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## Bi-directional signaling: Extracellular Matrix and Integrin Regulation of Breast Tumor Progression

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### Abstract

Cell transformation and tumor progression involves a common set of acquired capabilities, including increased proliferation, failure of cell death, self-sufficiency in growth, angiogenesis, and tumor cell invasion and metastasis <sup>(1)</sup>. The stromal environment consists of many cell types, including fibroblasts, macrophages, and endothelial cells, in addition to various extracellular matrix (ECM) proteins that function to support normal tissue maintenance, but have also been implicated in tumor progression <sup>(2)</sup>. Both the chemical and mechanical properties of the ECM have been shown to influence normal and malignant cell behavior. For instance, mesenchymal stem cells differentiate into specific lineages that are dependent on matrix stiffness <sup>(3)</sup>, while tumor cells undergo changes in cell behavior and gene expression in response to matrix stiffness <sup>(4)</sup>. ECM remodeling is implicated in tumor progression and includes changes in both the chemical and mechanical properties of the ECM <sup>(5)</sup> that can be a result of 1.) increased deposition of stromal ECM, 2.) enhanced contraction of ECM fibrils, and 3.) altered collagen alignment and ECM stiffness. In addition, remodeling of the ECM may alter whether tumor cells employ proteolytic degradation mechanisms during invasion and metastasis. Tumor cells respond to such changes in ECM remodeling through altered intracellular signaling and cell cycle control that lead to enhanced proliferation, loss of normal tissue architecture, and local tumor cell migration and invasion into the surrounding stromal tissue <sup>(6)</sup>. This review will focus on the bi-directional interplay between the mechanical properties of the ECM and changes in integrin-mediated signal transduction events in an effort to elucidate cell behaviors during tumor progression.

## Keywords

Mechanotransduction; mechanosensor; integrins; morphogenesis; invasion; tumor progression; matrix remodeling; RhoA; FAK; extracellular matrix; bi-directional signaling

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## Integrin Signaling in Tumor Progression

Integrins are transmembrane cell surface receptors that provide external links to the ECM and are the major receptors responsible for cell-matrix adhesions<sup>(7, 8)</sup>. Integrins consist of one  $\alpha$  subunit and one  $\beta$  subunit that assemble into 24 different heterodimers, each with different binding properties for a variety of ECM proteins<sup>(7, 9, 10)</sup>. The large ectodomain of integrins is responsible for ligand binding, while the cytoplasmic tails of integrin heterodimers form multi-molecular complexes containing adaptor proteins involved in signaling pathways and scaffolding proteins that couple the ECM to the actin cytoskeleton<sup>(7, 8, 11, 12)</sup>.

Extracellular ligand binding to the ectodomain induces integrin clustering and conformational rearrangements of the cytoplasmic tail, resulting in changes in binding interactions with the actin cytoskeleton and signal transduction pathways<sup>(13, 14)</sup>. Corresponding conformational changes in the cytoplasmic tail are transmitted to the ligand-binding regions of the ectodomain, via the transmembrane domain, resulting in enhanced binding affinity and additional integrin clustering<sup>(7, 8, 14)</sup>. For adherent cells, integrin clustering can lead to local remodeling of the actin cytoskeleton to form focal adhesions. Consequently, cell-generated contractile forces transmitted through integrins can remodel the surrounding matrix and increase matrix stiffness<sup>(15–18)</sup>. This bi-directional signaling through outside-in and inside-out mechanisms has important implications in tumor progression. For instance, matrix deposition can occur through a feedback loop involving both outside-in and inside-out signaling. Specifically, intracellular actin-myosin generated force applied to  $\alpha 5\beta 1$  integrin results in an increase in fibronectin matrix formation<sup>(19, 20)</sup>. Thus, as the tumor microenvironment undergoes changes in both the chemical and mechanical composition of the ECM integrins play a key role in bidirectional signaling.

Integrins can be considered multisensory receptors that sense two general elements of the ECM; chemical composition (which ligands are present) and mechanical properties (what is the physical context of the ligands that are present). The variety of chemical cues that comprise the ECM of the basement membrane and stroma has been extensively reviewed elsewhere<sup>(21, 22)</sup>. Changes in ECM composition associated with mammary carcinoma alter the expression profile of integrins and downstream signaling to enhance cell invasion and tumor metastasis<sup>(23)</sup>. The expression profile of many integrins is altered in the transition of cells from normal to neoplastic and this is believed to be a necessary step for tumor cell invasion<sup>(23, 24)</sup>. Specifically, adhesion to periostin, osteopontin and tenascin-C alter cell signaling pathways to enhance cancer cell invasion<sup>(25–27)</sup>. Despite changes in integrin levels, integrins remain necessary, as tumorigenic mammary cells treated with a function-blocking antibody for  $\beta 1$  integrin demonstrated a decrease in cell proliferation and an increase in apoptosis<sup>(28)</sup>. Ultimately, modifications in tissue composition alter the local

microenvironment, resulting in dramatic changes in integrin engagement and downstream signaling associated with the progression of many types of cancers <sup>(29–32)</sup>.

## Increased Deposition of Stromal ECM during Tumor Progression

One of the single biggest risk factors for breast carcinoma is an increase in mammographic tissue density. While the underlying cause of increased mammographic tissue density remains largely unknown, there are several factors associated with changes in breast density, including diet, hormone exposure, genetics, age, body mass index, and parity <sup>(33–35)</sup>. In the normal adult mammary gland, several types of collagen (I, III, and V) and collagen crosslinking proteins are found abundantly and are regulated by reproductive state <sup>(36)</sup>. Dense breast tissue is comprised primarily of type I collagen <sup>(37–39)</sup> but also involves upregulation of other ECM proteins, such as, type III and V collagens, fibronectin (FN), tenascin-C, and periostin. Increased deposition of ECM results in tumor tissue being stiffer than normal tissue, as determined by Atomic Force Microscopy (AFM) measurements *in vivo* comparing normal tissue to tumor centers <sup>(40)</sup>. Consequently, the increased deposition of ECM proteins alters both the chemical composition and the mechanical properties of the ECM. An increase in the stiffness of the tumor microenvironment is functionally significant, as it promotes tumor progression through a variety of signaling pathways <sup>(41, 42)</sup>.

During tumor progression the deposition of these additional matrix proteins, a process termed desmoplasia, is associated with poor patient prognosis <sup>(43)</sup>. Thus, these extracellular proteins can be used as predictive markers for carcinoma. For example, Jahkola et al. determined that tenascin-C found at invasive mammary tumor borders is a predictor of both local and distant recurrence <sup>(44–46)</sup>. Additionally, periostin expression has also been associated with tumor size and with poor outcome of ER-positive tumors <sup>(47, 48)</sup>. Consequently, current research is aimed at better understanding the cellular mechanisms underlying the association of altered ECM composition and matrix stiffness with patient prognosis.

## Matrix Stiffness and Integrin Signaling

Mechanical parameters of the ECM, such as ligand density, porosity, cross-linking, and ECM orientation, all influence matrix stiffness and the counter-balancing tensional forces that the matrix exerts on cells. However, the mechanisms by which matrix tension regulates integrin-mediated changes in signaling and cytoskeletal reorganization are not known. Normal tissue homeostasis requires reciprocal interactions between the counter-balancing forces produced by the matrix and cell-generated contractile forces. Mechanotransduction is the process by which mechanical forces are converted into biochemical signals and thus the mechanism by which cells adjust to changes in the microenvironment during tumor progression.

Studies have shown that matrix stiffness strengthens integrin-cytoskeletal linkages and integrin clustering <sup>(49–53)</sup>, as well as increases integrin expression, activity, and focal adhesion formation <sup>(42, 54, 55)</sup>. As depicted in Figure 1, mechanosensitive proteins must undergo conformational changes that alter signal transduction events, intracellular

localization, or cytoskeletal reorganization in response to changes in the mechanical properties of the matrix. Cells sense external forces via integrin adhesions and respond through actomyosin contractile forces that are equal to that of the surrounding matrix to maintain normal tissue architecture<sup>(56–59)</sup>. However, an imbalance in the reciprocal force interactions between the matrix and the cells can result in pathological conditions, such as fibrosis, atherosclerosis, and cancer<sup>(56, 57, 60–63)</sup>.

Changes in the mechanical properties of the matrix, such as increased stiffness or ECM protein density, have been shown to enhance malignant and non-malignant cell growth and proliferation through integrin-mediated mechanisms<sup>(42, 58, 64–67)</sup>. Tissue morphogenesis is also regulated by the biophysical properties of the ECM *in vitro* and *in vivo* through integrin-mediated mechanisms. For instance, human breast cancer cells cultured in compliant matrices exhibit cell phenotypes similar to normal differentiated structures<sup>(42, 55, 58, 65, 68)</sup>. However, when the cells are cultured in a stiffer matrix, their tissue architecture is altered. Provenzano et al. (2008) demonstrate that high mammary collagen density promotes tumor formation *in vivo*, as quantified by an increased area of hyperplasia<sup>(69)</sup>. However, manipulating  $\beta 1$  integrin function using function-blocking antibodies causes tumorigenic mammary cells to undergo phenotypic reversion, such that they form structures similar to normal mammary acini<sup>(70)</sup>, while genetic ablation of  $\beta 1$  integrin inhibits mammary tumorigenesis *in vivo*<sup>(71)</sup>.

Matrix stiffness has also been shown to regulate integrin signaling during cell motility. Cell migration and integrin adhesions are influenced by matrix stiffness<sup>(52, 66, 72, 73)</sup>, while mechanical stretching of a collagen matrix or increased matrix stiffness can enhance cancer cell invasion<sup>(74, 75)</sup>. Moreover, Levental et al., (2009) showed that enhanced collagen cross-linking, which increases matrix stiffness, promotes breast tumorigenesis through increased invasion and enhanced integrin signaling. Data suggest that mechanotransduction in breast cancer cells involves  $\beta 1$ ,  $\beta 3$  and  $\beta 4$  integrins, which are major receptors for cell-matrix interactions involved in migration and invasion in 2D and 3D models<sup>(28, 76)</sup>. Furthermore, transformed mammary epithelial cells treated with either a  $\beta 1$  integrin function-blocking antibody or manipulated to decrease integrin signaling exhibit reduced cell invasion when cultured in cross-linked collagen gels. The outcome of this *in vivo* is likely to be enhanced metastasis, as changes in mammary collagen density and resulting stiffness are positively correlated with an increased number of lung metastases *in vitro* and *in vivo*<sup>(69, 77)</sup>.

## Integrin Signaling and Cell Tuning

Increased matrix stiffness activates integrin-mediated signaling events to increase traction forces in cells through actomyosin contractility<sup>(42, 73, 78)</sup>. The amount by which cells pull on the surrounding matrix is influenced by the stiffness of the matrix through a feedback mechanism<sup>(32, 62)</sup>. Solon et al. have demonstrated that fibroblasts alter the amount of actomyosin contractility to match the stiffness of the ECM<sup>(79)</sup>. If the stiffness of the ECM exceeds the abilities of cells to tune their contractility in order to produce equally reciprocal forces, then cell behavior is altered. For instance, when cell contractility is reduced through inhibition of RhoA or myosin, there is a disruption of integrin clustering, focal adhesion formation, and breast cell morphogenesis in response to matrix stiffness<sup>(29, 42)</sup>. However,

studies have shown that even when increased matrix tension is reciprocated by cell-generated contractile forces, there is less matrix contraction and remodeling, which correlates with disrupted tissue phenotype (58, 65, 68). These observations suggest cells constantly evaluate the stromal environment and modify, or tune, their internal tension to match that of the external environment (42, 57).

While it is established that integrins play a role in responding to mechanical properties in the ECM, it is not clear how mechanical cues facilitate changes in integrin-based multimolecular complexes to initiate biochemical signaling events and actin cytoskeletal reorganization in order to regulate cell behavior during cancer progression. External force induces tension-dependent conformational changes in many integrin-associated proteins, such as vinculin, p130Cas, FAK, src, and filamin A (50, 54, 80–82), suggesting these intracellular signaling molecules play an important role in the mechanosensing capabilities of integrins. For instance, Friedland et al. demonstrated that mechanical tension on  $\alpha 5\beta 1$  integrin increases integrin affinity and FAK phosphorylation, while the stretch-sensitive protein p130Cas undergoes conformational extension in response to cell stretching through activation of Rap1 GTPase (54, 83). In addition to regulating biochemical signaling events, multimolecular complexes have also been shown to couple integrins to the cytoskeleton in response to mechanical cues. For instance, talin has been shown to be required for the reinforcement of integrin-cytoskeletal linkages in response to external force application (84, 85), while zyxin, which directly interacts with p130Cas, has been shown to mobilize from focal adhesions to actin filaments in response to mechanical force (86). These studies support a role for multiple proteins in assembling a mechanosensitive complex to initiate biochemical signaling pathways and to regulate the actin cytoskeleton in response to the external force.

Filamin A (FLNa), an actin binding protein that directly couples  $\beta 1$  integrin to the actin cytoskeleton, has been suggested to play a mechanosensitive role in tuning tissue morphogenesis (87). Gehler et al. (2009) demonstrated that FLNa exhibits increased binding interactions with  $\beta 1$  integrin when breast epithelial cells are cultured in a stiffer collagen matrix (68). Interestingly, when  $\beta 1$  integrin-FLNa interactions are reduced, then cells cultured in a compliant matrix (which usually promotes normal tissue morphogenesis) exhibit reduced matrix contraction and disrupted tissue architecture. However, when  $\beta 1$  integrin-FLNa interactions are enhanced above that normally found in a stiff matrix, cells exhibited increased matrix contraction and displayed a reverted phenotype similar to normal morphogenesis (Figure 2).

The observation that enhanced FLNa binding to  $\beta 1$  integrin reverts cell phenotype in a stiff matrix suggests FLNa may be part of a mechanosensor that tunes cell contractility to reciprocate the external tension created. In support of this notion, mechanical deformation of F-actin/FLNa networks has been shown to enhance FLNa binding to the cytoplasmic domain of  $\beta 7$  integrin and Filamin A-binding RhoGTPase-activating protein (FilGAP), which is a regulator of Rac activity and actin assembly (82). This suggests FLNa binding to  $\beta 1$  integrin not only provides a link between the ECM and the actin cytoskeleton, but can also regulate signaling pathways that may be important for actomyosin contractility (Figure 2). Interestingly, Paszek et al. (2005) demonstrated that transformed epithelial cells with elevated levels of Rho activity exhibit disorganized tissue structures (42). However, when

Rho-generated contractility is reduced, these cells undergo phenotypic reversion, thus restoring tissue polarity *in vitro*. Conversely, when collagen crosslinking was reduced, thus decreasing matrix stiffness, tumor cell proliferation and focal adhesion formation was decreased *in vivo* <sup>(55)</sup>. Although our understanding of mechanotransduction continues to evolve, integrins likely form multimolecular mechanosensitive complexes that regulate both biochemical signaling events and cell-generated contractility through bi-directional mechanisms to respond to matrix stiffness during tumor progression.

## Rho Signaling During Tumor Progression

Rho GTPases are one branch of the larger superfamily of Ras-related small GTPases. To date, 22 human genes encoding at least 25 GTPase-containing proteins have been identified, and of these, Rho, Rac and CDC42 have been the most widely studied mainly due to their role in regulating the actin cytoskeleton and coordinating cell migration <sup>(88, 89)</sup>. Like all Ras-related small GTPases, Rho GTPases are activated downstream of integrins and growth factor receptors and they function as molecular switches that cycle between the active GTP-bound form and the inactive GDP-bound form. In their active form they bind to downstream effector molecules which regulate changes in adhesive state, actin cytoskeleton organization, cellular contractility, and cell motility <sup>(90–92)</sup>. The cycling of Rho activity is regulated by three classes of proteins: GAPs (GTP Activating proteins), GEFs (guanine nucleotide exchange factors) and GDIs (GDP dissociation inhibitors). RhoGAPs facilitate the hydrolysis of GTP to GDP, thereby inactivating Rho, whereas activation of Rho by GEFs occurs by phosphorylation of GDP to GTP. RhoGDIs bind and maintain Rho in its inactive form by blocking localization to the plasma membrane and inhibiting the release of GDP. While Rho is activated downstream of growth factor and integrin signaling, the exact mechanisms of regulation involving GAPs, GEFs, and GDIs largely remains under investigation.

Many tumors carry a mutation in RasGTPases leading to overexpression or increased activation of Ras. While no mutations have been identified in the Rho family of GTPases, several groups have reported overexpression of RhoGTPases in many types of cancer including breast cancers. In fact, increased levels of RhoA protein in breast cancer is associated with increased invasiveness <sup>(93, 94)</sup>. Like RhoA, RhoC is also overexpressed in ductal carcinoma and inflammatory breast cancer and it has been identified as a prognostic marker of mammary tumors and metastasis <sup>(95)</sup>. Furthermore, CDC42 and Rac1 have also been found to be overexpressed in breast carcinoma <sup>(93, 94)</sup>. Rac1 has been implicated in cell transformation *in vitro* and constitutive activation of Rac1 has been suggested to play a role in aggressive breast carcinoma <sup>(96)</sup>. Thus, identifying the mechanisms regulating GTPase activity is important to understanding the progression of breast carcinoma.

Several types of mechanical signals, including the increase in matrix stiffness and collagen alignment <sup>(29, 69)</sup>, are associated with mammary carcinoma, and have been identified as regulators of Rho GTPases <sup>(97–100)</sup>. Rho GTPase activation in response to matrix stiffness plays an important role in altering cell migration within the changing tumor associated microenvironment <sup>(29, 92)</sup>. Subsequently, force generated by Rho-mediated cellular contractility causes a feedback loop to enhance matrix stiffness and alignment by inducing a

conformational change in FN, which is necessary for fibrillar collagen deposition and assembly<sup>(101, 102)</sup>. Additionally, enhanced actomyosin contractility, through increased Rho-ROCK activation, increases collagen deposition and matrix stiffness, which also promotes tumor cell proliferation<sup>(103)</sup>. Alternately, inhibition of ROCK or myosin reduced gel contraction and disrupted tubulogenesis (Figure 3). Thus, integrin signaling to RhoGTPases is a key mechanism by which cells tune to changes in the microenvironment during tumor progression (Figure 3).

Rho GTPases have been implicated at all stages of cancer progression. The initial finding of their role in regulating cytoskeletal dynamics implicated their primary function in coordinating cell migration and adhesion, in addition to matrix reorganization. During cell migration within three-dimensional matrices, tumor cells can transition between mesenchymal, amoeboid, and collective cell migration. All three of these migratory phenotypes involve signaling through RhoGTPases (reviewed in<sup>(92)</sup>). Additionally, activation of the cell cycle via cyclin-D1 and promotion of epithelial to mesenchymal transition (EMT) by increased MMP expression and decreased stability of adherens junctions can be attributed to Rac<sup>(104–106)</sup>. Recent findings have given light to the function of Rho GTPases in other processes relevant to tumor progression including cell survival, protein secretion, vesicle trafficking and gene transcription<sup>(104)</sup>. Thus, elucidating the exact mechanisms of the RhoGTPases in tumor progression remains quite challenging however, their role as potential therapeutic targets is promising.

## Physical Cues and Cell Cycle Control during Tumor Progression

Integrin signaling has been linked to cellular proliferation through its activation of the FAK—Ras—ERK pathway under conditions of stiff matrices<sup>(65)</sup>. Activation of focal adhesion kinase (FAK) is one of the dominant integrin-mediated signaling events, and FAK is emerging as a central regulator of mammary phenotype. Targeted loss of FAK in mammary epithelial cells results in a gland that is hypoplastic, and corresponds to diminished activation of ERK and reduced expression of cyclin D1, suggesting the defect is due in part to diminished cell proliferation during lobuloalveolar development<sup>(107)</sup>. The role for FAK in regulating cell proliferation is further supported by the finding that FAK is strongly implicated in tumor formation. Several independent investigators demonstrated that loss of FAK suppresses tumor formation in mouse models<sup>(108–111)</sup>. This is consistent with the finding that FAK is often over-expressed in human breast carcinomas<sup>(112)</sup>. Analysis of genes regulated by FAK loss in mouse mammary tumors link FAK to proliferation genes involved in both G1 and G2/M<sup>(109, 113)</sup>. FAK loss resulted in the down-regulation of several signaling pathways that link to proliferation including ERK, PI3K, and Rho/ROCK<sup>(109–111)</sup>.

FAK levels are increased in many breast carcinoma samples<sup>(114)</sup>, and FAK is associated with increased progression in several tumors including breast and colon carcinoma, and astrocytoma<sup>(115–118)</sup>. FAK transcriptional expression is increased by the action of NF- $\kappa$ B, which is an important survival mediator<sup>(119, 120)</sup>. However, the finding that FAK levels diminish in some liver and cervical carcinomas<sup>(121, 122)</sup> and that FAK phosphorylation does not always correspond to tumor progression and metastasis<sup>(123)</sup> suggests that extracellular signals impinge upon FAK and determine its role in tumor suppression or progression.

Several investigators have demonstrated that activation of FAK downstream of integrins signals directly to the Ras/ERK pathway and also regulates growth-factor signaling to this pathway<sup>(124–128)</sup>. In the context of matrix stiffness, a dense or stiff matrix activates ERK in mammary epithelial cells, while a compliant matrix does not (Figure 3)<sup>(42, 65)</sup>. Moreover, a stiff matrix results in dramatic upregulation of a proliferation signature that is found in human breast cancer, which can be reversed by inhibition of MEK/ERK<sup>(65)</sup>. A striking feature of cells that are cultured in compliant 3D collagen matrices is that cell proliferation is markedly attenuated in a compliant matrix compared to stiff 3D or 2D matrices, which is linked to the down-regulation of this proliferation signature<sup>(65)</sup>. Importantly, the regulation of cell cycle progression has been specifically linked to tissue stiffness, rather than integrin ligation *per se*, demonstrating the ability of the ECM stiffness to regulate how integrins signal to proliferation<sup>(64)</sup>.

Stiff matrices also induce expression of several genes that are pro-invasive, including MMPs, integrin subunits, and migratory chemokine receptors. As with proliferation, inhibition of MEK reverts this gene expression, reverts the invasive phenotype, and promotes normal tubulogenesis in a stiff 3D collagen matrix<sup>(65)</sup>. These findings support the notion that one way a stiff matrix promotes tumor progression to invasiveness is by activating gene expression through the MEK/ERK pathway (Figure 3).

## Collagen Alignment during Tumor Progression

Recent findings have demonstrated that not only does the chemical and physical composition of the ECM affect mammary tumor progression, but the re-organization of matrix proteins into aligned fibers is also an important contributor to tumor progression. In mouse mammary tumors, the deposition and organization of collagen fibers changes dramatically as tumors progress. Provenzano et al (2006) described these changes as Tumor Associated Collagen Signatures (TACS) with TACS-3 representing perpendicular alignment of stromal collagen to the tumor in MMTV-PyVT mice<sup>(129)</sup>. In mice with collagen-dense mammary glands, the presence of TACS-3 increases and correlates with increased tumor burden, invasion, and metastasis to the lung<sup>(69)</sup>. These findings strongly implicate that aligned collagen fibers provide “tracks” by which tumor cells can escape from a primary tumor, thus facilitating metastasis. This is supported by observations that collagen aligned perpendicular to a tumor-explant boundary promotes the invasion of mammary epithelial cells *in vitro*<sup>(130)</sup>. Conklin et al. (2011) corroborated these results in a study of human breast cancer, where TACS-3 fibers surrounding hyperplastic mammary ducts were identified in human breast tumor histological sections, and were associated with poor patient outcome<sup>(131)</sup>. These initial observations of tumor-induced matrix reorganization provided some of the first evidence that matrix topographical cues in developing tumors may direct cell migration away from a primary tumor, and may reveal some insight into potential mechanisms of metastasis. However, the mechanism by which ECM alignment is produced and maintained *in vivo*, particularly with regard to TACS-3 aligned fibers surrounding breast tumors, is not well understood.



## Generation of Collagen Alignment

The ability of cells to create regions of aligned fibers *in vitro* has been well established in fibroblast-collagen gel cultures and occurs concomitantly with gel contraction by cells <sup>(132)</sup>. In this contraction-induced model, cells exert strain on surrounding matrix fibers such that when matrix stiffness is less than the force imparted by cells, the matrix deforms and undergoes changes in local alignment but the cells do not exhibit substantial levels of migration. However, when matrix stiffness exceeds cell contractile forces, cells are then able to migrate <sup>(18)</sup>. These findings further suggest that matrix stiffness determines the cellular tuning response, and that an appropriate amount of stiffness is required for cells to generate traction and subsequent cell migration (Figure 4).

A study of vascular smooth muscle cells cultured in collagen gels showed that integrin linkages to collagen fibers are required for gel contraction by cells. When the  $\alpha 2$  and  $\beta 1$  integrin subunits are blocked, contraction is halted, and the contraction-induced matrix tension is lost <sup>(133)</sup>. Similarly, Provenzano et al. (2008) showed that the generation of alignment between tissue explants or cell-seeded collagen gels is dependent on actin-myosin contractility and requires signaling via Rho GTPase and its effector, ROCK. This contractile mechanism, however, is only required to set up the alignment; when cells are confronted with a matrix that is already aligned, they migrate along fibers even when Rho is inhibited, further suggesting that cells must first utilize contractile means downstream of integrin linkages to align fibers, after which may then provide the necessary traction to support migration <sup>(130)</sup>.

It is unclear what cells in the tumor microenvironment are responsible for creating aligned matrices around developing tumors. Although carcinoma cell lines and cells from tumor explants have been shown to align collagen *in vitro*, multiple cell types may work synergistically to produce matrix alignment *in vivo*. Additionally, generation of alignment may also result from *de novo* collagen deposition by stromal fibroblasts. Indeed, fibroblasts can be exploited *in vitro* to deposit highly aligned collagen matrices, and the orientation of the fibroblasts determines that of the newly deposited aligned matrix. In turn, fibroblast orientation can be controlled by the initial matrix on which they are seeded. For example, when fibroblasts are allowed to adhere to a matrix that is already aligned, the cells will deposit more aligned matrix <sup>(134–137)</sup>.

One mechanism by which stromal cells deposit aligned matrices appears to involve the proteoglycan, syndecan-1. The cell surface proteoglycan, syndecan-1, interacts with integrins among other cell surface proteins to aid in adhesion and migration <sup>(138)</sup>. Yang et al (2011) showed that fibroblasts expressing elevated levels of syndecan-1 generate parallel-organized ECM fibers that allow seeded tumor cells to adhere to and migrate directionally along fibers. This occurs in contrast to cells cultured on randomly organized matrices generated by fibroblasts lacking syndecan-1 expression <sup>(135)</sup>. Furthermore, increased expression of syndecan-1 in human breast carcinomas is associated with the aligned matrix architecture present in tumors <sup>(131, 135)</sup>. These findings indicate syndecan-1 is a key player in the process of generating aligned fibers, which may shed some additional light on other potential signaling pathways; however, more work must be done to further understand this process.

The presence of TACS-3 aligned collagen fibers in breast cancer is strongly correlated with cancer cell invasion and metastasis, however, little is known how cells sense and respond to these changes in ECM topography. When cancer cells are cultured in matrices of pre-aligned collagen, they exhibit more directionally persistent migration compared to cells cultured in random collagen matrices<sup>(130)</sup>. Although the mechanisms of directional sensing are not yet fully understood, several hypotheses exist for how alignment of the ECM guides migrating cells. Clusters of  $\beta 1$  integrin localize along collagen fibers (unpublished data, Keely Lab) and fiber alignment likely guides newly formed integrin clusters along the leading edge of the cell. In addition to the effects of increased integrin signaling on cell motility and invasion, the channels created by parallel-aligned fibers provide less spatial impedance for migrating cells<sup>(139)</sup>. Furthermore, aligned collagen may be stiffer than randomly organized collagen<sup>(140)</sup>, suggesting that the increase in ECM stiffness may provide enhanced traction for motility and restrict motility to the direction of greatest traction, namely along parallel aligned ECM fibers.

In addition to mechanical recognition of aligned collagen, recent findings show that the ability of cells to recognize patterned fibronectin substrates is dependent on syndecan-4. Bass et al (2007) demonstrated that cells lacking syndecan-4 expression fail to migrate directionally when cultured on matrices containing aligned fibronectin. This response was shown to depend on the ability of syndecan-4 to bind to and localize PKC $\alpha$  to points of ECM engagement at the leading edge, followed by subsequent rac localization by PKC $\alpha$ <sup>(141)</sup>. In this scenario, cells are guided by the organization of the matrix via their points of contact with the matrix. These findings reinforce the concept that cells tune their responses through integrins to changes in matrix mechanics, composition, and organization, allowing them to constantly adapt to and modify their ever-changing environment.

## Matrix Metalloproteinases in Breast Cancer Invasion and Metastasis

While matrix remodeling that leads to changes in the mechanical properties of the ECM plays an important role during tumor progression, proteolytic degradation is another key component in this process. Matrix metalloproteinases (MMPs) have broad implications in many cancers and their functions involving tumor promotion and invasion have been extensively studied, although our understanding of this complex regulatory network remains limited. MMPs are most widely known for degrading ECM proteins, however, they have a diverse group of substrates and are implicated in many aspects of cell signaling. MMPs can bind integrins, release surface-bound growth factors and growth factor receptors, as well as cleave cell-cell and cell-matrix adhesion molecules<sup>(142)</sup>. In so doing, MMPs can directly regulate proliferation, migration, and apoptosis.

Expression of MMPs is markedly increased in many cancers including breast carcinomas, and is associated with poor prognosis<sup>(143, 144)</sup>. In mouse xenograft models, elevated expression of MMPs increases tumor burden and metastasis, whereas decreased levels of MMPs in more invasive cell lines reduces tumor malignancy and lung metastases<sup>(142)</sup>. Several *in vitro* experiments show a positive correlation between MMP expression and invasion of carcinoma cell lines through collagen type I matrices or matrigel<sup>(145–147)</sup>. While basement membrane and to a lesser degree, stromal collagen, largely serve as a barrier to

migration, MMPs allow cells to navigate through dense fibrillar networks. In addition to simply severing ECM fibers that may be obstructing a cell's trajectory, the cleavage of ECM proteins can expose cryptic binding sites that bind integrins and promote migration <sup>(142)</sup>.

### Evidence for MMP-dependent migration

Cancer cells cultured in 3D matrices employ a mesenchymal migration phenotype that is dependent on integrin-ECM engagement. In dense collagen matrices or matrigel, where the ECM poses a significant structural barrier, cells will extend pseudopods into the matrix, generate traction force, and locally secrete MMPs to sever fibers adjacent and posterior to the leading edge <sup>(148)</sup>, <sup>(149)</sup>. This allows cells to maintain traction on fibers where integrins are engaged, and subsequently enables translocation of the cell body through the matrix. Cleaved fibers are then displaced and become aligned as cells exert tension and push their way through the matrix <sup>(139)</sup>. The resulting aligned ECM tracks represent a permanent matrix deformation, which can then promote subsequent cancer cell migration in a protease-independent manner <sup>(150)</sup>, <sup>(148)</sup>.

### Evidence for MMP-independent migration

There is mounting evidence that tumor cells exhibit remarkable plasticity in their ability to change migration modes. When MMPs are inhibited *in vitro*, tumor cells can adopt a more amoeboid migration profile that is primarily dependent on Rho-mediated actin-myosin contractility (Figure 4), allowing cells to change shape and squeeze through pores in the matrix <sup>(151)</sup>, <sup>(152)</sup>. Additionally, cells that employ amoeboid migration do not permanently deform matrix fibers, but rather leave the matrix relatively undisturbed <sup>(153)</sup>. For this mode of migration, matrix density plays a much more significant role in that migration is arrested if the matrix pore size is sufficiently small <sup>(154)</sup>, <sup>(155)</sup>.

Conversely, numerous reports have also shown that a mesenchymal-like migration phenotype can prevail independent of MMP action. In nested collagen gel assays, cells retain their ability to mechanically deform matrix fibers via Rho and Rock allowing subsequent migration even when proteases are inhibited <sup>(130)</sup>. In these scenarios, matrix stiffness may play a larger role in allowing cells' contractile machinery to reorganize matrix fibers, rendering MMPs unnecessary. If, however, the matrix stiffness exceeds the amount of force cells are capable of producing, protease-dependent migration would likely dominate (Figure 4). The presence of aligned collagen fibers is expected to alleviate some of the restrictions that a dense matrix might create, and instead create an open track complete with adhesive ligand along which cells can migrate. Thus, it is not surprising that migration along aligned collagen fibers appears to be protease independent <sup>(130)</sup>.

Further support for protease independent cancer cell migration comes from recent work with mice that have a mutation in the collagenase cleavage site of the *coll1a1* gene, and results in an overabundance of collagenase-resistant stromal collagen <sup>(156)</sup>. These mice, in a PyMT background, have much more aggressive mammary tumors and more lung metastases, suggesting that proteases are not required for invasion through collagen-rich mammary glands <sup>(69)</sup>. Of interest, in *Col1a1<sup>tm1jae</sup>* animals with protease resistant collagen, collagen alignment is augmented <sup>(69)</sup>. These findings highlight an important distinction between the

role of proteases and the respective ECM compartments in which their function is necessary for cells to effectively migrate. Conceivably, proteases may only be necessary for invasion through dense basement membrane, or during intra- or extravasation of the vasculature, where a cell cannot penetrate the ECM utilizing any other means. Once cells have crossed these superficial barriers to migration, however, they may then migrate independently of proteases.

## Conclusions

Cells interact with their microenvironment through reciprocal interactions that involve both chemical AND mechanical properties of the ECM, such as matrix stiffness, topography, organization, and alignment. Together, these ECM properties regulate integrin signaling to influence cell behavior that promotes growth and differentiation, proliferation, and invasion and metastasis during tumor progression. For instance, cells can tune their response, within a range, to changes in the mechanical properties of the ECM through integrins and adjust the force they exert upon the matrix. This process likely involves numerous signaling molecules that comprise a mechanosensitive complex. This cellular tuning profoundly affects the cellular response, such as proliferation and invasion, to the ECM that can lead to tumor progression. Conversely, cell-mediated activities, such as myosin-generated traction forces, cross-linking, and protease digestion, modify and alter the chemical and structural composition of the ECM that in turn influences cell behavior. While our understanding of the mechanotransduction is incomplete, further elucidating the mechanisms involved in the reciprocal interactions between cells and various properties of the microenvironment will provide valuable insight to how mechanical cues regulate cell behavior.

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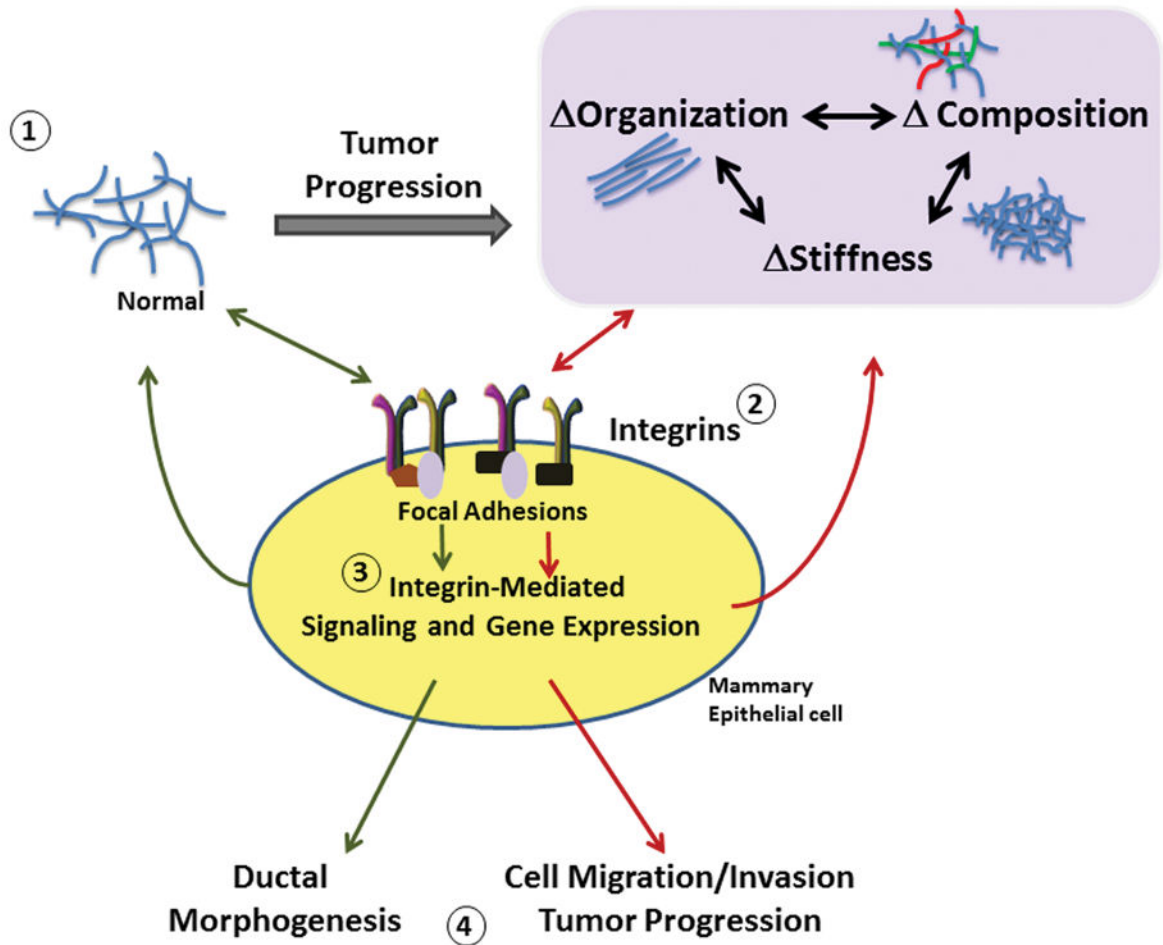


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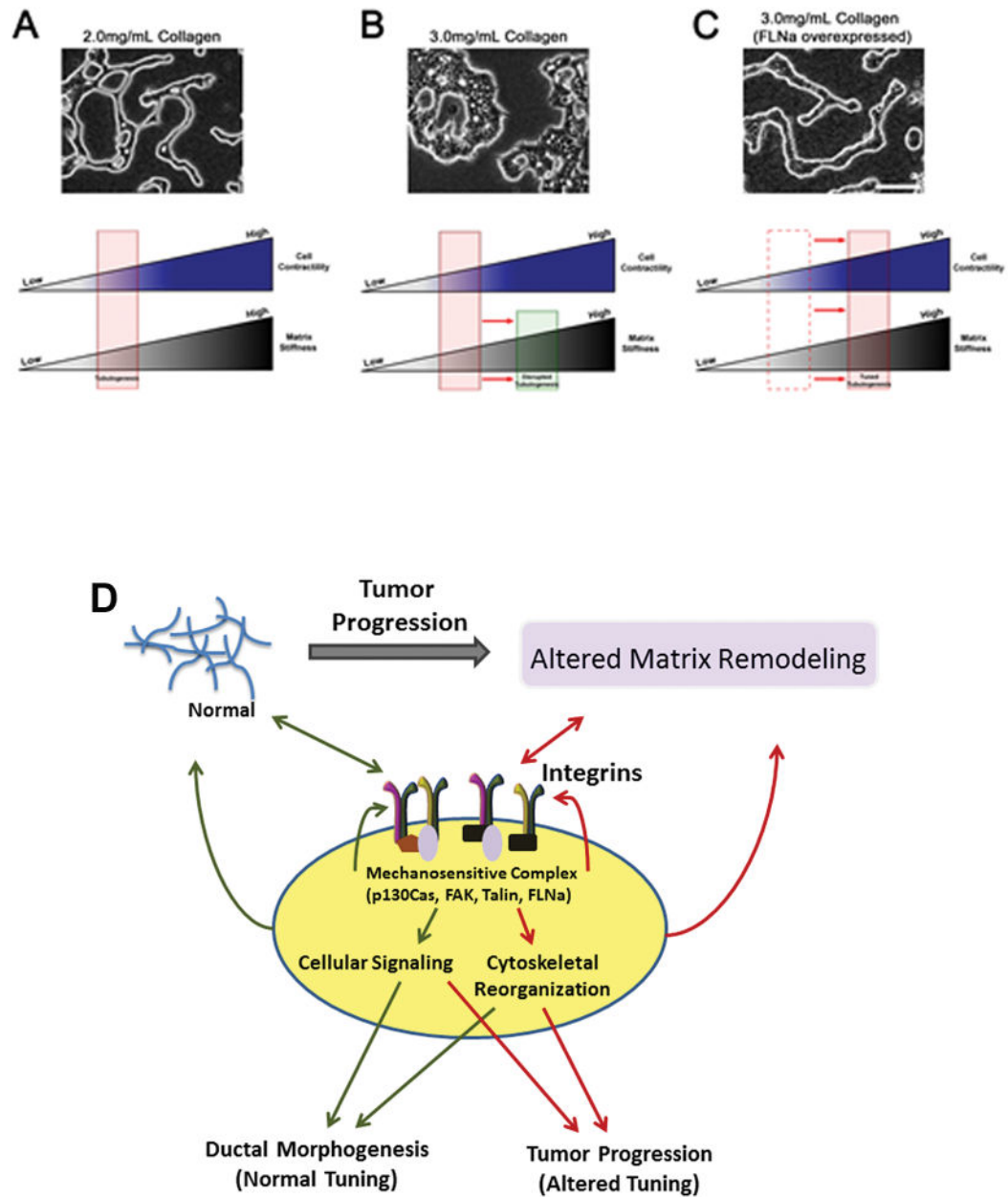
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**Figure 1.**

Bi-directional signals from integrins and the ECM during mammary tumor progression. 1. The extracellular matrix changes in both physical and chemical composition during breast cancer progression. 2. The integrins respond to and signal back to the matrix through altered integrin engagement, increased adhesion, and focal adhesion signaling. 3. Downstream integrin signaling changes in response to cues from the ECM. Integrin signaling pathways feed back to the matrix through enhancing deposition of matrix components and by altering cell contractility to induce matrix organization. 4. Ultimately, mammary epithelial cells respond to the ECM by tuning their phenotype toward ductal morphogenesis under normal matrix conditions or toward enhanced proliferation, migration and invasion in response to abnormal matrix conditions.



**Figure 2.**

Cell contractility can be tuned to reciprocate matrix stiffness to result in phenotypic reversion. A) T47D cells cultured in a 2.0mg/mL collagen gel exhibits normal tubulogenesis. This suggests cell contractility reciprocates the stiffness of the matrix. B) When matrix stiffness increases (3.0mg/mL collagen gel), cells undergo disrupted tubulogenesis as a result of the inability of the cells to counter the increased matrix tension. C) T47D cells that overexpress FLNa, which exhibit enhanced cell contractility, undergo phenotypic reversion when cultured in a 3.0mg/mL collagen gel. Enhanced FLNa binding with  $\beta 1$  integrin produces similar results. Scale bar = 100 $\mu$ m. D) Matrix-induced changes in integrin-mediated cellular signaling and cytoskeletal organization involve a mechanosensitive

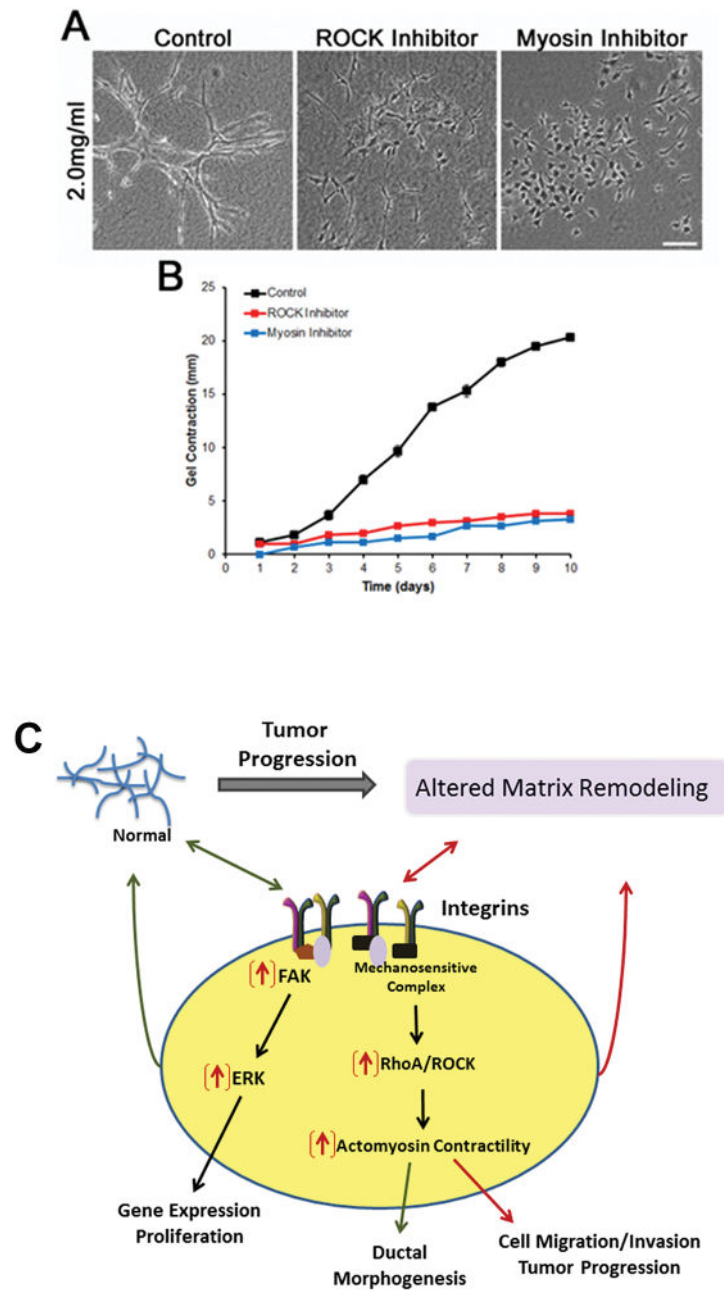
complex that can undergo reciprocal signaling to alter matrix remodeling and integrin clustering.

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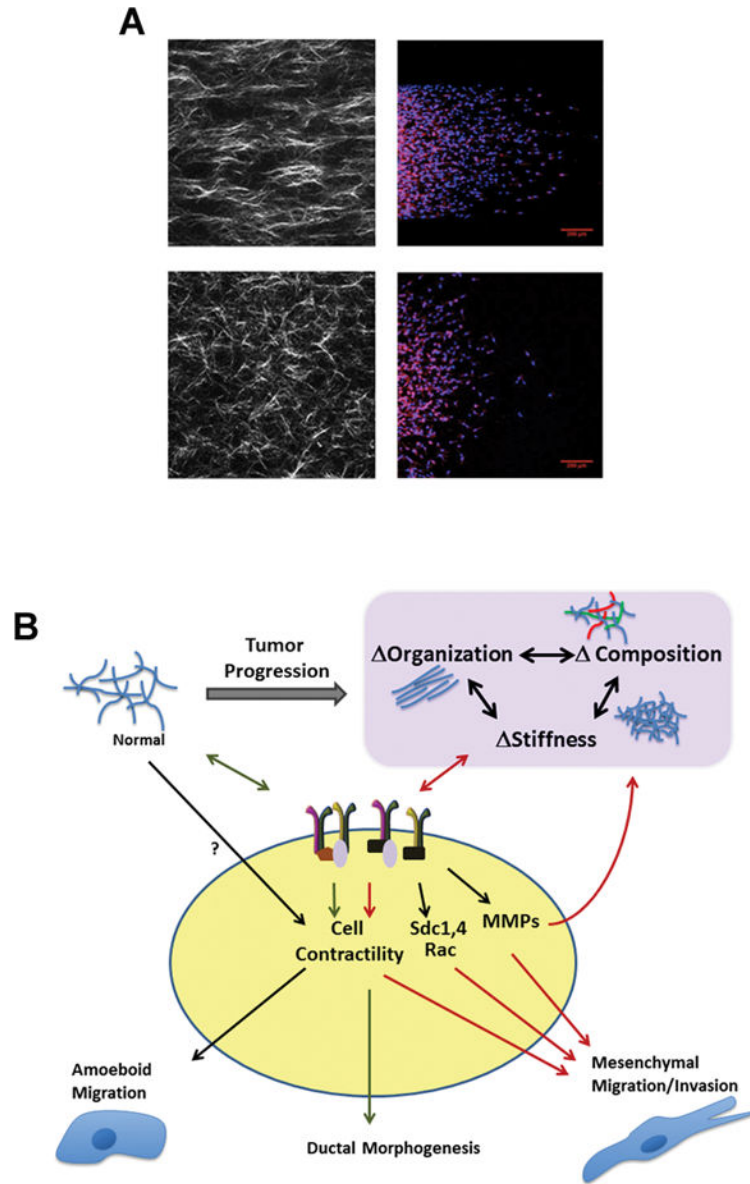
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**Figure 3.**

RhoA-mediated cell contractility is essential for branching morphogenesis and gel contraction. Inhibition of ROCK (2.5 $\mu$ M H-1152) or myosin (10 $\mu$ M Blebbistatin) disrupt branching morphogenesis (A) and gel contraction (B) in NMuMG breast epithelial cells cultured in a 2.0mg/mL collagen gel. Scale bar = 100 $\mu$ m. C) Matrix-induced changes in integrin-mediated cell signaling and actomyosin contractility which are important for cell behaviors, such as proliferation and morphogenesis, and reciprocal matrix remodeling. Increased FAK/ERK and RhoA/ROCK signaling (indicated by red arrows) promotes tumor progression through enhanced cell migration/invasion and proliferation.





**Figure 4.**

Bi-directional signals from integrins and extracellular matrix composition, organization, and stiffness lead to altered cell behavior in tumor progression. A. MDA-MB-231 cells cultured in microchannels containing aligned or random collagen matrices migrate more when collagen is aligned (top panel). Collagen is visualized via second harmonic generation (left) and cells are stained for nuclei and actin with DAPI and Phalloidin, respectively (right). B. Cells respond to changes in ECM chemical and physical composition by altering signaling through integrins, and in tumor progression, regulate further changes in the ECM by increasing the expression and activation of MMPs or by enhancing Rho-mediated contractility. In so doing, tumor cells rapidly adapt to changes in their environment, and consequently promote invasion and metastasis.