Filamin A–β1 Integrin Complex Tunes Epithelial Cell Response to Matrix Tension

Scott Gehler,*† Massimiliano Baldassarre,‡ Yatish Lad,‡ Jennifer L. Leight,§ Michele A. Wozniak,*§ Kristin M. Riching,* Kevin W. Eliceiri,† Valerie M. Weaver,‖ David A. Calderwood,‡ and Patricia J. Keely*†

*Department of Pharmacology and †Laboratory for Optical and Computational Instrumentation, University of Wisconsin, Madison, WI 53706; ‡Department of Pharmacology and Interdepartmental Program in Vascular Biology and Transplantation, Yale University School of Medicine, New Haven, CT 06520; §Department of Bioengineering, University of Pennsylvania, Philadelphia, PA 19104; and ‖Center for Bioengineering and Tissue Regeneration, Department of Surgery, University of California, San Francisco, CA 94143

Submitted December 11, 2008; Revised April 22, 2009; Accepted May 13, 2009
Monitoring Editor: Yu-Li Wang

The physical properties of the extracellular matrix (ECM) regulate the behavior of several cell types; yet, mechanisms by which cells recognize and respond to changes in these properties are not clear. For example, breast epithelial cells undergo ductal morphogenesis only when cultured in a compliant collagen matrix, but not when the tension of the matrix is increased by loading collagen gels or by increasing collagen density. We report that the actin-binding protein filamin A (FLNa) is necessary for cells to contract collagen gels, and pull on collagen fibrils, which leads to collagen remodeling and morphogenesis in compliant, low-density gels. In stiffer, high-density gels, cells are not able to contract and remodel the matrix, and morphogenesis does not occur. However, increased FLNa–β1 integrin interactions rescue gel contraction and remodeling in high-density gels, resulting in branching morphogenesis. These results suggest morphogenesis can be “tuned” by the balance between cell-generated contractility and opposing matrix stiffness. Our findings support a role for FLNa–β1 integrin as a mechanosensitive complex that bidirectionally senses the tension of the matrix and, in turn, regulates cellular contractility and response to this matrix tension.

INTRODUCTION

The compliance of the extracellular matrix (ECM) and cellular regulation of matrix remodeling are critical determinants of tissue morphogenesis, tumor invasion, and wound healing (Larsen et al., 2006). Increasing the matrix tension by culturing cells in a stiff or a loaded three-dimensional (3D) matrix perturbs morphogenesis of fibroblasts and breast epithelial cells (Wozniak et al., 2003; Paszek et al., 2005; Guo et al., 2006). Cells exert mechanical force on the ECM, which results in matrix remodeling and collagen fibril translocation (Miron-Mendoza et al., 2008) and myosin-mediated contractility (Discher et al., 2005; Giannone and Sheetz, 2006). Along these lines, inhibition of myosin activity disrupts cell morphogenesis and collagen matrix organization (Wozniak et al., 2003; Meshel et al., 2005; Paszek et al., 2005; Guo et al., 2006). Consequently, cells are able to “feel” the stiffness of their microenvironment and respond through mechanical feedback to regulate cell contractility and cell behavior. Although the interplay between matrix stiffness and cell contractility is essential for morphogenesis, the signaling mechanisms by which cells detect mechanical cues and transduce this information into biochemical signals is not well understood.

Because integrins link the ECM to the cytoskeleton through scaffolding proteins, integrins and focal adhesions have been proposed to play an integral role in mechanotransduction. Cells respond to mechanical cues by strengthening integrin–cytoskeletal attachments (Choquet et al., 1997; Pelham and Wang, 1997). Furthermore, changes in ECM stiffness or internal force generation alter integrin–cytoskeletal connections to induce changes in cell morphogenesis and matrix remodeling (Giannone and Sheetz, 2006). For example, inhibition of α2β1 integrin function affects collagen matrix contraction and organization and disrupts cell morphology (Schiro et al., 1991). Although it is apparent that cells encountering matrices of different physical properties alter their integrin–cytoskeletal linkages, it is not clear mechanistically how this occurs.

The actin-binding protein filamin A (FLNa) interacts with the cytoplasmic domain of β1 integrin to regulate integrin function (Loo et al., 1998; Pfaa et al., 1998; Calderwood et al., 2001). Not only does FLNa scaffold several signaling molecules but also it is postulated to act as a mechanosensor in cells (Stossel et al., 2001). In support of this, FLNa accumulates at adhesion sites in response to mechanical tension and is important for tension-induced actin accumulation at these sites (Glogauer et al., 1998; D’Addario et al., 2002). Moreover, FLNa cross-links the actin cytoskeleton and regulates the tension of polymerized actin networks (DiDonna and Levine, 2006; Gardel et al., 2006). FLNa can potentiate actomy-
osin ATPase activity in vitro (Sosinski et al., 1984; Janson et al., 1991) and bind regulators of myosin-mediated contractility (Ohta et al., 1999; Pi et al., 2002; Ueda et al., 2003). Therefore, we hypothesize that FLNa-β1 integrin complexes could serve as a mechanical or biochemical link that couples the actin cytoskeleton to the ECM and regulate cell morphogenesis in response to the stiffness of the extracellular matrix.

High breast density is linked to an increased risk of breast carcinoma (Boyd et al., 2001), and it is associated with a significant increase in the deposition of extracellular matrix components, especially collagen and fibronectin (Guo et al., 2001). It has been demonstrated that the stiffness of a collagen matrix increases with increasing collagen concentration (Roeder et al., 2002; Paszek et al., 2005). Thus, understanding how cells respond to matrix stiffness could inform an understanding of how breast density links to carcinoma risk. Using 3D collagen gels, we show that FLNa binding to β1 integrin is essential for cells to contract and remodel collagen matrices, and this is in turn essential for ductal morphogenesis. Furthermore, enhanced FLNa-β1 integrin interactions are sufficient to “tune” branching morphogenesis in stiffer, high-density gels. Our results suggest FLNa-β1 integrin complexes serve as part of the mechanosensitive machinery that both senses matrix tension and regulates collagen matrix contraction and cell morphogenesis in response to the physical properties of the matrix.

MATERIALS AND METHODS

Reagents

Collagen type I was obtained from BD Biosciences Discovery (Bedford, MA). Antibodies used include the following: human (h)FLNa and β1 integrin (Millipore, Billerica, MA), anti-human β1 integrin 12C10, mouse (m)FLNa (Cell Signaling Technology, Beverly, MA), rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit green fluorescent protein (GFP) (Sigma-Aldrich, St. Louis, MO), and phospho-myosin light chain (MLC) antibodies (Ser19 and Thr18/Ser19) and total MLC (Cell Signaling Technology). Bisbenzimide was purchased from Sigma-Aldrich, and AlexaFluor594 phalloidin was obtained from Invitrogen (Carlsbad, CA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Jackson Immunoresearch Laboratories, West Grove, PA. MTC=1 integrin shRNA, or luciferase shRNA control vectors (Open Biosystems, Huntsville, AL). DMEM containing 10% fetal bovine serum. Cells were stably transfected with murine mammary gland (NMuMG) cells were kindly provided by Dr. Caro-

Cell Culture and Transfection

T47D cells were obtained from American Type Culture Collection (Manassas, VA). T47D cells were maintained as described previously (Keely et al., 1999). Normal murine mammary gland (NMuMG) cells were kindly provided by Dr. Caro-

Western Blotting and Immunoprecipitations

Protein expression was assessed through immunoblotting. In brief, cells were lysed in denaturing Laemmli buffer followed by protein separation using SDS-PAGE. After proteins were transferred onto PVDF membrane, membranes were blocked using 5% milk plus 0.05% Tween 20 in TBS. Membranes were probed with either 1:1,000 anti-hFLNa or 1:2,000 anti-mFLNa, followed by incubation with 1:5,000 HRP-conjugated secondary antibodies. Membranes were visualized using ECL reagents (GE Healthcare). Immunoprecipitations of β1 integrin were performed as described previ-

Collagen Matrix Contraction Measurements

Gel contraction measurements were performed as described previously (Keely et al., 2007). Gels were incubated at 37°C for 2 weeks. The time at which the gels were rendered floating was considered day 0. Gel diameter was measured every day for 10 d and was presented as total contraction (in millimeters) over time or as contraction at day 10 (T47D) or day 4 (NMuMGs).

Second harmonic generation and multiphoton microscopy was performed on collagen gels that were fixed with 2% paraformaldehyde for 15 min before imaging. All images were acquired using a custom-designed multiphoton laser-scanning optical workstation (Wokosin et al., 2003). Cells were imaged using a TE300 inverted microscope (Nikon, Tokyo, Japan) equipped with a 40× Plan Fluor oil immersion objective (numerical aperture 1.3; Nikon) by using a mode-locked Ti:sapphire laser (Spectra Physics Millenia/Tsunami, Mountain View, CA) with excitation wavelength tuned at 890 nm. A 445-nm glass band pass filter (Thin Film Imaging Inc.) was used to detect the second-harmonic generation (SHG) signal of collagen, whereas a 520/35 nm filter (Semrock, Rochester, NY) was used to detect cell autofluorescence or GFP signal. Serial image planes were acquired at 1-μm steps and stacked to form a 3-D structure. Image acquisition was performed using WiscanScan (http://www.loci.wisc.edu/wiscanscan/). Images were ana-

Myosin Activity Assay

Phosphorylated (p)MLC levels were used to assess the amount of myosin activity of cells in collagen gels. Cells were detached using 0.5 mM EDTA in phosphate-buffered saline (PBS) and suspended in serum-free medium plus 5 mg/ml bovine serum albumin (BSA) to eliminate the effects of serum stimulation. Gels (400 μl) containing 2 million cells were poured in 12-well plates and allowed to incubate 1 h at 37°C. Gels were released and incubated an additional 90 min. Cells in gels were lysed using an equal volume of 2× sample buffer [125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 100 mM dithiothreitol, and 0.02% bromphenol blue] followed by heating the sample for 15 min at 95°C. Samples were separated using SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% nonfat milk plus 0.1% Tween 20 in Tris-buffered saline (TBS) for 1 h at room temperature and then incubated with 1:1,000 pMLC(Ser19) or pMLC(Thr18/Ser19) overnight at 4°C. After rinsing, membranes were incubated with a HRP-conjugated rabbit secondary and then visualized using enhanced chemiluminescence (ECL) reagents (GE Healthcare, Chalfont St. Giles, Buckinghamshire, United Kin-

Elastic Modulus Measurements

Collagen-I HC (high concentration BD) samples were tested on a controlled strain rheometer (RFS-II; Rheometric Scientific, Piscataway, NJ) at 37°C, 2% strain, and a frequency of 10 rad/s. Samples were preformed in a plastic washer (d = 8.5 mm, t = 1.45 mm) (McMaster-Carr, Cleveland, OH), polymeric chamber at 37°C for 65 min, and tested with an 8-mm parallel plate geometry. Elastic modulus was calculated from shear modulus measurements (Poisson’s ratio = 0.5).

Vol. 20, July 15, 2009 3225

3225
an additional 1 h at 37°C. Cells in gels were lysed with 2× lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM EDTA, 0.2% BSA, 0.2% Triton X-100, 10 mM NaF, 1 mM pependate, and protease inhibitors) and incubated at 4°C for 20 min. After centrifugation, supernatants were incubated with β1 integrin antibody plus 30 μl of GammaBind G-Sepharose (GE Healthcare) overnight at 4°C. Samples were washed extensively with lysis buffer followed by denaturation with Laemmli buffer. Samples were separated using SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked with 3% BSA plus 0.3% Tween 20 in TBS and then incubated with FLNa or β1 integrin antibodies. After incubation with secondary antibodies, membranes were rinsed and then visualized using ECL reagents (GE Healthcare). FLNa was normalized to β1 integrin by densitometry using ImageJ. All digital images for micrographs and blots were produced and processed using Adobe Photoshop CS2 (Adobe Systems).

**RESULTS**

**Increased Collagen Density and Stiffness Disrupts Collagen Matrix Contraction and Ductal Morphogenesis**

We previously showed that the density of a collagen gel, which can be altered by increasing the collagen concentration, regulates breast epithelial cell morphogenesis (Wozniak et al., 2003). Well-differentiated human T47D breast carcinoma cells underwent ductal morphogenesis only when cultured in low-density (1.0 mg/ml), floating collagen gels (Figure 1Aa). If these same gels were left attached to the dish, such that the gel cannot be contracted, then morphogenesis was disrupted (Figure 1Bb). Because attached gels are typically thought of as mechanically loaded because cellular contraction on the gel is met with resistance, the failure of cells to form tubes in an otherwise identical collagen matrix demonstrates that these cells must be in a compliant matrix to undergo this type of morphogenesis. In this case, tubulogenesis is defined by the formation of thin, hollow tubes composed of one cell layer and a lumen (Keely et al., 1995). Similar to cells in mechanically loaded gels, cells cultured in high-density (2.0 mg/ml) floating gels also fail to undergo tubulogenesis (Figure 1, C and D, c and d). This likely reflects an increased stiffness in high-density gels, because the elastic modulus increased with increasing collagen concentration (Figure 1E). This finding is consistent with the results of others, in which increased collagen gel concentrations result in an increased modulus, as measured using both compression or tensile testing (Roeder et al., 2002; Paszek et al., 2005). It is likely that the response of cells to the high-density (2.0 mg/ml) gel is due to the effect of matrix stiffness rather than due to increased ligand concentration, because cells in the attached low-density (1.0 mg/ml) gel phenocopy the high-density gel (compare Figure 1B with C). Thus, increased matrix stiffness, whether due to constraint, or due to density, inhibits ductal morphogenesis. Collectively, these findings reinforce the notion that matrix tension is a crucial regulator of cell morphogenesis.

The ability of a cell to contract and remodel the matrix is influenced by a balance between the stiffness of the matrix and cell contractility (Giannone and Sheetz, 2006; Larsen et al., 2006). Consistent with the finding that high-density gels are stiffer than low-density gels, T47D cells cultured 10 d in high-density floating gels contracted the gels 61% less than cells cultured in low-density floating gels (change in gel diameter, 6 mm in low-density vs. 2.33 mm in high-density gels) (Figure 1F). These results suggest that high-density gels are too stiff for cells to adequately contract. Floating gels did not contract when cells were absent (data not shown), verifying that gel contraction is a cell-mediated process.

Transduction of the physical properties of the ECM depends on myosin-mediated cell contractility (Pelham and Wang, 1997; Clark et al., 2007; Alexander et al., 2008). Furthermore, it has been shown that myosin II is critical for collagen fibril translocation and contraction of the collagen matrix through α2β1 integrin in fibroblasts (Meshel et al., 2005). Blebbistatin, an inhibitor of nonmuscle myosin II, disrupted tube formation in low-density floating gels (Figure 1, G–J). Furthermore, treatment with blebbistatin reduced gel contraction by 55% and 50% in low- and high-density gels, respectively (Figure 1K). Interestingly, blebbistatin disrupted not only tube formation but also the binding of cells to one another, suggesting that myosin-mediated contractility is necessary to maintain cell–cell junctions in this system. Thus, myosin-mediated gel contraction is necessary for cell–cell attachment as well as ductal morphogenesis, consistent with other findings that link cellular contractility to morphological regulation (Wozniak et al., 2003; Paszek et al., 2005; Engler et al., 2006; Guo et al., 2006; Fischer et al., 2009). Inhibition of gel contraction was not due to an effect on cell number, because blebbistatin did not inhibit cell proliferation (data not shown). These results suggest myosin is a key regulator of collagen matrix contraction in response to the stiffness of the ECM during ductal morphogenesis.

To determine whether myosin activity can be regulated by the density of the collagen matrix, we assessed myosin activity by using phosphospecific antibodies to MLC, which regulates the motor activity of myosin II (Adelstein and Eisenberg, 1980; Sellers et al., 1985). Phosphorylation of MLC correlates to an increase in contraction of actomyosin (Chranowska-Wodnicka and Burridge, 1996), and is an indication of activity, as phosphorylation of MLC on serine 19 is required for force production (Sellers et al., 1985; Umemoto et al., 1989), whereas additional phosphorylation at threonine 18 has been shown to enhance the actin-activated ATPase activity of myosin (Tanaka et al., 1985; Ikebe et al., 1986; Umemoto et al., 1989). Cells cultured in high-density gels exhibited an ~60% increase in monophosphorylated (Ser19) and a 45% increase in diphosphorylated (Thr18/Ser19) MLC (Figure 1L). However, even though the cells have increased pMLC levels in high-density gels, the elevated level of myosin activity was still not sufficient to contract these stiffer gels. Instead, it is probable that the cells have set up tension within the cell to balance the higher stiffness outside the cells, and this tension results in ongoing myosin activity. Conversely, that blebbistatin inhibits tubulogenesis suggests that myosin II is needed for the contraction of a compliant matrix. It is likely that myosin activity is tightly regulated during tubulogenesis, because pMLC levels are lower (but not absent) in a low-density collagen gel that is permissive for tubulogenesis to occur. Together, these results demonstrate the important balance that occurs between intracellular contractility and the stiffness of the matrix to regulate morphogenic processes.

**Filamin A Levels Regulate Collagen Gel Contraction in Response to Matrix Stiffness**

Filamin A binds to the cytoplasmic domain of β1 integrin and undergoes localization to integrin-induced adhesions in response to force application (Glogauer et al., 1998; D’Addario et al., 2002), suggesting FLNa may be an impor-
Figure 1. Cells must contract a compliant collagen matrix to undergo tubulogenesis. (A–D) T47D cells form tubules after 10 d when cultured in a compliant, low (1.0 mg/ml)-density floating collagen gel but not when the gel remains attached to the dish or when the collagen density is increased (2.0 mg/ml). Bar, 50 μm. (a–d) Fluorescent images of T47D cells in collagen gels labeled with AlexaFluor594 phalloidin (red) and bisbenzimide (blue). Bar, 20 μm. Cells exhibit protrusions in low- and high-density attached conditions (arrows), which is consistent with previous observations (Wozniak et al., 2003). (E) Elastic modulus (e) of cell-free collagen gels of increasing collagen concentration, measured by rheology as described in Materials and Methods. Collagen gels of greater concentration (density) have a stiffer elastic modulus. (F) Time course of gel contraction over 10 d. High-density gels are contracted 61% less than low-density gels at day 10. Statistics were performed on the mean contraction at day 10 from six experiments and demonstrated that contraction of a 2.0-mg/ml gel was statistically less than a 1.0-mg/ml gel (data not shown; p < 0.001 by a two-sample t test). (G–J) Actomyosin contractility is important for matrix contraction in response to collagen density. Blebbistatin (20 μM) disrupted tubule formation of T47D cells in low-density floating collagen gels. Bar, 50 μm. (K) Treatment with blebbistatin caused approximately a 55 and 50% reduction in the contraction of low- and high-density floating collagen gels at day 10, respectively, relative to dimethyl sulfoxide (DMSO) control for each collagen concentration. The mean contraction at day 10 from six independent experiments showed a statistically significant decrease in collagen gel contraction when blebbistatin was added to the culture compared with control cultures (data not shown; p < 0.001 by a two-sample t test). (L) Myosin activity is enhanced in cells cultured in high-density gels, as shown through Western blot analysis of MLC phosphorylation. pMLC(Ser19) increased by ~60%, whereas pMLC(Thr18/Ser19) was enhanced by ~45% in response to high-density gels. Left, representative Western blot. Right, data were graphed representing the mean ± SEM for eight experiments. *p < 0.01; statistical difference relative to low-density control (two-sample t test).
Figure 2. Filamin levels control the extent of collagen gel contraction and MLC phosphorylation. (A) Stable expression of FLNa shRNA reduced FLNa in T47D cells, demonstrated by Western blot analysis. GAPDH was used as a loading control. (B) Cells expressing FLNa shRNA exhibited a 42 and 38% reduction in gel contraction by day 10 when cultured in low- or high-density gels, respectively, relative to vector control for each collagen concentration. Data are represented over 10 d and are graphed as a mean for day 10 ± SEM for six experiments. *p < 0.001, **p < 0.01 statistical difference (two-sample t test). (C) Endogenous FLNa levels were increased by stably expressing FILIP shRNA, as demonstrated by Western blot with anti-FLNa antibodies. (D) Expression of FILIP shRNA increased gel contraction over 10 d, and by day 10 there was an increase of ~50 and 62% in low- and high-density floating gels, respectively, relative to control cells for each collagen concentration.
Figure 2 (cont). Each collagen concentration. Bar graphs represent the mean ± SEM for at least eight experiments. *p < 0.001 statistical difference (two-sample t test). (E) Perturbing FLNa levels down or up regulates myosin activity, as measured by pMLC. T47D cells expressing FLNa shRNA exhibited a 65–70% reduction in pMLC(Ser19) and pMLC(Thr18/Ser19) in both low- and high-density gels (Figure 2H). It is important to note that increasing FLNa expression, using either approach (FILIP shRNA or expression of FLNa-GFP), enhanced pMLC above that observed in control cells (Figure 2E). Expression of FLNa-GFP significantly enhanced both the rate and extent of gel contraction (Figure 2G) and correlated to an increase in both pMLC(Ser19) and pMLC(Thr18/Ser19) in both low- and high-density gels (Figure 2H). The increased contraction of both FLNa-GFP and FLNa-GFP cell lines was not due to changes in cell proliferation (data not shown). These results suggest that increased FLNa expression supports increased myosin light chain phosphorylation, thus facilitating gel contraction in high-density gels.

FLNa A Levels Tune Tubulogenesis in Stiffer Collagen Gels

The observation that the level of FLNa affected matrix contraction and phosphorylation of MLC suggested that FLNa might regulate the ability of cells to adjust to stiffer matrix environments. To determine whether this was the case, we determined the effects of modulating FLNa levels on tubulogenesis. Knockdown of hFLNa by shRNA disrupted tubule formation in low-density floating collagen gels (Figure 3B), supporting the important role FLNa plays in this process. As a control, T47D cells stably transfected with luciferase shRNA exhibited normal phenotypes in both low- and high-density gels (Figure 3, A and C), meaning that they formed tubules in low-density gels but not in high-density gels. Importantly, when endogenous FLNa levels were increased by expression of FILIP shRNA, tubulogenesis occurred in the stiffer 2.0 mg/ml collagen gels (Figure 3H). The same result was observed when FLNa levels were increased by expression of FLNa-GFP (Figure 3L). It was of interest that increased FLNa levels disrupted tubulogenesis in the compliant 1.0 mg/ml collagen gels (Figure 3, F and J), again suggesting that it is the balance of contractile forces within the context of the matrix compliance that regulates cellular behavior, controlling morphogenesis. Because of these results, note that we do not include here “rescue” of FLNa shRNA-expressing cells by re-expressing FLNa, because changes in FLNa levels due to re-expression affect the outcome and make the experiments difficult to interpret.

FLamin a-β1 Integrin Interactions Are Regulated by Collagen Density

β1 integrin is an important regulator of breast epithelial cell morphogenesis in reconstituted matrices in vitro and in the mammary gland in vivo (Keely et al., 1995; Zutter et al., 1995; Weaver et al., 1997; Chen et al., 2002; White et al., 2004; Naylor et al., 2005). Because FLNa binds to the cytoplasmic domain of β1 integrin and accumulates at adhesion sites in response to mechanical tension (Glogauer et al., 1998; Loo et al., 1998; Pfaff et al., 1998; D’Addario et al., 2002), we determined whether the density of the matrix can regulate FLNa binding to β1 integrin. FLNa-β1 integrin coprecipitation was enhanced in T47D cells cultured in high-density collagen gels (Figure 4A). Although culture of cells in low-density attached gels enhanced FLNa-β1 integrin association by ~56% compared with low-density floating gels, culture in either high-density floating or attached gels produced a 96 and 103% increase in FLNa-β1 integrin association, respectively (Figure 4A). These results suggest FLNa binding to β1 integrin is enhanced in a stiffer matrix.

Although increased levels of FLNa enhanced contraction of collagen gels (Figure 2), it is unclear whether this effect on contraction is due to enhanced FLNa-β1 interactions or to other functions of FLNa. Therefore, we first determined
whether cells expressing elevated levels of FLNa exhibit enhanced FLNa-β1 integrin interactions in response to changes in collagen density. Using T47D FILIP shRNA cells, we observed that FLNa-β1 integrin interactions were enhanced by ~160 and 240% in low- and high-density floating collagen gels, respectively (Figure 4B). These results suggest that the FLNa-β1 integrin interaction may mediate the elevated collagen gel contraction noted in Figure 2 for FILIP shRNA-expressing cells. Note that on a quantitative level, the relative amount of FLNa/β1 integrin is higher in cells expressing FILIP shRNA than in parental T47D cells cultured in a high-density 2.0 mg/ml gel. This suggests that, even though cells respond to a stiffer matrix by increasing FLNa association with β1 integrin, this interaction may still not be enough to rescue tubulogenesis of parental cells in the high-density gels.

**FLNa Binding to β1 Integrin Mediates the Effects of FLNa on Collagen Matrix Contraction and Tubulogenesis**

In addition to directly binding to β1 integrin, FLNa binds to several proteins in the cytosol (Stossel et al., 2001). Thus, it is possible that up or down-regulation of FLNa levels affects several pathways. To determine whether it is the specific binding of FLNa to β1 integrin that influences gel contraction and morphogenesis, we competitively inhibited endogenous FLNa binding to β1 integrin by overexpressing domain 21 of FLNa, which contains the integrin-binding region (Loo et al., 1998; Kiema et al., 2006). T47D cells were transfected with enhanced EGFP-IgFLNa21 (termed GFP-F21) to compete with endogenous FLNa for binding to β1 integrin. As a control, T47D cells were transfected with EGFP-IgFLNa21(I/C) (termed GFP-F21(I/C)) containing a point mutation at Ile2283 that impairs β1 integrin binding (Kiema et al., 2006). This fragment is an appropriate control because it does not bind to β1 integrin and does not block FLNa-β1 integrin interactions. Fluorescence-activated cell sorting (FACS) analysis was carried out to isolate cells expressing equal levels of GFP-F21(I/C) and GFP-F21 (data not shown). Expression of GFP-F21(I/C) or GFP-F21 did not alter endogenous FLNa expression in these cells (Figure 5A). Cells expressing GFP-F21 displayed reduced levels of binding between endogenous FLNa and β1 integrin in both low- and high-density collagen gels (Figure 5B), whereas GFP-F21(I/C) cells showed “normal” FLNa-β1 integrin interactions that were increased in high-density gels relative to low density gels, consistent with untransfected T47D cells (Figure 4A). Expression of GFP-F21 diminished gel contraction compared with control GFP-F21(I/C) cells (Figure 5C). By day 10, GFP-F21 cells exhibited a 26 and 28% reduction in gel contraction in low- and high-density gels, respectively, supporting the idea that FLNa-β1 integrin interactions regulate gel contraction. Expression of IgFLNa21 blocked the density-dependent increases in pMLC(Ser19) and pMLC(Thr18/Ser19) (Figure 5D). These results suggest that reducing FLNa-β1 integrin interactions reduces collagen matrix contraction and activation of myosin.

Consistent with our finding that contraction, FLNa, and tubulogenesis are linked, blocking FLNa-β1 integrin interactions with IgFLNa21 disrupted tubulogenesis (Figure 5I). In contrast, control IgFLNa21(I/C) cells formed tubules in low-density gels (Figure 5G). All cell types exhibited disrupted tubule formation when cultured in high-density collagen gels (Figure 5, F, H, and J). Thus, FLNa binding to β1 integrin mediates the effects of FLNa on tubulogenesis.

**Increased Binding of FLNa to β1 Integrin Enhances Collagen Matrix Contraction and Tunes Tubulogenesis in a Stiffer Collagen Matrix**

Reduction of FLNa-β1 integrin binding disrupted gel contraction and tubulogenesis (Figure 5). Therefore, we next determined whether enhanced FLNa-β1 integrin interactions also regulate matrix contraction and morphogenesis. To do this, we used a human β1 integrin containing two point mutations (V787,791I) in the cytoplasmic domain filamin-binding site that enhance FLNa binding (Calderwood et al., 2001). This mutant β1 integrin, hβ1(V787,791I), was stably expressed in NMuMG cells. Because hβ1(V787,791I) integrin was expressed in mouse NMuMG cells, we were able to identify and select cells that express the human form of β1 integrin using a human-specific β1 integrin antibody. As a control, wild-type human β1 [hβ1(WT)] integrin was stably expressed in NMuMG cells to similar levels as hβ1(V787,791I), which was confirmed using FACS analysis (data not shown). Expression of hβ1(WT) or hβ1(V787,791I) integrin did not affect the expression of FLNa in NMuMG cells (Figure 6A). For this set of experiments, it should be noted that NMuMG cells, which have an increased ability to contract collagen matrices compared with T47D cells, require a higher density of collagen matrix relative to T47D cells to undergo branching morphogenesis, and thus 2.0 mg/ml is a compliant matrix for these cells, whereas 3.0 mg/ml is a stiff matrix. hβ1(V787,791I) integrin exhibited a 147 and 152% increase in binding to mouse FLNa in both 2.0 and 3.0 mg/ml floating collagen gels, respectively, as determined by coimmunoprecipitation with β1 integrin (Figure 6B).

To test whether expression of hβ1 integrin affects gel contraction by NMuMG cells, we compared both hβ1(WT) and hβ1(V787,791I) cell lines with untransfected NMuMG
Figure 5. Decreased FLNa-β1 integrin binding reduces collagen matrix contraction and disrupts tubule formation. To inhibit FLNa-β1 integrin interactions, the integrin-binding region of FLNa coupled to GFP (GFP-F21) was expressed in T47D cells. As a control, the same construct containing a point mutation that abolishes FLNa binding to β1 integrin, GFP-F21(I/C), was used. (A) T47D cells expressing GFP-F21(I/C) or GFP-F21 have similar levels of endogenous FLNa, demonstrating that these constructs do not alter FLNa levels. Equal numbers of cells were lysed and used for comparison of FLNa expression. (B) Coimmunoprecipitation with anti-β1 integrin antibody demonstrates that GFP-F21, which binds to β1 integrin, reduced coprecipitation of FLNa with β1 integrin, whereas GFP-F21(I/C), which does not bind β1 integrin, did not block FLNa-β1 integrin coprecipitation. Graphed data (right) represent the mean of three similar experiments quantified ± SEM, *p < 0.05 for all conditions; statistical significance relative to GFP-F21(I/C) control cells in low-density collagen gels (two-sample t test). (C) Disruption of FLNa binding to β1 integrin reduces contraction of collagen gels. Compared with GFP-F21(I/C) controls, GFP-F21 cells display a 26 and 28% reduction in the contraction of low- and high-density floating gels, respectively, at day 10. Bar graph data represent the mean contraction at day 10 ± SEM from a minimum of 11 experiments. *p < 0.001 statistical difference (two-sample t test). (D) Expression of GFP-F21 blocked the density-dependent increase in both pMLC(Ser19) and pMLC(Thr18/Ser19). However, GFP-F21 did not have a significant effect on pMLC levels in low-density gels. Quantified data represent the mean ± SEM for seven experiments. *p < 0.02; statistical difference compared with control cells in low-density collagen gels (two-sample t test). (E–J) Cells expressing GFP-F21(I/C) formed tubules in low-density collagen gels and exhibited disrupted tubulogenesis in high-density floating gels, similar to GFP vector control. However, expression of GFP-F21 disrupted tubulogenesis in both low- and high-density floating gels. Bar, 50 μm.

Filamin-β1 Integrin Regulates Collagen Gel Remodeling

Our results suggest that tubulogenesis occurs only when cells are in a matrix that is compliant enough for the cells to contract it and that tuning the contractile response through FLNa binding to β1 integrin allows cells to form tubules in gels that are stiffer. When cells contract a collagen matrix, they remodel the individual collagen fibrils and translocate the fibrils toward the cells, resulting in condensed regions of collagen surrounding cell bodies (Yamato et al., 1995; Tamariz and Grinnell, 2002; Miron-Mendoza et al., 2008). Therefore, we next examined the fate of the collagen fibrils as a consequence of the remodeling that occurs during tubulogenesis. To investigate how collagen fibers are reorganized during tubulogenesis, we used multiphoton laser scanning micros-
copy (MPLSM) (Denk et al., 1990) and SHG imaging (Campaognola and Loew, 2003) to directly observe the rearrangement of type I collagen under matrix conditions that facilitate cell morphogenesis. Because collagen is noncentrosymmetric, we can use SHG to examine the orientation and density of fibrillar collagen without the need for indirect labeling using fluorescently tagged proteins or antibodies (Mohler et al., 2003; Provenzano et al., 2006). Moreover, the intensity of the SHG signal corresponds in a linear manner to collagen concentration (Brown et al., 2003; Mohler et al., 2003).

Using MPLSM and SHG, we visualized cell–ECM boundaries to examine the local organization of the collagen matrix under conditions that facilitate breast epithelial cell morphogenesis. Collagen gels without cells exhibited randomly organized collagen fibrils (Figure 7, A and B). Consistent with previous studies, increasing the collagen concentration increased the density of visible collagen fibrils (Roeder et al., 2002) and increased the intensity of the collagen SHG signal (Figure 7, A vs. B). When T47D cells were added to low-density floating gels, collagen fibrils were relocalized into condensed regions between and directly adjacent to tubular structures, as indicated by an increase in fluorescence intensity (Figure 7, C and E). Interestingly, this resulted in a local collagen matrix with a greater concentration than the high-density gels, which again suggests that it is not collagen ligand density per se to which cells respond when comparing a low-density gel to a high-density gel. Rather, these results suggest it is the tension of the matrix in which the cell resides that determines subsequent cellular behavior. In contrast, condensation of collagen fibrils in high-density floating gels was diminished and limited to regions immediately adjacent to the cell structures (Figure 7, D and F), consistent with the limited amount of global gel contraction observed.
for dense gels (Figure 2). Notably, morphogenesis did not occur in high-density gels, strengthening the link between collagen gel contraction leading to matrix reorganization and tubulogenesis (Wozniak et al., 2003).

To quantify collagen rearrangement, a region of interest along a line scan was used to measure the collagen fluorescence intensity from the edge of the cell–ECM boundary (0 µm) out 50 µm into the collagen matrix. Although the entire gel had been reorganized in the presence of cells, these measurements indicate collagen condensation was most enhanced within 15 µm of the cell boundary in floating gels (Figure 7I). Consistent with the global gel contraction results, collagen fibril condensation is cell dependent, because no collagen fibril condensation occurred when cells were absent in floating gels (note flat lines in Figure 7I). Importantly, changes in collagen fluorescence intensity were more robust in low-density floating gels relative to high-density floating gels, consistent with the observation that there was also quantitatively more global gel contraction in low-density gels (compare with Figure 1F). Although gel contraction is not possible in attached gels due to the nature of the assay, it is interesting to note that some reorganization did occur in attached gels of both low- and high-density, although this was much less than that observed in floating collagen gels, and collagen fibrils did not condense near regions adjacent to cell structures (Figure 7, G and H).
To verify that the collagen fibril remodeling and condensation was due to contractile forces, cultures were treated with blebbistatin to inhibit myosin II activity. Blebbistatin treatment reduced the extent of collagen fibers that condensed near cell–ECM boundaries (Figure 7, J–N). These treatments confirmed an increase in the condensation of collagen gels (Figure 8C). Average fluorescence intensity measurements were taken from the edge of the cell–ECM boundary (0 μm) into the collagen matrix. Data are averaged from a minimum of six images, three measurements per image. At 5 μm, statistical difference p < 0.01 (1.0 control vs. 1.0 FLNa shRNA), p < 0.05 (2.0 control vs. 2.0 FLNa shRNA) (two-sample t test) and p < 0.0001 by regression analysis. (C) FLIP shRNA enhances collagen fibril condensation. Cell autofluorescence (red) and collagen (green) were imaged using MPLSM and SHG imaging, respectively. Bar, 50 μm. (D) Average fluorescence intensity of collagen fibrils in low- and high-density floating gels. Line scans were taken from the edge of the cell–ECM boundary (0 μm) into the collagen matrix. Data are averaged from a minimum of eight images, three measurements per image. p < 0.05 statistical difference at 5 μm (1.0 control vs. 1.0 FILIP shRNA; 2.0 control vs. 2.0 FILIP shRNA) (two-sample t test), and p < 0.0001 by regression analysis. (E) FLNa-GFP enhances collagen fibril condensation near cell structures. GFP-labeled cells (pseudocolored red) and collagen (green) were imaged using MPLSM and SHG imaging, respectively. Bar, 50 μm. (F) Fluorescence intensity measurements were taken from the edge of the cell–ECM boundary (0 μm) into the collagen matrix of low and high-density floating gels. Data are averaged from a minimum of six images, three measurements per image. p < 0.05 statistical difference at 5 μm (1.0 control vs. 1.0 FLNa-GFP; 2.0 control vs. 2.0 FLNa-GFP) (two-sample t test), and p < 0.0001 by regression analysis.

DISCUSSION

The compliance of the ECM and cellular regulation of matrix remodeling are critical determinants of tissue morphogene-
Figure 9. The interaction of FLNa with β1 integrin regulates collagen matrix remodeling. (A–D) GFP-F21 reduced the ability of cells to reorganize the collagen matrix, compared with control cells expressing GFP-F21(I/C). GFP-labeled cells (pseudocolored red) and collagen (green) were imaged using MPLSM and SHG imaging, respectively. Bar, 50 μm. Line scans (not shown) taken from the edge of the cell-ECM boundary from 6 images, three measurements per image demonstrated that there is a statistical difference between 1.0 GFP-F21(I/C) versus 1.0 GFP-F21, p < 0.05 by two-sample t test and by regression analysis. (E–H) FLNa binding to β1 integrin was enhanced in NMuMG cells by expression of human β1 integrin containing two point mutations that specifically enhance binding of FLNa, hβ1(V787,971I). Expression of hβ1 integrin wild type (WT) served as a control. Note that increased FLNa-β1 integrin interactions in hβ1(V787,971I) enhanced matrix remodeling compared with hβ1(WT) even in the high-density (3.0 mg/ml) collagen gel.

FLNa-β1 Tunes Response to Matrix Tension

This study demonstrates that FLNa binding to β1 integrin is an important regulator of matrix contraction during breast epithelial cell morphogenesis. We show that increased collagen density results in a stiffer matrix that regulates cell morphogenesis, gel contraction, and FLNa-β1 integrin interactions. Interaction of FLNa with β1 integrin is important for these processes, because disruption of FLNa-β1 integrin complexes disrupts collagen gel contraction and morphogenesis. Conversely, increasing FLNa-β1 integrin interactions enhances gel contraction and allows cell morphogenesis in high-density gels that are otherwise too stiff for morphogenesis to occur. Collagen gel contraction and morphogenesis are accompanied by remodeling of collagen fibers around the forming tubules, and this remodeling is likewise regulated by the level of FLNa-β1 integrin interactions. These results support a role for FLNa binding to β1 integrin both as a mechanosensor of matrix tension and as an effector that tunes the balance between matrix stiffness and cellular contraction against that matrix. The degree by which cells pull on their surroundings is influenced by the stiffness of the matrix through a feedback mechanism (Discher et al., 2005; Giannone and Sheetz, 2006). For example, a balance between traction and adhesion forces in response to the stiffness of 3D matrices has been shown to regulate cell migration (Zaman et al., 2006). The observation that both decreased and increased FLNa expression disrupts collagen gel contraction and morphogenesis in low-density gels (Figures 2 and 3) suggests that an optimal level of FLNa expression is important during morphogenesis. Interestingly, increasing FLNa binding to β1 integrin, which correlates with enhanced myosin activity, increases matrix contraction and shifts the optimal range for cell morphogenesis to a higher density matrix (Figures 3 and 6). This observation supports the notion that the effects of matrix stiffness can be overcome by enhancing myosin-mediated matrix contraction through FLNa-β1 integrin interactions to tune morphogenesis. A recent study demonstrated a relationship between the biophysical properties of the ECM and myosin activity in regulating branching morphogenesis (Fischer et al., 2009). It was demonstrated that endothelial cell branch initiation in a 3D collagen matrix can be inhibited by increased collagen stiffness and myosin activity. However, this study demonstrated that local inhibition of myosin II activity could induce branch initiation in both compliant and stiff collagen gels. Our results imply that epithelial phenotype is regulated by an overall balance of forces outside the cell and those from inside the cell exerted on the surrounding matrix. Our observations further demonstrate that matrix remodeling through contraction is a key process during epithelial cell morphogenesis. Although gel contraction is a measure to infer contractile activity of cells in the context of global collagen remodeling, SHG imaging is a powerful tool to complement global gel contraction measurements by allowing for the direct observation of structural aspects of type I collagen, such as fibrillar orientation and density, without the need for indirect labeling of collagen using antibodies or fluorescently-tagged proteins (Mohler et al., 2003; Provenzano et al., 2006). Features of SHG imaging depend on many factors, including the size, density, and orientation of fibrillar collagen (Campagnola and Loew, 2003). Our results suggest manipulation of FLNa binding to β1 integrin affects the local collagen reorganization and fibrillar condensation as indicated by changes in signal intensity adjacent to cell-ECM boundaries. Although matrix remodeling can also include proteolytic degradation, herein we focused specifically on collagen reorganization during cell morphogenesis. It is worth noting that adding a cocktail to inhibit matrix metalloproteinases and other proteases had no effect on collagen fiber remodeling, matrix contraction, nor morphogenesis in either low- or high-density gels (data not shown), demonstrating these processes can occur even in the absence of proteolysis. However, additional studies would be necessary to fully understand the contributions of proteolysis during these collagen matrix remodeling events.

Our findings suggest that culture in a stiff, dense collagen matrix causes an increase in FLNa binding to β1 integrin, although it is not clear how this interaction is regulated. Phosphorylation of FLNa on serine 2152 has been suggested to potentially influence the binding of FLNa to β1 integrin (Vadlamudi et al., 2002; Jay et al., 2004; Woo et al., 2004). However, although FLNa undergoes enhanced phosphorylation in response to force application, the phosphorylation state of serine 2152 does not affect integrin binding to FLNa (19-24), which contains the integrin-binding domain (Glogauer et al., 1998; Travis et al., 2004). Recently, it was demonstrated that FLNa undergoes intramolecular autoinhibition of integrin binding (Lad et al., 2007). Although a mechanism to regulate the autoinhibition of the integrin-binding site of FLNa is not presently known, it is conceivable that mechanical forces acting on filamin, either through
changes in ECM stiffness or internal force generation, might alter the conformation of IgFLNa20, thus releasing its auto-inhibitory effect on integrin binding. In support of this, mechanical forces applied through β1 integrins enhance FLNa recruitment to integrin-induced adhesion sites (Glogauer et al., 1998; D’Addario et al., 2001, 2002). Consistent with this finding, we find that cells cultured in high-density collagen gels undergo enhanced FLNa-β1 integrin interactions (Figure 4).

Although this study demonstrates that FLNa-β1 integrin complexes regulate myosin-mediated gel contraction, the mechanism by which this is accomplished remains a subject for future investigation. One possibility might be that integrin-bound FLNa could enhance the local concentration of cross-linked actin and so increase the number of sites accessible for myosin binding. The amount of actin cross-linking has been shown to be an important determinant of filament contraction and actomyosin ATPase activity (Stendahl and Stossel, 1980; Janson et al., 1991). Alternatively, or in addition, FLNa serves as a scaffold for many signaling molecules, including known regulators of myosin-mediated contractility RhoA and Rho-kinase (ROCK) (Ohita et al., 1999; Pi et al., 2002; Ueda et al., 2003), guanine nucleotide exchange factors Trio and Lbc (Bellanger et al., 2000; Pi et al., 2002), and FilGAP (Ohita et al., 2006). Thus, FLNa may alter the signaling that governs myosin-generated contractility. Breast epithelial cell morphogenesis is regulated by RhoA-mediated contraction (Stendahl and Stossel, 1980). Hence, FLNa signaling through the ROCK disrupts collagen gel contraction and branching morphogenesis of hβ1(V787,791)expressing cells in high-density gels (data not shown).

We propose that FLNa-β1 integrin is a bidirectional mechanosensitive complex that both regulates collagen matrix contraction during cell morphogenesis in response to changes in collagen density and that it tunes cellular responses to high-density gels through a balance of myosin activity modulated by FLNa-β1 integrin interactions. Although it is likely that multiple proteins are implicated in a mechanosensitive complex, the identification of FLNa-β1 integrin as a component of the complex provides a mechanism that couples the ECM to the cytoskeletal contractile machinery. Furthermore, FLNa signaling through β1 integrin may modulate gel contraction by serving as a scaffold for various signaling molecules. However, further investigation is required to distinguish whether the FLNa-β1 integrin complex serves as a mechanosensor or whether FLNa regulates the β1 integrin mechanosensor complex. Other studies have identified p130Cas and Zyxin as mechanosensitive proteins that undergo changes in conformation and signaling that are implicated in the cellular response to mechanical stretch (Yoshigi et al., 2005; Sawada et al., 2006). Future work aimed at elucidating how FLNa cooperates with other mechanosensitive proteins will further our understanding of the mechanisms that are involved in matrix contraction and regulation of cell morphogenesis.

These observations may ultimately be of pathological importance, because they may explain some of the underlying mechanisms by which increased mammographic and stromal density might contribute to altered breast epithelial phenotype and breast carcinoma. Breast density is linked to an increased risk of breast carcinoma (Boyd et al., 2001) and is associated with a significant increase in the deposition of extracellular matrix components, especially collagen and fibronectin (Guo et al., 2001). Furthermore, matrix density could alter treatment efficacy by hindering the delivery of therapeutic macromolecules (Netti et al., 2000). Understanding the mechanisms of how environmental factors, such as collagen density, regulate cell phenotype will help elucidate the role of breast density on the development of breast carcinoma.

ACKNOWLEDGMENTS

We thank Drs. Gianluca Gallo, Paul Letourneau, and Suzanne Ponik for critically reading the manuscript and Carolyn Pehrke and Dr. Paolo Provenzano for helpful discussion. Also, we are grateful to the University of Wisconsin Paul Carbone Cancer Center Flow Cytometry Facility for assistance in cell sorting and flow cytometry. This work was supported by National Institutes of Health and National Institute on Aging grant T32 AG-20013 (Sanjay Ashana, principal investigator); to S. G., National Institutes of Health National Cancer Institute grant RO1 CA-076537 (to P.J.K.), National Institutes of Health National Institute of Biomedical Imaging and Bioengineering grant R01 EB-00184 (to K.W.E.), National Institutes of Health grant R01 GM-06860 (to D.A.C.), and American Cancer Society grant RSC-00-339-04 (to P.J.K.).

REFERENCES


