Supplementary methods for:

Breast cancer cells produce tenascin-C as a metastatic niche component to colonize the lungs

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Oncosphere analysis. We cultivated oncospheres for two weeks and analyzed gene expression by quantitative Real-Time PCR. We analyzed signaling pathways by treating oncospheres with recombinant human Wnt3a (R&D systems) at 200 ng ml^{-1} for 6 h, JAK2 inhibitor AG490 (Calbiochem) at 100 μM for 7 h, or nicotinoyl hydrazone-based STAT5 inhibitor (Santa Cruz) at 150 μM for 12 h. DKK1 (0–300 ng ml^{-1}) (R&D systems) was used to antagonize Wnt3a stimulation. Secondary oncospheres were derived by dissociating primary oncospheres, plate as single cell suspension and grow for additional 2 weeks on ultra-low adhesive plates. Oncospheres grown under stressed conditions were cultivated in the absence of supplemental factors.

Generation of knockdown cells. We used Mission TRC lentiviral shRNA vectors (Sigma-Aldrich) to knock down TNC, MSI1 and LGR5. The following TRC numbers and shRNA sequences were used.

TNC:
TRCN0000157688,
5'-CCGGCCAGGAATCTTCGACGTGTTTCTCGAGAAACACGTCGAAGATTCCTGGTTTTTTG-3'
TRCN0000154001,
5'-CCGGCCACTGGAATAACCCTACTTCTCGAGAAGTAGGGTTATTTCCAGTGGTTTTTTG-3'
MSI1:
TRCN0000064004,
5'-CCGGCCCTTTGATTGCCACAGCCTTCTCGAGAAGGCTGTGGCAATCAAAGGGTTTTTG-3'
TRCN0000064007,
5'-CCGGCACGTTTGAGAGTGAGGACATCTCGAGATGTCCTCACTCTCAAACGTGTTTTTG-3'
LGR5:
TRCN0000011586,
5'-CCGGCCATAGCAGTTCTGGCACTTACTCGAGTAAGTGCCAGAACTGCTATGGTTTTT-3'
TRCN0000011589,
5'-CCGGGCTCTACTGCAATTTGGACAACTCGAGTTGTCCAAATTGCAGTAGAGCTTTTT-3'

We used lentiviral particles to infect subconfluent cell cultures over night in the presence of 8 μg ml^{-1} polybrene (Sigma-Aldrich). Selection of virally infected cells expressing the shRNA was done using 2 μg ml^{-1} puromycin (Sigma-Aldrich) in the media. For conditional TNC knockdown, we used a doxycycline inducible shRNA against TNC

expressed by a lentiviral vector (TRIPZ Clone ID V2THS_133229, Open Biosystems). In vitro conditional knockdown was induced by adding 1 μg ml^{-1} doxycycline (Sigma-Aldrich) to the media and gene expression analyzed 72 hours later.

MSI1 expressing cells. We generated MSI1 expressing cells by PCR amplifying MSI1 from a cDNA clone (ID 100014977, Open Biosystems), and subclone into pBabe-hygromycin retroviral vector. PCR primers:

Forward, 5'-CAGGATCCGTACAAAAAAGCAGAAGGGCC-3'

Reverse, 5'-ACGCGTCGACGTACAAGAAAGCTGGGCCCA-3'

Retroviral particles were packed using GPG29 packaging cell line. GPG29 cells were maintained in DMEM with 2 mM L-Glutamine, 50 IU ml^{-1} penicillin, 50 μg ml^{-1} streptomycin, 20 ng ml^{-1} doxycycline, 2 μg ml^{-1} puromycin and 0.3 mg ml^{-1} G418. We transfected the cells with retroviral constructs using Lipofectamine 2000 transfection agent (Invitrogen). Selection for MSI1 expressing cells was done using 500 μg ml^{-1} hygromycin (Calbiochem) in media.

mRNA and microRNA expression analysis. We used Taqman gene expression assays (Applied Biosystems) to analyze mRNA expression. Assays used: TNC (Hs00233648_m1), NANOG (Hs02387400_g1), OCT4 (Hs00742896_s1) (Ref. 1), SOX2 (Hs01053049_s1), LGR5 (Hs00969420_m1), MSI1 (Hs00159291_m1), GATA3 (Hs00231122_m1), HEY2 (Hs00232622_m1), DTX1 (Hs00269995_m1). LEF1 (Hs01547250_m1) and AXIN2 (Hs00610344_m1). Relative gene expression was normalized to the "housekeeping" gene β2M (Hs99999907_m1). Taqman gene expression assays used to analyze miRNA expression were miR335 (TM546), normalized to RNU6B (TM1093).

Immunofluorescence. Tissues were fixed in 4% paraformaldehyde at 4 °C overnight. After PBS wash, we mounted the tissue, froze in OCT and stored at –80°C. We incubated 8 μm thick cryosections with a blocking buffer (Mouse on mouse- MOM kit, Vector Laboratories) and incubated with the primary antibody of interest at 4 °C overnight in diluent (MOM kit, Vector Laboratories). The sections were incubated at room temperature for 30 min with the corresponding fluorochrome conjugated secondary antibodies (Molecular Probes). We used species matched isotype antibodies as negative controls. Slides were mounted in aqueous mounting media containing DAPI (Fluorogel II

from Electron Microscopy Sciences). Stained tissue sections were visualized under a Carl Zeiss Axioimager Z1 microscope.

Immunohistochemistry. We fixed tissues in 4% paraformaldehyde at 4 °C overnight. Paraffin mounting and cutting was done at Histoserv, Inc. 5 μm thick tissue sections were baked at 56 °C for 1 h and deparaffinized and treated with 1% hydrogen peroxide for 10 min. For tenascin staining, sections were subsequently treated with pepsin dissolved in 0.2N HCl (Dako) for 5 min at 37°C for antigen retrieval. For fascin-1 staining, antigen retrieval was done in a citrate buffer (pH 6.0). We incubated sections with a blocking buffer followed by primary antibody of interest as mentioned in the previous section. Corresponding biotinylated secondary antibody and an ABC avidin-biotin-DAB detection kit (all from Vector laboratories) were used for detection and visualization of staining following manufacturer's instructions. Sections were analyzed under Zeiss Axio2Imaging microscope.

Migration and invasion assays. We performed transwell migrations assays as previously described[2]. Briefly, we labeled serum starved (0.2% FBS) cancer cells with 5 μM cell tracker green (Invitrogen) for 30 min at 37°C. We seeded cells (50,000) onto membrane inserts with 3 μm pore size fluorescence blocking filter (Falcon) (migration assay) or onto matrigel coated transwell inserts (BD Biosciences) (invasion assay). Migration and invasion were scored following 5-6 hours and 24 hours respectively, We analyzed migration and quantified under a Zeiss Axio2Imaging fluorescence microscope.

Flow cytometry. We preincubated cancer cells with antibodies against CD44 (coupled to APC, BD Biosciences) and CD24 (coupled to PE, BD Biosciences) for 20 min on ice and washed with PBS. Samples were analyzed on FACSCalibur flow cytometer (BD Biosciences). We analyzed data with FlowJo software (Tree Star, Inc).

TNC binding receptor analysis. We identified potential TNC binding receptors through literature search (reviewed in Refs. 3 and 4). Expression was determined by Affymetrix Gene Chip analysis. We made presence call by MAS5 normalization algorithm implemented by the Affimetrix package R.

Microarray dataset analysis: TNC - and STAT5 - signatures. We obtained a TNC signature from Ruiz et al [5] and employed it to cluster microarray data of 344 primary breast tumors and 67 metastatic breast tumors using heatmap.2 function in the R statistical package. The most robust clustering was achieved with Pearson correlation as the distance function and complete linkage as the hierarchical clustering function. One of the major subcluster exhibited expression pattern of TNC signature (see **Supplementary Fig. 19**). Specifically, compared to the other clusters this cluster overexpressed 75% and underexpressed 80% of genes whose fold change were >1 and <1 in TNC signature, respectively. We therefore defined this cluster as TNCS+ cluster. To further test the similarity between this group of tumors and cancer cells interacting with TNC, we calculated Pearson correlation coefficients using TNC signature as a "template vector" comprised by 1's and -1's. The median correlation coefficient of the TNCS+ tumors is 0.19 ($p < 0.001$) whereas that of the rest of tumors is -0.03. We used a published gene expression profile (Ref. 6) to generate STAT5 gene-expression classifier. Statistical scores were determined by calculating Pearson correlation coefficients using the significant genes between each tumor and a template vector of "1" and "-1" as described by others[7].

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