Force Engages Vinculin and Promotes Tumor Progression by Enhancing PI3K Activation of Phosphatidylinositol (3,4,5)-Triphosphate

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Abstract
Extracellular matrix (ECM) stiffness induces focal adhesion assembly to drive malignant transformation and tumor metastasis. Nevertheless, how force alters focal adhesions to promote tumor progression remains unclear. Here, we explored the role of the focal adhesion protein vinculin, a force-activated mechanotransducer, in mammary epithelial tissue transformation and invasion. We found that ECM stiffness stabilizes the assembly of a vinculin–talin–actin scaffolding complex that facilitates PI3K-mediated phosphatidylinositol (3,4,5)-triphosphate phosphorylation. Using defined two- and three-dimensional matrices, a mouse model of mammary tumorigenesis with vinculin mutants, and a novel super resolution imaging approach, we established that ECM stiffness, per se, promotes the malignant progression of a mammary epithelium by activating and stabilizing vinculin and enhancing Akt signaling at focal adhesions. Our studies also revealed that vinculin strongly colocalizes with activated Akt at the invasive border of human breast tumors, where the ECM is stiffest, and we detected elevated mechanosignaling. Thus, ECM stiffness could induce tumor progression by promoting the assembly of signaling scaffolds, a conclusion underscored by the significant association we observed between highly expressed focal adhesion plaque proteins and malignant transformation across multiple types of solid cancer.

See all articles in this Cancer Research section, "Physics in Cancer Research."

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Introduction
The extracellular matrix (ECM) is remodeled and stiffened in tandem with the malignant transformation of tissues (1–5). Enhanced collagen cross-linking stiffens the ECM in vivo and in vitro, increases phosphorylation of focal adhesion kinase (FAK), and promotes mammary tumor progression, whereas preventing ECM cross-linking and decreasing tissue tension reduce FAK activity and prevent tumor cell invasion and metastasis (1, 6). Although these findings implicate force-induced focal adhesions in malignant progression, the molecular mechanisms remain unclear. Moreover, ECM concentration and organization also influence focal adhesion assembly, and ECM density differs in tumors, while the invasive front of a transformed epithelium frequently contains tracts of perpendicularly oriented, collagen fibers (1, 7–10). These data suggest that the altered ECM topology and density in tumor tissue could also promote integrin focal adhesion assembly to drive malignant progression. Along these lines, Kubow and colleagues argue that adhesion-mediated cell migration in three dimensions (3D) is determined not only by myosin activity, but also by the architecture and density of the local ECM (11). Indeed, whether a cell can even assemble a focal adhesion in a 3D ECM and in tissues has recently been debated (12–14).

Focal adhesions are the conduits through which cells process extrinsic mechanical signals (15). Vinculin is a critical component of focal adhesions and has been shown to regulate cell spreading and stabilize focal adhesions (16–19). The phosphorylation and mechanical unmasking of a cryptic autoinhibited binding site in vinculin favor a conformational change that permits binding to talin and actin (17, 20–22). Vinculin also mechanically couples integrin adhesions to actin retrograde motion and increases force transmission at focal adhesions (18, 23, 24). Thus, vinculin is a mechanically activated mechanotransducer that is ideally posed to mediate force-dependent cell invasion. Consistent with this hypothesis, vinculin is upregulated in primary invasive human cancers, mediates single-cell invasion within a 3D collagen hydrogel, and its loss compromises cell migration during embryonic development (25–27). Nevertheless, whether and how vinculin mediates force-induced tumor cell invasion has yet to be determined.
ECM adhesion is critical for cell growth and survival, and integrins can directly and indirectly enhance growth factor receptor signaling (28, 29). Integrins are a major molecular constituent of focal adhesions, and focal adhesions facilitate actin assembly and actomyosin stimulation and regulate growth factor–dependent ERK and PI3K activation (30, 31). We showed that ECM stiffness promotes growth factor–dependent PI3K activation of Akt in culture (1). Using a mouse model of HER2/Neu-induced mammary cancer, we also demonstrated that inhibiting collagen cross-linking and reducing tissue tension prevent malignant transformation and repress tumor cell invasion while simultaneously decreasing PI3K signaling through Akt (1). These findings suggest that ECM stiffness could promote malignant progression and tumor cell invasion by enhancing growth factor receptor signaling. Here, we asked whether vinculin, as a major mechanically sensitive focal adhesion molecule that binds actin and talin and transmits mechanical cues, could translate ECM stiffness to drive malignant transformation by potentiating growth factor receptor signaling through PI3K. We found that ECM stiffness stabilizes a mechanosensitive vinculin–talin–actin adhesion complex that facilitates PI3K-mediated phosphatidylinositol (3,4,5)-trisphosphate (PI3P) production. Thus, by driving the assembly of a molecular scaffold at the focal adhesion, ECM stiffness potentiates oncogenic signaling through PI3K to drive malignant transformation.

Materials and Methods

Cell culture and reagents

Mammary epithelial cells (MEC), including nonmalignant MCF10A [ATCC, 2007; verified by epithelial cell morphology in two dimensions (2D), ability to form hollow polarized acini in 3D rBM gels, and expression of epithelial markers including E-cadherin and cytokeratins 8 and 14] and premalignant Ha-ras and vinculin-GFP retrovirus (gifted by Dr. Kris DeMali, University of Iowa, Iowa City, IA, 2013; verified by epithelial morphology in 2D), were cultured (7), and 3D multicellular spheroids were generated in recombinant basement membrane (rBM; Matrigel) suspension culture (32). MCF10A vinculin knockdown (KD)– and wild-type (WT)–recovered MECs were generated using vinculin shRNA and vinculin-GFP retrovirus (gifted by Dr. Kris DeMali, University of Iowa, Iowa City, IA, 2013; verified by epithelial cell morphology in 2D; ref. 33). Vinculin homozygous null mouse embryonic fibroblasts were cultured as previously described (gifted by Dr. Andres Garcia, Georgia Institute of Technology, Atlanta, GA, 2013; verified by mesenchymal cell morphology in 2D; ref. 18). The following small-molecule inhibitors were used: Y27632 rho-associated protein kinase (ROCK) inhibitor at 10 μmol/L (Cayman Chemical), FAK Inhibitor I at 1 μmol/L (Tocris), GDC-0941 PI3K inhibitor at 1 μmol/L (Selleckchem), NSC 23766 Rac1 inhibitor at 50 μmol/L (Cayman Chemical), ML7 MLCK inhibitor at 10 μmol/L (Sigma Aldrich), and U0126 MEK1/2 inhibitor at 10 μmol/L (LC Laboratories).

Expression constructs

N-terminus GFP tagged vinculin (Gallus) constructs (gifted by Dr. Susan Craig, Johns Hopkins University, Baltimore, MD; ref. 24), including pEGFP-C1/Gg V1-258 WT, pEGFP-C1/Gg V1-1066 T12, pEGFP-C3/Gg V884-1066, pEGFP-C1/Gg V1-1066 A50I, and pEGFP-C1/Gg V1-851. Vinculin tension sensor components 26019, 26020, and 26021 were acquired from Addgene. Other plasmid constructs used included N- and C-terminus–tagged vinculin-mEmerald, paxillin-mCherry, farnesyl-mEmerald, and farnesyl-mCherry. Transient transfection was performed using the Lonza Nucleofector Kit V, with program T024 for MECs and program U024 for fibroblasts. Lentiviral construct PGK-H2B-EGFP was used in combination with Clontech mCherry-CAX and mEmerald CAAX vectors and the plLV MCS vector to generate plLV hPGK mCherry-CAAX. Stable expression of paxillin-mEmerald, vinculin-mCherry, mEmerald-Farnesyl, and mCherry-Farnesyl recombinant lentivirus was prepared as described (Supplementary Methods). The phosphatidylinositol 4,5-bisphosphate (PIP2; GFP-PH-PLC5) and PIP3 (mKO2-PH-Gpr1) activity probes were prepared as described (gifted by Keith Mostov, University of California, San Francisco, San Francisco, California; ref. 34).

Substrate and hydrogel preparation

Collagen gels were fabricated by diluting 3.8 mg/mL acid-solubilized rat-tail collagen (BD) in 1:1 DMEM-F12 medium (Invitrogen), neutralizing to pH 7.4 with 1 mol/L NaOH (37°C, 1 hr). Self-assembling peptide (SAP) gels were fabricated by suspending cells in presonicated 0.8 mg/mL or 5.0 mg/mL SAP (BD PuraMatrix Peptide Hydrogel), with 10% sucrose, 0.1% bovine serum albumin, human plasma fibronectin (Millipore), and growth medium (32). 2D polyacrylamide (PA) gels (height ~200 μm) were fabricated by varying the acrylamide and cross-linker concentration and assessing stiffness by Atomic Force Microscopy or using a shear rheometer (3, 35). N-type [100]-orientation silicon wafers with 1.933-nm silicon oxide (Addison Engineering; 1 cm × 1 cm) and borosilicate glass coverslips (~150 μm thick) were cleaned by successive sonication in acetone and 1 mol/L KOH (20 minutes) followed by chemical activation with silane [0.5% (3-aminopropyl)trimethoxysilane] and glutaraldehyde (0.5% in water). The substrates were then UV and ethanol sterilized and incubated overnight (4°C) in ECM (human fibronectin; 10 μg/mL). Before use, substrates were washed in PBS (5 times; pH 7.4), treated with sodium borohydride (20 mg/mL), and washed with PBS. Scanning angle interference microscopy (SAIM) calibration wafers were prepared by sonicating carboxylate-modified red fluorescent spheres (100 nm; Invitrogen), following by bead deposition (5 × 10^8 beads per mL) in NaCl (100 mmol/L) PBS solution.

Immunofluorescence

Cultures were fixed with 4% paraformaldehyde (2D: 20 minutes, room temperature; 3D: overnight, 4°C), and staining was performed as described (1). Primary antibodies against vinculin (hVIN-1, Sigma Aldrich; 700062, Invitrogen; V284, Santa Cruz Biotechnology), β1 integrin (AIIB2, isolated from rat hybridoma), β4 integrin (3E1; Millipore MAB1964), pThr18/Ser19-FAK (44625G; Invitrogen), pS314-Akt (Cell Signaling; 9271S), Akt(p)-Alexa488 (Cell Signaling; C67E7), E-cadherin (610181; BD Transduction), β-catenin (610153; BD), pan-laminin (1:5933; Sigma), laminin 5 (P3H9, isolated from mouse hybridoma), β6 integrin (GoH3; Ebiosciences), Phospho-Myosin Light Chain Kinase 2—Thr18/Ser19 (3674; Cell Signaling), talin (T3287; Sigma), zyxin (BD; 610521), and pEGFP-C1/Gg V1-851. Vinculin tension sensor components 26019, 26020, and 26021 were acquired from Addgene. Other plasmid constructs used included N- and C-terminus–tagged vinculin-mEmerald, paxillin-mCherry, farnesyl-mEmerald, and farnesyl-mCherry. Transient transfection was performed using the Lonza Nucleofector Kit V, with program T024 for MECs and program U024 for fibroblasts. Lentiviral construct PGK-H2B-EGFP was used in combination with Clontech mCherry-CAX and mEmerald CAAX vectors and the plLV MCS vector to generate plLV hPGK mCherry-CAAX. Stable expression of paxillin-mEmerald, vinculin-mCherry, mEmerald-Farnesyl, and mCherry-Farnesyl recombinant lentivirus was prepared as described (Supplementary Methods). The phosphatidylinositol 4,5-bisphosphate (PIP2; GFP-PH-PLC5) and PIP3 (mKO2-PH-Gpr1) activity probes were prepared as described (gifted by Keith Mostov, University of California, San Francisco, San Francisco, California; ref. 34).
Mammary epithelial cells (MECs) maintain tissue integrity, even 24 hours after embedment within the compliant collagen/rBM gels, as indicated by the maintenance of spherical acini, intact adherens junctions, and tissue polarity, as revealed by basally localized laminin and cell–cell–localized β-catenin (Fig. 1A, left column). However, immunostaining revealed that after 48 hours in the stiffer gels, the basal polarity and cell–cell junctions in the nonmalignant structures were severely compromised (Fig. 1A, center column and Supplementary Fig. S1A). Importantly, the nonmalignant cells at the periphery of the colonies in the stiffer gels probed the local ECM, as indicated by prominent cell protrusions (39), although they never invaded into the gel (Fig. 1A, left column). By contrast, although the Ha-ras premalignant mammary spheroids retained a semblance of tissue polarity when embedded within the softest collagen/rBM gels (Fig. 1A, center column), indicated by retention of cell–cell–localized β-catenin and minimal protrusions in the stiffer gels, the structures completely collapsed and the transformed MECs invaded both collectively and individually (Fig. 1A, center column; ref. 1). Interestingly, two-photon imaging revealed that Ha-ras–transformed MCF10AT MECs invaded collectively and as single cells along collagen bundles that seemed to project perpendicularly from the colonies (Supplementary Fig. S2A), and atomic force microscopy illustrated that collagen bundles can be quite stiff under compression (Supplementary Fig. S2B and S2C; refs. 1, 40). Thus, although these findings do not rule out the possibility that ECM invasion into these collagen gels was also induced by the topological features of the collagen, the data strongly implicate ECM stiffness.

To explore functional links among focal adhesions, ECM stiffness, and tumor cell invasion, we stained 3D multicellular structures for the transmembrane protein β1 integrin, which connects the ECM to cell adhesions, and the focal adhesion proteins vinculin and pY972FAK, as well as fluorescently labeled

Statistical analysis

Statistical analysis for two groups was performed with an unpaired, two-tailed student’s t test. For multiple comparisons, an ANOVA test and Holm–Bonferroni t test method were performed. Calculations were implemented in Python.

Results

ECM stiffness and ligand density regulate focal adhesions to promote tumor cell invasion in 3D

Most studies linking ECM stiffness to focal adhesion assembly and cell motility have been conducted using single transformed cells or mesenchymal fibroblasts on 2D substrates (37, 38). Here, we examined the importance of tension-induced focal adhesions in nonmalignant versus transformed epithelial cell invasion both as single cells and tissue-like structures in 3D. We transplanted 5-day-old, rBM preassembled, proliferating nonmalignant MCF10A and premalignant Ha-ras MCF10AT–transformed MEC spheroids into collagen/rBM gels, with compliances calibrated to match normal (0.5 kPa; 0.5 mg/mL), premalignant (1.5 kPa; 2.0 mg/mL), and malignant (2.5 kPa; 5.0 mg/mL) mammary tissue (1, 3, 7). Phase contrast (Fig. 1A, top 2 left plots) and confocal immunofluorescence imaging (Fig. 1A, bottom 2 left plots) revealed that the transplanted nonmalignant mammary spheroids retained their integrity, even 24 hours after embedment within the compliant collagen/rBM gels, as indicated by the maintenance of spherical acini, intact adherens junctions, and tissue polarity, as revealed by basally localized laminin and cell–cell–localized β-catenin (Fig. 1A, left column). However, immunostaining revealed that after 48 hours in the stiffer gels, the basal polarity and cell–cell junctions in the nonmalignant structures were severely compromised (Fig. 1A, center column and Supplementary Fig. S1A). Importantly, the nonmalignant cells at the periphery of the colonies in the stiffer gels probed the local ECM, as indicated by prominent cell protrusions (39), although they never invaded into the gel (Fig. 1A, left column). By contrast, although the Ha-ras premalignant mammary spheroids retained a semblance of tissue polarity when embedded within the softest collagen/rBM gels (Fig. 1A, center column), indicated by retention of cell–cell–localized β-catenin and minimal protrusions in the stiffer gels, the structures completely collapsed and the transformed MECs invaded both collectively and individually (Fig. 1A, center column; ref. 1). Interestingly, two-photon imaging revealed that Ha-ras–transformed MCF10AT MECs invaded collectively and as single cells along collagen bundles that seemed to project perpendicularly from the colonies (Supplementary Fig. S2A), and atomic force microscopy illustrated that collagen bundles can be quite stiff under compression (Supplementary Fig. S2B and S2C; refs. 1, 40). Thus, although these findings do not rule out the possibility that ECM invasion into these collagen gels was also induced by the topological features of the collagen, the data strongly implicate ECM stiffness.

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Microscopy and analysis

Imaging was performed on a motorized TIRF-inverted microscope system (Ti-E Perfect Focus System; Nikon) controlled by Metamorph software (Molecular Devices), equipped with 488-, 561-, and 640-nm lasers, 350/50 epifluorescence, a CSU-X1 spinning disc confocal unit (Yokogawa Electric Company), electronic shutters, motorized stage, a scientific sCMOS camera (Zyla 5.5 megapixel; Andor), and an electron-multiplying charged-coupled device camera (QuantEM 512; Photometrics). Temperature and CO2 were controlled by an environmental chamber and PID-controlled heater (In Vivo Scientific). Samples were imaged with a 100×1.49 numerical aperture (NA) total internal reflection fluorescence (TIRF) oil immersion. For 3D imaging, confocal image slices were taken at 0.2 μm or 1.0 μm, and the individual planes were maximum intensity z-projected for 2D visualization. A custom software package for image analysis was written in Python, using Imagej and the Eclipse Development Environment in Linux. All images were subject to a Gaussian blur of 1.0 pixel to smooth background noise. DAPI images were subject to a local background subtraction of approximately 5 μm to reduce fringing from epifluorescence imaging through the spinning disc unit. Intensity and colocalization measurements of single confocal planes were calculated on a pixel-by-pixel basis. 3D image rendering was performed in Imaris (Bitplane). Cell projections and adhesions were counted manually. Acini cross-sectional area was quantified by basally localized laminin and cell–cell–localized β-catenin (Fig. 1A, left column). However, immunostaining revealed that after 48 hours in the stiffer gels, the basal polarity and cell–cell junctions in the nonmalignant structures were severely compromised (Fig. 1A, center column and Supplementary Fig. S1A). Importantly, the nonmalignant cells at the periphery of the colonies in the stiffer gels probed the local ECM, as indicated by prominent cell protrusions (39), although they never invaded into the gel (Fig. 1A, left column). By contrast, although the Ha-ras premalignant mammary spheroids retained a semblance of tissue polarity when embedded within the softest collagen/rBM gels (Fig. 1A, center column), indicated by retention of cell–cell–localized β-catenin and minimal protrusions in the stiffer gels, the structures completely collapsed and the transformed MECs invaded both collectively and individually (Fig. 1A, center column; ref. 1). Interestingly, two-photon imaging revealed that Ha-ras–transformed MCF10AT MECs invaded collectively and as single cells along collagen bundles that seemed to project perpendicularly from the colonies (Supplementary Fig. S2A), and atomic force microscopy illustrated that collagen bundles can be quite stiff under compression (Supplementary Fig. S2B and S2C; refs. 1, 40). Thus, although these findings do not rule out the possibility that ECM invasion into these collagen gels was also induced by the topological features of the collagen, the data strongly implicate ECM stiffness.

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and AlexaFluor phallolidin (633-conjugate; Invitrogen) were used. Secondary antibodies used include AlexaFluor goat anti-mouse, anti-rabbit, and anti-rat (488, 568, and 633 conjugates).

Mouse and human studies

Cohorts of PyMT (polyoma middle T) and FVB mice were maintained in accordance with University of California Institutional Animal Care and Use Committee guidelines under protocol AN092125. Starting at 4 weeks of age, mice were treated with a LOX function-blocking polyclonal antibody (3 mg/kg; OpenBiosystems) injected intraperitoneally twice per week (1). Mice were sacrificed at 80 to 95 days of age, at which time the fourth mammary gland was paraformaldehyde fixed. Under protocol 10-05046 and in accordance with the UCSF Committee on Human Research, formalin-fixed and paraffin-embedded human breast cancer biopsies containing normal, DCIS (ductal-carcinoma-in-situ), and estrogen receptor– and progesterone receptor–positive invasive cancer tissue were obtained. Mouse mammary tissue (10 μm) and human breast biopsy (6 μm) sections were analyzed for histology (hematoxylin and eosin, H&E), and parallel tissue was stained for the focal adhesion proteins vinculin, pY972FAK, and β1 integrin and for the PI3K downstream target p473Akt.

Statistical analysis

Statistical analysis for two groups was performed with an unpaired, two-tailed student’s t test. For multiple comparisons, an ANOVA test and Holm–Bonferroni t test method were performed. Calculations were implemented in Python.
phalloidin to image filamentous actin. Confocal imaging revealed that MECs at the periphery of the Ha-ras premalignant colonies, as well as the individual transformed MECs invading into the stiffer collagen/rBM gels, stained robustly for all three focal adhesion proteins (Fig. 1B, right columns) as well as F-actin (data not shown). Moreover, treatment of the
premalignant spheroids with 1 μmol/L FAK-Inhibitor 14 ablated the invasive behavior of these transformed mammary tissues within the stiffer gels (Fig. 1A, right column). However, we also detected abundant levels of these adhesion proteins arranged as punctate structures reminiscent of focal adhesions in the nonmalignant MECs at the periphery of the spheroids, where the cells interact directly with the stiff ECM (Fig. 1B, left column). Thus, although these findings suggest ECM stiffness activates focal adhesions to promote MEC invasion in 3D, the data also indicate that focal adhesions are not by themselves sufficient, and MEC invasion also requires the activation of pathways engaged by oncogenic transformation (1, 7).

To directly test the role of ECM stiffness on tumor cell invasion in 3D, in the absence of changes in ECM pore size, topology, density, or composition, we used 3D RADA-16 SAP gels doped with saturating concentrations of laminin 111 or fibronectin (32). By increasing polymer concentration, the compliance of these SAP gels can be varied across a range of stiffness (0.5–5 kPa) appropriate for mammary and other epithelial tissues, with negligible effects on pore size and ECM topology, and we and others showed that cells embedded in these gels ligate the ECM protein(s) passively adhered to the peptides and respond to the stiffness of the gel (32, 41). Consistently, we observed that MECs embedded in SAP gels with saturating concentrations of ECM ligand (fibronectin; 6.2 mg/mL) increased their protrusive activity in proportion to the stiffness of the gel (Fig. 1C and Supplementary Fig. S1B), similar to what we observed in 3D collagen gels (Supplementary Fig. S1D). Confocal imaging also revealed that MECs coexpressing GFP-tagged vinculin and mCherry paxillin assembled small punctate structures reminiscent of focal contacts in the softest SAP gels, and larger focal adhesion-like structures in the stiffer SAP gels (Fig. 1D). Furthermore, the size and number of these adhesions as well as the number of cellular protrusions, which is a proxy for invasive behavior, increased in proportion to ligand concentration (Fig. 1E and Supplementary Fig. S1C). Importantly, after embedment into the stiffer SAP gels, immunofluorescence imaging revealed that the integrity and polarity of the nonmalignant MEC organoids were severely compromised, as indicated by loss of basally localized α6 integrin and basally deposited laminin 5 (Fig. 1C). Moreover, in marked contrast to their behavior in the collagen/rBM gels, where pore size limited invasion (Supplementary Fig. S1B, bottom row, fourth column), phase contrast imaging revealed that the invasive behavior of the premalignant mammary colonies increased further in the stiffest SAP gels (Supplementary Fig. S1B). These observations show that ECM stiffness and ligand density regulate focal adhesions to permit the invasion of an oncogenically transformed epithelium in 3D.

**ECM stiffness activates vinculin to promote an invasive phenotype**

Vinculin is a major focal adhesion plaque protein whose structure function is exquisitely sensitive to mechanical force, and vinculin can act as a mechanical clutch to stabilize adhesions (18, 23). This prompted us to ask if ECM stiffness promotes tumor cell invasion by activating vinculin to stabilize focal adhesions. Consistently, we noted that MECs expressing a wild-type vinculin (vinculin WT) that were plated on a soft fibronectin-conjugated PA gel assembled small focal contacts, showed only modest protrusive activity, and failed to spread (Fig. 2A, top left plot; ref. 7). By contrast, parallel cultures of MECs that expressed a constitutively active vinculin T12, which lacks the autoinhibition domain when plated on soft gels, had increased adhesion area, exhibited robust protrusive activity, and spread appreciably (Fig. 2A, top right plot and Supplementary Fig. S1E). In addition, MEC expressing vinculin T12 on stiff substrates had prominent stress fibers and localized more vinculin at the focal adhesions (Fig. 2B; ref. 17). Moreover, MECs in which vinculin levels were reduced using shRNA had significantly reduced protrusive activity, reflecting invasive behavior, even when the cells were embedded within a stiff, fibronectin-saturated, SAP gel (Fig. 2C). By contrast, the protrusive activity of these MECs was fully restored following reexpression of an RNAi-resistant vinculin (Fig. 2C). In this regard, we observed that the ability of vinculin to restore the protrusive activity in vinculin-null murine fibroblasts in response to ECM stiffness required a critical level of cellular vinculin, where the greatest protrusive activity was noted in cells with the highest vinculin expression (Fig. 2D). Thus, fibroblasts expressing high amounts of vinculin assembled punctate adhesive-like structures analogous to focal adhesions, and increased their protrusive activity in response to a stiff SAP gel (Fig. 2B; ref. 27). These data demonstrate that ECM-induced invasion requires the engagement of a critical threshold of vinculin that stabilizes focal adhesions.

**Extrinsic and intrinsic forces activate vinculin at focal adhesions**

We next explored the relationship between force, vinculin activation, and focal adhesion stabilization. We first demonstrated that 15 to 45 minutes following ROCK inhibition (Y27632; 10 μmol/L), the size and number of the vinculin-positive focal adhesions were significantly decreased in the nonmalignant MECs expressing a GFP-tagged vinculin WT (Fig. 3A, bottom left graph). By contrast, no quantifiable change in either the size or the number of adhesions was observed in the ROCK inhibitor–treated MECs expressing the GFP-tagged vinculin T12 (Fig. 3A, bottom left graph). These findings agree with prior data obtained in fibroblasts, which established a role for actomyosin tension in vinculin-mediated focal adhesion stabilization (17, 19).

Focal adhesions are composed of over 200 proteins, which are segregated into stratified functional layers (42, 43). Yet, SAIM, which accurately records the nanoscale position of the molecular constituents of focal adhesions, indicates that the spatial organization of many of adhesion plaque proteins, including the scaffolding molecule paxillin, changes dramatically and dynamically during focal adhesion assembly and cell invasion (36). Therefore, to understand the interplay between extrinsic and intrinsic force and vinculin-mediated stabilization of focal adhesions and cell invasion, we monitored vinculin dynamics during focal adhesion assembly and disassembly using SAIM. Imaging showed that vinculin WT localized at 100 nm (Fig. 3B), which is a composite of the talin–actin and actin only–bound molecules. Indeed, we observed that
a constitutively active vinculin T12, all of which is bound to a talin–actin complex, resides at 95 nm and a vinculin tail–only mutant, which binds entirely to actin, localized to a height of 105 nm (Fig. 3B). To rule out the effect of cytoplasmic vinculin, we removed the dorsal cell membrane and cytoplasm in MEC and were then able to measure a significant change in vinculin T12 axial position compared with vinculin WT in the ventral cell surface (Supplementary Fig. S3A). We also observed similar behavior of several vinculin mutants between vinc–/– mouse fibroblasts and MEC, and were able to measure the separation of vinculin’s N- and C-terminus (Supplementary Fig. S4A and S4B). Moreover, ablating actomyosin intrinsic tension in the MECs, by treatment with a ROCK inhibitor (Fig. 3A), shifted the bulk of the vinculin WT to the actin-associated fraction, whereas vinculin T12 remained localized to the focal adhesions with altered axial localization (Fig. 3A). The vinculin WT that remained at the adhesions after ROCK or myosin II inhibition (Blebbistatin; 25 μmol/L) was bound at a stable height of 100 nm, which is a composite of talin–actin-bound and actin-bound (Fig. 3A and Supplementary Fig. S3B). In contrast, a different adhesion plaque protein, paxillin, shifted from 60 nm to a height of 90 nm following myosin inhibition (Fig. 3C). These findings illustrate that once engaged, the vinculin–talin–actin complex is remarkably stable.

To determine if ECM stiffness promotes cell invasion by stably increasing cell-generated force over the vinculin–actin–talin complex, we used a vinculin intramolecular tension sensing probe (21) with the pbFRET method (Fig. 3D, top plot). We then examined the response of vinculin WT and mutant expressing cells to ECM stiffness using fibronectin-conjugated PA gels. We found in MECs on either soft (1.1 kPa) or stiff (13.8 kPa) fibronectin-conjugated PA gels that vinculin WT incorporated into focal adhesions was under the same tension (Fig. 3D, bottom plot). However, the tension on the autoactivated vinculin T12, which forms a stable talin–actin–vinculin complex, increased significantly in MECs on the stiffer gels as compared with level on the softer gels, presumably because more force-activated talin becomes available to bind (Fig. 3D). Thus, force activates vinculin to facilitate its assembly into a highly stable talin–actin complex that in turn applies...
activation alters the nanoscale topography of the plasma sions (Fig. 5A). To directly examine if force-induced vinculin al-farnesyl assembled more and longer membrane protru-
stiffness, MECs expressing an inert membrane probe mEmer-
membrane (46). We therefore hypothesized that vinculin adhe-
this regard, recent

activated p473Akt and to a lesser extent p397FAK to the sites of

precursor stimulation, we observed that PIP2 was decreased and

and we found that this phenotype is lost if ECM cross-linking

premalignant mammary colonies with the PI3K inhibitor GDC-

cant increase in

signaling molecules critical for cell invasion. We observed that 10

minutes after EGF stimulation, Akt was recruited to adhesions

stiffened (Fig. 4A). ECM stiffness also increased the quantity of

mice, these

levels measured at the adhesions in the WT vinculin expressing fibroblasts (Fig. 4B). These findings suggest that ECM stiffness promotes the assembly of a stable vinculin–actin–talin complex at the focal adhesion that then permits the nucleation and subsequent efficient activation of signaling molecules critical for cell invasion. We observed that 10 minutes after EGF stimulation, Akt was recruited to adhesions on both soft and stiff 2D PA matrices in MECs, but importantly, we were only able to document a significant increase in activated p473Akt in vinculin-enriched focal adhesions in MECs on a stiffer substrate (Fig. 4C). Moreover, treating MCF10AT premalignant mammary colonies with the PI3K inhibitor GDC-0941 (1 μmol/L) repressed their prosurvival activity and invasion into a stiffened 3D collagen/RBM gel (1.5 kPa; Fig. 4D, rightmost plot), while there was no reduction in vinculin and β1 integrin–positive focal adhesions, even after 48 hours of treatment (Fig. 4D). These data indicate that ECM stiffness stimulates tumor cell invasion by activating vinculin to assemble a stable complex with talin and actin that enhances PI3K signaling.

Force activates vinculin to increase membrane protrusions and nucleate PIP3

We next asked how the force-induced assembly of a stable vinculin–talin–actin complex potentiates PI3K signaling. In this regard, recent findings by Wang and colleagues showed that PIP3 clusters into distinct nanodomains in the plasma membrane (46). We therefore hypothesized that vinculin adhesions could enhance the ability of PI3K to convert PIP2 to PIP3 in the plasma membrane. Consistently, in response to ECM stiffness, MECs expressing an inert membrane probe mEmer-

membrane, MECs expressing vinculin WT or a vinculin T12 together with an inert membrane probe (mCherry-farnesyl) were seeded on fibronectin-coated silicon wafers, and SAIM was performed to visualize the nanoscale position of vinculin and the associated ventral plasma membrane (Fig. 5B). Imaging studies revealed that although the height of the plasma membrane varied widely across the cell (up to ± 70 nm), the height of the membrane significantly decreased by approximately 20 nm at sites of vinculin-rich focal adhesions, where vinculin remained constant at 100 nm (Fig. 5C). These findings indicate that vinculin-positive focal adhesions correlate to areas of altered membrane topography and suggest that the assembly of a stable vinculin–talin–actin complex likely modifies membrane topology. To directly determine whether a vinculin-stabilized focal adhesion facilitates PIP3 accumulation to enhance Akt activation, MECs expressing a probe for PIP3 (mKO2-PH-Grp1) with either a probe for PIP2 (PLCδ-PH-EGFP), GFP vinculin WT, or GFP vinculin T12 were seeded on fibronectin-coated coverslips and imaged. Following growth factor stimulation, we observed that PIP2 was decreased and PIP3 was increased via PI3K activity (Fig. 5D). Data revealed that enhancing the assembly of the vinculin–talin–actin complex in MECs, by expressing activated vinculin T12, significantly increased the levels of PIP3 at the focal adhesion site, as compared with MECs expressing vinculin WT (Fig. 5E). These results build upon previous findings that PIP3 is localized to the cell–ECM basal border in epithelial acini embedded within a 3D ECM (34), and suggest the force-induced assembly of a stable vinculin–talin–actin complex potentiates Akt activation via PI3K by facilitating membrane changes that favor the accumulation of PIP3 at the adhesion complex. Given that many oncogenes enhance PI3K activity, this mechanism may explain why ECM stiffness specifically induces invasion in a transformed cell.

ECM stiffness regulates vinculin and p473Akt activity in experimental mouse tumors and colocalizes with vinculin-rich adhesions in invasive human breast cancer, and adhesome proteins are upregulated in solid tumors

We and others have shown that p473Akt and p397FAK colocalize at the invasive front of experimental mammary tumors, and we found that this phenotype is lost if ECM cross-linking and stiffening is prevented (1, 47). Here, we determined that ECM stiffness mediates the localization and levels of these signaling molecules at the invasive front by activating vinculin. Thus, while confocal immunofluorescence imaging revealed strong colocalization of vinculin and p473Akt at the invasive front of PyMT mammary tumors, where the ECM is stiffest, in tissue from mice that had been treated with either a pharmacologic lysyl oxidase inhibitor (data not shown) or a function-blocking antibody to inhibit lysyl oxidase activity (Fig. 6A) to prevent collagen cross-linking and stiffening, p473Akt and vinculin levels were greatly reduced and little to no colocalization of these molecules was observed. The clinical relevance of these findings was illustrated by showing a progressive increase in the levels and colocalization of vinculin and p473Akt.
Figure 3. Vinculin activity depends on extracellular and intracellular tension. A, nonmalignant MEC plated on 2D fibronectin-coated glass 45 minutes after ROCK inhibition (10 μmol/L Y27632); left, representative image of vinculin WT and T12 fluorescence; middle, axial position of vinculin WT and T12 via SAIM; right, diagram of vinculin WT and T12 after ROCK inhibition; bottom left, quantification of adhesion area per cell after ROCK inhibition (\textsuperscript{t}, \(P < 0.01\); ±SD, \(n > 18\) cells per condition); bottom right, axial distribution of vinculin and vinculin T12 after ROCK inhibition. Histogram constructed from 3,500 single pixel (0.012 μm\(^2\)) SAIM measurements per condition (\(n > 18\) cells per condition). ROCK inhibition reduced the size of vinculin WT-positive adhesions but did not perturb axial position, whereas vinculin T12-positive adhesions were not reduced in size, but localized to axial positions of 80 and 120 nm. B, vinculin nanoscale axial position in nonmalignant MEC plated on a 2D glass surface via SAIM measurements of whole cell averages (\textsuperscript{t}, \(P < 0.01\); ±SD, \(n > 26\) cells per condition) and diagram of vinculin axial position relative to other adhesion proteins. (Continued on the following page.)
as well as β1 integrin and p95-FAK (Fig. 6B and C) in normal, DCIS, and ER/PR-positive invasive human breast cancer.

Having implicated force-regulated vinculin in tumor invasion, we next asked if other adhesion proteins might also be similarly involved in malignant progression. We used a bioinformatics approach that involved the analysis of pathologically scored immunohistochemistry data compiled by the Human Protein Atlas database (Supplementary Methods, Supplementary Table S1, and Supplementary Fig. S5A; ref. 48). Using an unbiased approach to pair each tumor type to its corresponding normal cell, we determined the most common expression level observed across all samples within each tumor type to generate composite profiles of "typical" protein expression levels for each tumor type. Using this approach, we observed that focal adhesion proteins (42) that were normally expressed at low to negligible levels in healthy tissues were significantly likely to be overexpressed in their corresponding cancer tissue (Fig. 6D and Supplementary Fig. S5B). Interestingly, we also found that many of the oncogenes implicated in cancer were also similarly upregulated (Supplementary Methods and Supplementary Fig. S5C). These bioinformatics results indicate that adhesomes proteins as an aggregate are upregulated at the protein level across many different solid cancer types, in a manner that is analogous to known oncogenes, suggesting they may play a more significant role in malignant progression than previously appreciated.

Discussion

The tumor microenvironment and specifically the ECM is an important regulator of malignant progression (1, 3, 5–7, 49). Using defined 2D and 3D matrices, mouse models, and human biopsies together with a series of molecular mutants and a novel super resolution imaging approach, we demonstrate for the first time in 3D and in tissues that ECM stiffness regulates the activation of vinculin and its nanoscale organization at focal adhesions. This research provides the first detailed molecular mechanism to explain how tumor-associated ECM cross-linking and stiffening stabilizes the vinculin–talin–actin complex, promoting malignant transformation and invasion in tissue (Fig. 7A and B; refs. 1, 11, 27, 50). Our data illustrate how the induction and stabilization of the talin–vinculin–actin scaffolding complex facilitate oncogenic signaling through pathways such as growth factor receptor–induced PIP3 generation of PIP3 (Fig. 7B). Thereby our results emphasize vinculin's role as a nucleator of cell signaling and highlight a vinculin-dependent signaling circuit through which cancer cells both gather information about their altered environment and alter intracellular signaling in response to this input (18, 19, 23, 51). Indeed, by demonstrating a central role for force regulation of a vinculin–talin–actin complex, we provide the first definitive evidence for why malignant transformation and tumor cell invasion require the cooperative interplay between oncogenic transformation and a stiffened ECM (Fig. 7A; refs. 1, 3, 7, 52).

Although most available work on focal adhesions has been performed in 2D in vitro systems, our studies link and extend existing knowledge to cell behavior in a physiologically relevant context. We combine nanoscale characterization with 3D and in vivo analysis to provide a holistic view of how ECM stiffness changes the context in which intracellular signaling takes place. Our data show that by driving molecular scaffolding at focal adhesions, increased ECM stiffness promotes signaling in pathways that are important in both growth and invasion. On the molecular level, our data establish vinculin as a versatile adhesion molecule involved in many aspects of cell–ECM interactions. We confirm that vinculin's autoinhibition domain reacts in a force-dependent manner and operates as a mechanical clutch, connecting the actomyosin network with the ECM. This tantalizingly suggests similar mechanisms of regulation for other plaque proteins containing cryptic binding sites, including talin and α-actinin at cell–ECM junctions, cadherins and platelet endothelial cell adhesion molecule at cell–cell junctions, and ECM proteins such as fibronectin may also play an equally important role in regulating malignancy (53–57). At the tissue level, our data suggest that cells migrating as a collective also likely employ vinculin as a force sensor at cell–cell junctions and force responsive actin cross-linking proteins, including CAS and FERM family proteins (44, 58). More broadly, our results confirm the existence of focal adhesions and focal adhesion activity in 3D and in vivo and demonstrate their functional importance by showing that they are instrumental in regulating growth factor receptor signaling (11, 13, 59, 60). Indeed, our data showed that neither matrix stiffness nor oncogenic transformation are sufficient for malignant transformation and tumor cell invasion, suggesting that ECM stiffness collaborates with key oncogenic pathways to exacerbate the potentiating mutations found in cancer. Indeed, our findings highlight vinculin in particular at the intersection of extrinsic mechanical properties and intracellular growth factor signaling (61). Moreover, our bioinformatics findings, which show amplification of adhesome molecules at the protein level across 20 solid tumor types, suggest that further scrutiny of the role of upregulated adhesion proteins may be another method by which cancers may be categorized and screened (48, 62, 63).

The clinical relevance of our culture studies was demonstrated by our findings that there is a progressive increase in colocalized vinculin and p473-Akt at the invasive tumor border in human breast cancer where the ECM is stiffest and mechanosignaling is elevated, as revealed by increased p95-FAK (64). Functional links between tissue mechanics and the vinculin-P3K phenotype were illustrated by our studies in the PyMT mouse model of mammary carcinogenesis by the loss of vinculin–p473-Akt association and p95-FAK when collagen
Figure 4. Vinculin stabilizes focal adhesions to facilitate FAK and PI3K/Akt signaling, drivers of cellular invasion in 3D. A, vinculin, pAkt473, and pFAK397 recruitment to adhesions in nonmalignant MEC on 2D PA gels of different stiffness, coated with fibronectin. Left, protein recruitment measured by quantitative immunofluorescence at single pixels (0.011 μm²) and averaged over whole cell (\( P < 0.01; \pm SD, n > 18 \) cells per condition); right, quantification of signaling protein colocalization to vinculin per pixel cell, averaged over whole cell (\( P < 0.01; \pm SD, n > 18 \) cells per condition). B, pFAK recruitment to adhesions in vinculin homozygous null mouse fibroblasts expressing vinculin WT or T12 plated on a 2D PA gel. Vinculin T12 significantly increases pFAK measurements made at single pixels and averaged over whole cell (\( P < 0.05; \pm SD, n > 9 \) cells per condition). C, vinculin, Akt, and pAkt473 recruitment to adhesions in nonmalignant MEC on 2D PA gels. Akt is recruited to adhesions on soft and stiff gels, whereas p473Akt is dramatically increased in adhesions on stiff PA gels. D, premalignant MEC spheroids in 1.5-kPa 3D collagen gels for 48 hours, with FAK (1 μmol/L FAK Inhibitor 14) or PI3K (1 μmol/L GDC-0941) inhibition. Before fixation, the cross-sectional area of spheroids was measured to quantify 3D cell invasion (\( P < 0.01; \pm SD, n > 26 \) spheroid per condition). Spheroids were then cryosectioned and immunostained for vinculin and integrin \( \beta_1 \) to demonstrate that focal adhesions are not ablated after PI3K or FAK inhibition.
Figure 5. Vinculin-positive focal adhesions are colocalized to areas of altered membrane topography and composition. A, nonmalignant MEC in 3D collagen gels for 24 hours. Membrane protrusions are dependent on high (2.5 kPa) matrix stiffness; visualized via mEmerald-farnesyl membrane probe. B, organization of vinculin and the ventral plasma membrane of MEC plated on a 2D glass surfaces, via SAIM measurements of vinculin-GFP and mCherry-farnesyl. Axial position is plotted relative to the mean height of the membrane and vinculin, respectively. C, quantification of the significant decrease in membrane axial position at focal adhesions ( *, P < 0.01; ±SD, n > 18 cells per condition). D, line graphs showing relative abundance of PIP2 and PIP3 in the ventral membrane of nonmalignant MEC between 0 to 30 minutes following EGF stimulation. Ten minutes after stimulation, PIP2 significantly decreases and PIP3 significantly increases. However, inhibiting PI3K activity (1 μmol/L GDC-0941) is able to abrogate this increase in PIP3 ( *, P < 0.05; ±SE, n > 25 cells per condition). E, confocal images of premalignant MEC plated on 2D PA gels of increasing stiffness expressing vinculin-GFP activity mutants and a probe for PIP3 activity (mKO2-PH-Grp1), where red arrows indicate areas enriched in PIP3 activity. Measurements of all pixels in adhesions were averaged over whole cell; PIP3 was significantly enriched in focal adhesions of cells expressing activated vinculin T12 ( *, P < 0.05; ±SD, n > 7 cells per condition).
Figure 6. In vivo, vinculin is localized to cell–ECM borders and areas of high FAK and Akt signaling. A, p397 FAK and β1 integrin immunostaining of an ER/PR-positive human breast cancer tumor biopsy and representative H&E staining of these tissues. B, vinculin and immunostaining of an ER/PR-positive human breast cancer tumor biopsy. 397 FAK, β1 integrin, vinculin, and 473 Akt are enriched at the cell–ECM border in invasive tissue. C, vinculin, 397 FAK, and 473 Akt immunostaining of mammary gland tissue from FVB (normal) and PyMT background mice ± LOX cross-linking inhibition. Cross-linking reduces vinculin and 473 Akt recruitment. D, changes in integrin adhesome protein expression in 20 cancer types as compared with normal tissue and quantified from pathologically scored immunohistochemical data compiled by the Human Protein Atlas database. Adhesome proteins expressed at low levels in normal tissues were significantly more likely to be upregulated within cancer type (*, P < 0.01).
cross-linking and ECM stiffening were prevented by inhibiting lysyl oxidase activity (1, 65). It is therefore feasible that the concentration of vinculin at the invasive front tumors also affects the signaling of other growth- and survival-enhancing pathways, including enhanced Src-dependent growth or PI3K/PIP3/Akt-dependent activation of mTOR and altered cell metabolism or apoptosis resistance through Bcl-2 activation of Bim (66–69). Accordingly, strategies to target vinculin’s scaffolding function may offer a new therapeutic approach to treat premalignant lesions and may even help to identify high-risk, noninvasive lesions (70).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Figure 7. Model of how MEC invasion is dependent on ECM cues, vinculin engagement, and malignant transformation. A, model of mammary duct organization, which can be perturbed by malignant transformation or changes in ECM stiffness. Together, these events lead to cancer cell invasion through PI3K, FAK, and vinculin activation. B, biophysical model of vinculin-dependent adhesion signaling. Extracellular cues drive integrin activation, talin binding, and vinculin activation. Vinculin–talin–actin stabilization facilitates PI3K conversion of PIP2 to PIP3, activation of FAK and Akt, and intracellular signaling that leads to cancer cell invasion.
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References


Force Engages Vinculin and Promotes Tumor Progression by Enhancing PI3K Activation of Phosphatidylinositol (3,4,5)-Triphosphate
