

Mammary Gland ECM Remodeling, Stiffness, and Mechanosignaling in Normal Development and Tumor Progression

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Cells of the mammary gland are in intimate contact with other cells and with the extracellular matrix (ECM), both of which provide not only a biochemical context, but a mechanical context as well. Cell-mediated contraction allows cells to sense the stiffness of their micro-environment, and respond with appropriate mechanosignaling events that regulate gene expression and differentiation. ECM composition and organization are tightly regulated throughout development of the mammary gland, resulting in corresponding regulation of the mechanical environment and proper tissue architecture. Mechanical regulation is also at play during breast carcinoma progression, as changes in ECM deposition, composition, and organization accompany breast carcinoma. These changes result in stiffer matrices that activate mechanosignaling pathways and thereby induce cell proliferation, facilitate local tumor cell invasion, and promote progression. Thus, understanding the role of forces in the mammary gland is crucial to understanding both normal developmental and pathological processes.

While it has long been appreciated that the biochemical environment provided by the extracellular matrix (ECM) is a key determinant of normal and pathological progression in the mammary gland, recently the realization that there is also a mechanical aspect to cell responses to the ECM has emerged. The signal-transduction response of cells to the physical aspects of their environment is termed “mechanosignaling,” and is the general subject of this article. It is anticipated that the mammary

gland will prove to be a powerful developmental model to investigate mechanosignaling due to its postnatal development, which extends the time-course and provides a large tissue source for biochemical studies. However, ultimately, the power of this model lies in the fact that the normal mammary gland exists in many different developmental states, each with unique tissue tension requirements. For this review, we include studies from non-mammary cells to help inform what may be occurring in the





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context of the mammary gland, and try to indicate where information is specifically obtained in a mammary system.

The recognition that a cell is in a physical continuum with its ECM was discerned early on from images obtained by quick freeze, deep-etch electron microscopy, a rapid, chemical-fixation-free method that preserves native macromolecular structure with high fidelity. Using this approach to study cells within their tissue context, fine ECM fibers were found to radiate orthogonally from the plasma membrane surface and to exist as a continuum with the cytoplasmic cytoskeleton (Mecham and Heuser 1990; Singer 1979; Singer et al. 1984). The clear registry of cellular microtubules and actin cytoskeleton with extracellular matrix fibers in fibroblasts (Tomasek et al. 1982) and corneal epithelium (Sugrue and Hay 1981) led Dr. Elizabeth D. Hay to propose that the physical continuum between ECM and cytoskeletal organization reflects a functional continuum (Bissell 1981; Emerman et al. 1981; Hay 1981).

Independently, involvement of the cytoskeletal components in growth regulation (Teng et al. 1977), the connection between extracellular matrix (ECM) and gene expression in mammary epithelial cells (Emerman et al. 1981; Bissell 1981), and the importance of 3D context in functional differentiation (Hall et al. 1982; Hall and Bissell 1986) led Mina Bissell to state that the microenvironment regulates gene expression (Bissell 1981; Bissell et al. 1982). Subsequent studies evaluating human embryonic lung epithelial cells showed concomitant organization of fibronectin fibers secreted by and deposited beneath the cell and the micro-filament bundles within the cell (Hynes and Yamada 1982) as well as collagen fibers with intermediate filaments (Hall and Bissell 1986). This non-random orientation of ECM fibers with respect to the cell surface is called anisotropy and leads to spatially oriented matrix networks that serve as adhesion sites, migration routes, as well as concentration gradients of fibrils that generate differential tension and distinct gene expression patterns.

The major structural protein in the mammary gland, and indeed in the entire body, is

fibrillar collagen. In addition to providing a biochemical ligand for several receptors, collagen provides structural support for the gland, which is appreciated when one sees the relationship of collagen fibers to the epithelial cells (Fig. 1A). Fibrillar collagen is closely associated with the basal lamina, a highly organized and specialized ECM region that separates the epithelium from the less structured underlying collagen1-rich stromal compartment (Monaghan et al. 1983). The proteins comprising the basal lamina were classically identified as collagen IV, the laminins, entactin, and proteoglycans. While the mechanical properties of the basal lamina per se are currently not clear, no doubt several basal lamina proteins contribute to the mechanical properties of the ECM, and their roles are expected to emerge in coming years. In this article, we focus predominantly on the stromal ECM and the fibrillar collagens as the major structural proteins that affect the mechanical environment of mammary epithelial cells, as this is the aspect that is best understood.

Cells adhere to the ECM through several different receptors, the most prominent of which belong to the integrin family. Several reviews of integrin structure and function exist, and the reader is referred there for more information (Liu et al. 2000; Hynes 2002; Katsumi et al. 2004). Beta-1 family integrins in particular are implicated in maintaining the normal phenotype of breast epithelial cells (Streuli et al. 1991), and contribute to the architecture and branching morphogenesis of the gland. Knockout of the $\beta 1$ integrin subunit results in disrupted differentiation and alveologensis, presumably through loss of critical adhesion sites (Li et al. 2005; Naylor et al. 2005). Moreover, knockout of the $\alpha 2$ subunit, a key receptor for collagens in the mammary gland, leads to a loss of branching complexity (Chen et al. 2002). While a classic biochemical receptor/ligand relationship between integrins and ECM molecules has been the model, emerging is the concept that receptors for ECM work at the interface of linking mechanical signals into biochemical signals in the cell.

The mechanical properties of the ECM are regulated by numerous growth factors acting on cells such as fibroblasts, which deposit

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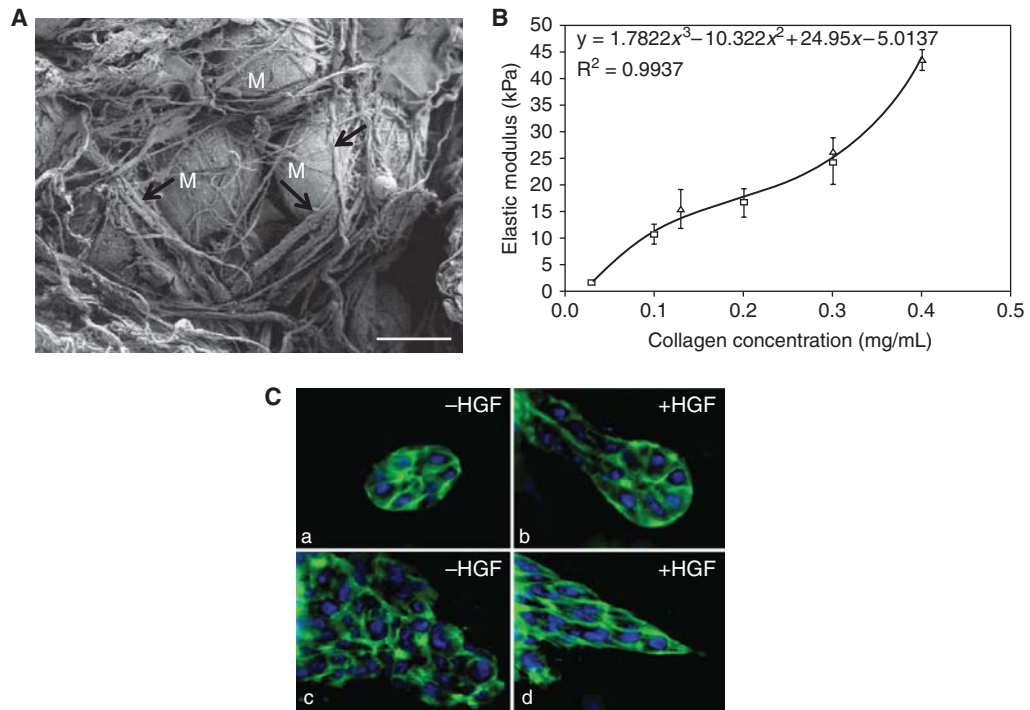


Figure 1. Mammary epithelial cells respond to the stiffness of a collagen matrix. (A) Scanning electron microscopy image of mammary acini showing individual mammary epithelial cells (M) surrounded by oriented collagen fibers (arrows). (SEM image courtesy of Paolo Provenzano.) Bar, 30 μ m. (B) Elastic modulus of collagen gels increases with increased collagen concentration. Modulus was measured by tension as described (Provenzano et al. 2009). Triangles represent data from Provenzano et al. (2009), squares from Roeder et al. (2002). Data was fitted and the resulting equation shown. (C) MCF10A cells cultured in a low-density (1.0 mg/ml) collagen gel form polarized acinar structures (a), while the same cells cultured in a high-density (2.0 mg/ml) collagen gel proliferate in a disorganized manner (c). Addition of HGF to low-density cultures results in tubule formation (b), while HGF added to cells in high-density culture results in an invasive phenotype (d). (Panels B and C reprinted from Provenzano et al. 2009 with permission from Nature Publishing Group © 2009.)

stroma and arrange collagen. TGF- β 1 is a primary activator of quiescent fibroblasts to highly contractile myofibroblasts involved in tissue fibrosis. Using culture conditions that permit tension modulation of the ECM, primary rat lung fibroblasts activated TGF- β 1 stored in the ECM only under high tension conditions (Wipff et al. 2007). The authors propose that a requirement of high tissue tension for TGF- β activation restricts generation of myofibroblasts to a stiffened ECM, thus imposing a physical limitation to fibrosis. Relevance to the mammary gland is likely, as latent TGF- β stored within the mammary matrix, and known to be activated in response to irradiation-induced

fibrosis (Barcellos-Hoff and Ravani 2000), is ovarian-hormone regulated.

Changes in the composition of the ECM during both mammary development and with tumor progression will affect the mechanical environment of the cells. Tumor progression in particular is characterized by increased ECM deposition, termed “desmoplasia.” Moreover, collagen V is increased in desmoplastic stroma (Barsky et al. 1982), which is mechanically significant as collagen V changes the structure of collagen I fibrils (Wenstrup et al. 2004; Berendsen et al. 2006; Breuls et al. 2009). In addition, several other proteins such as fibronectin and proteoglycans bind to



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collagens and affect the organization of collagen fibers, and thereby have effects on the mechanical milieu. The properties of the ECM are further changed by remodeling and the function of several matrix proteases, which will not be extensively covered in this article. The reader is referred to a couple of recent reviews on this topic (Page-McCaw et al. 2007; Rowe and Weiss 2008; Wolf and Friedl 2009). Finally, cross-linking of collagen fibers and collagen arrangement will also affect the mechanical properties of the ECM (Raub et al. 2007, 2008).

In addition to the ECM, several other aspects of mammary gland structure contribute to the mechanical environment. Normal mammary epithelial cells are polarized, and adhere in part via $\alpha 6 \beta 4$ integrin to the basement membrane at hemidesmosomes, which will have their own mechanical properties. In addition, tight junctions and adherens junctions between cells provide sites of attachment and mediate forces exerted between cells. This aspect of mechanosignaling is not well understood in the mammary gland, and will not be extensively covered here. The reader is referred to recent reviews on this subject regarding other cell types and developmental systems (Pokutta and Weis 2007; Wozniak and Chen 2009). The actin cytoskeleton, linked to force and contractility by interaction with myosin, links the adherens junctions with the integrin-mediated adhesions and hemidesmosomes, creating a potentially integrated tensile structure. Finally, myoepithelial cells, which tightly surround ductal epithelium and loosely encase the luminal epithelial cells, are specifically designed for contractility, and likely create mechanical events within the gland that differ between ductal and alveolar cells and with hormone status. Thus, every aspect of the mammary gland is a source of possible mechanical stimuli that can impact the behavior of mammary epithelial cells.

DYNAMIC NATURE OF TENSILE FORCES IN THE MAMMARY GLAND

Forces exerted on cells can occur in multiple dimensions: Tissues can be compressed or stretched in axial or multiaxial directions. The

mammary gland is subject to several forces of both types in the course of daily activities such as walking, exercising, or physical work. Compression in the mammary gland is probably most relevant during lactation and tumor growth, where the cells may be pushing out on their local environment, as well as during mammography and other examinations where external pressure is applied. In order to sense a tensile mechanical environment, a cell needs to adhere, experience force exerted upon it, and meet that force with some level of resistance. It is proposed that forces within the cell reach a tensional balance with the stiffness outside the cell, a concept termed “tensional homeostasis” (Paszek et al. 2005), and thus understanding and measuring cellular forces and matrix stiffness has become an area of intense interest.

DEFINING MATRIX STIFFNESS

Each ECM will have material properties based on the structure and arrangement of its component parts. Collagen itself is a viscoelastic material, meaning that it exhibits behaviors of both viscous and elastic materials, and its deformation depends on the rate at which it is strained. The viscoelastic property is quantified by applying force as tension, shear, or compression, and measuring the relationship between stress and displacement in order to determine a property termed the elastic modulus. A full description is beyond the scope of this article, and the reader is referred to a few excellent texts on the subject (Fung 1993; Humphrey and Delang 2004; Janmey et al. 2007).

The tensional moduli of gels composed of predominantly collagen I have been reported (Roeder et al. 2002; Provenzano et al. 2009) and demonstrate that an increase in the concentration of collagen from 1.0 to 4.0 mg/ml results in a non-linear increase in the elastic modulus of the gel (Fig. 1B). Compression studies of collagen gels across the same concentration range give a similar finding: Modulus increases as collagen concentration increases (Paszek et al. 2005; Gehler et al. 2009). Note that the modulus measured by tensional forces

results in a value in the kPa range, while modulus measured by compression is in the Pa range. Importantly, when actual mammary tissue, more complex because it contains heterogeneous stroma and cells, is measured by compression, there is an increase in the modulus for mammary tumors compared to normal tissue (Paszek et al. 2005). This makes sense, as mammary tumors can be found by their increased stiffness upon palpation. It is important to note here that the compression modulus of normal mammary tissue is similar to the compression modulus of lower concentration collagen gels, while the compression modulus of tumor tissue is similar to the compression modulus of higher concentration collagen gels (Paszek et al. 2005). Engineered environments differing in stiffness can also be created by coating flexible polyacrylamide gels with collagen, fibronectin, or other proteins. Further, by altering the proportion of bis-acrylamide, the modulus can be altered in precise ways (Wang et al. 2000).

Functionally, differences in matrix stiffness result in profound cellular responses. Mammary epithelial cells cultured in lower concentration 3D collagen gels organize into polarized acinar and ductal structures, while those in higher concentration collagen gels, which are stiffer, lose this polarization and instead become locally invasive (Fig. 1C). Cells cultured in stiffer matrices have an increase in cellular proliferation and changes in gene expression compared to cells in less stiff or compliant matrices. This finding is true in mammary epithelial cells (Wozniak et al. 2003; Paszek et al. 2005; Provenzano et al. 2009) as well as several other cell types (Chen et al. 1997; Wang et al. 2000; Discher et al. 2005). Moreover, stem cells differentiate in a manner that parallels the stiffness of their environment, such that stem cells cultured in a matrix with a stiffness similar to muscle become myogenic, while those in even stiffer matrices that mimic bone become osteogenic (Engler et al. 2006). This differentiation may be related to the Wnt pathway, as canonical Wnt signaling is enhanced when bone is mechanically loaded (Robinson et al. 2006). Moreover, cells migrate

preferentially onto stiffer surfaces (Wang et al. 2001), a process fundamental to embryonic morphogenesis and termed “durotaxis.”

MEASUREMENTS OF CELL FORCE

Several means have emerged to measure cell-generated forces. Force applied to cells with “laser tweezers” to optically trap beads coated with fibronectin results in a strengthening of the integrin-cytoplasmic linkages (Choquet et al. 1997). By altering this force, the point at which the bond to the bead is broken occurs at 2 pN (picoNewtons), suggesting this is the force of the integrin-mediated link between the ECM and the cytoskeleton (Jiang et al. 2003). Because contraction of a collagen gel is a balance between the stiffness of the collagen gel and the force exerted by the cells therein, a change in collagen gel contraction suggests a change in the force exerted by the cells. Thus, another force measure can be implied by cellular displacement of collagen fibers (Miron-Mendoza et al. 2008). Using this approach, it was found that enhanced filamin binding to integrins results in an increased ability to contract a stiff collagen gel, and relates to increased contractility signaling (Gehler et al. 2009). When the elastic modulus of the environment is known, force can also be computed from the displacement of beads within that environment. Such measurements demonstrate that cellular forces are greatest and more dynamic at the leading edge of a migrating cell compared to the trailing edge (Pelham and Wang 1997, 1999). Cell-mediated forces also have been measured by placing cells on micro-patterned flexible posts that vary in geometry and stiffness, and measuring the displacement of those posts, which demonstrates that cells exert greater forces as they become more spread (Tan et al. 2003). Similarly, as cells crawl over a micromachined device, cell forces are demonstrated to move toward the center of the cell (Galbraith and Sheetz 1997).

The question of whether cell force exerted on matrix influences large-scale anisotropic patterning of ECM, such as that observed in the mammary gland, is of obvious interest. In vivo, individual mammary acini and ducts are

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frequently circumscribed with 10–100 μm or larger bands of organized collagen-rich fibrillar stroma. In vitro, force exertion experiments, such as those described above, may provide insight into this macro-scale patterning. Pellets of fibroblast cells on an isotropic collagen-1 gel and separated by distances of 1000 μm reorganize the collagen into bundles running parallel between the cell foci (Sawhney and Howard 2002, 2004). The initial rate of collagen traction was 0.28 $\mu\text{m}/\text{min}$ with an estimated collagen translocation of $\leq 10 \mu\text{m}$ at the cell surface. Surprisingly, this localized cell surface force was sufficient to rearrange the collagen over the 1000 μm gap between cell pellets. Further, while the fibroblasts interacted with the collagen at their cell surface, the induction of the bands of collagen was transmitted simultaneously throughout the gel, rather than progressively from the cell surface as predicted if the gel was a collection of distinct fibers. Instead, these data indicate that the collagen gel has properties of an interconnected mesh, where tension exerted in one location is rapidly transmitted to distant sites due to its interconnected nature (Sawhney and Howard 2002, 2004).

Global self-organization of ECM by mammary epithelial cells has been observed in a 3D culture model designed to evaluate rapid

effects of endogenous matrices on acinar organization in the absence of cell proliferation. Using Matrigel spiked with either FN or mammary matrix isolated from nulliparous rats, highly organized bands of matrix were found to extend 20–30 μm from the basal side of the acini, with further matrix organization persisting even deeper into the matrix pad (Fig. 2A) (Schedin et al. 2004, 2000b). Thus, organizing antistrophic ECM at the cell periphery and at a distance appears to be an intrinsic property of mammary epithelial cells, much like the properties of cell and acinar size regulation and polarity. Further, these observations indicate that tissue-extracted matrix, whether from EHS sarcoma or from rat mammary gland, retains viscoelastic and mechanosignaling properties and acts more like an interconnected mesh than as a collection of individual matrix components.

CONTRACTILE PATHWAYS GENERATE FORCE

In large part, cells sense the stiffness of their environment by pulling against the ECM using intracellular contractile mechanisms derived from actin-myosin interactions. Thus, in addition to the concept that external forces may

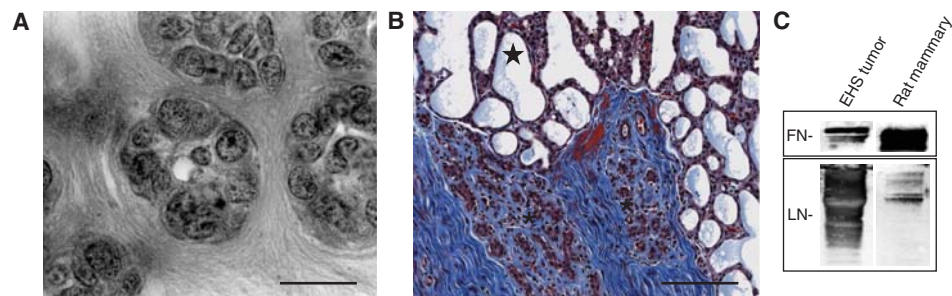


Figure 2. Anisotropic organization of matrix is an intrinsic property of MECs and is developmentally regulated. (A) Macro-patterning of anisotropic ECM by mammary epithelial cells in 3D culture. Bar, 15 μm . (B) Collagen fibers in blue (Trichrome stain) show increased deposition in regressing mammary lobules (white asterisks) in comparison to lactating lobules (black star). Bar, 100 μm . (C) Comparison of fibronectin and laminin ratios between rat mammary ECM and EHS reconstituted basement membrane (Matrigel). Lane 1: 10.8 μg EHS tumor matrix. Lane 2: 10.8 μg Day 4 involution rat mammary matrix. Based on scanning densitometry, rat mammary matrix has \sim five-fold more FN per μg protein than EHS matrix, whereas EHS matrix has \sim 10-fold more LN per μg protein than rat mammary matrix, with a FN/LN ratio of 50 or higher in rat mammary matrix compared to EHS matrix.



be exerted upon cells, cells exert forces upon themselves when they contract against the ECM. In a compliant matrix, the cells will pull the components of that matrix in toward themselves, and when in a stiffer matrix, the cells will generate forces that result in focal adhesions and stress fiber formation (Grinnell 2000; Wozniak et al. 2003; Miron-Mendoza et al. 2008; Gehler et al. 2009).

Contractility is positively affected by phosphorylation of the myosin regulatory light chain (MLC). Both MLC-kinase and the Rho effector, Rho-Kinase (ROCK), can directly phosphorylate MLC (Amano et al. 1996). Also, ROCK can inhibit the phosphatase that acts on MLC, further enhancing phosphorylation of MLC (Kimura et al. 1996). Of these two pathways, the Rho-ROCK pathway is the one predominantly linked to mammary epithelial cell response to matrix stiffness (Wozniak et al. 2003; Paszek et al. 2005; Wyckoff et al. 2006). Rho activity is itself linked to matrix stiffness, as a compliant matrix leads to down-regulation of Rho-GTP (Wozniak et al. 2003). It appears that part of the mechanism by which Rho plays a role involves controlling the degree of cell spreading, as endothelial cells confined in their shape on a patterned 2D surface have increased proliferation only if allowed to spread (Chen et al. 1997). This effect involves a bi-modal regulation of focal adhesion kinase (FAK) function, such that phosphorylated FAK promotes proliferation, while unphosphorylated FAK in rounded cells prevents proliferation (Pirone et al. 2006). Moreover, cell shape regulates the differentiation of mesenchymal stem cells in a Rho-ROCK dependent manner (McBeath et al. 2004). The relationship of cell spreading to Rho is also bi-directional, as spread cells are able to activate the Rho-ROCK pathway (Bhadriraju et al. 2007).

FOCAL COMPLEXES AS MECHANICAL SENSORS

Mechanosignaling entails the conversion of a mechanical event, such as cellular deformation, into a biochemical event typically thought of

in signal transduction, such as protein phosphorylation or generation of second messengers (Fig. 3). There is much speculation regarding the identity of putative mechano-responsive sensors within cells, but much more information is needed before understanding this process fully. It makes intuitive sense that mechanosensing proteins or cellular structures should have the property of being conformationally regulated when forces are exerted upon them and that these conformational changes should alter the signaling properties of the sensor, for example by regulating catalytic activity or by exposing binding sites for other molecules.

Integrin-mediated focal complexes as a unit could represent a mechanical sensor, as these structures are assembled in response to mechanical strain and substratum stiffness (Choquet et al. 1997; Pelham and Wang 1997; Katz et al. 2000) (for review, see Galbraith et al. 2002). While some have suggested that focal adhesions do not exist in three-dimensional matrices (Fralley et al. 2010), it should be noted that this observation was made in the context of a compliant 3D matrix. In stiff matrices, specialized focal adhesions termed “3D matrix adhesions” (Cuikerman et al. 2001) have been noted (Wozniak et al. 2003; Paszek et al. 2005; Provenzano et al. 2009). Integrins are proposed to themselves represent mechano-sensors (Katsumi et al. 2004, 2005), as they have profound conformational changes upon ligand binding. Moreover, integrin-cytoskeletal linkages are enhanced when force is exerted on integrins, suggesting that binding sites within the complex are exposed under strain (Choquet et al. 1997). Many of the molecules resident in focal complexes are implicated in mechanosensing, including talin (Giannone et al. 2003; Jiang et al. 2003), vinculin, FAK, Src, and p130Cas (Felsenfeld et al. 1999; Wang et al. 2001; Li et al. 2002; Frame 2004; Kostic and Sheetz 2006; Sawada et al. 2006), and indeed the proteins resident in focal adhesions may function cooperatively as a mechanosensing complex.

Talin in particular is a candidate mechano-sensor in focal complexes. Talin is uniquely involved in direct activation of integrin function, as talin binding to integrin β cytoplasmic tails

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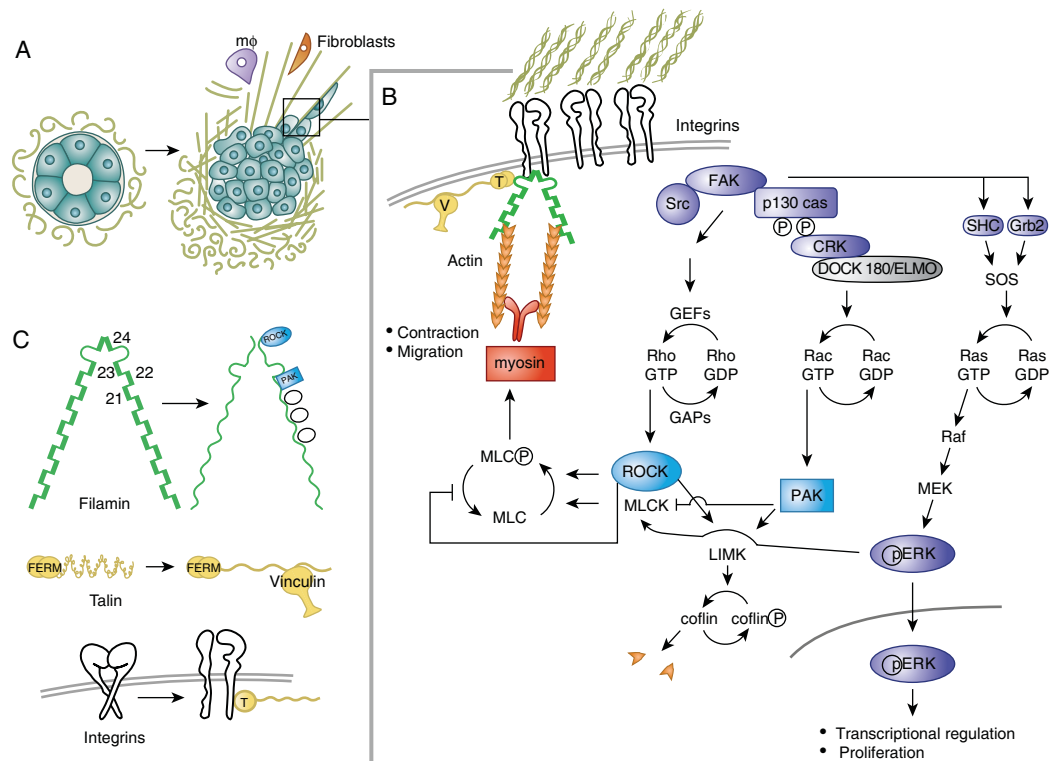


Figure 3. Mechanical signaling pathways. (A) Mammary cells in a compliant matrix organize into polarized acinar and ductal structures. Local deposition and remodeling of extracellular matrices accompanies tumor progression (green = collagen fibers). (B) Diagram of signaling pathways related to focal contacts and cellular contractility. (T) talin, (V) vinculin. (C) Diagrams representing conformational changes in putative mechanosensors: filamin, talin, and integrins.

induces conformational changes that propagate across the membrane and enhance ligand binding (Critchley 2000; Calderwood and Ginsberg 2003; Garacia-Alvarez et al. 2003; Calderwood et al. 2004; Wegener et al. 2007; Himmel et al. 2009). Talin binding to integrins involves both the N-terminal FERM domain (Calderwood et al. 1999; Wegener et al. 2007) as well as a C-terminal binding site in the rod domain (Gingras et al. 2009). The N- and C-terminal sites work in a potentially cooperative manner, as talin is auto-inhibitory when not bound to integrins (Goksoy et al. 2008). Calpain cleavage can release some of this auto-inhibition (Yan et al. 2001). As well, there is likely a mechanical component to talin function. Force applied to the talin rod domain stretches a series of helical bundles in the rod domain, opening up not only

the cryptic C-terminal integrin binding site, but also binding sites for vinculin and actin (Hytonen and Vogel 2008; del Rio et al. 2009). Thus, integrin activation by talin is likely mechanically regulated and indeed is necessary for vinculin recruitment and cytoskeletal strengthening when force is applied to integrins (Giannone et al. 2003). Vinculin, too, is conformationally regulated such that it is active at focal adhesions (Chen et al. 2005).

The scaffolding molecule, p130Cas, has recently emerged as a putative mechanosensor. p130Cas contains a highly phosphorylated substrate domain in the middle of the molecule that is phosphorylated predominantly by Src, and may be the means by which Src participates in mechano-signaling (Honda et al. 1999; Sawada et al. 2006). Stretching of p130Cas



exposes this domain, and results in increased phosphorylation through the Src-family kinase, fyn (Kostic and Sheetz 2006; Sawada et al. 2006). Once phosphorylated, p130Cas scaffolds several molecules, and results in signaling through myosin light chain to promote cellular contractility (Cheresh et al. 1999), as well as activation of Dock180 to promote Rac activation (Kiyokawa et al. 1998). Importantly, exposure of the p130Cas substrate domain occurs at protrusive edges of spreading cells (Sawada et al. 2006), suggesting spatial regulation that may allow p130Cas to translate mechanical cues into migration, and may explain part of the mechanism underlying durotaxis.

Filamin is a large scaffolding molecule that has several folded domains, and is hypothesized to unfold upon mechanical forces placed on the molecule, exposing several binding sites for signaling molecules such as Rho, ROCK, PAK, and PKC (Stossel et al. 2001; Vadlamudi et al. 2002; reviewed in Feng and Walsh 2004). Filamins are poised to mediate mechanical cues from the ECM, as they bind directly on one end to integrins and on the other to the actin cytoskeleton. Importantly, filamin-integrin linkages regulate mechanical responses such as cell migration and collagen gel contraction, and may serve to “tune” the response of mammary epithelial cells to matrix stiffness (Calderwood et al. 2001; Gehler et al. 2009). Moreover, filamin alters the mechanical properties of actin networks (DiDonna and Levine 2006; Gardel et al. 2006).

FAK is also implicated in mechanosensing. In particular, phosphorylation of FAK at Y397 is up-regulated when cells are on stiff matrices compared to compliant matrices (Yano et al. 1996; Cukierman et al. 2001; Wozniak et al. 2003). Cells devoid of FAK lose the ability to migrate in response to mechanical cues (Wang et al. 2001; Li et al. 2002). FAK is also recruited to focal adhesions in cells undergoing mechanical strain within stiff 3D matrices (Wozniak et al. 2000; Provenzano et al. 2009), where it is necessary for subsequent mechanically responsive signaling (Schober et al. 2007). Src is intimately linked to FAK function, and has also emerged as a molecule that is

both responsive to and necessary for cellular responses to mechanical cues (Felsenfeld et al. 1999; Frame 2004). Src and focal adhesion regulation are implicated in cellular responses to osmotic forces (Volonte et al. 2001). Src activation has been used as an in situ mechanosensitive reporter, with data suggesting that mechanical signaling to Src propagates through the cell via both actin filaments and microtubules (Wang et al. 2005).

Integrating these various signaling pathways (Fig. 3), the model that emerges is one in which cells pull against the ECM using Rho-ROCK-mediated contraction of the actin cytoskeleton. If the ECM around the cell is stiff and the cell meets with resistance, the forces generated result in tension across the adhesion receptors and focal complexes. This in turn results in adhesion strengthening, recruitment of signaling molecules into the focal complexes, and focal adhesion signaling. Moreover, a stiff matrix provides enough traction that the cell is able to spread and proliferate, or to migrate. If the matrix is compliant, the cells will instead deform the matrix and round up, resulting in different signaling and phenotypic outcomes.

MECHANICAL SIGNALING REGULATES PROLIFERATION AND DIFFERENTIATION

Signaling events related to mechanical stimuli clearly have effects on gene expression downstream from these signals. A predominant outcome found in cells of all types when they are in rigid matrices or allowed to spread is cellular proliferation (Chen et al. 1997; Fringer and Grinnell 2001; Thery et al. 2005). Specifically, mammary cells in a stiff matrix are more proliferative (Wozniak et al. 2003; Paszek et al. 2005) and up-regulate proliferative signals including cyclin D1 (Klein et al. 2009) as well as an entire set of genes identified as a proliferation signature in human breast carcinoma (Provenzano et al. 2009). The pathway leading to proliferation is linked to function of MEK/ERK, as inhibition of this pathway reverses expression of the proliferation signature induced by rigid substratum (Assoian and Klein 2008; Provenzano et al. 2009). Involvement of the MEK/ERK



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pathway also mediates the proliferation of fibroblasts cultured in a stiff matrix (Fringer and Grinnell 2001, 2003).

In contrast to proliferation, mammary cells within compliant matrices demonstrate growth control, organization of glandular architecture, and express proteins that are consistent with a more “differentiated” phenotype (Emerman and Pitelka 1977; Streuli et al. 1995; Wozniak et al. 2003; Paszek et al. 2005; Provenzano et al. 2009). For example, the expression of β -casein, a marker of breast epithelial differentiation, is coordinately regulated by both adhesion to laminin and a compliant matrix (Alcaraz et al. 2008). The polarization of the epithelial cell is thought to drive these events, with compliant matrices supporting and stiff matrices abolishing polarity. During lactation, the mammary epithelial cells is at its most polarized state, as tight junction complexes close with lactation, absolutely restricting transport between the basolateral and luminal compartments of the gland (Neville 2009). Proper lactogenesis and signaling involves contact with the BM, as well as the down-regulation of Rho activity (Lee et al. 2009). The role of integrins in this regulation is demonstrated by the finding that inhibition of β 1 integrin adhesion to the ECM that mimics a more compliant environment reverts the disorganized phenotype of mammary carcinoma cells, resulting in restoration of acinar structure (Weaver et al. 1997). Conversely, matrix cross-linking, which is associated with and enhances tumor progression, acts to stimulate integrin signaling (Levental et al. 2009). Compliance works in concert with growth factor pathways to regulate cellular proliferation and phenotype. For example, when the ErbB2 receptor is driven to dimerize in cells within compliant rBM, otherwise normal MECs lose growth control and proliferate into the lumen of ascini (Muthuswamy et al. 2001). This result demonstrates that compliance can be “overruled” by strong mitogenic signals, but intriguingly raise the possibility that compliance may also normally down-regulate these pathways.

Given that apical/basal polarity defines epithelium, the role of cell adhesion in regulating

polarity has been investigated in retinal pigment epithelial cells using defined micro-patterned ECM substrates that force single cells to have patterned areas of adhesion and non-adhesion. In this study, geometry of the ECM determined positioning of the nucleus-centrosome complex, with orientations reproducibly directed toward cell adhesive edges. These data add the property of ECM geometry, in concert with composition and mechanical properties, in regulating gene expression patterns critical for cell fate decisions (Thery et al. 2006).

CHANGES IN MAMMARY ECM DURING NORMAL DEVELOPMENT—FOCUS ON COLLAGEN AND FIBRONECTIN

While the mammary anlagen is formed embryologically, the majority of ductal development occurs with onset of sexual maturation and alveolar development with pregnancy. The mammary gland is also unique in that terminal differentiation does not occur unless lactation ensues, and further, with each estrous cycle, the “immature” gland undergoes a cyclic expansion followed by a modest regression phase (Schedin et al. 2000a; Fata et al. 2001). Consequently, the normal adult mammary gland exists in many different developmental states that vary with time.

Based on pioneering work from Mina Bissell’s laboratory showing mammary epithelial cells differentiate to a milk-secreting phenotype when cultured on floating collagen pads (Lee et al. 1984) or embedded in thick pads of reconstituted basement membrane proteins (Matrigel) (Li et al. 1987), the functional unit of the mammary gland was defined as the epithelial cell plus its ECM (Bissell and Barcellos-Hoff 1987). A corollary to this hypothesis is the ECM tensional requirements of the gland would change to accommodate the distinct demands required of the nulliparous, pregnant, lactating, or involuting mammary epithelium. Indeed, the microenvironment of the mammary gland is highly dynamic as each developmental state has a unique ECM protein signature, and further, the responsiveness of the epithelium to systemic hormones is facilitated through

concomitant modification of the ECM (Warburton et al. 1982; Silberstein and Daniel 1984; Schedin et al. 2004).

A role for fibrillar collagen and thus tissue tension in the development of the rudimentary mammary anlagen to the fully elaborated ductal tree was observed early on. Terminal end buds (TEBs) are transient, mitotically active, and motile structures that drive ductal elongation and penetration through the mammary fat pad. TEBs are characterized by a basement membrane devoid of collagen-1 bundles. Collagen deposition occurs along the flank of the nascent ducts, where epithelial cell proliferation is inhibited (Silberstein and Daniel 1982). By implanting a slow release pellet containing exogenous TGF- β at the tip of the growing ductal tree, ductal growth was rapidly inhibited concurrent with thick fibrillar collagen deposition around the TEBs (Silberstein and Daniel 1987). However, not only the presence, but also the orientation of collagen is important for proper mammary gland development. Aligned collagen fibers are found radiating from the terminal end bud (TEB) of the developing mammary gland just prior to the invasion of the cells into the mammary fat pad (Ingman et al. 2006). That these fibers might assist morphogenesis of the mammary gland is suggested by the finding that branching morphogenesis follows patterns of ECM topography in engineered matrices (Nelson et al. 2006).

To date, a relatively limited number of mammary ECM proteins have been quantitatively evaluated across gland development. Nonetheless, evidence that developmentally regulated changes in mammary ECM composition result in distinct viscoelastic and mechanical properties is compelling. Gene expression of numerous collagens are differentially regulated by reproductive state, including fibrillar collagens type I, III, and V, bead-filament collagen VI, FACIT family member collagen IX, and basal lamina collagen IV (Schedin et al. 2007). Collagen-associated proteins known to influence cross linking are similarly regulated including elastin, fibrillin 1, decorin, lumican, and biglycan (Schedin et al. 2007). Recently, fibrillar collagen deposition has been quantitated

in the rat mammary gland across the pregnancy, lactation, and involution cycle, and the ratio of collagen to epithelial cells differs by an order of magnitude depending on developmental state (O'Brien et al. 2010). The lactating gland has very few fibrils of collagen between individual acini, an observation consistent with previous studies, demonstrating that a compliant ECM is required for MEC differentiation in vitro. Following weaning, high levels of fibrillar collagen are deposited between involuting acini. This increase has been observed in mouse, rat and human, suggesting the associated tensional changes are required for the massive remodeling that occurs with involution (Fig. 2B). In addition, numerous ECM proteins including LN5 (Giannelli et al. 1997), entactin (Alexander et al. 1996), FN (Schedin et al. 2000b), and collagen 1 (O'Brien et al. 2010) are targeted for partial proteolysis during involution, which alters the viscoelastic properties of the matrix significantly, as well as releasing cryptic ECM fragments capable of engaging additional integrin and non-integrin signaling pathways (Werb et al. 1989; Schenk and Quaranta 2003; Mott and Werb 2004). In conclusion, when considering the single ECM protein collagen 1 and the myriad of regulatory controls such as expression, cross linking, proteolysis, and cellular engagement, the potential for fine tuning mechanosignaling in the mammary gland is evident and likely integral to tissue function.

FN IS UNIQUELY POISED TO REGULATE MAMMARY MECHANICAL PROPERTIES

FN is found in abundant quantities within the normal rat mammary gland intra- and interlobular stroma (Schedin et al. 2004). By Western blot analysis, the relative abundance of FN in mammary ECM has been compared to reconstituted basement membrane preparations derived from EHS tumors (Matrigel), and mammary stroma has 50–100 fold higher levels of FN than Matrigel (Fig. 2C) (Schedin et al. 2004). In what was somewhat of a surprise, given that FN is not classically considered a basal lamina protein, FN is also found in the

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highly organized and specialized mammary basal lamina (Monaghan et al. 1983), implying that FN can interact directly with $\alpha 5 \beta 1$ -integrin expressing mammary epithelial cells. Like mammary epithelial cells themselves, FN and $\alpha 5 \beta 1$ -integrin expression are under endocrine control, with expression up-regulated during the pubertal and pregnancy windows of gland expansion (Haslam and Woodward 2001; Woodward et al. 2001), and precipitously down-regulated at late pregnancy after completion of the proliferative phase, and with full differentiation of the lactating gland (Schedin et al. 2004). This is in contrast to mammary LN mRNA, which appears to be constitutively expressed across the pregnancy, lactation, and involution cycle (Woodward et al. 2001; Schedin et al. 2004). The in vivo FN studies are supported by in vitro experiments where intact FN induced proliferation, increased acinar size in 3D culture, and stimulated proliferation of growth-arrested mammary epithelial cells (Barkan et al. 2008; Williams et al. 2008).

Results from embryonic lethal FN knockout mice demonstrating a requirement for anisotropic FN fibrils in embryonic cell migration has peaked interest in the potential role of FN in mechanosignaling (Yang et al. 1999). Recently, force generation has been identified as the mechanism by which FN is required for branching morphogenesis in the salivary gland (Larsen et al. 2006). In an elegant series of experiments, FN was observed to translocate directionally by addition of new FN to the ends of older FN fibrils. Old FN fibril accumulated at the sites of cleft formation in a pattern consistent with FN, driving a physical wedge between cells at the cleft, resulting in bifurcation.

Numerous physical attributes enable fibrillar FN to contribute to tensional status of the microenvironment. FN is a large approximately 440,000 kD glycoprotein consisting of two similar 220,000 kD subunits bridged by a disulfide bond. FN has a modular structure and interacts directly with cells through two heparin-binding domains and two distinct RGD containing sites. Assembly of secreted FN into fibrils appears to be nucleated through $\alpha 5 \beta 1$ integrin binding, as $\alpha 5 \beta 1$ blocking

antibodies inhibit FN matrix formation in vitro (Fogerty et al. 1990; Mao and Schwarzbauer 2005). It is thought that the requirement for $\alpha 5 \beta 1$ permits precise tempo-spatial integration between location of FN matrix assembly, local tissue tension, and specific requirements of the cell or tissue. FN fibrils also incorporate directly into the ECM due to specific heparin, fibrin I, fibrin II, and multiple collagen binding domains. In fact, while fibrillar collagen formation in vitro is driven by self assembly thermodynamics, in vivo fibrillar collagen formation has been demonstrated to be FN-dependent due to a required conformational change in FN (Kadler et al. 2008). This conformational change is likely induced by contractile forces exerted on FN by cells, as FN assembly and thus subsequent collagen assembly is dependent on Rho-mediated contractility (Zhang et al. 1997). Evidence from biochemical studies shows that FN directly modifies mechanical and structural properties of collagen fibers. The addition of FN to type I collagen prior to collagen fibrillogenesis increases the percentage of linear fibers and reduces the number of collagen cross-links, suggesting that FN shifts the balance towards linear collagen growth (Guarnieri et al. 2007). This shift correlates with a reduction in elasticity at high FN concentrations (Guarnieri et al. 2007). Recent data from Viola Vogel's laboratory has shown that FN fibers display extraordinary extensibility, which is reversible (Klotzsch et al. 2009). Further, they demonstrate that FN extension increases matrix rigidity and cryptic epitope exposure (Klotzsch et al. 2009), directly implicating FN in mechanotransduction processes (Smith et al. 2007). Finally, bioactivity of FN fragments was initially demonstrated in rabbit synovial fibroblasts, where exposure to FN fragments, but not intact FN, induced collagenase and stromelysin gene expression (Werb et al. 1989). FN fragments were subsequently demonstrated to up-regulate MMP2 activity in human breast cancer cell lines as well as induce cell motility and invasion (Schedin et al. 2000b). More recently, the ability of FN fragments and a splice variant named migration-stimulating factor (MSF) to stimulate fibroblast migration has



been mapped to specific sites (Schor et al. 2003). Further, FN domains that mask the motogenic site when FN is intact have been identified (Ellis et al. 2010). Questions such as how matrix tension influences protease access to cryptic cleavage sites, and how specific FN fragments, in turn, alter matrix tension remain intriguing and to date, unanswered.

In summary, in the mammary gland, FN fibers present at the epithelial cell membrane and within the intra and inter-lobular stromal compartment can exert influence on micro-patterning at the cellular scale, such as actin cytoskeleton organization, cell polarity, signal transduction, and altered gene expression, as well as macropatterning at the tissue level, including matrix rigidity, collagen fiber density, orientation, and gradient formation. Further studies to understand the relationships between FN fibril thickness, length, orientation, partial proteolysis, and mechanical strength are clearly required to more fully understand its importance in mammary epithelial cell function.

CANCER IMPLICATIONS FOR HORMONAL CONTROL OF MATRIX ASSEMBLY

The endocrine control of FN expression and subsequent hormone-dependent matrix assembly observed in the normal mammary gland and described above has been documented in breast cancer cells in vitro (Quinn et al. 2009). Additional mechanisms for ECM regulation of estrogen signaling in breast cancer have been reported as well. MCF-7 cells cultured on stiff collagen 1 matrix responded to estrogen stimulation with up-regulation of the Rac1/JNK/c-Jun pathway, cyclin D1 expression, and proliferation, whereas this response was significantly dampened in cells cultured on compliant LN (Xie and Haslam 2008). ECM regulation of ER expression has also been reported, with ER β expressed when MDA-MB-231 cells are cultured on rigid plastic, and expression lost when cultured on basal lamina proteins collagen IV and Laminin-111 (Neubauer et al. 2009). While these studies clearly demonstrate ECM control over endocrine responsiveness in both normal and transformed mammary

epithelial cells, the specific role mechanosignaling plays remains to be confirmed. Nonetheless, a recurrent theme is that substrata with higher tension correlate with increased ER signaling, even in the absence of ligand. The implications for collagen deposition as one primary determinant of increased breast cancer risk associated with high mammographic density are clear, especially since mammographic density is hormonally responsive (Boyd et al. 2009).

ECM DENSITY AND ALIGNMENT IN TUMOR PROGRESSION

Mammographic density is associated with a 4–6 fold increased relative risk of developing breast carcinoma and is largely associated with an increase in stromal collagen (Boyd et al. 2001; Guo et al. 2001). While the underlying mechanism for the risk factor is not entirely known, it was possible that density could be merely a co-associated risk factor, but itself had no contribution to carcinoma. However, recent studies suggest that density per se contributes to carcinoma progression, as tumor formation and metastasis is enhanced in the background of a mouse mammary gland enriched in collagen because the collagen is protease resistant (Provenzano et al. 2008b). A collagen-rich ECM is mechanically stiffer (Paszek et al. 2005; Provenzano et al. 2009), and this will promote the pro-tumorigenic changes in signaling and gene expression discussed above. While it is not currently known whether ECM stiffness is an initiating event in mammary carcinoma, this possibility is consistent with the fact that normal MECs cultured on stiff substratum activate proliferation genes and oncogenic signaling pathways such as ERK (Paszek et al. 2005; Provenzano et al. 2009).

During breast carcinoma progression, additional ECM deposition, or desmoplasia, occurs and is associated with a poorer predicted outcome (Walker 2001). In a study of triple-negative breast carcinomas, it was found that fibrosis was associated with distant metastasis (Kreike et al. 2007). Not only does the total amount of ECM components increase, but the anisotropy of the ECM also increases. Collagen

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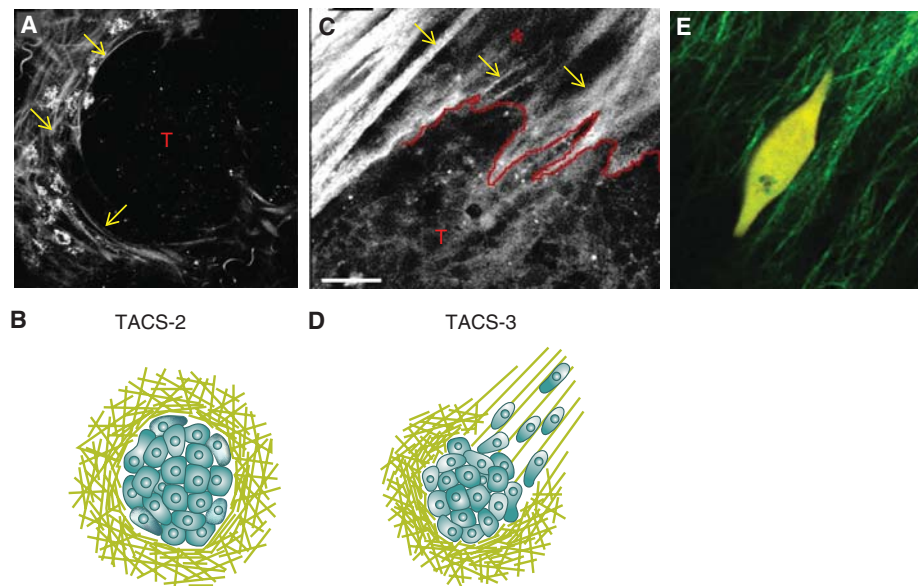


Figure 4. Collagen alignment facilitates invasion. (A) Parallel alignment of collagen fibers (arrows) around a non-invasive tumor (T), imaged in a live mammary tumor ex vivo by multiphoton laser scanning microscopy and second harmonic generation (SHG) to image collagen. (B) Diagram of TACS-2 (example in A), in which collagen fibers are denoted by tan-colored lines. (C) Perpendicular alignment of collagen fibers (arrows) at a tumor (T) boundary, which is depicted by the red line. (Panel reprinted from Provenzano et al. 2006 with permission from BioMed Central Ltd. © 2006.) Live tumor was imaged as in A. (D) Diagram of TACS-3 (example in C), with cells invading out from the tumor along aligned collagen fibers. (E) Human T47D breast carcinoma cells (yellow) aligns relative to collagen fibers (green) within an engineered collagen matrix. The orientation of collagen fibers is depicted by the arrow. Live cell in matrix was imaged as in A.

fibers thicken and straighten during tumor progression, and ultimately align perpendicular to the tumor boundary concordant with tumor invasion (Fig. 4) (Provenzano et al. 2006). These changes, termed “Tumor Associated Collagen Signatures” (TACS) manifest in specific ways during mammary tumor progression in mice (Provenzano et al. 2006). Changes in collagen fiber organization may be due in part to FN as described above or to an increase in the intermolecular cross-links between collagen fibers. Lysyl oxidase, which catalyzes collagen cross-links, is increased during tumor progression (Peyrol et al. 1997; Decitre et al. 1998; Kirschmann et al. 1999). Cross-linking is expected to make the ECM stiffer, and therefore would have consequences for mechanosignaling.

Studies in vitro help to suggest mechanisms by which collagen might become aligned in vivo. Collagen fibers are aligned when subjected

to externally applied mechanical load (Vader et al. 2009). The load placed on collagen fibers by cellular contractility can also align the collagen in a Rho-ROCK-myosin-dependent manner (Provenzano et al. 2009). This result suggests that tumor cells themselves may help set up the matrix alignment associated with cellular invasion. Alignment of the ECM may occur during the process of cell invasion, as mammary carcinoma cells invading through 3D collagen matrices can simultaneously move and align collagen fibers (Wolf et al. 2007).

Carcinoma-associated fibroblasts (CAFs) also contribute to the ECM changes that accompany tumor progression. CAFs differ from normal mammary fibroblasts in their ability to promote cellular proliferation and tumor progression (Olumi et al. 1999; Orimo et al. 2005; Orimo and Weinberg 2006). In addition to the mechanisms discussed above for $\alpha 5 \beta 1$

integrin-mediated FN deposition, there is also a role for the cell-surface proteoglycan, syndecan-1 (Sdc-1), which is expressed on CAFs but not on normal mammary fibroblasts (Su et al. 2007). Sdc-1 expressing fibroblasts promote tumor progression in vivo, and lead to a desmoplastic stroma (Maeda et al. 2006). The signal that induces CAFs to express Sdc-1 in mammary stroma is not yet known, but may be a response of fibroblasts to matrix density or early desmoplasia, as mechanical strain induces Sdc-1 expression in smooth muscle cells (Julien et al. 2007). Since FN can scaffold and direct collagen fiber assembly (Kadler et al. 2008), it is likely that the aligned matrix observed near tumors is due, at least in part, to the role of CAFs. Physiologically regulated changes in FN fibrillogenesis, as discussed above, may inadvertently provide signals for Sdc-1 expression and anisotropic FN assembly by CAFs. Relevance to cancer progression is suggested, as FN is a classic marker of epithelial to mesenchymal transition (EMT) as well as mammary cancer initiating cells (Mani et al. 2008).

CELL INVASION AND MECHANOSIGNALING

Aligned matrices found near tumors are not only a sign of a reactive stroma, but likely act to facilitate tumor cell invasion. Carcinoma cells preferentially invade along aligned fibers, versus randomly organized fibers (Provenzano et al. 2008c). Elegant intra-vital imaging demonstrates that mammary carcinoma cells traverse along radial collagen fibers in vivo (Wyckoff et al. 2004). It is intriguing that tumor cells may have co-opted the normal process of TEB invasion that occurs along aligned fibers in the developing mammary gland (see above) (Ingman et al. 2006). In addition to carcinoma cells, fibroblasts and myeloid cells also demonstrate increased migration proximal to the tumor (Egeblad et al. 2008), suggesting that the local increase in ECM deposition and alignment may serve to recruit additional stromal cells. This is significant for metastasis, as macrophages promote the local invasion of tumor cells and their extravasation into blood vessels

(Goswami et al. 2005). Moreover, the production of an aligned matrix may be facilitated by macrophages (Ingman et al. 2006). Among the many areas that are currently understudied, the question of how collagen fibers are oriented with respect to the involuting mammary epithelium is of intense interest, given the relationships between collagen fiber orientation (O'Brien et al. 2010), cancer metastasis, and the poor prognosis of breast cancers diagnosed in the post-partum window (Lyons et al. 2009; O'Brien et al. 2010). Further research separating roles of collagen deposition from alignment is highlighted by recent evidence showing that collagen levels increase in mammary glands of rats treated with doses of tamoxifen that prevent tumor progression, in part due to decreased MMP activity (Hattar et al. 2009).

The precise mechanisms by which cells recognize and migrate along aligned ECMs are not fully known. In vitro, cells preferentially migrate along stiffer substrata in a manner dependent on mechanical signaling through FAK, as FAK-depleted cells lose their preference for stiff substrata and will migrate as well on compliant surfaces (Wang et al. 2001). A role for FAK in tumor cell invasion in vivo was recently demonstrated by four independent groups (Lahlou et al. 2007; Provenzano et al. 2008a; Pylayeva et al. 2009). Tumors can arise in the FAK^{-/-} mouse mammary epithelium, but are largely non-invasive hyperplasias (Lahlou et al. 2007; Provenzano et al. 2008a). Interestingly, this points to a specific role for FAK in the epithelium, as these animals have normal FAK expression in the stromal compartment (Provenzano et al. 2008a).

Recent findings suggest that specific cytoskeletal regulators, including Arp2/3, cofilin, and vinculin, are associated with an invasive phenotype (Wang et al. 2004). An intriguing possibility is that expression of these molecules in invasive tumors reflects a role in sensing ECM stiffness or alignment. A recent exciting finding is the identification of splice variants of the actin-organizing protein, Mena, that are specifically up-regulated in breast carcinoma cells and that increase invasion in 3D matrices (Philippart et al. 2008; Goswami et al. 2009).

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Mena serves to alter the branching of the actin cytoskeleton, and regulates migration of several cell types (Bear et al. 2001). Mena is also implicated in regulation of adherens junctions (Vasioukhin and Fuchs 2001), suggesting an important role in epithelial polarity as well. Moreover, the expression of several cytoskeletal-regulating proteins is altered in non-invasive FAK^{-/-} mammary carcinoma cells (Provenzano et al. 2008a). Because of the role of actin-myosin contractility in sensing matrix stiffness, molecules that regulate the cytoskeleton may contribute to invasion along aligned matrices.

SUMMARY

While it is understood that the extracellular microenvironment around normal tissues affects cellular behavior, more recently it has become clear that the ECM serves not only scaffolding and biochemical functions, but also affects the mechanical environment. The demonstration that cells within the mammary gland respond to changes in the stiffness of their environment points to an important role for cellular force and mechanosignaling events in the normal development and differentiation of the gland at puberty, pregnancy, lactation, and involution. The complexity and potential for fine-tuning of tissue tension is highlighted by the distinct yet interconnected roles of ECM deposition, alignment, cross-linking, and partial proteolysis. Moreover, force also plays an important role in tumor progression, as stiff matrices are associated with increased risk of breast carcinoma, and promote cellular proliferation as well as progression. In addition to a general increase in the deposition of ECM components with cancer progression, collagen and fibronectin are deposited as aligned matrices with various levels of cross-linking and proteolysis that provide preferred surfaces on which carcinoma cells invade, thus promoting breast cancer metastasis. As our understanding of mechanical signaling events evolves, we may find novel means by which to predict outcome and hopefully target breast carcinoma progression.

ACKNOWLEDGMENTS

The authors would like to thank members of their laboratories, Ori Maller, Traci Lyons, Paolo Provenzano, Steve Trier, and Kristin Riching for providing data presented in Figures 1, 2, and 4, and for critical review of the manuscript. Work pertinent to this article has been funded by grants to P.J.K. (American Cancer Society RPG-00-339; Susan G Komen Foundation, and DOD Postdoctoral Fellowship W81XWH-04-1-042 to P. Provenzano) and to P.S. (DOD Synergistic Idea Award #BC060531 and Susan G Komen, Avon and Mary Kay Ash Foundations).

REFERENCES

- Alcaraz J, Xu R, Mori H, Nelson CM, Mroue R, Spencer VA, Brownfield D, Radisky DC, Bustamante C, Bissell MJ. 2008. Laminin and biomimetic extracellular elasticity enhance functional differentiation in mammary epithelia. *Embo J* **27**: 2829–2838.
- Alexander CM, Howard EW, Bissell MJ, Werb Z. 1996. Rescue of mammary epithelial cell apoptosis and entactin degradation by a tissue inhibitor of metalloproteinases-1 transgene. *J Cell Biol* **135**: 1669–1677.
- Amano M, Ito M, Kimura K, Fukata Y, Chihara K, Nakano T, Matsuura Y, Kaibuchi K. 1996. Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *J Biol Chem* **271**: 20246–20249.
- Asoian RK, Klein EA. 2008. Growth control by intracellular tension and extracellular stiffness. *Trends Cell Biol* **18**: 347–352.
- Barcellos-Hoff MH, Ravani SA. 2000. Irradiated mammary gland stroma promotes the expression of tumorigenic potential by unirradiated epithelial cells. *Cancer Res* **60**: 1254–1260.
- Barkan D, Kleinman H, Simmons JL, Asmussen H, Kamaraju AK, Hoenorhoff MJ, Liu ZY, Costes SV, Cho EH, Lockett S, et al. 2008. Inhibition of metastatic outgrowth from single dormant tumor cells by targeting the cytoskeleton. *Cancer Res* **68**: 6241–6250.
- Barsky SH, Rao CN, Grotendorst GR, Liotta LA. 1982. Increased content of Type V Collagen in desmoplasia of human breast carcinoma. *Am J Pathol* **108**: 276–283.
- Bear JE, Krause M, Gertler FB. 2001. Regulating cellular actin assembly. *Curr Opin Cell Biol* **13**: 158–166.
- Berendsen AD, Bronckers AL, Smit TH, Walboomers XF, Everts V. 2006. Collagen type V enhances matrix contraction by human periodontal ligament fibroblasts seeded in three-dimensional collagen gels. *Matrix Biol* **25**: 515–522.
- Bhadriraju K, Yang M, Alom Ruiz S, Pirone D, Tan J, Chen CS. 2007. Activation of ROCK by RhoA is regulated by cell adhesion, shape, and cytoskeletal tension. *Exp Cell Res* **313**: 3616–3623.

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- Bissell MJ. 1981. The differentiated state of normal and malignant cells or how to define a 'normal' cell in culture. *Int Rev Cytol* **70**: 27–100.
- Bissell MJ, Barcellos-Hoff MH. 1987. The influence of extracellular matrix on gene expression: Is structure the message? *J Cell Sci Suppl* **8**: 327–343.
- Bissell MJ, Hall HG, Parry G. 1982. How does the extracellular matrix direct gene expression? *J Theor Biol* **99**: 31–68.
- Boyd NF, Martin LJ, Rommens JM, Paterson AD, Minkin S, Yaffe MJ, Stone J, Hopper JL. 2009. Mammographic density: A heritable risk factor for breast cancer. *Methods Mol Biol* **472**: 343–360.
- Boyd NF, Martin LJ, Stone J, Greenberg C, Minkin S, Yaffe MJ. 2001. Mammographic densities as a marker of human breast cancer risk and their use in chemoprevention. *Curr Oncol Rep* **3**: 314–321.
- Breuls RG, Klumpers DD, Everts V, Smit TH. 2009. Collagen type V modulates fibroblast behavior dependent on substrate stiffness. *Biochem Biophys Res Commun* **380**: 425–429.
- Calderwood DA, Ginsberg MH. 2003. Talin forges the links between integrins and actin. *Nat Cell Biol* **5**: 694–697.
- Calderwood DA, Huttenlocher A, Kiosses WB, Rose DM, Woodside DG, Schwartz MA, Ginsberg MH. 2001. Increased filamin binding to β -integrin cytoplasmic domains inhibits cell migration. *Nat Cell Biol* **3**: 1060–1068.
- Calderwood DA, Tai V, Di Paolo G, De Camilli P, Ginsberg MH. 2004. Competition for talin results in trans-dominant inhibition of integrin activation. *J Biol Chem* **279**: 28889–28895.
- Calderwood DA, Zent R, Grant R, Rees DJG, Hynes RO, Ginsberg MH. 1999. The talin head domain binds to integrin β subunit cytoplasmic tails and regulates integrin activation. *J Biol Chem* **274**: 28071–28074.
- Chen CS, Mrksich M, Huang S, Whitesides GM, Ingber DE. 1997. Geometric control of cell life and death. *Science* **276**: 1425–1428.
- Chen H, Cohen DM, Choudhury DM, Kioka N, Craig SW. 2005. Spatial distribution and functional significance of activated vinculin in living cells. *J Cell Biol* **169**: 459–470.
- Chen J, Diacovo TG, Grenache DG, Santoro SA, Zutter MM. 2002. The α_2 integrin subunit-deficient mouse: A multifaceted phenotype including defects of branching morphogenesis and hemostasis. *Am J Pathol* **161**: 337–344.
- Cheresh DA, Leng J, Klemke RL. 1999. Regulation of cell contraction and membrane ruffling by distinct signals in migratory cells. *J Cell Biol* **146**: 1107–1116.
- Choquet D, Felsenfeld DP, Sheetz MP. 1997. Extracellular matrix rigidity causes strengthening of integrin–cytoskeleton linkages. *Cell* **88**: 39–48.
- Critchley DR. 2000. Focal adhesions—the cytoskeletal connection. *Curr Opin Cell Biol* **12**: 133–139.
- Cukierman E, Pankov R, Stevens DR, Yamada KM. 2001. Taking cell-matrix adhesions to the third dimension. *Science* **294**: 1708–1712.
- Decitre M, Gleyzal C, Raccourt M, Peyrol S, Aubert-Foucher E, Csiszar K, Sommer P. 1998. Lysyl oxidase-like protein localizes to sites of de novo fibrinogenesis in fibrosis and in the early stromal reaction of ductal breast carcinomas. *Lab Invest* **78**: 143–151.
- del Rio A, Perez-Jimenez R, Liu R, Roca-Cusachs P, Fernandez JM, Sheetz MP. 2009. Stretching single talin rod molecules activates vinculin binding. *Science* **323**: 638–641.
- DiDonna BA, Levine AJ. 2006. Filamin cross-linked semiflexible networks: Fragility under strain. *Phys Rev Lett* **97**: 068104.
- Discher DE, Janmey P, Wang YL. 2005. Tissue cells feel and respond to the stiffness of their substrate. *Science* **310**: 1139–1143.
- Egeblad M, Ewald AJ, Askautrud HA, Truitt ML, Werb Z. 2008. Visualizing stromal cell dynamics in different tumor microenvironments by spinning disk confocal microscopy. *Dis Model Mech* **1**: 155–167; discussion 165.
- Ellis IR, Jones SJ, Staunton D, Vakonakis I, Norman DG, Potts JR, Milner CM, Meenan NAG, Raibaud S, Schor AM, et al. 2010. Multi-factorial modulation of IGD motogenic potential in MSF (migration stimulating factor). *Exp Cell Res* doi: 10.1016/j.jyexcr.2010.04.003.
- Emerman JT, Bartley JC, Bissell MJ. 1981. Glucose metabolite patterns as markers of functional differentiation in freshly isolated and cultured mouse mammary epithelial cells. *Exp Cell Res* **134**: 241–250.
- Emerman JT, Pitelka DR. 1977. Maintenance and induction of morphological differentiation in dissociated mammary epithelium on floating collagen membranes. *In Vitro* **13**: 316–328.
- Engler AJ, Sen S, Sweeney HL, Discher DE. 2006. Matrix elasticity directs stem cell lineage specification. *Cell* **126**: 677–689.
- Fata JE, Chaudhary V, Khokha R. 2001. Cellular turnover in the mammary gland is correlated with systemic levels of progesterone and not 17 β -estradiol during the estrous cycle. *Biol Reprod* **65**: 680–688.
- Felsenfeld DP, Schwartzberg PL, Venegas A, Tse R, Sheetz MP. 1999. Selective regulation of integrin–cytoskeleton interactions by the tyrosine kinase Src. *Nature Cell Biology* **1**: 200–206.
- Feng Y, Walsh CA. 2004. The many faces of filamin: A versatile molecular scaffold for cell motility and signalling. *Nat Cell Biol* **6**: 1034–1038.
- Fogerty FJ, Akiyama SK, Yamada KM, Mosher DF. 1990. Inhibition of binding of fibronectin to matrix assembly sites by anti-integrin ($\alpha_5 \beta_1$) antibodies. *J Cell Biol* **111**: 699–708.
- Fraley SI, Feng Y, Krishnamurthy R, Kim DH, Celedon A, Longmore GD, Wirtz D. 2010. A distinctive role for focal adhesion proteins in three-dimensional cell motility. *Nat Cell Biol* **12**: 598–604.
- Frame MC. 2004. Newest findings on the oldest oncogene; how activated src does it. *J Cell Sci* **117**: 989–998.
- Fringer J, Grinnell F. 2001. Fibroblast quiescence in floating or released collagen matrices: Contribution of the ERK signaling pathway and actin cytoskeletal organization. *J Biol Chem* **276**: 31047–31052.
- Fringer J, Grinnell F. 2003. Fibroblast quiescence in floating collagen matrices—decrease in serum activation of MEK and RAF but not Ras. *J Biol Chem* **278**: 20612–20617.



P. Schedin and P.J. Keely

- Fung Y. 1993. *Biomechanics: Mechanical properties of living tissue* Springer-Verlag, New York.
- Galbraith CG, Sheetz MP. 1997. A micromachined device provides a new bend on fibroblast traction forces. *Proc Natl Acad Sci U S A* **94**: 9114–9118.
- Galbraith CG, Yamada KM, Sheetz MP. 2002. The relationship between force and focal complex development. *J Cell Biol* **159**: 695–705.
- Garcia-Alvarez B, de Pereda JM, Calderwood DA, Ulmer TS, Critchley D, Campbell ID, Ginsberg MH, Liddington RC. 2003. Structural determinants of integrin recognition by talin. *Mol Cell* **11**: 49–58.
- Gardel ML, Nakamura F, Hartwig JH, Crocker JC, Stossel TP, Weitz DA. 2006. Prestressed F-actin networks cross-linked by hinged filamins replicate mechanical properties of cells. *Proc Natl Acad Sci U S A* **103**: 1762–1767.
- Gehler S, Baldassarre M, Lad Y, Leight JL, Wozniak MA, Ricking KM, Eliceiri KW, Weaver VM, Calderwood DA, Keely PJ. 2009. Filamin A- β 1 integrin complex tunes epithelial cell response to matrix tension. *Mol Biol Cell* **20**: 3224–3238.
- Giannelli G, Falk-Marzillier J, Schiraldi O, Stetler-Stevenson WG, Quaranta V. 1997. Induction of cell migration by matrix metalloprotease-2 cleavage of laminin-5. *Science* **277**: 225–228.
- Giannone G, Jiang G, Sutton DH, Critchley DR, Sheetz MP. 2003. Talin1 is critical for force-dependent reinforcement of initial integrin-cytoskeleton bonds but not tyrosine kinase activation. *J Cell Biol* **163**: 409–419.
- Gingras AR, Ziegler WH, Bobkov AA, Joyce MG, Fasci D, Himmel M, Rothenmund S, Ritter A, Grossmann JG, Patel B, et al. 2009. Structural determinants of integrin binding to the talin rod. *J Biol Chem* **284**: 8866–8876.
- Goksoy E, Ma YQ, Wang X, Kong X, Perera D, Plow EF, Qin J. 2008. Structural basis for the autoinhibition of talin in regulating integrin activation. *Mol Cell* **31**: 124–133.
- Goswami S, Philippart U, Sun D, Patsialou A, Avraham J, Wang W, Di Modugno F, Nistico P, Gertler FB, Condeelis JS. 2009. Identification of invasion specific splice variants of the cytoskeletal protein Mena present in mammary tumor cells during invasion in vivo. *Clin Exp Metastasis* **26**: 153–159.
- Goswami S, Sahai E, Wyckoff JB, Cammer M, Cox D, Pixley FJ, Stanley ER, Segall JE, Condeelis JS. 2005. Macrophages promote the invasion of breast carcinoma cells via a colony-stimulating factor-1/epidermal growth factor paracrine loop. *Cancer Res* **65**: 5278–5283.
- Grinnell F. 2000. Fibroblast-collagen-matrix contraction: Growth-factor signalling and mechanical loading. *Trends Cell Biol* **10**: 362–365.
- Guarnieri D, Battista S, Borzacchiello A, Mayol L, De Rosa E, Keene DR, Muscarello L, Barbarisi A, Netti PA. 2007. Effects of fibronectin and laminin on structural, mechanical and transport properties of 3D collagenous network. *J Mater Sci Mater Med* **18**: 245–253.
- Guo YP, Martin LJ, Hanna W, Banerjee D, Miller N, Fishell E, Khokha R, Boyd NF. 2001. Growth factors and stromal matrix proteins associated with mammographic densities. *Cancer Epidemiol Biomarkers Prev* **10**: 243–248.
- Hall HG, Bissell MJ. 1986. Characterization of the intermediate filament proteins of murine mammary gland epithelial cells. Response to collagen substratum. *Exp Cell Res* **162**: 379–89.
- Hall HG, Farson DA, Bissell MJ. 1982. Lumen formation by epithelial cell lines in response to collagen overlay: A morphogenetic model in culture. *Proc Natl Acad Sci U S A* **79**: 4672–4676.
- Haslam SZ, Woodward TL. 2001. Reciprocal regulation of extracellular matrix proteins and ovarian steroid activity in the mammary gland. *Breast Cancer Res* **3**: 365–372.
- Hattar R, Maller O, McDaniel S, Hansen KC, Hedman KJ, Lyons TR, Lucia S, Wilson RS Jr, Schedin P. 2009. Tamoxifen induces pleiotrophic changes in mammary stroma resulting in extracellular matrix that suppresses transformed phenotypes. *Breast Cancer Res* **11**: R5.
- Hay ED. 1981. *Cell biology of the extracellular matrix* Plenum Press, New York.
- Himmel M, Ritter A, Rothenmund S, Pauling BV, Rottner K, Gingras AR, Ziegler WH. 2009. Control of high affinity interactions in the talin C terminus: How talin domains coordinate protein dynamics in cell adhesions. *J Biol Chem* **284**: 13832–13842.
- Honda H, Nakamoto T, Sakai R, Hirai H. 1999. p130(Cas), an assembling molecule of actin filaments, promotes cell movement, cell migration, and cell spreading in fibroblasts. *Biochem Biophys Res Commun* **262**: 25–30.
- Humphrey J, Delang S. 2004. *An introduction to biomechanics: Solids and fluids, analysis and design* Springer-Verlag, New York.
- Hynes RO. 2002. Integrins: Bidirectional, allosteric signaling machines. *Cell* **110**: 673–687.
- Hynes RO, Yamada KM. 1982. Fibronectins: Multifunctional modular glycoproteins. *J Cell Biol* **95**: 369–377.
- Hytonen VP, Vogel V. 2008. How force might activate talin's vinculin binding sites: SMD reveals a structural mechanism. *PLoS Comput Biol* **4**: e24.
- Ingman WV, Wyckoff J, Gouon-Evans V, Condeelis J, Pollard JW. 2006. Macrophages promote collagen fibrillogenesis around terminal end buds of the developing mammary gland. *Dev Dyn* **235**: 3222–3229.
- Janmey PA, Georges PC, Hvidt S. 2007. Basic rheology for biologists. *Methods Cell Biol* **83**: 3–27.
- Jiang GY, Giannone G, Critchley DR, Fukumoto R, Sheetz MP. 2003. Two-piconewton slip bond between fibronectin and the cytoskeleton depends on talin. *Nature* **424**: 334–337.
- Julien MA, Haller CA, Wang P, Wen J, Chaikof EL. 2007. Mechanical strain induces a persistent upregulation of syndecan-1 expression in smooth muscle cells. *J Cell Physiol* **211**: 167–173.
- Kadler KE, Hill A, Canty-Laird EG. 2008. Collagen fibrillogenesis: Fibronectin, integrins, and minor collagens as organizers and nucleators. *Curr Opin Cell Biol* **20**: 495–501.
- Katsumi A, Naoe T, Matsushita T, Kaibuchi K, Schwartz MA. 2005. Integrin activation and matrix binding mediate cellular responses to mechanical stretch. *J Biol Chem* **280**: 16546–16549.
- Katsumi A, Orr AW, Tzima E, Schwartz MA. 2004. Integrins in mechanotransduction. *J Biol Chem* **279**: 12001–12004.

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- Katz BZ, Zamir E, Bershadsky A, Kam Z, Yamada KM, Geiger B. 2000. Physical state of the extracellular matrix regulates the structure and molecular composition of cell-matrix adhesions. *Mol Biol Cell* **11**: 1047–1060.
- Kimura K, Ito M, Amano M, Chihara K, Fukata Y, Nakafuku M, Yamamori B, Feng J, Nakano T, Okawa K, et al. 1996. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science* **273**: 245–248.
- Kirschmann DA, Seftor EA, Nieva DR, Mariano EA, Hendrix MJ. 1999. Differentially expressed genes associated with the metastatic phenotype in breast cancer. *Breast Cancer Res Treat* **55**: 127–136.
- Kiyokawa E, Hashimoto Y, Kobayashi S, Sugimura H, Kurata T, Matsuda M. 1998. Activation of Rac1 by a Crk SH3-binding protein, DOCK180. *Genes Dev* **12**: 3331–3336.
- Klein EA, Yin L, Kothapalli D, Castagnino P, Byfield FJ, Xu T, Levental I, Hawthorne E, Janmey PA, Assoian RK. 2009. Cell-cycle control by physiological matrix elasticity and in vivo tissue stiffening. *Curr Biol* **19**: 1511–1518.
- Klotzsch E, Smith ML, Kubow KE, Muntwyler S, Little WC, Beyeler F, Gourdon D, Nelson BJ, Vogel V. 2009. Fibronectin forms the most extensible biological fibers displaying switchable force-exposed cryptic binding sites. *Proc Natl Acad Sci U S A* **106**: 18267–18272.
- Kostic A, Sheetz MP. 2006. Fibronectin rigidity response through Fyn and p130Cas recruitment to the leading edge. *Mol Biol Cell* **17**: 2684–2695.
- Kreike B, van Kouwenhove M, Horlings H, Weigelt B, Peterse H, Bartelink H, van de Vijver MJ. 2007. Gene expression profiling and histopathological characterization of triple-negative/basal-like breast carcinomas. *Breast Cancer Res* **9**: R65.
- Lahlou H, Sanguin-Gendreau V, Zuo D, Cardiff RD, McLean GW, Frame MC, Muller WJ. 2007. Mammary epithelial-specific disruption of the focal adhesion kinase blocks mammary tumor progression. *Proc Natl Acad Sci U S A* **104**: 20302–20307.
- Larsen M, Wei C, Yamada KM. 2006. Cell and fibronectin dynamics during branching morphogenesis. *J Cell Sci* **119**: 3376–3384.
- Lee EY, Parry G, Bissell MJ. 1984. Modulation of secreted proteins of mouse mammary epithelial cells by the collagenous substrata. *J Cell Biol* **98**: 146–155.
- Lee YJ, Hsu TC, Du JY, Valentijn AJ, Wu TY, Cheng CF, Yang Z, Streuli CH. 2009. Extracellular matrix controls insulin signaling in mammary epithelial cells through the RhoA/Rok pathway. *J Cell Physiol* **220**: 476–484.
- Levental KR, Yu H, Kass L, Lakins JN, Egeblad M, Erler JT, Fong SE, Csiszar K, Giaccia A, Weninger W, et al. 2009. Matrix crosslinking forces tumor progression by enhancing integrin signaling. *Cell* **139**: 891–906.
- Li ML, Aggeler J, Farson DA, Hatier C, Hassell J, Bissell MJ. 1987. Influence of a reconstituted basement membrane and its components on casein gene expression and secretion in mouse mammary epithelial cells. *Proc Natl Acad Sci U S A* **84**: 136–140.
- Li N, Zhang Y, Naylor MJ, Schatzmann F, Maurer F, Wintermantel T, Schuetz G, Mueller U, Streuli CH, Hynes NE. 2005. $\beta 1$ integrins regulate mammary gland proliferation and maintain the integrity of mammary alveoli. *Embo J* **24**: 1942–1953.
- Li S, Butler P, Wang Y, Hu Y, Han DC, Usami S, Guan JL, Chien S. 2002. The role of the dynamics of focal adhesion kinase in the mechanotaxis of endothelial cells. *Proc Natl Acad Sci U S A* **99**: 3546–3551.
- Liu SC, Calderwood DA, Ginsberg MH. 2000. Integrin cytoplasmic domain-binding proteins. *J Cell Sci* **113**: 3563–3571.
- Lyons TR, Schedin PJ, Borges VF. 2009. Pregnancy and breast cancer: When they collide. *J Mammary Gland Biol Neoplasia* **14**: 87–98.
- Maeda T, Desouky J, Friedl A. 2006. Syndecan-1 expression by stromal fibroblasts promotes breast carcinoma growth in vivo and stimulates tumor angiogenesis. *Oncogene* **25**: 1408–1412.
- Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, Shipitsin M, et al. 2008. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* **133**: 704–715.
- Mao Y, Schwarzbauer JE. 2005. Fibronectin fibrillogenesis, a cell-mediated matrix assembly process. *Matrix Biol* **24**: 389–399.
- McBeath R, Pirone DM, Nelson CM, Bhadriraju K, Chen CS. 2004. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. *Dev Cell* **6**: 483–495.
- Mecham RP, Heuser J. 1990. Three-dimensional organization of extracellular matrix in elastic cartilage as viewed by quick freeze, deep etch electron microscopy. *Connect Tissue Res* **24**: 83–93.
- Miron-Mendoza M, Seemann J, Grinnell F. 2008. Collagen fibril flow and tissue translocation coupled to fibroblast migration in 3D collagen matrices. *Mol Biol Cell* **19**: 2051–2058.
- Monaghan P, Warburton MJ, Perusinghe N, Rudland PS. 1983. Topographical arrangement of basement membrane proteins in lactating rat mammary gland: Comparison of the distribution of type IV collagen, laminin, fibronectin, and Thy-1 at the ultrastructural level. *Proc Natl Acad Sci U S A* **80**: 3344–3348.
- Mott JD, Werb Z. 2004. Regulation of matrix biology by matrix metalloproteinases. *Curr Opin Cell Biol* **16**: 558–564.
- Muthuswamy SK, Li D, Lelievre S, Bissell MJ, Brugge JS. 2001. ErbB2, but not ErbB1, reinitiates proliferation and induces luminal repopulation in epithelial acini. *Nat Cell Biol* **3**: 785–792.
- Naylor MJ, Li N, Cheung J, Lowe ET, Lambert E, Marlow R, Wang P, Schatzmann F, Wintermantel T, Schuetz G, et al. 2005. Ablation of $\beta 1$ integrin in mammary epithelium reveals a key role for integrin in glandular morphogenesis and differentiation. *J Cell Biol* **171**: 717–728.
- Nelson CM, Vanduijn MM, Inman JL, Fletcher DA, Bissell MJ. 2006. Tissue geometry determines sites of mammary branching morphogenesis in organotypic cultures. *Science* **314**: 298–300.
- Neubauer H, Ruoff A, Paessler N, Solomayer E, Wallwiener E, Fehm T. 2009. A laminin-rich basement membrane matrix influences estrogen receptor β expression and morphology of MDA-MB-231 breast cancer cells. *Oncol Rep* **21**: 475–481.

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- Neville MC. 2009. Introduction: Tight junctions and secretory activation in the mammary gland. *J Mammary Gland Biol Neoplasia* **14**: 269–270.
- O'Brien J, Lyons T, Monks J, Lucia MS, Wilson RS, Hines L, Man YG, Borges V, Schedin P. 2010. Alternatively activated macrophages and collagen remodeling characterize the postpartum involuting mammary gland across species. *Am J Pathol* **176**: 1241–1255.
- Olumi AF, Grossfeld GD, Hayward SW, Carroll PR, Tlsty TD, Cunha GR. 1999. Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. *Cancer Res* **59**: 5002–5011.
- Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL, Weinberg RA. 2005. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell* **121**: 335–348.
- Orimo A, Weinberg RA. 2006. Stromal fibroblasts in cancer: A novel tumor-promoting cell type. *Cell Cycle* **5**: 1597–1601.
- Page-McCaw A, Ewald AJ, Werb Z. 2007. Matrix metalloproteinases and the regulation of tissue remodelling. *Nat Rev Mol Cell Biol* **8**: 221–233.
- Paszek MJ, Zahir N, Johnson KR, Lakins JN, Rozenberg GI, Gefen A, Reinhart-King CA, Margulies SS, Dembo M, Boettiger D, et al. 2005. Tensional homeostasis and the malignant phenotype. *Cancer Cell* **8**: 241–254.
- Pelham RJ Jr, Wang Y. 1997. Cell locomotion and focal adhesions are regulated by substrate flexibility. *Proc Natl Acad Sci U S A* **94**: 13661–13665.
- Pelham RJ Jr, Wang Y. 1999. High resolution detection of mechanical forces exerted by locomoting fibroblasts on the substrate. *Mol Biol Cell* **10**: 935–945.
- Peyrol S, Raccurt M, Gerard F, Gleyzal C, Grimaud JA, Sommer P. 1997. Lysyl oxidase gene expression in the stromal reaction to in situ and invasive ductal breast carcinoma. *Am J Pathol* **150**: 497–507.
- Philippart U, Roussos ET, Oser M, Yamaguchi H, Kim HD, Giampieri S, Wang Y, Goswami S, Wyckoff JB, Lauffenburger DA, et al. 2008. A Mena invasion isoform potentiates EGF-induced carcinoma cell invasion and metastasis. *Dev Cell* **15**: 813–828.
- Pirone DM, Liu WF, Ruiz SA, Gao L, Raghavan S, Lemmon CA, Romer LH, Chen CS. 2006. An inhibitory role for FAK in regulating proliferation: A link between limited adhesion and RhoA-ROCK signaling. *J Cell Biol* **174**: 277–288.
- Pokutta S, Weis WI. 2007. Structure and mechanism of cadherins and catenins in cell–cell contacts. *Annu Rev Cell Dev Biol* **23**: 237–261.
- Provenzano PP, Eliceiri KW, Campbell JM, Inman DR, White JG, Keely PJ. 2006. Collagen reorganization at the tumor-stromal interface facilitates local invasion. *BMC Med* **4**: 38.
- Provenzano PP, Inman DR, Eliceiri KW, Beggs HE, Keely PJ. 2008a. Mammary epithelial-specific disruption of focal adhesion kinase retards tumor formation and metastasis in a transgenic mouse model of human breast cancer. *Am J Pathol* **173**: 1551–1565.
- Provenzano PP, Inman DR, Eliceiri KW, Knittel JG, Yan L, Rueden CT, White JG, Keely PJ. 2008b. Collagen density promotes mammary tumor initiation and progression. *BMC Med* **6**: 11.
- Provenzano PP, Inman DR, Eliceiri KW, Trier SM, Keely PJ. 2008c. Contact guidance mediated three-dimensional cell migration is regulated by rho/ROCK-dependent matrix reorganization. *Biophys J* **95**: 5374–5384.
- Provenzano PP, Inman DR, Eliceiri KW, Keely PJ. 2009. Matrix density-induced mechanoregulation of breast cell phenotype, signaling and gene expression through a FAK-ERK linkage. *Oncogene* **28**: 4326–4343.
- Pylayeva Y, Gillen KM, Gerald W, Beggs HE, Reichardt LF, Giancotti FG. 2009. Ras- and PI3K-dependent breast tumorigenesis in mice and humans requires focal adhesion kinase signaling. *J Clin Invest* **119**: 252–266.
- Quinn JA, Graeber CT, Frackelton AR Jr, Kim M, Schwarzbauer JE, Filardo EJ. 2009. Coordinate regulation of estrogen-mediated fibronectin matrix assembly and epidermal growth factor receptor transactivation by the G protein-coupled receptor, GPR30. *Mol Endocrinol* **23**: 1052–1064.
- Raub CB, Suresh V, Krasieva T, Lyubovitsky J, Mih JD, Putnam AJ, Tromberg BJ, George SC. 2007. Noninvasive assessment of collagen gel microstructure and mechanics using multiphoton microscopy. *Biophys J* **92**: 2212–2222.
- Raub CB, Unruh J, Suresh V, Krasieva T, Lindmo T, Gratton E, Tromberg BJ, George SC. 2008. Image correlation spectroscopy of multiphoton images correlates with collagen mechanical properties. *Biophys J* **94**: 2361–2373.
- Robinson JA, Chatterjee-Kishore M, Yaworsky PJ, Cullen DM, Zhao W, Li C, Kharode Y, Sauter L, Babij P, Brown EL, et al. 2006. Wnt/ β -catenin signaling is a normal physiological response to mechanical loading in bone. *J Biol Chem* **281**: 31720–31728.
- Roeder BA, Kokini K, Sturgis JE, Robinson JP, Voytk-Harbin SL. 2002. Tensile mechanical properties of three-dimensional type I collagen extracellular matrices with varied microstructure. *J Biomech Eng* **124**: 214–222.
- Rowe RG, Weiss SJ. 2008. Breaching the basement membrane: Who, when and how? *Trends Cell Biol* **18**: 560–574.
- Sawada Y, Tamada M, Dubin-Thaler BJ, Cherniavskaya O, Sakai R, Tanaka S, Sheetz MP. 2006. Force sensing by mechanical extension of the Src family kinase substrate p130Cas. *Cell* **127**: 1015–1026.
- Sawhney RK, Howard J. 2002. Slow local movements of collagen fibers by fibroblasts drive the rapid global self-organization of collagen gels. *J Cell Biol* **157**: 1083–1091.
- Sawhney RK, Howard J. 2004. Molecular dissection of the fibroblast-traction machinery. *Cell Motil Cytoskeleton* **58**: 175–185.
- Schedin P, Mitrenga T, Kaech M. 2000a. Estrous cycle regulation of mammary epithelial cell proliferation, differentiation, and death in the Sprague–Dawley rat: A model for investigating the role of estrous cycling in mammary carcinogenesis. *J Mammary Gland Biol Neoplasia* **5**: 211–225.
- Schedin P, Mitrenga T, McDaniel S, Kaech M. 2004. Mammary ECM composition and function are altered by reproductive state. *Mol Carcinog* **41**: 207–220.

Mammary Gland ECM Remodeling, Stiffness, and Mechanosignaling

- Schedin P, O'Brien J, Rudolph M, Stein T, Borges V. 2007. Microenvironment of the involuting mammary gland mediates mammary cancer progression. *J Mammary Gland Biol Neoplasia* **12**: 71–82.
- Schedin P, Strange R, Mitrenga T, Wolfe P, Kaeck M. 2000b. Fibronectin fragments induce MMP activity in mouse mammary epithelial cells: Evidence for a role in mammary tissue remodeling. *J Cell Sci* **113** (Pt 5): 795–806.
- Schenk S, Quaranta V. 2003. Tales from the crypt[ic] sites of the extracellular matrix. *Trends Cell Biol* **13**: 366–375.
- Schober M, Raghavan S, Nikolova M, Polak L, Pasolli HA, Beggs HE, Reichardt LF, Fuchs E. 2007. Focal adhesion kinase modulates tension signaling to control actin and focal adhesion dynamics. *J Cell Biol* **176**: 667–680.
- Schor SL, Ellis IR, Jones SJ, Baillie R, Seneviratne K, Clausen J, Motegi K, Vojtesek B, Kankova K, Furrie E, et al. 2003. Migration-stimulating factor: A genetically truncated onco-fetal fibronectin isoform expressed by carcinoma and tumor-associated stromal cells. *Cancer Res* **63**: 8827–8836.
- Silberstein GB, Daniel CW. 1982. Glycosaminoglycans in the basal lamina and extracellular matrix of the developing mouse mammary duct. *Dev Biol* **90**: 215–222.
- Silberstein GB, Daniel CW. 1984. Glycosaminoglycans in the basal lamina and extracellular matrix of serially aged mouse mammary ducts. *Mech Ageing Dev* **24**: 151–162.
- Silberstein GB, Daniel CW. 1987. Reversible inhibition of mammary gland growth by transforming growth factor- β . *Science* **237**: 291–293.
- Singer II. 1979. The fibronexus: A transmembrane association of fibronectin-containing fibers and bundles of 5 nm microfilaments in hamster and human fibroblasts. *Cell* **16**: 675–685.
- Singer II, Kawka DW, Kazazis DM, Clark RA. 1984. In vivo co-distribution of fibronectin and actin fibers in granulation tissue: Immunofluorescence and electron microscope studies of the fibronexus at the myofibroblast surface. *J Cell Biol* **98**: 2091–2106.
- Smith ML, Gourdon D, Little WC, Kubow KE, Eguiluz RA, Luna-Morris S, Vogel V. 2007. Force-induced unfolding of fibronectin in the extracellular matrix of living cells. *PLoS Biol* **5**: e268.
- Stossel TP, Condeelis J, Cooley L, Hartwig JH, Noegel A, Schleicher M, Shapiro SS. 2001. Filamins as integrators of cell mechanics and signalling. *Nat Rev Mol Cell Biol* **2**: 138–145.
- Streuli CH, Bailey N, Bissell MJ. 1991. 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* **115**: 1383–1395.
- Streuli CH, Schmidhauser C, Bailey N, Yurchenco P, Skubitz AB, Roskelley C, Bissell MJ. 1995. Laminin mediates tissue-specific gene expression in mammary epithelia. *J Cell Biol* **129**: 591–603.
- Su G, Blaine SA, Qiao D, Friedl A. 2007. Shedding of Syndecan-1 by stromal fibroblasts stimulates human breast cancer cell proliferation via FGF2 activation. *J Biol Chem* **282**: 14906–14915.
- Su G, Blaine SA, Qiao D, Friedl A. 2008. Membrane type 1 matrix metalloproteinase-mediated stromal syndecan-1 shedding stimulates breast carcinoma cell proliferation. *Cancer Res* **68**: 9558–9565.
- Sugrue SP, Hay ED. 1981. Response of basal epithelial cell surface and cytoskeleton to solubilized extracellular matrix molecules. *J Cell Biol* **91**: 45–54.
- Tan JL, Tien J, Pirone DM, Gray DS, Bhadriraju K, Chen CS. 2003. Cells lying on a bed of microneedles: an approach to isolate mechanical force. *Proc Natl Acad Sci U S A* **100**: 1484–1489.
- Teng MH, Bartholomew JC, Bissell MJ. 1977. Synergism between anti-microtubule agents and growth stimulants in enhancement of cell cycle traverse. *Nature* **268**: 739–741.
- Thery M, Racine V, Pepin A, Piel M, Chen Y, Sibarita MB, Bornens M. 2005. The extracellular matrix guides the orientation of the cell division axis. *Nat Cell Biol* **7**: 947–953.
- Thery M, Racine V, Piel M, Pepin A, Dimitrov A, Chen Y, Sibarita JB, Bornens M. 2006. Anisotropy of cell adhesive microenvironment governs cell internal organization and orientation of polarity. *Proc Natl Acad Sci U S A* **103**: 19771–19776.
- Tomasek JJ, Hay ED, Fujiwara K. 1982. Collagen modulates cell shape and cytoskeleton of embryonic corneal and fibroblast fibroblasts: Distribution of actin, α -actinin, and myosin. *Dev Biol* **92**: 107–122.
- Vader D, Kabla A, Weitz D, Mahadevan L. 2009. Strain-induced alignment in collagen gels. *PLoS One* **4**: e5902.
- Vadlamudi RK, Li F, Adam L, Nguyen D, Ohta Y, Stossel TP, Kumar R. 2002. Filamin is essential in actin cytoskeletal assembly mediated by p21-activated kinase 1. *Nat Cell Biol* **4**: 681–690.
- Vasioukhin V, Fuchs E. 2001. Actin dynamics and cell-cell adhesion in epithelia. *Curr Opin Cell Biol* **13**: 76–84.
- Volonte D, Galbiati F, Pestell RG, Lisanti MP. 2001. Cellular stress induces the tyrosine phosphorylation of caveolin-1 (Tyr¹⁴) via activation of p38 mitogen-activated protein kinase and c-Src kinase. Evidence for caveolae, the actin cytoskeleton, and focal adhesions as mechanical sensors of osmotic stress. *J Biol Chem* **276**: 8094–8103.
- Walker R. 2001. The complexities of breast cancer desmoplasia. *Breast Cancer Res* **3**: 143–145.
- Wang HB, Dembo M, Hanks SK, Wang Y. 2001. Focal adhesion kinase is involved in mechanosensing during fibroblast migration. *Proc Natl Acad Sci U S A* **98**: 11295–11300.
- Wang HB, Dembo M, Wang YL. 2000. Substrate flexibility regulates growth and apoptosis of normal but not transformed cells. *Am J Physiol Cell Physiol* **279**: C1345–C1350.
- Wang W, Goswami S, Lapidus K, Wells AL, Wyckoff JB, Sahai E, Singer RH, Segall JE, Condeelis JS. 2004. Identification and testing of a gene expression signature of invasive carcinoma cells within primary mammary tumors. *Cancer Res* **64**: 8585–8594.
- Wang Y, Botvinick EL, Zhao Y, Berns MW, Usami S, Tsien RY, Chien S. 2005. Visualizing the mechanical activation of Src. *Nature* **434**: 1040–1045.
- Warburton MJ, Mitchell D, Ormerod EJ, Rudland P. 1982. Distribution of myoepithelial cells and basement



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- membrane proteins in the resting, pregnant, lactating, and involuting rat mammary gland. *J Histochem Cytochem* **30**: 667–676.
- Weaver VM, Petersen OW, Wang F, Larabell CA, Briand P, Damsky C, Bissell MJ. 1997. Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. *J Cell Biol* **137**: 231–245.
- Wegener KL, Partridge AW, Han J, Pickford AR, Liddington RC, Ginsberg MH, Campbell ID. 2007. Structural basis of integrin activation by talin. *Cell* **128**: 171–182.
- Wenstrup RJ, Florer JB, Brunskill EW, Bell SM, Chervoneva I, Birk DE. 2004. Type V collagen controls the initiation of collagen fibril assembly. *J Biol Chem* **279**: 53331–53337.
- Werb Z, Tremble PM, Behrendtsen O, Crowley E, Damsky CH. 1989. Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression. *J Cell Biol* **109**: 877–889.
- Williams CM, Engler AJ, Slone RD, Galante LL, Schwarzbauer JE. 2008. Fibronectin expression modulates mammary epithelial cell proliferation during acinar differentiation. *Cancer Res* **68**: 3185–3192.
- Wipff PJ, Rifkin DB, Meister JJ, Hinz B. 2007. Myofibroblast contraction activates latent TGF- β 1 from the extracellular matrix. *J Cell Biol* **179**: 1311–1323.
- Wolf K, Friedl P. 2009. Mapping proteolytic cancer cell-extracellular matrix interfaces. *Clin Exp Metastasis* **26**: 289–298.
- Wolf K, Wu YI, Liu Y, Geiger J, Tam E, Overall C, Stack MS, Friedl P. 2007. Multi-step pericellular proteolysis controls the transition from individual to collective cancer cell invasion. *Nat Cell Biol* **9**: 893–904.
- Woodward TL, Mienaltowski AS, Modi RR, Bennett JM, Haslam SZ. 2001. Fibronectin and the α (5) β (1) integrin are under developmental and ovarian steroid regulation in the normal mouse mammary gland. *Endocrinology* **142**: 3214–3222.
- Wozniak M, Fausto A, Carron CP, Meyer DM, Hruska KA. 2000. Mechanically strained cells of the osteoblast lineage organize their extracellular matrix through unique sites of α v β 3-integrin expression. *J Bone Miner Res* **15**: 1731–1745.
- Wozniak MA, Chen CS. 2009. Mechanotransduction in development: a growing role for contractility. *Nat Rev Mol Cell Biol* **10**: 34–43.
- Wozniak MA, Desai R, Solski PA, Der CJ, Keely PJ. 2003. ROCK-generated contractility regulates breast epithelial cell differentiation in response to the physical properties of a three-dimensional collagen matrix. *J Cell Biol* **163**: 583–595.
- Wyckoff J, Wang W, Lin EY, Wang Y, Pixley F, Stanley ER, Graf T, Pollard JW, Segall JE, Condeelis JS. 2004. A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. *Cancer Res* **64**: 7022–7029.
- Wyckoff JB, Pinner SE, Gschmeissner S, Condeelis JS, Sahai E. 2006. ROCK- and myosin-dependent matrix deformation enables protease-independent tumor-cell invasion in vivo. *Curr Biol* **16**: 1515–1523.
- Xie JW, Haslam SZ. 2008. Extracellular matrix, Rac1 signaling, and estrogen-induced proliferation in MCF-7 breast cancer cells. *Breast Cancer Res Treat* **110**: 257–268.
- Yan B, Calderwood DA, Yaspan B, Ginsberg MH. 2001. Calpain cleavage promotes talin binding to the β 3 integrin cytoplasmic domain. *J Biol Chem* **276**: 28164–28170.
- Yang JT, Bader BL, Kreidberg JA, Ullman-Cullere M, Trevi-thick JE, Hynes RO. 1999. Overlapping and independent functions of fibronectin receptor integrins in early mesodermal development. *Dev Biol* **215**: 264–277.
- Yano Y, Geibel J, Sumpio B. 1996. Tyrosine phosphorylation of pp125^{FAK} and paxillin in aortic endothelial cells induced by mechanical strain. *Am J Physiol* **271**: C635–C649.
- Zhang Q, Magnusson MK, Mosher DF. 1997. Lysophosphatidic acid and microtubule-destabilizing agents stimulate fibronectin matrix assembly through Rho-dependent actin stress fiber formation and cell contraction. *Mol Biol Cell* **8**: 1415–1425.





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Mammary Gland ECM Remodeling, Stiffness, and Mechanosignaling in Normal Development and Tumor Progression

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Cold Spring Harb Perspect Biol 2011; doi: 10.1101/cshperspect.a003228 originally published online October 27, 2010

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