Supplementary Information for

Exploiting Differential Effects of Actomyosin Contractility to Control Cell Sorting Among Breast Cancer Cells

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This PDF file includes:

Supplementary text
Figures S1 to S14
Legends for Movies S1 to S3
SI References

Other supplementary materials for this manuscript include the following:

Movies S1 to S3

Supporting Text

Materials and Methods

1.1 Cell Lines and Reagents

MDA-MB-231, MDA-MB-468, MDA-MB-436, MDA-MB-157, and ZR-75-1 cells were obtained from the American Type Culture Collection (Manassas, VA). MCF-10A and MCF-10AHRas cells were a gift from Professor Carol Prives (Columbia University, NY). DMEM high glucose liquid media, RPMI 1640 liquid media, and phosphate buffered saline (PBS) were obtained from GE Healthcare HyClone (Logan, Utah). Fetal bovine serum (FBS), horse serum, DMEM/F12, HEPES buffer, and sodium bicarbonate ($NaHCO_3$) solution were obtained from Gibco (Grand Island, NY). Ultra-low attachment plates were obtained from Thermo Fisher Scientific (Nunclon Sphera microplates) or were prepared in house using a previously described polyHEMA coating method(Kuroda *et al.*, 2013). Pepsin-treated (PT) bovine collagen I (~6 mg/mL solution) and acid-solubilized (AS) rat tail collagen I (~4 mg/mL solution) were obtained from Advanced BioMatrix (San Diego, CA). Except where specified, all collagen I used in this work is PT collagen. Matrigel growth factor reduced basement membrane extract (BME) as a ~7-10 mg/mL solution, and 100x MEM nonessential amino acid solution were obtained from Corning (Corning, NY). Cell Recovery Solution was obtained from Corning. Accutase and 100x penicillin-streptomycin-amphotericin B were obtained from MP Biomedicals (Santa Ana, CA). 10x DMEM, sodium hydroxide solution, hydrocortisone, cholera toxin, epidermal growth factor (EGF), and insulin as a 10 mg/mL solution were obtained from Sigma Aldrich (St. Louis, MO). CellTracker Green CMFDA and CellTracker Orange CMRA were obtained from Invitrogen Life Technologies (Grand Island, NY). Silencer select siRNAs were obtained from Thermo Fisher (Watham, MA) as annealed duplexes. β_1 integrin: Sense 5'- GGAGGAAUGUUACACGGCUtt-3/Antisense 5- AGCCGUGUAACAUUCCUCCag-3 and negative control #2 siRNA (catalog#4390846). Opti-MEM medium and lipofectamine RNAiMax were obtained from Thermo Fisher. β_1 integrin monoclonal inhibitory antibody (clone 4B4) was obtained from Beckman-Coulter (Brea, CA). β_1 integrin active conformation monoclonal antibody (clone 12G10) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). E-cadherin monoclonal inhibitory antibody (clone SHE78-7) was obtained from Invitrogen (Carlsbad, CA). E-cadherin antibody used for immunofluorescence (clone 67A4) was obtained from Thermo Fisher. Phospho-myosin light chain (Ser19) antibody was obtained from Cell Signaling Technology (Beverly, MA). Goat-anti-mouse and goat-anti-rabbit secondary antibodies labeled with either AlexFluor 488 or AlexaFluor 647 were obtained from Abcam (Cambridge, MA). Bovine serum albumin (BSA) was obtained from Thermo Fisher. (-)-blebbistatin, (+)-blebbistatin, ML-7, Y27632 ROCK inhibitor, and urea were all obtained from Sigma-Aldrich. Para-aminoblebbistatin and para-nitroblebbistatin were obtained from Optopharma (Budapest, Hungary). Prolong Glass mounting media, AlexaFluor 568 phalloidin, methanol-free formaldehyde, and phosphate-buffered formalin were all obtained from Thermo Fisher. Triton-X-100 was obtained from EMD Millipore Chemicals (Billerica, MA).

1.2 Cell Culture

All cell lines were maintained on tissue culture plastic in a humidified incubator at 37°C with 5% carbon dioxide. MDA-MB-231, MDA-MB-468, and MDA-MB-436 were maintained in DMEM high glucose growth medium supplemented with 10% (v/v) FBS, 1% (v/v) 100x penicillin-streptomycin-amphotericin B, and 1% (v/v) 100x MEM nonessential amino acid solution. MDA-

MB-157 and ZR-75-1 were similarly maintained in RPMI 1640 growth medium containing the same supplements. MCF-10A and MCF-10AHRas were maintained in DMEM/F12 growth medium containing 5% (v/v) horse serum, 1% (v/v) 100x penicillin-streptomycin-amphotericin B solution, 0.5 µg/mL hydrocortisone, 10 µg/mL insulin, 0.1 µg/mL cholera toxin, and 20 ng/mL EGF. Cells were detached using Accutase when 80-90% confluent prior to experiments. MCF-10A and MCF-10HRas cells were not used beyond passage 40. All other cells were not used beyond passage 20.

1.3 Gel Contraction Assay

PT collagen I gel solutions were prepared by combining 10% (v/v) 10x DMEM, 2.5% (v/v) 1 M HEPES, 2.5% (v/v) 0.89 M $NaHCO_3$, and collagen I stock solution. The solution was neutralized to pH 7.4 with 1 N NaOH. Equal volumes of deionized (DI) water and cell suspension were added to the neutralized solution such that the final collagen concentration was 1 mg/mL and the final concentration of cells was $5x10^5$ cells/mL. 500 µL of the solution was added to 35 mm glass bottom dishes, which were transferred to a 37°C incubator to gel for 1 hour. After gelation, a 2 mL media overlay was added on top of each gel, and the dish was gently tapped to release the gel from the glass surface so that it was floating. Floating gels were allowed to contract for 4 hours. Contraction was documented using digital photography and quantified by measuring the area of the gel before and after contraction. Images were taken at t=0 (just before gel release) and t=4 h. Contractility is expressed as a percent reduction in gel area after the contraction period.

1.4 Collagen Invasion Assay

Spheroids were prepared by plating cells lines in 96-well ultra-low adhesion plates (2000 cells/well) in complete media. Media was supplemented with compaction enhancers BME (0.2575 mg/mL), blebbistatin (5-20 µM), or AS collagen I (10 µg/mL) when appropriate and as specified in the text. Plates were centrifuged at 1000 x g for 10 minutes at 4°C. Spheroids were allowed to form for 24-72 hours. Prior to use, BME-formed spheroids were transferred to a suspension 96-well containing Cell Recovery Solution on ice. Spheroids were left in Cell Recovery Solution to remove BME for 1 hour on ice. Spheroids formed without BME were implanted directly into collagen solutions without Cell Recovery Solution treatment. Collagen gels were prepared similarly to as described in Section 1.3 above: 10% (v/v) 10x DMEM, 2.5% (v/v) 1 M HEPES, 2.5% (v/v) 0.89 M $NaHCO_3$, collagen I stock solution, and a sufficient volume of DI water to dilute the collagen concentration to 1 mg/mL was added. The resulting solution was neutralized to pH 7.4 with 1 N NaOH. The neutralized solution was added in 200 µL portions to 10 mm cloning cylinders glued to 35 mm glass bottom dishes. The inside of the cloning cylinder was lined with a small strip of Nylon mesh to anchor the gel to the sides and prevent gel collapse. Spheroids were transferred to gel solutions in 5 µL volumes. Plates were transferred to a 37°C incubator and allowed to gel for 1 hour. Gels were overlaid with 50 µL of complete media immediately after the gelation period. A transmitted light image was taken at t=1 h for invasive distance analysis to capture the initial size of the spheroids. A second image was taken at t=24 h. Invasive distance was quantified by considering the maximum radial distance traveled by cells beyond the starting diameter of the spheroid.

1.5 Microscopy

Imaging was performed on an inverted confocal laser-scanning microscope (Olympus Fluoview 300, Zeiss LSM 700, or Zeiss LSM 800) in scanning transmittance or confocal fluorescence mode with 10x air (NA 0.4), 20x air (NA 0.75), 60x oil (NA 1.42), or 63x oil (NA 1.4) objectives.

Microscopes were equipped with 488 nm, 532 nm (Olympus), 561 nm (Zeiss), 633 nm (Olympus), and 639 nm (Zeiss) laser lines for excitation of fluorophores. Photomultiplier tubes were used for detection on the Olympus and Zeiss LSM 700, while an Airyscan detection system was used on the Zeiss LSM 800.

Live cell imaging was performed on an inverted confocal laser-scanning microscope (Olympus Fluoview 300) or multi-mode plate reader (Biotek Cytation 5 equipped with BioSpa). All live cell movies were taken at 37°C and 5% CO_2. Shorter-term movies (overnight, Video S1) were taken on the Olympus using a 10x objective, where imaging conditions were maintained using a stage top incubator (Neue Biosciences). Longer-term imaging (>1 day, Video S2, S3) was performed using the Cytation 5 with 4x (NA 0.13, Video S2) and 10x (NA 0.3, Video S3) objectives. For each imaging step, plates were automatically transported from the incubation chamber to the imaging chamber, both of which were maintained at 37°C and 5% CO2 for the duration of the experiment.

1.6 Sorting Experiments

Sorting experiments are described fully in Materials and Methods in the main text.

1.7 Analysis of Sorting Dynamics

To analyze sorting behaviors, a previously described method was employed (Cochet-Escartin *et al.*, 2017). In brief, images obtained as described in Section 1.6 were thresholded, a mask was created for the thresholded image, and speckling and cellular debris in the image were removed by applying a size cutoff using the Analyze Particles function in ImageJ. The thresholded image was subjected to k-means clustering within the pre-defined mask area. This assigned all pixel values in the area to a color corresponding to one cell line or the other. This yielded a final segmented image (**Fig. S4**) from which blob size and boundary ratios could be determined. K-means clustering, boundary ratio, and blob size determinations were performed using custom written scripts in Python and Matlab. Briefly, blob size is the invert of the weighted average of frequencies obtained from a two-dimensional Fourier transform of the image, while boundary ratio is the length of the boundary between the two cell types divided by the total perimeter of the aggregate. Both quantities are normalized and unitless. Blob size is normalized to the initial state of the mixed aggregate, while the boundary length between cell types is normalized to the total perimeter of the aggregate at each stage of sorting.

1.8 Spheroid Compaction and Inhibitory Treatments

To assess compaction, cells were plated in a 96-well ultra-low adhesion plate using the same conditions for the sorting experiments (2000 cells per well, no media supplements). Aggregates were imaged every 12-24 hours. For quantification of aggregate area, transmitted light images were taken at the widest point of each aggregate. Aggregate areas were measured in ImageJ by outlining each aggregate and measuring the area at each timepoint. For BME-supplemented spheroids, BME was added to cells at a final concentration of 0.2575 mg/mL. β_1 integrin (clone 4b4) and E-cadherin (clone SHE-78-7) inhibitory antibodies were added at 10 µg/mL to each well to assess spheroid compaction mechanisms. For modification of actomyosin contractility during spheroid formation, (-)-blebbistatin (1-20 µM), (+)-blebbistatin (20 µM), para-nitroblebbisatin (1-20 µM), para-aminoblebbistatin (20-100 µM), ML-7 (20 µM), or Y-27632 (10 µM) were added to cell culture media in 96-well plates. For calcium depletion, EGTA was added to culture media at a final concentration of 1 mM. For serum starvation, serum concentration in the media was reduced to 0% after plating cells in low attachment plates.

1.9 siRNA Mediated β_1 Integrin Knockdown

Prior to transfection, complete culture medium was removed and replaced with serum free Opti-MEM. siRNA and lipofectamine RNAiMax were mixed in Opti-MEM according to manufacturer instructions, added to cells at a final RNA concentration of 10 nM, and incubated for either 6 (MDA-MB-468) or 16 hours (MDA-MB-231). Media was replaced with DMEM containing 10% FBS after the 6-16 hour initial incubation. After approximately 72 hours, cells were returned to complete growth medium containing antibiotics. Approximate knockout efficiency was assessed by detaching cells 72 hours post-transfection and plating on a glass bottom dish. Cells were allowed to attach for 24 hours and were then fixed and stained for β_1 integrin. Cells were imaged under identical laser power and PMT voltage settings as negative control siRNA transfected cells and a threshold intensity value was set using these negative control cells that was applied to all images. β_1 integrin siRNA treated cells displaying fluorescence after such thresholding were counted as β_1 integrin positive, and cells displaying no fluorescence were counted as β_1 integrin negative. We determined a KO efficiency of 95% and 89% for MDA-MB-231 and MDA-MB-468, respectively.

1.10 Immunocytochemistry

Cells were fixed using either 10% phosphate-buffered formalin or freshly prepared 4% methanol-free formaldehyde. For higher quality imaging of co-culture spheroids, spheroids were implanted in 1 mg/mL collagen and immediately fixed with 10% formalin after gelation. To achieve maximum imaging depth, gels were tissue cleared by soaking in freshly prepared 8 M urea for 4 days prior to imaging. Gels were imaged on a 20x air objective. Alternatively, imaging depth was enhanced by directly mounting spheroids on glass sides with Prolong Glass hard set mounting media after fixation. Spheroids mounted on slides were imaged using a 63x oil objective. For imaging of the actin cytoskeleton, spheroids were fixed with 4% methanol-free formaldehyde, permeabilized with 0.2% Triton-X-100 for 10 minutes, and labeled with AlexaFluor 568 phalloidin (1:100 dilution) overnight at 4°C. For staining of β1 integrin, cells were fixed with 10% formalin, blocked with 1% BSA for 1 hour at room temperature, and incubated with β1 integrin antibody clone 4b4 (1:200 dilution) or β1 integrin antibody clone 12G10 (1:50 dilution) in 1% BSA overnight. Spheroids were incubated with fluorophore labeled secondary antibody (1:500 dilution) in 1% BSA for 3 hours at room temperature. For staining of E-cadherin, spheroids were fixed with 10% formalin, permeabilized with 0.2% Triton-X-100 for 10 minutes, blocked with 1% BSA for 1 hour at room temperature, and incubated with E-cadherin antibody clone 67A4 (1:200 dilution). Incubation with secondary antibody was the same as described above. For staining of phospho-myosin light chain (p-MLC), spheroids were fixed with 4% methanol-free formaldehyde, permeabilized with 0.2% Triton-X-100 for 10 minutes, blocked with 1% BSA for 1 hour at room temperature, and incubated with p-MLC antibody (1:50 dilution). Incubation with secondary antibody was the same as described above. For double-staining of actin and p-MLC, fluorescent phalloidin was added along with the fluorescent secondary antibody. All spheroids stained with antibody/phalloidin were mounted on coverglass with Prolong mounting media, and were imaged with a 63x oil objective (LSM 700 and LSM 800).

1.11 Additional Image Analysis

Analysis of gel contraction, aggregate area, spheroid invasion in monoculture, and cell sorting metrics are described in earlier sections. Beyond this, additional image analysis is performed as described here.

For quantification of cell shape, images of cells in fixed, phalloidin stained spheroids were

manually segmented, and the perimeter, area, and aspect ratios were measured using ImageJ. Cells for quantification were selected only from central slices of the spheroid to ensure accurate identification of surface and interior cells. Cell shape was defined as $\frac{\text{Perimeter}}{\sqrt{\text{Area}}}$.

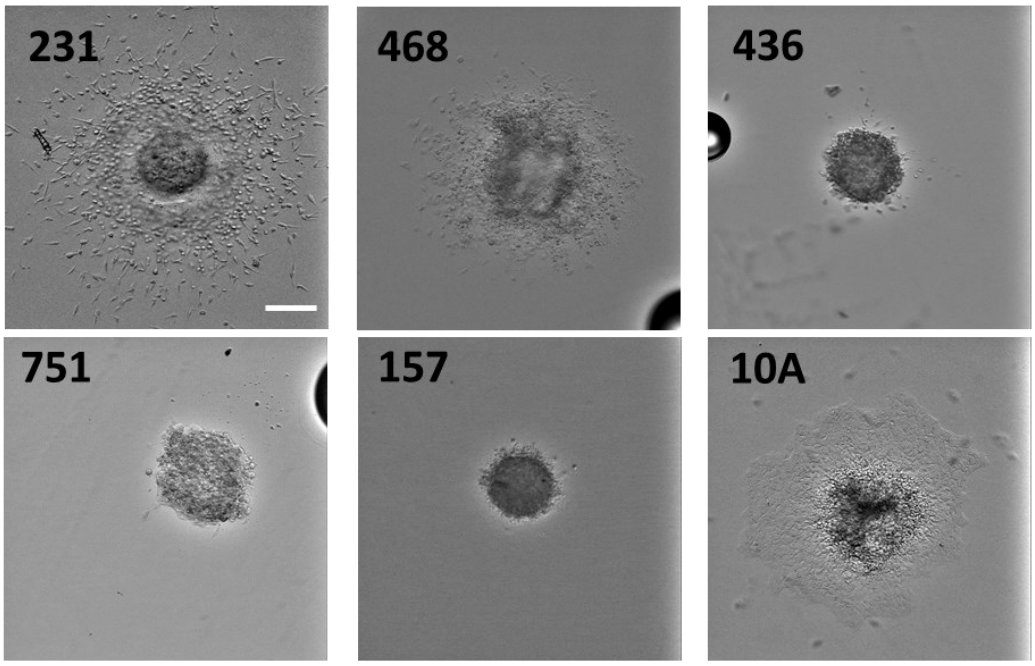
Relative amounts of ligand bound β_1 integrin between spheroids was determined by considering the fluorescence intensity differences between surface and interior cells in fixed spheroids stained with β_1 integrin 12G10 antibody. Cell borders were manually outlined in ImageJ using a line with a width of 2 pixels. The average intensity along this selection was determined for each cell. For interior cells the full border was considered while for exterior cells, only the surface in contact with the medium was considered. Interior/exterior cells were randomly paired and the ratio of fluorescence intensity for interior:exterior for each pair was calculated.

Spheroid invasion in co-culture spheroids was analyzed by thresholding the image and using the Analyze Particles feature in ImageJ to set a minimum size cutoff such that objects smaller than cells (speckling, debris, etc.) would be excluded from analysis. The number of features found beyond the center spheroid mass was counted and the x,y coordinates of each were recorded in ImageJ. The distances each recorded cell traveled relative to the center of the spheroid were calculated.

1.12 Statistical Analysis

All statistical tests were run in R. Statistical significance is indicated by *p<0.05, **p<0.01, and ***p<0.001. Lack of statistical significance is denoted by “ns”, indicating not significant. Descriptors of sorting dynamics were tested for normality using the Shapiro-Wilk test. Depending on the result of normality testing, statistical significance was determined between sorting metrics through either a t test or non-parametric equivalent Mann-Whitney test. Cell shape distributions in spheroids under different pharmacological treatments were compared using the Kolmogorov–Smirnov test. Spheroid invasion data was tested for normality with the Shapiro-Wilk test. Student’s t tests or Mann-Whitney tests were used to test for significance depending on the result of normality testing.

a



b

Cell Line	MDA-MB-231	MDA-MB-468	MDA-MB-436	ZR-75-1	MDA-MB-157	MCF-10A
Contraction (%)	49 ± 4	26 ± 9	11 ± 3	29 ± 3	54 ± 10	13 ± 2

Figure S1. Invasive capacity and contractile capacity of cell lines. (a) Representative transmitted light images of each cell line in collagen I (1 mg/ml) 24 hours post-implantation. Scale bar = 200 μm. MDA-MB-231 and MDA-MB-468 show robust invasion while the other cell lines do not. MCF-10A does exhibit an epithelial spreading pattern that is distinct from that of cancer cell lines and is not considered invasive. Cell lines are identified by shortened forms, which are the last 3 alpha numeric digits of the full cell line name. (b) Cellular contractility for each cell type as determined by gel contraction in collagen I (1 mg/mL) gels over 4 hours. Contraction is expressed as a percent which is calculated by comparing the initial area to the final area of the gel. Gel contraction is expressed as mean ± standard deviation. n = 6 for each cell line.

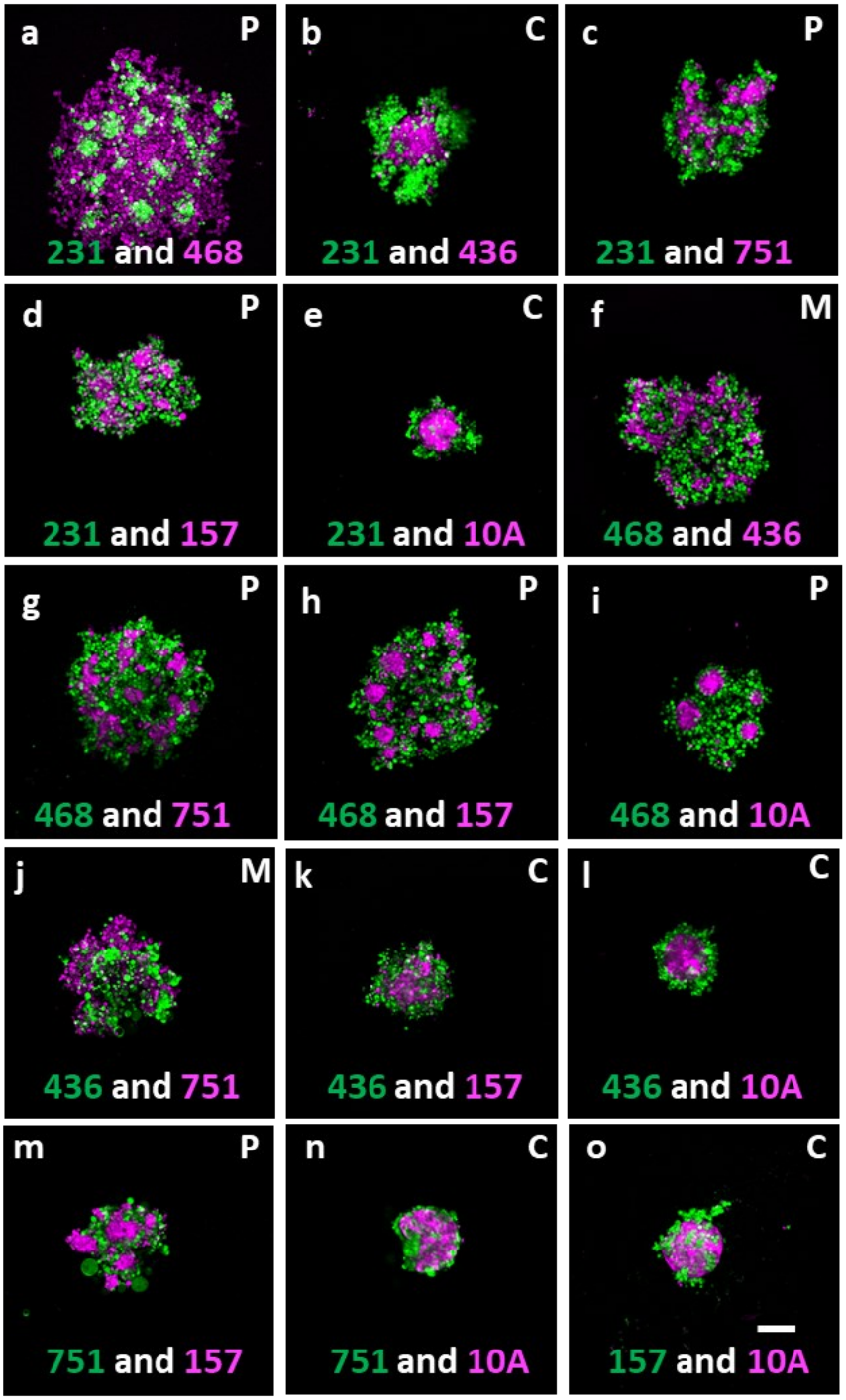


Figure S2. (a-o) Representative confocal fluorescence images of all final sorted states for each cell line combination. Scale bar = 200 μm. For each combination, cell sorting is followed for up to 72 hours. The images shown are the final sorted state, which may occur at (e,i,l) 24, (b,d,f,h,j,k,m,n,o) 48, or (a,c,g) 72 hours depending on the combination. Cell lines are identified by shortened forms, as in Fig. S1. C, P, and M indicate sorting categorized as C (complete sorting), P (partial sorting), or M (no sorting; mixed).

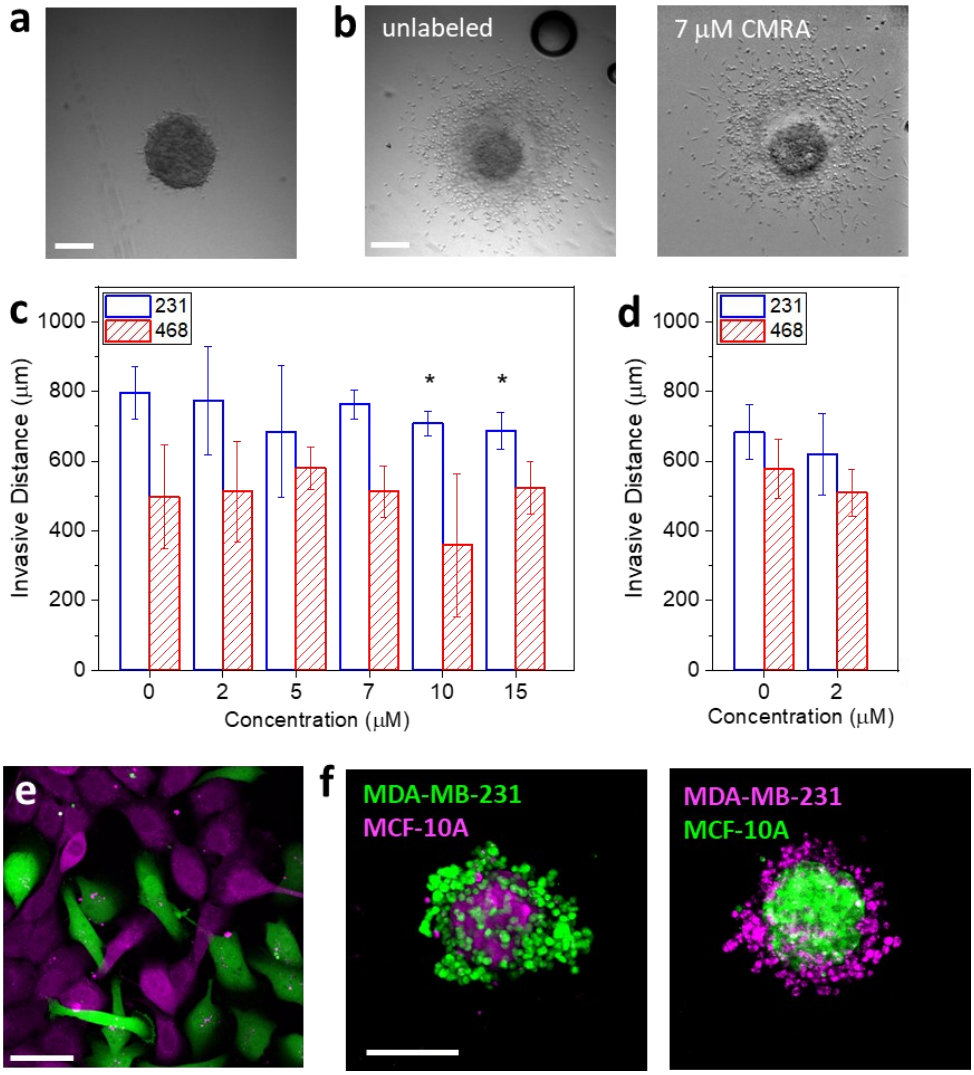


Figure S3. (a) Representative transmitted light image of an unlabeled MDA-MB-231 spheroid 1 hour post-implantation in a 1 mg/ml collagen gel. Scale bar = 200 μm. (b) Representative transmitted light images of (left) unlabeled and (right) labeled with CellTracker CMRA MDA-MB-231 spheroids after 24 hours in the gel. (c,d) Invasive distance of MDA-MB-231 and MDA-MB-468 spheroids over 24 hours as a function of CellTracker (c) CMRA and (d) CMFDA dye concentration. Significant effects on invasion were observed at concentrations of 10 and 15 μM for CMRA in MDA-MBA-231 cells. No other significant differences from control were found. (* indicates $p<0.05$; unlabeled bars indicate no significant change relative to control; n=6, errors bars are standard deviations). Final dye concentrations used during sorting experiments for CellTracker CMRA and CMFDA were 7 μM and 2 μM, respectively. (d) MDA-MB-231 (green) and MCF-10A (magenta) cells labeled with 2 μM CellTracker CMFDA and 7 μM CMRA, respectively, plated on glass bottom dishes, allowed to adhere, and imaged after 48 hours. Dye retention was high and minimal transfer between cells was evident. Scale bar = 50 μm. (e) Sorting experiments with MDA-MB-231 and MCF-10A labeled with CellTracker CMFDA (green) and CMRA (magenta). The same sorting, cell and general aggregate morphology are seen regardless of which cell line is labeled with which dye. Scale bar = 200 μm.

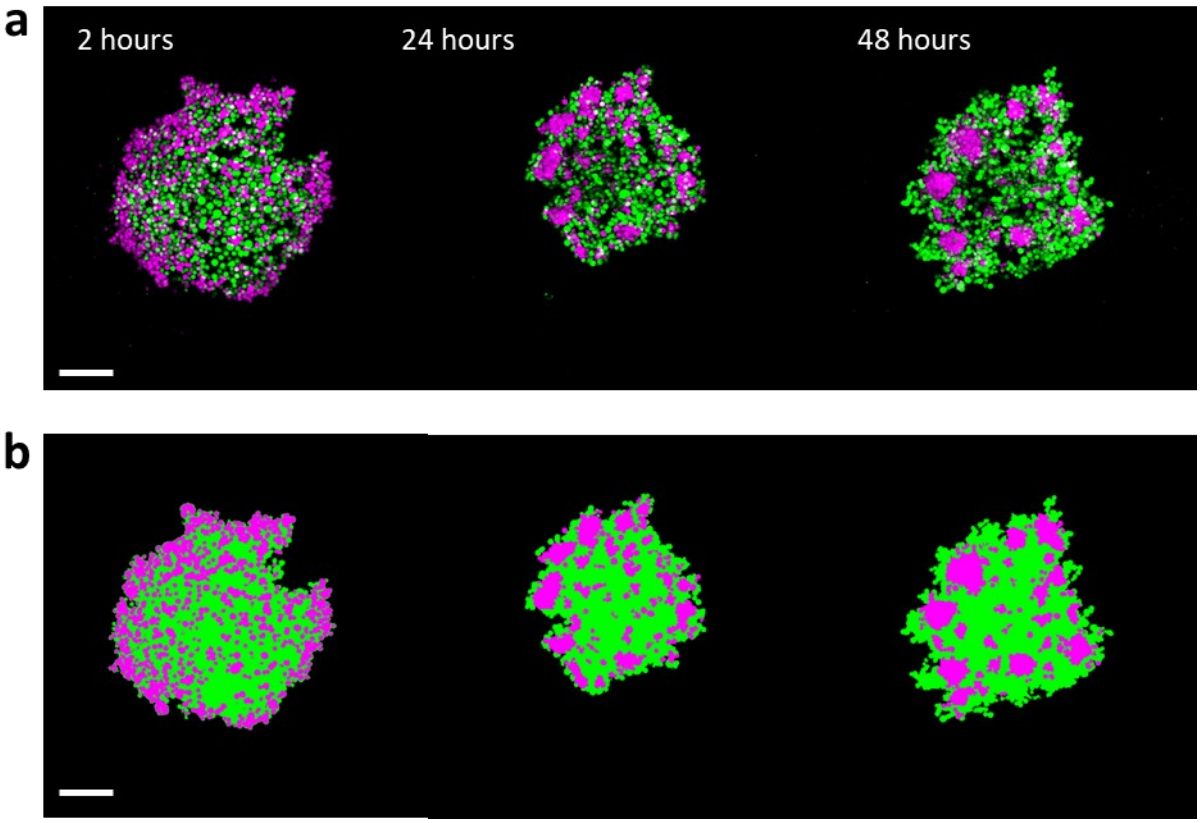


Figure S4. Depiction of the image segmentation process. (a) Raw images taken throughout the sorting process of (green) MDA-MB-468 and (magenta) MDA-MB-157 cells. (b) Post-processed segmented binarized images used to determine blob size and boundary ratio. Scale bar = 200 μm.

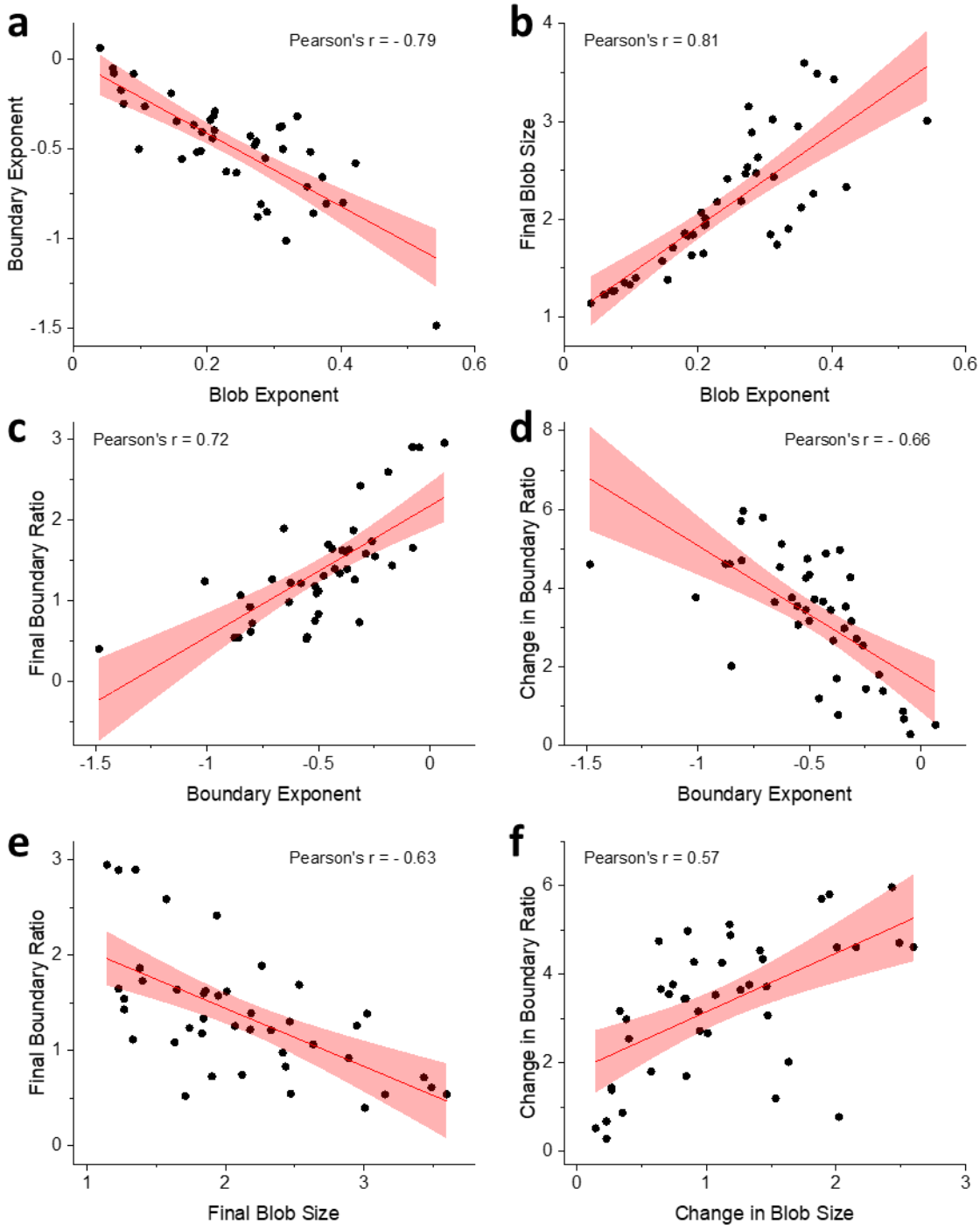


Figure S5. Correlations between sorting descriptors. Moderately strong correlations are shown (as indicated by Pearson's r correlation coefficients) between (a) fitted exponents, which describe the sorting rate, (b-d) exponents and blob sizes/boundary ratios, which describe the rate and degree of sorting, respectively, and (e-f) blob sizes/boundary ratios, which describe the degree of sorting. Red shaded areas indicate 95% confidence intervals for the regression line.

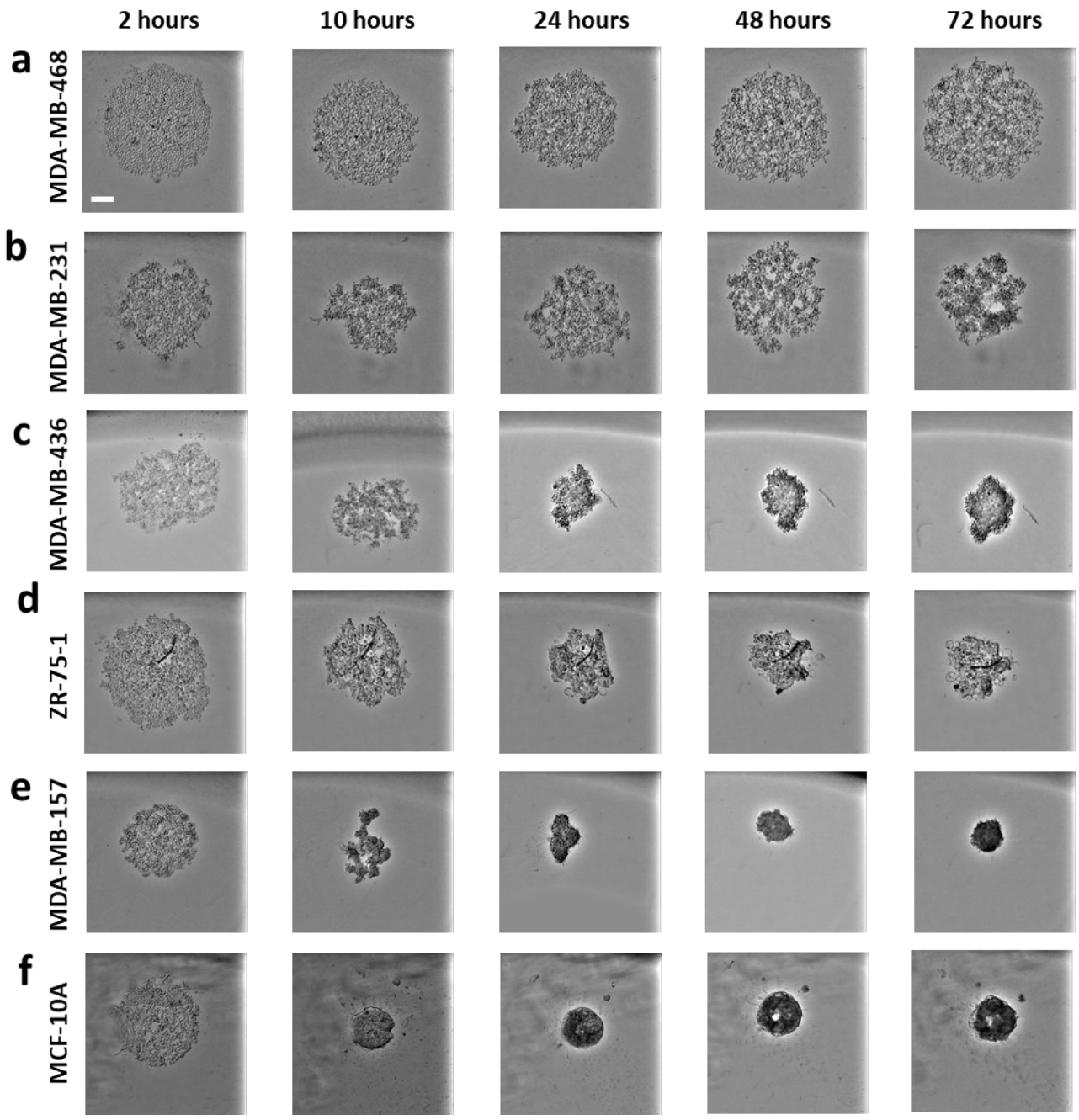


Figure S6. Representative transmitted light images of each cell line during aggregate formation in suspension, with data shown at 2, 10, 24, 48, and 72 hours. A subset of these images is also shown in Fig. 2 in the main text. Scale bar = 200 μm.

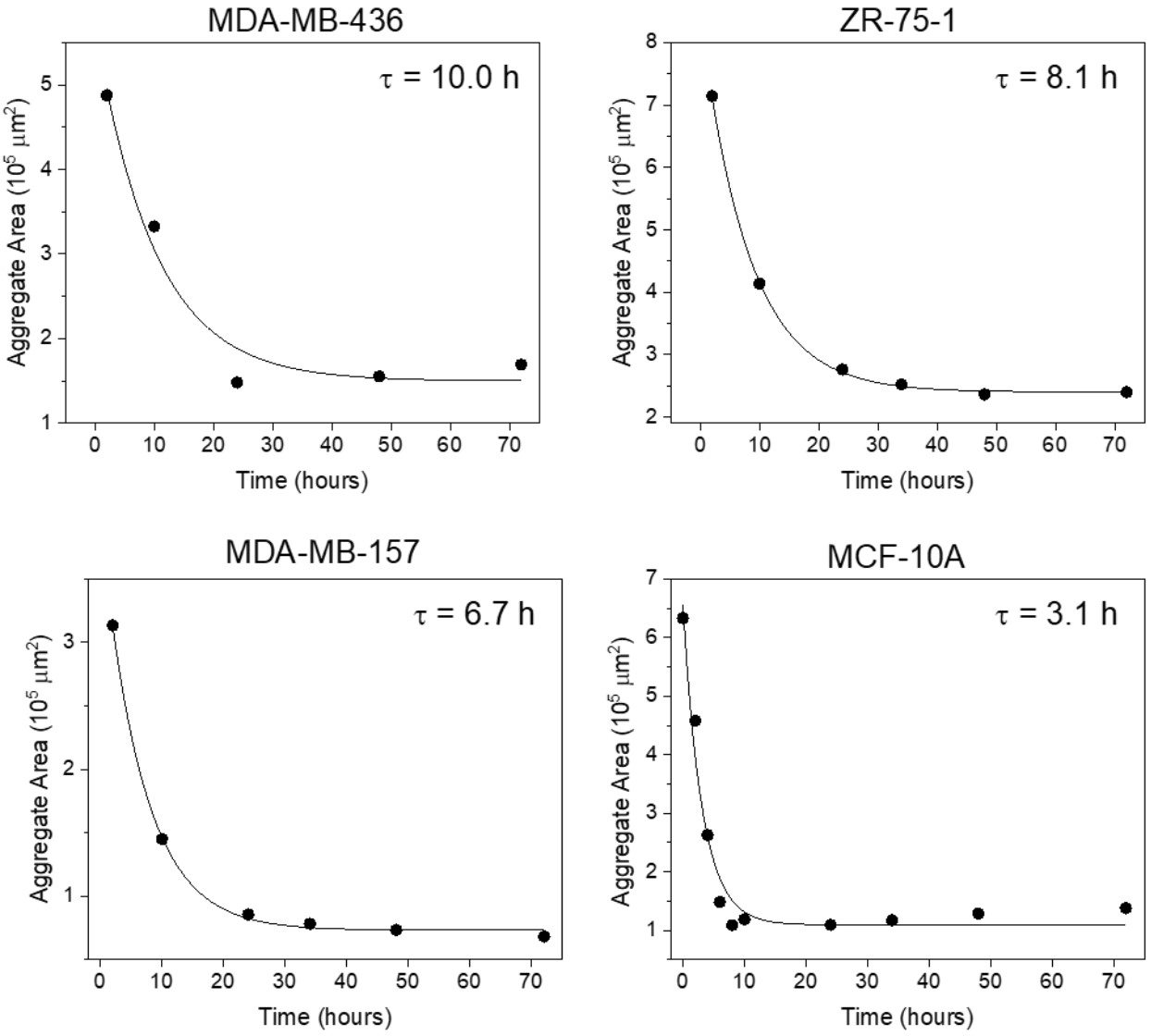


Figure S7. Aggregate area (shown also in Fig. 2 of the main text) and fits of aggregate area to $A^*e^{-t/\tau} + c$ for the four cell lines whose aggregation is well-described by that functional form. Characteristic time for compaction, τ, is reported in Fig. 2 of the main text.

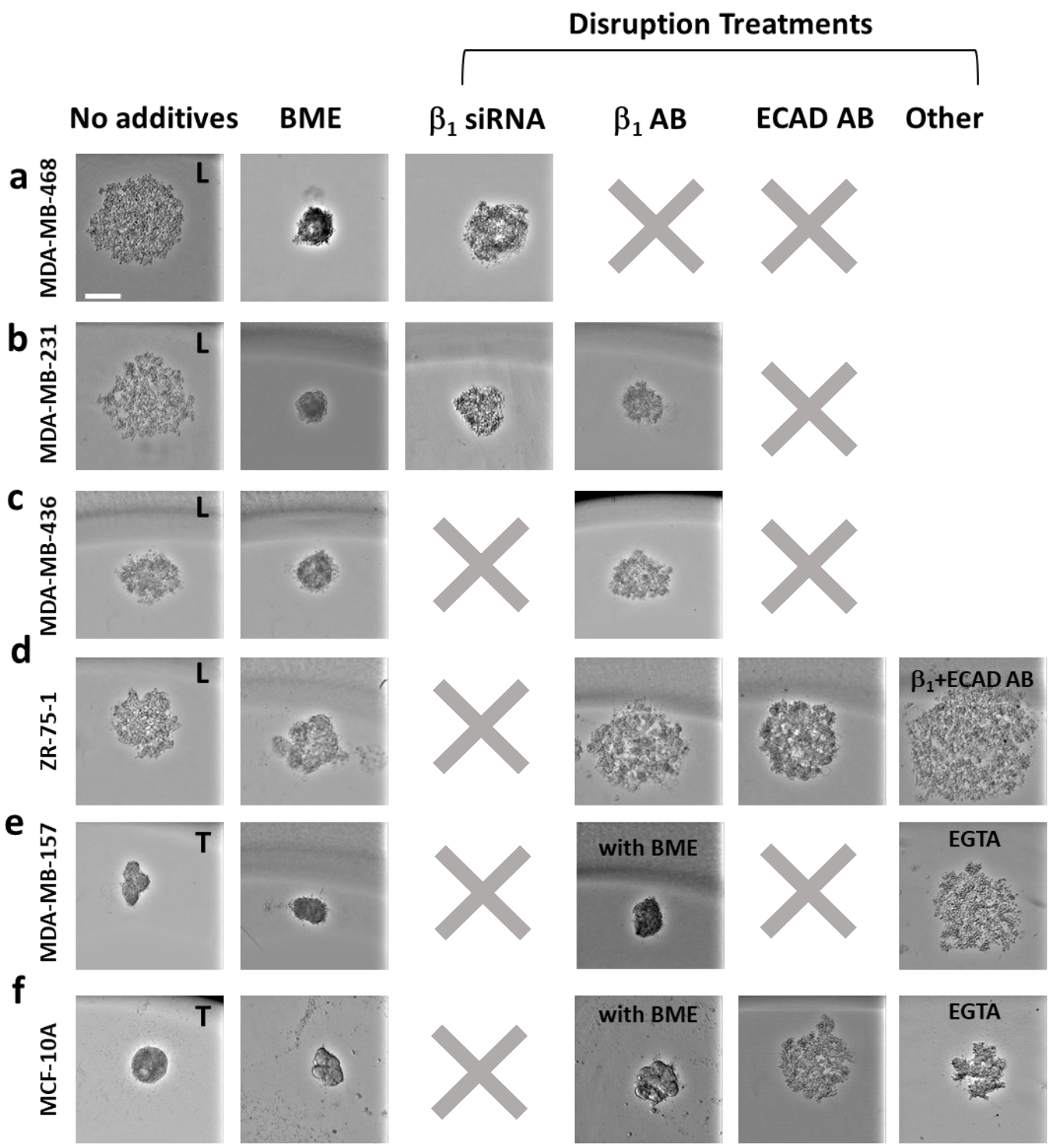

Disruption Treatments
No additives
BME
β1 siRNA
β1 AB
ECAD AB
Other
a
MDA-MB-468
L
b
MDA-MB-231
L
c
MDA-MB-436
L
d
ZR-75-1
L
β1+ECAD AB
e
MDA-MB-157
T
with BME
EGTA
f
MCF-10A
T
with BME
EGTA

Figure S8. Representative transmitted light images demonstrating spheroid compaction mechanisms. (first column) Aggregates formed in suspension culture for 24 hours, which are used to identify loose and tight aggregate formers, as identified by L and T, designations respectively. (second column) Aggregates formed with the addition of 0.2575 mg/ml BME for 24 hours to facilitate compaction in cell lines that do not otherwise tightly aggregate. (remaining columns) Treatments that disrupted aggregate formation in each cell line. Treatments included β1 integrin inhibition (via $\beta_1$integrin siRNA KO [labeled β_1 siRNA] or 4b4 antibody [β_1 AB]), cadherin inhibition (via ECAD inhibitory antibody SHE-787 [ECAD AB] or Ca^{2+} depletion with 1 mM EGTA [EGTA]). Disruption treatments with cell lines that utilize β_1 integrin (231, 468, 436, 751) were performed in the presence of BME, while cadherin inhibition is done without additional media supplementation (157,10A). For 157 and 10A, β_1 integrin antibody inhibition with BME supplementation [with BME] was also performed to confirm cadherin dependence. Inhibitory antibodies were used at 10 μg/mL and 10 nM siRNA was used for KO. All images shown are at 24 hours. Large gray Xs indicate experiments performed with no notable change in spheroid size or shape relative to control. Scale bar = 200 μm and applies to all images.

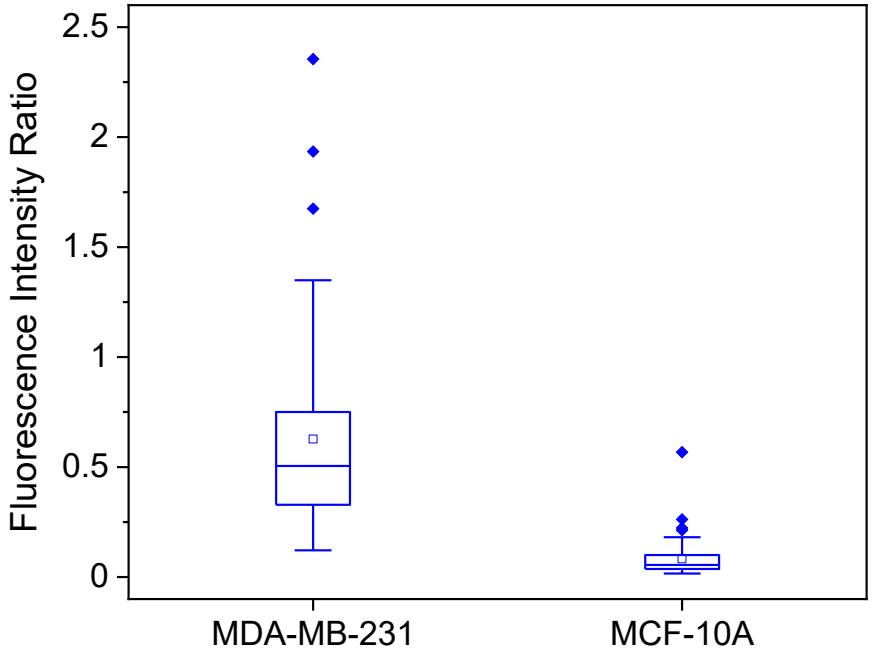


Figure S9. Ratio of fluorescence intensity for active integrin at cell-cell interfaces compared to cell-media interfaces in spheroids such as those shown in images **Fig. 3b** and **3c** in the main text. n=60 cells, measured over several spheroids. Bars represent the interquartile range (IQR), whiskers represent 1.5*IQR range, and outliers (blue diamonds) are points that fall beyond the first/third quartile -/+ 1.5*IQR. Median is represented by a line and mean by a square.

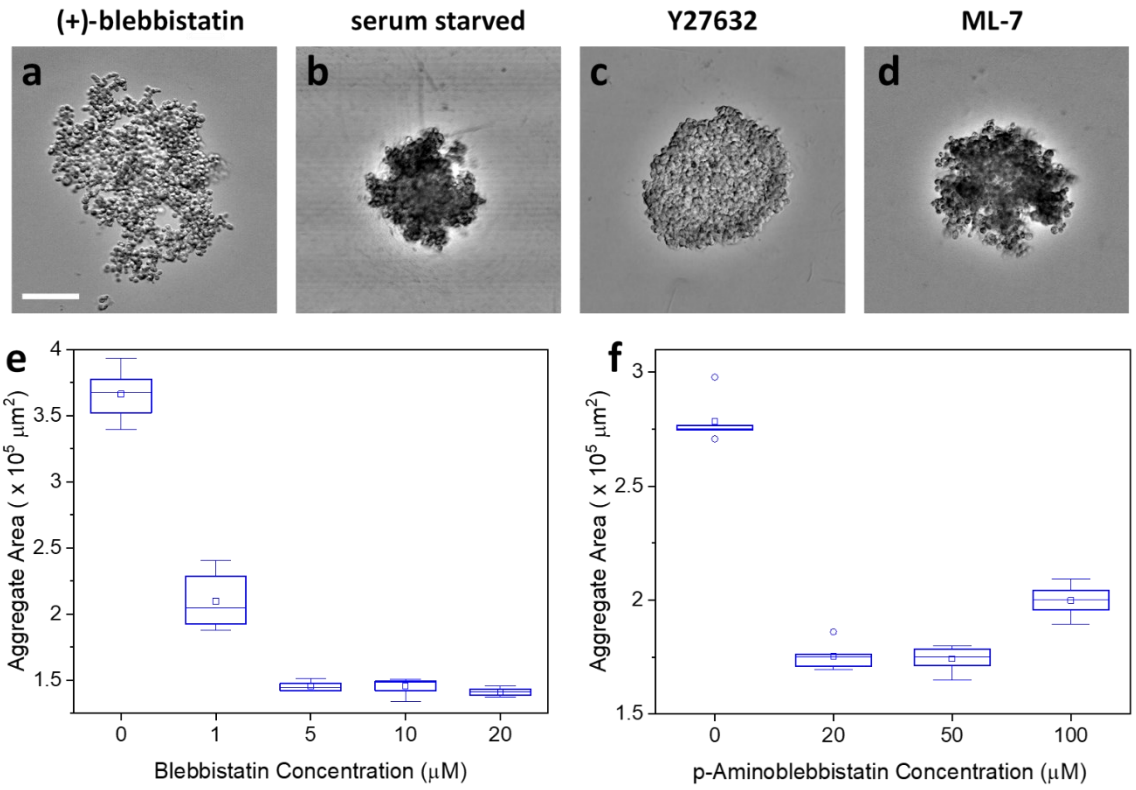


Figure S10. (a-d) Representative transmittance images of MDA-MB-231 cells (a) treated with 20 μM (+)-blebbistatin, (b) serum starved, (c) treated with 10 μM Y-27632, or (d) 20 μM ML-7. All images are taken after 24 hours of treatment except (d), where 48 hours was required to observe an appreciable effect. Scale bar = 200 μm. (e-f) Area of MDA-MB-231 cells treated over a range of (e) blebbistatin or (f) para-aminoblebbistatin concentration over 24 hours. The filled in bars represent the interquartile range (IQR), whiskers represent 1.5*IQR, outliers are points that fall beyond first/third quartile -/+ 1.5*IQR.

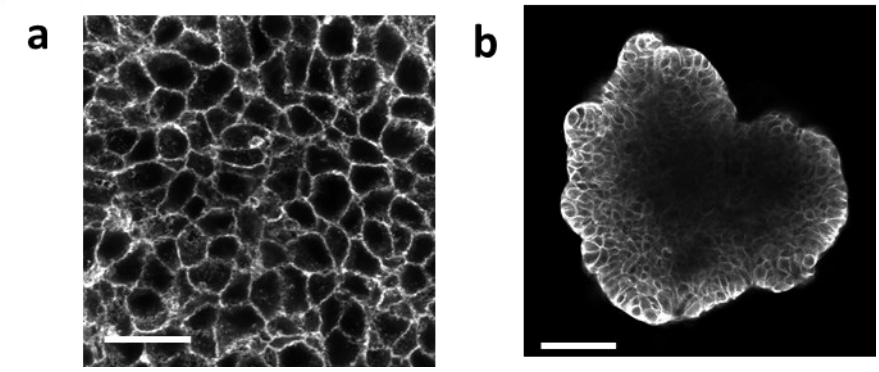


Figure S11. (a) Representative interior cells in a MCF-10A spheroid immunostained for actin. (b) MCF-10A spheroid formed with BME supplementation and immunostained for actin. Scale bars are (a) 20 μm and (b) 100 μm.

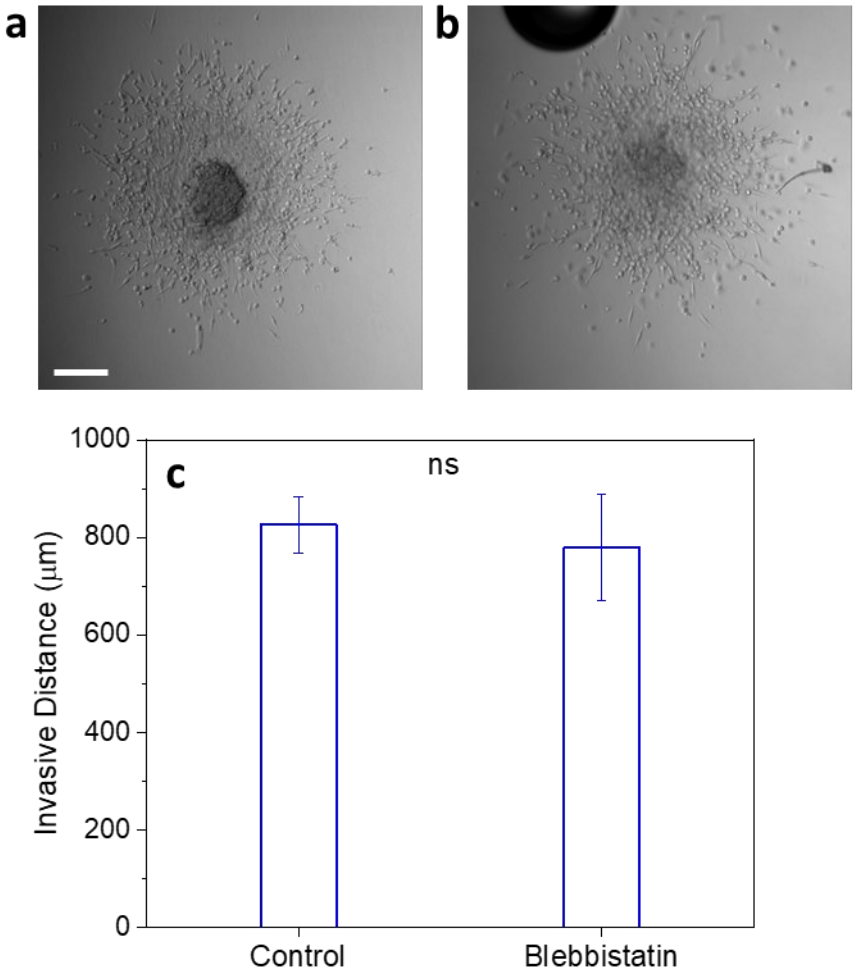


Figure S12. Representative MDA-MB-231 spheroids formed with (a) BME or (b) 20 µM blebbistatin and implanted in 1 mg/mL collagen I and allowed to invade for 24 hrs. (c) Invasive distance for MDA-MB-231 spheroids formed with BME or blebbistatin and assessed after 24 hours. No significant difference was found. (n=11, error bars show standard deviation; t test, ns=not significant). Scale bar = 200 µm.

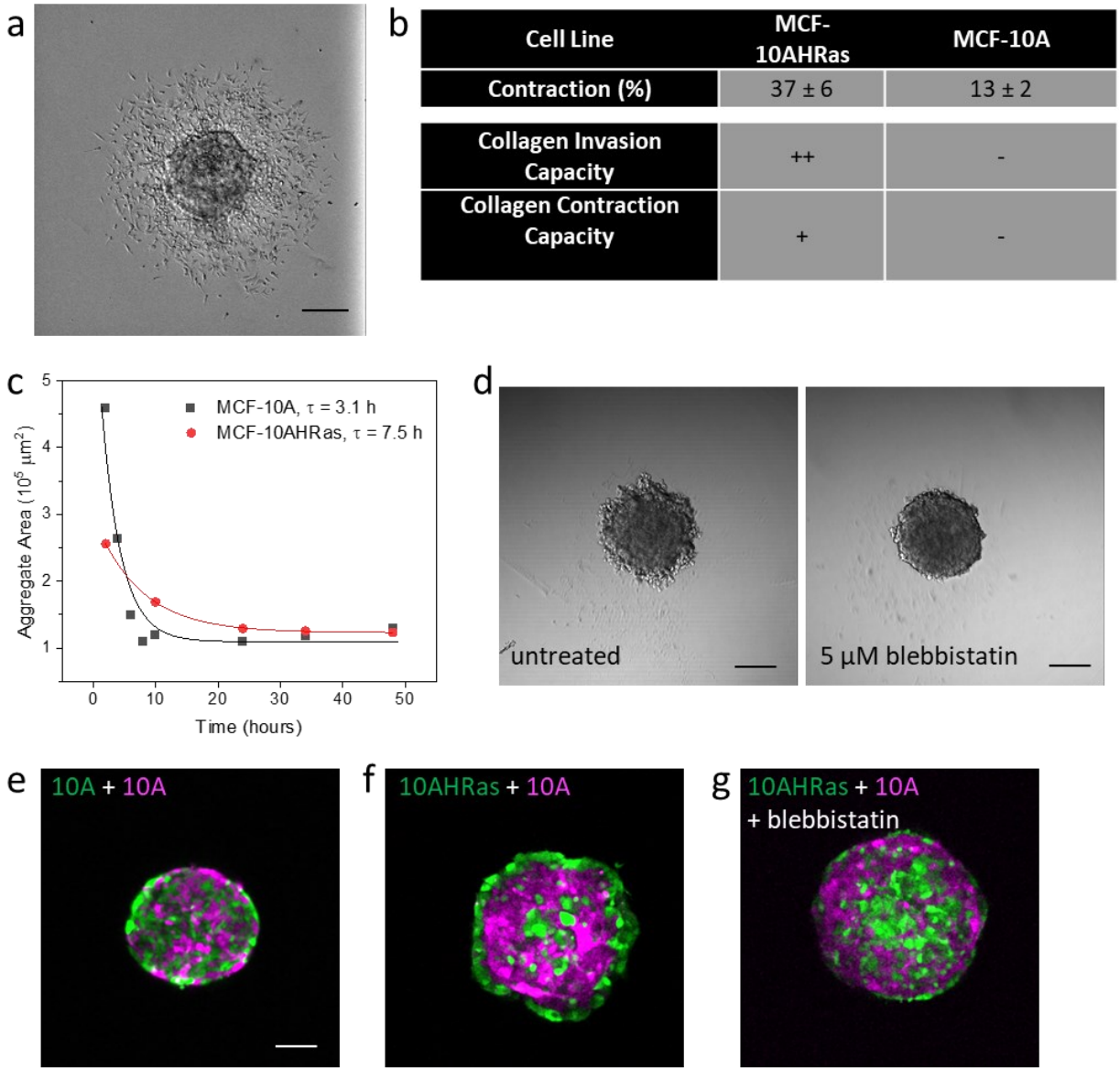


Figure S13. MCF-10AHRas characteristics and sorting behaviors. (a) Representative transmitted light image of MCF-10AHRas monoculture spheroid invasion in collagen I (1 mg/mL) at 24 hours post-implantation. Scale bar is 200 μm. (b) Cellular contractility for MCF-10HRas cells and comparison to MCF-10A cells as determined by gel contraction in collagen I (1 mg/mL) gels over 4 hours (as shown in Fig. S1 for other cell lines, n = 6 for each cell type). Measures of collagen invasion capacity and contractility for MCF-10HRas in comparison with MCF-10A, as reported for other cell lines in Table 1 are also presented. (c) Aggregate area (shown also in Fig. 2 of the main text for MCF-10A) and fits of aggregate area to $A^*e^{-t/\tau} + c$ (as described in Fig. 2 and S7) for MCF-10A and MCF-10AHRas cells. (d) Representative transmitted light images of MCF-10AHRas aggregate formation at 48 hours (right) with and (left) without 5 μM blebbistatin supplementation during the formation period. Scale bars are 200 μm. (e-g) Sorting patterns observed in MCF-10A and MCF-10AHRas mixed spheroids under different formation conditions. Spheroids composed of a 50:50 mixture of (d) identical MCF-10A cells labeled with different dyes, (e) MCF-10AHRas (green) and MCF-10A (magenta), and (f) MCF-10AHRas and MCF-10A treated with 5 μM blebbistatin during formation. Scale bar is 100 μm and the same in all panels.

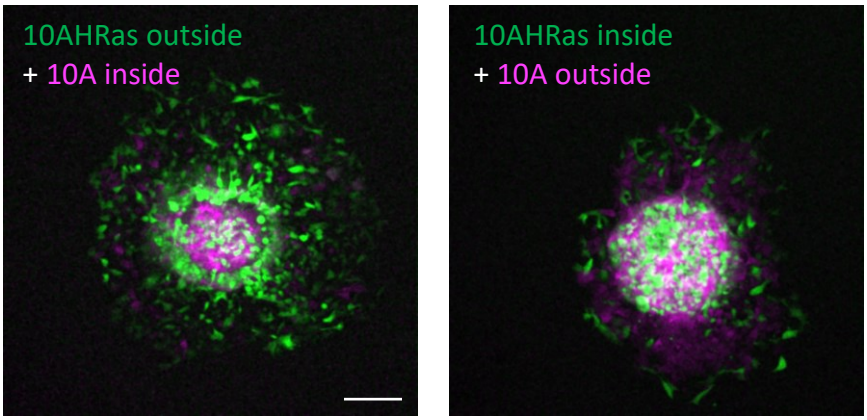


Figure S14. Confocal fluorescence maximum projections of MCF-10AHRas (green) and MCF-10A (magenta) mixed spheroid invasion in 4 mg/ml collagen gels 24 hours after implantation. (left) Representative image of invasion of an untreated mixed spheroid, starting from a configuration with MCF-10AHRas cells outside. (right) Representative image of invasion of a mixed spheroid prepared with 5 μm blebbistatin, starting from a configuration with MCF-10AHRas cells inside. Scale bar is 200 μm and the same in both panels.

Supporting Movie Captions

Movie S1 (separate file). MDA-MB-231 (green) and MCF-10A (magenta) co-culture spheroid formed in a low adhesion plate. Images are taken every 10 min for 20 hr.

Movie S2 (separate file). (left) Untreated MDA-MB-231 cells allowed to aggregate and imaged every 30 minutes for 30 hours. (right) MDA-MB-231 cells treated with 5 μM para-nitroblebbistatin and imaged under the same conditions. Para-nitroblebbistatin was used in place of blebbistatin in live-cell imaging due to poor photostability of and background fluorescence contributed by conventional blebbistatin.

Movie S3 (separate file). MDA-MB-231 (magenta) and MDA-MCF-157 (green) co-culture spheroid formed with BME for 24 hours, treated with cell recovery solution for 1 hour, and subsequently treated with 5 μM para-nitroblebbistatin. Images are taken every 6 hrs for 120 hrs. Para-nitroblebbistatin was used in place of blebbistatin in live-cell imaging due to poor photostability of and background fluorescence contributed by conventional blebbistatin.

Supporting References

Cochet-Escartin, O, Locke, TT, Shi, WH, Steele, RE, and Collins, EMS (2017). Physical Mechanisms Driving Cell Sorting in Hydra. **Biophys J** *113*, 2827–2841.

Kuroda, Y, Wakao, S, Kitada, M, Murakami, T, Nojima, M, and Dezawa, M (2013). Isolation, culture and evaluation of multilineage-differentiating stress-enduring (Muse) cells. **Nat Protoc** *8*, 1391–1415.