INVENTORY OF SUPPLEMENTAL INFORMATION

Collective Invasion in Breast Cancer Requires a Conserved Basal Epithelial Program

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Figure S1 (related to Figure 1). Leader Cells are Molecularly Distinct from Normal Differentiated Myoepithelial Cells and Co-express Luminal Epithelial Markers

(A-C) Leader cells from MMTV-PyMT tumor organoids stained with K14 and K8 (A), K8, p63, and phalloidin (B), or E-cadherin (Ecad) and phalloidin (C). Arrow in (A), a K14+K8+ cell. Arrow in (B), a K8+p63+ cell. 75% of leaders were K14+K8+ (n=21/28) and 34% of leaders were K8+p63+ (n=22/65). Blue arrows in (C), E-cadherin staining at cell-cell contacts between leader and follower cells.

(D) Leader cells from MMTV-PyMT tumor organoids were stained with K14, SMA, and phalloidin (left), K14, MYH11, and phalloidin (middle), and K14, CNN1, and phalloidin (right).

(E) mRNA gene expression of core basal/myoepithelial genes between normal and tumor organoids grown in collagen I matrix for 4 days. Core genes were divided into basal epithelial genes and those specific to the myoepithelial contractility program. Data presented as mean ± sd. n=3 normal and tumor organoids. ** P-value < 0.01, * P-value < 0.05; P-values determined by two-sided t-test.

(F) Schema of oxytocin contractility experiment. Normal mammary organoids and MMTV-PyMT tumor organoids were imaged by DIC microscopy and treated with oxytocin at nM concentrations. Maximal contraction was observed at 25 min post incubation.

(G) The reduction in organoid area calculated in (F) provides a proxy for oxytocin-dependent contractility. Organoids were incubated in oxytocin at the specified doses, and the change in area was determined. Data are presented as box plots. N=23-30 organoids per condition from 3 independent experiments. * P-value < 0.005, ** P-value < 1 x 10^-4; P-values determined by two-sided t-test.

(H) Leader cells from MMTV-PyMT tumor organoids were stained with K14, Twist, and DAPI (left), K14, Slug, and DAPI (middle), or E-cadherin (Ecad), Vimentin (Vim), and phalloidin (right).

The scale bars represent 50 μm in (F), 20 μm in (A-C), and 10 μm in (D,H).

Figure S2 (related to Figure 2). K14+ Cells are Enriched at the Tumor Invasive Border and in Lung Metastases

(A) Area of tumor invasion into muscle, stained with K14, K8, and phalloidin. Insert highlights a group of K14+K8+ cells. Arrow, protrusive tip of a K14+K8+ cell; M, F-actin+ muscle fibers.

(B-C) Micrographs of reconstructed metastatic lung lesions stained with K14, SMA, and phalloidin. Both K14+ micrometastatic (B) and large lesions (C) are negative for SMA.

(D) MMTV-PyMT mammary tumor that express GFP driven by the K14 promoter were dissociated and counted by FACS. Representative FACS plot (n=3 independent experiments).

(E) Schema to determine the enrichment for K14+ cells at the tumor-stromal border. mTomato+ tumor organoids were transplanted orthotopically into non-fluorescent congenic hosts. Transplanted tumors > 1cm were harvested to generate montages of the tumor-stromal border.

(F) Micrographs of a representative mTomato+ tumor-stromal border stained with K14 and phalloidin. (S), a tumor-stromal interface at the outside edge of the tumor. (IS), an “internal” region of tumor revealed by mTomato fluorescence to be a tumor-stromal interface.

(G) Schema to quantify the frequency of K14+ cells relative to distance from the tumor-stromal interface. A Euclidian distance map was calculated, such that the distance to the nearest stromal pixel (defined as an mT-, F-actin+ pixel) was determined for each K14+ and K14- pixel. N=5 reconstructions from 4 tumors.

(H) Frequency plot of the percentage of K14+ and K14- cells that are located as a function of distance from the tumor-stromal border, calculated from (G). Cell counts were pooled into 50 μm bins.

The scale bars represent 20 μm in (A-C) and 500 μm in (F).

Figure S3 (related to Figure 3). K14+ Invasion in Primary Mouse Mammary Carcinomas and Human Breast Tumors

(A) Micrograph of a leader cell from a MMTV-Neu tumor organoid stained with K14 and K8.

(B) Micrograph of leader cells from a C3(1)/Tag tumor organoid stained with K14 and K8.

(C-D) Micrographs of leader cells in primary human breast tumor organoids stained with K14, p63 and DAPI (C), or K14, K18, and K8 (D). In (D), star, a K14+ leader cell; vertical bracket, K14+K8+K18+ cells just proximal to the leader cell.

(E) Frequency of leader cells expressing K14 or SMA in human breast tumor organoids. 95% confidence intervals for each proportion denoted in parentheses.

(F) Micrographs of K14+ cells in vivo from primary human samples (S1, S3, and S4). Insets demonstrate collective strands of individually protrusive K14+ cells.

The scale bar represents 20 μm in (A-D, and F: middle and right panels) and 100 μm in (F: left panel).

Figure S4 (related to Figure 3). K14 is Expressed in Primary Human Breast Cancers In Vivo

(A) Representative micrographs of low (+), medium (++), or high(++) K14 staining intensity among grade 3 luminal B human breast tumors.

(B) Representative micrographs of the distinct K14 immunohistochemical staining patterns observed in primary human breast specimens. Insets, collective invasion fronts.

(C) The frequency of cases with cytoskeletal or cytoplasmic only K14 staining patterns stratified by IHC-defined breast cancer subtype.

(D-G) Representative micrographs of K14+ collective invasion fronts from archival specimens representing the major subtypes of breast cancer. Red bar, leader cells.

Scale bars in (A-B,D-G) represent 100 μm.

Figure S5 (related to Figure 4). K14+ Leaders Originate from Luminal Tumor Cells

(A) K14 intensity was quantified into 0 (no or few), 1 (intermediate), 2 (bright) K14 signal. N=920 orgs, from 5 to 8 mice per day.

(B) K8 intensity was quantified into 0 (no or few), 1 (intermediate), 2 (bright) K8 signal. N=269 orgs, from 3 mice per day.

(C) Predicted probability function of invasion as a function of K14 intensity and day in culture. Logistic regression was conducted fitting the dependent variable invasion to the product of independent variables [K14 intensity] x [day in culture]. K14 intensity and day in culture contribute to the model with P-values < 0.05.

(D) Micrographs of mammary tumor organoids grown in 3D collagen matrix, stained with K14, pH3, and DAPI.

(E) The number of mitoses (pH3+) per tumor organoid (Org) as a function of time. Data presented as boxplots. $n \geq 31$ orgs per time-point. *** P-value < 0.0001. * P-value < 0.05. P-value determined by two-sided t-test. N=137 orgs in total, from 2 mice per condition.

(F) Time-lapse microscopy of tumor organoids derived from MMTV-PyMT; K14-GFP mice at day 0 post plating (en face view). Colored arrowheads, luminal tumor cells initially GFP- which become GFP+. See Movie S4.

(G) Micrographs of human tumor organoids (sample S4) grown in 3D collagen matrix, stained with K14, K8 and phalloidin.

(H) Micrographs of human tumor organoids (sample S1) grown in 3D collagen matrix, stained with p63, DAPI and phalloidin.

(I) Bar graphs of K14 intensity in human tumor organoids versus day in culture for human tumor organoids (for samples S1,S3,S4). N=44-78 orgs per human tumor. K14 intensity was quantified into 0 (no or few), 1 (intermediate), 2 (bright) K14 signal.

Scale bars are 40 μm in (D), 20 μm in (G-H), and 10 μm in (F).

Figure S6 (related to Figure 5). K14+ Cells Acquire Leader Cell Behaviors Specifically in Collagen I Rich Local Microenvironments

(A) Schema of the 3D Matrigel organoid assay. Tumor organoids are embedded in 3D Matrigel.

(B) Time-lapse DIC microscopy of an embedded MMTV-PyMT mouse mammary tumor organoid. The tumor organoid does not initiate protrusive collective invasion into Matrigel.

(C) Invasion was quantified by scoring protrusive morphology of cancer cells in contact with the ECM. Data presented as mean ± sd. N=431 organoids, 3-4 mice. P-value determined by two-sided t-test.

(D and D') Micrographs of normal mammary organoids and mammary tumor organoids cultured in Matrigel and stained with K14, SMA and phalloidin.

(E) Time-lapse sequence of K14-GFP; MMTV-PyMT tumor organoid embedded in 3D Matrigel. Arrows, single GFP negative cell that becomes GFP positive. See Movie S5.

(F) Schematic of matrix switching experiment. MMTV-PyMT mammary tumor was digested into tumor organoids and embedded in 3D Matrigel. Following K14+ conversion, tumor organoids were freed and re-embedded in 3D collagen I.

(G) Time-lapse microscopy of MMTV-PyMT; K14-GFP tumor organoid preconditioned in Matrigel and then re-embedded in collagen I. Arrowheads, K14-GFP+ cells that switch to protrusive morphology and lead collectively invading strands of cells. See Movie S6.

Scale bars represent 50 μm in (B) and 20 μm in (D,E,G).

Figure S7 (related to Figure 6). Basal Epithelial Genes K14 and p63 Are Required for Collective Invasion in 3D Culture

(A) Western blotting for K14 expression was conducted on total cell lysates from MMTV-PyMT tumor organoids transduced with lentiviral particles encoding shRNAs against luciferase control (labeled shL) or one of five unique K14 constructs (labeled A-E).

(B) Western blotting for p63 expression was conducted on total cell lysates from MMTV-PyMT tumor organoids transduced with lentiviral particles encoding shRNAs against luciferase control (labeled shL) or one of five unique p63 constructs (labeled A-E).

(C) The number of protrusive cells in Luc shRNA, K14 shRNA, and p63 shRNA transduced organoids presented as boxplots. The number of protrusive cells was determined from fixed collagen I embedded organoids. N=54-122 organoids from 3 independent experiments. All comparisons against Luc shRNA yielded P-values < 1x10^-5. P-values determined by 2-sided t-test.

(D) Micrographs of MMTV-PyMT tumor organoids transduced with Luc shRNA and p63 shRNA stained with p63, DAPI, and phalloidin.

Scale bar represents 20 μm in (D).

SUPPLEMENTAL MOVIE LEGENDS

Movie S1 (related to Figure 1). This movie shows a time-lapse sequence of a representative MMTV-PyMT mouse mammary tumor organoid embedded in 3D collagen I. Collectively migrating cells emerge from the primary tumor organoid. Protrusive leader cells are readily identified at the front of these invasive strands. Scale bar represents 50 μm.

Movie S2 (related to Figure 2). 3D projection of collectively organized K14+ leaders invading into muscle in vivo. K14+ cells direct strands of cells along different planes and are in close proximity to an SMA+K14- structure, likely vascular. K14 denoted in green, SMA in red, and phalloidin in blue. Scale bar represents 20 μm.

Movie S3 (related to Figure 3). This movie shows time-lapse sequences for representative tumor organoids embedded in 3D collagen I derived from left to right: MMTV-Neu, MMTV-PyMT, and C3(1)/Tag mouse mammary carcinomas and a human luminal breast cancer (Sample S4, ER+Her2+). Collectively migrating cells emerge from the primary tumor organoid. Protrusive leader cells are readily identified at the front of these invasive strands. Scale bar represents 50 μm.

Movie S4 (related to Figure 4). This movie shows time-lapse sequences of K14-GFP; MMTV-PyMT tumor organoids embedded in 3D collagen I matrix. The first sequence shows phenotypic conversion in a tumor organoid imaged in cross-section. K14-GFP negative cells convert to K14-GFP positive cells over 48 hrs. Scale bar is 20 μm. The second sequence shows phenotypic conversion in a tumor organoid imaged en face. Scale bar is 10 μm. The third sequence shows collectively migrating K14-GFP positive cells extend from the primary tumor organoid ahead of trailing K14-GFP negative cells. Scale bar represents 20 μm.

Movie S5 (related to Figure 5). This movie shows a time-lapse sequence of phenotypic conversion K14-GFP; MMTV-PyMT tumor organoid embedded in 3D Matrigel. GFP negative cells convert to GFP positive cells over 18 hr. Scale bar is 20 μm.

Movie S6 (related to Figure 5). This movie shows a time-lapse sequence of a K14-GFP; MMTV-PyMT tumor organoid cultured initially in 3D Matrigel and then switched to collagen I matrix. GFP positive cells switch to protrusive morphology and lead collectively invading strands of cells. Scale bar is 20 μm.

Movie S7 (related to Figure 6). This movie shows a time-lapse sequence of a representative MMTV-PyMT tumor organoids transduced with control luciferase shRNA (Luc-kd), K14 shRNA (K14-kd), or p63 shRNA (p63-kd), and then embedded in 3D collagen I. Scale bars represent 50 μm.

EXTENDED EXPERIMENTAL PROCEDURES

Antibodies and Immunofluorescence

For immunofluorescence of tumor organoids cultured in 3D gels, gels were fixed in 4% paraformaldehyde, permeabilized with Triton-X/1x PBS, and blocked in 1x PBS + 10% fetal bovine serum and 1% BSA. Primary antibodies were incubated ~ 8 hours at room temperature or overnight at 4 C at the dilutions listed below in antibody diluent, 1x PBS + 1% fetal bovine serum and 1% BSA. Secondary antibodies coupled to Alexa Fluor 488, 562, or 647 (Invitrogen) were incubated ~ 4 hours at room temperature. Where indicated, phalloidin was added at 1:100 dilution to stain F-actin positive cell membranes.

For immunofluorescence experiments of thick tumor tissues (100 μm or greater), tissue organoids were fixed overnight in 4% paraformaldehyde, embedded in OCT, and cut into sections on a cryostat. In this case, primary antibody incubation was performed in antibody diluent, for 2 to 3 days at 4 C, followed by secondary antibody incubation for 1 to 2 days also at 4 C. Overlapping z-stacks were captured using a high speed confocal spinning disc microscope, and the z-stacks were assembled using Fiji software, and custom scripts. To estimate the relative distance of cell populations from the tumor-stromal border, pixels were classified as K14+, K14- (mTomato+K14-), or stroma (F-actin+mTomato-). For each K14+ or K14-pixel we then identified the nearest neighboring stromal pixel. Using Fiji software, we calculated the Euclidian distance map. Then using R software, we tabulated the total number of pixels at any given distance from stroma. All custom scripts available upon request.

Primary antibodies were Cytokeratin 14 (Covance, PRB-155P), Cytokeratin 5 (Covance, PRB-160P), p63 (Epitomics, 5353-1, or Santa-Cruz, sc-8431), SMA (Sigma, A5228), K8 (TROMA-1c)

(Developmental Studies Hybridoma Bank, 386-113626), K18 (Epitomics, 1924-1), P-cadherin (Life Technologies, Clone PCD-1 or Novus, NBP1-59222), Myh11 (Santa-Cruz, sc-6956), E-cadherin (Invitrogen, 13-1900), Phospho-histone H3 (Ser10) (Cell Signaling Technologies, #9701), and Calponin (Millipore, 04-589).

Immunohistochemistry of Primary Human Tumor Sections

K14 protein expression was evaluated in formalin-fixed paraffin-embedded primary tumors by immunohistochemistry in accordance to IRB protocol NA_00003308. Five-μm sections were treated with DAKO target retrieval solution for 20 minutes according to the manufacturer's recommendations and then incubated for 1 hour with an anti-human-K14 rabbit polyclonal antibody (Covance PRB-155P) at a concentration of 2.5 μ g/ml, using an automated slide stainer (DAKO). Secondary reagents (LSAB2 secondary reagent system) were supplied by DAKO and used according to the manufacturer's specifications.

The intensity of staining in the cancer cells was scored as + (low to no staining), ++, or +++ (intense staining). The pattern of staining was scored as primarily cytoplasmic or cytoskeletal with or without enrichment at the border of collective invasive units. Scoring was conducted in a blinded manner by a pathologist observer (EG). The breast cancer subtype was determined for each specimen according to St. Gallen Consensus Guidelines using surrogate IHC criteria, including estrogen receptor (ER), progesterone receptor (PR), HER2, and Ki-67 status (Goldhirsch et al. 2013). Luminal A tumors were defined as those that were ER positive and Her2 negative and had PR > 20% and Ki-67 < 20%. Luminal B tumors were defined as those tumors that were ER positive and any of the following: PR ≤ 20%, Ki-67 ≥ 20%, or Her2 amplified. TNBC tumors were defined as those tumors that were negative for ER, PR, and Her2.

Isolation and 3D Culture of Murine Organoids

We isolated organoids from primary mammary tumors and normal mammary gland using previously described techniques (Nguyen-Ngoc et al. 2012). MMTV-PyMT tumors were harvested from mice at 12-16 weeks of age, C3(1)-Tag tumors were harvest at 22-24 weeks of age, and MMTV-Neu tumors were harvested at 40-50 weeks of age, coinciding with palpable solid tumors. For each mouse, we surgically isolated the largest tumor and processed it as above. Briefly, we dissected tissue into epithelial organoids by a combination of mechanical and collagenase/trypsin digestion. We then separated these organoids from single cells by differential centrifugation. Any incompletely digested large tumor organoids were removed prior to differential centrifugation. We also added additional rounds of differential centrifugation as needed to remove single cells. The final pellet was composed of epithelial organoids, each containing 200-1000 cells.

We then embedded organoids in type I rat tail collagen gels (BD Biosciences 354236) or 3D Matrigel (BD Biosciences 354230). All cultures were set up in 24-well coverslip bottom plates (E&K Scientific EK-42892), or 2-well or 4-well coverslip bottom chambers (Nunc 155383). Acid solubilized rat tail collagen I gels (3 mg/ml collagen I, pH 7-7.5) were prepared as previously described (Nguyen-Ngoc et al. 2012). For each matrix, organoids were mixed to yield a suspension of 1-2 organoids/μl. A 100 μl suspension of organoids was plated in each well on the 37^{o}C heating block, followed by incubation at 37 ^{o}C to allow polymerization. All murine samples were cultured in 1 ml of 2.5 nM FGF2 in murine organoid media.

Image Acquisition and Quantification

Confocal images were acquired using a custom spinning disc microscope using a 40x cAPO oil objective. For z-stacks, 2 μm spacing was used. Representative single z planes are presented

unless noted. Images were recorded at 1024x1024 square pixels representing 0.17 μm per pixel. Second-harmonic generation microscopy was conducted using a Zeiss LSM 710 laser-scanning microscope at 63X water objective. Red-green-blue (RGB) images were assembled using Fiji software and custom scripts.

Time-lapse Imaging

Live differential interference contrast (DIC) imaging of tumor organoids was conducted using a Zeiss Cell Observer system with a Zeiss AxioObserver Z1 and a AxioCam MRM camera. In general, images were collected at 20-min intervals with exposure times of ~250 ms. Live confocal time-lapse imaging was performed using a custom spinning disc microscope (Ewald, 2013). Temperature was held at 37°C and CO_2 at 5%.

Flow Cytometry

Primary tumors from MMTV-PyMT mice carrying a K14-GFP reporter were harvested for tumor organoids. Organoids were subsequently digested down to single cell suspensions in trypsin-versene according to established protocols (Smalley, 2010). Cells were resuspended in HBSS/1%BSA. The resulting cell suspensions were filtered through 100 μm cell strainers and analyzed on a BD FACSAria or a Beckman-Coulter MoFlo Cytometer. Dead cells were excluded by propidium iodide fluorescence.

Basal and Myoepithelial Gene Expression

mRNA expression from relevant probes were identified from a previously published microarray dataset by our laboratory (Nguyen-Ngoc et al. 2012). Normal mammary epithelial organoids and mammary tumor epithelial organoids were cultured in 3D collagen for 4 days and harvested for

mRNA. Each sample represented at least 3 independent biologic replicates. Sample preparation, labeling, and array hybridizations were performed according to standard protocols from the UCSF Shared Microarray Core Facilities and Agilent Technologies (http://www.arrays.ucsf.edu and http://www.agilent.com). Equal amounts of Cy3-labeled target were hybridized to Agilent whole mouse genome 4x44K Ink-jet arrays (Agilent). Arrays were scanned using the Agilent microarray scanner (Agilent), and raw signal intensities were extracted with Feature Extraction v9.1 software (Agilent). Microarray data was deposited to the NCBI GEO repository and are accessible through GEO series accession number GSE39173.

Oxytocin Contractility Experiment

Normal mammary epithelial organoids and MMTV-PyMT tumor organoids were embedded in 3D collagen I matrix and cultured in FGF2 organoid media for 4 days. Normal and tumor organoids were then monitored by DIC time-lapse microscopy collecting images every 5 minutes. Oxytocin (Sigma, 03251) was dissolved to a final concentration of 5 and 50 nM in FGF2 organoid media. The medium was replaced for normal and tumor samples and contraction was observed by time-lapse imaging, again collecting images every 5 minutes. The area defined by the tissue organoid boundary pre and post oxytocin treatment was measured using Zeiss software, and plotted using R and the ggplot2 package.

Second Harmonic Generation Microscopy to Measure Collagen Fiber Density

Tumor sections from 8 tumors were stained for K14, phalloidin and collagen IV. Confocal sections from optically cleared specimens were imaged on a Zeiss 710 with a Chameleon laser set to 880 nm, set to max pinhole. Random fields were collected at 25X resolution and classified into one of three morphologies: K14+ cells with invasive morphology, K14+ cells with non-invasive morphology, or K14- regions. Mean pixel intensity for SHG and collagen IV channels were then determined in Fiji using a custom macro. We defined the zero point to be the mean

pixel intensity averaged over all K14- regions, which did not express collagen IV and had weak SHG signal. Mean pixel intensities were then plotted in R using ggplot and are presented as box-plots. Statistics were determined using an unpaired two-sided t-test.

Matrix Switching Experiments

MMTV-PyMT; K14-GFP; mT/mG tumor organoids were cultured in 3D Matrigel for four days, and then freed from the gel by gentle agitation and resuspension in DMEM. Intact tumor organoids were spun down by differential centrifugation and re-embedded in 3D collagen I. Fluorescent tumor organoids were then imaged used a spinning disc confocal microscope in time-lapse mode with membrane Tomato outlining cell membranes, and GFP marking K14+ cells.

Lentiviral Transduction of Tumor Organoids

Under standard conditions, epithelial organoids have extremely low lentiviral transduction efficiency. We achieved efficient viral transduction in mammary epithelium by using a magnet-based approach, in which magnetic bead-coupled virus is brought into direct contact with organoid structures (Oz Biosciences). Lentiviral particles encoding shRNAs against luciferase, K14, and p63 were purchased at a titer of 1x10^6 PFU/mL from the MISSION pLKO library (Sigma). Organoids were infected with lentivirus in complex with magnetic nanoparticles, and after a 1-day growth period, were selected with puromycin to enrich for shRNA expressing cells. Selected organoids were then harvested for Western blotting or embedded in 3D collagen for time-lapse DIC microscopy.

Mammary Fat Pad Transplantation

Tumor organoids were harvested and either directly transplanted the following morning or infected and selected following lentiviral transduction. Mammary organoid transplantation was

conducted using a JHU IACUC approved protocol. 3-4 week old congenic FVB host mice were anesthetized with isofluorane. Following a midline excision, each #4 mammary gland was exposed, cleared of the fat pad extending to the mammary lymph node, and ~300-500 organoids either in 20 uL of DMEM or 50:50 DMEM/Matrigel were transplanted into the residual fat pad. Cleared areas were treated with bupivicane for analgesia, and the mouse was closed up with surgical staples. Mice were followed for recovery every day for 1 week, and monitored twice weekly thereafter for tumor growth. Mice were generally harvested with tumors exceeding 1 cm in size.

SUPPLEMENTAL REFERENCES

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