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Fibroblast subtypes define a metastatic matrisome in breast cancer
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ABSTRACT
Small primary breast cancers can show surprisingly high potential for metastasis. Clinical decision making for tumor aggressiveness, including molecular profiling, relies primarily on analysis of the cancer cells. Here we show that this is insufficient; that the stromal microenvironment of the primary tumor plays a key role in tumor-cell dissemination and implantation at distant sites. We previously described two cancer-associated fibroblasts (CAFs) that either express (CD146\textsuperscript{pos}) or lack (CD146\textsuperscript{neg}) CD146 (official symbol MCAM; alias MUC18). We now find that when mixed with human breast cancer cells, each fibroblast subtype determines the fate of cancer-cells: CD146\textsuperscript{neg} fibroblasts promote increased metastasis compared to CD146\textsuperscript{pos} fibroblasts. Novel quantitative and qualitative proteomic analyses show that CD146\textsuperscript{pos} CAFs produce an environment rich in basement membrane proteins, while CD146\textsuperscript{neg} CAFs exhibit increases in FN1, LOX, and TNC; all over-expressed in aggressive disease. We also show clinically, that CD146\textsuperscript{neg} CAFs predict for likelihood of lymph node involvement even in small primary tumors (<5 cm). Clearly small tumors enriched for CD146\textsuperscript{neg} CAFs require aggressive treatments.
INTRODUCTION

Development of breast cancer metastasis requires a tumor microenvironment (TME) that allows cancer cells to invade, migrate and ultimately disperse from the primary disease site. Dysregulation of both structural (i.e., Collagen I and IV, proteoglycans and glycoproteins) and matricellular components of the TME (i.e., growth factors, metalloproteinases, tissue inhibitors of metalloproteinases, chemokines and cytokines) has been linked with development of metastases in multiple cancer types (1, 2). For example, in breast tissue, the glycoprotein Tenascin-C (TNC) is often over-expressed at the invasive front of aggressive and metastatic breast tumors (3, 4). TNC utilizes its FN1 and EGF-like repeat domains to change cancer cell adhesion, proliferation and migration. TNC is one of many proteins and signaling molecules in the TME that are influenced by the dynamic relationship between cancer cells and stroma, which both contribute to extracellular matrix (ECM) composition. Ultimately, these interactions help shape how aggressive a tumor becomes and they can initiate and maintain signaling pathways that support invasion and metastasis formation.

Several stromal cell types contribute to the establishment and maintenance of the TME; however, most commonly, cancer-associated fibroblasts (CAFs) represent the predominant non-epithelial component of primary breast cancers. We and others have shown that CAFs represent a heterogeneous cell population known to influence treatment response and metastatic potential of cancer cells by secreting matricellular proteins and signaling molecules (5, 6). Previously we described two subtypes of breast cancer fibroblasts, based on the presence or absence of the melanoma cell adhesion molecule (MCAM; alias, MUC18 or CD146) (5) and demonstrated that CD146$^{neg}$ CAFs promoted loss of estrogen receptor (ER) expression and decreased response to anti-endocrine therapy in a model of ER-positive breast cancer. Such mounting evidence lends to the hypothesis that improved understanding of CAF subtypes and their influence on ECM composition, will ultimately lead to an improvement in breast cancer treatment strategies. To that end, our lab and others have begun isolating and defining subpopulations of CAFs based on several cell markers including Caveolin 1, alpha-Smooth Muscle Actin, Fibroblast Specific Protein-1, alpha and beta-Platelet Derived Growth Factor, vimentin and others (7, 8).

Here, we hypothesized that influence from CAF subtypes, identified by the presence or absence of CD146 expression, would drive the production of qualitatively different TME matrixes in the presence of the same
breast cancer cell types. In order to describe the contributions of each CAF subtype to the TME, we developed a new metastatic model of ER+ disease and employed an innovative ECM-focused mass spectrometry based approach (9-12). Here we describe how CD146\textsuperscript{neg} CAFs produce an increased metastasis enhancing TME compared to the CD146\textsuperscript{pos} CAF derived TME. We further show that CD146\textsuperscript{neg} CAF dependent cancer cell invasion and metastatic phenotype is dependent on TNC expression and epidermal growth factor receptor (EGFR) pathway activation.
RESULTS

The TME of tumors bearing CD146\textsuperscript{neg} CAFs or CD146\textsuperscript{pos} CAFs are defined by unique ECM profiles.

We injected a mixture of 150,000 ZS-green labelled ER+ MCF-7 cells with either 150,000 CD146\textsuperscript{pos} CAFs or CD146\textsuperscript{neg} CAFs into the mammary fat pad of intact female NSG mice supplemented with 1mg estrogen pellets. All tumors were collected at the same time with restriction of any single tumor reaching 2 cm in size by caliper measurement. This corresponded to approximately 5 weeks for establishment of primary tumors and 6 weeks of measurable growth. In order to determine the effects of CD146\textsuperscript{pos} CAFs and CD146\textsuperscript{neg} CAFs on the TME, we modified the standard ECM-focused mass spectrometry-based approach for quantifying ECM proteins. We utilized a library of stable isotope labeled reporter peptides containing a mixture of human (H) specific, mouse (M) specific, and shared (S) species ECM probes. This unique method afforded us the ability to quantitatively measure and specifically attribute host versus human contributions to the TME. Liquid chromatography select reaction monitoring (LC-SRM) was applied to quantitatively assess differences in the abundance of ECM and ECM-associated components of the TME. In order to obtain the most comprehensive analysis of these components, each sample was processed using three serial extractions including cellular (CHAPS with high salt), soluble ECM (Gnd-HCl), and insoluble ECM (NH\textsubscript{2}OH digestion) fractions. Each of the three fractions were spiked with stable-isotope labeled peptide standards specific to ECM and ECM-associated proteins at a known concentration (Figure 1A) (13). This experiment resulted in the absolute quantification of 127 ECM and ECM-associated proteins of interest in our tumor samples: 24 specific to human (H), 36 specific to mouse (M) and 67 with shared (S) peptide sequences (Figure 1B). CD146\textsuperscript{pos} CAF and CD146\textsuperscript{neg} CAF derived tumors were distinguished from one another based on hierarchical clustering of the complete proteome - all 127 proteins - (Supplemental Figure 1), as well as clustering based on the human or shared proteins. Interestingly, the CAF derived tumors were not hierarchically clustered based on the mouse preoteome. Principle Component Analysis (PCA) confirmed the establishment of two unique TME profiles dependent on CAF subtype (Figure 1C). These data suggest that in our model the human (i.e. CAFs and tumor cells) secretome dictated the TME composition to a greater extent than the host (i.e. mouse).

\textbf{The TME of CD146\textsuperscript{neg} CAF derived tumors is enriched for pro-metastatic proteins.}
We used functional matrisome analysis to group ECM proteins according to their gene ontology functional classification as previously described (10, 12). Organizing the data in this way allowed us to observe broad differences in CD146\textsuperscript{neg} CAF versus CD146\textsuperscript{pos} CAF derived TME. In CD146\textsuperscript{neg} CAF influenced tumors, the analysis revealed significantly decreased abundance of basement membrane proteins and significantly increased abundance of ECM regulators (i.e. LOX) and structural ECM proteins (i.e. FN1, TNC, collagens) (Figure 2A). These changes indicate that CD146\textsuperscript{neg} CAF containing tumors underwent substantial ECM remodeling supportive of cancer cell invasion, disease progression, and metastatic development. Of the 127 detected proteins, 73 proteins were identified as having greater than 1.5-fold difference (p<0.055) between CD146\textsuperscript{pos} CAF and CD146\textsuperscript{neg} CAF tumors (Figure 2B and Supplemental Table 1). In MCF-7/CD146\textsuperscript{pos} CAF tumors, 48 proteins were expressed significantly more compared to MCF-7/CD146\textsuperscript{neg} CAF tumors; 3 human specific ECM proteins were increased (THBS1 – 2.17-fold, p = 0.009; COL18A1 – 1.96-fold, p = 0.014; and Agrin – only expressed in tumors with CD146\textsuperscript{pos} CAFs), 27 were identified as shared and 18 coming from mouse. In contrast, MCF-7/CD146\textsuperscript{neg} CAF tumors showed significant increase in 25 proteins, of these 15 were human specific and 10 were identified as shared, none from mouse. These data support the conclusion that interactions between the human CD146\textsuperscript{neg} CAFs and MCF-7 cells promoted an increasingly reactive TME compared to interaction between CD146\textsuperscript{pos} CAFs and MCF-7 cells.

MCF-7/CD146\textsuperscript{pos} CAF tumors expressed significantly more laminin proteins which are major components of the basement membrane and act as an important barrier to tumor cell invasion (Laminin \(\alpha_{1}^{M}\) and Laminin \(\alpha_{2}^{S}\) were restricted to CD146\textsuperscript{pos} CAF tumors and Laminin \(\beta_{2}^{M}\), Laminin \(\gamma_{1}^{M}\), Laminin \(\beta_{1}^{S}\), Laminin \(\beta_{2}^{S}\), Laminin \(\gamma_{1}^{S}\) and increased respectively by: 3.2-fold, p = 0.033; 12.5-fold, p = 0.049; 12.5-fold, p = 0.055; 3.6-fold, p = 0.011; and 9.1-fold, p = 0.046). In contrast, CD146\textsuperscript{neg} CAF containing tumors expressed significantly more human specific collagens (COL) associated with tumor progression and metastasis (COL4A1 and COL5A3 were only detected in CD146\textsuperscript{neg} CAF tumors and others were strongly upregulated including COL1A2 – 14.8-fold, p = 0.031; COL6A1 – 10.1-fold, p = 0.021; COL6A2 – 12.2-fold, p = 0.021; and COL6A3 – 4.3-fold, p = 0.060) (14-17). Furthermore, lysyl oxidase (LOX), which catalyzes formation of collagen cross-links, was expressed exclusively in tumors mixed with CD146\textsuperscript{neg} CAFs. Human produced ECM glycoproteins Fibronectin 1 (FN1) and Tenascin-C (TNC) were increased 50-fold and 21-fold respectively (p = 0.001 and p =
0.002) (Figure 2B and Supplemental Table 1). Over-expression of FN1 has been shown to increase both tumor migration and invasion and is linked to higher risk for metastasis based on its ability to align and interact directly with collagen, TNC (18, 19) and cancer cell integrins (18, 20). TNC has been shown to co-localize with fibers perpendicular to the tumor border (12), a structural feature associated with poor prognosis in hormone receptor positive breast cancer. However, the contribution of TNC in ER+ breast cancer metastasis formation has not been fully elucidated (21, 22).

**CD146<sup>neg</sup> CAFs promote ER+ breast cancer metastasis in vivo.**

Several studies have shown that the metastatic process is accompanied by significant changes in abundance and organization of collagen fibers deposited within the ECM (23, 24). We used Gomori’s Trichrome stain to observe changes in collagen deposition in tumors excised (25) from the mammary fat pad in our cohort of mice (Figure 2C). Arrangement of the collagen fibrillar networks in the center of the tumors were similar between CAF subtypes. However, differences were observed along the tumor edge. For example, in tumors mixed with CD146<sup>neg</sup> CAFs the border areas contained thin fibers oriented perpendicular to the tumor edge. This was in contrast to the more parallel fibrillar arrangement along the borders of tumors mixed with CD146<sup>pos</sup> CAFs.

To better quantify these observations, we quantified sections stained with Picrosirius Red under polarized light. Birefringence in the inner portion of tumors was not significantly different between CAF subtypes (Figure 3A); however, there was a significant decrease of birefringence along the tumor edges in tumors with CD146<sup>neg</sup> CAFs (Figure 3B). These results are consistent with our observations of a network of aligned fibers associated with the edges of CD146<sup>neg</sup> CAF containing tumors.

Several studies have illustrated that distinctive patterns of collagen reorganization occur during breast cancer progression, termed Tumor-Associated Collagen Signatures (TACS) (25-27). We applied second harmonic generation (SHG) imaging to visualize stromal collagen and infer mechanical properties based on the appearance of wavy (i.e. relaxed) or straight (i.e. stiff) collagen fibers, followed by blinded post-hoc evaluation of TACS (28-30). The TACS scale is based on scoring the abundance and orientation of collagen fibers around the tumor border, with TACS-1 defined as collagen that appears wavy, curly and random, with increased fiber accumulation; TACS-2 defined as straightened collagen fibers that align tangentially to the tumor border, and
TACS-3 defined as straight collagen fibers that align perpendicular to the tumor border. Increased TACS scores correlate with disease progression and worse patient outcomes (26). Tumors with CD146\textsuperscript{pos} CAFs had significantly more TACS1 than TACS3 scoring (Figure 3C) which is indicative of decreased metastatic potential, whereas tumors with CD146\textsuperscript{neg} CAFs had equal distribution between all TACS scores. In combination with our proteomics analysis and collagen staining results, our data indicate that CAF subtypes drive ECM remodeling in tumors.

We next wanted to determine if these ECM changes would lead to differences in metastatic outcome. We chose MCF-7 cells for \textit{in vivo} studies because this ER+ cell line has a well documented low potential for development of distant organ metastases in animal models. As expected, lymph node metastases were more common, observed in 33% of mice with MCF-7/CD146\textsuperscript{pos} CAF tumors, compared to 86% of mice with MCF-7/CD146\textsuperscript{neg} CAF tumors. This difference did not reach statistical significance (Figure 4). However, 86% of mice bearing tumors mixed with CD146\textsuperscript{neg} CAFs developed lung metastases, compared to 16% of those with CD146\textsuperscript{pos} CAFs (p = 0.021) (Figure 5A-B).

In order to further quantify the \textit{in vivo} metastatic disease burden, we paraffin embedded the whole lung of each mouse (N = 7 for CD146\textsuperscript{neg} CAF and N = 6 for CD146\textsuperscript{pos} CAF), stained for human specific pan-cytokeratin and counted positive cells in all five lung lobes each section (Figure 5C). In this analysis, we identified positive cells in lungs from mice with both CAF subtypes. However, mice with MCF-7/CD146\textsuperscript{neg} CAF tumors had significantly more lesions per lung (69% increase, p<0.05; Figure 5D). Furthermore, we counted the number of cells per lesion and classified each lesion as single cell, micro-metastasis (≤20 cells) or macro-metastasis (>20 cells) (Figure 5E). We found that the lungs from mice with CD146\textsuperscript{neg} CAF bearing tumors had significantly more micro-metastases compared to those with CD146\textsuperscript{pos} CAFs (86% increase, p<0.01). We anticipate that the micro-metastases would have further developed into larger lesions if given time to grow, but this study was restricted to 11 weeks growth or primary tumors reaching 2 cm in any direction, at which time all samples were collected. Our data show that CD146\textsuperscript{neg} CAFs enhanced the metastatic potential of MCF-7 cells compared to CD146\textsuperscript{pos} CAFs.

We also found evidence that compared to CD146\textsuperscript{pos} CAFs, CD146\textsuperscript{neg} CAFs may encourage tumor growth, as the average volume of excised tumors was significantly different (MCF-7/CD146\textsuperscript{neg} CAF, 570 +/-
313 mm$^3$ versus MCF-7/CD146$^{pos}$ CAF, 195 +/- 99 mm$^3$, p = 0.0171; Figure 5F). Surprisingly, when we graphed CD146$^{pos}$ CAF bearing tumors alone (blue dots), CD146$^{neg}$ CAF bearing tumors alone (red dots) or combined, there was no correlation between final tumor volume and the number of total lesions per lung (Figure 5G). Our experimental findings are in agreement with the recent extensive analysis where metastatic outcomes and nodal involvement in a cohort of 819,647 breast cancer patients did not correlate with primary tumor size at the time of diagnosis (31).

**TME based gene signature predicts lymph node involvement in patients.**

Breast cancer is recognized as a systemic disease with metastatic potential regardless of the size of the primary tumor. We hypothesized that the potential of small primary tumors (T1) to develop early metastases is partly dependent on their ECM composition. Therefore, we generated a signature from our ECM proteomics data set, based on significant difference (p < 0.05) between the CAF subtypes (identified as human specific or shared, Supplemental Table 2). We then used data from 1,009 breast cancer patients described in The Cancer Genome Atlas (TCGA Research Network, https://www.cancer.gov/tcga) and classified primary tumors based on their ECM signature as high or low expressers. The gene signature of ECM proteins corresponding to an CD146$^{neg}$ CAF containing tumor was highly predictive of lymph node involvement at time of diagnosis for both T1 (tumor size <2cm, p = 0.015) and T2 (tumor size between 2-5cm, p = 0.006) tumors. High gene expression demonstrated a metastatic ECM (derived from the proteome of CD146$^{neg}$ CAFs) and low gene expression was representative of a non-metastatic ECM (derived from the proteome of CD146$^{pos}$ CAFs) (Figure 5H). The relationship did not hold for T3 tumors. These data support the role of ECM in development of early breast cancer metastases. In order to perform further mechanistic studies, we next established an *in vitro* spheroid assay.

**CD146$^{neg}$ CAFs promote breast cancer cell invasion.**

We used a three-dimensional spheroid assay in which 600 red CD146$^{pos}$ CAFs or CD146$^{neg}$ CAFs were mixed with 3000 nuclear-green tagged MCF-7 (Figure 6A) or MDA-MB-231 (Figure 6B) tumor cells, representing one breast cancer cell line with low invasive potential and one breast cancer cell line with high invasive potential. The mixed cells formed tight spheres with the outer most shell being comprised mostly of
tumor cells and the inner area generally divided into two components; one dense region of fibroblasts and a second diffuse area of tumor cells mixed with fibroblasts. Over a defined time course (21 days for MCF-7 cultures; 6 days for MDA-MB-231 cultures), CD146\textsuperscript{neg} CAF protrusions were documented exiting the spheres while tumor cells closely followed and invaded into the surrounding matrix. Both MCF-7 and MDA-MB-231 invaded significantly more in spheroids mixed with CD146\textsuperscript{neg} CAFs. MCF-7 invasion was not inhibited by CD146 over-expression in CD146\textsuperscript{neg} CAFs (Supplemental Figure 2A-C). To further confirm results we used two ER+ patient derived breast cancer cell lines UCD65 and UCD46, which were gfp-labelled. Both UCD65 (Figure 7A-C) and UCD46 (Figure 7D-F) cells demonstrated significantly less invasion when cultured alone or with CD146\textsuperscript{pos} CAFs compared to co-culture spheroids with CD146\textsuperscript{neg} CAFs.

**Loss of TNC significantly decreases spheroid invasion.**

FN1 was the most abundant differentially expressed protein in our ECM analysis. Its role in the TME is permissive for breast cancer metastasis and has been well documented (18, 20, 32). However, the role of TNC specifically regarding metastatic progression is not as well understood and has rather been suggested to be more closely associated with onset of Tamoxifen resistance (21). Proteomic analysis revealed TNC as the second most abundant differentially expressed ECM protein, encouraging further investigation of TNC’s role in our invasion phenotype. We first verified our proteomic data by staining for TNC in our in vivo cohorts (Supplemental Figure 3A). Indeed, tumors containing CD146\textsuperscript{neg} CAFs had significantly higher TNC protein expression than tumors with CD146\textsuperscript{pos} CAFs. Next, we co-cultured MCF-7 cells on a monolayer of CAFs consisting of 50% CD146\textsuperscript{pos} CAFs and 50% CD146\textsuperscript{neg} CAFs and stained the cultures for the MCF-7 cell marker pan-cytokeratin, CDCP1, which specifically marks CD146\textsuperscript{neg} CAFs, TNC and nuclear fast red to demonstrate the presence of CD146\textsuperscript{pos} CAFs (Supplemental Figure 3B-C). As expected, TNC staining in these cultures was strictly confined to areas populated by CD146\textsuperscript{neg} CAFs. We next generated 3 different CD146\textsuperscript{neg} CAF cell lines expressing shRNA against TNC. Quantitative qPCR and western blot analysis confirmed between 40-80% loss of TNC expression in the 3 cell lines compared against the control line which expressed shRNA against GFP (GFP was not expressed in the CD146\textsuperscript{neg} CAF cell line; Supplemental Figure 3D). In spheroids grown under serum-starved conditions (0.5% FBS), invasion of MCF-7 and MDA-MB-231 cells was significantly inhibited by the loss of TNC expression in CD146\textsuperscript{neg} CAFs (Figure 8A-B). The decrease in MCF-7 spheroid co-cultures was
by an average of 27\%, or 58\% when analyzing only shRNA TNC 88 and 88b. For MDA-MB-231 spheroid co-cultures, the average decreased invasion was 19\%.

TNC has been shown to activate the extracellular signal-regulated kinase (ERK) pathway (33). We used immunohistochemistry to stain spheroid co-cultures, generated using CD146\textsuperscript{neg} CAFs expressing either control shRNA or shRNA against TNC, for phosphorylated extracellular signal-regulated kinase (pERK) and TNC. The invasion fronts of spheroids generated with shRNA control CD146\textsuperscript{neg} CAFs had significantly increased expression of TNC and phospho-ERK compared to spheroids generated with TNC shRNA CD146\textsuperscript{neg} CAFs (Figure 9 and Supplemental Figure 4). Taken together, these data suggest that loss of TNC expression leads to decreased ERK signaling and significantly decreased invasion of breast cancer cells.

**Loss of epidermal growth factor receptor (EGFR) signaling significantly decreases spheroid invasion.**

TNC contains 14.5 EGF-like repeats and is a low affinity EGFR ligand (34). Activation of EGFR is linked to increased invasion and tumor metastasis (35). The positive correlation between TNC expression and increased phosphorylated ERK, which is a common downstream effector protein of the EGFR pathway (36) suggested that EGFR signaling was a component of our invasion phenotype. When spheroid co-cultures were generated in serum-starved conditions with nuclear-gfp tagged MCF-7 or MDA-MB-231 breast cancer cells, both cancer cell types were significantly less invasive with CD146\textsuperscript{pos} CAFs compared to CD146\textsuperscript{neg} CAFs (89\% and 47\% decrease respectively; Figure 10A-B). These data suggest that invasion in spheroid co-cultures with CD146\textsuperscript{pos} CAFs requires components supplied by the serum that can be compensated for in the spheroid co-cultures generated with CD146\textsuperscript{neg} CAFs. To test if invasion in the serum-starved spheroid co-cultures with CD146\textsuperscript{neg} CAFs required EGFR signaling, we treated them with gefitinib, an EGFR inhibitor. Due to the loss of signaling pathways dependent on cell-to-cell interactions that might support the invasion phenotype, spheroids consisting solely of tumor cells or CAFs were not generated for treatment with gefitinib. Addition of 5μM gefitinib, resulted in decreased invasion in MCF-7/CD146\textsuperscript{neg} CAF spheroid co-cultures by 63\% and by 18\% in MDA-MB-231/CD146\textsuperscript{neg} CAF spheroid co-cultures (Figure 10A-B). These data demonstrate an important role for the EGFR signaling pathway in the invasion phenotype of the spheroid co-cultures.
DISCUSSION

While it is widely accepted that TME remodeling is an enabling characteristic in breast cancer progression and treatment resistance, the generation of in vivo models and quantitative techniques capable of assessing the role of TME components in metastatic ER+ positive breast cancer has been the limiting factor. Historically, promising pre-clinical testing of drugs, such as EGFR inhibitors, designed to treat metastatic breast cancer all too often disappointed in the clinic. Many novel targeting agents, including immune check point inhibitors, are clearly dependent on TME and by extension on ECM composition. Our data reinforces the idea that TME remodeling is closely tied to metastatic progression and that we have a need to develop a deeper understanding of the interplay between tumor cells, CAFs, immune cells and the signaling changes pursuant to these interactions.

For ER+ breast cancer in particular, one of the impediments to studying the TME, is a lack of metastatic models that recapitulate natural disease evolution from the orthotopic site to distant organs (37). Through the use of CD146\textsuperscript{pos} CAFs or CD146\textsuperscript{neg} CAFs, we have been able to develop a model that consistently forms metastasis from the orthotopic site and has utility for comparative analysis of the TME. Our model produced increased macro-metastases when tumors were influenced by CD146\textsuperscript{neg} CAFs compared to CD146\textsuperscript{pos} CAFs and similar numbers of micro-metastases. Further investigation is required to determine if the micro-metastases in tumors influenced by CD146\textsuperscript{pos} CAFs have the appropriate stimuli to further develop into macro-metastases over time. Our model reinforces previous findings that the type of collagen deposited in the TME along with its organization around the tumor boundary are indicators of prognosis in an ER positive breast tumor. In addition, one of the unique aspects of our study is documentation of the ability to go beyond histologic description and use precise quantification of ECM components, including the ability to identify human versus host contributions. We show how consideration of TME components is predictive of lymph node involvement at the time of diagnosis in early stage breast tumors. These findings are independent of the clinically used IHC and genomic assays focusing on receptor status and proliferative index in hormone receptor positive breast cancer. Further refinement of this signature in a manner that also correlates with recurrence rates would add new information that may benefit patients and physicians who are weighing treatment options and deciding on the intensity of the treatment plan.
Development of metastatic disease in ER+ breast cancer is commonly associated with increased EGFR signaling (38). Our in vitro results support a role for EGFR signaling in breast cancer metastasis and showed that the type of CAF present in the TME determined involvement of EGFR mediated invasion. We also found that loss of TNC expression specifically in the CAF population resulted in decreased invasion which correlated with decreased phospho-ERK expression, suggesting that TNC activates EGFR and partly mediates an invasive phenotype in spheroid cultures. Although more studies are required to directly link the invasion phenotype with direct EGFR activation by TNC, our results recapitulate previous reports correlating increased TNC expression to higher risk for invasive breast cancer and several other cancer types (39-43). Interestingly, in a cohort of 1,286 primary ER+ breast tumors, TNC expression was also linked to Tamoxifen resistance (21). We previously showed that influence of CD146neg CAFs leads to Tamoxifen resistance, which potentially places TNC as a central player in the development of endocrine resistance as well as metastatic progression of hormone receptor positive breast cancer. TNC is a highly reactive TME protein and can mediate intracellular signaling in tumor cells through interactions with fibronectin and other cell integrins (44). Although our in vitro results suggest that interaction between EGFR and TNC represent an important axis in the invasion phenotype, the proteomic TME profile of tumors with CD146neg CAFs expressed several proteins capable of governing EGFR activation which indicates a complex in vivo signaling environment that is likely governed by more than TNC/ERK/EGFR interactions.

Our data highlights the importance for placing the TME in context with other known drivers of breast cancer progression such as endocrine receptor status. Taken together, this work provides a model to study the role of the TME in development of breast cancer metastasis and supports the need to consider and target cell-matrix interactions in future drug development.
METHODS

Cell culture

The human MCF-7 (p53 wildtype, ER-positive, luminal subtype), MDA-MB-231 (triple negative, EGFR-positive) breast cancer cell lines and the human stromal HS-5 fibroblast cell line were cultured in Modified Eagle’s Medium (MEM) supplemented with 10% fetal calf serum, L-glutamine, and HEPES buffer at 37 °C with a 5% CO₂/95% atmospheric air. The human stromal HS-27A fibroblast cell line was cultured in RPMI 1640 basal media supplemented with 10% fetal calf serum, L-glutamine, and HEPES buffer at 37 °C with a 5% CO₂/95% atmospheric air. In all co-culture experiments, the base media used was MEM. HS-5 cell lines expressing shRNA for TNC or the scrambled control were produced using standard viral transduction methods. Knockdown efficiency was determined by real time quantitative PCR. All cell lines were authenticated by STR profile testing in May 2016. All cell lines were validated to be mycoplasmā free prior to use in any in vitro or in vivo experiments using the Universal Mycoplasma Detection Kit from the American Type Culture Collection (ATCC, Manassas, VA).

We previously validated the HS-5 and HS27-A fibroblasts as genetically akin to primary human fibroblasts which we isolated from ER+ breast cancer patient tissue (5). However, unlike the HS27-A and HS5 fibroblast cell lines, patient CAFs are passage limited and difficult to expand in sufficient quantities for multiple in vitro studies and for large in vivo studies. Given these limitations and our previous validation, we chose to use HS5 and HS27-A fibroblasts as a reasonable substitute for our experiments.

Antibody and shRNA Sources

Lung IHC – Primary antibody to detect human tumor cells (MCF-7) was Pancytokeratin from Biorbyt (San Francisco, CA; orb386219; dilution 1:150). Secondary detection was with Anti-rabbit Alkaline Phosphatase IgG followed by Vector Blue Alkaline Phosphatase Substrate Kit from Vector Labs (Burlingame, CA; MP-5401 and SK-5300; dilutions according to manufacturer recommendations).

Co-cultures – Primary antibody to detect MDA-MB-231 and MCF-7 cells was Pancytokeratin from Bioss Antibodies (Woburn, MA; Bs-1712R; dilution 1:100). Primary antibody to detect HS-27A cells was CD146 from
Abcam (Cambridge, MA; ab75769; dilution 1:100). Primary antibody to detect HS-5 cells was CDCP1 from R&D Systems (Minneapolis, MN; AF2666; dilution 1:40). Primary antibody to detect TNC was from Abcam (Cambridge, MA; ab3970; dilution 1:100). Primary antibody to detect phospho-ERK was from ThermoFisher Scientific Invitrogen (Waltham, MA; 14-9109-80; dilution 1:200). All secondaries and chromogens were used according to manufacturer recommendations and purchased from Vector Labs (Burlingame, CA; MP-7401, SK-4105, MP-5405, SK-5300, MP-7402, SK-4605).

For shRNA and over-expression experiments, all vectors were purchased from the Functional Genomics Facility, a University of Colorado Cancer Center Shared Resource. Two TNC directed shRNA were used, TNC85 (TRCN0000230785) and TNC88 (TRCN0000230788). TNC88b is a second population of HS5 cells transduced with the TNC88b viral particles. The non-targeting control was SHC002. One MCAM (CD146) open reading frame (ORF) over-expression vector was used (06567).

**Invasion Assay**

Invasion was measured by generating single spheroids of either MDA-MB-231 or MCF-7 cell lines mixed with either HS-5 (CD146<sup>neg</sup> CAFs) or HS-27A (CD146<sup>pos</sup> CAFs) fibroblasts. We used the Cultrex 3D Cell Invasion Assay (Trevigen, Gaithesburg, MD) following manufacturer’s guidelines for generating spheres in a 96-well round bottom plate. Spheroids were then transferred to a flat-bottom plate with a set layer of invasion matrix, and sandwiched with a second layer of invasion matrix.

Nuclear-green tagged MCF-7 cells were mixed with uncolored CAFs and invasion was quantified on day 19. MCF-7 invasion was analyzed with FIJI (https://fiji.sc) for corrected total cell fluorescence (CTCF) to quantify the green fluorescent signal in the invasion region (https://theolb.readthedocs.io/en/latest/imaging/measuring-cell-fluorescence-using-imagej.html). Briefly, invasion CTCF was measured via freeform drawing using an Intuos2 tablet (Wacom, Portland, OR) to define the total tumor with invasion, the main sphere body and a non-fluorescent background sample (Supplemental Figure 4A). Invasion was calculated using the following formula:

\[
\text{Invasion CTCF} = (\text{Total tumor integrated density} - (\text{area of invasion} \times \text{background mean})) \\
- (\text{Spheroid Integrated Density} - (\text{area of spheroid} \times \text{background mean}))
\]


Invasion in spheroids generated by mixing nuclear-green tagged MDA-MB-231 with uncolored CD146\textsuperscript{pos} or CD146\textsuperscript{neg} was quantified on day 6 using Zen 2.3 lite. A circle was drawn around the main spheroid, and length of invasion arms were measured from the most distant tumor cell per arm to the spheroid body (Supplemental Figure 4B). To validate the methods, we re-analyzed MDA-MB-231 co-culture spheroids using the method for MCF-7 spheroids (Supplemental Figure 4C-D). Both methods produced highly reproducible results for the MDA-MB-231 spheroids which provide validation for the method used to measure MCF-7 spheroids. Images for these analyses measurements were captured on a Zeiss Axio Vert.A1 fluorescent microscope (Carl Zeiss Microscopy, Peabody, MA).

We next generated ds-red tagged CD146\textsuperscript{pos} and CD146\textsuperscript{neg} using viral transduction to generate more precise images of CAF and tumor cell interactions in the mixed spheroids and for comparison with higher magnification imaging on the Zeiss Axio Vert.A1 microscope. In order to obtain high magnification images without interference from the concentrated fluorescence in the sphere body, we had to close the aperture to focus zs-green labelled MCF-7 cells invaded away from the main sphere body in co-culture with CD146\textsuperscript{neg} CAFs. A single uncolored fibroblast can be seen at the tip of the invasion arm (Supplemental Figure 4E). In comparison, high resolution live imaging of gfp-labelled MDA-MB-231 with rfp-labelled CD146\textsuperscript{neg} (Supplemental Figure 4F) or CD14\textsuperscript{pos} (Supplemental Figure 4G) CAFs similarly demonstrate the generation of invading cells in arms comprised of CAFs and MDA-MB-231 cells with several arms of the MDA-MB-231/CD146\textsuperscript{neg} spheroid culture being tipped by CAFs. High-resolution live imaging was captured using an Olympus IX83 P2ZF (Olympus, Waltham, MA) Images using the two-color model were captured on day 21 for MCF-7/CAF spheroids and on day 6 for MDA-MB-231/CAF spheroids.

**Animal Experiments**

MCF-7 tumors labeled with ZS-green were established by mixing 1 x 10\textsuperscript{6} cells in Cultrex and injecting them into the mammary fat pad of NOD scid gamma (NSG) female mice. HS-27A or HS5 cells were mixed with the tumor cells at a 1:1 ratio (N = 7 mice per stroma subtype). Tumors were allowed to grow for 11 weeks or until they reached protocol limits (2cm measured in any direction) prior to removal. All tumors received continuous estrogen supplementation throughout the study, as previously described (45). Tumor measurements were
taken weekly throughout the duration of the experiment. Metastasis was monitored by examining excised organs with an Illumatool Light source (Lightools Research) attached to an Olympus camera. Organs were collected for IHC validation of whole mount results. Lymph node tumor validation was completed by Hematoxylin and Eosin staining. Lung tumor validation was monitored by staining for human specific pan-cytokeratin. IHC imaging was captured using (Olympus IX83 P2ZF, Waltham, MA). Second harmonic generation microscopy was performed using a Zeiss LSM780 confocal microscope equipped with a tunable infrared Coherent Chameleon Ultra II laser (Oberkochen Germany).

**IHC Image Capture and Analysis:** IHC imaging for lymph and lung tumor validation was captured using an Olympus IX83 P2ZF microscope (Waltham, MA). Picrosirius red staining was captured using a Nikon Eclipse Ni equipped with a Nikon DS-Ri2 camera and a polarized light filter (Melville, NY). Second harmonic generation microscopy was performed using a Zeiss LSM780 confocal microscope equipped with a tunable infrared Coherent Chameleon Ultra II laser (Oberkochen, Germany). IHC was quantified using FIJI (46).

**Proteomics Analysis**

**Proteomic Sample Preparation:** Tissue samples were flash frozen in liquid nitrogen and powderized using a ceramic mortar and pestle. Tissue was dried overnight in a lyophilizer and weighed tissue (approximately 1 mg of each) was homogenized in freshly prepared high-salt buffer (50 mM Tris-HCl, 3 M NaCl, 25 mM EDTA, 0.25% w/v CHAPS, pH 7.5) containing 1x protease inhibitor (Halt Protease Inhibitor, Thermo Scientific) at a concentration of 10 mg/mL. Homogenization took place in a bead beater (Bullet Blender Storm 24, Next Advance, 1 mm glass beads) for 3 min at 4 °C. Samples were then spun for 20 min 18,000 x g at 4 °C, and the supernatant removed and stored as the cellular fraction. Sequential tissue extraction on the remaining pellet were performed as previously described (13).

**Trypsin Digestion:** 100 µL of the cellular fraction (combined fractions 1, 2 and 3) and 200 µL of fractions 4 & 5 of all samples were subsequently subjected to reduction, alkylation, and enzymatic digestion with trypsin. 100 fmols of each stable isotope labeled (SIL) peptide (770 peptides total, 170 mouse, 188 human, 412 shared) were spiked into 100 µL of sample to allow for four injections per sample (50 fmols eQ 1-6 per injection) (47,
A filter-aided sample preparation (FASP) approach, as well as C18 cleanup, was performed as previously described (49).

**LC-SRM Analysis:** Samples were analyzed by LC-SRM and LC-MS/MS as described (48). Equal volumes from each post-digestion sample were combined and injected every third run and used to monitor technical reproducibility. Skyline (version 3.7) was used for method development and to extract the ratio of endogenous light peptides to heavy internal standards from LC-SRM data for protein quantification as described (50). Limits of detection, quantification, and dynamic range were determined for each peptide as previously described (47). Final fmol values are expressed as fmol/mg where milligrams represent milligrams of starting dry tissue weight.

**Proteomic Data Analysis:** Principal component analysis (PCA) was performed using GraphPad Prism (version 8) and partial least squares-discriminant analysis (PLS-DA) was performed using the MetaboAnalyst (version 3.0) with sum and range scaling normalizations (51). For proteomic analysis, two group comparisons were made and we used the false discovery rate (FDR) method for multiple measurement correction. Heat maps were generate with Heatmapper using Average Linkage for clustering and Pearson for Distance Measurement (52).

**Statistical Analysis**

Statistical analysis was completed using R-package software for the gene expression data sets and with GraphPad Prism 8 analytical software (La Jolla, CA) for all other experiments. For single comparisons we used unpaired two-tailed t-tests with assumptions of parametric Gaussian distribution and equal standard deviations. For multiple comparisons we used ordinary one-way ANOVA analysis with Tukey multiple comparisons tests. For contingency analysis we used Fishers Exact Test. Significance was set at p<0.05. All cell culture experiments consisted of at least N = 4 or more and were repeated at least once. Our in vivo experiment consisted of N = 7 animals per stromal subtype. Outliers were identified using the GraphPad Prism Outlier ROUT function with Q = 1%. All data are presented as mean +/- the standard deviation.
Study Approval

All animal experiments were conducted in an AAALAC accredited facility at the University of Colorado Denver under an IACUC approved protocol.

AUTHOR CONTRIBUTIONS

HMB, KH and PK designed experiments. HMB, ASB, EK, JCH, ALH and JF-S conducted experiments. HMB, ASB, EK, AEG, KH and PK analyzed results. HMB, DMC, PO, KBH, CAS, KH and PK wrote and edited the manuscript. HMB, KH and PK conceived the study. PK supervised the study.
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Figure 1. Tumors bearing CD146\textsuperscript{neg} or CD146\textsuperscript{pos} CAFs were identified based on their TME proteomic profiles. (A) Pictorial representation of the proteomics approach used to quantitatively measure human produced versus host derived proteins in the tumor microenvironment. (B) Heatmap illustrations show how tumors cluster according to CAF subtype by the Human Secretome and Shared Secretome (* = Arrestin/Core Protein; ** = H2A-A-A-K), but not according to the Mouse Secretome. Proteomics was completed in triplicate (CD146\textsuperscript{pos}.1-.3 and CD146\textsuperscript{neg}.1-.3). Low to high expression = green to red; grey indicates no expression. (C) Principle component analysis of total extracellular matrix (ECM) scores demonstrates distinct TME environments that classify tumors based on CAF subtypes.
Figure 2. Tumors bearing CD146<sup>neg</sup> CAFs had increased abundance of pro-metastatic proteins. (A) Functional class plot based on gene ontology and functional classification indicates significant changes in the TME of tumors based on CAF subtype. Each bar in the functional matrisome graph represents the total abundance of all proteins found in each functional class of matrisome proteins. Analyzed by multiple t-tests, *p<0.05, **p<0.01. (B) Volcano plot distribution of all detected proteins. Blue dots represent proteins detected with significantly more abundance in MCF-7/CD146<sup>pos</sup> compared to MCF-7/CD146<sup>neg</sup> tumors. Red dots represent proteins detected with significantly more abundance in MCF-7/CD146<sup>neg</sup> compared to MCF-7/CD146<sup>pos</sup> tumors. (C) Representative images of tumors stained with Gomori’s Trichrome in the tumor center and at the tumor edge of MCF-7/CD146<sup>neg</sup> and MCF-7/CD146<sup>pos</sup> tumors. Scale bars in tumor center = 200µm; tumor edge = 60µm. The inset is zoomed 200% to show blue fiber arrangement along the tumor edge. N = 6 tumors stained for CD146<sup>pos</sup> and N = 7. tumors stained for CD146<sup>neg</sup>. 
Figure 3. ECM structural organization is determined by CAF subtype. Picrosirius Red (PSR) staining quantified under polarized light. (A) Birefringence in the tumor center was not significantly different between CAF subtypes. (B) Tumors influenced by CD146<sup>pos</sup> CAFs had significantly increased birefringence compared to CD146<sup>neg</sup> CAFs along tumor edges. (C) Second Harmonic Generation (SHG) Microscopy and quantified TACS scoring showed CD146<sup>pos</sup> CAFs influenced tumors had significantly more regions scored as TACS1 compared to TACS3. For PSR analysis at least five images were quantified per tumor region on N = 6 tumors for CD146<sup>pos</sup> and N = 7 tumors for CD146<sup>neg</sup>; *p < 0.05 by unpaired two-tailed t test. For SHG analysis at least 14 images per tumor were quantified on N = 4 CD146<sup>neg</sup> and N = 4 CD146<sup>pos</sup> tumors; *p < 0.05 by 2-way ANOVA followed by Tukey’s multiple comparisons restricted to within groups. All scale bars are 100 µM.
Figure 4. **CD146\textsuperscript{neg}** promote metastasis in vivo. MCF-7 tumors mixed with either CD146\textsuperscript{neg} or CD146\textsuperscript{pos} CAFs were grown in the mammary fat pad of mice. Representative images of positive lymph nodes in whole tissue (green fluorescence) or by Hematoxylin and Eosin in mice with MCF-7/CD146\textsuperscript{pos} or MCF-7/CD146\textsuperscript{neg} tumors. The presence of lymph node metastasis was not significantly different between CAF subtypes. Scale bar = 1cm. Contingency analysis used Fisher's exact test. N = 7 mice with CD146\textsuperscript{neg} and N = 6 mice with CD146\textsuperscript{pos}.
Figure 5. CD146\textsuperscript{neg} promote metastasis in vivo. MCF-7 tumors mixed with either CD146\textsuperscript{neg} or CD146\textsuperscript{pos} CAFs were grown in the mammary fat pad of mice. (A-B) Representative image and quantification of metastasis to the lung of mice with MCF-7/CD146\textsuperscript{neg} or MCF-7/CD146\textsuperscript{pos} tumors in whole tissue (green fluorescence). Scale bars = 100\textmu m. Contingency analysis used Fisher’s exact test. (C) Lesions were detected by immunohistochemistry staining with human specific pan-cytokeratin (blue) and counted in all lobes. (Blue arrows show single cell, micro-metastasis (\leq 20 cells) or macro-metastasis (>20 cells)). (D) Analysis of the total number of metastatic lesions showed that mice with MCF-7/CD146\textsuperscript{neg} tumors had significantly more lesions than those with MCF-7/CD146\textsuperscript{pos} tumors. Total analysis used Mann-Whitney test. (E) Lesions were grouped according to the number of cells per lesion. Mice bearing MCF-7/CD146\textsuperscript{neg} tumors had significantly more micro-metastases compared to MCF-7/CD146\textsuperscript{pos} tumors. Lung lesion by size analysis used a 2-way ANOVA. Scale bars = 100\textmu m. (F) Tumors with CD146\textsuperscript{neg} CAFs grew significantly larger than tumors with CD146\textsuperscript{pos} CAFs. Analysis was by 2-way ANOVA followed by multiple comparison with Tukey test. (G) The number of metastatic lung lesions did not correlate with the size of the tumor when the tumors were combined and analyzed as a total group (p = 0.6616, R\textsuperscript{2} = 0.01806) or separated by CD146\textsuperscript{neg} (red; p = 0.3574, R\textsuperscript{2} = 0.1563) or CD146\textsuperscript{pos} (blue; p = 0.4953, R\textsuperscript{2} = 0.1231) CAF influence. Correlation was tested using Pearson Correlation analysis. (H) Gene signature based on proteomic analysis is predictive of the presence of lymph node metastasis at diagnosis in breast cancer patients with small tumors. N = 7 mice with CD146\textsuperscript{neg} and N = 6 mice with CD146\textsuperscript{pos}; *p<0.05; **p<0.01; ****p<0.0001.
Figure 6. CD146\textsuperscript{neg} CAFs promote breast cancer cell invasion. (A) MCF-7 breast cancer cells expressing green fluorescent protein (GFP) mixed with CD146\textsuperscript{neg} or CD146\textsuperscript{pos} CAFs (expressing dsRed – red) and grown in spheroid invasion assays. MCF-7/CD146\textsuperscript{pos} spheroids were significantly less invasive compared to MCF-7/CD146\textsuperscript{neg} spheroids. Scale bars = 400 µm (B) MDA-MB-231 breast cancer cells, expressing nuclear GFP were mixed in a 3D-spheroid assay with either CD146\textsuperscript{pos} or CD146\textsuperscript{neg} CAFs expressing dsRed. MDA-MB-231/CD146\textsuperscript{pos} spheres invade significantly less compared to MDA-MB-231/CD146\textsuperscript{neg}. Scale bars = 200 µm. ****p < 0.0001 by t-test; Experiments were individually repeated three times for MCF-7 and twice for MDA-MB-231. Symbols with the same shape represent replicates within the same experiment, symbols with different shapes represent repeated experiments. Individual symbols represent replicates within the same experiment, different symbols represent repeated experiments.
Figure 7. CD146\textsuperscript{neg} CAFs, but not CD146\textsuperscript{pos} CAFs promote invasion of ER\textsuperscript{+} patient-derived breast cancer cell lines UCD46 and UCD65. Representative image series showing minimal invasion for (A-B) patient-derived UCD65 ER\textsuperscript{+} cancer cell line in spheroid co-cultures. UCD65 cells were GFP-labelled and cultured with unlabeled CD146\textsuperscript{neg} or CD146\textsuperscript{pos} CAFs. Insets show zoomed in regions demonstrating areas of invasion with arrows pointing to representative invasive cells. (C) Quantification of invasion showing significantly increased invasion by UCD65 cells in spheroid co-cultures with CD146\textsuperscript{neg} CAFs compared to co-cultures alone or with CD146\textsuperscript{pos} CAFs. (D-F) Repeat experiment shown in panels A-B using GFP-labelled ER\textsuperscript{+} patient derived breast cancer cells UCD46. N = 4-6 spheroids quantified per group. Scale bars are 200 µm. Statistical analysis was ordinary 1-way ANOVA followed by Dunnet’s multiple comparisons test **** = p < 0.0001.
Figure 8. Loss of tenascin-C expression decreases breast cancer cell invasion. (A) MCF-7 or (B) MDA-MB-231 spheroids formed in serum starved conditions (0.5% FBS) with CD146$^{\text{neg}}$ CAFs expressing control shRNA (shCont) had significantly more invasion than spheroids with shRNA against TNC (85, 88, 88b). Experiments were individually repeated twice for MCF-7 and five times for MDA-MB-231. Symbols with the same shape represent replicates within the same experiment, symbols with different shapes represent repeated experiments. Scale bars = 500 µm. *p<0.05; **p<0.01; ****p<0.0001; spheroids were analyzed ordinary one-way ANOVA followed by Dunnett’s multiple comparison test of each column mean with the mean of shCont.
Figure 9. MCF-7 spheroid invasion fronts expressing TNC have significantly more phospho-ERK expression. MCF-7 spheroids were co-cultured with CD146\textsuperscript{neg} CAFs (HS5) expressing (A) control shRNA (shCont) or (B) shRNA against TNC (shTNC88). Immunohistochemistry for phospho-ERK (pERK, blue) and TNC (brown) in 3 representative spheroids and counterstained with Nuclear Fast Red. Insets are marked by a blue box. (C) Intensity scores on a scale of 1-3 (1 = minimal staining, 2 = moderate staining, 3 = intense staining) for pERK and TNC. 4-6 serial images were scored per spheroid with N = 3 spheroids per group. Scale bars are 100mm. ****p<0.0001; statistics were completed using an ordinary 1-way ANOVA followed by Tukey's multiple comparison test.
Figure 10. Loss of EGFR signaling decreases breast cancer cell invasion. (A) Compared to MCF-7 or (B) MDA-MB-231 spheroids mixed with CD146\textsuperscript{neg} CAFs, spheroids with CD146\textsuperscript{pos} CAFs or treated with the EGFR inhibitor gefitinib (Gefit) were significantly less invasive. Scale bars = 500µm. *p<0.05; **p<0.01; ****p<0.0001, spheroids were analyzed with One-way Anova followed by Tukey’s multiple comparison test. All experiments were repeated 3 times. Symbols with the same shape represent replicates within the same experiment, symbols with different shapes represent repeated experiments.