Journal of Mammary Gland Biology and Neoplasia, Vol. 9, No. 4, October 2004 (© 2004)
DOI: 10.1007/s10911-004-1403-y

A Visual-Quantitative Analysis of Fibroblastic Stromagenesis in Breast Cancer Progression

Edna Cukierman[1,2]

One fundamental difference between normal and transformed cells is the way they interact with their immediate environment. Exploring this difference is crucial for understanding the pathobiology of cancer progression. Benign epithelial tumors are constrained by a surrounding stroma consisting, among other cells, of fibroblasts embedded within fibrillar three-dimensional matrices. However, at a critical point in tumor progression, tumor cells become altered and overcome the barrier, inducing changes in the stroma, which promote, rather than impede, tumor progression. Inherited or acquired genetic aberrations affecting mammary gland epithelia are usually blamed for promoting neoplasia in individuals at "high risk" for breast cancer. However, in addition to these epithelial aberrations certain individuals possess permissive breast stroma. The occurrence of this permissive stroma results in a predisposition for cancer initiation or progression. Here we review stromagenic stages, experimental 3D systems, and discuss digital imaging analyses suitable for uncovering the mechanisms behind fibroblastic breast stromagenesis.

KEY WORDS: breast stromagenesis; fibroblastic stroma; mesenchymal 3D systems; digital imaging analyses.

BREAST STROMAGENESIS

During epithelial tumor progression, the tumor microenvironment undergoes a number of dynamic and regulated alterations that occur in parallel to transformation. In many cases, as proliferating epithelial cells progress and become aggressive, the host microenvironment evolves too, inducing basement membrane discontinuation, severe immune responses, and the formation of new blood vessels. These microenvironmental host responses are accompanied by additional dynamic alterations of the mesenchyme in the near vicinity of the progressing tumor. The mesenchymal alterations that occur parallel to tumor progression are similar to those that accompany certain developmental processes, pathological fibrotic reactions, and fibrotic wound healing responses *(1–4)*. The "normal stroma," which include quiescent mesenchymal fibroblastic cells and their extracellular matrix (ECM), are believed to restrain tumor progression by acting as a natural barrier *(5,6)*. However, at a critical time during epithelial transformation, the early progressive tumor triggers mesenchymal changes "priming" or provoking the fibroblastic stroma, which in turn further stimulate and support the progression of tumors *(7,8)*. These mesenchymal host responses engage in a vicious cycle of paracrine and autocrine signals between progressive tumor and encouraging microenvironment *(9)*. Eventually, the parallel tumor and stroma progression disturb the tissue homeostasis, disrupting intrinsic regulatory cues to further aggravate both tumor and stroma. During this parallel progression the stroma become fully "activated" and

[1] Division of Basic Science/Tumor Cell Biology, Fox Chase Cancer Center, Philadelphia, Pennsylvania.
[2] To whom correspondence should be addressed at Basic Science/Tumor Cell Biology, Fox Chase Cancer Center, 333 Cottman Avenue, Room W428, Philadelphia, Pennsylvania 19111-2497; e-mail: Edna.Cukierman@fccc.edu.

Abbreviations used: 3D, three-dimensional; ECM, extracellular matrix; FAP, fibroblast activation protein; TAF, tumor-associated fibroblast; TGF, transforming growth factor.

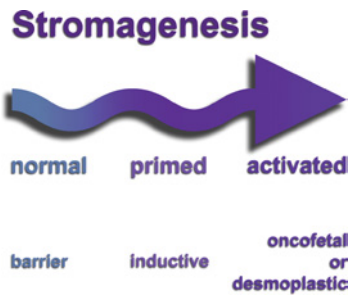

Fig. 1. A working model describing the three stromagenic stages. A descriptive illustration emphasizing the barrier capabilities of "normal" stroma, the inductive, supportive, and promoting tumorigenic effects of "primed" stroma, and the pathological identifiable oncofetal or desmoplastic phenotypes of "activated" stroma.

pathologically evident "desmoplastic" or "oncofetal" microenvironments arise, while the tumor progression is further accelerated and driven toward microenvironmental independence *(2–4)*.

In the course of this review, the mesenchymal fibroblastic stroma reactions induced by, and regulated parallel to, epithelial neoplastic transformation, are termed "stromagenesis." This section specifically focuses on breast cancer stromagenesis with an emphasis on the dynamic changes of its fibroblastic cells, ECMs, and the interactions among cells and matrix components during this process. To clarify the stromagenic events in this review, we sorted breast stromagenesis into three stages: "normal," "primed," and "activated" (Fig. 1).

Normal Stroma: A Neoplastic Progression-Restraining Environment

Mechano-sensing of the microenvironment plays a critical role in regulating cell migration and growth during normal developmental processes and tumor formation *(5,7–13,10–16)*. The complexity of signal transduction pathways that are sensed by fibroblastic *(17)* or epithelial cells *(18)* due to specific microenvironmental settings can influence the physiological state *(19)*. It has become clear that the structural components of the stromal ECM influence physiological cell behavior *(17,18,20)*. A tumor clone resulting from cell transformation is initially under growth-regulation by the microenvironment and might be in a dormant state for a long time. For the hyperplastic clone to develop into an evident tumor, it must overcome the microenvironmental constraint imposed by the normal stroma by distorting intercellular contacts as well as cell interactions with ECM required for maintenance of an intact differentiated epithelium *(21)*. Integrin-ECM interactions constitute the main cell-to-matrix adhesion structures and are critical for building a definitive normal tissue architecture and for controlling both epithelial and mesenchymal cell proliferation *(21)*. Of great importance is the restriction of tumor cell (carcinomas and sarcomas) growth by normal fibroblasts within the microenvironment *(6)*. Transformed tumor cells must overcome this normally suppressive stromal barrier in order to spread *(5)*.

The restrictive character of stroma is evident in breast cancer initiation. For example, primary myoepithelial cells from the normal breast can reduce breast cancer cell invasion *(22)*. In two independent transgenic mouse models overexpressing either transforming growth factor (TGF) *(23)* or nuclear peroxisome proliferator-activated receptor γ*(24)*, the mammary tumor phenotypes observed were found to be completely dependent upon whether the background strain of mice utilized for the production of the transgenic animals were permissive or restrictive. Both studies indicate natural host restraints and the need to surmount normal barriers to favor breast cancer progression. In a xenotransplant model, tumor growth was initially not achieved within unmodified murine fat-pads, suggesting the presence of a natural murine restrictive stroma. Even after humanizing the murine fat-pads by pre-inoculating them with normal human fibroblasts, no tumor was evident. Successful human neoplastic transformation within murine fat-pads was achieved only if the stroma were humanized using pre-aggravated or primed human fibroblasts before inoculating the epithelial component into the fat pads *(25)*. In the above-mentioned humanized system, normal stromal fibroblasts suppress abnormal cell growth and provide signals inducing normal morphogenesis, suggesting that transformed cells cannot overcome the constraints imposed by the normal microenvironment unless the stroma are primed or assertively modified *(25,26)*. Thus, stromal and tumor cells are inexorably linked together throughout tumor progression. Moreover, another report showed that when treated with carcinogens, neoplastic transformation of rat mammary epithelial cells occurred just after exposing the stroma to the carcinogens, regardless of whether or not the epithelial cells were exposed to the aggravating agent *(27)*. In support of this study, it was stated that alterations of the

stroma are necessary and also sufficient for induction of malignant behavior in genetically normal cells, indicating that normal stroma behaves as a natural barrier until primed, at that point becoming permissive for breast cancer progression *(27,28)*. Furthermore, in a three-dimensional (3D) model for cell–cell interactions, reduction mammoplasty fibroblasts were capable of inhibiting morphological conversion and growth of normal and pre-neoplastic breast cells while tumor associated fibroblasts (TAFs) evoked ductal-alveolar morphogenesis of both cell types. The growth and morphogenesis inhibitory effects of normal fibroblasts remain even in the presence of the growth inductive hormone estrogen, whereas TAFs supported and maintained estrogen responsiveness of the pre-neoplastic epithelial breast cells *(29)*.

Various individuals among a given population might be better protected genetically than others because their breast stroma is more restrictive and more capable of containing breast cancer initiation or progression. Yet, a distinct group of individuals seem to be predisposed by virtue of harboring a host permissive stroma, which facilitates mammary cell transformation *(30)*. As a result of uncovering the constraining characteristics of an effective barrier stroma, investigators should gain valuable information applicable in breast cancer prevention.

Primed Stroma: A Permissive and Supportive Landscape for Tumor Progression

Several reports support the hypothesis that a transformed epithelium is incapable of maintaining normal differentiation of adjacent stroma. The presence of abnormal stroma leads to loss of stromal control over the epithelium, resulting in cell propagation and support of epithelial proliferation or invasion *(9,31–34)*. Instead of behaving as a passive bystander, the stroma actively aid the hyperplastic progression toward tumorigenesis. In turn, the stroma itself become reciprocally modified by the tumor cells in a process known as "stromagenesis." Activation of the local invasive environment at early stages of tumor progression creates a permissive field for the malignant cell *(7)*. A given microenvironment can affect the efficiency of tumor formation, the rate of tumor growth, the extent of invasiveness, and the ability of tumor cells to metastasize *(8)*. Emerging and established carcinomas respond to stromal signals that drive progression to malignancy via a vicious cycle mediated by soluble and insoluble molecules secreted by carcinoma cells and stroma *(9)*.

In mammary glands, modulation of surface receptors, including those for the ECM and growth factors, can provide signals reverting malignant mammary epithelial cells to a normal morphology and behavior or, in reciprocal fashion, converting epithelial breast cellular characteristics from benign to malignant *(32)*. Evidence for the ability of breast primed stroma to promote tumorigenesis by the production of stromal growth factors is provided by several reports, such as the previously-mentioned study establishing humanized breast development in murine fat pads *(25)*. In this system, breast carcinomas at all stages emerged when human organoids were engrafted into a humanized primed stroma overexpressing growth factors to provide a permissive terrain for tumor growth. This study illustrates the need for better understanding the priming events, which are permissive for breast cancer initiation and/or progression even under conditions when the epithelial components initially appear as phenotypically normal. In additional studies, it has been shown that the dynamic influence of stroma on tumor cells, at least at an early stage of tumor progression, provides an opportunity to target stromal cells to control the malignant behavior of genetically-unstable epithelial cells *(32)*.

The primed breast stroma are not merely a passive barrier restricting invasive tumors but rather represent a host response to epithelial tumors that cause fibroblast proliferation and ECM remodeling, similar to wound healing processes that have lost their controls *(1,35)*. Carcinoma cells stimulate the release of matrix regulatory proteins from fibroblasts (but not vice versa). Moreover, tumor-derived, but not normal fibroblasts, initiate key physiological cell responses in matrix dynamics that result in a permissive environment for cancer progression *(36)*. Stromal–epithelial interactions modulate mammary epithelial cell growth and apoptosis by influencing cell adhesion and tissue organization. Therefore, any significant change in the breast stromal ECM, or in the adhesion structures and molecules expressed by the breast epithelium, should have an impact on mammary epithelial cell survival or apoptosis *(37)*. Furthermore, breast tumor cells can, by paracrine influences, affect stromal-dynamics, thus manipulating the availability or activation states of key regulatory molecules. For example, epithelial-activated metalloproteinases, extracellular enzymes known for their

substrate regulatory capabilities, can trigger ECM modifications that favor stromal priming *(22,38–45)*. The presence of distinct ECM-modulating enzymes, such as heparanase, within breast stroma is frequently correlated with tumor size *(46)*. The expression of additional regulatory molecules, such as cathepsin B *(47)* or urokinase type plasminogen activator *(48)*, is modulated by integrin-ECM dependent interactions in breast fibroblasts and contributes to breast cancer progression. Moreover, the acquisition of hormone responsiveness in the normal breast and the loss of responsiveness in advanced breast cancer are also ECM stromal- and integrin-dependent *(49)*.

Additional evidence for the presence of a primed permissive stroma is derived from studies indicating that genetic alterations in nonhereditary invasive human colon and breast cancers (e.g., loss of heterozygosity and TP53 mutations) occur not only in the neoplastic epithelial cells, but also in the adjacent fibroblastic tumor stroma, both of which probably share clonal features *(50)*. It is clear that the primed stroma fibroblasts, genetically modified *(30)* or affected only via paracrine factors, have a profound effect on stromagenesis. Besides angiogenic support, TAFs influence the behavior of tumor cells as well as the behavior of additional host cells via direct and indirect mechanisms *(4)*. In some instances the tumor will initiate an immune reaction that induces dynamic changes within the stromal ECM, which in turn will result in a more permissive microenvironment for breast cancer progression *(51)*. It has also been shown that mesenchymal adipocytes and adipokines are involved in stromal supportive and promoting effects inducing ductal epithelial cell malignancies in mammary glands *(52)*. Nevertheless, as we previously discussed, in a study observing mammary gland growth and morphogenesis, it was shown that TAFs, but not normal fibroblasts, fail to suppress the estrogen-induced growth of pre-neoplastic cells *(29)*. It is possible that stromal ECM might control the synthetic activity and polarity of epithelial cells, by means of integrins, which are the main cell-matrix adhesion receptors *(53)*.

The activation of integrins has been implicated in both emerging and progressing carcinomas *(54)*. Integrins have been shown to be responsible for both fibronectin fibrillogenesis *(55,56)* and differential cellular responses to mesenchymal microenvironments *(17,53,57)*. Stromagenic fibroblasts effectively utilize integrins in collaboration with growth factor receptors to actively sense and modify their immediate microenvironment *(58)*. Moreover, it has been recently shown that TAFs efficiently promote the expansion and dissemination of a pre-neoplastic epithelial cell population by determining specific ECM stroma profiles *(4)*. ECM stroma profiles are characterized by differential expression and physical organization of soluble factors and insoluble molecules within a given ECM. They comprise fibroblastic-dependent microenvironments with tumor-tailored alterations that directly influence cancer permissiveness. For example, in highly fibrotic tumors presenting altered ECM profiles, the interaction between tumor cells and stromal fibroblasts plays an important role in the formation of fibrotic foci *(59)*. Therefore, it is not surprising that the metastatic ability of breast invasive ductal carcinomas containing fibrotic foci is greatly dependent on the proliferative activity of the fibroblasts forming these fibrotic aggregates *(60)*. Moreover, the stroma are thought to interact with ductal structures in breast cancer initiation. Breast density, assessed by mammographic evaluation, is thought to relate more to stromal rather than epithelial composition *(61)*. Breast density might represent a predisposed, primed, or permissive microenvironmental setting facilitating neoplastic transformation, thus explaining why individuals with denser breasts are at greater risk of developing breast cancer even in the absence of epithelial hyperplasia. All the above-mentioned studies clearly reinforce the idea that fibroblastic-dependent and tumor-tailored primed stroma support and promote tumorigenesis. These studies underline the importance of both stromal fibroblasts and matrices and implicate integrin dependent cell–matrix interactions as possible regulators for stroma priming and stromagenic dynamics.

Utilizing the term "primed stroma" to describe the early predisposed microenvironment which initiates and supports tumor progression *(21,33,62–64)* and distinguishes it from both normal and activated stroma (Fig. 1). In the presence of normal stroma, the tumor is contained *(34)* and, in some cases, may even be reverted *(65,66)*. In contrast, the activated stroma are pathologically identifiable when the tumor has in most cases progressed to develop anchorage independence, invasion, and metastases *(20)*. In comparison to normal and activated stroma, permissive primed stroma surround the early stage tumor but do not exhibit a characteristic pathological phenotype. Nevertheless, primed stroma can be revealed by a few well-defined biological markers. For example, fibroblast activation protein (FAP) *(67–70)*, is a dipeptidyl peptidase and member of the serine protease family that is expressed in wound-healing

tissue. The selective expression of FAP in primed stroma at very early stages of cancer progression, but its absence in normal stroma of benign tumors, normal mesenchyme, and epithelial cells regardless of their transformation stage, indicates that FAP may be a marker protein for primed breast stroma *(67,68)*. Indeed, in a study characterizing FAP stromal expression, FAP was detected on 100% of transformed breast cancer samples analyzed *(67)*. In an additional study that attempted to implicate FAP in the process of tumorigenesis, FAP-transfected cells, but not their catalytically-inactive mutant FAP counterparts, induced tumor formation when xenografted into scid mice *(71)*. This dynamic influence of stroma on tumor cells at an early stage of tumor progression provides an opportunity to target stromal cells to control the malignant behavior of genetically unstable epithelial cells *(32)*. Interestingly, antibodies against FAP were among the first candidates in the emerging class of therapeutics targeting the stroma *(72)*, though there are now others *(73)*. Taken together, the evidence indicates that primed stroma might be a reversible stage that is both induced and supportive of neoplastic transformation. Thus, primed stroma might be a promising target for developing new therapeutics in an attempt to contain breast cancer.

Activated Stroma: The Advanced Neoplastic Microenvironment

The concept of "breast tumor microenvironment" emerged from the observation that tumor cells (e.g., carcinomas) do not exist in isolation but instead exhibit a range of behaviors regulated by host cells comprising the tumor stroma *(74)*. The tumor stroma contain TAFs, vasculature, cells of the immune system, and TAF-derived ECM *(3,4)*. Stromal cells, together with the spectrum of paracrine and autocrine mediators within the tumor microenvironment, play critical roles in the biology of the malignant cells *(3,4,20,62,74,75)*. The normal stromal microenvironment is known to regulate epithelial differentiation, motility, growth, and function during development *(62,75)*. Likewise, the tumor microenvironment plays a critical role during stromagenesis and in tumor differentiation, motility, growth, invasion, angiogenesis, and ultimately, in metastasis *(21,76)*. Thus, the specific normal organ microenvironment or stroma can either inhibit or facilitate tumorigenesis depending on the specific tissue and stromagenic stage *(77)*.

In breast stromagenesis, terms like tumor stroma, desmoplasia, oncofetal stroma, TAF, myofibroblast, and fetal-type fibroblast have been defined *(4)*. Great interest is being directed toward the functional impact of TAF on tumor cells *(4)*, as well as toward the fibroblast–host cell interactions within the tumor tissues *(2)*. It has been shown that diverse fibroblast-derived cytokines and ECMs can affect the invasion of breast carcinoma cells *(78)*. In coculture experiments utilizing immortalized human epithelial cells and stromal cells derived from human carcinomas, the tumor stromal cells could induce permanent carcinogenic changes in the epithelial cells, eventually leading to tumor independence from the microenvironment *(76)*. Breast TAFs exhibit greater proliferative profiles than their normal counterparts *(79)*, suggesting that breast tumors can also influence TAFs. In a study analyzing a variety of breast fibroblasts derived from various stromagenic stages, investigators observed that fibroblasts from patients with benign breast lesions display a different migratory phenotype than breast carcinoma-associated fibroblasts, suggesting phenotypic variation in stromagenic stages *(80)*. The above-mentioned study revealed heterogeneity among fibroblast derived from stromal interlobular samples compared to intralobular fibroblasts, which are more tightly associated with epithelial breast tissue. The study also presented statistical evidence for the presence of functionally aberrant intralobular fibroblasts in histologically normal breast tissue neighboring carcinomas *(80)*. When confronted with tumor cells, fibroblasts convert into a graded pattern of embryonic-like or myogenic-differentiated in the immediate vicinity of tumor cells *(81)*. Furthermore, multiple reports screening pathological cancerous stromas have observed hyper-differentiated or de-differentiated stromal phenotypes. Hyper-differentiated stroma or desmoplasia *(3,4,82–87)* consists of senescent TAFs presenting a myofibroblast morphology. Desmoplastic stroma are enriched in strong condensed microfilaments, resembling those observed in chronic inflammatory diseases, granulation tissue, and scar-forming tissue found during wound healing *(88)*. Although this pathology has been implicated in invasive tumor growth *(89,90)*, the presence of desmoplasia may also be linked to a controversial prognosis of a relatively elongated lifetime *(91,92)*. Smooth muscle actin, vimentin, desmin, and tenascin-C are classic molecular markers upregulated in desmoplastic stroma *(93)*. In comparison to desmoplasia,

de-differentiated or oncofetal stroma show a different morphology *(3,4,34)*. Oncofetal stroma are thick, flaky, and largely disorganized *(4,34)*. TAFs in oncofetal stroma share similarities with embryonic fibroblasts, appear to proliferate more, and their stroma often lose differentiation markers such as smooth muscle actin. Instead of differentiation markers, oncofetal stroma express fetal mesenchymal autocrine (and paracrine) factors found early in cancer-progression, such as the fetal splice variant form of fibronectin known as oncofetal-fibronectin *(94–96)*, and migration-stimulating factors normally present on developing mesenchyme that induce fast migration and proliferation *(34,97)*. In breast cancer, re-expression of laminin $\beta 2$ chain, which is normally present within embryonic matrices, and the ability of tumor cells to grow and metastasize are affected by the presence of adjacent host cells, particularly fibroblasts and in some instances myofibroblasts (1). Extensive ECM accumulation, including collagen types I, III, and V, fibronectin, elastin, and proteoglycans, are increased within activated stromas (1). Although desmoplastic and oncofetal TAFs share similar phenotypic characteristics *(98–101)*, it is not clear if they are derived from each other or arise independently. However, desmoplasia is restricted to the tumor microenvironment, while oncofetal stroma can be observed as an "extended field effect" within the skin of about 50% of patients with breast, colon, lung, prostate carcinomas, melanomas, or soft tissue sarcomas *(97)*. Current therapeutic strategies targeted at the tumor microenvironment provide a novel means of controlling tumor growth and metastasis *(20)* and are directed primarily to the control of "activated" desmoplastic or oncofetal stroma. However, since the presence of activated stroma usually indicates an advanced stage of cancer progression with little or no potential for reversion *(3,4,34,102)*, intervention at an earlier stage of stromagenesis may hold greater therapeutic promise.

VISUAL-QUANTIFICATION OF STROMAGENESIS

Recently, a call made by the National Cancer Institute to focus on tumor–stroma interactions in cancer research resulted in a special publication of the journal *Differentiation* dedicated to the tumor microenvironment (Vol. 70, 2002). The crucial contribution of the tumor microenvironment to tumor development and progression needs to be brought to the forefront of cancer research. The editors declared that elucidating the molecular mechanisms underlying these interactions should be a high priority of cancer research *(20)*. A significant lack of information in the literature that describes the mechanistic alterations within primed and activated stroma highlights the need for technological tools enabling direct visual observations of stromal dynamics and cell–matrix interactions within a physiologically relevant 3D system. In this part of the review we will emphasize identifying the availability of both fibroblastic and ECM stromal markers that are differentially quantifiable throughout the stromagenic stages, offering means for digital imaging in diagnostics. We will describe some visual quantitative approaches and technologies proposing new means of studying stromagenic mechanisms and stromagenic-dependent breast cancer permissiveness.

Quantifiable Breast Stromal Fibroblastic Markers

Fibroblastic cells from normal, primed, or activated breast stromal tissues present multiple quantitative differences in gene expression even when grown in isolation *(75,103)*. Some stromal genes, such as CD34 *(104)* or decorin *(105)*, are downregulated during breast stromagenesis, while others, such as the smooth muscle actin indicative of myofibroblastic differentiation *(75,104)*, are upregulated. The protein SPARC, also known as osteonectin (51), and the migration stimulating factor *(106)* have been shown to be overexpressed by stromal cells. Moreover, phenotypic and genetic alterations in mammary stroma similar to the presence of the above mentioned markers have been implicated in tumor progression *(107)*. Syndecan-1 has been found to be required for the growth-promoting activities of reactive breast stroma. This transmembrane receptor is expressed in developing mammary breast fibroblastic stroma and during stromagenesis. Moreover, a glycanated syndecan-1 dependent growth-promoting loop between breast cancer cells and their stroma has been postulated *(108)*, suggesting that stromal induction of syndecan-1 promotes breast carcinoma cell growth. Other visually detected components within stromagenic fibroblasts are markers for stromagenic processes, such as the loss of stromagenic quiescence and loss of homeostasis *(3,4)*, or the morphological changes in stromagenic fibroblasts *(81,90,109)*. Perhaps the most clearly identifiable fibroblastic stromagenic marker is the protein FAP *(67,72)*, which,

incidentally, was the first therapeutic stromal target *(72)*. Unfortunately, although it is differentially expressed in tumor stroma, FAP use as a marker is controversial because it has also been associated with a positive prognostic outcome when expressed within stromagenic tissue *(91)*, emphasizing the need for more detailed studies describing FAP activity and its effects during stromagenesis. Thus, most fibroblastic stromagenic markers are either over- or under- but not differentially-expressed during stromagenesis, or, if specifically expressed, their functional significance within the stroma is not clear. Therefore, developing stromagenic systems that can accurately mimic the three stromagenic stages are needed in order to assist in the study of stromagenic processes and consequently identify fibroblastic stromal markers. These markers should not only serve as tools for uncovering the fibroblastic mechanisms during stromagenesis but could potentially be brought back to the clinic and targeted in an attempt to restrict cancer progression by stromal regression, thus eliminating breast cancer as a life-threatening disease and relegating it to the status of a chronic disorder.

Quantifiable Breast Stromal ECM Components

Most stromagenic processes are evident within the stromagenic ECM. One of the most dynamic and abundant mesenchymal ECM proteins is the glycoprotein fibronectin. There is a well known pathological association with the plasma increase of fibronectin, since fibronectin goes through a series of breakdowns by neutral proteases. Several of its proteolytic breakdown products exhibit unexpected and mostly harmful biological activities which trigger effects that induce and support malignant transformation *(110)*. Moreover, one of the early signs of malignant transformation is the fragmentation of pericellular fibronectin concomitant with altered production of this protein in the tumor stroma *(110)*. The amount of fibronectin mRNA detected in tumor stroma is different within carcinomas and normal tissues. The fibrillar organization of fibronectin and other ECM proteins is tightly regulated and modified during stromagenesis *(111)*. It is known that the physical state of fibronectin regulates the composition and stability of ECM fibrils and cell-matrix adhesions *(112)*. Therefore, one could assume that the patterning or topographical organization of a tumor-affected stromagenic ECM is directly dependent on its fibroblastic state and, at the same time, clearly reflected in its ECM characteristics. Fibronectin can be expressed not only in different amounts and patterns but as differentially spliced variants that are indicative of stromagenic stage and type *(80,95,106,107,113)*. The presence or absence of these differentially spliced variants within stroma can assist in both sorting and predicting the stromagenic potential of a given tumor. Fibronectin provides a supporting scaffolding and a positive feedback stimulator of tumor growth; high expression of fibronectin, as well as collagen and tenascin-C among others, alters cell–matrix interactions of the stroma. These interactions are modified by soluble factors, such as TGF-β_1, that play important roles in tumor stroma dynamics *(110)*. We suggest that the amount of fibronectin within a given stroma and the fibronectin to collagen and fibronectin to tenascin-C ratios can be used for characterizing stromagenic ECMs by type (desmoplastic or oncofetal) and stage (normal, primed and activated).

As previously mentioned, tenascin-C and its splice variants have been found to be differentially expressed during breast stromagenesis *(114)*. Tenascin-C not only induces metalloproteinase-9 expression directly and by collaboration with TGF-β, but it also alters the stromal ECM dynamics *(87,114,115)*. Even more important, tenascin-C can be converted from a nonadhesive into an adhesive substrate for inducing T cells by the matrix dynamic modulator urokinase plasminogen activator *(111)*. So, fibronectin, collagen, tenascin-C, as well as additional ECM associated glycoproteins, are all quantifiable stromagenic markers that should provide mechanistic-stromagenic information as identification tools for all the stromagenic stages.

Soluble factors, such as TGF-β, are also important stromagenic components. TGF-β has been described as having both tumor-suppressing and oncogenic functions. In epithelial cells, TGF-β has tumor-suppressing effects by initially controlling tumor growth; loss of TGF-β signaling in stromal fibroblasts leads to intraepithelial neoplasia *(116)*. Only later, during tumor progression, epithelial cells cease to respond to TGF-β growth inhibition, though they may still overproduce this cytokine. Thus, TGF-β can not only induce an epithelial to mesenchymal transition, enhancing the invasive and metastatic behavior of the tumor cells, but it also can create a stromagenic immunosuppressive environment that fosters tumor growth and promotes differential ECM dynamics that upregulate collagen I deposition *(117)*. These ECM changes might result in variations in

plasticity and pliability of the stromagenic environment that can further increase the stromal tumor permissiveness *(118)*. An additional group of resident ECM soluble factors are the proteases, more specifically the metalloproteinases, which are believed to play important roles in stromagenic dynamics and in facilitating angiogenesis, as well as in tumor invasion *(22,39,40,42–45,48)*. Telomere length has been associated with malignant breast cancer phenotypes but not TAFs *(119)* and alterations in proteases, as well as their inhibitors during stromagenesis, have been shown to cause changes in breast epithelial telomere length *(48)*. The overexpression of the endoglycosidase heparanase in mammary glands is associated with basement membrane degradation and ECM remodeling, which leads to excess branching and abnormal formation of alveolar structures, as well as in widening of the ducts resulting in an activated fibrotic stroma *(120)*.

All the above-mentioned markers are detectable and visually quantifiable and could serve for stromagenic-related studies. As a result, it might be possible to relate the quantitative protein content of a given molecule during stromagenesis to properties of the stroma, such as pliability. Establishing these relationships may assist in understanding not only the mechanisms behind stromagenesis that are responsible for inducing breast cancer progression but also provide insights into methods for manipulating the stroma affecting tumor permissiveness for future therapeutic intervention.

Quantitative Visual Measuring Strategies and Technologies Applicable in Stromagenic Studies

In order to improve physiological relevance, many investigators utilize a variety of approaches that mimic various aspects of stromagenesis and tumor–stroma interactions. These approaches have led to developing a series of *in vivo*-like 3D systems *(121,122)* that improve disease target identification, drug design, and drug resistance *(123–125)*. Some systems reconstitute epithelial basement membranes in 3D cultures and have shown that they can regulate mammary differentiation *(126)*, while others test 2D versus 3D basement membrane effects on epithelial cancer progression *(127)*. Selected systems utilize mesenchymal-like 3D collagen gels *(53,128)*, while others use fibroblast-derived 3D matrices *(17,57,129–131)*, all of which are believed to be physiologically relevant for studying fibroblast biology while offering new opportunities to understand the reciprocal and adaptive interactions that occur between breast cells and the surrounding matrix in a tissue-like environment. Various other culture systems recapitulate other aspects of tumor–stroma interactions and ECM stromagenic dynamics, such as heterotypic cocultures *(29,132–134)*. Others use animal models for xenografts of various cells *(25,71,135,136)*. We believe that a variety of these systems can be used in a visually quantitative manner in order to enable researchers to conduct mechanistic studies uncovering stromagenic mechanisms and their involvement in stromagenic-induced breast cancer progression. The need for quantitative digital analyses stems from the difficulty of applying classic biochemical and molecular approaches to heterogeneous and diverse cell populations. Moreover, in many instances the importance of the dynamic localization of the proteins or cells requires a visual approach.

Several of the putative markers expressed during stromagenesis can be differentially identified and semiquantified during stromagenesis by immunohistochemical methods. Moreover, multi-photon microscopy provides an opportunity to image specific molecular distributions and signals in living cells with improved sensitivity and diversity over the classic laser scanning confocal microscopy. Since multi-photon microscopy does not involve the need to excite the tissue outside the desired focal area, cells tolerate repeated images of protein auto-fluorescence. Most multi-photon microscopy studies in biological systems have relied on two-photon excited fluorescence to produce images. The broadly used multi-photon microscopy uses nonlinear light-to-matter interactions to provide contrast and optical sectioning capabilities for high-resolution imaging, offering deep penetrating and real time imaging capabilities. With increasing applications of multi-photon microscopy to thick-tissue "intravital" imaging, second-harmonic generation from structural proteins has emerged as a potentially important new contrast mechanism. Thus, it is possible to image cells and extracellular matrices *in vivo* by using second-harmonic generation and two-photon excited fluorescence *(137–139)*. In addition, dual or multi-channel approaches can be used to differentiate *in vivo* stroma from tumor or angiogenic markers. The most accepted approach for quantifying differential expression of markers is the use of indirect immunochemical or immunofluorescent methods. For example, dual-color fluorescence imaging visualizes the tumor–host interaction by whole-body imaging

and at the cellular level in fresh tissues, dramatically expanding previous studies in fixed and stained preparations *(140)*. The dual-color approach has paved the way for introducing multi-channel acquisitions utilizing the various commercially available Meta- or Spectra-imaging systems. These systems have the added capability of simultaneously exciting samples and emitting images from customizable multi-wavelength settings.

Many investigators use imaging techniques to follow changes of molecules and cells migrating within 3D mesenchymal systems. Following morphological changes and migratory patterns of cancer cells within collagen gels has permitted investigators to study the complexity of tumor cell migration, allowing the incorporation of not only knowledge of intracellular pathways, but also fundamental parameters of migratory behavior into the theories of tumor cell migration and metastasis *(141)*. This approach has, for example, described various strategically interchangeable mechanisms by which cells migrate within fibrous 3D microenvironments. Invading cells can utilize either "mesenchymal" or "amoeboid" movements by turning on and off distinct signaling pathways, such as those involving matrix proteases or the Rho/ROCK pathway *(142–148)*.

Using fibroblastic stromal-derived 3D matrices for measuring the effects of stromagenic matrices on cell morphology and motility can be performed by quantitative digital analyses (Fig. 2, *(17,130)*). Moreover, morphological changes such as cell shape change can be quantified within physiologically relevant 3D microenvironments. These measurements have shown that the morphology of a given cell is dependent on the molecular content, the three-dimensionality, and the pliability of the substrate *(17,149)*. Digital analyses of attachment and morphology of cells within fibroblast-derived 3D matrices, correlated with measurements of stroma-induced cell proliferation and phosphorylation or activation state of key signaling molecules *(17,131)*,

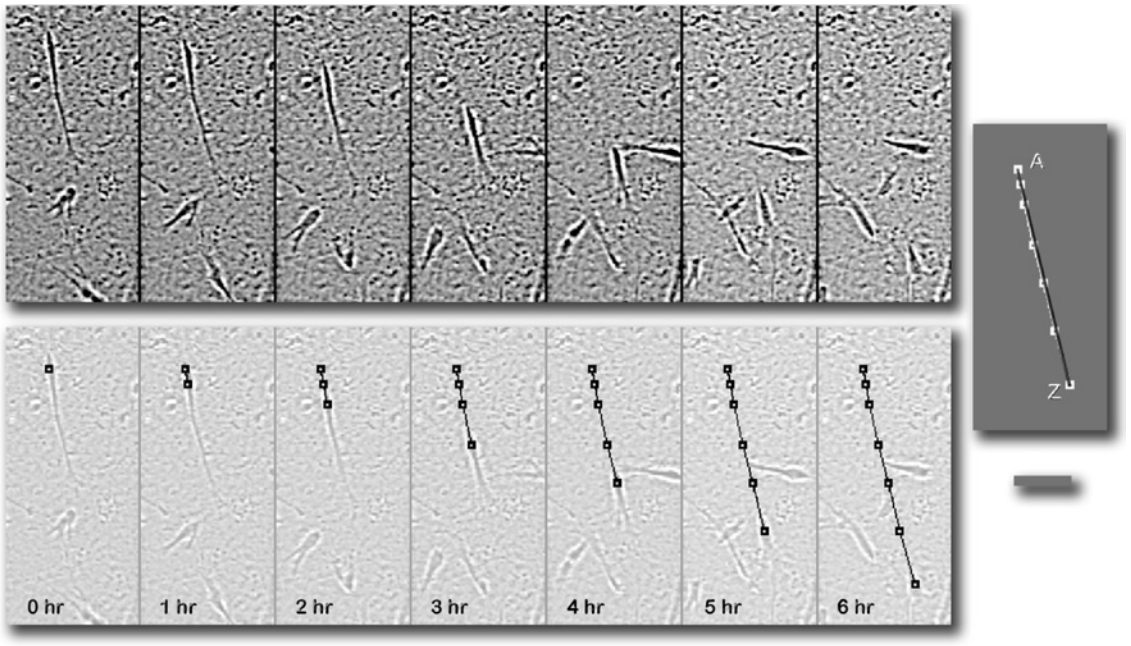

Fig. 2. Fibroblastic motility within stromal-derived 3D matrices. The figure shows the tracking motility and directionality of a human fibroblast within a murine fibroblast-derived 3D matrix for a period of 6 h. The top panel represents a chronological montage of seven digital images acquired at 1-h intervals following a specific cell. The bottom panel represents the same images on which the path trajectory of the individual cell has been systematically traced. The right panel represents the final path track and relative distance. Note that the trajectory-path length and the measured relative distance (A–Z) are 349.7 μm and 349.5 μm, respectively, rendering a directionality rate of 1.0006, which is very close to 1, indicating a considerable directional motility, whereas the calculated motility rate for the given example is 58.3 μm/h. Bar represents 100 μm. This figure is a reproduction from "Cell migration analyses within fibroblast-derived 3D matrices. In: Guan J, editor. Cell migration: Developmental methods and protocols. Totowa, NJ: Humana Press." *(130)*.

provide means for deciphering mechanistic events that trigger stromagenesis.

We trust that this review provides compelling evidence for concluding that during stromagenesis a battery of changes are observed which induce and support tumor progression. Furthermore, investigators now have the tools necessary for conducting breast stromagenic studies utilizing systems and technologies for uncovering the mechanisms behind stromagenic permissiveness during tumor progression. Hence, we are confident that it is only a matter of time before novel therapeutic targets for stromagenic obstruction are revealed, and that this will produce strategies for maintaining breast cancer in a chronically innocuous state.

ACKNOWLEDGMENTS

We thank Drs. L. J. Goldstein, and J. Chernoff for their critical comments, Dr. D. A. Beacham for her useful suggestions, and K. B. Buchheit for the assertive proofreading of this manuscript. This work is supported by a Tobacco Formula grant from the Pennsylvania Department of Health, a grant from the National Pancreas Foundation, an AACR-Pennsylvania Department of Health Career Development Award in Basic Cancer Research, and a NIH/NCI R21 grant (1 R21 CA109442-01).

REFERENCES

(1) Noel A, Kebers F, Maquoi E, Foidart JM. Cell–cell and cell–matrix interactions during breast cancer progression. Curr Top Pathol 1999;93:183–193.

(2) Tuxhorn JA, Ayala GE, Rowley DR. Reactive stroma in prostate cancer progression. J Urol 2001;166(6):2472–83.

(3) Kunz-Schughart LA, Knuechel R. Tumor-associated fibroblasts (Part II): Functional impact on tumor tissue. Histol Histopathol 2002;17(2):623–37.

(4) Kunz-Schughart LA, Knuechel R. Tumor-associated fibroblasts (Part I): Active stromal participants in tumor development and progression? Histol Histopathol 2002;17(2):599–621.

(5) Schaefer D, Jurgensmeier JM, Bauer G. Catechol interferes with TGF-beta-induced elimination of transformed cells by normal cells: Implications for the survival of transformed cells during carcinogenesis. Int J Cancer 1995;60(4):520–6.

(6) Bauer G. Elimination of transformed cells by normal cells: A novel concept for the control of carcinogenesis. Histol Histopathol 1996;11(1):237–55.

(7) Park CC, Bissell MJ, Barcellos-Hoff MH. The influence of the microenvironment on the malignant phenotype. Mol Med Today 2000;6(8):324–9.

(8) Elenbaas B, Weinberg RA. Heterotypic signaling between epithelial tumor cells and fibroblasts in carcinoma formation. Exp Cell Res 2001;264(1):169–84.

(9) Sung SY, Chung LW. Prostate tumor–stroma interaction: Molecular mechanisms and opportunities for therapeutic targeting. Differentiation 2002;70(9–10):506–21.

(10) Brown RA, Sethi KK, Gwanmesia I, Raemdonck D, Eastwood M, Mudera V. Enhanced fibroblast contraction of 3D collagen lattices and integrin expression by TGF-beta1 and -beta3: Mechanoregulatory growth factors? Exp Cell Res 2002;274(2):310–22.

(11) Geiger B, Bershadsky A, Pankov R, Yamada KM. Transmembrane crosstalk between the extracellular matrix and the cytoskeleton. Nat Rev Mol Cell Biol 2001;2(11):793–805.

(12) Graf R, Freyberg M, Kaiser D, Friedl P. Mechanosensitive induction of apoptosis in fibroblasts is regulated by thrombospondin-1 and integrin associated protein (CD47). Apoptosis 2002;7(6):493–8.

(13) Jalali S, del Pozo MA, Chen K-D, Miao H, Li Y-S, Schwartz MA, *et al*. Integrin-mediated mechanotransduction requires its dynamic interaction with specific extracellular matrix (ECM) ligands. PNAS 2001;98(3):1042–46.

(14) Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA. Myofibroblasts and mechano-regulation of connective tissue remodelling. Nat Rev Mol Cell Biol 2002;3(5):349–63.

(15) Zhang HJ, Cai SX, Lu X. Role of integrins in cellular responses to mechanical stress. Prog Biochem Biophys 2002;29(3):359–62.

(16) Katsumi A, Orr AW, Tzima E, Schwartz MA. Integrins in mechanotransduction. J Biol Chem 2004;279(13):12001–4.

(17) Cukierman E, Pankov R, Stevens DR, Yamada KM. Taking cell-matrix adhesions to the third dimension. Science 2001;294(5547):1708–12.

(18) Ingber DE. Cancer as a disease of epithelial–mesenchymal interactions and extracellular matrix regulation. Differentiation 2002;70(9–10):547–60.

(19) Schatzmann F, Marlow R, Streuli CH. Integrin signaling and mammary cell function. J Mammary Gland Biol Neoplasia 2003;8(4):395–408.

(20) Cunha GR, Matrisian LM. It's not my fault, blame it on my microenvironment. Differentiation 2002;70(9–10):469–72.

(21) Abelev GI. Differentiation antigens: Dependence on carcinogenesis mechanisms and tumor progression (a hypothesis). Mol Biol 2003;37(1):2–8.

(22) Jones JL, Shaw JA, Pringle JH, Walker RA. Primary breast myoepithelial cells exert an invasion-suppressor effect on breast cancer cells via paracrine down-regulation of MMP expression in fibroblasts and tumour cells. J Pathol 2003;201(4):562–72.

(23) Rose-Hellekant TA, Gilchrist K, Sandgren EP. Strain background alters mammary gland lesion phenotype in transforming growth factor-alpha transgenic mice. Am J Pathol 2002;161(4):1439–47.

(24) Saez E, Rosenfeld J, Livolsi A, Olson P, Lombardo E, Nelson M, *et al*. PPARgamma signaling exacerbates mammary gland tumor development. Genes Dev 2004;18(5):528–40.

(25) Kuperwasser C, Chavarria T, Wu M, Magrane G, Gray JW, Carey L, *et al*. Reconstruction of functionally normal and malignant human breast tissues in mice. PNAS 2004;101(14):4966–71.

(26) Laconi E, Oren R, Mukhopadhyay DK, Hurston E, Laconi S, Pani P, *et al.* Long-term, near-total liver replacement by transplantation of isolated hepatocytes in rats treated with retrorsine. Am J Pathol 1998;153(1):319–29.

(27) Maffini MV, Soto AM, Calabro JM, Ucci AA, Sonnenschein C. The stroma as a crucial target in rat mammary gland carcinogenesis. J Cell Sci 2004;117(8):1495–1502.

(28) Weaver VM, Gilbert P. Watch thy neighbor: Cancer is a communal affair. J Cell Sci 2004;117(8):1287–90.

(29) Shekhar MP, Werdell J, Santner SJ, Pauley RJ, Tait L. Breast stroma plays a dominant regulatory role in breast epithelial growth and differentiation: Implications for tumor development and progression. Cancer Res 2001;61(4):1320–26.

(30) Moinfar F, Man YG, Arnould L, Bratthauer GL, Ratschek M, Tavassoli FA. Concurrent and independent genetic alterations in the stromal and epithelial cells of mammary carcinoma: Implications for tumorigenesis. Cancer Res 2000;60(9):2562–66.

(31) Bissell MJ, Radisky D. Putting tumours in context. Nat Rev Cancer 2001;1(1):46–54.

(32) Bissell MJ, Radisky DC, Rizki A, Weaver VM, Petersen OW. The organizing principle: Microenvironmental influences in the normal and malignant breast. Differentiation 2002;70(9–10):537–46.

(33) Mueller MM, Fusenig NE. Tumor–stroma interactions directing phenotype and progression of epithelial skin tumor cells. Differentiation 2002;70(9–10):486–97.

(34) Wong YC, Tam NN. Dedifferentiation of stromal smooth muscle as a factor in prostate carcinogenesis. Differentiation 2002;70(9–10):633–45.

(35) Cheng JD, Weiner LM. Tumors and their microenvironments: Tilling the soil. Commentary re: A. M. Scott *et al.*, A phase I dose-escalation study of sibrotuzumab in patients with advanced or metastatic fibroblast activation protein-positive cancer. Clin Cancer Res 2003;9:1639–47, 9(5):1590–95.

(36) Boyd RS, Balkwill FR. MMP-2 release and activation in ovarian carcinoma: The role of fibroblasts. Br J Cancer 1999;80(3–4):315–21.

(37) Chrenek MA, Wong P, Weaver VM. Tumour-stromal interactions: Integrins and cell adhesions as modulators of mammary cell survival and transformation. Breast Cancer Res 2001;3(4):224–9.

(38) Tang Y, Kesavan P, Nakada MT, Yan L. Tumor–stroma interaction: Positive feedback regulation of extracellular matrix metalloproteinase inducer (EMMPRIN) expression and matrix metalloproteinase-dependent generation of soluble EMMPRIN. Mol Cancer Res 2004;2(2):73–80.

(39) Bisson C, Blacher S, Polette M, Blanc JF, Kebers F, Desreux J, *et al.* Restricted expression of membrane type 1-matrix metalloproteinase by myofibroblasts adjacent to human breast cancer cells. Int J Cancer 2003;105(1):7–13.

(40) Behrens P, Rothe M, Wellmann A, Krischler J, Wernert N. The Ets-1 transcription factor is up-regulated together with MMP 1 and MMP 9 in the stroma of pre-invasive breast cancer. J Pathol 2001;194(1):43–50.

(41) Werb Z, Sympson CJ, Alexander CM, Thomasset N, Lund LR, MacAuley A, *et al.* Extracellular matrix remodeling and the regulation of epithelial–stromal interactions during differentiation and involution. Kidney Int 1996;54(Suppl.):S68-74.

(42) Singer CF, Kronsteiner N, Marton E, Kubista M, Cullen KJ, Hirtenlehner K, *et al.* MMP-2 and MMP-9 expression in breast cancer-derived human fibroblasts is differentially regulated by stromal–epithelial interactions. Breast Cancer Res Treat 2002;72(1):69–77.

(43) Mari BP, Anderson IC, Mari SE, Ning Y, Lutz Y, Kobzik L, *et al.* Stromelysin-3 is induced in tumor/stroma cocultures and inactivated via a tumor-specific and basic fibroblast growth factor-dependent mechanism. J Biol Chem 1998;273(1):618–26.

(44) Saad S, Gottlieb DJ, Bradstock KF, Overall CM, Bendall LJ. Cancer cell-associated fibronectin induces release of matrix metalloproteinase-2 from normal fibroblasts. Cancer Res 2002;62(1):283–9.

(45) Heppner KJ, Matrisian LM, Jensen RA, Rodgers WH. Expression of most matrix metalloproteinase family members in breast cancer represents a tumor-induced host response. Am J Pathol 1996;149(1):273–82.

(46) Maxhimer JB, Quiros RM, Stewart R, Dowlatshahi K, Gattuso P, Fan M, *et al.* Heparanase-1 expression is associated with the metastatic potential of breast cancer. Surgery 2002;132(2):326–33.

(47) Koblinski JE, Dosescu J, Sameni M, Moin K, Clark K, Sloane BF. Interaction of human breast fibroblasts with collagen I increases secretion of procathepsin B. J Biol Chem 2002;277(35):32220–7.

(48) Martens JW, Sieuwerts AM, Vries JB, Bosma PT, Swiggers SJ, Klijn JG, *et al.* Aging of stromal-derived human breast fibroblasts might contribute to breast cancer progression. Thromb Haemost 2003;89(2):393–404.

(49) Haslam S, Woodward T. Reciprocal regulation of extracellular matrix proteins and ovarian steroid activity in the mammary gland. Breast Cancer Res 2001;3(6):365–72.

(50) Wernert N, Locherbach C, Wellmann A, Behrens P, Hugel A. Presence of genetic alterations in microdissected stroma of human colon and breast cancers. J Mol Med 2000;78(7):B30.

(51) Sangaletti S, Stoppacciaro A, Guiducci C, Torrisi MR, Colombo MP. Leukocyte, rather than tumor-produced SPARC, determines stroma and collagen Type IV deposition in mammary carcinoma. J Exp Med 2003;198(10):1475–85.

(52) Iyengar P, Combs TP, Shah SJ, Gouon-Evans V, Pollard JW, Albanese C, *et al.* Adipocyte-secreted factors synergistically promote mammary tumorigenesis through induction of anti-apoptotic transcriptional programs and proto-oncogene stabilization. Oncogene 2003;22(41):6408–23.

(53) Walpita D, Hay E. Studying actin-dependent processes in tissue culture. Nat Rev Mol Cell Biol 2002;3(2):137–41.

(54) Thiery JP. Cell adhesion in cancer. Comptes Rendus Physique 2003;4(2):289–304.

(55) Zamir E, Katz M, Posen Y, Erez N, Yamada KM, Katz BZ, *et al.* Dynamics and segregation of cell-matrix adhesions in cultured fibroblasts. Nat Cell Biol 2000;2(4):191–6.

(56) Pankov R, Cukierman E, Katz BZ, Matsumoto K, Lin DC, Lin S, *et al.* Integrin dynamics and matrix assembly. Tensin-dependent translocation of alpha(5)beta(1) integrins promotes early fibronectin fibrillogenesis. J Cell Biol 2000;148(5):1075–90.

(57) Cukierman E, Pankov R, Yamada KM. Cell interactions with three-dimensional matrices. Curr Opin Cell Biol 2002;14(5):633–40.

(58) Lee JW, Juliano R. Mitogenic signal transduction by integrin- and growth factor receptor-mediated pathways. Mol Cells 2004;17(2):188–202.

(59) Hasebe T, Imoto S, Ogura T, Mukai K. Significance of basic fibroblast growth factor and fibroblast growth factor receptor protein expression in the formation of fibrotic focus in invasive ductal carcinoma of the breast. Jpn J Cancer Res 1997;88(9):877–85.

(60) Hasebe T, Sasaki S, Imoto S, Ochiai A. Highly proliferative fibroblasts forming fibrotic focus govern metastasis of invasive ductal carcinoma of the breast. Mod Pathol 2001;14(4):325–37.

(61) Alowami S, Troup S, Al-Haddad S, Kirkpatrick I, Watson P. Mammographic density is related to stroma and stromal proteoglycan expression. Breast Cancer Res 2003;5(5):R129–135.

(62) Olumi AF, Grossfeld GD, Hayward SW, Carroll PR, Tlsty TD, Cunha GR. Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. Cancer Res 1999;59(19):5002–11.

(63) Pupa SM, Menard S, Forti S, Tagliabue E. New insights into the role of extracellular matrix during tumor onset and progression. J Cell Physiol 2002;192(3):259–67.

(64) Skobe M, Fusenig NE. Tumorigenic conversion of immortal human keratinocytes through stromal cell activation. Proc Natl Acad Sci USA 1998;95(3):1050–55.

(65) Hayashi N, Cunha GR, Wong YC. Influence of male genital tract mesenchymes on differentiation of Dunning prostatic adenocarcinoma. Cancer Res 1990;50(15):4747–54.

(66) Wong YC, Cunha GR, Hayashi N. Effects of mesenchyme of the embryonic urogenital sinus and neonatal seminal vesicle on the cytodifferentiation of the Dunning tumor: Ultrastructural study. Acta Anat (Basel) 1992;143(2):139–50.

(67) Garin-Chesa P, Old LJ, Rettig WJ. Cell surface glycoprotein of reactive stromal fibroblasts as a potential antibody target in human epithelial cancers. Proc Natl Acad Sci USA 1990;87(18):7235–69.

(68) Huber MA, Kraut N, Park JE, Schubert RD, Rettig WJ, Peter RU, *et al*. Fibroblast activation protein: Differential expression and serine protease activity in reactive stromal fibroblasts of melanocytic skin tumors. J Invest Dermatol 2003;120(2):182–8.

(69) Rettig WJ, Chesa PG, Beresford HR, Feickert HJ, Jennings MT, Cohen J, *et al*. Differential expression of cell surface antigens and glial fibrillary acidic protein in human astrocytoma subsets. Cancer Res 1986;46(12 Pt 1):6406–12.

(70) Rettig WJ, Garin-Chesa P, Healey JH, Su SL, Ozer HL, Schwab M, *et al*. Regulation and heteromeric structure of the fibroblast activation protein in normal and transformed cells of mesenchymal and neuroectodermal origin. Cancer Res 1993;53(14):3327–35.

(71) Cheng JD, Dunbrack RL, Jr., Valianou M, Rogatko A, Alpaugh RK, Weiner LM. Promotion of tumor growth by murine fibroblast activation protein, a serine protease, in an animal model. Cancer Res. 2002;62(16):4767–72.

(72) Mersmann M, Schmidt A, Rippmann JF, Wuest T, Brocks B, Rettig WJ, *et al*. Human antibody derivatives against the fibroblast activation protein for tumor stroma targeting of carcinomas. Int J Cancer 2001;92(2):240–8.

(73) Corsini C, Mancuso P, Paul S, Burlini A, Martinelli G, Pruneri G, *et al*. Stroma cells: A novel target of Herceptin activity. Clin Cancer Res 2003;9(5):1820–25.

(74) Noel A, Foidart JM. The role of stroma in breast carcinoma growth in vivo. J Mammary Gland Biol Neoplasia 1998;3(2):215–25.

(75) Kunz-Schughart LA, Heyder P, Schroeder J, Knuechel R. A heterologous 3-d coculture model of breast tumor cells and fibroblasts to study tumor-associated fibroblast differentiation. Exp Cell Res 2001;266(1):74–86.

(76) Cunha GR, Hayward SW, Wang YZ. Role of stroma in carcinogenesis of the prostate. Differentiation 2002;70(9–10):473–85.

(77) Fidler IJ. The organ microenvironment and cancer metastasis. Differentiation 2002;70(9–10):498–505.

(78) Arihiro K, Oda H, Kaneko M, Inai K. Cytokines facilitate chemotactic motility of breast carcinoma cells. Breast Cancer 2000;7(3):221–30.

(79) Valenti MT, Sartore S, Azzarello G, Balducci E, Amadio M, Sandri M, *et al*. Human fibroblasts from normal and malignant breast tissue grown in vitro show a distinct senescence profile and telomerase activity. Histochem J 2002;34(8–9):403–10.

(80) Schor AM, Rushton G, Ferguson JE, Howell A, Redford J, Schor SL. Phenotypic heterogeneity in breast fibroblasts: Functional anomaly in fibroblasts from histologically normal tissue adjacent to carcinoma. Int J Cancer 1994;59(1):25–32.

(81) Ronnov-Jessen L, Petersen OW, Koteliansky VE, Bissell MJ. The origin of the myofibroblasts in breast cancer. Recapitulation of tumor environment in culture unravels diversity and implicates converted fibroblasts and recruited smooth muscle cells. J Clin Invest 1995;95(2):859–73.

(82) Thomas GJ, Hart IR, Speight PM, Marshall JF. Binding of TGF-beta1 latency-associated peptide (LAP) to alpha(v)beta6 integrin modulates behaviour of squamous carcinoma cells. Br J Cancer 2002;87(8):859–67.

(83) Hauptmann S, Siegert A, Berger S, Denkert C, Kobel M, Ott S, *et al*. Regulation of cell growth and the expression of extracellular matrix proteins in colorectal adenocarcinoma: A fibroblast-tumor cell coculture model to study tumor–host interactions in vitro. Eur J Cell Biol 2003;82(1):1–8.

(84) Kaarteenaho-Wiik R, Soini Y, Pollanen R, Paakko P, Kinnula VL. Over-expression of tenascin-C in malignant pleural mesothelioma. Histopathology 2003;42(3):280–91.

(85) Slater MD, Lauer C, Gidley-Baird A, Barden JA. Markers for the development of early prostate cancer. J Pathol 2003;199(3):368–77.

(86) De Santis R, Anastasi AM, D'Alessio V, Pelliccia A, Albertoni C, Rosi A, *et al*. Novel antitenascin antibody with increased tumour localisation for Pretargeted Antibody-Guided RadioImmunoTherapy (PAGRIT(R)). Br J Cancer 2003;88(7):996–1003.

(87) Kalembeyi I, Inada H, Nishiura R, Imanaka-Yoshida K, Sakakura T, Yoshida T. Tenascin-C upregulates matrix metalloproteinase-9 in breast cancer cells: Direct and synergistic effects with transforming growth factor beta1. Int J Cancer 2003;105(1):53–60.

(88) Dvorak HF. Tumors: Wounds that do not heal. Similarities between tumor stroma generation and wound healing. N Engl J Med 1986;315(26):1650–59.

(89) Seemayer TA, Lagace R, Schurch W, Thelmo WL. The myofibroblast: Biologic, pathologic, and theoretical considerations. Pathol Annu 1980;15(Pt. 1):443–70.

(90) Barsky SH, Green WR, Grotendorst GR, Liotta LA. Desmoplastic breast carcinoma as a source of human myofibroblasts. Am J Pathol 1984;115(3):329–33.

(91) Ariga N, Sato E, Ohuchi N, Nagura H, Ohtani H. Stromal expression of fibroblast activation protein/seprase, a cell membrane serine proteinase and gelatinase, is associated with longer survival in patients with invasive ductal carcinoma of breast. Int J Cancer 2001;95(1):67–72.

(92) Wiksten JP, Lundin J, Nordling S, Lundin M, Kokkola A, von Boguslawski K, *et al*. Tenascin-C expression correlates with prognosis in gastric cancer. Oncology 2003;64(3):245–50.

(93) Powell DW, Mifflin RC, Valentich JD, Crowe SE, Saada JI, West AB. Myofibroblasts. I. Paracrine cells important in health and disease. Am J Physiol 1999;277(1 Pt. 1):C1–9.

(94) Matsuura H, Hakomori S. The oncofetal domain of fibronectin defined by monoclonal antibody FDC-6: Its presence in fibronectins from fetal and tumor tissues and its absence in those from normal adult tissues and plasma. Proc Natl Acad Sci USA 1985;82(19):6517–21.

(95) Loridon-Rosa B, Vielh P, Matsuura H, Clausen H, Cuadrado C, Burtin P. Distribution of oncofetal fibronectin in human mammary tumors: Immunofluorescence study on histological sections. Cancer Res 1990;50(5):1608–12.

(96) Wunderlich H, Reichelt O, Zermann DH, Schubert J, Berndt A, Kosmehl H. Fetal fibronectin: A new screening-marker for bladder cancer? Oncol Rep 2001;8(3):669–72.

(97) Schor SL. Fibroblast subpopulations as accelerators of tumor progression: The role of migration stimulating factor. Exs 1995;74:273–96.

(98) Brouty-Boye D, Raux H, Azzarone B, Tamboise A, Tamboise E, Beranger S, *et al*. Fetal myofibroblast-like cells isolated from post-radiation fibrosis in human breast cancer. Int J Cancer 1991;47(5):697–702.

(99) Berndt A, Kosmehl H, Katenkamp D, Tauchmann V. Appearance of the myofibroblastic phenotype in Dupuytren's disease is associated with a fibronectin, laminin, collagen type IV and tenascin extracellular matrix. Pathobiology 1994;62(2):55–8.

(100) Berndt A, Kosmehl H, Mandel U, Gabler U, Luo X, Celeda D, *et al*. TGF beta and bFGF synthesis and localization in Dupuytren's disease (nodular palmar fibromatosis) relative to cellular activity, myofibroblast phenotype and oncofetal variants of fibronectin. Histochem J 1995;27(12):1014–20.

(101) Ueki N, Taguchi T, Takahashi M, Adachi M, Ohkawa T, Amuro Y, *et al*. Inhibition of hyaluronan synthesis by vesnarinone in cultured human myofibroblasts. Biochim Biophys Acta 2000;1495(2):160–7.

(102) Liotta LA, Kohn EC. The microenvironment of the tumour–host interface. Nature 2001;411(6835):375–9.

(103) Spanakis E, Brouty-Boye D. Discrimination of fibroblast subtypes by multivariate analysis of gene expression. Int J Cancer 1997;71(3):402–9.

(104) Ramaswamy A, Moll R, Barth PJ. CD34+ fibrocytes in tubular carcinomas and radial scars of the breast. Virchows Arch 2003;443(4):536–40.

(105) Leygue E, Snell L, Dotzlaw H, Troup S, Hiller-Hitchcock T, Murphy LC, *et al*. Lumican and decorin are differentially expressed in human breast carcinoma. J Pathol 2000;192(3):313–20.

(106) Schor SL, Ellis IR, Jones SJ, Baillie R, Seneviratne K, Clausen J, *et al*. Migration-stimulating Factor: A genetically truncated onco-fetal fibronectin isoform expressed by carcinoma and tumor-associated stromal cells. Cancer Res 2003;63(24):8827–36.

(107) Schor SL, Schor AM. Phenotypic and genetic alterations in mammary stroma: Implications for tumour progression. Breast Cancer Res 2001;3(6):373–9.

(108) Maeda T, Alexander CM, Friedl A. Induction of syndecan-1 expression in stromal fibroblasts promotes proliferation of human breast cancer cells. Cancer Res 2004;64(2):612–21.

(109) Gudjonsson T, Ronnov-Jessen L, Villadsen R, Rank F, Bissell MJ, Petersen OW. Normal and tumor-derived myoepithelial cells differ in their ability to interact with luminal breast epithelial cells for polarity and basement membrane deposition. J Cell Sci 2002;115(Pt. 1):39–50.

(110) Labat-Robert J. Fibronectin in malignancy: Effect of aging. Semin Cancer Biol 2002;12(3):187–95.

(111) Silzle T, Randolph GJ, Kreutz M, Kunz-Schughart LA. The fibroblast: Sentinel cell and local immune modulator in tumor tissue. Int J Cancer 2004;108(2):173–80.

(112) Sottile J, Hocking DC. Fibronectin polymerization regulates the composition and stability of extracellular matrix fibrils and cell-matrix adhesions. Mol Biol Cell 2002;13(10):3546–59.

(113) Fukuda T, Yoshida N, Kataoka Y, Manabe R, Mizuno-Horikawa Y, Sato M, *et al*. Mice lacking the EDB segment of fibronectin develop normally but exhibit reduced cell growth and fibronectin matrix assembly in vitro. Cancer Res 2002;62(19):5603–10.

(114) Tsunoda T, Inada H, Kalembeyi I, Imanaka-Yoshida K, Sakakibara M, Okada R, *et al*. Involvement of large tenascin-C splice variants in breast cancer progression. Am J Pathol 2003;162(6):1857–67.

(115) Martin D, Brown-Luedi M, Chiquet-Ehrismann R. Tenascin-C signaling through induction of 14–3-3 tau. J Cell Biol 2003;160(2):171–5.

(116) Bhowmick NA, Chytil A, Plieth D, Gorska AE, Dumont N, Shappell S, *et al*. TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. Science 2004;303(5659):848–51.

(117) Siegel PM, Massague J. Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. Nat Rev Cancer 2003;3(11):807–21.

(118) Beacham DA, Cukierman E. Stromagenesis: The changing face of fibroblastic microenvironments during tumor progression. Semin Cancer Biol 2005 (in press).

(119) Meeker AK, Hicks JL, Gabrielson E, Strauss WM, De Marzo AM, Argani P. Telomere shortening occurs in subsets of normal breast epithelium as well as in situ and invasive carcinoma. Am J Pathol 2004;164(3):925–35.

(120) Zcharia E, Metzger S, Chajek-Shaul T, Aingorn H, Elkin M, Friedmann Y, *et al*. Transgenic expression of mammalian heparanase uncovers physiological functions of heparan sulfate in tissue morphogenesis, vascularization, and feeding behavior. Faseb J 2004;18(2):252–63.

(121) Kim JB, Stein R, O'Hare MJ. Three-dimensional in vitro tissue culture models of breast cancer—A review. Breast Cancer Res Treat 2004;85(3):281–91.

(122) Bissell MJ, Rizki A, Mian IS. Tissue architecture: The ultimate regulator of breast epithelial function. Curr Opin Cell Biol 2003;15(6):753–62.
(123) Schmeichel KL, Bissell MJ. Modeling tissue-specific signaling and organ function in three dimensions. J Cell Sci 2003;116(12):2377–88.
(124) Morin PJ. Drug resistance and the microenvironment: Nature and nurture. Drug Resist Updates 2003;6(4):169–72.
(125) Hazlehurst LA, Landowski TH, Dalton WS. Role of the tumor microenvironment in mediating de novo resistance to drugs and physiological mediators of cell death. Oncogene 2003;22(47):7396–402.
(126) Streuli CH, Bailey N, Bissell MJ. Control of mammary epithelial differentiation - basement- membrane induces tissue-specific gene-expression in the absence of cell cell-interaction and morphological polarity. J Cell Biol 1991;115(5):1383–95.
(127) Weaver VM, Howlett AR, Langton-Webster B, Petersen OW, Bissell MJ. The development of a functionally relevant cell culture model of progressive human breast cancer. Semin Cancer Biol 1995;6(3):175–84.
(128) Grinnell F. Fibroblast biology in three-dimensional collagen matrices. Trends Cell Biol 2003;13(5):264–9.
(129) Yamada KM, Pankov R, Cukierman E. Dimensions and dynamics in integrin function. Braz J Med Biol Res 2003;36(8):959–66.
(130) Cukierman E. Cell migration analyses within fibroblast-derived 3D matrices. In: Guan J, editor. Cell migration: Developmental methods and protocols. Totowa, NJ: Humana; 2004. p. 79–93.
(131) Cukierman E. Preparation of extracellular matrices produced by cultured fibroblasts. In: Bonifacino JS, Dasso M, Lippincott-Schwartz J, Harford JB, Yamada KM, editors. Current Protocols in Cell Biology. Hoboken, NJ: Wiley; 2002. p. 10.9.1–14.
(132) Ogmundsdottir HM, Petursdottir I, Gudmundsdottir I, Amundadottir L, Ronnov-Jessen L, Petersen OW. Effects of lymphocytes and fibroblasts on the growth of human mammary carcinoma cells studied in short-term primary cultures. In Vitro Cell Dev Biol Anim 1993;29A(12):936–42.
(133) Beaupain R. A method for three-dimensional coculture of cancer cells combined to any other type of cells maintained organotypically. Methods Cell Sci 1999;21(1):25–30.
(134) Seidl P, Huettinger R, Knuechel R, Kunz-Schughart LA. Three-dimensional fibroblast-tumor cell interaction causes downregulation of RACK1 mRNA expression in breast cancer cells in vitro. Int J Cancer 2002;102(2):129–36.
(135) Shao ZM, Nguyen M, Barsky SH. Human breast carcinoma desmoplasia is PDGF initiated. Oncogene 2000;19(38):4337–45.
(136) Tahtis K, Lee F-T, Wheatley JM, Garin-Chesa P, Park JE, Smyth FE, *et al*. Expression and targeting of human fibroblast activation protein in a human skin/severe combined immunodeficient mouse breast cancer Xenograft model. Mol Cancer Ther 2003;2(8):729–37.
(137) Brown E, McKee T, DiTomaso E, Pluen A, Seed B, Boucher Y, *et al*. Dynamic imaging of collagen and its modulation in tumors in vivo using second-harmonic generation. Nat Med 2003;9(6):796–801.
(138) Stoller P, Reiser KM, Celliers PM, Rubenchik AM. Polarization-modulated second harmonic generation in collagen. Biophys J 2002;82(6):3330–42.
(139) Zoumi A, Yeh A, Tromberg BJ. Imaging cells and extracellular matrix in vivo by using second-harmonic generation and two-photon excited fluorescence. PNAS 2002;99(17):11014–19.
(140) Yang M, Li L, Jiang P, Moossa AR, Penman S, Hoffman RM. Dual-color fluorescence imaging distinguishes tumor cells from induced host angiogenic vessels and stromal cells. Proc Natl Acad Sci USA 2003;100(24):14259–62.
(141) Niggemann B, Drell I, Theodore L., Joseph J, Weidt C, Lang K, Zaenker KS, *et al*. Tumor cell locomotion: Differential dynamics of spontaneous and induced migration in a 3D collagen matrix. Exp Cell Res 2004;298(1):178–87.
(142) Deryugina EI, Ratnikov BI, Strongin AY. Prinomastat, a hydroxamate inhibitor of matrix metalloproteinases, has a complex effect on migration of breast carcinoma cells. Int J Cancer 2003;104(5):533–41.
(143) Friedl P, Noble PB, Walton PA, Laird DW, Chauvin PJ, Tabah RJ, *et al*. Migration of coordinated cell clusters in mesenchymal and epithelial cancer explants in vitro. Cancer Res 1995;55(20):4557–60.
(144) Friedl P, Brocker EB. The biology of cell locomotion within three-dimensional extracellular matrix. Cell Mol Life Sci 2000;57(1):41–64.
(145) Friedl P, Brocker EB. T cell migration in three-dimensional extracellular matrix: Guidance by polarity and sensations. Dev Immunol 2000;7(2–4):249–66.
(146) Friedl P, Wolf K. Tumour-cell invasion and migration: Diversity and escape mechanisms. Nat Rev Cancer 2003;3(5):362–74.
(147) Sahai E, Marshall CJ. Differing modes of tumour cell invasion have distinct requirements for Rho/ROCK signalling and extracellular proteolysis. Nat Cell Biol 2003;5(8):711–9.
(148) Wolf K, Mazo I, Leung H, Engelke K, von Andrian UH, Deryugina EI, *et al*. Compensation mechanism in tumor cell migration: Mesenchymal-amoeboid transition after blocking of pericellular proteolysis. J Cell Biol 2003;160(2):267–77.
(149) Geiger B. Cell biology: Encounters in Space. Science 2001;294(5547):1661–63.