

# 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 more hypoxic than 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 met-

astatic 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 were expressed 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. *Cancer Res*; 76(18): 5277–87. ©2016 AACR.

## 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 transformation of mammary epithelial cells (7). In 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 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 (CSC; ref. 13), a distinct population of cancer cells with enhanced proliferative and invasive character-

istics (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  $\beta$ 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

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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 Biotechnology) 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% CO<sub>2</sub>.

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/cm<sup>2</sup> on synthetic substrata and cultured for 72 hours at 37°C in either humidified normoxic conditions (95% air and 5% CO<sub>2</sub>) or in a modular incubator chamber (Billups-Rothenberg, Inc.) filled with hypoxic gas (94% N<sub>2</sub>, 1% O<sub>2</sub>, and 5% CO<sub>2</sub>).

#### Quantitative real-time 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 quantitative real-time PCR (qRT-PCR) using a Bio-Rad 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 Supplementary Table S1 (Supplementary Information). The expression level of each mRNA was normalized to that of 18S in the same sample.

#### Time-lapse imaging and cell tracking

Time-lapse imaging was performed using a Nikon Ti-U inverted microscope equipped with a stage top incubator maintained at 37°C in a 5% CO<sub>2</sub>, 90% relative humidity atmosphere (Pathology Devices, Inc.). Images were acquired every 30 minutes 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 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 (1:1,000; Abcam), rabbit anti-ILK (1:1,000; Abcam), rabbit anti-Nanog (1:1,000; Novus Biologicals), mouse anti-CD44 (1:1,000; Novus Biologicals), and rabbit anti-GAPDH (1:1,000; Cell Signaling Technology). After washing, blots were probed with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse secondary antibodies (1:5,000; Cell Signaling Technology) for 1 hour. Blots were incubated with ECL Plus Western Blotting Detection System (GE Healthcare) for 5 minutes and signals were detected with a FluorChemE Imager (Cell Biosciences, Inc.).

#### Soft agar assay

Approximately 5,000 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 minutes: 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 ALDEFUOR fluorescent reagent system (Stem Cell Technologies) was used according to the manufacturer's instructions. Stained cells were analyzed using an LSRII flow cytometer (BD Biosciences) and data were analyzed using FlowJo.

#### Secondary mammosphere formation assay

A single-cell suspension was seeded at a density of 3,000 cells per well into 12-well ultra-low attachment plates (BD Biosciences). Cells were cultured in DMEM/F12 medium containing 20 ng/mL EGF 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 minutes 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 1,000 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 hours, 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 minutes 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 minute 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 assay

Chicken chorioallantoic membrane (CAM) assays were performed as described previously (27). Cells were transduced with recombinant adenovirus encoding GFP at an MOI of 100 for 24 hours 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- $\mu$ L 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, as per approval by the Mayo Clinic Institutional Review Board. Tissue sections were deparaffinized 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 hour 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 minutes. For fluorescent detection, tissue sections were rinsed 3 times for 5 minutes each with PBS containing 1.43  $\mu$ mol/L 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, HRP for 15 minutes. Tissue sections were rinsed with TBST wash buffer and then incubated in 3,3'-diaminobenzidine (DAB+; DAKO), and counterstained with modified Schmidt 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  $\pm$  SEM of at least three independent experiments conducted in triplicate. For CAM assays, data represent mean  $\pm$  SEM 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  $t$  test.  $P < 0.05$  was considered to represent a significant difference between conditions.

## Results

### Substratum stiffness and oxygen tension regulate the expression of $\beta$ 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 modulus,  $E \sim 130$  Pa, "soft") and in breast tumors ( $E \sim 4020$  Pa, "stiff"; ref. 4). On soft substrata, 4T1 mouse mammary carcinoma cells (Fig. 1A) and MDA-MB-231 human breast carcinoma cells (Supplementary Fig. 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  $\beta$ 1-integrin and ILK in the cells cultured on stiff substrata at the transcript (Fig. 1B and C) and protein (Fig. 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  $\beta$ 1-integrin (Fig. 1B and D) and ILK (Fig. 1C and D) in both 4T1 and MDA-MB-231 cells (Supplementary Fig. S1). A stiff, hypoxic microenvironment induced the highest expression of these markers (Fig. 1B–D and Supplementary Fig. S1). These results suggest that stiff and hypoxic microenvironments potentiate signaling downstream of  $\beta$ 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 (Fig. 1E–H and Supplementary Fig. 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 (Fig. 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

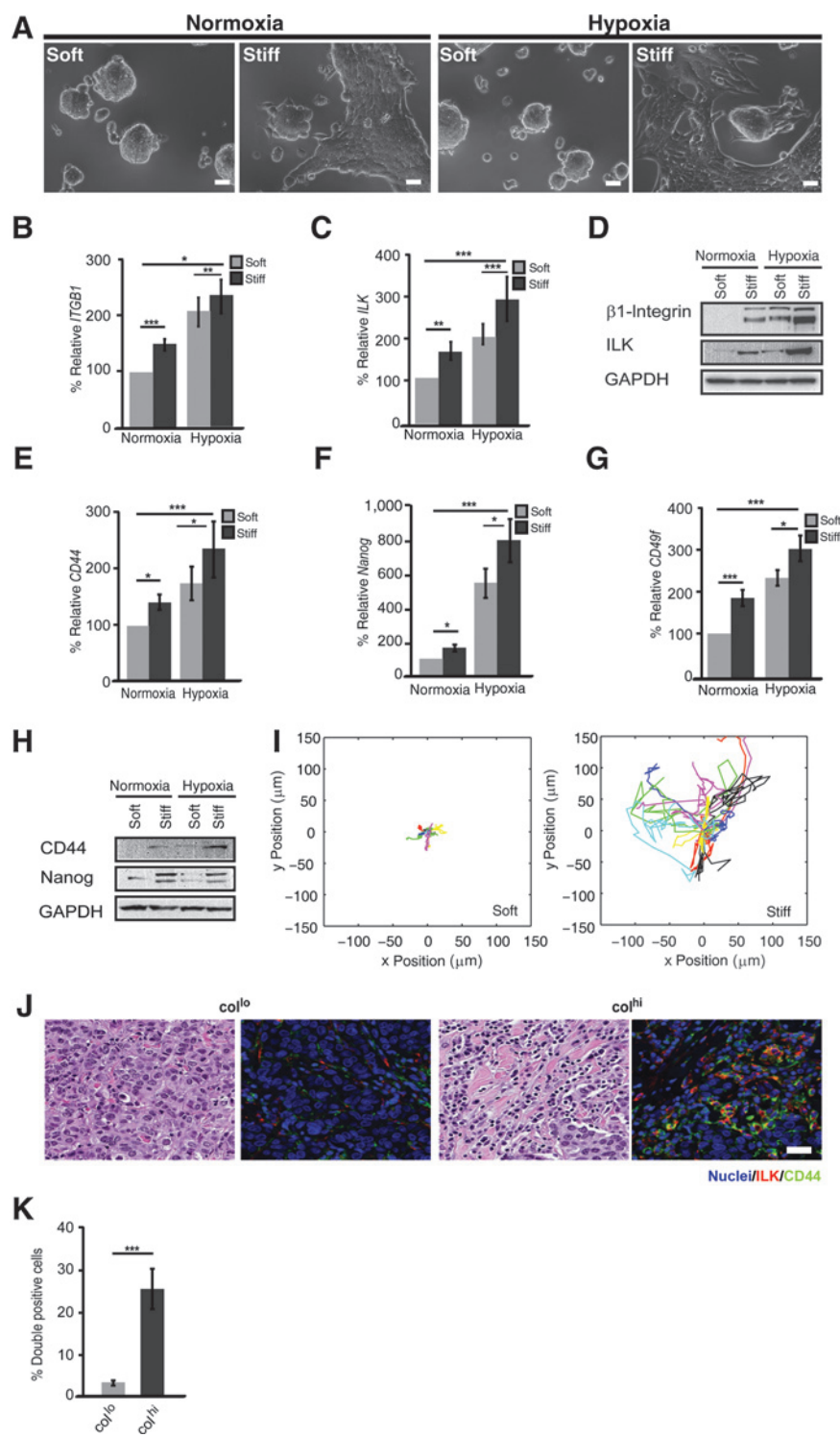
To evaluate the relationship between stiffness, ILK expression, and CSC marker induction *in vivo*, we examined the expression of ILK and CD44 in human breast cancer samples. We compared these to relative collagen content, which is highly correlated with stiffness in mammary cancers (30, 31). Immunofluorescence 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 ( $col^{hi}$ ) (Fig. 1). In  $col^{hi}$  regions, more than 25% of the cells expressed both ILK and CD44. In contrast, only approximately 4% of the cells expressed both markers in  $col^{lo}$  regions (Fig. 1K). These data are consistent with the results of our cell

culture experiments, and suggest that breast CSCs are preferentially located in stiff microenvironments.

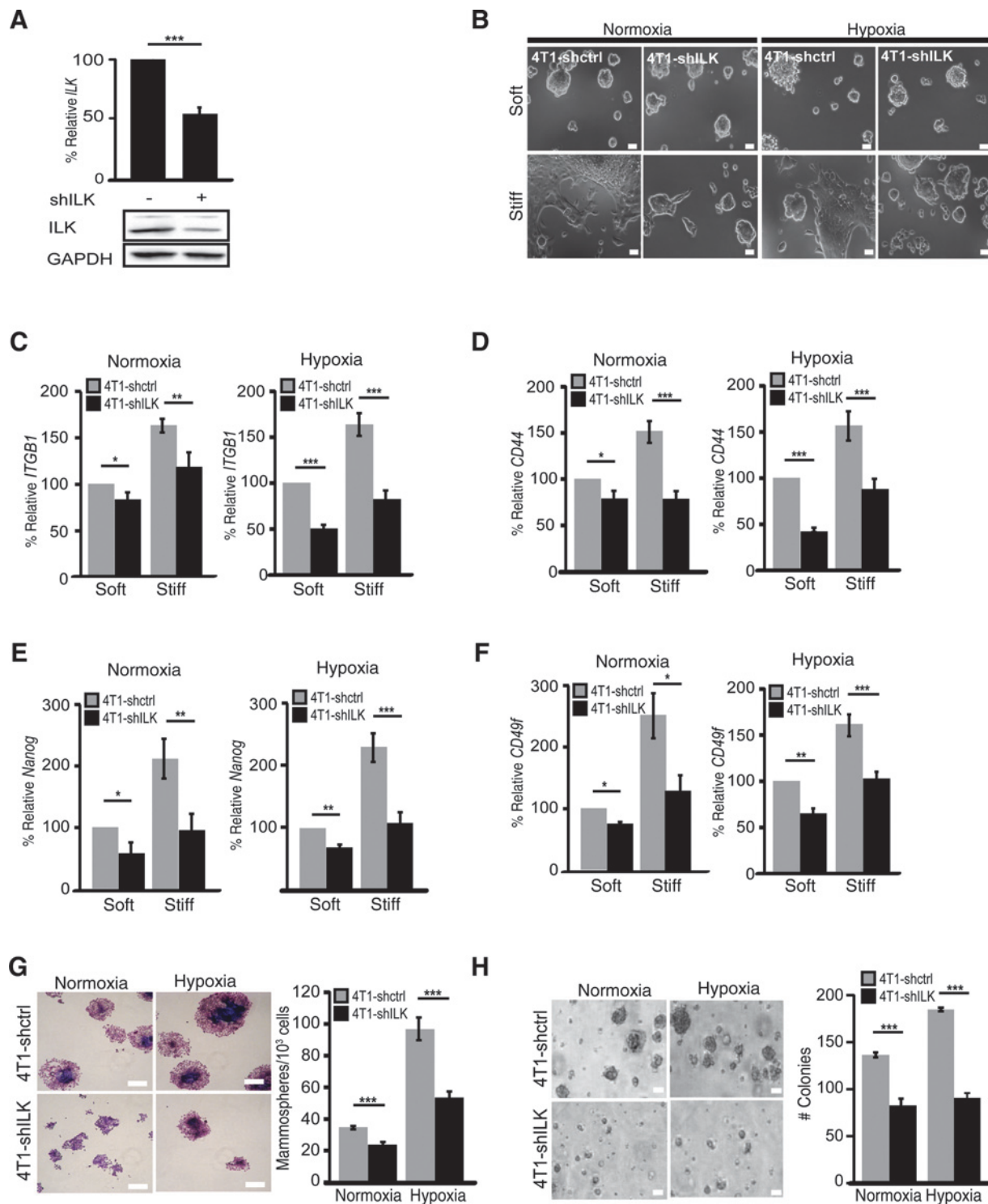
### Depleting ILK prevents CSC development in cells on stiff substrata

$\beta$ 1-integrin transmits cues from the ECM through ILK to control cellular behavior (22), and enhanced signaling through ILK has



**Figure 1.**

Stiff and hypoxic microenvironments promote the expression of  $\beta$ 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  $\beta$ 1-integrin (*ITGB1*; **B**) and *ILK* (**C**) in 4T1 cells cultured on soft or stiff substrata under normoxia or hypoxia. **D**, immunoblotting analysis for  $\beta$ 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 *CD44* (**E**), *Nanog* (**F**), and *CD49f* (**G**) 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**, IHC 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  $\mu$ m. Shown are mean  $\pm$  SEM. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

**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 *ITGB1* (**C**), *CD44* (**D**), *Nanog* (**E**), and *CD49f* (**F**) in 4T1-shctrl or 4T1-shILK cells cultured on soft or stiff substrata under normoxia or hypoxia. Phase-contrast images and quantification of secondary mammospheres (**G**) and colonies formed in soft agar (**H**) by 4T1-shctrl and 4T1-shILK cells. Scale bars, 50  $\mu$ m. Shown are mean  $\pm$  SEM. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

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) (Fig. 2A) and MDA-MB-231 cells (Supplementary Fig. S2). Depleting ILK led to a significant change in the morphology of 4T1 cells cultured on stiff substratum, as compared with scrambled controls (4T1-shctrl; Fig. 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  $\beta$ 1-integrin (Fig. 2C) and the CSC markers *CD44* (Fig. 2D), *Nanog* (Fig. 2E), and *CD49f* (Fig. 2F) in 4T1 and MDA-MB-231 cells (Supplementary Fig. 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 (Fig. 2G and Supplementary Fig. S2) and colonies in soft agar (Fig. 2H; Supplementary Fig. S2). Together, these data suggest that ILK is necessary for the induction of 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 (Fig. 3A) and MDA-MB-231 cells (Supplementary Fig. S3), and increased phosphorylation of Akt (pAkt; S437; Fig. 3B and Supplementary Fig. S3). qRT-PCR analysis revealed that ectopic expression of ILK increased the transcript levels of  *$\beta$ 1-integrin* (Fig. 3C), *CD44* (Fig. 3D), *Nanog* (Fig. 3E), and *CD49f* (Fig. 3F) in 4T1 and MDA-MB-231 cells (Supplementary Fig. S3) cultured on soft or stiff substrata under normoxia or hypoxia. Ectopic expression of ILK also enhanced the formation of secondary mammospheres (Fig. 3G and Supplementary Fig. S3) and colonies in soft agar (Fig. 3H and Supplementary Fig. 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 (Fig. 4A and Supplementary Fig. S4). Disrupting signaling through PI3K by treating cells with the selective inhibitor, LY294002 (Fig. 4B and Supplementary Fig. S4), also decreased the transcript levels of  *$\beta$ 1-integrin* (Fig. 4C) and *ILK* (Fig. 4D) in 4T1 and MDA-MB-231 cells (Supplementary Fig. 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* (Fig. 4E), *Nanog* (Fig. 4F), and *CD49f* (Fig. 4G) decreased

significantly in response to treatment with LY294002 in 4T1 and MDA-MB-231 cells (Supplementary Fig. S4) under all conditions.

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

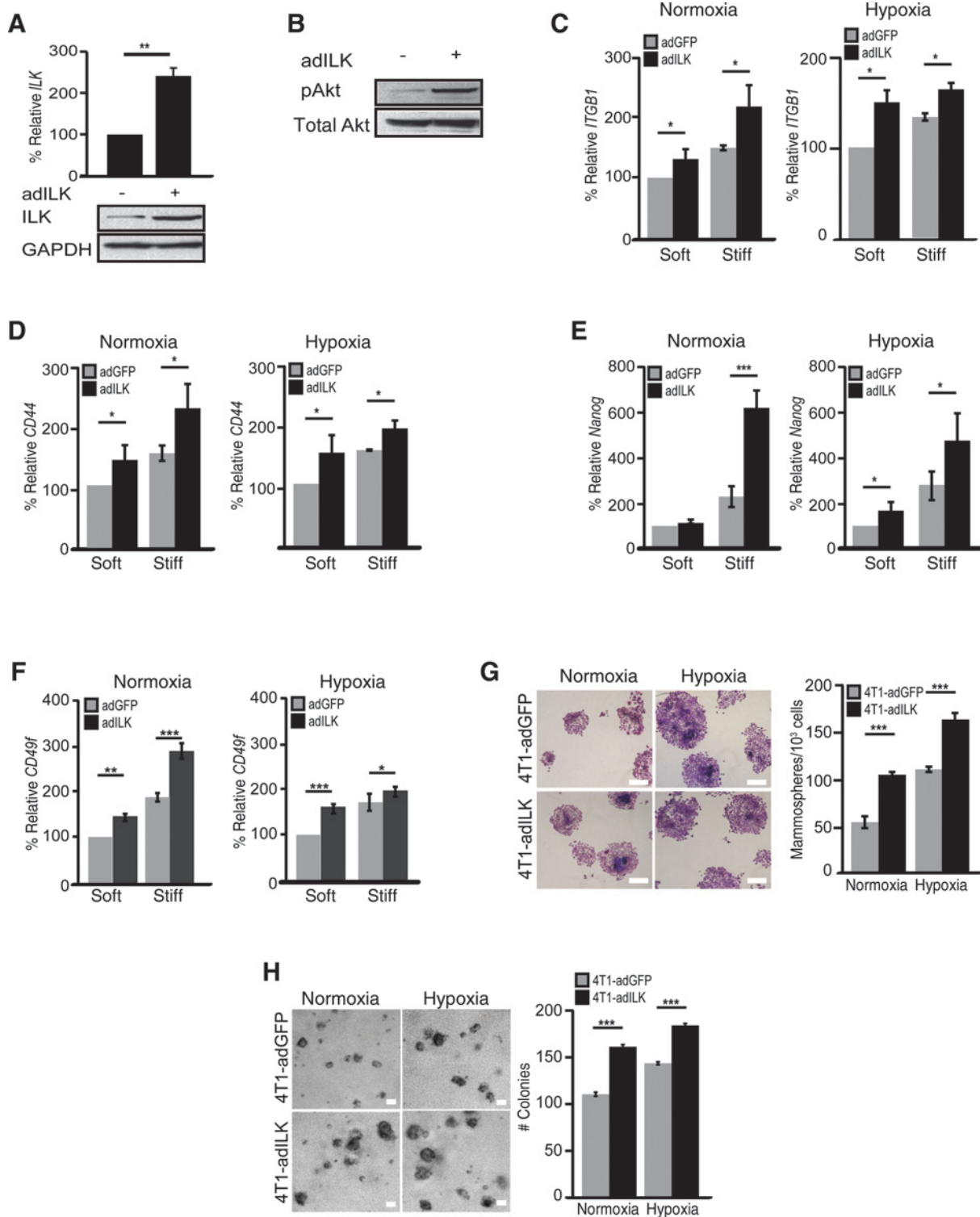
#### Stiff substratum promotes angiogenesis and dissemination of tumor cells through ILK

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 (Fig. 5A) and then grafted them onto the 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 of 6 of the  $col^{med}$ -grafted CAMs developed primary tumors (Fig. 5B). The tumors that formed in high collagen developed multiple micrometastases near blood vessels adjacent to the graft sites (Fig. 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 (Fig. 5D). However, the 4T1-shILK primary tumors were half the size of the 4T1-shctrl tumors (Fig. 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 (Fig. 5E). These data suggest that matrix stiffness and ILK affect tumor formation as well as tumor cell dissemination.

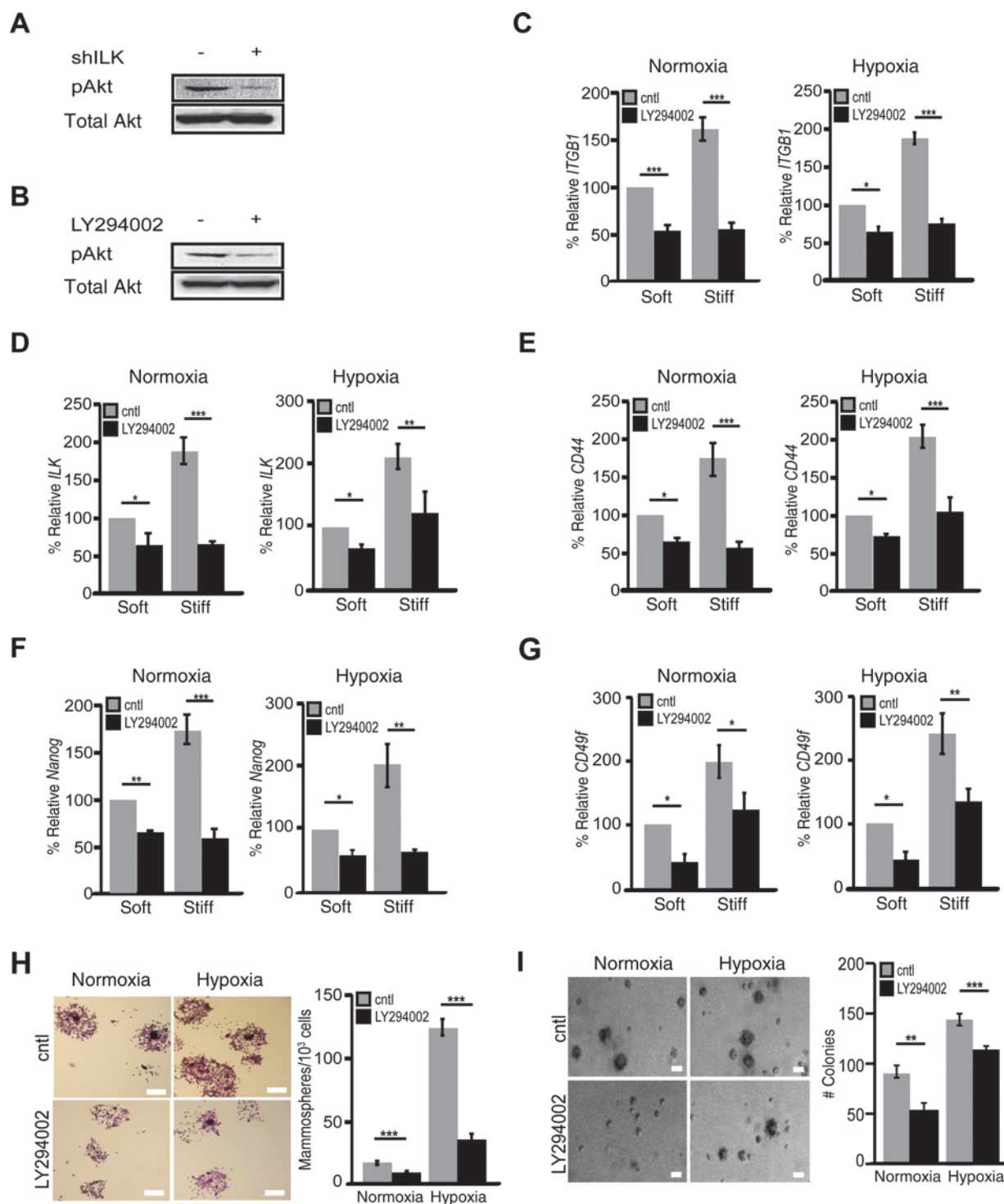
Angiogenesis is a hallmark of cancer that is critical for tumor growth and metastasis (41). To investigate how substratum stiffness affects hypoxia-dependent angiogenesis, we examined the expression of *VEGF-A* in 4T1 cells cultured on soft or stiff substrata under normoxia or hypoxia. We found that culture on stiff substratum upregulated the expression of *VEGF-A* (Fig. 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 (Fig. 5F). Depleting ILK significantly reduced *VEGF-A* expression in 4T1 cells cultured on soft or stiff substrata under normoxia or hypoxia (Fig. 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 (Fig. 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* (Fig. 5I), whereas ectopic expression of ILK increased the levels of *VEGF-A* (Fig. 5I) 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

**Figure 3.**

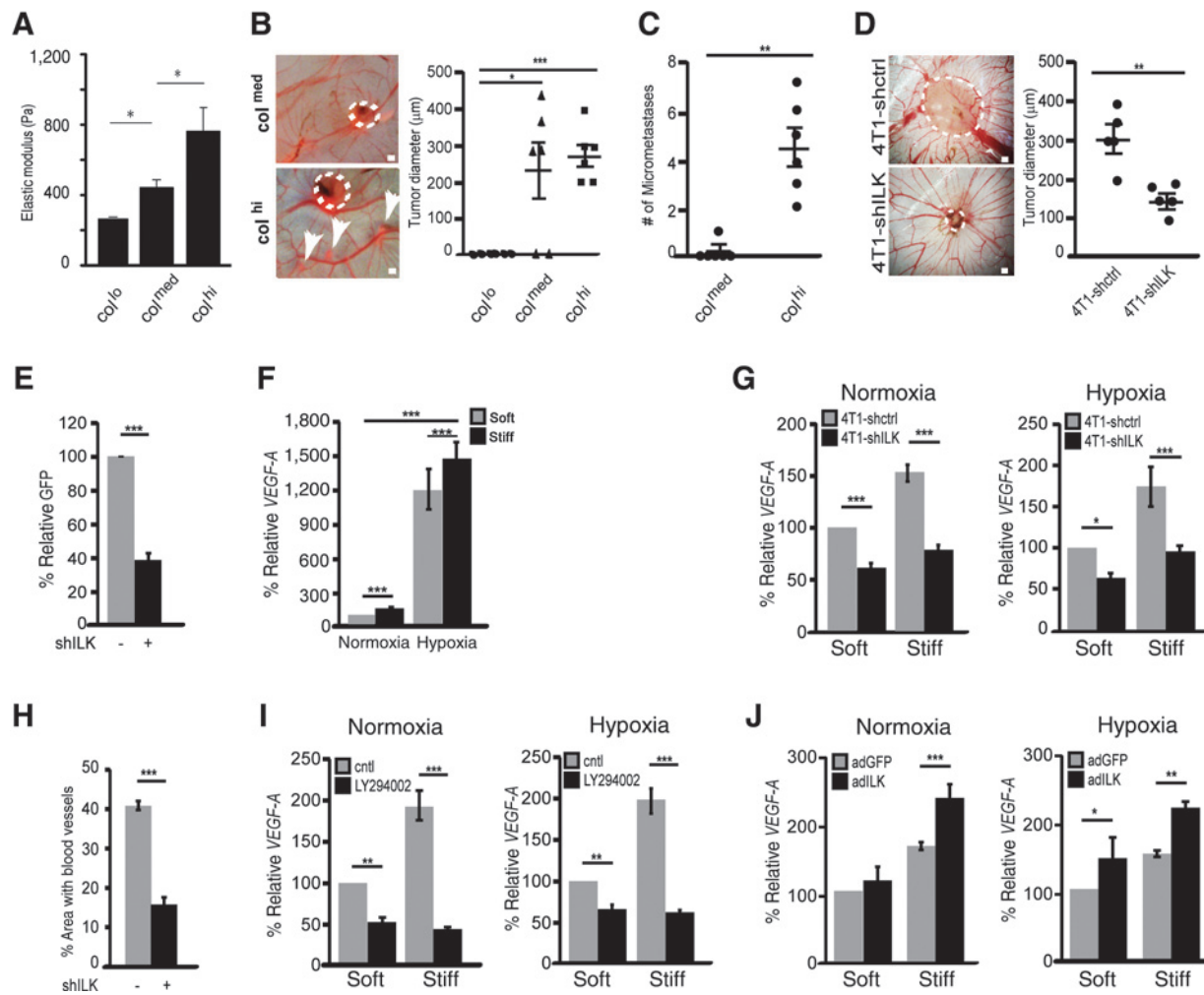
Ectopic expression of ILK induces breast CSCs. **A**, qRT-PCR and immunoblotting analysis for ILK in 4T1 cells transduced with adGFP or adILK. **B**, immunoblotting analysis for phosphorylated and total Akt in 4T1 cells transduced with adGFP or adILK. Transcript levels of *ITGB1* (**C**), *CD44* (**D**), *Nanog* (**E**), and *CD49f* (**F**) in 4T1 cells transduced with adGFP or adILK cultured on soft or stiff substrata under normoxia or hypoxia. Phase-contrast images and quantification of secondary mammospheres (**G**) and colonies formed in soft agar (**H**) by 4T1 cells transduced with adGFP or adILK. Scale bars, 50  $\mu$ m. Shown are mean  $\pm$  SEM. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .



**Figure 4.**

ILK induces breast CSCs by signaling through PI3K. Immunoblotting analysis for phosphorylated and total Akt in 4T1-shctrl and 4T1-shILK cells (A) or 4T1 cells treated with or without LY294002 (50  $\mu$ mol/L; B). Transcript levels of *ITGB1* (C), *ILK* (D), *CD44* (E), *Nanog* (F), and *CD49f* (G) in 4T1 cells treated with or without LY294002 cultured on soft or stiff substrata under normoxia or hypoxia. Phase-contrast images and quantification of secondary mammospheres (H) and colonies formed in soft agar (I) by 4T1 cells treated with or without LY294002 under normoxia or hypoxia. Scale bars, 50  $\mu$ m. Shown are mean  $\pm$  SEM. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .



**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 tumor diameter (**B**) and number of micrometastases (**C**) formed by 4T1 cells embedded in low, medium, or high concentrations of collagen implanted on CAMs. Scale bars, 50  $\mu\text{m}$ . **D**, representative images and quantification of tumor diameter on CAMs grafted with 4T1-shctrl and 4T1-shILK cells. Scale bars, 25  $\mu\text{m}$ . **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 4T1 cells (**F**) or 4T1-shctrl and 4T1-shILK cells (**G**) 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 treated with or without LY294002 (**I**) or transduced with adGFP or adILK (**J**) and cultured on soft or stiff substrata under normoxia or hypoxia. Shown are mean  $\pm$  SEM. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

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  $\beta$ 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  $\beta$ 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 $\alpha$ -dependent manner (54). Recent studies indicate that HIF1 $\alpha$  can drive breast cancer metastasis through ECM stiffening and collagen fiber alignment (55). It is possible that hypoxia promotes ECM remodeling 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.

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## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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