Supplemental Data

Extracellular Matrix Rigidity Promotes Invadopodia Activity

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Supplemental Experimental Procedures

Reagents

The antibodies used were as follows: Cortactin, 4F11 (Upstate Biotechnology, Lake Placid, NY), non-muscle myosin IIA and IIB (Sigma, St. Louis, MO), phosphorylated serine 19-myosin light chain 2 and pY165-p130Cas (Cell Signaling, Danvers, MA), pY397 FAK (BioSource, Carlsbad, CA), and Alexa Fluor 633 goat anti-mouse IgG, coumarin phallacidin, and Alexa Fluor 546 phalloidin (Invitrogen). Blebbistatin, ML-7 and Y-27632 were from CalBiochem (San Diego, CA).

Cell culture and Invadopodia analyses

MCF10A-CA1d breast cancer cells were obtained from Dr. Fred Miller (Karmanos Institute, MI) and has been previously described [1]. Cells were cultured in DMEM/F12 supplemented with 5% horse serum (HyClone), 0.1 ug/ml cholera toxin (Calbiochem), 10 ug/ml insulin (Gibco), 0.5 ug/ml hydrocortisone (Sigma), and 20 ng/ml EGF (Invitrogen) at 37° C with constant humidity. The ECM degradation assay has been previously described [2]. Briefly cells were cultured in a 1:1 ratio of DMEM:RPMI-1640 with 5% NuSerum (Gibco, Carlsbad, CA), 10% FBS, and 20 ng/ml EGF. MatTek culture dishes (MatTek Corp., Ashland, MA) were coated with a thin layer of gelatin (0.5 to 5%) that was crosslinked with 0.5% glutaraldehyde prior to addition of 50 μg/ml FITC-conjugated fibronectin (FITC-FN). Cells were cultured for 18 to 20 hours before fixation with 4% paraformaldehyde and immunostaining. Wide-field fluorescent images were captured on a Nikon Eclipse TE2000-E microscope with a 40X Plan Fluor oil immersion objective lens. Confocal images were taken using a Zeiss LSM 510 microscope with a Plan Apo 63X oil immersion objective lens, and Z-section images were captured using 0.05 μm optical sections.

Invadopodia were defined as: Functional: F-actin-positive (Fig 1,5) or F-actin/cortactin double positive (Figs 2,3) puncta that colocalized with areas of ECM degradation; Nonfunctional: F-actin-positive (Fig 5) or F-actin/cortactin double positive invadopodia without degradation (Fig 3); or Total: functional + nonfunctional (Fig 3). Cortactin was used as an additional marker to define invadopodia in Figs 2, 3 because while actin puncta associated with ECM degradation is a common indicator of invadopodia, we wanted to more rigorously define invadopodia on polyacrylamide gels and in the presence of drug treatments. Invadopodia were manually counted from images and reported as invadopodia per cell. Degradation area was determined by performing an inclusive threshold of the FITC channel to include the dark, degraded areas: then, the region of interest tool was used to calculate the thresholded area. Statistical analyses were performed using the Student's t-test, statistical significance set to $p<0.05$.

Polyacrylamide gels

Cells were cultured on glass coverslips coated with polyacrylamide gels according to a method from Pelham and Wang [3]. The gels used contained 8% acrylamide and 0.05% bis-acrylamide (soft) and 0.35% bis-acrylamide (hard). The thin (75 μm) PA gels were coated with 1% gelatin (~1 μm thickness) for one hour, at which point excess gelatin was wicked away from the surface of the PA gel and crosslinked with glutaraldehyde, followed by coating with FITC- or TRITC-Fibronectin. These coverslips were then used in the ECM degradation assay.

Rheology

Gelatin and PA gels (both hard and soft) were analyzed on a TA Instruments AR-G2 rheometer at 37°C using a 25 mm circular head. Strain sweeps were first performed at 1 Hz to identify the range where the storage modulus was constant as a function of strain, which ranged from 0.025 to 0.1% depending on the material. Frequency sweeps (0.1 – 10 Hz) were then performed for each material at constant applied strain. Storage modulus data reported in Figs 1C and Supp Fig 1 were measured at 1 Hz.

Figure S1. Gelatin and FN/gelatin layers do not substantially alter the storage moduli for soft and hard polyacrylamide (PA) gels. The storage moduli for the PA gels were measured at 1 Hz and and a constant strain of 0.05% using a parallel plate rheometer. PA gels were prepared as described for the invadopodia assays. Data are presented as mean +/- SEM with n=6 per condition and * indicating $p<0.05$.

Figure S2. Inhibition of Rho kinase decreases ECM degradation. A. Images of CA1D cells treated with the Rho kinase inhibitor Y-27632 after plating on FITC-FN/2.5% gelatin on glass coverslips. **B.** Quantification of the dose dependent inhibition of ECM degradation area/cell area following treatment with various concentrations of Y-27632. Data are represented as mean+/- SE. Asterisks indicate $p < 0.05$, compared with control cells.

Figure S3. Mean cell area measurements under diverse rigidity and contractile regimes. Mean cell areas were measured using the regions measurement tool in Metamorph software in CA1d cells plated on FITC-FN overlaying: (**A**) various concentrations of gelatin on glass; (**B**) 1% gelatin on soft or hard polyacrylamide (PA) gels; (**C**) FITC-Fn/2.5% gelatin/glass with various concentrations of blebbistatin or ML-7, as indicated; or (**D**) control cells (LZRS) or cells overexpressing focal adhesion kinase (FAK-OE) or p130Cas (Cas-OE) plated on 1% gelatin on soft or hard polyacrylamide (PA) gels. Asterisks = $p<0.05$

Figure S4. The contractile proteins myosin IIA, IIB, and pMLC do not localize to invadopodia. A. Confocal immunofluorescent localization of actin filaments (Actin, red in merges) and myosin IIA (MyoIIA, blue in merge) in invadopodia-producing cells. Invadopodia are identified by colocalization of actin filaments with dark areas of degradation in FITC-Fibronectin (FITC-FN, green in merges) images. **B.** Immunolocalization of myosin IIB (MyoIIB, blue in merge) in invadopodia-producing

cells. **C.** Immunolocalization of phosphomyosin light chain (pMLC, blue in merge) in invadopodia-producing cells. No or weak localization of contractile proteins to invadopodia is pointed out with red arrows. A ring-like structure of myosin IIA around an invadopodium is indicated with a yellow arrow in A and further shown in the enlarged inset in the upper left portion of the image. All images present CA1d cells plated on FITC-FN overlying 2.5% gelatin. Scale bars = 10 µm. Data are represented as mean+/- SE.

Figure S5. Coomassie-stained SDS-polyacrylamide gel of lysates from control (LZRS), FAK-overexpressing (FAK-OE) or Cas overexpressing (CAS-OE) CA1d cells.

Supplemental References

1. Santner, S.J., Dawson, P.J., Tait, L., Soule, H.D., Eliason, J., Mohamed, A.N., Wolman, S.R., Heppner, G.H., and Miller, F.R. (2001). Malignant MCF10CA1 cell lines derived from premalignant human breast epithelial MCF10AT cells. Breast Cancer Res Treat *65*, 101-110.
2. Chen, W.T. (1989). Proteolytic activity of specialized surface protrusions formed at rosette contact sites of transformed cells. J Exp Zool *251*, 167-185.
3. Pelham, R.J., Jr., and Wang, Y. (1997). Cell locomotion and focal adhesions are regulated by substrate flexibility. Proc Natl Acad Sci U S A *94*, 13661-13665.

Figure S1

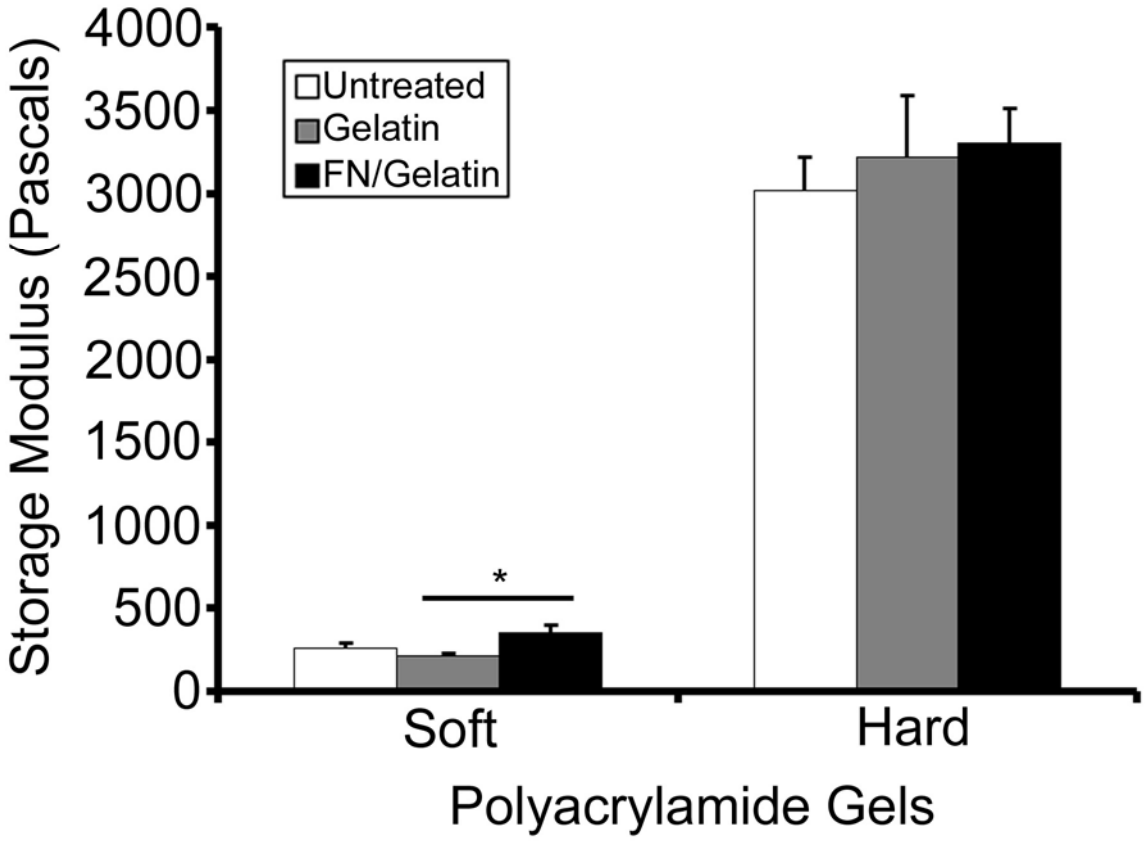
Storage Modulus (Pascals)
4000
3500
3000
2500
2000
1500
1000
500
0
Untreated
Gelatin
FN/Gelatin
*
Soft
Hard
Polyacrylamide Gels

Figure S2

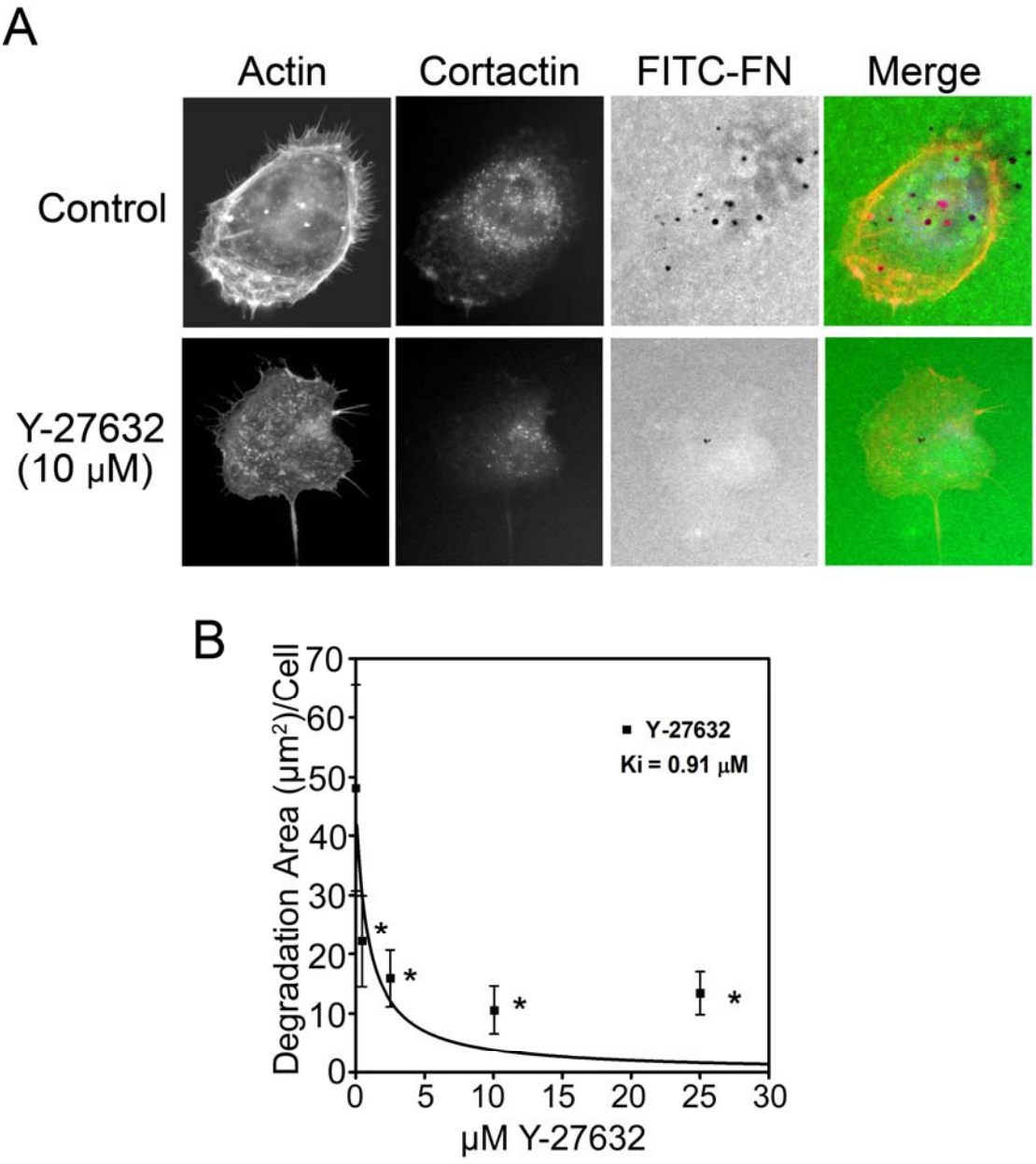
A
Actin
Cortactin
FITC-FN
Merge
Control
Y-27632
(10 μM)
B
Degradation Area (μm²)/Cell
μM Y-27632
Y-27632
Ki = 0.91 μM
0
10
20
30
40
50
60
70
0
5
10
15
20
25
30

Figure S3

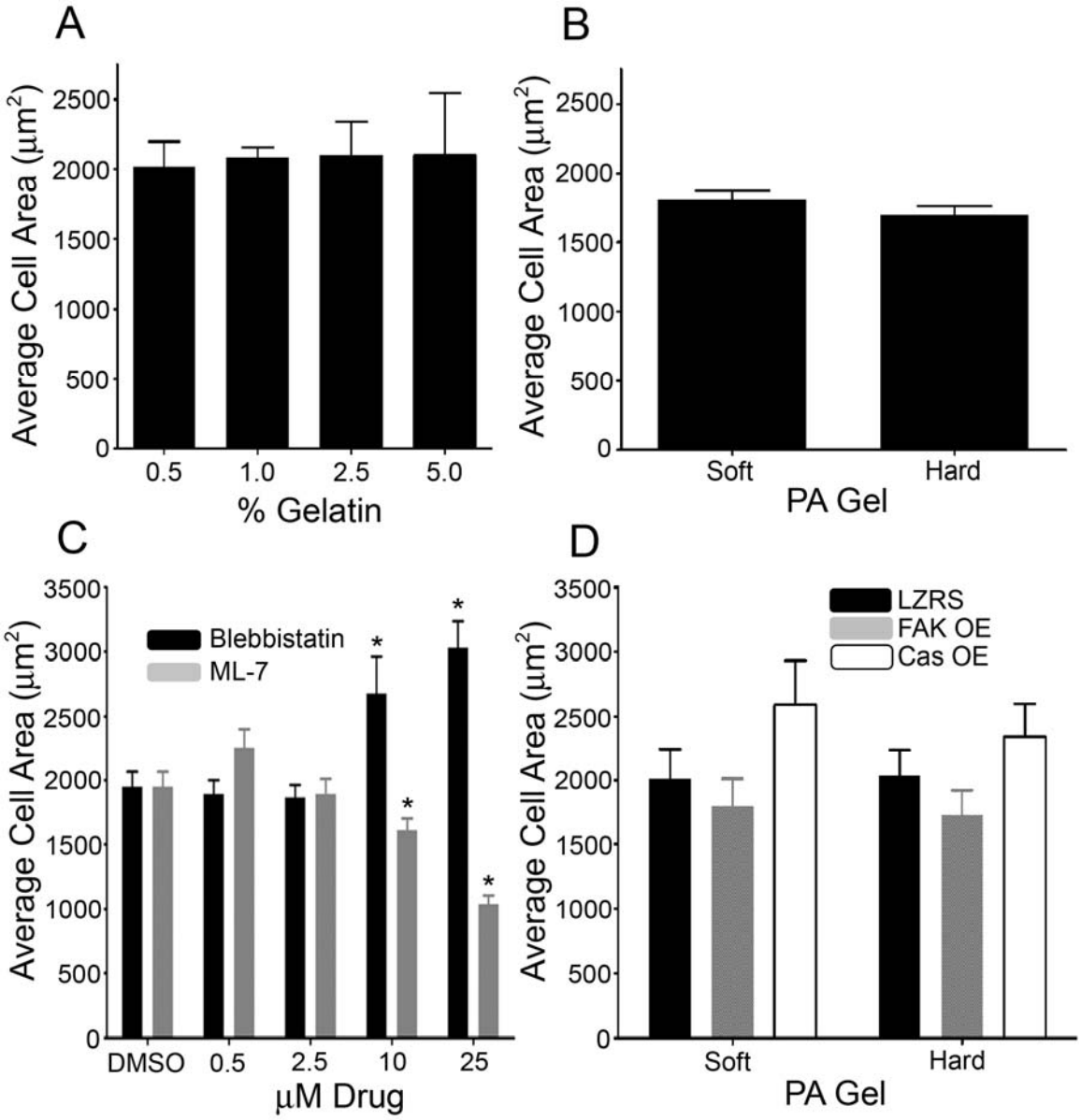
A
Average Cell Area (μm²)
2500
2000
1500
1000
500
0
0.5
1.0
2.5
5.0
% Gelatin
B
Average Cell Area (μm²)
2500
2000
1500
1000
500
0
Soft
Hard
PA Gel
C
Average Cell Area (μm²)
3500
3000
2500
2000
1500
1000
500
0
Blebbistatin
ML-7
*
*
*
DMSO
0.5
2.5
10
25
μM Drug
D
Average Cell Area (μm²)
3500
3000
2500
2000
1500
1000
500
0
LZRS
FAK OE
Cas OE
Soft
Hard
PA Gel

Figure S4

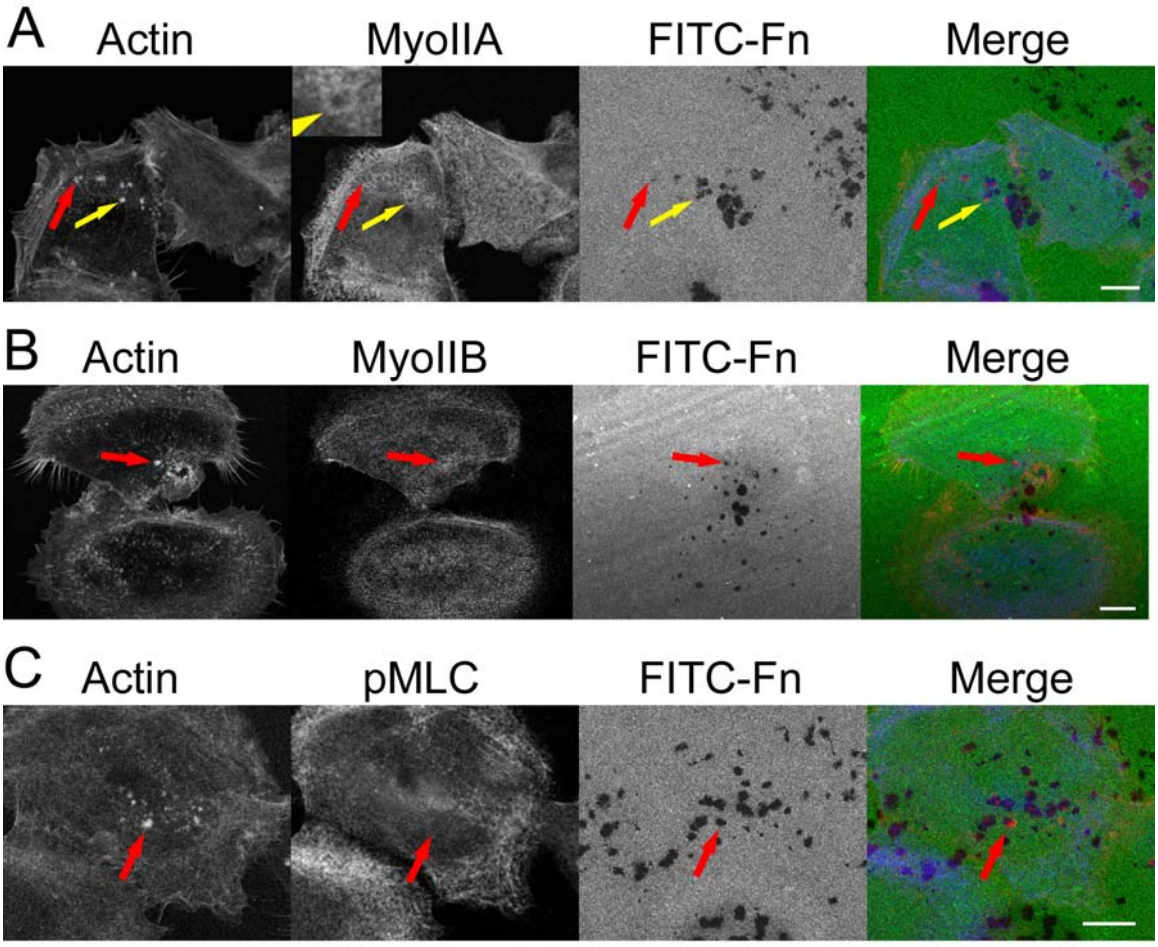
A
Actin
MyoIIA
FITC-Fn
Merge
B
Actin
MyoIIB
FITC-Fn
Merge
C
Actin
pMLC
FITC-Fn
Merge

Figure S5