CO_2 at 37 °C for 2 mo. Fresh culture medium was added every other day. Plates were photographed on a Zeiss Axiovert 200 microscope to verify colony formation, stained with 0.01% crystal violet for 30 min, and counted.

Matrigel Invasion Assay. Matrigel invasion chambers (BD Biosciences) were prepared by incubation with 500 μL serum-free media for 2 h at 37 °C. Serum-starved cells were washed with HBSS, dissociated by nonenzymatic HBSS, centrifuged to remove the dissociation medium, resuspended in DMEM plus 0.2% FCS, and counted with a Vi-CELL cell analyzer. The medium in the Matrigel invasion chambers was removed and replaced with 500 μL DMEM plus 10% (vol/vol) FCS; 5×10^4 cells suspended in DMEM plus 0.2% FCS were added into top inserts in triplicate, and then DMEM plus 0.2% FCS was added to achieve a final volume of 300 μL. Chambers were maintained in a humidified CO_2 incubator at 37 °C for 1 d. Cells that had migrated onto/invaded the filter face in the lower invasion chamber were stained with 0.125% Coomassie Blue in methanol: acetic acid:H_2O (45:10:45, vol/vol/vol) for 20 min. Inserts were then rinsed with water, air-dried for 20 min, and photographed. The cell-surface area of invaded cells was quantified using the particle analyzer module of ImageJ.

Ex Ovo F-HA Uptake and Extravasation in the Chick Chorioallantoic Membrane. On day 13 of embryonic development, tumor cells in PBS were i.v. injected into a vein within the chorioallantoic membrane (CAM) ($n = 5$ each group). Before injection, cancer cells were pretreated with HA (100 mg/mL) or vehicle (PBS) for 1 h on ice. Immediately after injection, four aluminum foil square markers (5-mm diameter) were placed on the surface of the CAM to form a large rectangular region of interest (ROI) for macroscopic imaging. Stitched images of the ROI were acquired on an upright Zeiss Axio Examiner Z1 microscope and a Hamamatsu 9100–02 electron microscopy CDD camera using Volocity software (Perkin-Elmer, Inc.). Stitched images of each animal's ROI were taken immediately after injection and at 24 h postinjection. For the MDA-MB-231 cells experimental condition, cells were grown to subconfluence in 2D as explained above. Released cells were washed in DMEM plus 10% (vol/vol) FCS, resuspended in 1 mL DMEM, then counted with a hemacytometer; 7.5×10^4 tumor cells were incubated with 100 μL A^{647}-HA in PBS (1 mg/mL) and immediately injected into a vein in the CAM of day-13 chicken embryos as above. Time-lapse confocal images of MDA-MB-231 cells that were arrested within the vein were taken 30 min after injection. To confirm successful extravasation, the vascular lumen of the CAM was labeled by i.v. injection of fluorescent *Lens culinaris* agglutinin (LCA) (Vector Laboratories) at 24 h postinjection, and cancer cells were visualized using a spinning disk confocal microscope (Quorum Technologies) (9). Intravascular cells were identified as being present only within the CAM lumen as labeled by LCA. Extravascular cells were identified as being present within the underlying stroma, and not within the same Z plane as the CAM lumen (10). At least 200 cells for each ROI at $T = 0$ were analyzed and enumerated.

Preparation of Tumor Xenografts in Severely Immunocompromised Mice. Mice were maintained at five per cage with unlimited chow and water in a controlled aseptic animal barrier; 62.5×10^3 tumor cells were suspended in 20 μL of Matrigel/DMEM plus 10% FCS (1:1 vol) on ice then injected into the left fourth mammary fat pad of mice anesthetized under isofluorane gas (level 1–1.5 mixed with O_2). Six to 12 mice were randomly assigned to 4 cohorts: 1 = unsorted cells without F-HA, 2 = unsorted cells with F-HA, 3 = sorted $HA^{-/low}$ subpopulation, and 4 = sorted HA^{high} subpopulation. Mice received antibiotics in their drinking water for 2 wk postsurgery. Tumor size was monitored using vernier calipers for 3–8 wk, and the tumor volume was calculated [$L \times W^2 \times 0.5$ (where 1 cm^3 = 1 g)]. At 8 wk postinjection, mice were euthanized in CO_2 chambers and tumors were harvested, weighed to obtain wet tumor weight, and fixed in 2% (wt/vol) paraformaldehyde in PBS overnight, followed by serial dehydration in 30% (vol/vol; 45–60 min), 50% (vol/vol; 45–60 min), and 70% ethanol (vol/vol; 45–60 min) on a shaker. Fixed tissues were paraffin-processed and histology sections were prepared at the Mouse Pathology Core of the Helen Diller Family Comprehensive Cancer Center (University of California, San Francisco). Stained slides were imaged by a Zeiss Axioskop microscope.

Immunofluorescence Detection of Human Ki67. Tissues were deparaffinized and rehydrated before immersion in citrate buffer (4.5 mL of 0.21 g citric acid monohydrate/10 mL H_2O plus 20.5 mL of 2.94 g trisodium citrate/100 mL dH_2O plus 225 mL H_2O) solution for 10 min at boiling temperature. After cooling down to 25 °C for 10 min, they were quenched using 50-mL of 3% (vol/vol) H_2O_2 in 100% MeOH for 10 min, permeabilized in a 250-mL solution of 0.2% Triton X-100 in PBS for 12 min, and washed by a 250-mL solution of 0.2% Tween-20 in PBS for 5 min before blocking with 150 μL serum-free protein block (Dako) per slide for 45–60 min at 25 °C. Anti-Ki67 antibody (Abcam) was diluted to 1:500 in a background-reducing antibody diluent (Dako). The block was wicked off and replaced with the diluted anti-Ki67 antibodies, and slides were incubated overnight at 4 °C on a level surface. The following day, the primary antibody was removed by washing slides with three 5-min cycles of PBS. Slides were incubated with anti-Rb Hrp secondary antibody (diluted to 1:2,000 in PBS; Sigma) for 1 h at 25 °C then washed 3× with PBS for 5 min each. Diaminobenzidine (Dako) was used to detect bound secondary antibody, and hematoxylin (Sigma) was used as a counter stain. Slides were washed in H_2O for 30 s then dehydrated through alcohol series. Mounted slides were dried overnight in a fume hood. A ScanScope Digital Slide Scanner (Aperio) was used to image the tissue, and groups (two or more cells) of positive cells were counted per tissue section.

Oncomine Query. Oncomine data banks were investigated for significantly altered ($P < 0.05$) mRNA expression of CD44, RHAMM/HMMR, lymphatic vessel endothelial HA receptor 1 (LYVE1), layillin (LAYN), and stabilin 2 (STAB2) in primary cancer vs. normal breast tissue and for an association of elevated mRNA expression with poor clinical outcome measured by death, primary tumor recurrence or appearance of metastases.

SI Results

mRNA expression of CD44 and RHAMM was significantly higher than that of LYVE1, LAYN, or STAB2 in primary breast tumors, and elevated CD44/RHAMM expression was linked to poor clinical outcome in more studies than the other three HA receptors (Table S1). Therefore, we determined whether significantly elevated mRNA expression levels of CD44 and RHAMM were found in all or only restricted molecular subtypes, respectively (Table S2). Although CD44 mRNA expression was elevated in ER^+/PR^+ and $ERBB2^+$, it was most frequently elevated in the

triple-negative basal-like subtype, in agreement with evidence that CD44 protein expression may be a marker for triple-negative breast cancer (BCa) (11). RHAMM mRNA expression was increased across all subtypes, and high CD44 and RHAMM mRNA expression was significantly linked to high levels of HA synthase 2 (HAS2) mRNA in triple-negative BCa. Therefore, we measured the cell-surface CD44 and RHAMM protein displays and then F-HA binding in malignant basal-like (ER^-/PR^-/$HER2^-$) and luminal (ER^+/PR^+/$HER2^-$, ER^-/PR^-/$HER2^+$, and ER^+/PR^-/$HER2^+$) BCa lines, and in nonmalignant breast mammoplasty-derived cell lines (Table S3), used as controls. CD44 cell-surface display was highest in triple-negative basal-like BCa lines in agreement with mRNA expression profiles of Oncomine datasets. However, display was low or absent in luminal BCa lines. Cell-surface RHAMM was displayed in all examined subtypes, although, in contrast to CD44, levels were higher in luminal than in basal-like lines. Therefore, displays of these two HA receptors in BCa lines of different molecular subtypes are in general agreement with mRNA expression levels in clinical BCa samples.

1. Briand P, Nielsen KV, Madsen MW, Petersen OW (1996) Trisomy 7p and malignant transformation of human breast epithelial cells following epidermal growth factor withdrawal. *Cancer Res* 56(9):2039–2044.
2. Petersen OW, Rønnov-Jessen L, Howlett AR, Bissell MJ (1992) Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells. *Proc Natl Acad Sci USA* 89(19): 9064–9068.
3. Weaver VM, Howlett AR, Langton-Webster B, Petersen OW, Bissell MJ (1995) The development of a functionally relevant cell culture model of progressive human breast cancer. *Semin Cancer Biol* 6(3):175–184.
4. Debnath J, Muthuswamy SK, Brugge JS (2003) Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. *Methods* 30(3):256–268.
5. Lee GY, Kenny PA, Lee EH, Bissell MJ (2007) Three-dimensional culture models of normal and malignant breast epithelial cells. *Nat Methods* 4(4):359–365.
6. Wang F, et al. (2002) Phenotypic reversion or death of cancer cells by altering signaling pathways in three-dimensional contexts. *J Natl Cancer Inst* 94(19):1494–1503.
7. Veiseh M, et al. (2012) Imaging of homeostatic, neoplastic, and injured tissues by HA-based probes. *Biomacromolecules* 13(1):12–22.
8. Hamilton SR, et al. (2007) The hyaluronan receptors CD44 and Rhamm (CD168) form complexes with ERK1,2 that sustain high basal motility in breast cancer cells. *J Biol Chem* 282(22):16667–16680.
9. Leong HS, et al. (2010) Intravital imaging of embryonic and tumor neovasculature using viral nanoparticles. *Nat Protoc* 5(8):1406–1417.
10. Arpaia E, et al. (2012) The interaction between caveolin-1 and Rho-GTPases promotes metastasis by controlling the expression of alpha5-integrin and the activation of Src, Ras and Erk. *Oncogene* 31(7):884–896.
11. Idowu MO, et al. (2012) CD44(+)/CD24(-/low) cancer stem/progenitor cells are more abundant in triple-negative invasive breast carcinoma phenotype and are associated with poor outcome. *Hum Pathol* 43(3):364–373.

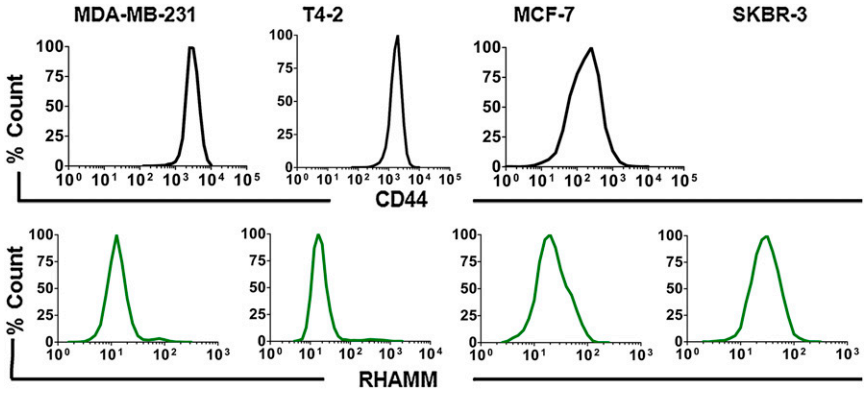

Fig. S1. CD44 and RHAMM display profiles do not recapitulate F-HA–binding heterogeneity. Flow cytometry profiles of (*Upper*) CD44 and (*Lower*) RHAMM displays after incubating MDA-MB-231, T4-2, MCF-7, and SKBR-3 with anti-CD44 and anti-RHAMM antibodies, respectively. n = 3–8. Of note, profiles do not span from 0 to 10^5 fluorescent signals and do not contain a second peak or positively skewed tails.

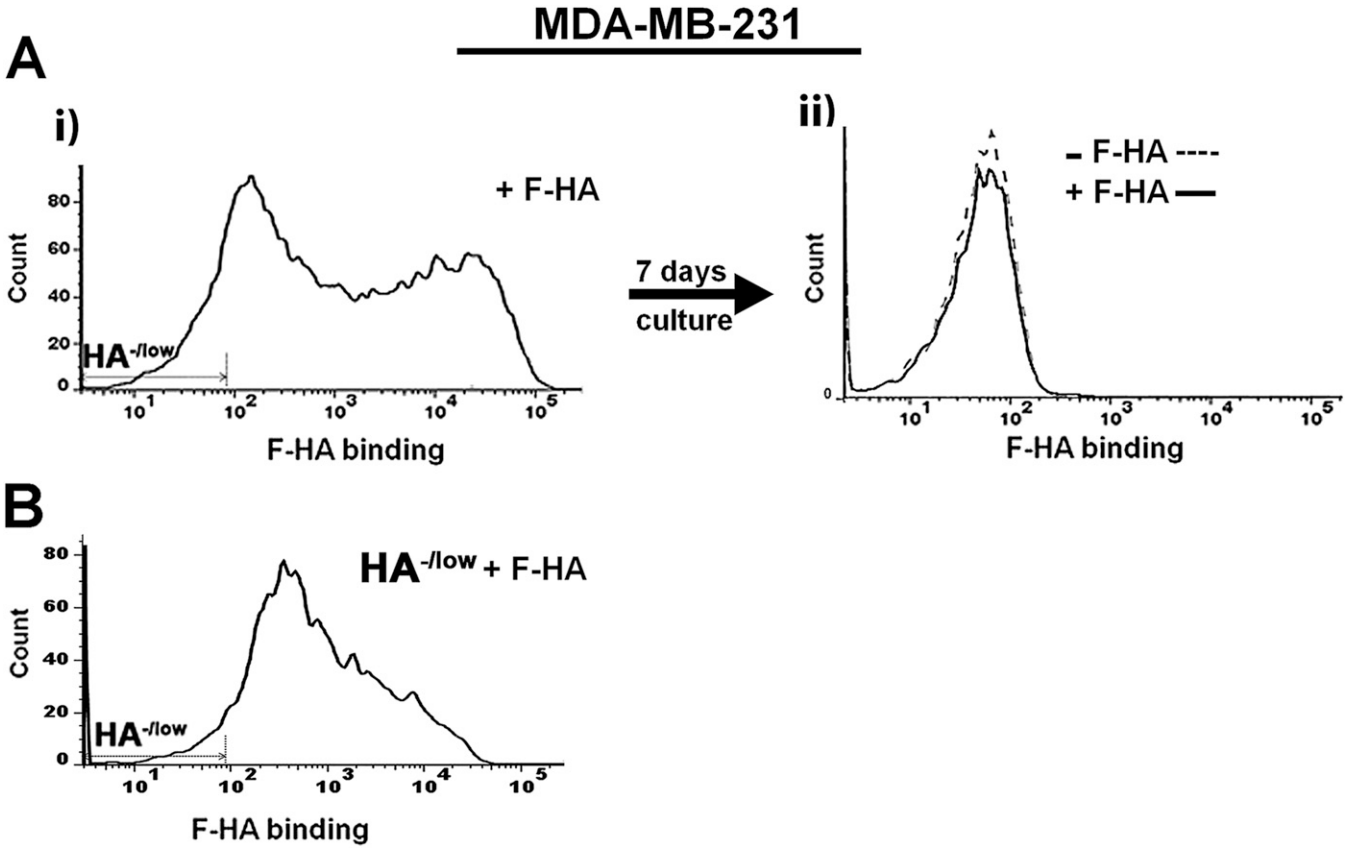

Fig. S2. $HA^{-/low}$ bind F-HA stochastically, but the subpopulation of parental tumor cells that binds high levels of F-HA appears to be stable. (*A*, *i*) Representative F-HA–binding profile of parental MDA-MB-231 cells and identification/isolation of a subpopulation that bound no or low amounts of HA ($HA^{-/low}$) from HA-bound MDA-MB-231 cells. $n = 3$. (*A*, *ii*) FACS analysis of retained cell-surface F-HA after 7 d in culture. (*B*) HA-binding profile of a $HA^{-/low}$ subpopulation after 7 d growth in culture and reexposure to F-HA. $n = 3$.

Table S1. mRNA expression of HA receptors in BCa

Phenotype	CD44	RHAMM	LAYN	LYVE1	STAB2
Cancer vs. normal	3/51	10/14	1/10	0	0
Poor clinical outcome	8/29	19/29	3/13	5/25	5/25

Values are presented as the number of data sets reported in separate studies, where mRNA expression is significantly ($P < 0.05$) elevated and the gene of interest is ranked in the top 10% of expressed genes.

Table S2. mRNA expression of CD44 and RHAMM in molecular subtypes of BCa

Breast cancer subtype	CD44	RHAMM	CD44 and RHAMM coexpressed	CD44, RHAMM, and HAS2 coexpressed
Triple-negative basal-like	11/35	7/49	5/7	4/11
ER^+/PR^+ luminal	4/35	10/49	1/10	1/04
$ERBB2^+$ luminal	3/35	11/49	2/11	1/03

Values are presented as the number of data sets reported in separate studies, where mRNA expression is significantly ($P < 0.05$) elevated and the gene of interest is ranked in the top 10% of expressed genes.

Table S3. Breast-derived cell lines display CD44 and/or RHAMM proteins on their surfaces

Cell lines	Subtype	CD44	RHAMM
MDA-MB-231 (ER^-,PR^-,$HER2^-$)	Basal-like	+++	+
HS-578T (ER^-,PR^-,$HER2^-$)	Basal-like	++++	NA
T4-2 (ER^-,PR^-,$HER2^-$)	Basal-like	++	+
S1 (ER^-,PR^-,$HER2^-$)	Basal-like	++	–/low
MCF-10A (ER^-,PR^-,$HER2^-$)	Basal-like	++	+
MCF-7 (ER^+,PR^+,$HER2^-$)	Luminal	Low/+	++
SKBR-3 (ER^-,PR^-,$HER2^+$)	Luminal	–	++
MDA-MB-361 (ER^+,PR^-,$HER2^+$)	Luminal	–	NA

Scores are determined by FACS geometric mean and median values of cell-surface CD44 and RHAMM display (n = 3–8). The degree of positivity is measured by comparing the geometric mean of sample data with that of control (isotype-matched nonimmune IgG treated) sample data. NA, not applicable; –, 0–100; Low, 100–500; +, 500–1,000; ++, 1,000–2,000; +++, 2,000–3,000; ++++, more than 3,000 for CD44. FACS signal values of RHAMM surface proteins were nearly 50-fold less than those of CD44. ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor-2.