Laminin and biomimetic extracellular elasticity enhance functional differentiation in mammary epithelia

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ABSTRACT

In the mammary gland, epithelial cells are embedded in a 'soft' environment, and become functionally differentiated in culture when exposed to a laminin-rich extracellular matrix gel. Here we define the processes by which mammary epithelial cells integrate biochemical and mechanical extracellular cues to maintain their differentiated phenotype. We used cells cultured on top of gels in conditions permissive for β-casein expression using atomic force microscopy to measure the elasticity of the cells and their underlying substrata. We found that maintenance of β-casein expression required both laminin signaling and a 'soft' extracellular matrix as is the case in normal tissues in vivo, and biomimetic intracellular elasticity as is the case in intact primary mammary epithelial organoids. Conversely two hallmarks of breast cancer development, stiffening of the extracellular matrix and loss of laminin signaling, led to loss of β-casein expression and non-biomimetic intracellular elasticity. Our data indicate that tissue-specific gene expression is controlled by both the the tissues' unique biochemical milieu and the distinct mechanical properties of the extracellular matrix, processes involved in both maintenance of tissue integrity and protection against tumorigenesis.

INTRODUCTION

Signals from the microenvironment are essential to direct normal tissue development and, in the adult organism, to maintain tissue-specific functions (Nelson and Bissell, 2006). Studies in three-dimensional (3D) cultures have identified key biochemical microenvironmental cues underlying mammary-specific structure and function. The extracellular matrix (ECM) component laminin-111 (LM1) is necessary to induce polarity and acinar morphogenesis (Gudjonsson et al., 2002), whereas both LM1 and lactogenic hormones are required to express β-casein (Streuli et al., 1991; Xu et al., 2007) in mammary epithelial cells (MECs). In addition to a unique biochemical milieu, different tissue microenvironments exhibit distinct mechanical properties (Discher et al., 2005). Rather than being a passive property of the tissue, there is growing evidence that the mechanical properties of the microenvironment have a direct impact on tissue-specific morphogenetic programs in MECs (Paszek et al., 2005; Wozniak et al., 2003) and other cell types (Engler et al., 2006). Furthermore, because abnormally high stiffness and loss of tissue function are hallmarks of solid tumors (Paszek et al., 2005), and increased mammographic density is a risk factor for breast cancer (Boyd et al., 1998), it has been suggested that aberrant tissue stiffness may facilitate the acquisition of a malignant phenotype (Paszek et al., 2005; Provenzano et al., 2006; Wozniak et al., 2003). Tissue elasticity is thought to be maintained by a mechanical homeostatic mechanism largely determined by the reciprocal mechanochemical interactions between cells and their surrounding ECM (Bissell et al., 1982; Discher et al., 2005; Paszek et al., 2005). Previous studies have examined the effects of either the biochemical composition or the elasticity of the substrata on functional differentiation or cell mechanics individually; however a comprehensive approach aiming to dissect how these signals modulate each other, and how the whole controls tissue-specific gene expression has been lacking (Alcaraz et al., 2004).

To determine how the mechanochemistry of the cellular microenvironment affects tissue-specific gene expression, we used two mammary epithelial cell lines (SCp2 and EpH4) that in culture can be induced to functionally differentiate (defined here as expression of an abundant mouse milk protein, β-casein) in presence of appropriate ECM. Because in the presence of a LM1-containing gels cell-cell contact is not required for β-casein expression (Streuli et al., 1991) (Supplementary

Figure 1), we examined single cells cultured on top of gels under these conditions permissive for β-casein expression. This "cell-on-top" assay allowed us to employ atomic force microscopy (AFM) to assess the elasticity or stiffness (defined by the Young's elastic modulus *E*) of single cells and that of their underlying substrata. Using this quantitative and comprehensive approach we unraveled the intimate interrelationship among LM1 binding, ECM stiffness, cell shape and cell stiffness, as well as the synergistic effects of these mechanochemical properties on tissue-specific gene expression. Furthermore we show that variations of these properties from biomimetic values, i.e. comparable to normal physiologic conditions, are potentially linked to tumorigenesis.

RESULTS

Laminin-111 and biomimetic extracellular elasticity induce β-casein expression, and a cell shape and elasticity comparable to cells within primary mammary organoids

Previous studies have reported that normal mouse mammary tissue is very soft ($E \sim 100$ Pa) (Table I) (Paszek et al., 2005), and have suggested that this elasticity is caused mainly by the ECM. In contrast the actual elastic modulus of epithelial cells within the mammary gland still remains unknown. To estimate the elasticity of fully differentiated MECs *in vivo*, we isolated primary mammary organoids from early pregnant mice and cultured them on top of Matrigel in differentiation medium for 24h. In these conditions, mammary organoids can express and secrete milk proteins (Barcellos-Hoff et al., 1989). Matrigel is a suitable substratum because it is rich in LM1 and exhibits biomimetic elastic modulus, as measured by AFM and rheometry (Table I). A schematic representation of this experimental setup is shown in Figure 1A. Mammary organoids are often morphologically heterogeneous in culture. To account for this heterogeneity, we measured the elasticity of small and medium sized organoids (Figure 1B), which typically contain about a dozen or several dozen cells, respectively. We found that, irrespective of size, cells exhibited comparable average elastic moduli between 400 and 800 Pa (Figure 1C). We used this range as a reference for biomimetic cellular elasticity throughout this work.

As shown in Figure 1F, the elastic moduli of either single SCp2 or EpH4 cells cultured on top of Matrigel for 24h fell within the biomimetic range defined by cells in mammary organoids (parallel dashed lines). Since MECs exhibit a round morphology both *in vivo* and when exposed to LM1 in culture (Roskelley et al., 1994), it is possible that their similar elastic moduli is due to the round shape *per se*. To address this question, we measured the elasticity of both cell types rounded in the absence of LM1 signaling by culturing them on top of a substratum coated with the non adhesive poly(2-hydroxyethyl methacrylate) (polyHEMA) (Figure 1D). Unlike cells on Matrigel, cells cultured on polyHEMA were significantly stiffer (Figure 1F), and did not express β-casein (Figure 1E). The stiffening of MECs on polyHEMA was not due to increased cell death (Muschler et al., 1999). These data confirm that LM1 signaling is necessary for functional differentiation of MECs,

and indicate that biomimetic cellular elasticity in MECs is cell line-independent and downstream of cell-ECM rather than cell-cell adhesion or cell rounding *per se*.

Increasing extracellular elasticity beyond normal mammary tissue values inhibits β-casein expression and promotes spreading and stiffening in MECs

Because normal mammary tissue is soft (Table I), we hypothesized that strong functional differentiation in culture would only be achieved by using substrata the elasticity of which mimics normal tissue. Accordingly we used two culture assays (Alcaraz et al., 2004) that allow increasing extracellular stiffness beyond biomimetic values while holding biochemical signaling constant. In the first assay, EpH4 cells were cultured on top of gels containing LM1 mixed with collagen type I (COL I) (3 g/l) (40:60% v/v). Four hours after plating, differentiation media was added and half of the gels were gently detached from their container along the gel's edges and rendered floating in the medium (Michalopoulos and Pitot, 1975). Since AFM requires samples to be somewhat anchored, caution was taken to avoid complete gel detachment by leaving the bottom-center of the gel attached to the underlying glass surface. AFM measurements revealed that the average floating gel elasticity was close to that of bulk mammary tissue, whereas the attached gel was three-fold stiffer (Figure 2A). Such gel stiffening was sufficient to dramatically downregulate β-casein (Figure 2A) and increase the elastic modulus of the cells (Figure 2C). In the second assay, EpH4 were plated on top of polyacrylamide gels exhibiting elastic moduli either close to mammary tissue (soft), or comparable or even higher than mammary tumors (stiff). Only 24h after plating on the stiffer substrata, cells displayed a spread morphology (Figure 2D) and non-biomimetic elasticity (Figure 2F). To induce β-casein, cells were overlaid with 2% Matrigel diluted in differentiation medium. In agreement with the first assay, stiffer substrata downregulated β-casein transcription, measured both by quantitative RT-PCR (Figure 2E) and by the fluorescence of cells transfected with a construct containing 16 copies of the mouse β-casein gene promoter driving cyan fluorescent protein (CFP) expression (Figure 2G). These findings support our hypothesis and indicate that LM1-dependent functional differentiation is modulated by the extracellular elasticity.

Loss of LM1 signaling downregulates β-casein expression and induces non-biomimetic cellular elasticity and/or morphology

MECs *in vivo* are in basal contact with a basement membrane, a specialized ECM rich in LM1 that physically separates mammary epithelium from the stroma; the latter is rich in COL I (Provenzano et al., 2006) and contains much less LM1 (Klinowska et al., 1999). During tumor cell invasion, the integrity of the basement membrane is often compromised (Wetzels et al., 1989) and MECs can contact the stroma directly. To investigate how this loss of LM1 signaling affects functional differentiation and the mechanical properties of MECs, we examined SCp2 plated on top of LM1 gels mixed with increasing concentrations of COL I (2 g/l). We found that ~10% LM1 could override COL I signaling in terms of maintaining biomimetic substratum and cellular elasticity as well as β-casein expression (Figure 3A,B). Interestingly reducing LM1 concentration below 10% led to a sudden increase of gel's elastic moduli (Figure 3B), induced non-biomimetic cellular elasticity (Figure 3A, black dots), and a dramatic downregulation of both β-casein expression (Figure 3A, white dots) and promoter activity (Figure 3D). The unexpected stiffening of SCp2 on softer gels (Figure 3C) confirms that our methodology to probe cell mechanics with AFM was not biased by the stiffness of the underlying substrata (further discussion of the lack of contribution of the underlying substratum elasticity on cell mechanical measurements is presented in Supplementary Materials). The relative changes in β-casein expression as a function of LM1 concentration in EpH4 were comparable to those in SCp2 (Figure 3E and Supplementary Figure 2). However, unlike SCp2, loss of LM1 signaling in EpH4 cells induced stiffening (Figure 3F) and spreading (Figure 3G) comparable to that found on glass substrata. The differences between SCp2 and EpH4 on these gels most probably arise from the distinct expression profiles of LM1 and COL I integrin receptors (Figure 4A). These experiments reveal that at 10% concentration or above, LM1 signaling is dominant over COL I signaling and that COL I-dependent loss of β-casein expression is associated with non-biomimetic extra- and intercellular elastic moduli and changes in cell shape.

Receptors involved in LM1-dependent biomimetic cellular elasticity

To begin to define the molecular mechanisms underlying LM1-dependent biomimetic cellular elasticity, we examined the role of laminin-specific receptors necessary for β-casein expression in culture: β1- and α6-integrins (Muschler et al., 1999) and dystroglycan (DG) (Weir et al., 2006). To inhibit integrin receptors, we used function-blocking antibodies against either β1- or α6-integrins as previously described (Muschler et al., 1999). Blocking β1-integrin in SCp2 dramatically

decreased their elastic moduli (Figure 4B), whereas blocking α6-integrin had only a weak effect (Figure 4C). Unlike β1-integrin blocking experiments, we did not observe a statistically significant difference between the elasticity of DG-/- and DG+/+ cells. These data suggest that a β1-integrin other than α6β1 mediates LM1-dependent biomimetic cellular elasticity.

Biomimetic cellular elasticity is associated with low non-muscle myosin II activity and/or a low actin polymerization

The actin-myosin cytoskeleton is the major contributor to cellular elasticity (Roca-Cusachs et al., 2008; Wakatsuki et al., 2003). When SCp2 cells cultured on glass for 24 h were treated with specific inhibitors of actin polymerization (latrunculin B), Rho kinase (ROCK) (Y27632) and myosin II ATPase activity (blebbistatin), cellular elasticity markedly decreased towards the biomimetic range, although the difference were significantly smaller than cells cultured on top of Matrigel (Figure 5A). In contrast, two different inhibitors of microtubule polymerization essentially had no effect on cellular elasticity. Immunoblot analysis of EpH4 showed that phosphorylation of Thr18 and Ser19 of myosin II light chain (MLCII), which are indicative of the specific activity of myosin II, were significantly lower on Matrigel than on tissue culture plastic (Figure 5B,C). Confocal visualization of F-actin staining in SCp2 showed that on Matrigel cells were round and F-actin was mostly cortical, whereas on glass, cells would spread and the corresponding F-actin was both cortical and cytoplasmic (Figure 5D-E). Increased F-actin on glass was confirmed by quantitative analysis of the average fluorescence intensity of phalloidin staining per cell (Figure 5E). Similar findings were obtained in EpH4 cells (data not shown). These results indicate that LM1-dependent cellular biomimetic elastic modulus is mediated at least in part by targeting the actin-myosin cytoskeleton through downregulation of actin polymerization and/or myosin II activity.

DISCUSSION

In vivo, signals from the microenvironment are essential for normal development and organ homeostasis, and abnormalities in these signals contribute to numerous pathologies including tumorigenesis (Ingber, 2003; Nelson and Bissell, 2006). Nevertheless the detailed mechanochemical signaling by which the microenvironment controls these processes are still ill defined (Alcaraz et al., 2004). Previous studies using cultured cells have examined the effects of extracellular biochemical or biophysical cues on differentiation (Engler et al., 2004b), morphology (Engler et al., 2004a), and mechanics (Solon et al., 2007), each in isolation. Here we used a comprehensive approach to demonstrate quantitatively the intimate connection among LM1 binding, ECM elasticity, cell shape and cell elasticity in controlling tissue-specific gene expression in single epithelial cells. In addition, we found that variations of these properties from biomimetic values induce a loss of functional differentiation, which could potentially be linked to tumorigenesis (Paszek et al., 2005).

Biochemical and mechanical extracellular cues synergize to maintain (or supress) functional differentiation in culture

We used both biologic and synthetic substrata to test the hypothesis that robust functional differentiation in culture requires substrata exhibiting biomimetic elasticity (i.e. mimicking normal tissue elasticity). The elastic modulus of the substrata was measured at the micro- and macroscopic scales by AFM and rheometry, respectively. In agreement with previous findings on synthetic gels (Engler et al., 2004a), both techniques provided comparable values on biological gels (Table I), indicating that either method is suitable to probe the mechanics of compliant materials. In support of our hypothesis, we found that β-casein expression was maximal in MECs cultured on biological gels containing ~40% LM1 or more and exhibiting elastic moduli values within 75-120 Pa (Supplementary Figure 3A), a range comparable to normal mammary tissue. Conversely reduced β-casein expression (< 3 fold) was observed in gels with less than 10% LM1 and a non-biomimetic elasticity of > 250 Pa. Likewise increasing the stiffness of synthetic polyacrylamide gels close to mammary tumor values and beyond was sufficient to downregulate functional differentiation induced upon cell's overlay with Matrigel, although the corresponding levels of β-casein expression were much less than those found using biological gels. This lower β-casein induction is

not surprising if one considers the important differences between the biological gels and the polyacrylamide gel assay in terms of how LM1 is supplied to the cells (within a gel or soluble) and the timing of LM1 binding (at plating time or 24h after, respectively). Despite the different nature of these assays, it is remarkable that all consistently reported that LM1-dependent functional differentiation is modulated by extracellular elasticity, and that, for a given LM1 concentration, β-casein expression is enhanced when the substrata exhibits biomimetic elasticity.

In addition to a requirement for biomimetic extracellular elasticity, we observed that strong β-casein expression (10-100 fold) was tightly associated with biomimetic cell elastic moduli (400-800 Pa), whereas β-casein expression was lost when values fell outside this range (Supplementary Figure 3B). Collectively our findings reveal that MECs are intrinsically programmed to be highly responsive - in terms of functional differentiation- in a narrow range of extra- and intercellular elasticity only, in agreement with recent findings on mechanical-dependent stem cell commitment (Engler et al., 2006). These findings also suggest that the high compliance of the mammary tissue *in vivo* is essential for its functional differentiation (our data) and morphogenesis (Paszek et al., 2005; Wozniak et al., 2003). It is worth noting that all these observations strongly support a strategy to potentially increase the efficacy of current scaffolds used in tissue engineering and regenerative medicine by mimicking the physiological elasticity of the target tissue (Engler et al., 2006).

Molecular mechanisms underlying LM1-dependent biomimetic cellular elasticity

The conditions of our 'cell-on-top' assay are not the traditional definition of 3D culture because cells are not completely surrounded by an ECM network. However we and others have amply demonstrated that the 'cell-on-top' conditions are much closer to 3D than commonly used two-dimensional glass or plastic substrata: MECs form multicellular 3D colonies that mimic *in vivo* acinar structures (Barcellos-Hoff et al., 1989; Lee et al., 2007) and express β-casein (Muschler et al., 1999) when cultured on top of Matrigel. Therefore, to our knowledge, these are the first mechanical measurements of cells within intact 'normal tissue structures' and in conditions that mimic much of the 3D microenvironment *in vivo*. Our findings suggest that LM1-dependent cell mechanics is largely dominated by cell-ECM interactions rather than by cell-cell interactions or changes in cell shape alone. Accordingly we found that compromising signaling of β1-integrin

family of ECM receptors using function blocking antibodies in SCp2 was sufficient to inhibit LM1 effects on cellular elasticity. Furthermore the elasticity of SCp2 on top of Matrigel treated with β1-integrin blocking antibodies was as low as that of the same cells cultured on COL I gels, thereby indicating that low LM1 signaling induces non-biomimetic cellular elasticity. Conversely it has been reported that upregulation of β1-integrin signaling has the opposite effects, since clustering of β1-integrin in human MECs is sufficient to increase cell contractility and prevent formation of acinar-like structures (Paszek et al., 2005). Among the different LM1 integrins, α3β1 is the major candidate in mediating LM1-dependent cell biomimetic elasticity in our experiments since SCp2 cells do not express α2-integrins (Figure 4A), inhibiting α6-integrin had only a weak effect on cell mechanics, and α1-integrins are expressed only in myoepithelial cells *in vivo* (Taddei et al., 2003). We previously showed that inhibition of α6- or β1-integrin were sufficient to down-regulate LM1-dependent β-casein expression in MECs, but did not prevent shape changes (Muschler et al., 1999). In contrast inhibiting a non-integrin LM1 receptor that binds to the laminin LG4-5 domain was sufficient to inhibit both cell rounding and β-casein expression in MECs (Muschler et al., 1999). This non-integrin receptor has been recently identified as DG (Weir et al., 2006). We could not observe any effect of DG in the LM1-control of cell mechanics, thereby indicating the specific role for β1-integrins in controlling cellular elasticity. All these findings suggest that there is a division of labor between different LM1 receptors in controlling cell shape, elasticity and β-casein expression.

Signals downstream of β1-integrin have been implicated in the control of the actin-myosin cytoskeleton in MECs (Paszek et al., 2005; Wozniak et al., 2003) and other cell types (Galbraith et al., 2002; Wakatsuki et al., 2003; Wang and Ingber, 1994). Actin-myosin contractility is the main mechanism by which non-muscle cells generate endogenous forces, and it is regulated in part by the Rho/ROCK pathway and its downstream effects on MLCII phosphorylation (Wakatsuki et al., 2003; Wozniak et al., 2003). However the role of these pathways on the mechanics of MECs have not been quantitatively examined in detail yet. We found that inhibiting ROCK, myosin II ATPase activity or actin polymerization in cells on glass led to biomimetic cellular elasticity, although none of these inhibitors fully mimicked the mechanical effects induced by LM1-rich ECM gels (Figure 5A). These data suggest that LM1-dependent cell biomimetic elasticity is partially

mediated by downmodulating actin polymerization and/or myosin II activity. Our findings also reveal that downregulation of microtubule polymerization is unlikely to be involved in LM1-control of cell mechanics. Nonetheless we cannot rule out a role for microtubules in functional differentiation, since microtubule integrity (Zoubiane et al., 2004) and tubulin (Houdebine, 1990) have been implicated in β-casein expression. Based on the strong correlation found between β-casein expression and biomimetic cell elastic moduli (Supplementary Figure 3B), it is conceivable that this mechanical signal is necessary, although not sufficient, for functional differentiation of MECs. Defining the mechanisms by which cellular elasticity regulates β-casein expression is beyond the scope of this study. However it should be noted that a low MLCII activity in functionally differentiated MECs is consistent with observations that Rac1 signaling -which is associated with low contractility in different cell types (Nimnual et al., 2003)- is necessary for β-casein expression in primary MECs (Akhtar and Streuli, 2006).

Does normal tissue mechanics have tumor suppressor-like functions?

Because tissue mechanics is tissue-specific (Discher et al., 2005), it is conceivable that there homeostatic mechanisms that maintain such specificity. In support of this hypothesis, we found a strong association between extra- and intracellular elasticity and functional differentiation in MECs. In addition, we showed clearly that LM1 signaling and biomimetic cell and extracellular elasticity are necessary for functional differentiation of MECs. As these signaling pathways go awry in tumorigenesis (Gudjonsson et al., 2002; Paszek et al., 2005; Wetzels et al., 1989), it is tempting to speculate that, in addition to LM1 signaling, tissue mechanics and/or its underlying homeostatic mechanisms have protective (i.e. tumor suppressor) roles associated with maintenance of function in MECs *in vivo*.

MATERIAL AND METHODS

Culture and transfection of cell lines

SCp2 (Desprez et al., 1993), EpH4 (Pujuguet et al., 2001) and MEpL DG-/- and DG+/+ cells (Weir et al., 2006) (kind gift from Dr. J. Muschler) were propagated in growth medium as previously described. Unless noted otherwise, cells were seeded at a density of ~ 10,000 cells/cm^2 on top of different substrata in growth medium supplemented with hydrocortisone (1 μg/ml) (Sigma). After 4h, cells were treated with differentiation medium consisting of DMEM/F12, insulin, gentamicin, hydrocortisone and prolactin (3 μg/ml). Cell mechanics and β-casein expression were probed 24h and 48h after adding differentiation medium, respectively. To pre-round cells in the absence of exogenous ECM signaling, cells were cultured on the nonadhesive substratum polyHEMA (Sigma) (Muschler et al., 1999). EpH4 cells were transfected with a plasmid containing 16 copies of the mouse β-casein promoter fused to the CFP gene using Lipofectamine 2000 (Invitrogen). To screen for positive clones, 2% Matrigel (BD Biosciences) diluted in differentiation medium (~15-200 μg/ml) was added to cells 24h after plating on glass.

Mammary organoids

Primary organoids were isolated as previously described (Fata et al., 2007). Briefly, mammary glands were removed from 9 weeks old pregnant (day 5) FVB mice and minced with two parallel razor blades (approved by the Animal Welfare and Research Committee (AWRC) at Lawrence Berkeley Labs; animal protocol #0510). Minced tissue (4–8 glands) was treated with collagenase/trypsin solution, DNAse and washed, leaving a final pellet rich in epithelial pieces with virtually no stromal components or single cells. The pellet was plated on top of Matrigel in basal media (Fata et al., 2007) supplemented with 5% fetal bovine serum. Differentiation medium was added 24h after plating.

Preparation of substrata

For AFM experiments, 100 μl of ECM solutions were added to glass-bottomed culture dishes (MatTek) and incubated for 30 min at 37^{o}C to allow gelation unless otherwise indicated.

Biological gels included Matrigel, LM1 (incubated overnight) (Trevigen), and neutralized COL I (2 or 3 g/l) (ICN Biomedicals) (see Supplementary materials for more details) mixed with increasing concentration of LM1. Traditional two-dimensional substrata included uncoated borosilicate glass coverslips and polystyrene, referred to as glass and plastic, respectively. In the floating gel assay, gels containing 40% LM1 and 60% COL I (3 g/l) were detached from the container edge with a spatula, while the gel center remained attached to the underlying substratum for AFM measurements. The polyacrylamide gel assay was performed as described elsewhere (Pelham and Wang, 1997). Briefly different volumes of 30% w/v acrylamide and 2% w/v bis-acrylamide solution (BioRad) were mixed to form gels attached to a glass coverslip, with *E* comparable to normal rodent mammary tissue, average tumors or stiffer (Paszek et al., 2005). Gels were subsequenty coated with 40 μg/cm^2 human fibronectin (BD) to facilitate cell adhesion.

Inhibitors

Actin polimerization was inhibited with latrunculin B (1 μM, Sigma). Microtubule polymerization was inhibited with nocodazole (10 μM, Sigma) and colcemid (23 μM, Sigma). ROCK and non-muscle MLCII activity were inhibited using Y27632 (10 μM, Calbiochem) and blebbistatin (10 μM, Sigma), respectively. All drugs were added to the cells 30 min prior to AFM measurements. To inhibit the function of specific integrin subunits, cells were plated in the presence of 5 μg/ml azide- and endotoxin-free function-blocking antibodies against β1- (Ha2/5), α6-integrin (GoH3), or the corresponding isotype matched control (IgM and IgG2, respectively) (BD Pharmingen) (Muschler et al., 1999).

RT-PCR analysis

Total cellular RNA was extracted using RNeasy kit (Qiagen). cDNA was synthesized with Superscript first strand synthesis kit (Invitrogen) from 0.1-0.5 μg RNA samples. To assess the transcription prolife of LM1 and COL I specific integrin receptors in SCp2 and EpH4, cDNA was used as a template for amplification α2-, α6-, β1-, and β4-integrin, and GAPDH as an equal loading control, using an annealing temperature of 56 ºC for 36 cycles. Quantitative real-time PCR analysis of β-casein and 18S rRNA (used as loading control) was performed with the Lightcycler System (Lightcycler FastStart DAN Master SYBR Green I kit, Roche). Description of the primers

and protocol used to amplify β-casein cDNA and 18S rRNA can be found in the supplementary materials. All β-casein data were normalized to the corresponding 18S and averaged from 3 measurements for each independent experiment ($n \geq 2$). To compare β-casein values from different experiments, we included cells on COL I in each experiment and used their β-casein expression level as a common reference. Accordingly all β-casein data (mean ± SE) are given as fold with respect to COL I.

Immunoblot analysis

Cells were cultured on uncoated tissue culture plastic or on top of Matrigel. Total cell lysates were obtained as described elsewhere (Lee et al., 2007) and treated with urea buffer (8M Urea, 0.01M Tris-HCl pH8.0, 0.01M NaH_2PO_4 and 150mM NaCl) supplemented with phosphatase (set I, Sigma) and protease inhibitor cocktails (set I, Calbiochem). Equal protein amounts were separated on reducing SDS-PAGE gels (Invitrogen), transferred onto nitrocellulose membrane, blocked and incubated overnight with antibodies that recognize either total or phosphorylated (Thr18/Ser19) non-muscle MLCII (Cell Signaling). Primary antibodies were detected with the Pierce SuperSignal detection kit and resulting bands were imaged with FluorChem 8900 analysis system (Alpha Innotech).

AFM elasticity measurements

We used a stand-alone AFM (Bioscope, Veeco) coupled to an inverted microscope as described in detail elsewhere (Alcaraz et al., 2003; Roca-Cusachs et al., 2008). In brief, force measurements were conducted using low spring constant cantilevers (k = 0.03 N/m) (Microlever, Veeco). For cell measurements, the tip was positioned above the central part of the cell, and approached to the cell surface using a stepping motor. Following the initial tip contact with the cell surface, three force vs piezo displacement recordings (*F-z* curves) were acquired at moderate loading force (~ 1 nN) and low speed (~ 5 μm/s). A similar procedure was used to probe the elasticity of the substrata. E and sample indentation d were computed from *F-z* curves as described elsewhere (Alcaraz et al., 2003). Recordings from cells subjected to d larger than 15% of the total height were discarded to rule out any artifactual contribution from the underlying substratum (Sridhar and Sivashanker, 2003). Data from cells or gels that yielded non-reproducible E values (coefficient of variation CV

(CV=SD/mean) higher than 15%) were also discarded. *E* data were screened for outliers using Chauvenet's criterion (Taylor, 1997). Further details on *F-z* curve analysis, discarded *E* data and the contribution of the stiffness of the underlying substratum on cell measurements are given as Supplementary Materials. All mechanical data are given as mean ± SE and were calculated from at least 9 measurements for each independent experiment ($n \geq 2$).

Rheometry measurements

The bulk elasticity of biological gels prepared as in AFM experiments was assessed by measuring the complex modulus at low frequencies (~ 1 Hz) using a parallel plate rheometer (Paar Physica MCR 300, kindly provided by Prof K. Healy at UCB). Data are given as mean ± SE and were calculated from at least 6 repeated measurements for each independent experiment ($n \geq 2$).

Imaging

Bright-field images of cells and AFM cantilevers, and CFP fluorescent images were acquired using an inverted microscope coupled to a CCD camera and a 10X and 40X objective, respectively. To visualize F-actin in cells cultured on glass or on top of Matrigel, cells were fixed in formaldheyde solution (Formalin, Sigma), blocked, permeabilized and labeled with Texas red-conjugated phalloidin (Molecular Probes). Labeled cells were examined on a spinning disk confocal system (Solamere Technology Group). Images were taken using a 63X oil immersion objective (Zeiss). The average phalloidin fluorescence intensity per cell was calculated by adding the total fluorescence intensity of each confocal section after subtracting the background. All image processing was carried out using Image J.

Supplementary data

Supplementary information is available at *The EMBO Journal* Online.

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FIGURE LEGENDS

FIGURE 1

LM1 and biomimetic extracellular elasticity induce robust β-casein expression, and a cell shape and elasticity comparable to cells within mammary organoids. (A) Schematic representation of the experimental setup: either mammary organoids or single SCp2 or EpH4 cells were plated on top of an ECM gel. An AFM probe was used to quantify the elasticity of either the cell or the underlying ECM gel. (B-C) Morphology (B) and elastic moduli (C) of single cells within primary mammary organoids isolated from mice in early pregnancy (5 days) and cultured on top of LM1-rich ECM gels (Matrigel) in differentiation medium for 24h. The elastic modulus of the substrata is indicated at the bottom of each image. (D-F) Effect of cell shape and cell-ECM interactions in the elasticity and functional differentiation of two MECs: SCp2 and EpH4. Cells were cultured in the presence (Matrigel) or absence (PolyHEMA) of ECM signaling. Both culture conditions produced a similarly round morphology (D), but differed dramatically in their effects on β-casein (E) and cellular elasticity (F). Dashed lines (F) correspond to the lower and upper values defined by the elasticity of cells within mammary organoids (C), and were used as a reference for biomimetic cell elastic moduli throughout this study. * $p< 0.1$, ** $p<0.05$ and *** $p<0.01$ were determined by two-tailed Student t-test with respect to (w.r.t.) Matrigel or control throughout this work unless otherwise indicated.

FIGURE 2

Increasing extracellular elasticity beyond normal mammary tissue inhibits β-casein expression and promotes spreading and stiffening in MECs. We used two independent culture assays to increase extracellular elasticity beyond normal mammary tissue values while holding biochemical composition constant. (A-C) Floating gel assay: EpH4 cells were cultured on top of LM1 mixed with COL I (3 g/l) (40:60% v/v). 4h after plating, differentiation media was added and half of the gels were gently detached from the container. The elastic modulus of the floating gel was comparable to normal tissue (A, top image), whereas that of the attached gel was 3-fold stiffer (A, bottom image). Corresponding β-casein expression (B) and cell stiffness (C) in these culture conditions. (D-M) Polyacrylamide gel assay: EpH4 cells were cultured on top of gels coated with

equal fibronectin concentration but exhibiting a stiffness comparable to normal tissue (referred to as 'soft') or within the range of mammary tumors (referred to as 'stiff'). Morphology (D) and elasticity (F) 24h after plating. β-casein expression (E) and promoter activity (G) 48h after overlaying cells with 2% Matrigel diluted in differentiation media. Both assays consistently reported downregulation of β-casein and non-biomimetic cell shape and elasticity in gels with non-biomimetic elastic moduli.

FIGURE 3

Loss of LM1 signaling downregulates β-casein expression and induces non-biomimetic cellular elasticity and/or morphology. (A) β-casein expression and elastic moduli of SCp2 cultured on top of COL I (2 g/l) gels mixed with decreasing concentrations of LM1. (B) Elasticity of LM1:COL I mixed gels. (C) Cellular elasticity as a function of gel elasticity. (D) Visualization of the activity of the β-casein promoter in EpH4 cells cultured as in (A). (E-F) Comparison of β-casein expression and elasticity of both SCp2 and EpH4 in different LM1:COL I gels. (G) Representative images of the morphology of SCp2 and EpH4 in LM1:COL I gels and on glass substrata.

FIGURE 4

Role of LM1 receptors in LM1-induced cell biomimetic elasticity. (A) Transcription prolife of LM1 and COL I specific integrin receptors in SCp2 and EpH4 cultured in 2D assessed by RT-PCR. (B-C) Elasticity of SCp2 cultured on top of Matrigel in the presence of function blocking antibodies against β1- (B) and a6-integrins (C) or corresponding isotype controls. (D) Elasticity of MEpL expressing (DG++) or not (DG--) the dystroglycan gene. Cells remained fairly round under all conditions.

FIGURE 5

Role of actin-myosin cytoskeleton in LM1-induced cell biomimetic elasticity. (A) Comparison of the elasticity of SCp2 cells cultured for 24h on top of Matrigel or on glass treated for at least 30 min with either vehicle (DMSO) or inhibitors of microtucule (nocodazole, colcemid), actin polymerization (latrunculin B), ROCK (Y26732) and myson II ATPase (blebbistatin) activities using concentrations described in the text. (B) Immunoblot of total and phosphorylated (Thr18/Ser19) MLC II in MECs cultured on a tissue-plastic dish and on top of Matrigel, and

corresponding quantification by densitometry analysis (C). (D-F) F-actin organization and morphology of SCp2 spread on glass or round on top of Matrigel studied with confocal microscopy. (D) Confocal sections showing F-actin organization and (E) corresponding quantification of the average fluorescence intensity of F-actin phalloidin staining per cell. (F) Box plot of cell spreading. ** $p<0.05$ was determined using Mann-Whitney Rank Sum Test.

TABLES

Table I Summary of mechanical parameters: comparison between rodent mammary tissue, different biological substrata and single MECs cultured on top these substrata.

Sample	Tissue or substratum		Single cells [c]
	E_{AFM} (Pa) [a]	E_{bulk} (Pa) [b]	E_{AFM} (Pa) [a]
Normal mammary tissue	n.a.	170 ± 30 [d]	
Average mammary tumor	n.a.	4050 ± 940 [d]	
Mammary organoids on Matrigel	120 ± 20	220 ± 10	600 ± 200
Matrigel	120 ± 20	220 ± 10	700 ± 100
Laminin-111	110 ± 30	n.a.	730 ± 110
Laminin-111 + Collagen I (2 g/l) (40:60% v/v)	72 ± 8	150 ± 40	600 ± 90
Collagen I (2 g/l)	290 ± 100	240 ± 40	400 ± 100
PolyHEMA	n.a.	n.a.	1700 ± 300
Borosilicate glass (2D)	n.a.	63×10^{9} [f]	1300 ± 200

data are mean ± SE; n.a., not available; [a] Young's modulus measured by AFM; [b] Young's modulus measured with a rheometer; [c] Mean of all cell lines; [d] From reference (Paszek et al., 2005); [f] According to manufacturer

FIGURE 1

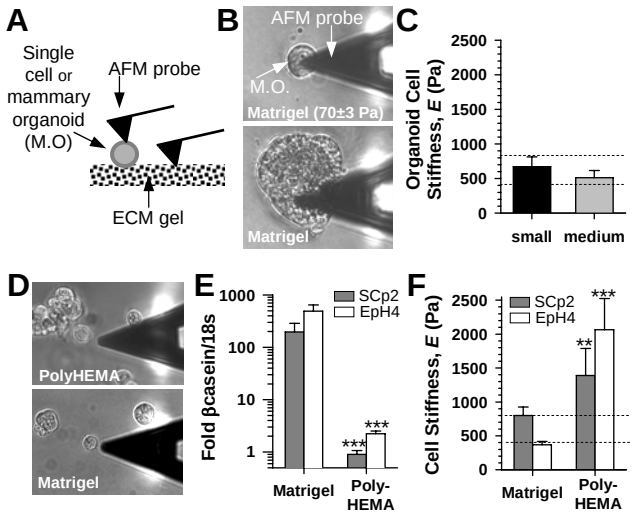

A
Single cell or mammary organoid (M.O)
AFM probe
ECM gel
B
AFM probe
M.O.
Matrigel (70±3 Pa)
Matrigel
C
Organoid Cell Stiffness, E (Pa)
2500
2000
1500
1000
500
0
small
medium
D
PolyHEMA
Matrigel
E
Fold βcasein/18s
1000
100
10
1
SCp2
EpH4
***
***
Matrigel
Poly-HEMA
F
Cell Stiffness, E (Pa)
2500
2000
1500
1000
500
0
SCp2
EpH4
**
***
Matrigel
Poly-HEMA

FIGURE 2

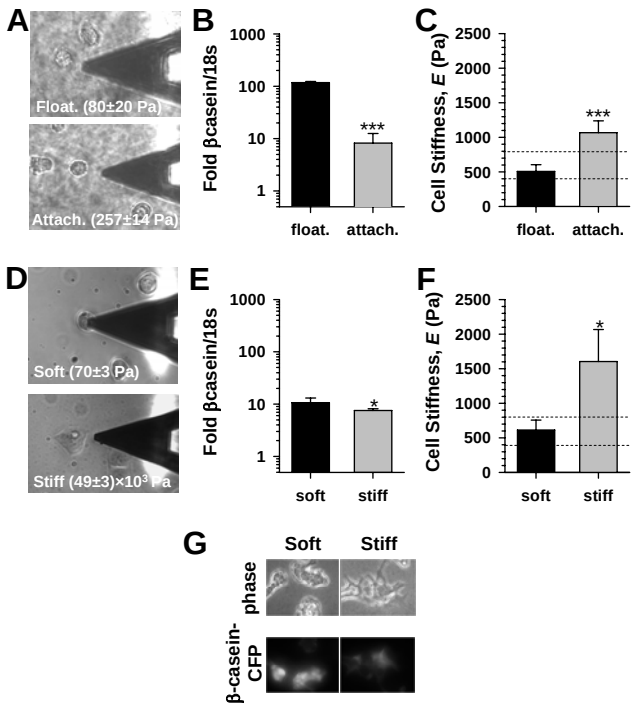

A
Float. (80±20 Pa)
Attach. (257±14 Pa)
B
Fold βcasein/18s
1000
100
10
1
***
float.
attach.
C
Cell Stiffness, E (Pa)
2500
2000
1500
1000
500
0
***
float.
attach.
D
Soft (70±3 Pa)
Stiff (49±3)×10³ Pa
E
Fold βcasein/18s
1000
100
10
1
*
soft
stiff
F
Cell Stiffness, E (Pa)
2500
2000
1500
1000
500
0
*
soft
stiff
G
Soft
Stiff
phase
β-casein-CFP

FIGURE 3

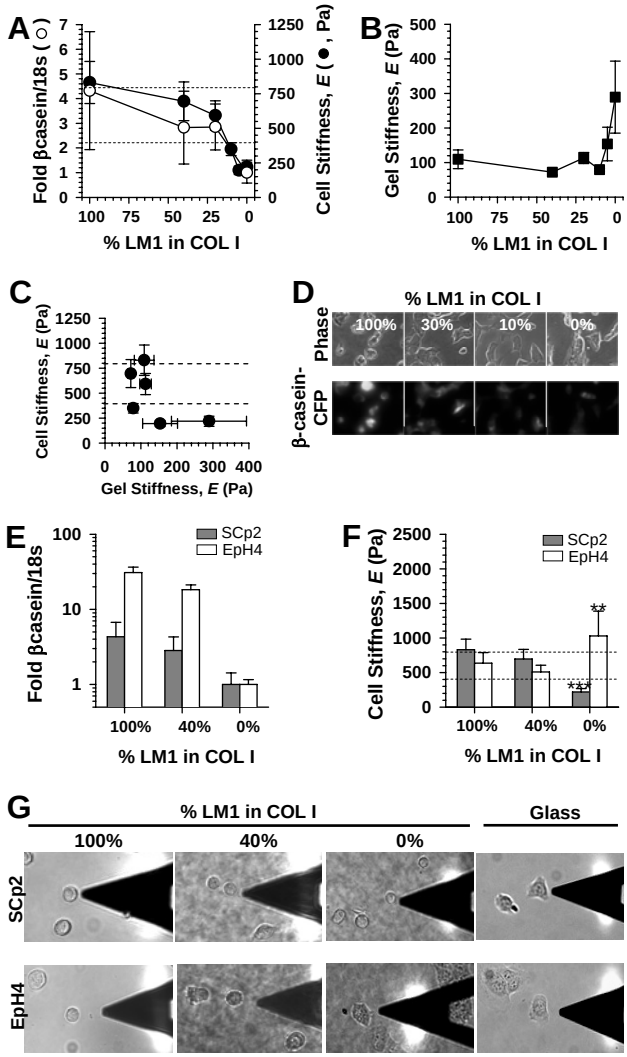

A
Fold βcasein/18s (○)
Cell Stiffness, E (●, Pa)
% LM1 in COL I
B
Gel Stiffness, E (Pa)
% LM1 in COL I
C
Cell Stiffness, E (Pa)
Gel Stiffness, E (Pa)
D
% LM1 in COL I
100%
30%
10%
0%
Phase
β-casein-CFP
E
Fold βcasein/18s
SCp2
EpH4
100%
40%
0%
% LM1 in COL I
F
Cell Stiffness, E (Pa)
SCp2
EpH4
100%
40%
0%
% LM1 in COL I
G
% LM1 in COL I
Glass
100%
40%
0%
SCp2
EpH4

FIGURE 4

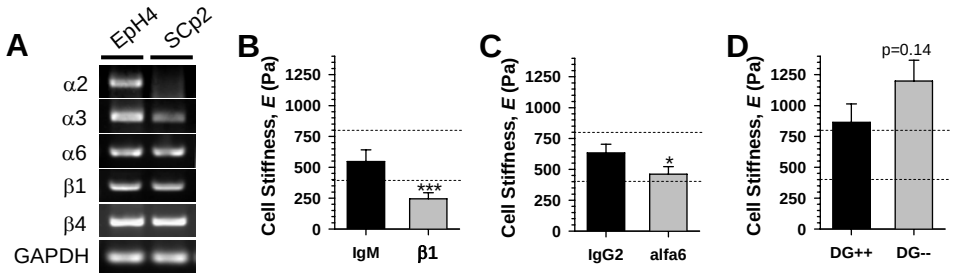
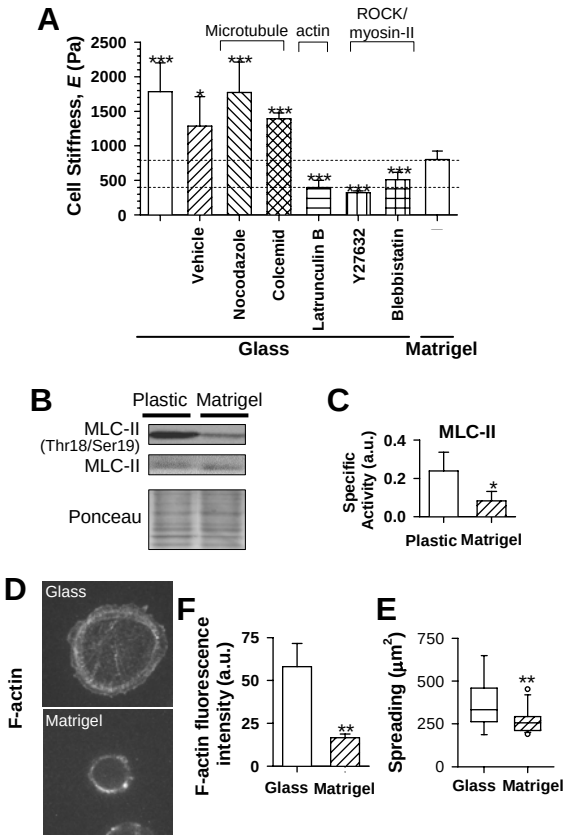

A
EpH4
SCp2
α2
α3
α6
β1
β4
GAPDH
B
Cell Stiffness, E (Pa)
1250
1000
750
500
250
0
***
IgM
β1
C
Cell Stiffness, E (Pa)
1250
1000
750
500
250
0
*
IgG2
alfa6
D
Cell Stiffness, E (Pa)
p=0.14
1250
1000
750
500
250
0
DG++
DG--

FIGURE 5


A
Microtubule
actin
ROCK/
myosin-II
Cell Stiffness, E (Pa)
2500
2000
1500
1000
500
0
Vehicle
Nocodazole
Colcemid
Latrunculin B
Y27632
Blebbistatin
Glass
Matrigel
B
Plastic
Matrigel
MLC-II
(Thr18/Ser19)
MLC-II
Ponceau
C
MLC-II
Specific
Activity (a.u.)
0.4
0.2
0.0
Plastic
Matrigel
D
F-actin
Glass
Matrigel
F
F-actin fluorescence
intensity (a.u.)
75
50
25
0
Glass
Matrigel
E
Spreading (μm²)
750
500
250
0
Glass
Matrigel