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Tissue stiffness and hypoxia modulate the integrin-linked kinase ILK to control breast cancer stem-like cells

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Abstract

Breast tumors are stiffer and hypoxic compared to nonmalignant breast tissue. Here we report that stiff and hypoxic microenvironments promote the development of breast cancer stem-like cells (CSC) through modulation of the integrin-linked kinase ILK. Depleting ILK blocked stiffness and hypoxia-dependent acquisition of CSC marker expression and behavior, whereas ectopic expression of ILK stimulated CSC development under softer or normoxic conditions. Stiff microenvironments also promoted tumor formation and metastasis *in ovo*, where depleting ILK significantly abrogated the tumorigenic and metastatic potential of invasive breast cancer cells. We further found that the ILK-mediated phenotypes induced by stiff and hypoxic microenvironments are regulated by PI3K/Akt. Analysis of human breast cancer specimens revealed an association between substratum stiffness, ILK and CSC markers, insofar as ILK and CD44 co-localized in cancer cells located in tumor regions predicted to be stiff. Our results define ILK as a key mechanotransducer in modulating breast CSC development, in response to tissue mechanics and oxygen tension.

Keywords

mechanobiology; mechanotransduction; tension

Introduction

The mechanical stiffness of the cellular microenvironment, dominated by the composition and crosslinking of the extracellular matrix (ECM), is a key modulator of cell fate (1,2). For example, human mesenchymal stem cells differentiate down distinct lineages depending on the stiffness of their underlying substratum (1,3), and matrix stiffness regulates epithelial plasticity by controlling the induction of epithelial-mesenchymal transition (4). Increased matrix stiffness enhances tumor cell invasiveness and dissemination (5,6), and can direct the

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transformation of mammary epithelial cells (7). By contrast, tumors grown in mice with disorganized, compliant ECM architecture are minimally invasive (8). Stiff ECM has been found to promote tumor progression through the induction of signaling pathways downstream of integrins and phosphatidylinositol 3-kinase (PI3K) (9).

In addition to increased stiffness, invasive breast cancers are frequently hypoxic (10). Tumor hypoxia correlates with poor prognosis and decreased survival in breast cancer patients (11,12). Hypoxia can activate signaling pathways that regulate cancer stem-like cells (CSCs) (13), a distinct population of cancer cells with enhanced proliferative and invasive characteristics (14). Under hypoxic conditions, breast cancer cells express CSC-associated markers, including CD44, Nanog, CD49f, and ALDH (15-19). Hypoxic conditions also promote pluripotency and viability of CSC populations (20,21).

Integrin-linked kinase (ILK) is a crucial mediator of signal transmission from the ECM. ILK interacts with β 1-integrin and transmits extracellular signals from the ECM to regulate cellular activities including anchorage-dependent growth and survival, migration, invasion, differentiation, and tumor angiogenesis (22). Elevated expression of ILK has been closely associated with high-grade human tumors (23,24) and ILK has been shown to activate oncogenic pathways to promote tumor progression (25). However, it remains unclear how the expression of ILK and its downstream signaling are regulated in the stiff, hypoxic microenvironments common to invasive cancers. Because ILK is a critical adaptor used by cancer cells to sense their surrounding microenvironment, we hypothesized that matrix stiffness and hypoxia could affect ILK signaling in breast cancer cells to regulate breast CSC-associated gene expression and cellular behaviors. Here, we used engineered synthetic substrata to recapitulate the mechanical properties of the normal mammary gland as well as that of breast tumors. We investigated how the mechanical properties and oxygen tension in the tumor microenvironment affect the formation of breast CSCs. We found that breast CSC markers are activated synergistically in response to stiff, hypoxic conditions, and that ILK is an essential regulator of breast CSCs.

Materials and Methods

Cell culture and reagents

MDA-MB-231 human breast carcinoma cells and 4T1 murine mammary carcinoma cells were obtained from the ATCC and maintained in DMEM/F12 or RPMI base medium (respectively) that was supplemented with 10% FBS and 1% gentamycin. Both cell lines were authenticated by short tandem repeat genotyping, tested for mycoplasma contamination (DDC Medical), and were used before passage 20 and within 6 months after resuscitation. To reduce the expression of ILK, cells were transduced with lentiviral particles carrying short hairpin RNA (shRNA) against *ILK* (sc-35667-V and sc-35666-V, Santa Cruz) or control lentivirus expressing a scrambled shRNA sequence. Stable shRNA-expressing clones were produced according to the manufacturer's instructions and selected using puromycin. All cells were maintained in a humidified incubator held at 37°C and 5% CO₂.

To investigate the effect of matrix stiffness, cells were cultured on synthetic substrata of different compliances conjugated with fibronectin, which were prepared as described

previously (4). Cells were seeded at a density of 500,000 cells/cm² on synthetic substrata and cultured for 72 h at 37°C in either humidified normoxic conditions (95% air and 5% CO₂) or in a modular incubator chamber (Billups-Rothenberg, Inc.) filled with hypoxic gas (94% N₂, 1% O₂, and 5% CO₂).

Quantitative real time-PCR (qRT-PCR) analysis

Total RNA was extracted using TRIzol® reagent according to the manufacturer's instructions, followed by cDNA synthesis using the Verso cDNA synthesis kit (Thermo Scientific). Transcript levels were measured by qRT-PCR using a BioRad Mini Opticon instrument and iTaq Universal SYBR Green Supermix (Bio-Rad). Amplification was followed by melting curve analysis to verify the presence of a single PCR product. Primers specific for GFP, ILK, ITGB1, CD44, Nanog, CD49f, VEGF-A and 18S rRNA are listed in Table S1 (Supplementary Information). The expression level of each mRNA was normalized to that of 18S in the same sample.

Timelapse imaging and cell tracking

Timelapse imaging was performed using a Nikon Ti-U inverted microscope equipped with a stage top incubator maintained at 37°C in a 5% CO₂, 90% relative humidity atmosphere (Pathology Devices, Inc.). Images were acquired every 30 min using a Plan Fluor 10×/0.3 NA air objective (Nikon) and a Hamamatsu Orca-100 camera. Individual GFP-labeled cells were tracked from the aligned image sequences using the "Manual Tracking" plugin in ImageJ.

Immunoblotting analysis

Samples were lysed in radioimmunoprecipitation assay (RIPA) buffer (Pierce Biotechnology) supplemented with a protease inhibitor cocktail (Roche). Equal amounts of total protein were separated by standard electrophoresis using 4-12% gradient NuPage gels (Invitrogen). Proteins were transferred onto nitrocellulose membranes, which were then blocked and incubated overnight with primary antibodies at 4°C. Antibodies used for immunoblotting were: rabbit anti-β1-integrin (1:1000; Abcam), rabbit anti-ILK (1:1000; Abcam), rabbit anti-Nanog (1:1000; Novus Biologicals), mouse anti-CD44 (1:1000; Novus Biologicals), and rabbit anti-GAPDH (1:1000; Cell Signaling). After washing, blots were probed with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (1:5000; Cell Signaling) for 1 h. Blots were incubated with ECL Plus Western Blotting Detection System (GE Healthcare) for 5 min and signals were detected with a FluorChem E Imager (Cell Biosciences, Inc.).

Soft agar assay

Approximately 5000 cells were resuspended in serum-free DMEM/F12 medium supplemented with 0.35% agar and plated in a 12-well plate that contained a base layer of DMEM/F12 medium supplemented with 0.7% agar. Cells were incubated for 14 days and the culture medium was changed twice per week. At the end of the experiment, colonies were fixed with 4% PFA, washed with PBS, and imaged. Colonies were counted if they were larger than 10 μm in diameter as measured using ImageJ.

Aldefluor assay and flow cytometry

Cells were harvested by trypsinization using Accutase (eBioscience), washed with PBS, and resuspended in flow cytometry staining buffer (eBioscience). Cells were filtered through a 40 μm cell strainer (Corning) to obtain single cell suspensions. For intracellular staining, cells were fixed and permeabilized using Foxp3 transcription factor staining buffer set (eBioscience) prior to staining. Cells were stained without fixation for cell surface markers. Cells were incubated with the following fluorochrome-conjugated antibodies at 4°C for 45 min: CD49f (1:200, clone GoH3; eBioscience), CD44 (1:100, clone IM7; eBioscience), and Nanog (1:100, clone eBioMLC-51; eBioscience). Stained cells were washed twice with flow cytometry buffer (eBioscience). To assess ALDH activity, the ALDEFLUOR™ fluorescent reagent system (Stem Cell Technologies) was used according to the manufacturer's instructions. Stained cells were analyzed using an LSRII flow cytometer (BD Bioscience) and data were analyzed using FlowJo.

Secondary mammosphere formation assay

A single cell suspension was seeded at a density of 3000 cells per well into 12-well ultra-low attachment plates (BD Biosciences). Cells were cultured in DMEM/F12 medium containing 20 ng/ml epidermal growth factor and 20 ng/ml basic fibroblast growth factor, and culture medium was changed twice per week. After 7 days, primary mammospheres were collected and disassociated enzymatically with 0.05% trypsin (Invitrogen) for 5 min at 37°C and mechanically by filtering through a 40 μm cell strainer (Corning). Single cell suspensions were replated onto 12-well ultra-low attachment plates at a density of 1000 cells per well. After 14 days, secondary mammospheres were transferred to a 12-well plate. Serum-containing medium was added and secondary mammospheres were allowed to attach to the bottom surface of the plate. After 12 h, cells were fixed with 4% PFA and stained with 0.05% crystal violet. Samples were imaged, analyzed using ImageJ, and mammospheres were counted if they had a diameter larger than 50 μm .

Mechanical testing of collagen gels

The elastic moduli of gels comprising different concentrations of collagen were estimated from unconfined compression experiments, as described previously (26). Briefly, collagen solutions containing 500 nm fluorescent beads were allowed to gel for at least 30 min at 37°C within circular polydimethylsiloxane (Sylgard 184) molds. After gelation, the mold was removed and the cylindrical gel was immersed in PBS. Confocal stacks of the gel were acquired before and 1 min after loading a glass coverslip of known weight to the top of the gel. The thickness of the gel was estimated from the fluorescence signal of the embedded beads and, as a first approximation, the measured change in thickness was used to estimate the elastic modulus of the gel.

Chicken chorioallantoic membrane (CAM) assay

CAM assays were performed as described (27). Cells were transduced with recombinant adenovirus encoding GFP at an MOI of 100 for 24 h prior to implantation. GFP-transduced cells were resuspended in neutralized rat tail type I collagen (BD Biosciences) at a density of 300,000 cells per 30 μl pellet. Cell-embedded collagen gels were implanted on the CAM of a

chicken embryo at day 7 of incubation. Tumor formation, angiogenesis, and metastasis were scored in live embryos 5 days later using a stereomicroscope. Images were acquired using a Nikon digital camera.

Human breast cancer samples

Breast cancer biopsies were derived from waste surgical material from de-identified patients, and were formalin-fixed and paraffin-embedded, per approval by the Mayo Clinic Institutional Review Board. Tissue sections were deparaffinised by placing them into three changes of xylene and rehydrated in a graded ethanol series. The rehydrated tissue samples were rinsed in water and sections were subjected to heat antigen retrieval as described by the manufacturer (DAKO). Slices were incubated with each primary antibody for 1 h at room temperature. Sections were then rinsed with Tris-buffered saline/Triton-X-100 (TBST) wash buffer, and incubated with each secondary antibody for 30 min. For fluorescent detection, tissue sections were rinsed 3 times for 5 min each with PBS containing 1.43 μM 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific). Sections were rinsed with PBS and coverslips mounted with anti-fade mounting medium (DAKO). Sections stained for collagen I were rinsed with TBST wash buffer, and secondary incubation was with DAKO Envision anti-rabbit, horseradish peroxidase (HRP) for 15 min. Tissue sections were rinsed with TBST wash buffer and then incubated in 3,3'-diaminobenzidine (DAB+; DAKO), and counterstained with modified Schmidt's hematoxylin. Each antibody and its corresponding fluorescent secondary antibody used were: ILK1 (rabbit polyclonal, Cell Signaling Technology #3862) detected by Alexa594-conjugated donkey anti-rabbit IgG (Invitrogen, #A21207); CD44 (mouse IgG2A, Abcam #ab6124) detected by Alexa488-conjugated donkey anti-mouse IgG (H+L) (Invitrogen, #A21202). For DAB staining, collagen I (Abcam ab138492) was used.

Whole slide digital images of each breast cancer sample were captured with the Aperio Scanscope AT2 slide scanner (collagen) and the Aperio Scanscope FL slide scanner (fluorescent images) using a 20 \times objective. Using the digitized images, areas of high and low collagen content were selected and circled. Cell number in each area was determined by manually counting the number of DAPI-positive nuclei. Similarly, double positive cells (488 and 594) were identified and counted in each selected area. Density of double positive cells was calculated as the total number of positively stained cells per total number of cells in each selected area.

Statistical analysis

Data represent mean \pm s.e.m of at least three independent experiments conducted in triplicate. For CAM assays, data represent mean \pm s.e.m with $n = 5$ chicken embryos per group. For patient samples, statistical significance was tested with the Mann-Whitney U test; for all others, statistical analysis was conducted using a two-way ANOVA followed by Bonferroni post-test or Student's t test. $P < 0.05$ was considered to represent a significant difference between conditions.

Results

Substratum stiffness and oxygen tension regulate the expression of β 1-integrin and ILK in invasive breast cancer cells

To investigate how ECM compliance affects breast cancer cells, we used synthetic polyacrylamide substrata to mimic the compliance of the microenvironment in the normal mammary gland (Young's modulus, $E \sim 130$ Pa, "soft") and in breast tumors ($E \sim 4020$ Pa, "stiff") (4). On soft substrata, 4T1 mouse mammary carcinoma cells (Figure 1A) and MDA-MB-231 human breast carcinoma cells (Figure S1) were rounded in morphology. In contrast, both 4T1 and MDA-MB-231 cells displayed an elongated morphology on stiff substrata. Consistent with the expectation that the elongated cell morphology on stiff substrata was associated with increased interactions with the ECM, RT-PCR and immunoblotting revealed increased expression of β 1-integrin and ILK in the cells cultured on stiff substrata at the transcript (Figure 1B, C) and protein (Figure 1D) levels.

Hypoxia is prevalent in solid tumors and has been shown to activate stem cell-like properties in several cancers, including breast cancer (28). We found that hypoxia also led to an increase in the transcript and protein levels of β 1-integrin (Figure 1B, D) and ILK (Figure 1C, D) in both 4T1 and MDA-MB-231 cells (Figure S1). A stiff, hypoxic microenvironment induced the highest expression of these markers (Figure 1B-D; Figure S1). These results suggest that stiff and hypoxic microenvironments potentiate signaling downstream of β 1-integrin and ILK in breast cancer cells.

The presence of breast CSCs correlates with metastasis and relapse in breast cancer patients (29). To determine whether the mechanical microenvironment and oxygen tension can regulate breast CSC gene expression, we examined the relative effects of substratum stiffness and hypoxia on the expression of the CSC markers, CD44, Nanog, CD49f, and ALDH. We found that substratum stiffness and hypoxia led to synergistic increases in the levels of each of these markers in both 4T1 and MDA-MB-231 cells (Figure 1E-H; Figure S1).

ILK has been found to regulate integrin function and cell motility. We evaluated the effect of stiff substrata on cell movement using time-lapse imaging, which revealed that breast cancer cells cultured on stiff substrata were more motile than those cultured on soft substrata (Figure 1I). Together, these data suggest that the increased stiffness and decreased oxygen tension of the tumor microenvironment enhances integrin signaling and activates CSC marker expression in invasive breast cancer cells.

ILK and CD44 are elevated in potentially stiff regions of human tumors

To evaluate the relationship between stiffness, ILK expression, and CSC marker induction *in vivo*, we examined the expression of ILK and CD44 in human breast cancer samples. We compared these to relative collagen content, which is highly correlated with stiffness in mammary cancers (30,31). Immunofluorescence analysis revealed that most tumor cells expressed low levels of ILK and were CD44-negative in regions of the tumors that contained low levels of collagen (col^{lo}), whereas ILK and CD44 double-positive tumor cells were found in regions that contained high levels of collagen (col^{hi}) (Figure 1J). In col^{hi} regions,

more than 25% of the cells expressed both ILK and CD44. In contrast, only ~4% of the cells expressed both markers in col^{lo} regions (Figure 1K). These data are consistent with the results of our cell culture experiments, and suggest that breast CSCs are preferentially located in stiff microenvironments.

Depleting ILK prevents CSC development in cells on stiff substrata

β 1-integrin transmits cues from the ECM through ILK to control cellular behavior (22), and enhanced signaling through ILK has been implicated in human cancer (23,24). Matrix stiffness has been shown to increase integrin signaling and promote tumor progression (9). To define the role of ILK in the CSC response to stiffness and hypoxia, we used short hairpin RNA (shRNA) to stably deplete *ILK* in 4T1 (4T1-shILK) (Figure 2A) and MDA-MB-231 cells (Figure S2). Depleting ILK led to a significant change in the morphology of 4T1 cells cultured on stiff substratum, as compared to scrambled controls (4T1-shctrl) (Figure 2B). On stiff substrata, 4T1-shILK cells exhibited a rounded morphology, similar to those on soft substrata, under both normoxia and hypoxia. We also found that knockdown of ILK reduced the expression of β 1-integrin (Figure 2C) and the CSC markers CD44 (Figure 2D), Nanog (Figure 2E), and CD49f (Figure 2F) in 4T1 and MDA-MB-231 cells (Figure S2) under both normoxia and hypoxia. To examine the role of ILK in the regulation of the CSC characteristics of anchorage-independent growth and self-renewal, we performed secondary mammosphere formation and soft agar assays. We found that depleting ILK abrogated the ability of both 4T1 and MDA-MB-231 cells to form secondary mammospheres (Figure 2G; Figure S2) and colonies in soft agar (Figure 2H; Figure S2). Together, these data suggest that ILK is necessary for the induction of the CSC marker expression and behavior in breast cancer cells in response to matrix stiffness and hypoxia.

ILK expression enhances breast CSC development

To determine whether ILK is sufficient to induce the development of breast CSCs in the absence of a stiff microenvironment, we expressed ILK ectopically using a bicistronic recombinant adenovirus encoding for ILK and GFP (adILK). As a control, we used adenovirus encoding for GFP alone (adGFP). Transduction with adILK approximately doubled the levels of ILK transcript and protein in 4T1 (Figure 3A) and MDA-MB-231 cells (Figure S3), and increased phosphorylation of Akt (pAkt; S437) (Figure 3B; Figure S3). qRT-PCR analysis revealed that ectopic expression of ILK increased the transcript levels of β 1-integrin (Figure 3C), CD44 (Figure 3D), Nanog (Figure 3E), and CD49f (Figure 3F) in 4T1 and MDA-MB-231 cells (Figure S3) cultured on soft or stiff substrata under normoxia or hypoxia. Ectopic expression of ILK also enhanced the formation of secondary mammospheres (Figure 3G; Figure S3) and colonies in soft agar (Figure 3H; Figure S3), demonstrating that increased expression of ILK can further activate CSC gene expression and behavior even when cells are cultured on stiff substrata under hypoxic conditions.

ILK signals through PI3K/Akt to regulate CSC development

Previous work has suggested that ILK regulates cell survival through the PI3K/Akt pathway (32), which has also been implicated in cancer cell (33,34) and CSC survival (35,36), cancer cell proliferation (37,38), and the CSC phenotype (39). We found that knockdown of ILK reduced the phosphorylation of Akt (Figure 4A; Figure S4). Disrupting signaling through

PI3K by treating cells with the selective inhibitor, LY294002 (Figure 4B; Figure S4), also decreased the transcript levels of β 1-integrin (Figure 4C) and ILK (Figure 4D) in 4T1 and MDA-MB-231 cells (Figure S4) cultured on soft or stiff substrata under normoxia or hypoxia. Consistent with the concept that Akt activation is necessary for the ILK-dependent induction of CSCs in response to hypoxia or a stiff microenvironment, the levels of CD44 (Figure 4E), Nanog (Figure 4F), and CD49f (Figure 4G) decreased significantly in response to treatment with LY294002 in 4T1 and MDA-MB-231 cells (Figure S4) under all conditions.

Similarly, treatment with LY294002 reduced the formation of both secondary mammospheres (Figure 4H) and colonies in soft agar (Figure 4I). These data suggest that a stiff, hypoxic microenvironment regulates development of CSCs in part by activating signaling through ILK and PI3K.

Stiff substratum promotes angiogenesis and dissemination of tumor cells through ILK

To test directly whether stiff substratum promotes tumorigenesis and tumor cell dissemination, we embedded 4T1 cells in collagen gels of low (col^{lo} , 3 mg/ml), medium (col^{med} , 4.5 mg/ml), or high (col^{hi} , 6 mg/ml) stiffness (Figure 5A) and then grafted them onto the chorioallantoic membranes (CAMs) of chicken embryos (40). We found that all col^{hi} -grafted CAMs formed primary tumors at the graft sites, whereas none of the col^{lo} - and only 4 out of 6 of the col^{med} -grafted CAMs developed primary tumors (Figure 5B). The tumors that formed in high collagen developed multiple micrometastases near blood vessels adjacent to the graft sites (Figure 5C). To define the effects of ILK on tumor formation, we performed CAM assays using 4T1-shctrl or 4T1-shILK cells embedded in high collagen gels. Both 4T1-shctrl and 4T1-shILK cells formed primary tumors on the CAM (Figure 5D). However, the 4T1-shILK primary tumors were half the size of the 4T1-shctrl tumors (Figure 5D). To examine how ILK affects metastatic potential, we performed qRT-PCR analysis for GFP in the lungs of the chicken embryos after 5 days of incubation with GFP-labeled 4T1 cells. We found significantly lower levels of GFP in the lungs of embryos with 4T1-shILK-grafted CAMs than in those with 4T1-shctrl-grafted CAMs (Figure 5E). These data suggest that matrix stiffness and ILK affect tumor formation as well as tumor cell dissemination.

Angiogenesis is a hallmark of cancer that is critical for tumor growth and metastasis (41). To investigate how substratum stiffness affects hypoxia-dependent angiogenesis, we examined the expression of vascular endothelial growth factor-A (VEGF-A) in 4T1 cells cultured on soft or stiff substrata under normoxia or hypoxia. We found that culture on stiff substratum upregulated the expression of VEGF-A (Figure 5F), suggesting that the mechanical properties of the microenvironment can regulate angiogenic signaling. As expected, hypoxia further increased the levels of VEGF-A in cells cultured on both soft and stiff substrata (Figure 5F). Depleting ILK significantly reduced VEGF-A expression in 4T1 cells cultured on soft or stiff substrata under normoxia or hypoxia (Figure 5G). Quantitative image analysis revealed that the blood vessel density in the CAM adjacent to 4T1-shILK tumors was significantly lower than that adjacent to 4T1-shctrl tumors (Figure 5H). These data suggest that loss of ILK impairs the angiogenic potential of breast cancer cells. Consistently, we found that inhibiting PI3K with LY294002 significantly reduced the levels of VEGF-A

(Figure 5I), whereas ectopic expression of ILK increased the levels of VEGF-A (Figure 5J) under all culture conditions. These data suggest that ILK signals through the PI3K pathway to regulate VEGF-A expression in response to matrix stiffness and hypoxia.

Discussion

It is well appreciated that the distinct physical properties of the tumor microenvironment can affect cancer cell fate. Tumor stiffness induces integrin clustering and downstream signaling that control cancer cell proliferation, gene expression, and invasiveness (8,42,43).

Separately, hypoxia promotes CSC properties, in part through activation of hypoxia-inducible factors (HIFs) that regulate the expression of stem cell markers (16,44). Here, we found that these two features of the tumor microenvironment, hypoxia and substratum stiffness, work synergistically through ILK to enhance breast CSC gene expression, tumor growth, and metastasis. Future work using limiting dilution assays *in vivo* with cells primed by hypoxia, stiff substrata, and/or ILK might clarify which subpopulation has the highest tumor-initiating potential, but experimental approaches that maintain the microenvironmental conditions of the injected cells need to be developed. Nonetheless, our findings are congruent with clinical data showing that hypoxia and stiffness correlate with a poor prognosis in breast cancer (45,46).

Also known as tumor-repopulating cells or tumor-initiating cells, CSCs represent a self-renewing subpopulation of cancer cells that promote tumor progression of many solid cancers, including those of the breast, colon, and brain (47). While our data suggest that stiff and hypoxic microenvironments promote the development of CSCs from breast cancer cells, this relationship is probably not generalizable to all tumor types and stages. Indeed, melanoma tumor-repopulating cells preferentially self-renew on soft substrata in a Sox2-dependent manner (48). In contrast, glioblastoma tumor-initiating cells are insensitive to matrix mechanics, and enhancing their cytoskeletal contractility causes a loss of their tumor-promoting properties (49). In the breast, mechanosensing and mechanotransduction are altered as a function of aging (50), so the effects of stiffness on CSCs likely also depends on age.

Matrix stiffness clearly activates signaling through β 1-integrin, which transmits information about the mechanical properties of the microenvironment through the ILK/PI3K/Akt pathway and eventually to CSC-related genes, including the CSC markers examined here – CD44, Nanog, CD49f, and ALDH. ILK is well appreciated for its roles in stemness and metastasis (33,34,37) and is known to stimulate tumor angiogenesis through VEGF-A (51). Our data suggest a positive feedback loop in which the signaling activated by ILK induces increased expression of mechanosensors, including β 1-integrin and ILK itself. Hypoxia enhances activation of this mechanotransduction pathway, and while the exact mechanism by which this occurs is not clear, hypoxia has been found to upregulate the expression of ILK in prostate cancer (52) and colorectal cancer cells (53) in a HIF1 α -dependent manner (54). Recent studies indicate that HIF1 α can drive breast cancer metastasis through ECM stiffening and collagen fiber alignment (55). It is possible that hypoxia promotes ECM remodelling and, concomitantly, elevates the levels of ILK to prime breast cancer cells to be more sensitive to mechanically stiff microenvironments. Our results clearly indicate that loss

of ILK abrogates the mechanosensing capability of tumor cells, prevents the development of CSCs, and blocks tumor growth and dissemination. These data suggest that ILK acts as a critical mechanosensor that signals through the PI3K/Akt pathway to mediate the formation of breast CSCs under stiff and hypoxic conditions. The stiff, hypoxic regions of a tumor might, therefore, be responsible for inducing both CSC gene expression and behavior in tumor cells residing within, as well as angiogenesis to facilitate tumor cell dissemination. Therapies targeted at microenvironment-induced signaling would need to address both physical properties to disrupt the formation and metastatic spread of breast CSCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CAM	chorioallantoic membrane
CSC	cancer stem-like cell
ECM	extracellular matrix
HIF	hypoxia-inducible factor
ILK	integrin-linked kinase
PI3K	phosphatidylinositol 3-kinase
shRNA	short hairpin RNA
VEGF	vascular-endothelial growth factor

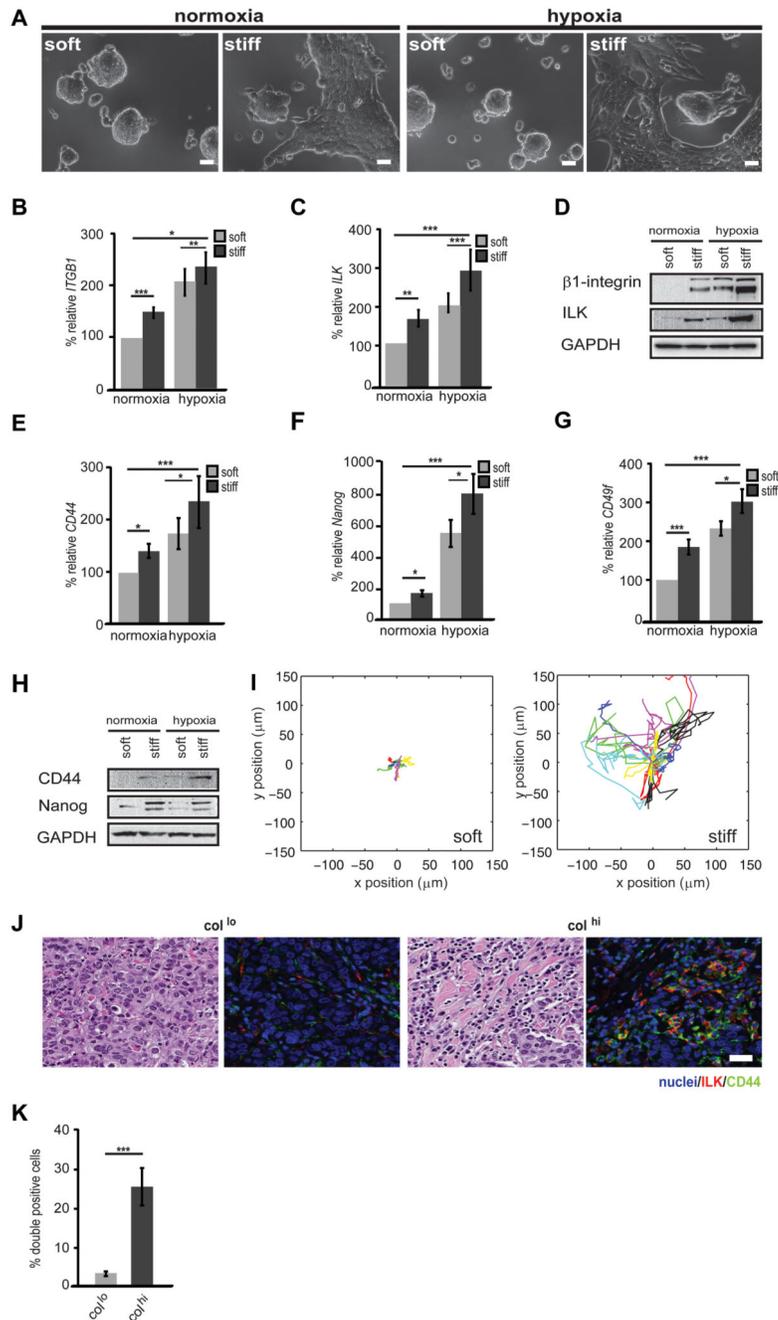
References

1. Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. *Cell*. 2006; 126(4):677–89. [PubMed: 16923388]
2. Butcher DT, Alliston T, Weaver VM. A tense situation: forcing tumour progression. *Nat Rev Cancer*. 2009; 9(2):108–22. [PubMed: 19165226]
3. Lui C, Lee K, Nelson CM. Matrix compliance and RhoA direct the differentiation of mammary progenitor cells. *Biomech Model Mechanobiol*. 2012; 11(8):1241–9. [PubMed: 22161021]
4. Lee K, Chen QK, Lui C, Cichon MA, Radisky DC, Nelson CM. Matrix compliance regulates Rac1b localization, NADPH oxidase assembly, and epithelial-mesenchymal transition. *Mol Biol Cell*. 2012; 23(20):4097–108. [PubMed: 22918955]

5. Boyd NF, Rommens JM, Vogt K, Lee V, Hopper JL, Yaffe MJ, et al. Mammographic breast density as an intermediate phenotype for breast cancer. *Lancet Oncol.* 2005; 6(10):798–808. [PubMed: 16198986]
6. Li T, Sun L, Miller N, Nicklee T, Woo J, Hulse-Smith L, et al. The association of measured breast tissue characteristics with mammographic density and other risk factors for breast cancer. *Cancer Epidemiol Biomarkers Prev.* 2005; 14(2):343–9. [PubMed: 15734956]
7. Paszek MJ, Zahir N, Johnson KR, Lakins JN, Rozenberg GI, Gefen A, et al. Tensional homeostasis and the malignant phenotype. *Cancer Cell.* 2005; 8(3):241–54. [PubMed: 16169468]
8. Goetz JG, Minguet S, Navarro-Lerida I, Lazzano JJ, Samaniego R, Calvo E, et al. Biomechanical remodeling of the microenvironment by stromal caveolin-1 favors tumor invasion and metastasis. *Cell.* 2011; 146(1):148–63. [PubMed: 21729786]
9. Levental KR, Yu H, Kass L, Lakins JN, Egeblad M, Erler JT, et al. Matrix crosslinking forces tumor progression by enhancing integrin signaling. *Cell.* 2009; 139(5):891–906. [PubMed: 19931152]
10. Axelson H, Fredlund E, Ovenberger M, Landberg G, Pahlman S. Hypoxia-induced dedifferentiation of tumor cells--a mechanism behind heterogeneity and aggressiveness of solid tumors. *Semin Cell Dev Biol.* 2005; 16(4-5):554–63. [PubMed: 16144692]
11. Bos R, Zhong H, Hanrahan CF, Mommers EC, Semenza GL, Pinedo HM, et al. Levels of hypoxia-inducible factor-1 alpha during breast carcinogenesis. *J Natl Cancer Inst.* 2001; 93(4):309–14. [PubMed: 11181778]
12. Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, et al. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer Res.* 1999; 59(22):5830–5. [PubMed: 10582706]
13. Miller KD. E2100: a phase III trial of paclitaxel versus paclitaxel/bevacizumab for metastatic breast cancer. *Clin Breast Cancer.* 2003; 3(6):421–2. [PubMed: 12636887]
14. Valent P, Bonnet D, De Maria R, Lapidot T, Copland M, Melo JV, et al. Cancer stem cell definitions and terminology: the devil is in the details. *Nat Rev Cancer.* 2012; 12(11):767–75. [PubMed: 23051844]
15. Xing F, Okuda H, Watabe M, Kobayashi A, Pai SK, Liu W, et al. Hypoxia-induced Jagged2 promotes breast cancer metastasis and self-renewal of cancer stem-like cells. *Oncogene.* 2011; 30(39):4075–86. [PubMed: 21499308]
16. Mathieu J, Zhang Z, Zhou W, Wang AJ, Heddleston JM, Pinna CM, et al. HIF induces human embryonic stem cell markers in cancer cells. *Cancer Res.* 2011; 71(13):4640–52. [PubMed: 21712410]
17. Louie E, Nik S, Chen JS, Schmidt M, Song B, Pacson C, et al. Identification of a stem-like cell population by exposing metastatic breast cancer cell lines to repetitive cycles of hypoxia and reoxygenation. *Breast Cancer Res.* 2010; 12(6):R94. [PubMed: 21067584]
18. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A.* 2003; 100(7):3983–8. [PubMed: 12629218]
19. Velasco-Velazquez MA, Homsí N, De La Fuente M, Pestell RG. Breast cancer stem cells. *Int J Biochem Cell Biol.* 2012; 44(4):573–7. [PubMed: 22249027]
20. Koh MY, Powis G. Passing the baton: the HIF switch. *Trends Biochem Sci.* 2012; 37(9):364–72. [PubMed: 22818162]
21. Yoshida Y, Takahashi K, Okita K, Ichisaka T, Yamanaka S. Hypoxia enhances the generation of induced pluripotent stem cells. *Cell Stem Cell.* 2009; 5(3):237–41. [PubMed: 19716359]
22. Hannigan G, Troussard AA, Dedhar S. Integrin-linked kinase: a cancer therapeutic target unique among its ILK. *Nat Rev Cancer.* 2005; 5(1):51–63. [PubMed: 15630415]
23. Graff JR, Deddens JA, Konicek BW, Colligan BM, Hurst BM, Carter HW, et al. Integrin-linked kinase expression increases with prostate tumor grade. *Clin Cancer Res.* 2001; 7(7):1987–91. [PubMed: 11448915]
24. Ito R, Oue N, Zhu X, Yoshida K, Nakayama H, Yokozaki H, et al. Expression of integrin-linked kinase is closely correlated with invasion and metastasis of gastric carcinoma. *Virchows Arch.* 2003; 442(2):118–23. [PubMed: 12596061]

25. Chan J, Ko FC, Yeung YS, Ng IO, Yam JW. Integrin-linked kinase overexpression and its oncogenic role in promoting tumorigenicity of hepatocellular carcinoma. *PLoS One*. 2011; 6(2):e16984. [PubMed: 21347395]
26. Varner VD, Gleghorn JP, Miller E, Radisky DC, Nelson CM. Mechanically patterning the embryonic airway epithelium. *Proc Natl Acad Sci U S A*. 2015; 112(30):9230–5. [PubMed: 26170292]
27. Palmer TD, Lewis J, Zijlstra A. Quantitative analysis of cancer metastasis using an avian embryo model. *J Vis Exp*. 2011; (51)
28. Favaro E, Lord S, Harris AL, Buffa FM. Gene expression and hypoxia in breast cancer. *Genome Med*. 2011; 3(8):55. [PubMed: 21875443]
29. Velasco-Velazquez MA, Popov VM, Lisanti MP, Pestell RG. The role of breast cancer stem cells in metastasis and therapeutic implications. *Am J Pathol*. 2011; 179(1):2–11. [PubMed: 21640330]
30. Cox TR, Erler JT. Remodeling and homeostasis of the extracellular matrix: implications for fibrotic diseases and cancer. *Dis Model Mech*. 2011; 4(2):165–78. [PubMed: 21324931]
31. Fenner J, Stacer AC, Winterroth F, Johnson TD, Luker KE, Luker GD. Macroscopic stiffness of breast tumors predicts metastasis. *Sci Rep*. 2014; 4:5512. [PubMed: 24981707]
32. Nho RS, Xia H, Kahm J, Kleidon J, Diebold D, Henke CA. Role of integrin-linked kinase in regulating phosphorylation of Akt and fibroblast survival in type I collagen matrices through a beta1 integrin viability signaling pathway. *J Biol Chem*. 2005; 280(28):26630–9. [PubMed: 15905178]
33. Garcia-Regalado A, Vargas M, Garcia-Carranca A, Arechaga-Ocampo E, Gonzalez-De la Rosa CH. Activation of Akt pathway by transcription-independent mechanisms of retinoic acid promotes survival and invasion in lung cancer cells. *Mol Cancer*. 2013; 12:44. [PubMed: 23693014]
34. Clark AS, West K, Streicher S, Dennis PA. Constitutive and inducible Akt activity promotes resistance to chemotherapy, trastuzumab, or tamoxifen in breast cancer cells. *Mol Cancer Ther*. 2002; 1(9):707–17. [PubMed: 12479367]
35. Hambardzumyan D, Becher OJ, Rosenblum MK, Pandolfi PP, Manova-Todorova K, Holland EC. PI3K pathway regulates survival of cancer stem cells residing in the perivascular niche following radiation in medulloblastoma in vivo. *Genes Dev*. 2008; 22(4):436–48. [PubMed: 18281460]
36. Dubrovskaya A, Kim S, Salamone RJ, Walker JR, Maira SM, Garcia-Echeverria C, et al. The role of PTEN/Akt/PI3K signaling in the maintenance and viability of prostate cancer stem-like cell populations. *Proc Natl Acad Sci U S A*. 2009; 106(1):268–73. [PubMed: 19116269]
37. Yang XL, Lin FJ, Guo YJ, Shao ZM, Ou ZL. Gemcitabine resistance in breast cancer cells regulated by PI3K/AKT-mediated cellular proliferation exerts negative feedback via the MEK/MAPK and mTOR pathways. *Onco Targets Ther*. 2014; 7:1033–42. [PubMed: 24966685]
38. Li X, Kong X, Wang Y, Yang Q. BRCC2 inhibits breast cancer cell growth and metastasis in vitro and in vivo via downregulating AKT pathway. *Cell Death Dis*. 2013; 4:e757. [PubMed: 23928696]
39. Bleau AM, Hambardzumyan D, Ozawa T, Fomchenko EI, Huse JT, Brennan CW, et al. PTEN/PI3K/Akt pathway regulates the side population phenotype and ABCG2 activity in glioma tumor stem-like cells. *Cell Stem Cell*. 2009; 4(3):226–35. [PubMed: 19265662]
40. Lokman NA, Elder AS, Ricciardelli C, Oehler MK. Chick chorioallantoic membrane (CAM) assay as an in vivo model to study the effect of newly identified molecules on ovarian cancer invasion and metastasis. *Int J Mol Sci*. 2012; 13(8):9959–70. [PubMed: 22949841]
41. Weis SM, Cheresh DA. Tumor angiogenesis: molecular pathways and therapeutic targets. *Nat Med*. 2011; 17(11):1359–70. [PubMed: 22064426]
42. Provenzano PP, Inman DR, Eliceiri KW, Knittel JG, Yan L, Rueden CT, et al. Collagen density promotes mammary tumor initiation and progression. *BMC Med*. 2008; 6:11. [PubMed: 18442412]
43. Paszek MJ, Weaver VM. The tension mounts: mechanics meets morphogenesis and malignancy. *J Mammary Gland Biol Neoplasia*. 2004; 9(4):325–42. [PubMed: 15838603]
44. Xiang L, Gilkes DM, Hu H, Takano N, Luo W, Lu H, et al. Hypoxia-inducible factor 1 mediates TAZ expression and nuclear localization to induce the breast cancer stem cell phenotype. *Oncotarget*. 2014; 5(24):12509–27. [PubMed: 25587023]

45. Schindl M, Schoppmann SF, Samonigg H, Hausmaninger H, Kwasny W, Gnant M, et al. Overexpression of hypoxia-inducible factor 1alpha is associated with an unfavorable prognosis in lymph node-positive breast cancer. *Clin Cancer Res.* 2002; 8(6):1831–7. [PubMed: 12060624]
46. Wei SC, Fattet L, Tsai JH, Guo Y, Pai VH, Majeski HE, et al. Matrix stiffness drives epithelial-mesenchymal transition and tumour metastasis through a TWIST1-G3BP2 mechanotransduction pathway. *Nat Cell Biol.* 2015; 17(5):678–88. [PubMed: 25893917]
47. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature.* 2001; 414(6859):105–11. [PubMed: 11689955]
48. Tan Y, Tajik A, Chen J, Jia Q, Chowdhury F, Wang L, et al. Matrix softness regulates plasticity of tumour-repopulating cells via H3K9 demethylation and Sox2 expression. *Nat Commun.* 2014; 5:4619. [PubMed: 25099074]
49. Wong SY, Ulrich TA, Deleyrolle LP, MacKay JL, Lin JM, Martuscello RT, et al. Constitutive activation of myosin-dependent contractility sensitizes glioma tumor-initiating cells to mechanical inputs and reduces tissue invasion. *Cancer Res.* 2015; 75(6):1113–22. [PubMed: 25634210]
50. Pelissier FA, Garbe JC, Ananthanarayanan B, Miyano M, Lin C, Jokela T, et al. Age-related dysfunction in mechanotransduction impairs differentiation of human mammary epithelial progenitors. *Cell Rep.* 2014; 7(6):1926–39. [PubMed: 24910432]
51. Tan C, Cruet-Hennequart S, Troussard A, Fazli L, Costello P, Sutton K, et al. Regulation of tumor angiogenesis by integrin-linked kinase (ILK). *Cancer Cell.* 2004; 5(1):79–90. [PubMed: 14749128]
52. Chou CC, Chuang HC, Salunke SB, Kulp SK, Chen CS. A novel HIF-1alpha-integrin-linked kinase regulatory loop that facilitates hypoxia-induced HIF-1alpha expression and epithelial-mesenchymal transition in cancer cells. *Oncotarget.* 2015; 6(10):8271–85. [PubMed: 25821081]
53. Xiao L, Yue X, Ming X, Xu L, Ding M, Xu J, et al. The integrin-linked kinase gene up-regulated by hypoxia plays its pro-survival role in colorectal cancer cells. *J Recept Signal Transduct Res.* 2014; 34(1):64–72. [PubMed: 24299190]
54. Abboud ER, Coffelt SB, Figueroa YG, Zwezaryk KJ, Nelson AB, Sullivan DE, et al. Integrin-linked kinase: a hypoxia-induced anti-apoptotic factor exploited by cancer cells. *Int J Oncol.* 2007; 30(1):113–22. [PubMed: 17143519]
55. Gilkes DM, Bajpai S, Chaturvedi P, Wirtz D, Semenza GL. Hypoxia-inducible factor 1 (HIF-1) promotes extracellular matrix remodeling under hypoxic conditions by inducing P4HA1, P4HA2, and PLOD2 expression in fibroblasts. *J Biol Chem.* 2013; 288(15):10819–29. [PubMed: 23423382]

**Figure 1.**

Stiff and hypoxic microenvironments promote the expression of β 1-integrin, ILK, and CSC markers. (A) Phase contrast images of 4T1 mouse mammary carcinoma cells cultured on soft ($E \sim 130$ Pa) or stiff ($E \sim 4020$ Pa) polyacrylamide substrata under normoxia or hypoxia. Transcript levels of (B) β 1-integrin (ITGB1) and (C) ILK in 4T1 cells cultured on soft or stiff substrata under normoxia or hypoxia. (D) Immunoblotting analysis for β 1-integrin and ILK in 4T1 cells cultured on soft or stiff substrata under normoxia or hypoxia. GAPDH was used as a loading control. Transcript levels of (E) CD44, (F) Nanog, and (G) CD49f in 4T1

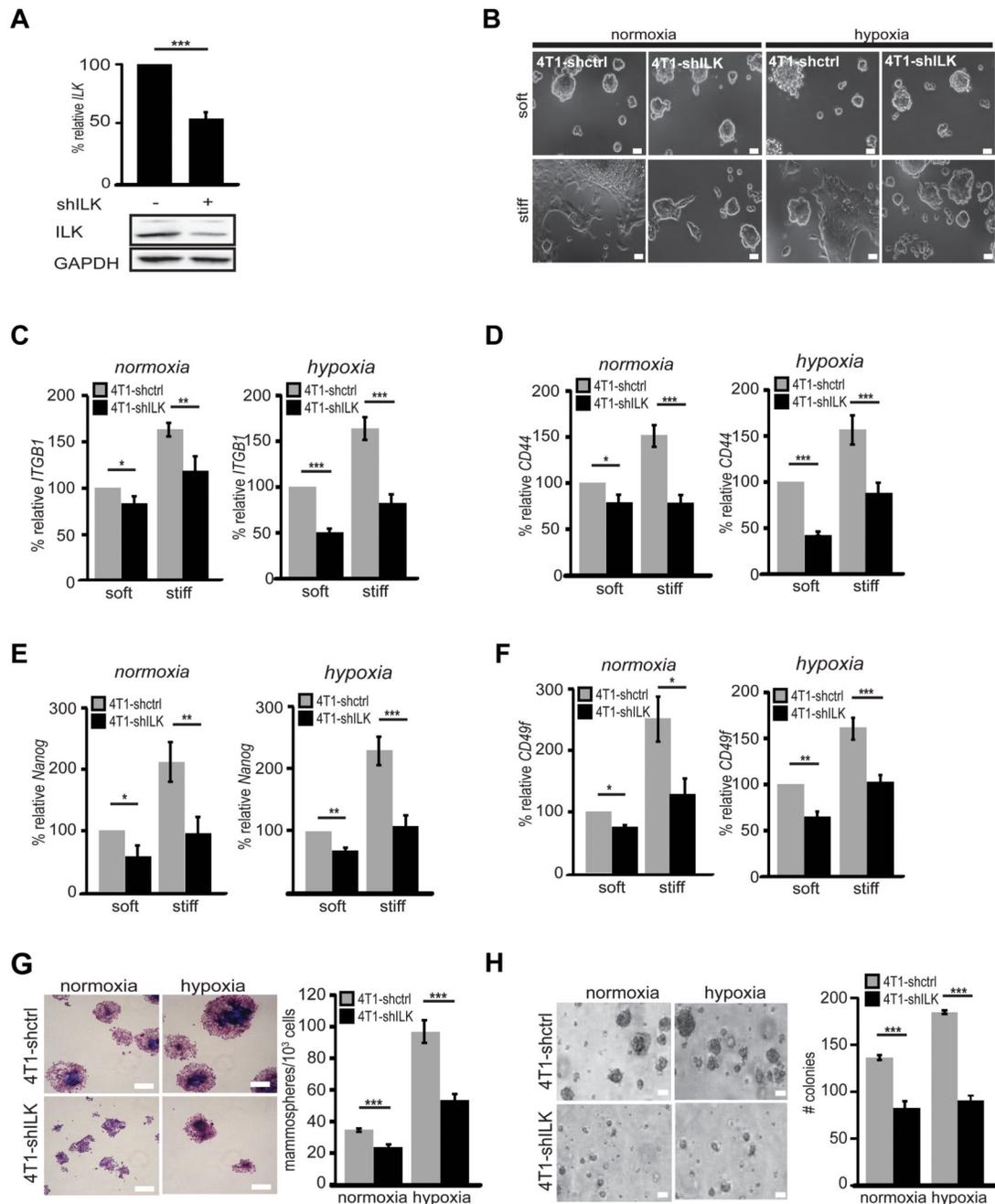
cells cultured on soft or stiff substrata under normoxia or hypoxia. **(H)** Immunoblotting analysis for CD44 and Nanog in 4T1 cells cultured on soft or stiff substrata under normoxia or hypoxia. **(I)** Tracks of 4T1 cells cultured on soft or stiff substrata. **(J)** Immunohistochemistry for ILK and CD44 in human breast cancer samples. **(K)** Quantification of ILK/CD44 double positive cells in human breast cancer samples. Scale bars, 50 μ m. Shown are mean \pm s.e.m. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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**Figure 2.**

ILK is required for CSC development from invasive breast cancer cells. (A) qRT-PCR and immunoblotting analysis for ILK in 4T1 cells stably expressing shRNA against ILK (shILK) or scrambled sequence control (shctrl). (B) Phase contrast images of 4T1-shctrl and 4T1-shILK cells cultured on soft or stiff substrata under normoxia or hypoxia. Transcript levels of (C) ITGB1, (D) CD44, (E) Nanog, and (F) CD49f in 4T1-shctrl or 4T1-shILK cells cultured on soft or stiff substrata under normoxia or hypoxia. Phase contrast images and quantification of (G) secondary mammospheres and (H) colonies formed in soft agar by

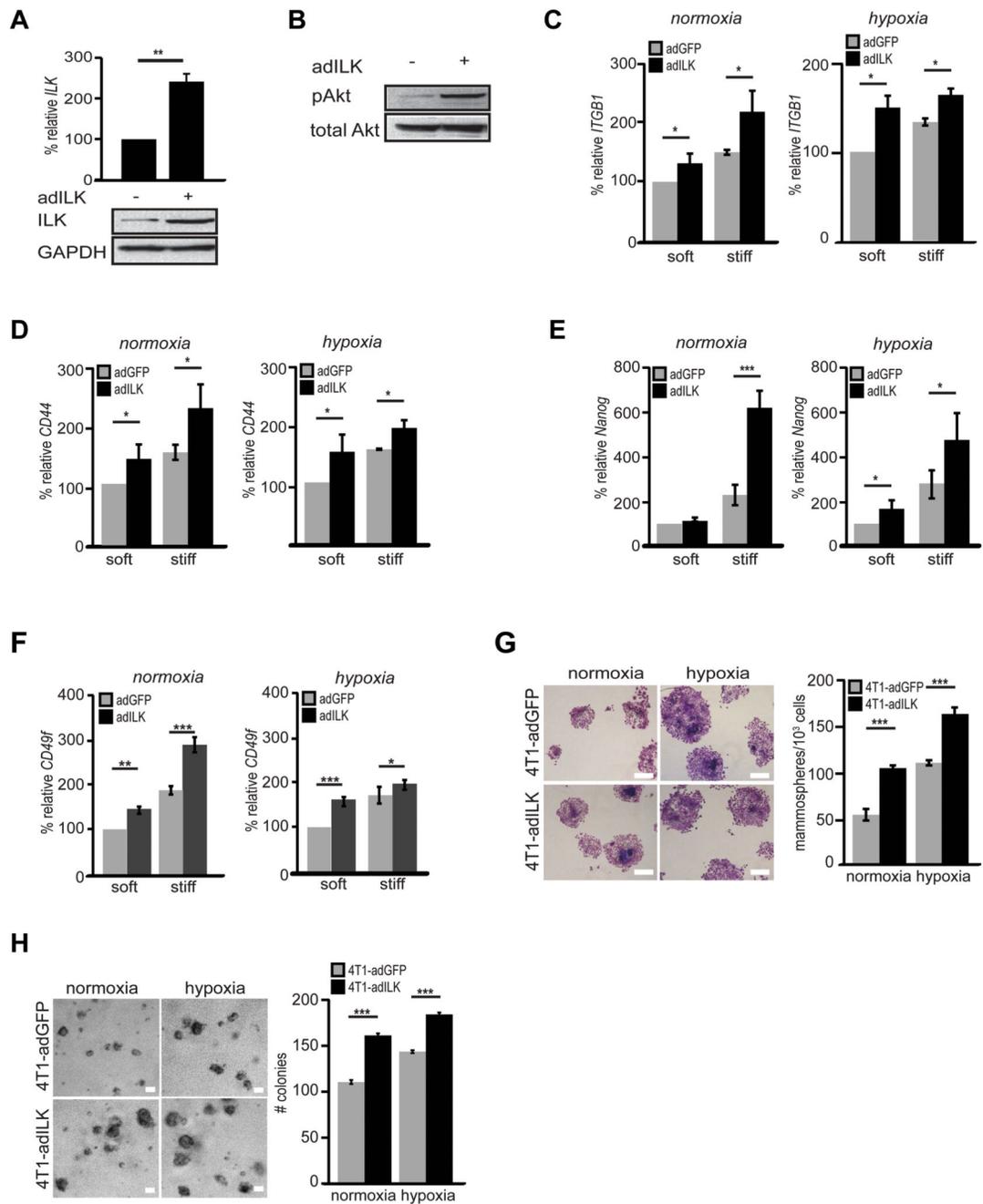
4T1-shctrl and 4T1-shILK cells. Scale bars, 50 μ m. Shown are mean \pm s.e.m. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

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**Figure 3.**

Ectopic expression of ILK induces breast CSCs. (A) qRT-PCR and immunoblotting analysis for ILK in 4T1 cells transduced with adGFP or adILK. (B) Immunoblotting analysis for phosphorylated and total Akt in 4T1 cells transduced with adGFP or adILK. Transcript levels of (C) ITGB1, (D) CD44, (E) Nanog, and (F) CD49f in 4T1 cells transduced with adGFP or adILK cultured on soft or stiff substrata under normoxia or hypoxia. Phase contrast images and quantification of (G) secondary mammospheres and (H) colonies

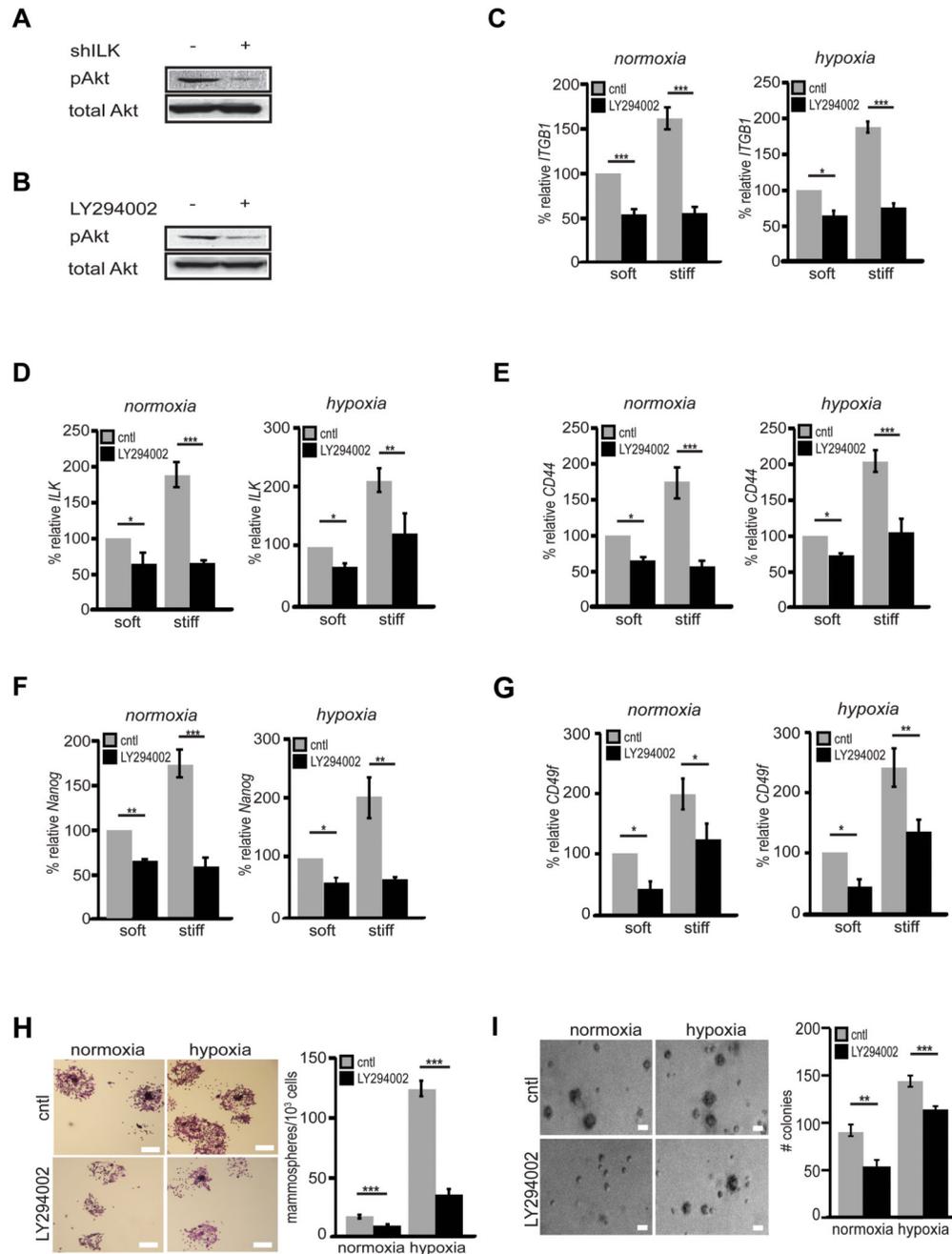
formed in soft agar by 4T1 cells transduced with adGFP or adILK. Scale bars, 50 μ m.
Shown are mean \pm s.e.m. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

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**Figure 4.**

ILK induces breast CSCs by signaling through PI3K. Immunoblotting analysis for phosphorylated and total Akt in (A) 4T1-shctrl and 4T1-shILK cells or (B) 4T1 cells treated with or without LY294002 (50 μ M). Transcript levels of (C) ITGB1, (D) ILK, (E) CD44, (F) Nanog, and (G) CD49f in 4T1 cells treated with or without LY294002 cultured on soft or stiff substrata under normoxia or hypoxia. Phase contrast images and quantification of (H) secondary mammospheres and (I) colonies formed in soft agar by 4T1 cells treated with or

without LY294002 under normoxia or hypoxia. Scale bars, 50 μ m. Shown are mean \pm s.e.m.
*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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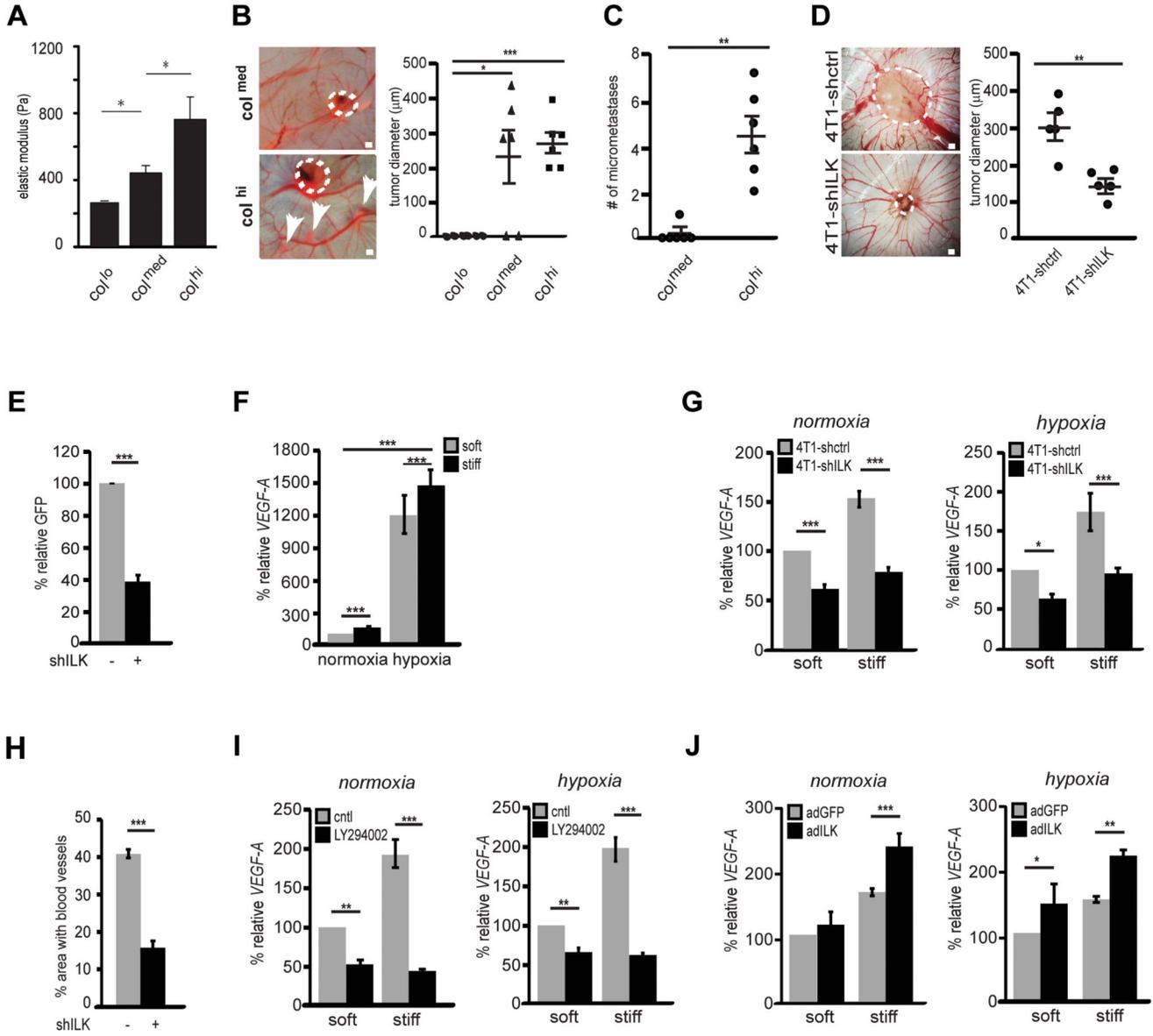


Figure 5. Stiff substratum and ILK signaling promote tumor growth, angiogenesis, and metastasis. (A) Estimated elastic modulus of low, medium, and high concentration collagen gels. Brightfield images and quantification of (B) tumor diameter and (C) number of micrometastases formed by 4T1 cells embedded in low, medium, or high concentrations of collagen implanted on CAMs. Scale bars, 50 μm. (D) Representative images and quantification of tumor diameter on CAMs grafted with 4T1-shctrl and 4T1-shILK cells. Scale bars, 25 μm. (E) Transcript levels of GFP in the lungs of chicken embryos whose CAMs were grafted with 4T1-shctrl or 4T1-shILK cells. Transcript levels of VEGF-A in (F) 4T1 cells or (G) 4T1-shctrl and 4T1-shILK cells cultured on soft or stiff substrata under normoxia or hypoxia. (H) Quantification of the relative area of the CAM covered with blood vessels 5 days after grafting with 4T1-

shctrl or 4T1-shILK cells. Transcript levels of VEGF-A in 4T1 cells (**I**) treated with or without LY294002 or (**J**) transduced with adGFP or adILK and cultured on soft or stiff substrata under normoxia or hypoxia. Shown are mean \pm s.e.m. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

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