Why the stroma matters in breast cancer
Insights into breast cancer patient outcomes through the examination of stromal biomarkers

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Survival and recurrence rates in breast cancer are variable for common diagnoses, and therefore the biological underpinnings of the disease that determine those outcomes are yet to be fully understood. As a result, translational medicine is one of the fastest growing arenas of study in tumor biology. With advancements in genetic and imaging techniques, archived biopsies can be examined for purposes other than diagnosis. There is a great deal of evidence that points to the stroma as the major regulator of tumor progression following the initial stages of tumor formation, and the stroma may also contribute to risk factors determining tumor formation. Therefore, aspects of stromal biology are well-suited to be a focus for studies of patient outcome, where statistical differences in survival among patients provide evidence as to whether that stromal component is a signpost for tumor progression. In this review we summarize the latest research done where breast cancer patient survival was correlated with aspects of stromal biology, which have been put into four categories: reorganization of the extracellular matrix (ECM) to promote invasion, changes in the expression of stromal cell types, changes in stromal gene expression, and changes in cell biology signaling cascades to and from the stroma.

Mechanical Forces are Key Regulators of the Mammary Gland Phenotype

In the adult mammary gland chemical cues (hormones), immune cell surveillance, extracellular matrices, stromal cells and mechanical forces are all present; the degree of influence each of these has on the tumor is an area of significant active study, and is expanding our understanding of how tumor biology encompasses much more than the properties of the tumor epithelium. This is particularly relevant when considering metastasis and the events that occur as cells invade into the stroma of their local environment.

The adult mammary gland is highly organized in terms of its stratification of cell and extracellular matrix (ECM) layers, which

is preserved throughout the arborization of ducts and lobules that comprise the breast. The epithelium itself is composed of luminal cells, the milk-producing cells, surrounded by a layer of basal or myoepithelial cells whose contraction aides in the expulsion of milk. This layer of myoepithelial cells is also responsible for creating and maintaining the next layer, the basement membrane, a specialized structure composed of collagen IV, laminin and proteoglycans that is extremely dense but is a mere 0.2 μm thick. This entire structure is then surrounded by a stromal extracellular matrix, comprised predominantly of collagen I.[1] The concept is emerging that the ECM provides both biochemical and mechanical signaling cues to the cells of the mammary gland. Cells bind specifically to ECM ligands through receptors that include the integrin family and cell-surface proteoglycans. It is well established that integrins and proteoglycans cluster into focal adhesions, which form a signaling complex able to activate numerous second messengers. More recently, it is appreciated that these same focal adhesion complexes exist under tension, balanced by contractile forces from within cells generated by the actin-myosin cell cytoskeleton and from without mediated by the stiffness of the ECM.[2] This theme of tensional homeostasis also applies to the layers of cells and ECM in the breast.[3-5] In the absence of a tumor, breast epithelial cells are tensionally "in tune" with the myoepithelial cell layer, which in turn is in tune with the basement membrane. Emerging is the concept that changes in tensional forces and extracellular matrix stiffness could be used to define disease progression. Indeed, during the early stages of tumor formation up to the carcinoma in situ stage, these layers are all still present, albeit slightly altered, and it is not until the tumor breaches the basement membrane, where mechanical forces between the cells and the ECM will need to adapt to this new tensional landscape, that the tumor begins to dramatically increase in size and invasion occurs. Therefore, the transition to the invasive phenotype may be in part a mechanical one.[6]

Changes to the Stroma are Predictive of Patient Survival

In breast cancer, the most well-established link between stromal biology and tumor progression has been made by Boyd et al., who found that women with mammographically dense breasts have a 2

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to 6-fold increase in their susceptibility to develop breast cancer, making it one of the highest risk factors among known biomarkers.[7] Strikingly, in heterogeneous breast tissue, tumors most often arise within the densest parts of the tissue.[8,9] The increase in mammographic density is associated with both an increase in cellularity, as well as increased concentration of collagen in the breast stroma, with the increased collagen representing the most significant correlation.[10] It has been shown that high mammographic density (> 75%) is an independent predictor of localized, but not distant, recurrence following radiotherapy (hazard ratio = 4.30, p = 0.071).[11] A separate study argues that density is not a predictor of recurrence at all in patients that have not received radiotherapy, but in women who have, recurrence is 5.7 times more likely in a dense breast, possibly indicating that the radiation itself is causing changes to the stroma that promote recurrence.[12] Evidence of a genetic basis for differences in breast density comes from studies of family history, familial aggregation and twin and segregation analyses (reviewed in Kelemen et al.) where it appears that circulating levels of hormones and insulin-like growth factor-1 (IGF-1) both play a role in altering gene expression in dense breasts.[13-15]

Despite the correlation, a causative link has not definitively been made between increased stromal collagen density and tumor formation. Animal studies have been useful to address this issue. Increased stromal density occurs in mice bearing a collagenase-resistant transgene for the α1 chain of collagen I, the Col1a1[tm1jae] mouse.[16] These animals show a 3-fold increase in tumor formation and metastasis driven by the MMTV-PyVT transgene compared with wild-type litter mates, suggesting a functional link between increased collagen and mammary tumor progression.[17] Similarly, increasing collagen cross-linking through the expression of lysyl oxidase, which makes the tissue stiffer, correspondingly increases mammary tumor formation.[18]

## Tumor Associated Collagen Signatures (TACS)

A major technical challenge in understanding the relationship between the ECM and cancer cells has been the traditional lack of tools for looking at cell-ECM interactions specifically and precisely. Traditional clinical imaging approaches for breast cancer do not provide the resolution needed to see these interactions at the cellular scale, nor the specificity for detecting both collagen and cells. Traditional research methods have also not offered this combination. The relatively recent application of nonlinear optics to breast cancer has offered a new set of tools that offer high cell resolution both spatially and temporally combined with the ability to image live tissue noninvasively.[19,20] The nonlinear optical approaches of second harmonic generation (SHG) allows collagen to be imaged within 2D and 3D samples with high resolution.[21] In particular, collagen changes at the tumor/stromal boundary have been imaged and classified as markers of mammary carcinoma progression, termed tumor-associated collagen signatures (TACS) (Figs. 1–3).[17,20] Using mouse models that recapitulate the histologic progression of human breast cancer,[22,23] mammary tumors exhibit a localized increase in the deposition of collagen near the tumor lesion
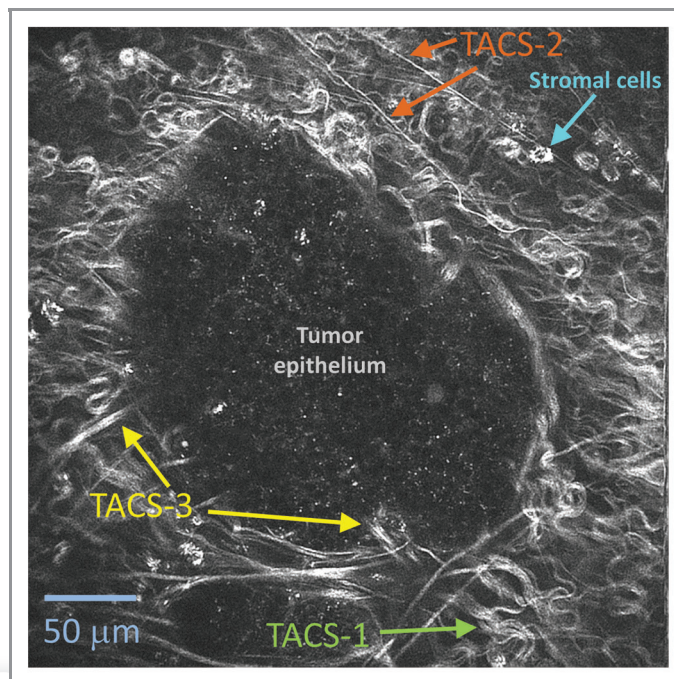
Figure 1. Tumor associated collagen signatures (TACS). Multiphoton image of an isolated ex vivo mouse breast tumor (MMTV-PyVT) containing distinct collagen organizational phenotypes. The weak autofluorescence signal present in the tumor epithelium is surrounded by the strong second harmonic generation (SHG) signal produced by collagen. This image contains all three of the archetypal tumor associated collagen signatures (TACS), as well as some brightly autofluorescent stromal cells. The first collagen signature (TACS-1) is defined as an increase in stromal collagen that retains a wavy appearance and is found throughout the tumor (arrow). The second signature (TACS-2) refers to the straightened appearance of individual collagen fibers, whereas the third signature (TACS-3) refers to the orientation of straightened collagen fibers. Specifically, TACS-3 is defined as collagen fibers oriented radially away from the tumor boundary (arrows). It has been shown that tumor cells will invade away from locations such as this along the straightened, perpendicularly aligned collagen fibers. Scale bar is 50 μm.

(termed TACS-1) that occurs very early in tumor formation. As tumors increase in size, a straightening of collagen fibers that are aligned parallel to the tumor boundary is noted (TACS-2[20]). Remodeling of the stroma progresses to the final stage, which is the reorientation of collagen such that multiple collagen fibers are bundled and aligned perpendicular to the tumor boundary (termed TACS-3[20]). The result of collagen fiber alignment is significant, as our group has shown that regions containing TACS-3 correspond to sites of focal invasion into the stroma,[20,24] and we and others have observed that tumor cells preferentially invade along straightened, aligned collagen fibers, which can promote intravasation (Figs. 2 and 3).[20,25-27] These specific definitions of TACS are consistent with the general and well-known feature termed "desmoplasia," a fibrous stromal deposition, surrounding tumors.[28-30]

As the TACS-3 phenotype is an avenue for metastasis in mice, we hypothesized that the survival of human patients diagnosed with breast carcinoma could be predicted by TACS-3 incidence.
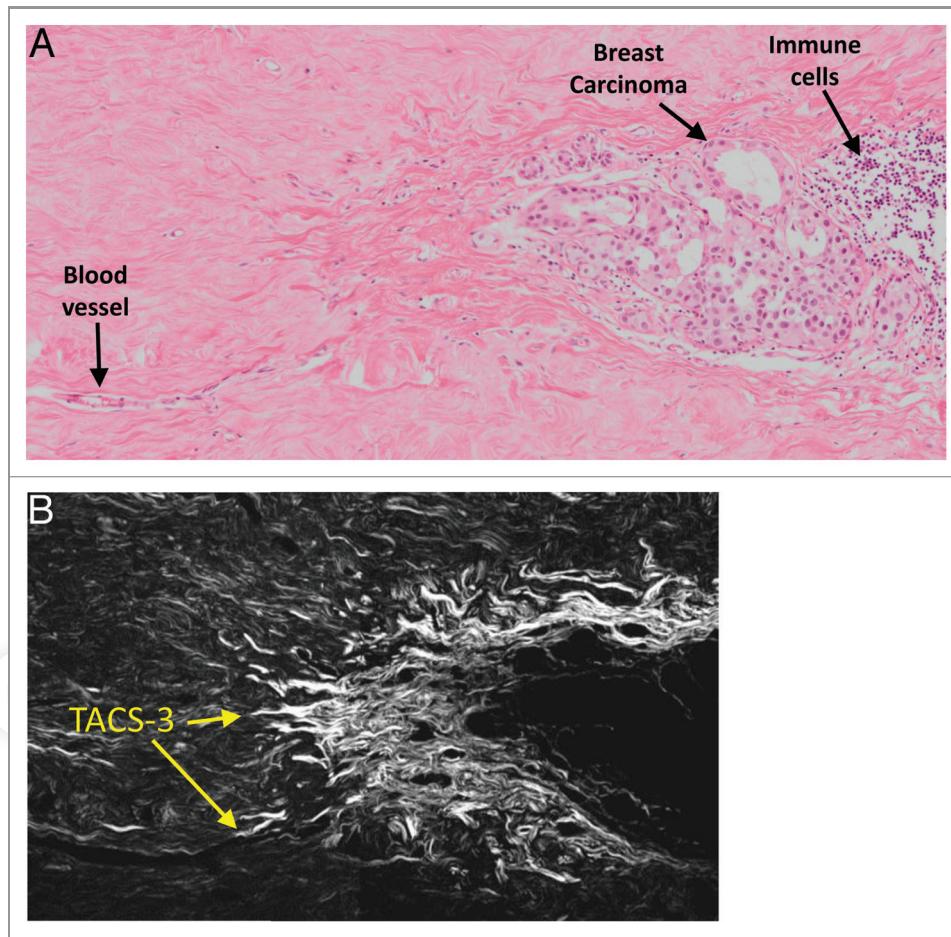
Figure 2. Aligned collagen is an avenue for invasion and metastasis. (A) H and E stained slide of human breast carcinoma. Disseminating cells can be observed migrating toward a nearby blood vessel. (B) Second harmonic generation (SHG) imaging of this same location reveals the underlying collagen matrix.

Imaging the second harmonic generation signal produced by collagen in a tissue microarray of samples from 196 patients diagnosed with invasive breast cancer, we measured the frequency of the TACS-3 phenotype. Univariate analysis of a Cox proportional hazard model demonstrates that the presence of TACS-3 is associated with poor disease-specific and disease-free survival, resulting in hazard ratios between 3.0 and 3.9 (see ref. 31 and **Table 1**). This biomarker was confirmed to be an independent prognostic indicator regardless of tumor grade, size, estrogen or progesterone receptor status, or human epidermal growth factor receptor-2 (HER-2) status. Surprisingly, TACS-3 was also independent of lymph node status. The preceding analysis was performed upon routinely prepared, hematoxylin and eosin (H and E) stained histopathology slides, which opens up the possibility of assessing direct interactions between the tumor epithelium and ECM as part of the standard histopathology workflow. This issue of the size scale of analysis is an important consideration, as invasion does not need to occur over a large distance for effective metastasis. In a sense, the alignment and deposition of collagen is a readout of the interactions between the tumor and the local combined stromal influences, and since TACS-3 alignment was so strongly correlated with patient outcome, we are interested in how any and all aspects of stromal biology could lead to the metastasis-promoting TACS-3 phenotype.

Syndecans contribute to an aligned collagen matrix. It is currently unclear exactly what mechanisms contribute to alignment of the collagen matrix. However, recent evidence points to the role of syndecan signaling. The four syndecans comprise a family of transmembrane cell surface heparan sulfate proteoglycans expressed on fibroblasts and epithelial cells. While syndecan-1 is expressed on normal mammary epithelial cells but not in the stroma, in breast cancers this expression switches, with ~70% of human breast carcinomas expressing syndecan in the stroma.[32] Stromal syndecan is found on carcinoma-associated fibroblasts (CAFs), which differ from normal fibroblasts in their ability to promote tumor progression.[33,34] The downstream effects of syndecan signaling on adhesion and migration are thought to be mediated through their binding to growth factors and the ECM itself via their heparan sulfate chains, as well as through the core protein.[35-37] Recent work has demonstrated that the presence of stromal syndecan-1 stimulates the proliferation of tumor epithelium and was correlated with the amount of tumor vascularization in a human tissue microarray of invasive breast
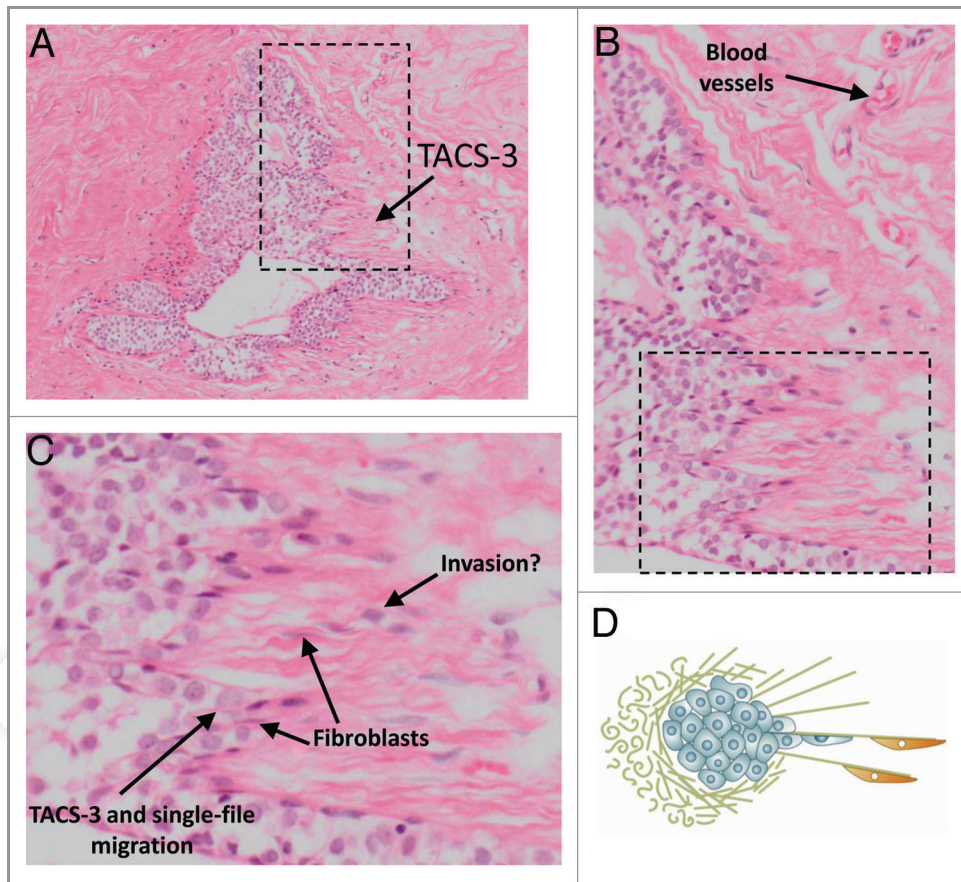
Figure 3. The TACS-3 phenotype isolates breast carcinoma for invasion. (A–C) A consequence of anchorage-independent growth of breast carcinoma is a co-mingling with the stromal matrix. Collagen can be observed to penetrate into the carcinoma cell mass, additionally some epithelial cells are disseminating away from the tumor, such infiltrations blur the tumor/stroma boundary, resulting in an irregular margin. The net effect is the isolation of cells into single-file columns of cells that have enhanced migration. Fibroblasts and other stromal cells are readily observable at these areas, and there is a great deal of evidence for their direct role in these activities. (D) An illustration depicting such a process, where the influences leading to such a phenotype will be detailed in **Figure 4**.

Table 1. Summary of clinical studies of stromal contribution to patient survival

Stromal component analyzed	Hazard ratio (95% CI)	p value	Authors
Breast density (≥ 75%)	4.30 (0.88–21.00)	0.071	Park et al., 2009[11]
Collagen alignment	3.18 (1.11–9.17)	0.032	Conklin et al., 2011[31]
Syndecan-1 expression	—	0.005	Baba et al., 2006[39]
Neutrophil gelatinase-associated lipocalin (NGAL)	2.32 (1.39–3.85)	0.001	Bauer et al., 2008[42]
High expression levels of COX-2 and COL1A1 (multivariate Cox analysis)	1.45 (1.08–1.95)	0.018	Lyons et al., 2011[52]
Macrophage presence (CD68 staining) (multivariate Cox analysis)	1.25 (1.13–1.38)	< 0.001	Mahmoud et al., 2011[71]
B lymphocyte presence (CD20+ staining)	—	0.001	Mahmoud et al., 2011[79]
Stroma-derived prognostic predictor (SDPP), stromal gene expression. (multivariate Cox regression of overall survival)	3.06 (1.42–6.58)	0.004	Finak et al., 2008[93]
Fibroblast wound response signature (multivariate Cox analysis of disease-specific survival)	11.18 (2.52–49.60)	0.001	Chang et al., 2005[94]
MMP-1 expression in tumor cells (disease-specific survival)	1.99 (1.12–3.53)	0.019	Bostrom et al., 2011[112]

All data shown was a univariate Cox proportional hazard model for disease-free survival unless otherwise specified. Not all studies provided hazard ratios (indicated by —). CI, confidence interval.

carcinoma patients.[38] Furthermore, syndecan-1 expression (but not syndecan-4 nor glypican-1) predicted patient outcome in the same tissue microarray (**Table 1**).[39]

Interestingly, TACS-3 was found to predict patient survival independent of all standard clinical variables with the exception of syndecan-1 expression, suggesting that these two factors are linked.[31] There appears to be a biological basis for this correlation, as it has been shown that syndecan-1 expression is necessary and sufficient to cause fibroblasts to deposit an aligned matrix in vitro.[40] However currently, the exact mechanism by which syndecan-1 promotes collagen alignment is unknown.

Studies of other glycoproteins have shown that chondroitin sulfate expression in tumor epithelium, but not in the stroma, is an independent predictor of shorter recurrence-free survival ($p < 0.05$).[41] Neutrophil gelatinase-associated lipocalin (NGAL) is a small, secreted glycoprotein that is involved in the transport of lipophilic substances and may also be involved in prostaglandin synthesis. NGAL is found in normal and tumor breast epithelium and forms a complex with MMP-9, preventing its degradation. Elevated expression of NGAL is an independent marker of decreased disease-free survival (**Table 1**). Although NGAL is an independent predictor of outcome in multivariate analysis, at the tissue level its expression by immunohistochemistry is strongly associated with HER-2 overexpression.[42]

ECM remodeling during involution. In addition to the stromal changes that occur during tumor formation, the cycle of pregnancy, lactation, and involution is another significant event in which the adult mammary gland undergoes changes to the strict stratification of cell and ECM layers. During the involution (weaning) period, the mammary gland undergoes massive cell death and radical remodeling of cell layers and ECM with the purpose of restoring normal glandular architecture,[43] a process that can take several weeks to complete in humans.

Evidence is mounting to suggest that the remodeling of involution creates a window of risk in which patients with tumors that arise during this period of remodeling or within 5 years postpartum have a significantly poorer survival than patients with breast cancer arising at other time points.[44-47] This process, termed Pregnancy Associated Breast Cancer (PABC) is a rare event (0.3 cases/1,000 pregnancies) but highly significant due to the low survivability.[48] The underlying reason for this may be due to the fact that the microenvironment of the involuting mammary gland shares many similarities to the inflammatory microenvironment associated with breast tumors including a "reactive" stroma that is associated with increased invasion and metastasis. One such similarity is that involuting glands contain an 8-fold increase in the number of macrophages present, an inflammatory response known to be associated with poor outcome.[49] ECM isolated from involuting rats was demonstrated to be chemotactic for macrophages (compared with nulliparous animals), and evidence presented that the chemoattractant was denatured collagen I.[49]

The involuting mammary gland stroma is linked to the effects of cyclooxygenase-2 (COX-2), the enzyme that catalyzes the synthesis of prostaglandin mediators of inflammation. Inhibition of COX-2 in rats with ibuprofen results in a stroma with severely diminished tumor promotional abilities.[50] Moreover, this is likely a positive feedback loop, as fibrillar collagen binding to β_1-integrins of epithelial cells stimulates the production of COX-2 and increased expression of the COX-2 message is associated with culture of mammary epithelial cells in a dense collagen matrix.[49,51] A clinical investigation of these results showed that in a multivariate analysis of relapse-free survival of 345 breast tumors diagnosed in women ≤ 45 years of age who relapsed within 5 years who had both high COX-2 and COL1A1 (the gene for type I collagen) levels had statistically significantly poorer survival compared with women with normal expression levels of these genes from the same group (**Table 1**).[52] Therefore increased deposition of collagen occurring as a result of the inflammatory response stimulated by the tumor causes the production of COX-2 and prostaglandin signaling, which in turn increases the inflammatory response and additional collagen deposition. Given the link between increased collagen density and survival described above, this signaling cascade may provide an explanation for decreased survival in PABC.

The role that COX-2 signaling plays in breast tumor progression appears to be significant. Several animal models of mammary tumorigenesis show that inhibition or deletion of COX-2 reduces tumor incidence,[53-56] and in rat models COX-2 inhibitors are chemopreventative and chemotherapeutic. In human breast cancer patients, moderate to strong expression of COX-2 protein occurs in ~40% of tumors and is associated with poor distant disease-free survival ($p < 0.0001$).[57] COX-2 is inhibited by NSAIDs and by celecoxib, but the side effects of these drugs, including gastrointestinal damage, prevent long-term use as a means to prevent breast cancer. A better alternative may be to target the downstream receptors of the specific prostaglandins produced in the breast as a result of COX-2 activity, therefore additional investigations are needed.

Is the Newfound Presence of Specific Cell Types in the Tumor Stroma a Portent of Future Biomarkers?

Carcinoma-associated fibroblasts (CAFs). Changes in the stroma surrounding tumors are largely due to the actions of activated fibroblasts in the stroma, termed carcinoma-associated fibroblasts, or CAFs. It is unclear whether CAFs are resident fibroblasts that are activated by paracrine tumor-secreted growth factors, or whether they are mesenchymal stem cells that have been recruited into the stroma.[58] Alternatively, adipocytes, the genetic precursors to fibroblasts, can be reverted to fibroblast-like cells in response to soluble tumor-derived factors.[59] These factors include TGF-β_1 and TNF-α, and may be a contributing factor as to why tumor progression and survival are linked to the expression levels of these molecules.[60-63] These CAFs have an increased ability to secrete several ECM proteins, including collagens, fibronectin and tenascin C.[64] CAFs also have increased expression of syndecan-1 as described above.[40]

As an example, tenascin C (TnC) is an extracellular glycoprotein that is deposited into the matrix as a hexamer that binds to collagen and fibronectin. Increased levels of TnC in the tumor stroma has been linked to decreased responsiveness to tamoxifen therapy,[65] increased distant recurrence[66] and

decreased disease-specific survival.[67] These outcomes may relate to the recent finding that TnC may help breast cancer cells colonize the lungs by providing a metastatic niche.[68] Moreover, specific splice variants have been linked to breast cancers in younger women.[69]

Immune cells. Although the presence of lymphocytes and macrophages trafficking into and out of breast tissue is a normal aspect of immune surveillance, several recent studies (discussed below) provide clear evidence that changes in the number and character of immune cells in tumors is associated with poor patient outcome. Understanding how such cells are recruited to the tumor in the first place, and what roles they play upon arrival, is currently a subject of much study as it has the potential for future development of drugs that inhibit their tumor-promotional effects (e.g., anti-VEGF or -EGF pharmaceuticals).[70]

Perhaps no other cell type is more strongly associated with pathological tumor progression than macrophages, where multiple studies have shown macrophage presence to be strongly correlated with poor patient prognosis.[71-73] At least some macrophages are present in most breast tumors, but they can also comprise a significant portion of the overall tumor mass. Mahmoud et al. examined 1322 breast cancer tumors for the presence of CD68-positive macrophages and found that higher numbers of macrophages were significantly associated with worse breast cancer-specific survival (**Table 1**).[71] Overexpression of the cytokine MIF (macrophage migration inhibitory factor) in breast tumor epithelium was associated with improved disease-free and overall survival.[74] Monocytes, the hematopoietic precursors to macrophages, are also linked to poor survival in human breast cancer patients, where it has been shown these cells facilitate metastasis through chemokine signaling.[75]

The poor survival of patients with large numbers of macrophages is attributable to both pharmacological and mechanical signaling pathways which promote tumor invasion. The primary pharmacological means by which macrophages promote poor patient survival is by increasing angiogenesis. A paracrine signaling loop in breast tumors occurs where tumor epithelium secretes colony stimulating factor-1 (CSF-1), a growth factor for macrophages, thereby recruiting them to the site of the tumor whereupon they secrete both EGF, which promotes the migration and invasion of tumor cells, and VEGF which recruits nearby endothelial cells to initiate and promote angiogenesis.[76]

Macrophages also stimulate collagen fibrillogenesis itself, as genetic ablation of macrophages reduced the amount of collagen fibrillogenesis by ~50%.[77] Moreover, macrophages appear to be capable of remodeling and aligning the collagen, as the normal pattern of aligned collagen near the tips of terminal end buds is severely reduced in mice that have a null mutation for CSF-1.[77] However, this is not to imply that macrophages are the sole cause of collagen alignment, as desmoplasia is observed in inflammatory breast cancers in which macrophages are rarely present. This has significant implications as invasion of tumor cells can be stimulated by macrophages, which track together along straightened collagen fibers in breast tumors.[25]

As part of the humoral immune response, B-cells (lymphocytes) are often observed to infiltrate tumors. Rody et al. found that the amount of B-cell presence [in combination with the amount of Interleukin-8 (IL-8) activity] was a powerful prognostic indicator for survival in triple negative breast cancer patients.[78] Those patients with high expression of B-cells and low expression of the IL8 metagene had significantly better prognosis than other patients with this same clinical diagnosis (HR = 0.37, p < 0.001). Similar results were obtained in an examination of CD20+ staining for B lymphocytes of 1,470 breast cancer patients, where it was found that an increased number of CD20+ cells is an independent marker for cancer-specific survival (**Table 1**) as well as an indicator of longer disease-free interval.[79] Although these results are intriguing, the role of the immune response in breast cancer is still controversial, as B cell antibody response may potentiate chronic inflammation, enhancing tumor progression.[80,81] In addition to B lymphocytes, T lymphocytes are also implicated in the progression of breast cancer.[82] Furthermore, there is evidence that natural killer (NK) cells may also be a predictor of recurrence for patients with early stage breast cancer.[83] Because so many immune cell types appear to play a role in breast cancer survival, no doubt additional information on this aspect will continue to emerge.

The Gene Expression Profile of the Stroma May be a Better Predictor of Patient Outcome than the Tumor Epithelium

Gene expression profiling approaches have been extensively used to determine not only the expression of individual genes, but also use hierarchal clustering to define sets of genes whose expression changes a significant degree, thereby creating a "signature" of genetic changes that occur with the onset of a tumor. Many of the gene signatures are associated with signaling pathways known to be important in tumor biology (Her-2 status, etc.).[84] Certain gene expression signatures and hierarchal clusters have been found to be indicative of poorer survival than others. The ultimate goal then is to identify (upon biopsy) which gene expression profile a patient has, which will help determine the standard of care options that are best suited for that individual patient.

The initial studies that identified gene expression signatures used tissue derived from whole tumors comprised of both the tumor epithelium and the stromal cells.[84-87] Therefore it is not possible to determine the cell type of origin of a particular gene expression change, and one cannot assume that all cell types within the tumor will express the same change in gene expression. Given that under some circumstances tumor epithelium only comprises ~50% of the cell mass of a tumor, this is a serious issue.[88] Furthermore, many of the subclassifications of gene expression signatures are a description of the cell of origin of the tumor (luminal or basal) and therefore not a description of the underlying biological changes or causes in tumor progression. As a result, such methodologies, even though they are predictors of survival, do not as of yet inform physicians of treatment options for patients. Efforts to fine-tune this approach are underway, where the categorization of subtypes of altered gene expression was based upon their responsiveness to adjuvant or neo-adjuvant therapy.[89] In this way, the actual signature developed was based

on the clinical outcome, and can then be used to prospectively sort new patients into treatment groups based on their signature. As such, customization of treatment can potentially take place where, for example, a given patient can be treated with hormone therapy alone, as it has been determined that for their molecular profile that further treatments were of no further clinical benefit. In another study, a stromal gene expression signature has been found that predicts the resistance to preoperative chemotherapy for patients with ER-negative tumors.[90] The presence of this signature was correlated with the amount of reactive stroma (i.e., collagen) present. Moreover, Losartan, an inhibitor of collagen I synthesis, improves the distribution of nanotherapeutics in tumors,[91] suggesting that antistromal agents applied prior to chemotherapy may result in higher efficacy.

In breast cancer, genes whose stromal expression increased in more aggressive tumors included Sdc-1, fibronectin and collagen X and XI.[92] It is interesting to note that a significant fraction of genes whose expression is altered encode secreted proteins and receptors, heavily implying the role of the stroma as the target for, or the effector of, changes in gene expression within the tumor. Taking these results a step further, a recent DNA microarray analysis of stromal cells of breast tumors was subsequently correlated with patient outcome.[93] This was achieved by using laser capture microdissection to isolate tumor and normal stroma specifically, thereby alleviating some of the issues discussed above. Initially, this approach identified a 26-gene stroma-derived prognostic predictor (SDPP) that stratified disease outcome independently of standard clinical prognostic factors and was particularly effective in those patients that were Her-2 positive (**Table 1**). Amazingly, the SDPP was applied to datamine multiple, published DNA microarray data sets acquired from whole tumors and was found to be a better predictor of survival than the signatures initially derived from this data. The SDPP was predictive in multiple clinical subtypes and was independent of lymph node status. Upon further analysis, five biological categories of changes in stromal gene expression were identified: matrix remodeling, hypoxia, fibroblast signaling, estrogen receptor signaling and the immune response. Therefore the stroma appears to be a more fruitful place to hunt for changes in gene expression initially, and the presence of a defined stromal signature allows whole tumors to be analyzed for patient diagnosis, bypassing the technologically challenging isolation of stromal cells for this purpose.

Other gene expression signatures have also been derived from studies of the stroma;[92] including identification of a "wound response signature" that predicts survival in whole tumors.[94] Patients that expressed this wound response had markedly diminished disease-specific survival and worse distant metastasis-free probability (**Table 1**). Others have found that the gene signature profile from the same tumor differs when the biopsy is performed using fine-needle aspiration (stroma-poor) or a core-needle (stroma-rich) extraction, with the latter containing the stromal metagene signature predictive of outcome.[95] Furthermore, gene expression changes in the stroma occur very early in tumor formation often preceding the onset of invasion, with 90% of the alterations occurring during the normal to DCIS stages.[96]

The origin of why gene expression signatures from the stroma appear to be better predictors of patient outcome may be rather simple. Maffini et al. provide evidence that the target of carcinogens in the breast may in fact be the stroma and not the epithelium in the first place.[97] Isolated normal mammary stroma and epithelium were exposed to either a carcinogen or vehicle control and then recombined. When the stroma was exposed to the carcinogen and combined with epithelium that was exposed to the vehicle, a neoplasm arises, whereas the opposite combination does not result in neoplasm.[97]

Stromal and Mechanical Signaling Pathways Associated with Matrix Remodeling that Lead to Poor Prognosis: Potential Mechanisms for Diminished Survival

Much recent in vitro data suggests mechanisms by which a mechanically stiff microenvironment could enhance tumor progression. Cells sense the stiffness of their local environment by exerting contractile force to pull against the matrix and several groups have shown a role for the small GTPase, Rho in this process.[4,98,99] Rho activation stimulates actin-myosin contractility through its effector, ROCK, which both phosphorylates the regulatory myosin light chain (MLC) directly and inhibits the phosphatase that would de-phosphorylate MLC. The model that emerges is that cells pulling on a compliant matrix will contract that matrix, while cells pulling against a stiff matrix will generate isometric tension due to the restraining force of the matrix. Indeed, when force is applied against integrins, the result is increased integrin avidity, recruitment of the actin cytoskeleton, and a mechanically stronger focal adhesion.[100] This is a dynamic process that is continuously monitored, as integrins at the cell surface that do not effectively bind ECM are endocytosed to endosomal compartments and recycled to other areas of the cell more actively engaged in adhesion.[101] Thus the underlying pathology of an increase in breast density in humans may be the inability of the normal breast epithelium to efficiently contract this high density of collagen, causing a tensional imbalance.

The result of focal adhesion signaling is activation of signaling pathways that are emerging as mechanically responsive and include focal adhesion kinase (FAK), the MEK/ERK pathway and activation of Rho itself.[4,51,98] Indeed, activation of FAK and ERK in breast epithelial cells cultured in a stiff matrix dramatically increases several genes associated with cellular proliferation and tumor progression.[51] Deletion of FAK results in suppression of a metastasis gene signature and tumors that are not locally invasive nor metastatic.[102-104]

Caveolin-1 and the regulation of Rho. A set of recent papers suggest an important role for stromal expression of caveolin-1 in breast tumor progression. Caveolin-1 is an integral membrane protein that is associated with the formation of lipid micro-domains. A recent paper by Goetz et al. found that knock out of caveolin-1 results in fibroblasts that are unable to create an elongated, aligned matrix typical of fibroblasts in culture.[105] Moreover, the stromal matrix, including fibronectin and collagen, are mis-organized around the normal mammary gland. Upon

tumor formation, the caveolin-1 knockout animals are unable to produce the aligned TACS-3 signature normally observed around breast tumors.[105] They further find that the stroma surrounding carcinomas of breast, colon and kidney in human tumors is abundant in caveolin-1, as is the stroma of metastatic melanoma lesions. In breast cancer patients, they find that patients with a caveolin-1 positive stroma have a 2.5 times higher 10 year mortality risk. Their findings are in contrast to those of Witkiewicz et al. and Sloan et al., who find that high levels of stromal caveolin-1 predict a better outcome in breast cancer patients.[106,107] This finding is consistent with a role for caveolin-1 as a tumor suppressor, which has been proposed, in spite of the fact that caveolin-1 expression is found to be elevated in breast and other tumors. Thus, although it appears that caveolin-1 has a role in the stroma that affects tumor outcome, further investigation is necessary to better understand that role.

The mechanism of stromal alignment appears to be linked to the activity of the small GTPase, Rho. Rho regulates intracellular contractility by activating ROCK and downstream regulation of myosin-light chain phosphorylation. Activated Rho and ROCK are necessary for cells to align a collagen matrix in vitro.[108] Interestingly, the mechanism by which caveolin-1 affects CAFs appears to also be through Rho regulation[105,109]. It is thought that in both breast epithelial cells and fibroblasts that phosphorylated caveolin-1 regulates Rho activation levels through its inactivation of the Src/p190RhoGAP pathway.[110] Therefore loss of caveolin-1 allows p190RhoGAP to become more activated at the plasma membrane, and downregulate Rho.[109] This results in cells that are less able to contract a collagen matrix in vitro, and less able to deposit an aligned matrix in vitro and in vivo. Importantly, inhibition of p190RhoGAP in Cav1$^{-/-}$ cells rescues the ability of the cells to contract a collagen gel and to deposit an aligned matrix.[105] Thus, these data demonstrate an important role for Rho-mediated contractility, and its regulation by caveolin-1 and p190RhoGAP, in the formation of aligned matrices. Because both caveolin-1 and matrix alignment are prognostic of breast cancer outcome,[31,105] the implication is that Rho activation in the stroma would also be associated with poor outcome in breast carcinomas.

Matrix metalloproteinases (MMPs). The specific roles that matrix metalloproteinases (MMPs) play in tumor progression is still a highly debated topic, and the targeting of MMPs in clinical scenarios has been disappointing.[111] Whether or not specific isoforms are required, be they secreted or membrane-bound, at what tumor stage they are expressed, and their enzymatic targets are all areas of active research. No matter the study, the hypotheses that emerge are that these enzymes are tumor-promotional because they contribute to the breakdown of the basement membrane and/or that they degrade stromal collagen to facilitate migration of tumor epithelial cells. Any controversy over MMP cell biology should not diminish their importance; as clinical studies have established a correlation between survival and MMP expression. Bostrom et al. performed immunohistochemical analysis of 125 breast cancer patients and found that tumor grade was positively correlated with MMP-1 expression.[112] The expression of MMP-1 in epithelial cells was correlated with p53 positivity, whereas stromal expression of MMP-1 was correlated

with HER-2 expression. MMP-1 expression was found to be an independent prognostic indicator (**Table 1**) and was significantly associated with triple-negative breast cancer subtypes. Other studies have shown that tumor-derived, but not stromal derived MMP-13 expression was correlated with decreased overall survival, and that MMP-14, but not MMP-2 was an independent factor for overall survival when adjusted for clinical prognosticators.[113,114] However others have shown that co-expression of MMP-9 with MMP-2 is an independent risk factor for survival, which raises the issue of whether MMPs are more effective promoters of tumor progression in concert with each other, and whether a survival analysis of the expression of certain combinations ("signatures") of MMPs at specific tumor stages is merited.[115] Matrix metalloproteinase activity in general may promote tumor progression as expression of stromelysin-3 (also known as MMP-11), which shares homology with other proteinases that have enzymatic targets beyond stromal collagen such as the basement membrane and proteoglycans, is significantly overexpressed in most breast cancer patients and is linked to patient outcome.[116] Although MMPs may represent useful biomarkers of outcome, they are not necessarily required for the TACS changes in collagen in vivo, and in vitro inhibition of MMPs does not prevent collagen alignment.[108]

Chemokines. Several secreted factors are also upregulated in breast tumors, including the CXCL14 and CXCL12 chemokines, which are specifically overexpressed in tumor myoepithelial cells and myofibroblasts, respectively.[117] These factors bind to receptors on epithelial cells and enhance their proliferation, migration and invasion.[117,118] Expression is elevated in both DCIS and invasive tumors where signaling is achieved through binding to specific receptors through an autocrine or paracrine fashion. Expression of chemokines and their receptors is sensitive to estrogen receptor status and hypoxia, and there are multiple signaling pathways activated downstream of receptor binding, not surprising given the wide variety of functions that chemokine signaling plays a role in. CXCL12 is also secreted from sites of metastasis and is capable of chemoattracting disseminated epithelial cells that express its receptor, CXCR4. It has been found that high expression levels of CXCL12, but not CXCR4, in breast tumor epithelium is correlated with improved disease-free and overall survival (HR = 0.79, p = 0.001), with the interesting hypothesis proposed that when CXCL12 levels are high, binding to their receptors is saturated in an autocrine fashion, thereby protecting against chemotaxis to distant sites.[119] Expression of the CXCR4 gene is upregulated in many types of cancer including breast where methylation of the promoter of this gene inhibits expression, which has also been shown to be a predictor of improved overall and disease-free survival.[120] Epigenetic regulation of tumor progression such as this is an emerging field of tumor biology, and is exciting especially in light of the finding that the COOH-terminal fragment of procollagen type I (C3) has been shown to induce the expression of CXCR4.[121] Considering the desmoplastic/inflammatory response to tumor formation, and the link between breast density and survival this result may be yet another example of how the stroma shapes the course of tumor progression.

Summary

There is a great deal of experimental and clinical evidence that points to the role of the stroma in tumor progression, underscoring the need for further studies in order to develop even more robust biomarkers. The aspects of stromal biology that predict patient survival are illustrated in **Figure 4**, where an aligned collagen matrix, the influx and crosstalk of various stromal cells, and the induction of altered gene expression are all depicted. Moreover, the observation that breast stromal density is a risk factor suggests that the stroma will become increasingly

appreciated in the initiation or formation of breast carcinomas. The challenge going forward is to assay the presence of these biomarkers as early as possible, and to find ways to make use of these biomarkers to assist surgeons and oncologists. In particular, the assessment of collagen density and alignment has the potential for application during surgical resection and biopsy, as the microscopy used to collect such images does not require any labeling and can be performed in fresh, unstained tissue. Moreover, collagen alignment as a biomarker is readily paired with the determination of other biomarkers. The degree of collagen alignment, for example, has not been directly correlated
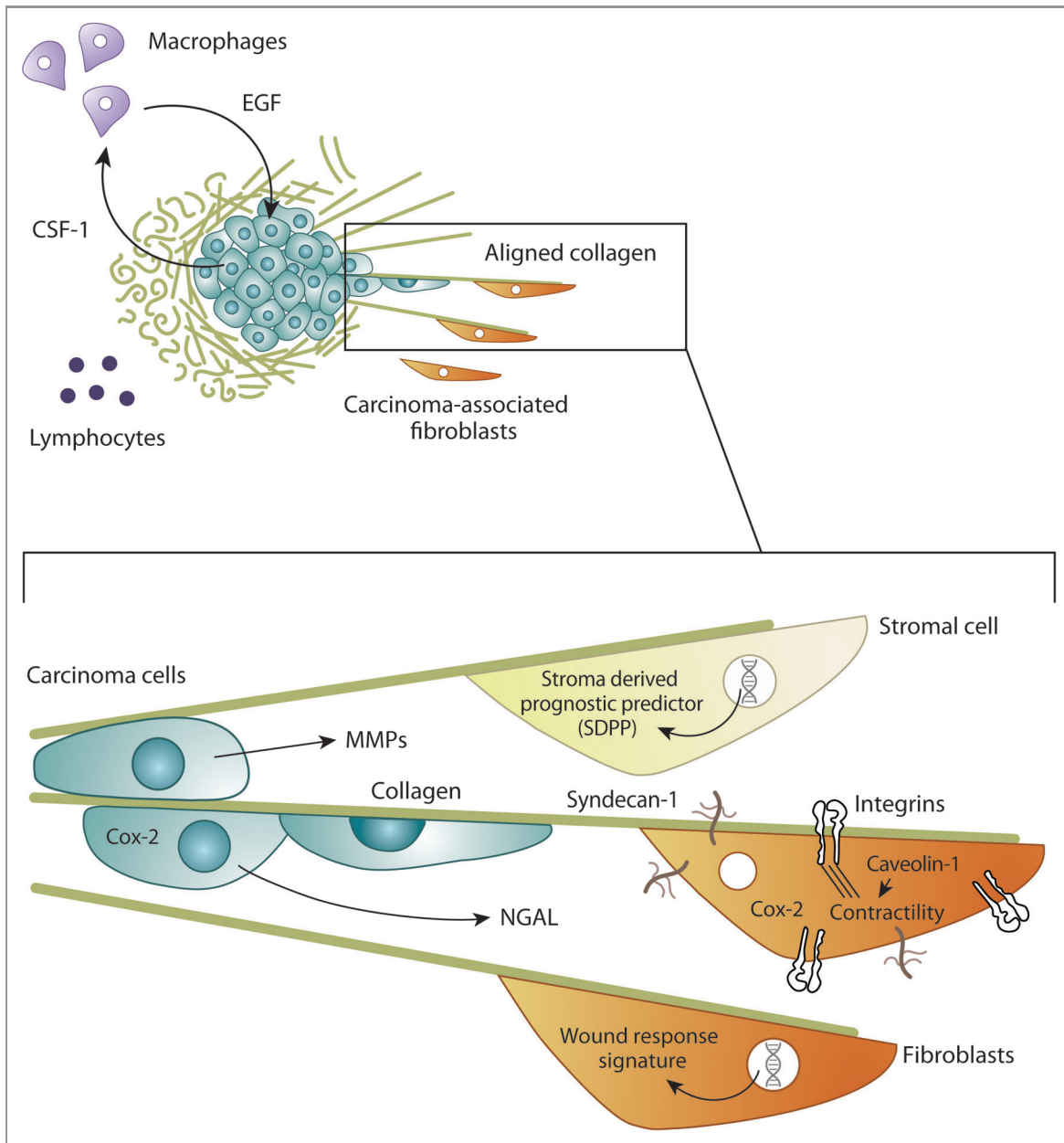
Figure 4. Stromal biomarkers that predict clinical outcome. An illustration of the biomarkers discussed in the manuscript text. The presence and influx of stromal cell types, alterations in gene expression, secreted factors and an altered collagen matrix are all depicted. The interplay of these features lead to a mechanism, aligned collagen, by which the stroma promotes tumor progression.

with the presence of fibroblasts or other stromal cells but it would be logical if this were so. The role of collagen alignment, the ECM, and various stromal cells in facilitating cell invasion links these predictors to underlying mechanisms of cell invasion. Further understanding of these processes should present new opportunities to diagnose, predict and treat breast cancer.

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