# Identification of Luminal Breast Cancers that Establish a Tumor Supportive Macroenvironment Defined by Pro-Angiogenic Platelets and Bone Marrow Derived Cells

Hanna S. Kuznetsov[1]*, Timothy Marsh[1]*, Beth A. Markens[1], Zafira Castaño[1,2], April Greene-Colozzi[3], Samantha A. Hay[1], Victoria E. Brown[4], Andrea L. Richardson[3,4], Sabina Signoretti[2,4], Elisabeth M. Battinelli[1,2], and Sandra S. McAllister[1,2,5,6]

[1]Hematology Division, Brigham & Women's Hospital, Boston, MA 02115, USA
[2]Department of Medicine, Harvard Medical School, Boston, MA 02115, USA
[3]Department of Cancer Biology, Dana-Farber Cancer Institute, Boston, Massachusetts, 02115, USA
[4]Department of Pathology, Brigham & Women's Hospital, Boston, MA 02115, USA
[5]Harvard Stem Cell Institute, Boston, MA 02115, USA
[6]Broad Institute of Harvard and MIT, Cambridge, MA USA

*These authors contributed equally

## SUPPLEMENTARY INFORMATION

## SUPPLEMENTAL METHODS

### Bone Marrow Harvest and Transplantation

BMCs were harvested from donor mice by flushing femurs with sterile Hanks' balanced salt solution (HBBS; GIBCO) with penicillin/streptomycin/fungisone. Cells were washed twice with sterile HBBS, dissociated with 18 g needle, and filtered through 70 μm nylon mesh. For transplantation experiments, 2 x $10^6$ BMCs from Rag1$^{-/-}$xEGFP transgenic donor mice (9) were injected into the retroorbital sinus 8–10 hr after irradiation of recipient mice (600 rads). Antibiotics were added to drinking water for 14 days following the procedure. Successful engraftment of GFP+ donor BMCs was tested 28-30 d after bone marrow transplantation using a small venous blood sample (~50 μl) from the retroorbital sinus of anesthetized recipient mice. Blood was treated with RBC lysis buffer (Sigma) to remove red blood cells, and flow cytometry was performed to detect GFP+ cells in the remaining white blood cell portion. At the end of each experiment, recipient mice were anesthetized by i.p. injection of Avertin, and vasculature was exsanguinated by perfusion of sterile PBS through the left ventricle.

### Platelet preparations

Human and mouse platelets were isolated from whole blood by differential centrifugation as described (47). Specifically, 600-1000 μl blood was collected into tubes containing 100 μl ACD buffer (130 mM citric acid, 125 mM trisodium citrate, 110 mM glucose) and centrifuged at 200 xg for 8 minutes. The

supernatant, buffy coat, and red blood cells were collected and wash buffer (140 mM sodium chloride, 12 mM trisodium citrate, 10 mM glucose, 12.5 mM sucrose, pH of 6.0) added to a volume of 800 μl, then centrifuged at 100 xg for 6 minutes. Supernantants containing platelets were collected and brought to a volume of 800 μl with wash buffer and centrifuged at 1200 x g for 5 minutes. Supernatants were asparated and the platelet pellet resuspended in 800μl of wash buffer and centrifuged at 1200 xg for 5 minutes. Platelet pellets were resuspended in 400 μl resuspension buffer (10 mM Hepes, 140 mM sodium chloride, 3 mM potassium chloride, 0.5 mM magnesium chloride, 5 mM sodium bicarbonate, 10 mM glucose, pH of 7.4). Platelets were allowed to rest for twenty to thirty minutes before further use. Platelet releasates were prepared by adding 50μM ADP to resting platelets at 37$^{o}$ C for 10 min. Platelets were pelleted by centrifugation at 1000xg for 5 minutes and releasate removed and stored in a clean eppendorf tube. Lysates were prepared by resuspending resting platelets in RIPA lysis buffer (Santa Cruz) at 100$^{o}$ C for 5 minutes.

**Platelet Immunofluorescence**

Resting platelets were fixed for 20 minutes in suspension by the addition of 1 vol of 8% formaldehyde. Solutions of fixed platelets in suspension were placed in wells of a 24-well microliter plate, each containing a polylysine-coated coverslip, and the plate was centrifuged at 250 g for 5 minutes to attach the cells to the coverslip. Megakaryocytes were fixed with 4% formaldehyde in Hanks' balanced salt solution (GIBCO BRL, Invitrogen, Carlsbad, CA) for 20 minutes, centrifuged

at 500 g for 4 minutes onto coverslips previously coated with poly-L-lysine, and permeabilized with 0.5% Triton X-100 in Hanks' balanced salt solution. Specimens were blocked overnight in phosphate-buffered saline (PBS) with 1% BSA, incubated in primary antibody for 2 to 3 hours, washed, and treated with appropriate secondary antibody for 1 hour, and then washed extensively. Primary antibodies were used at 1 μg/mL in PBS containing 1% BSA and secondary antibodies at 1:500 dilution in the same buffer. Controls were processed identically except for omission of the primary antibody. Controls consisted of either incubating cells with one or both primary antibodies without fluorescently labeled secondary antibodies, or cells incubated with one or both fluorescently labeled secondary antibodies in the absence of primary antibodies. Preparations were mounted in Aqua polymount from Polysciences (Warrington, PA) and analyzed at room temperature on a Nikon TE 2000 Eclipse microscope equipped with a Nikon 100×/1.4 NA objective and a 100-W mercury lamp. Images were acquired with a Hamamatsu (Bridgewater, NJ) Orca IIER CCD camera. Electronic shutters and image acquisition were under the control of Molecular Devices Metamorph software (Downington, PA). Images were acquired by fluorescence microscopy with an image capture time of 200 to 500 ms. Antibodies used: Human IL6 (R&D Systems MAB2061, 1:50), Mouse IL6 (R&D Systems AF-406-N, 1:20), VEGF (Fisher MS-1467-P), PLGF (Sigma P-3868), VWF (Dako M0616), TSP (Fisher MS-421-P), Fibrinogen (Santa Cruz sc-33917), and PF4 (PeproTech 500-P05).

**ELISA**

Conditioned media were prepared by changing growth medium 24 hours after passaging, collecting after 48 hours, centrifuging at 1500 RPM for 5 minutes. Mouse plasma was prepared by centrifugation of whole blood collected into EDTA microcontainer tubes (BD Pharmingen). Plasma levels of human OPN and IL6 levels were measured using Quantikine Immunoassay kits (R&D systems) according to manufacturer's instructions. Plasma levels of human TNF$\alpha$, IGF1, VEGF, IL6, FGFb, TGF$\beta$, EGF and Leptin were measured using the Human Angiogenesis ELISA Strip I (Signosis) according to manufacturer's instructions.

**Antibodies Used for Flow Cytometric Analysis**

In some cases, samples were blocked with an antibody to CD16/CD32 Fc$\gamma$ III/II receptor (250 ng/$10^6$ cells; BD Pharmingen). Antibodies used: PE-anti-Ly-6A/E/Sca-1 clone E13-161.7 (400 ng/$10^6$ cells; BD Pharmingen), APC-anti-CD117/c-Kit 2B8 (400 ng/$10^6$ cells; BD Pharmingen), APC-anti-CD45 (200 ng/$10^6$ cells; Caltag Laboratories), PE-anti-CD11b/Mac-1 clone M1/70 (400 ng/106 cells; BD Pharmingen), PE-anti-CD31 (600 ng/$10^6$ cells; Caltag), anti-Flt-1/VEGFR1 C-17 (200 ng/$10^6$ cells; Santa Cruz), anti-Flk-1/VEGFR2 clone Avas12a1 (250 ng/$10^6$ cells; eBioscience;), Alexa 647-goat anti-rabbit, Mol. Probes Alexa 647-goat anti-rat (200 ng/$10^6$ cells; Mol. Probes).

**Immunohistochemistry on Human cRCC Tissues**

Immunohistochemical analysis was performed using the EnVision™+ System horseradish peroxidase detection kit (Dako). Briefly, sections were deparaffinized and rehydrated in water, and antigen retrieval was done by heating slides in citrate buffer (pH 6.0) in a pressure cooker for 2 minutes. Sections were blocked with Peroxide Block (Dako) for 5 minutes and incubated with appropriate primary antibodies for 1 hour at room temperature. Sections were washed for 5 minutes in PBS and incubated with the anti-mouse HRP-conjugated antibody for 30 minutes. Sections were incubated with the DAB chromogen (Dako) for 5 minutes and lightly counterstained with hematoxylin.

**Antibodies used for Immunohistochemical Analysis**

αSMA (Vectorlabs VP-S281, 1:50), CD24 (Fisher Scientific MS-1279-P0, 1:100), CD44 (BD Biosciences 550538, 1:50), SV40 Large T (Santa Cruz sc-147, 1:75), Human mitochondria (Millipore MAB1273, 1:50), CD31 (BD Pharmingen 550274, 1:50), VEGFR2 (eBioscience 14-5821, 1:50), Meca-32 (U of Iowa, 1:50), Collagen IV (Abcam ab6586, 1:200), p-selectin (Santa Cruz sc-6941, 1:100), phospho-Tyr705-STAT3 (Cell Signaling 9145S, 1:50) and OPN (R&D Systems MAB1433 1:10), mouse-anti-human CAIX (1:15000, clone M75), rat-anti-mouse CD34 (1:100, Abcam).

**SUPPLEMENTAL FIGURE LEGENDS**

**Supplemental Figure 1.** Phenotypes of responding tumors and instigating luminal breast cancer tumor xenografts. **A,** Merged fluorescent images

visualizing expression of SV40 LgT antigen (green) expressed exclusively on responding tumor cells; nuclei counterstained with DAPI (blue). Scale bars = 100μm. **B,** H&E staining of indicated tumors; N denotes necrotic area, BV denotes large blood vessels. Scale bars = 100μm. **C,** Merged immunofluorescent images show proliferating cells (Ki67; green), responding tumor cells (human-specific mitochondrial stain; red), and cell nuclei (DAPI; blue). Scale bars = 100 μm. **D,** Staining for alpha-smooth muscle actin (αSMA, brown) in responding tumors under indicated systemic conditions. Nuclei counterstained with hematoxylin (blue); scale bars = 100μm. **E,** Concentrations of soluble human osteopontin (hOPN) secreted into conditioned medium of indicated instigating cell lines; n=5 different cell culture plates per group, run in duplicate.

**Supplemental Figure 2.** Visualization of tumor vasculature. **A,** Immunihistochemical stain for mouse endothelial antigen (MECA32, brown) and nuclei (hematoxylin, blue) of intra-tumoral (left) and peri-tumoral (right) blood vessels from a responding tumor that had grown in the LBC environment. **B,** Merged immunofluorescent images of peri-tumoral blood vessel in tissue from responding tumor grown in the LBC environment to visualize VEGFR2 (red), αSMA-positive pericytes (green), and cell nuclei (DAPI, blue). Bright orange staining in center of vessel represents autofluorescent red blood cells (RBC). Left: scale bar = 100μm. Right image is enhanced magnification of area selected in white box; scale bar = 20μm. **C,** Merged immunofluorescent image of responding tumor that had grown in the systemic environment established by

triple-negative breast cancer (TNBC) to visualize VEGFR2+ cells (red) and cell nuclei (DAPI, blue).

**Supplemental Figure 3.** Circulating GFP+ bone marrow cells and platelets. **A,** Peripheral blood samples from indicated groups of bone marrow transplant recipient mice were analyzed for percent of total GFP+ bone marrow-derived cells from donor mice. Samples were analyzed at the start of the experiment (4 wk after engraftment) and at the experimental end point. There were no statistical differences between different time points or between groups. **B,** Representative flow cytometric analysis of donor GFP+ bone marrow derived cells in dissociated tumor (top) and lung tissue (bottom) from a recipient mouse at the experimental end point. Gate M1 indicates non-GFP cells and M2 indicates GFP+ cells. **C,** Responding tumor that formed in LBC environment stained for CD24 (green) and DAPI (blue); BV denotes blood vessel. **D,** Average platelet counts in circulation of mice that were cancer free, bearing Matrigel control plugs (Mat Envt), or luminal breast cancer (LBC Envt); n=3 mice per group.

**Supplemental Figure 4.** Platelet analysis. **A,** Immunofluorescent images of platelets harvested from indicated mice to visualize human IL6 (green, top row), mouse IL6 (red, middle row), and human/mouse cross-reactive VEGF (green, bottom row). Images captured under 150x objective. **B,** Example of CellProfiler staining quantification. Platelets harvested from indicated mice were stained for

human IL6 (green, top panels) and staining was detected by the CellProfiler software (outlines, bottom panels). **C,** Results from quantification of immunostaining for various pro- and anti-angiogenic factors carried by circulating platelets from indicated mice. Data are expressed as staining intensity/staining area, relative to naïve platelets; **p<0.01. **D,** Western blots for phospho-STAT3 and β-actin in protein lystes from indicated BMC populations (n=3 mice per group). Tables represent chemiluminescence-based quantification of each sample; graphs represent average STAT3 signal relative to β-actin for each group.

**Supplemental Figure 5.** Responding tumor microvessel density. Images used to quantify microvessel density in responding tumors and tissues recovered after exposure to systemic environments of: Matrigel (**A**), TNBC (**B**), LBC (**C**), or hBRCA-LBC1 (**D**). Tissues were stained for the mouse endothelial antigen (MECA32, brown), captured under 40x magnification, and quantified using NIH ImageJ software. Areas that did not stain positively for MECA32 but had morphology consistent with blood vessels (BV) where red blood cell lakes were clearly evident were also counted.

**Supplemental Figure 6.** Instigating, non-instigating, and responding primary human breast tumors. **A,** Top panels: representative immunohistochemical images of pan cytokeratin (PanCK, brown) staining of fresh tumor specimens hBRCA-LBC1 (instigator) and hBRCA-LBC2 (non-instigator). Nuclei

counterstained with hematoxylin (blue). Bottom panels: H&E and panCK (inset, brown) stains of tumors of hBRCA-LBC1 and hBRCA-LBC2 tumors recovered from the mice. **B**, Human luminal breast cancer tumor specimens recovered from mice. H&E stains of two representative tumors from each indicated human tumor sample that was recovered from mice. Hormone receptor status and instigating ability are also noted. Numbers indicate experimental tumor sample identification and are not linked to any patient identifiers. **C,** Growth kinetics of the instigating hBRCA-LBC1 sample following implantation opposite Matrigel or the responding tumor cells. There were no statistical differences between groups at the experimental end point; n=3 mice per group. **D,** Hematoxylin and Eosin stain of hBRCA-LBC 5 responding tumor prior to xenograft implantation. **E**, Staining of responding tumor specimens from hBRCA-LBC5 under indicated conditions with a human-specific antibody against mitochondrial protein (red) to visualize the xenografted tissue; nuclei counterstained with DAPI; scale bar = 100μm. See also Fig. 5.

**Supplemental Figure 7.** Responding human primary renal cell carcinoma tumor xenografts. **A,** Representative images of freshly prepared human cRCC organoid specimens implanted into mice. Left: hematoxylin and eosin (H&E) staining; Right: staining for the human cRCC marker, CAIX (brown). **B,** Mass of cRCC tumor specimens after 80 days in indicated systemic environments; n=4 mice per group. See also Fig. 5.

**Supplemental Figure 8.** Aspirin does not affect instigating tumor growth or circulating platelet counts. **A,** Volume of instigating LBC tumors in indicated cohorts during experimental time course; n=10 (5 mice per group for 2 independent experiments); differences not significant (n.s.). **B,** Average circulating platelet count in indicated mice; n=10 per group; values not statistically different (n.s.). See also Fig. 6.