

# Soft Microenvironments Induce Chemoresistance by Increasing Autophagy Downstream of Integrin-Linked Kinase

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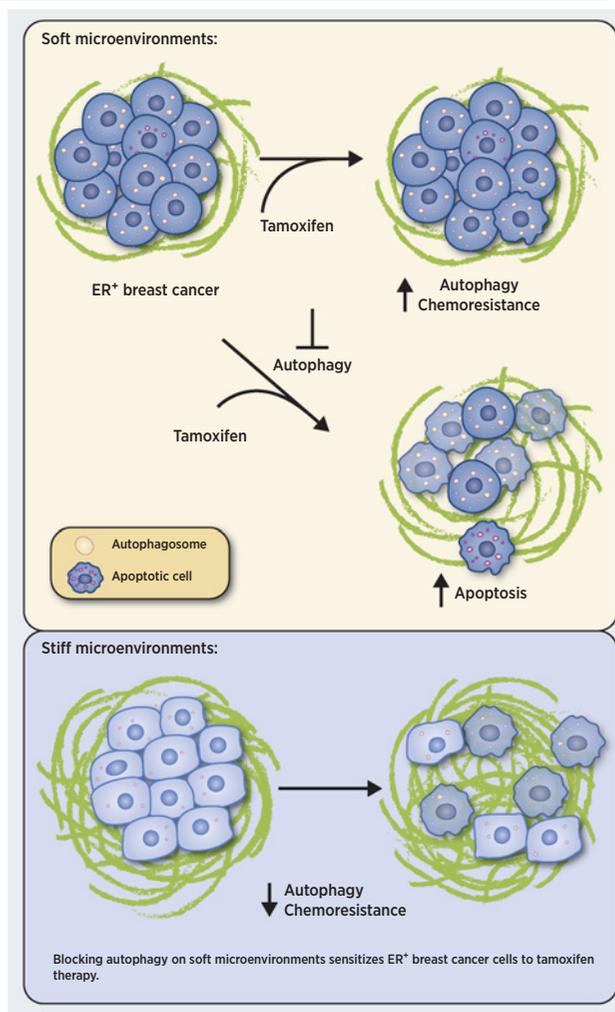


## ABSTRACT

Breast cancer relapse can develop over the course of years as a result of dormant cancer cells that disseminate to secondary sites. These dormant cells are often resistant to conventional hormone and chemotherapy. Although recurrence is the main cause of death from cancer, microenvironmental factors that may influence resistance to therapy and duration of dormancy are largely unknown. Breast cancer relapse is often detected in tissues that are softer than the normal mammary gland or the primary breast tumor, such as bone marrow, brain, and lung. We therefore explored how stiffness of the microenvironment at secondary sites regulates tumor dormancy and the response of breast cancer cells to hormone and chemotherapy. In soft microenvironments reminiscent of metastatic sites, breast cancer cells were more resistant to the estrogen receptor modulator tamoxifen as a result of increased autophagy and decreased expression of estrogen receptor- $\alpha$ . Consistently, pharmacologic inhibition or genetic downregulation of autophagy increased the response of breast cancer cells to tamoxifen on soft substrata. In addition, autophagy was decreased downstream of integrin-linked kinase on stiff substrata. Altogether, our data show that tissue mechanics regulates therapeutic outcome and long-term survival of breast cancer cells by influencing autophagy.

**Significance:** These findings characterize the persistence of dormant cells at metastatic sites, where soft microenvironments downregulate estrogen receptor expression and upregulate autophagy, thereby promoting therapy resistance in breast cancer cells.

**Graphical Abstract:** <http://cancerres.aacrjournals.org/content/cancerres/80/19/4103/F1.large.jpg>.



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**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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## Introduction

Approximately 90% of cancer-related deaths are due to malignant growths that appear at sites far removed from that of the primary tumor (1). Although the mechanisms that drive metastatic growth in secondary organs are still unclear, it is well appreciated that disseminated cancer cells can remain clinically asymptomatic for as long as decades. The time from the formation of the first micrometastasis to the appearance of a clinically detectable macrometastasis is known as “tumor dormancy,” the rate-limiting step in metastasis (2). Although tumor dormancy was initially attributed to temporary mitotic arrest or quiescence (3–5), alternative hypotheses have suggested that a

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micrometastasis can fail to increase in size due to poor vascularization or cytotoxic activity of the immune system (6–8). This makes it difficult to detect dormant cancer cells using current diagnostic tools or to target them using conventional chemo-, hormone-, or radiotherapy approaches. Therefore, clinical practices rely on estimating the probability of recurrence from prognostic factors such as the grade and stage of the disease, and limited understanding of the biology of dormancy prevents its effective treatment (9). To improve treatment of metastatic disease, it is necessary to elucidate the pathways that promote dormancy and to uncover how dormant cancer cells interact with their surrounding niche.

Cancer cell behavior is influenced by the tumor microenvironment, which is comprised of the extracellular matrix (ECM), neighboring host cells, the immune system, and soluble factors including hormones and cytokines. The ECM is a major regulator of cell function, as cancer cells receive biochemical and mechanical signals from the host tissue, interpret these signals, and tailor their microenvironment into a hospitable niche (10). Breast cancer metastases are most frequently detected in the bone, brain, liver, and lung (11), and it is likely that the distinct microenvironments offered by these sites affect the temporal and spatial progression of metastatic disease. Consistently, microenvironmental signals appear to regulate tumor dormancy. For example, stable microvasculature promotes quiescence in breast cancer cells, which switch to a proliferative phenotype when they encounter newly sprouted blood vessels (12). This transition from quiescence to proliferation depends on the production of fibronectin and signaling through  $\beta$ 1-integrin (13). Similarly, microenvironmental stresses such as hypoxia prime dormancy of head-and-neck squamous cell carcinoma and breast cancer cells, and posthypoxic disseminated tumor cells evade chemotherapy (14).

The mechanical signals that cancer cells receive from their microenvironment, including matrix stiffness as well as solid and fluid stress also influence cell proliferation, motility, differentiation, and resistance to apoptosis (15, 16). It is unclear, however, whether the mechanical properties of the microenvironment also regulate tumor dormancy. In hepatocellular carcinoma, matrix stiffness has been found to regulate cellular quiescence, response to chemotherapeutics, and clonogenic capacity following chemotherapy (17). Because breast cancer metastases are detected in tissues that are softer than a breast tumor or a healthy mammary gland (18–20), soft microenvironments could promote the survival of disseminated breast cancer cells at secondary sites.

Little is known about how micrometastases remain viable over prolonged periods of time. Recently, autophagy has emerged as a self-degradative process that has the potential to promote the survival of stem-like subpopulations of tumor cells (21–23) and that could contribute to the persistence of chemoresistant tumor cells at secondary sites (24). Monoallelic loss of *beclin1*, an essential autophagy gene, is common in breast cancer and is a precursor to tumorigenesis in mouse models of mammary carcinoma (25). These observations suggest that autophagy could play a tumor-suppressive role. Autophagy has also been demonstrated to promote the survival and chemoresistance of dormant ovarian and gastrointestinal cancer cells (26, 27). Overexpression of the tumor suppressor aplasia Ras homolog member I (*ARHI*) has been shown to induce dormancy and autophagy. In ovarian tumor xenografts, increased formation of autophagosomes is associated with reduced proliferation, and loss of *ARHI* expression correlates with a regain of proliferative potential (26). The fact that autophagy is a dynamic process that can be tumor-suppressive or tumor-promoting implies that its role in cancer progression might be influenced by the tumor microenvironment (28).

We show here that the mechanical properties of the surrounding microenvironment regulate tumor dormancy and autophagy in human breast cancer cells. Using synthetic substrata of tunable stiffness, we found that soft microenvironments reminiscent of metastatic sites harbor dormant estrogen receptor- $\alpha$  (ER $\alpha$ )-positive cancer cells that are resistant to treatment with the antiestrogen, tamoxifen. Soft microenvironments also cause an upregulation of autophagy, indicating that autophagy could serve as a survival mechanism for dormant cancer cells. In contrast, while breast cancer cells proliferate more on stiff microenvironments, they are also more prone to elimination by tamoxifen as well as common chemotherapeutics. Altogether, these results suggest that tissue mechanics regulate chemotherapeutic outcome and the long-term survival of breast cancer cells, in part, by influencing autophagy.

## Materials and Methods

### Cell culture and reagents

MCF7 and ZR-75-1 ER $\alpha$ -positive human breast cancer cells were obtained from the ATCC (in 2008 and 2018, respectively) and cultured in DMEM/F12 or RPMI medium respectively, supplemented with 10% heat-inactivated FBS (Atlanta Biologicals), 10  $\mu$ g/mL insulin (Sigma), and 50  $\mu$ g/mL gentamicin (Gibco) for 3 days unless otherwise specified. 5-fluorouracil (5  $\mu$ mol/L, Sigma) or tamoxifen (2  $\mu$ mol/L, Sigma) were added to fresh culture medium 24 hours after plating cells. To block autophagosome degradation, chloroquine (CQ; 0.1  $\mu$ mol/L, Sigma) was added to the culture medium 24 hours before fixing cells. Cell lines were authenticated by short tandem repeat genotyping (ATCC) and used before passage 20. MCF7 cells were tested for *Mycoplasma* (2017; Lonza); experiments with ZR-75-1 cells were completed within 6 months of thawing the purchased vial and therefore not tested for *Mycoplasma*.

### Viral transductions

A bicistronic recombinant adenovirus encoding ILK and GFP (AdILK) was obtained from Vector Biolabs (29). An adenovirus encoding GFP alone (AdGFP) was used as control. Each adenovirus was added to the cell culture medium at a multiplicity of infection of 100.

### Transient RNA interference

SMARTpool containing 4 siRNA duplexes per gene were used to knockdown the expression of ILK1, Beclin1, and Atg7 (L-004499-00-0005; L-010552-00-0005; L-020112-00-0005, Dharmacon). A nontargeting SMARTpool was used as a negative control (D-001810-10-05, Dharmacon). Cells were plated at a density of  $4 \times 10^5$  cells per mL and transfected using RNAiMAX transfection reagent (Invitrogen) to reach a final siRNA concentration of 20 nmol/L.

### Synthetic substrata

Shear moduli have been measured in live, normal human breast tissue, solid human breast tumors, and common metastatic sites (30–33). To mimic the mechanical microenvironments of these tissues, we used the shear moduli values to calculate elastic modulus, generated polyacrylamide (PA) substrata with these elastic moduli, and adapted them for cell culture as described previously (34). Glass coverslips (31-mm diameter) were washed with 0.1 N NaOH for 30 minutes and then washed three times with MilliQ water (Millipore) and dried. Coverslips were then immersed in 2% (vol/vol) aminopropyltrimethoxysilane (Sigma Aldrich) in acetone for 30 minutes, washed three times with acetone, and dried. Finally, coverslips were

immersed in 1% (vol/vol) glutaraldehyde (Sigma Aldrich) in PBS for 30 minutes, washed three times with MilliQ water, and dried. PA gels were synthesized on the treated coverslips as follows: 12.5% (vol/vol) acrylamide was mixed with either 0.5% (vol/vol) or 17.5% (vol/vol) bis-acrylamide in water. Polymerization was initiated by adding 10% ammonium persulfate (Bio-Rad) and N,N,N',N'-tetramethylethylenediamine (Sigma Aldrich). Thirty-six microliters of the mixture was placed onto treated coverslips and each sandwiched under an untreated glass coverslip. After drying for 1 hour at room temperature, PA gels were stored in PBS at 4°C. PA gels were characterized as described previously (35). The top coverslips were removed from the PA gels and functionalized with fibronectin using the heterobifunctional crosslinker, Sulfo-SANPAH (Thermo Fisher Scientific). Before plating cells, gels were washed three times with PBS and incubated with culture medium for 30 minutes at 37°C.

### Immunofluorescence analysis

Samples were fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature, washed three times with PBS, blocked with 10% (vol/vol) goat serum (Sigma Aldrich) in PBS for 2 hours, and incubated overnight at 4°C with rabbit anti-LC3B (1:200; Sigma) antibody. Samples were washed four times for 15 minutes each with 0.5% Triton X-100 in PBS (PBST), and then incubated with Alexa 488 goat anti-rabbit secondary antibody (Invitrogen) overnight at 4°C. To label nuclei, samples were incubated with Hoechst 33342 (1:1,000; Invitrogen) for 15 minutes at room temperature and washed three times with PBS for 10 minutes each. Apoptosis analysis was conducted using a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) kit (Thermo Fisher Scientific).

Images were acquired with a Hamamatsu Orca CCD camera attached to a Nikon Ti-U inverted fluorescence microscope using a  $\times 20$  magnification air objective (or  $\times 60$  magnification oil objective for autophagosome immunofluorescence). Phase-contrast and fluorescence images were merged using ImageJ. Proliferation analysis was based on EdU incorporation (Thermo Fisher Scientific). Briefly, the number of cells with EdU-positive nuclei was counted and recorded as the percentage of the total number of cells in each frame. The rolling average of the percentage of proliferating cells was calculated for each frame and for 400–500 cells for each condition. The size and number of autophagosomes (LC3B-labeled cytoplasmic vesicles) were quantified using a MATLAB code that employs Gaussian filtering to eliminate background noise.

### Immunoblotting analysis

Samples were lysed in RIPA lysis buffer (Thermo Fisher Scientific) supplemented with a protease inhibitor (Roche). Protein concentrations were measured using the Bradford Assay (Bio-Rad). Equal amounts of total protein were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes, which were then blocked in 5% nonfat milk and incubated overnight at 4°C in blocking buffer containing primary antibodies. Antibodies used for immunoblotting were: rabbit anti-LC3B (1:1,000; Sigma), rabbit anti-p21 (1:1,000; Cell Signaling Technology), rabbit anti-p27 (1:1,000; Cell Signaling Technology), rabbit anti-cyclin D1 (1:1,000; Cell Signaling Technology), mouse anti-p62 (1:1,000; Abcam), rabbit anti-ER $\alpha$  (1:1,000; Cell Signaling Technology), rabbit anti-Atg7 (1:1,000; Cell Signaling Technology), rabbit anti-Beclin1 (1:1,000; Cell Signaling Technology), rabbit anti-ILK1 (1:100; Cell Signaling Technology), and rabbit anti-GAPDH (1:2,000; Cell Signaling Technology). After three washes with TBST, blots were probed with horseradish peroxidase-conjugated anti-rabbit secondary antibodies for 45 minutes (1:5,000; Cell Signal-

ing Technology). Following incubation with ECL Plus Western Blotting Detection System (GE Healthcare), blots were imaged using a FluorChemE Imager (Cell Biosciences). For each protein, densitometry analysis was carried out in ImageJ by dividing the total intensity of each band by that of GAPDH in the same sample.

### Cell-cycle analysis

To assay for cell-cycle stage, the FUCCI cell-cycle sensor (Invitrogen) was added to culture medium at 50 particles per cell and the cells were cultured for another 24 hours. The cells were then fixed with 4% PFA, washed three times with PBS, and counterstained with Hoechst 33342 to visualize the nuclei. Images were acquired as described above using a  $\times 10$  magnification air objective. Nuclei with red, yellow, or green labeling were recorded as G<sub>0</sub>–G<sub>1</sub>, G<sub>1</sub>–S transition, or S–G<sub>2</sub>–M phases, respectively.

## Results

### Soft microenvironments harbor growth-arrested breast cancer cells

Breast cancer metastases are most often detected in tissues that are inherently softer than a breast tumor or a healthy mammary gland (18, 20, 30–32). This observation suggests a role for tissue stiffness in the regulation of dormancy, as well as in the response of disseminated breast cancer cells to commonly used cancer treatments. MCF7 or ZR-75-1 ER $\alpha$ <sup>+</sup> human breast cancer cells cultured on “soft” substrata ( $E \sim 0.1, 0.9, 2, 4$  kPa) that mimic metastatic sites including the bone marrow, the liver, and the brain (Fig. 1A; ref. 30) exhibit a rounded morphology (Fig. 1B; Supplementary Fig. S1A), and are significantly less proliferative than their counterparts cultured on “stiff” substrata ( $E \sim 45, 100$  kPa) that mimic the human mammary gland and breast tumors (Fig. 1C and D; Supplementary Fig. S1B and S1C), as indicated by EdU-incorporation assays. Cyclin D1, which regulates progression through the G<sub>1</sub> phase of the cell cycle, is down-regulated, while the cyclin-dependent kinase inhibitors p21 and p27 are upregulated (Fig. 1E) in MCF7 cells cultured on soft substrata. Consistently, cell-cycle analysis using the FUCCI reporter system revealed that the percentage of cells in the G<sub>0</sub>–G<sub>1</sub> phase of the cell cycle is increased on soft substrata (Fig. 1F). These data suggest that culture in soft microenvironments might induce quiescence in breast cancer cells.

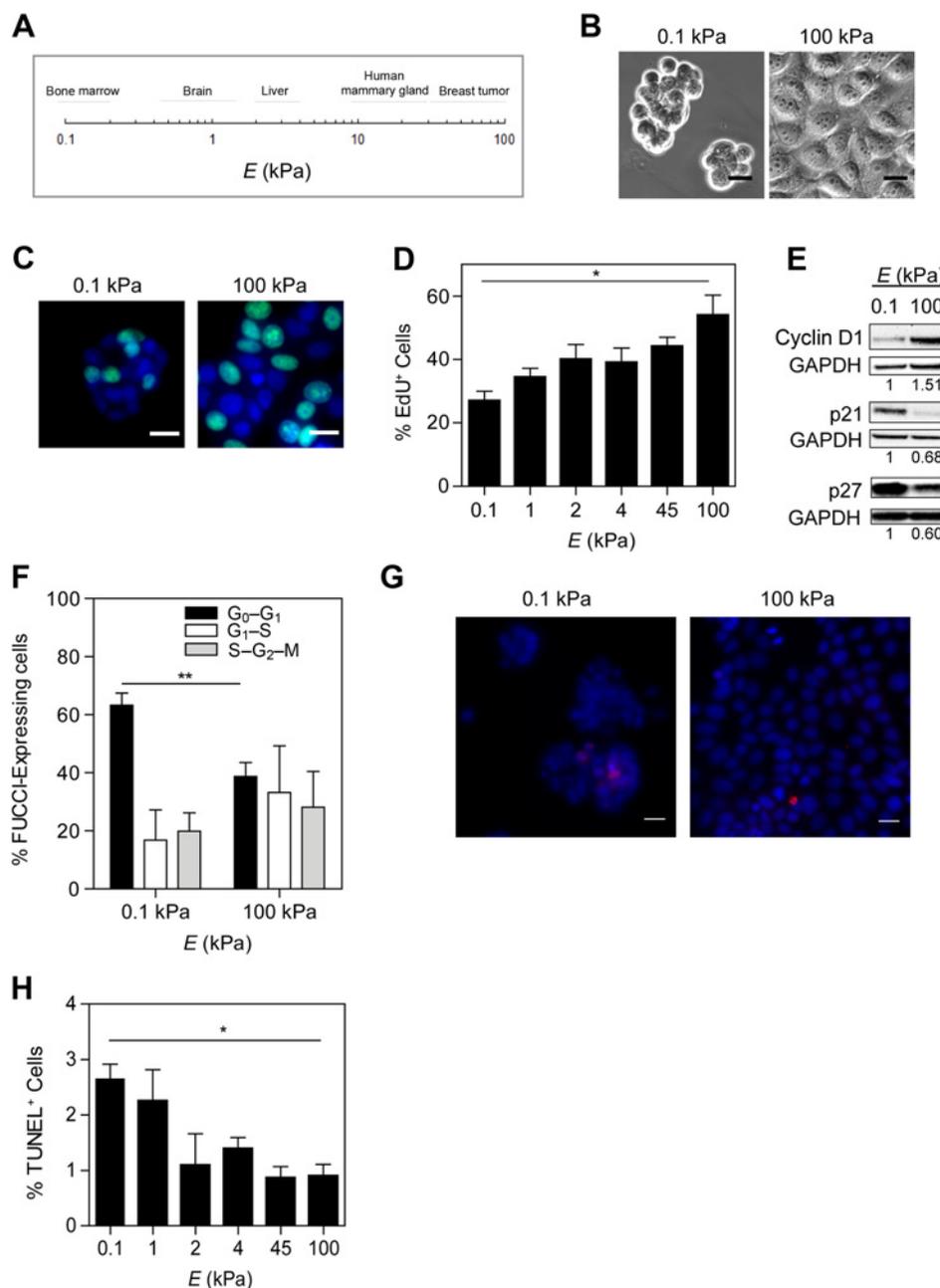
Given that dormant phenotypes can either result from cellular quiescence or from a balance between proliferation and cell death within micrometastases (8), we also investigated the effect of substratum stiffness on apoptosis. We cultured breast cancer cells on soft or stiff substrata and used the TUNEL assay to detect apoptosis (Fig. 1G; Supplementary Fig. S1D). TUNEL staining revealed that apoptosis is increased in both MCF7 and ZR-75-1 cells on soft microenvironments (Fig. 1H; Supplementary Fig. S1E).

Therefore, both proliferation and apoptosis are influenced by substratum stiffness in these ER<sup>+</sup> breast cancer cells. Prolonged periods of dormancy in breast cancer metastases could therefore result from the fact that soft microenvironments promote a balance between proliferation and apoptosis, or because they decrease cell-cycle progression.

### Substratum stiffness regulates the response of ER<sup>+</sup> breast cancer cells to tamoxifen, in part by modulating ER $\alpha$ expression

One of the biggest challenges in cancer treatment is resistance to conventional therapy. Because proliferation is altered by substratum stiffness, we explored how this microenvironmental parameter

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**Figure 1.**

Decreasing substratum stiffness reduces proliferation and increases apoptosis in MCF7 human breast cancer cells. **A**, Schematic of the elastic moduli ( $E$ ) of common breast cancer metastatic sites and breast tumors. **B**, Phase-contrast images of MCF7 human breast cancer cells cultured on soft (0.1 kPa) or stiff (100 kPa) substrata. **C**, EdU analysis of MCF7 cells on soft or stiff substrata. Green, EdU positive; blue, nuclei. **D**, Quantification of EdU incorporation in MCF7 cells on substrata of different stiffnesses. **E**, Immunoblotting analysis for cyclin D1, p21, p27, and GAPDH in MCF7 cells on soft or stiff substrata. **F**, Cell-cycle analysis of FUCCI-expressing MCF7 cells. **G**, TUNEL assay for apoptosis in MCF7 cells on soft or stiff substrata. Red, TUNEL positive; blue, nuclei. **H**, Quantification of TUNEL staining in MCF7 cells on substrata of different stiffnesses. Scale bars, 20  $\mu$ m. Shown are mean  $\pm$  SEM for 3–6 independent experiments. \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; **D** and **F**, one-way ANOVA.

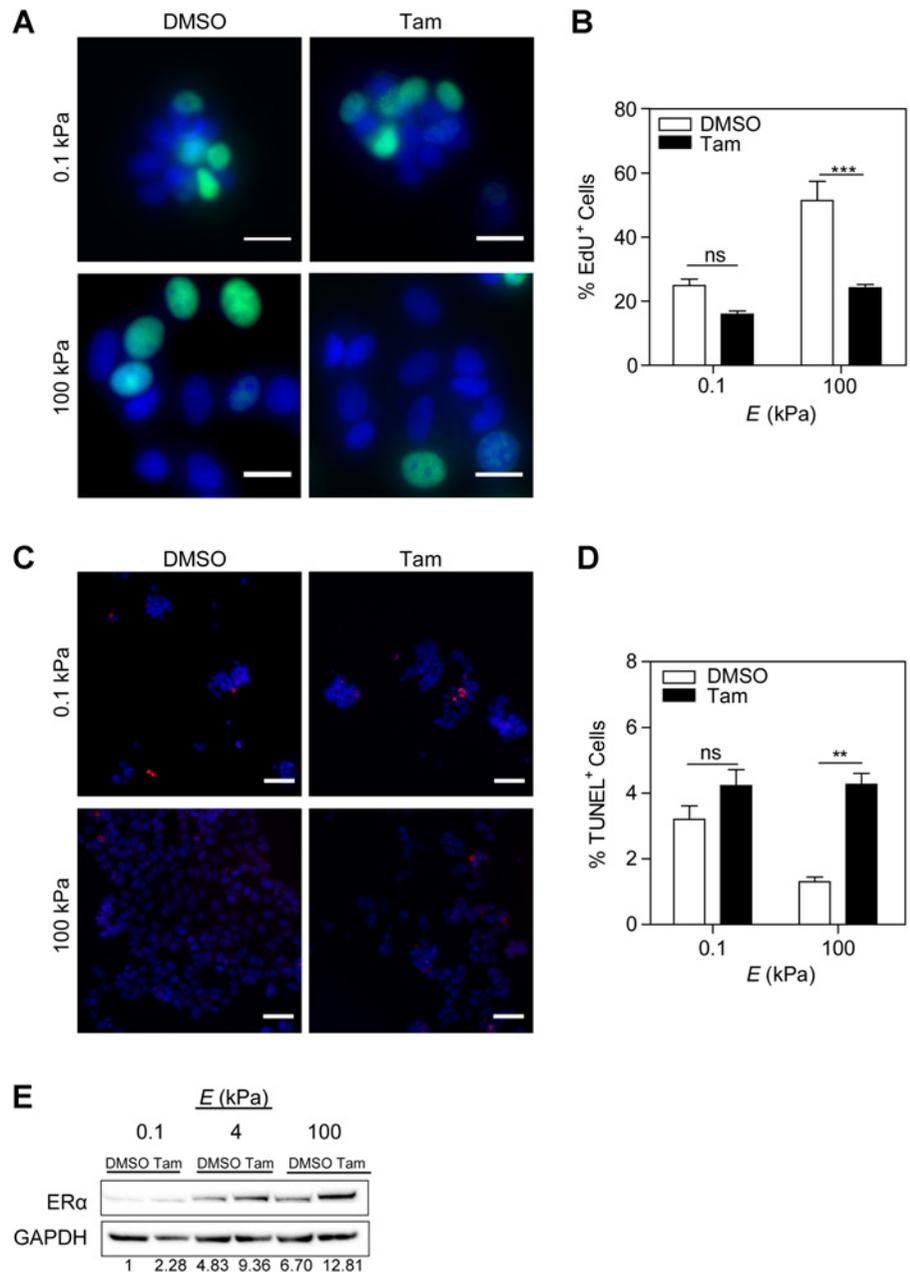
regulates the response of ER $\alpha$ <sup>+</sup> cells to the ER modulator tamoxifen (36). Breast cancer cells were cultured on soft or stiff substrata for 24 hours and treated with tamoxifen at a concentration that matches that found in the serum of breast cancer patients (2  $\mu$ mol/L for 72 hours; refs. 37, 38). We found that whereas tamoxifen inhibits the proliferation of MCF7 cells cultured on stiff microenvironments (Fig. 2A), those cultured on soft substrata are impervious to treatment with tamoxifen (Fig. 2B; Supplementary Fig. S2A). Given that breast cancer metastases are often detected in tissues softer than the primary tumor, our results suggest that conventional therapies may be ineffective at eliminating cells that have disseminated to softer secondary sites. We also assessed apoptosis in tamoxifen-treated MCF7 cells (Fig. 2C) and found that this treatment specifically increases apoptosis in the cells cultured on stiff

substrata, but does not affect those on soft substrata (Fig. 2D). These data show that tamoxifen decreases proliferation and enhances death in cells cultured on substrata that mimic the stiffness of primary tumors, but has no effect in cells on substrata that mimic soft metastatic sites.

Given that tamoxifen targets ER $\alpha$ , we performed immunoblotting analysis for ER $\alpha$  in MCF7 or ZR-75-1 cells treated with or without tamoxifen on soft or stiff substrata. This analysis revealed that culture on soft substrata decreases the expression of ER $\alpha$  (Fig. 2E; Supplementary Fig. S3A). We also found that the levels of ER $\alpha$  are increased in cells treated with tamoxifen (Fig. 2E), consistent with previous reports (39). These data suggest that cells on soft microenvironments downregulate ER $\alpha$  expression, which might partially account for their resistance to tamoxifen.

**Figure 2.**

Substratum stiffness tunes the response of MCF7 cells to tamoxifen. **A**, EdU analysis of MCF7 cells cultured on soft or stiff substrata and treated with either DMSO control or tamoxifen (Tam; 2  $\mu$ mol/L). Green, EdU-positive; blue, nuclei. Scale bars, 20  $\mu$ m. **B**, Quantification of EdU incorporation in cells on soft or stiff substrata treated with either DMSO or tamoxifen (2  $\mu$ mol/L). **C**, TUNEL assay for apoptosis in MCF7 cells cultured on soft or stiff substrata and treated with either DMSO control or tamoxifen. Red, TUNEL-positive; blue, nuclei. Scale bars, 50  $\mu$ m. **D**, Quantification of TUNEL staining in MCF7 cells on soft or stiff substrata treated with or without tamoxifen. **E**, Immunoblotting analysis for ER $\alpha$  in MCF7 cells on substrata of different stiffnesses in the presence or absence of tamoxifen. Shown are mean  $\pm$  SEM for three independent experiments. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ , two-way ANOVA; ns, nonsignificant.

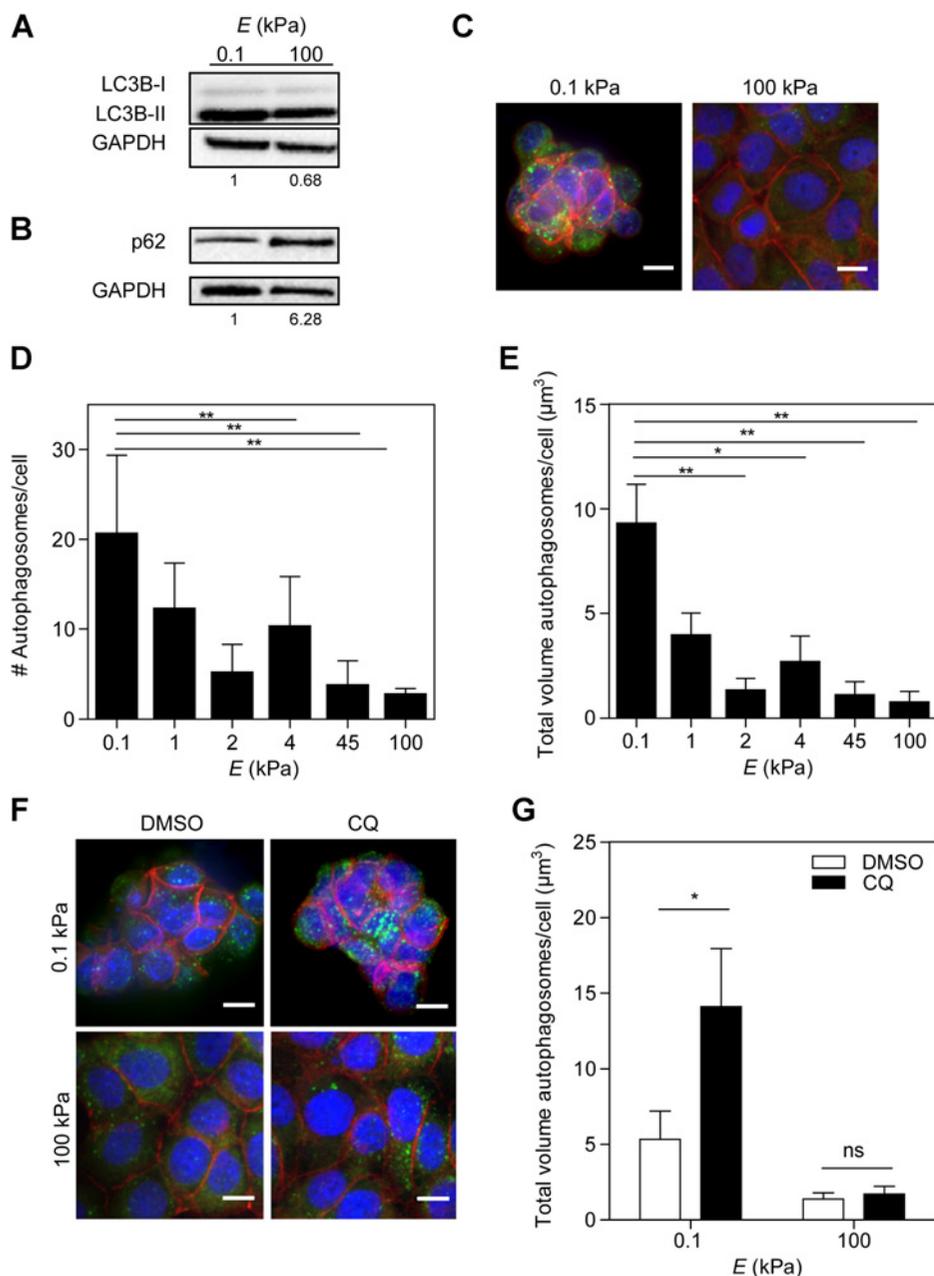


### Soft microenvironments induce chemoresistance by increasing autophagy

It is well appreciated that breast cancer cells can survive at secondary sites for prolonged periods of time, but how they do so remains a mystery. Autophagy was recently suggested as a mechanism to prevent slowly cycling ovarian or quiescent breast cancer cells from undergoing apoptosis (26, 40). To determine whether autophagy in breast cancer cells is affected by the mechanical properties of the microenvironment, we first examined how substratum stiffness regulates autophagosomes. LC3B-II, the lipidated version LC3B-I (cytosolic LC3B), is incorporated into autophagosomal membranes and serves as a marker for autophagosomes (41). We found that the expression of LC3B-II is elevated specifically in MCF7 or ZR-75-1 cells cultured on

soft substrata (Fig. 3A; Supplementary Fig. S3B), suggesting that autophagy may be increased in these microenvironments. We also examined the expression of the autophagy substrate p62, which serves as a link between LC3 and ubiquitinated substrates and is degraded by autophagy. The levels of p62 are therefore inversely correlated with induction of autophagy (41). As predicted, we found that the levels of p62 are decreased in cells on soft substrata (Fig. 3B; Supplementary Fig. S3C), consistent with an increase in autophagy on these microenvironments. Immunofluorescence analysis for LC3B (Fig. 3C; Supplementary Fig. S3D) revealed that the number (Fig. 3D) and total volume (Fig. 3E) of autophagosomes per cell are highest in MCF7 or ZR-75-1 cells on soft substrata, and decrease as substratum stiffness increases.

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**Figure 3.**

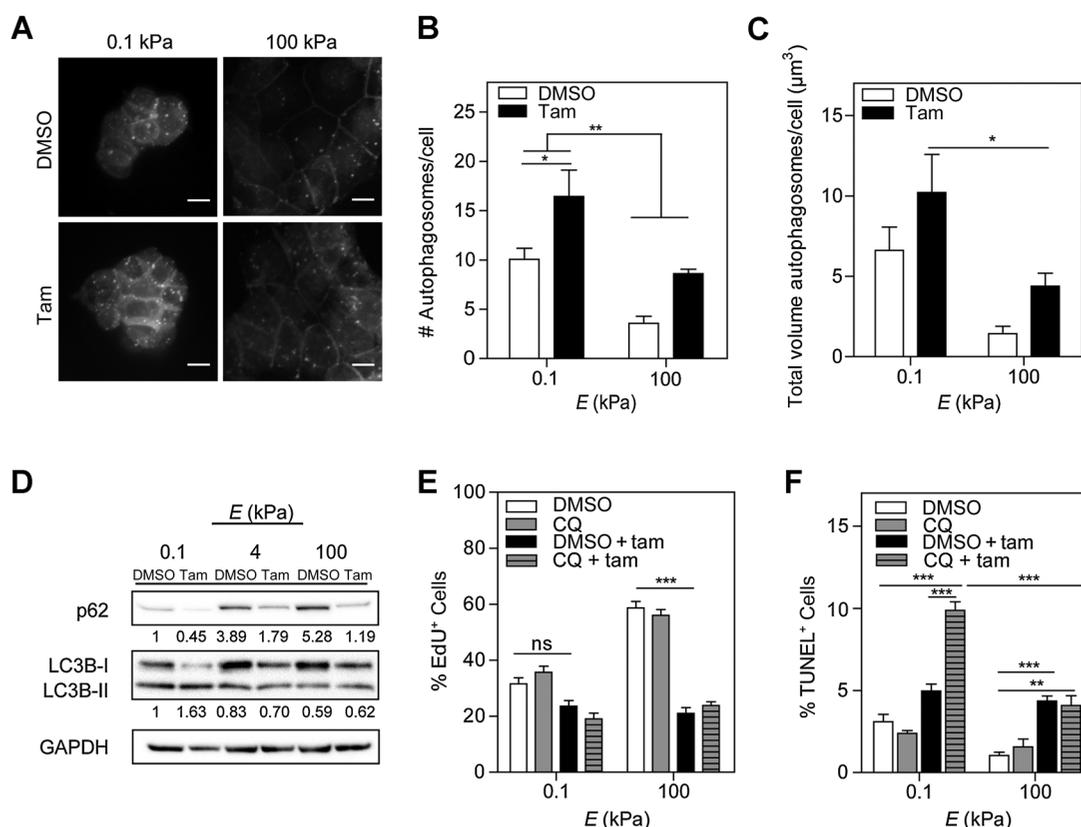
Substratum stiffness regulates autophagy in MCF7 cells. **A** and **B**, Immunoblotting analysis for LC3B (**A**) or p62 (**B**) in MCF7 cells cultured on soft or stiff substrata. The densitometry analysis for LC3B-II/GAPDH or p63/GAPDH are given below each blot. **C**, Immunofluorescence analysis for LC3B to label autophagosomes in MCF7 cells cultured on soft or stiff substrata. Red, E-cadherin; green, LC3B; blue, nuclei. **D** and **E**, Quantification of the number (**D**) and total volume (**E**) of autophagosomes in MCF7 cells cultured on substrata of different stiffnesses. **F**, Immunofluorescence analysis for LC3B in MCF7 cells cultured on soft or stiff substrata in the presence or absence of CQ. Red, E-cadherin; green, LC3B; blue, nuclei. **G**, Quantification of the volume of autophagosomes per cell in the presence or absence of CQ. Scale bars, 10  $\mu\text{m}$ . Shown are mean  $\pm$  SEM for three independent experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  (**D** and **E**, one-way ANOVA; **G**, two-way ANOVA); ns, nonsignificant.

### Inhibiting autophagy sensitizes breast cancer cells on soft microenvironments to tamoxifen

To determine whether the increase in autophagosomes on soft substrata is due to increased autophagosome formation or impaired autophagosome degradation, we performed an autophagosome turnover assay. We cultured MCF7 cells on soft or stiff substrata for 24 hours and then treated them with CQ (0.1  $\mu\text{mol/L}$  for 48 hours), which is a lysosomotropic agent that increases lysosomal pH and inhibits autophagosome-lysosome fusion, consequently blocking autophagosome degradation (41). We found that treatment with CQ leads to an increase in the number and volume of autophagosomes (Fig. 3F), specifically in MCF7 cells cultured on soft substrata (Fig. 3G). These data suggest that autophagosome formation is enhanced in MCF7 cells on soft microenvironments.

Altogether, our data show that autophagy is regulated by substratum stiffness in MCF7 cells, and reveal that autophagy may contribute to the survival of breast cancer cells on soft microenvironments.

To determine whether the increase in autophagy is responsible for resistance to tamoxifen on soft substrata, we treated MCF7 cells with tamoxifen and stained for LC3B as a marker of autophagosomes (Fig. 4A). We found that treatment with tamoxifen increases the number of autophagosomes on both soft and stiff substrata (Fig. 4B). However, the total volume of autophagosomes on soft substrata is significantly higher than that on stiff substrata upon exposure to tamoxifen (Fig. 4C). Immunoblotting analysis revealed that the expression of LC3B-II is increased while the expression of p62 is decreased upon tamoxifen treatment on soft substrata (Fig. 4D). To

**Figure 4.**

Blocking autophagy pharmacologically renders MCF7 cells on soft substrata sensitive to tamoxifen. **A**, Immunofluorescence analysis for LC3B in MCF7 cells cultured on soft or stiff substrata and treated with either DMSO control or tamoxifen (Tam; 2  $\mu$ mol/L). **B** and **C**, Quantification of the number (**B**) and total volume (**C**) of autophagosomes in MCF7 cells cultured on soft or stiff substrata in the presence or absence of tamoxifen. **D**, Immunoblotting analysis for p62 or LC3B in MCF7 cells cultured on substrata of different stiffnesses. **E**, Quantification of EdU incorporation in MCF7 cells treated with or without tamoxifen and CQ. **F**, Quantification of TUNEL staining in MCF7 cells treated with or without tamoxifen and CQ. Scale bars, 10  $\mu$ m. Shown are mean  $\pm$  SEM for three independent experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ , two-way ANOVA; ns, nonsignificant.

uncover whether autophagy promotes tamoxifen resistance on soft substrata, we treated MCF7 or ZR-75-1 cells with CQ and investigated their response to tamoxifen. While blocking autophagy does not affect proliferation (**Fig. 4E**; Supplementary Fig. S3E), our data suggest that apoptosis increases in autophagy-impaired cells in response to tamoxifen on soft substrata (**Fig. 4F**; Supplementary Fig. S3F). In contrast, blocking autophagy does not affect apoptosis in cells on stiff substrata (**Fig. 4F**; Supplementary Fig. S3G and S3H). Soft microenvironments therefore render ER $\alpha$ <sup>+</sup> breast cancer cells resistant to tamoxifen by upregulating autophagy.

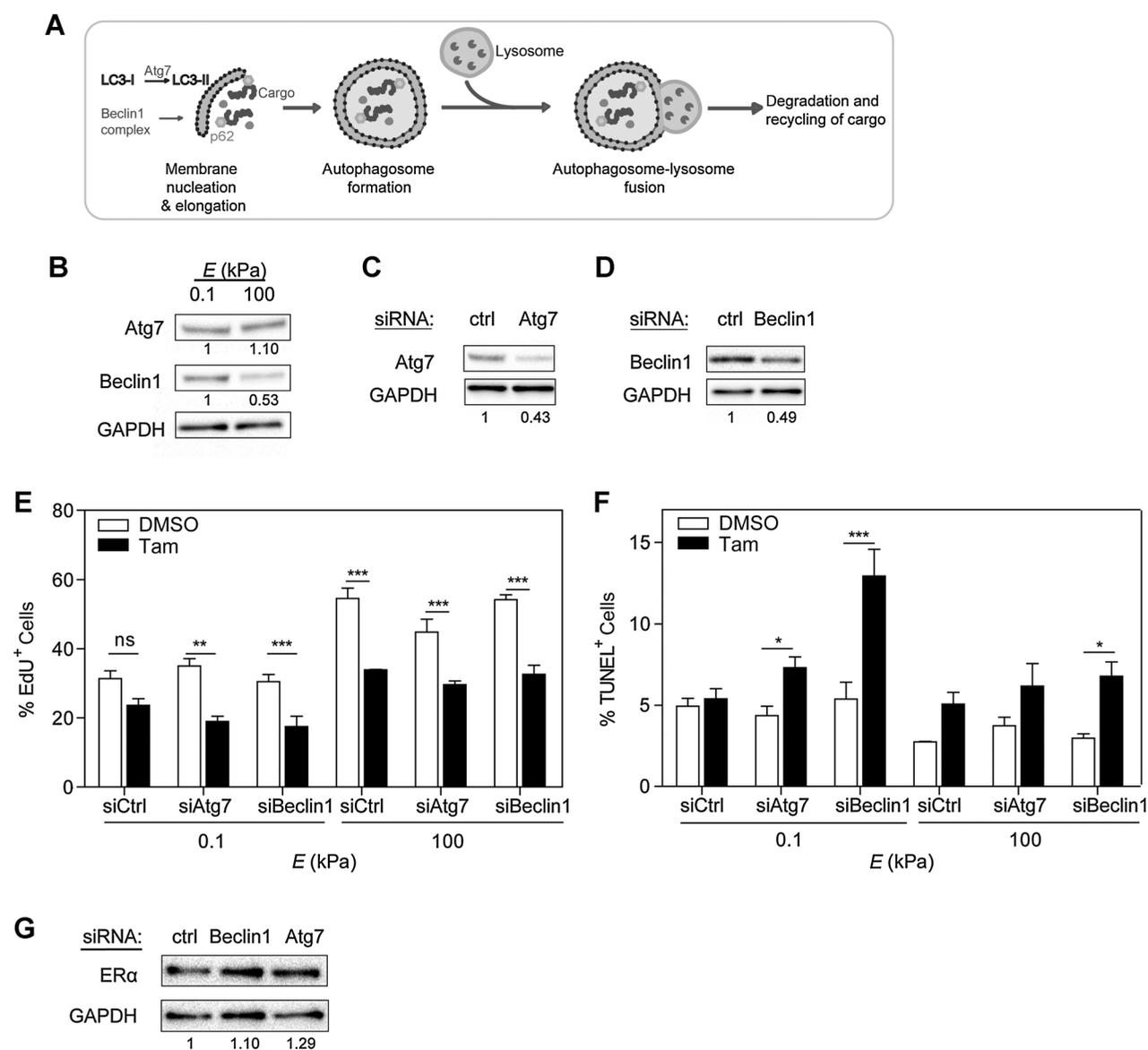
Formation of the autophagosome consists of initiation, nucleation, and elongation steps that are regulated by multiprotein complexes including those containing Beclin1 and Atg7 (**Fig. 5A**). Beclin1 interacts with class III type phosphoinositide 3-kinase/Vsp34 to coordinate membrane nucleation, whereas Atg7 catalyzes lipidation of LC3-I to LC3-II with phosphatidylethanolamine during elongation of the autophagosomal membrane (42). To define how the mechanical properties of the microenvironment affect autophagosome formation, we examined the expression levels of upstream regulators Beclin1 and Atg7 in cells cultured on soft or stiff substrata. Immunoblotting analysis revealed that the levels of Beclin1 are increased in cells on soft substrata (**Fig. 5B**), consistent with a possible increase in vesicle nucleation (43). To determine whether decreasing upstream initiation

of autophagy alters response to tamoxifen, we used siRNAs to deplete Atg7 (**Fig. 5C**) or Beclin1 (**Fig. 5D**) in MCF7 cells cultured on soft or stiff substrata in the presence or absence of tamoxifen. We found that decreasing the levels of Atg7 or Beclin1 sensitizes MCF7 cells to tamoxifen treatment. Specifically, we observed that proliferation decreases in autophagy-deficient cells on soft substrata in response to tamoxifen (**Fig. 5E**). We also found that apoptosis increases dramatically on soft substrata in Beclin1- or Atg7-deficient cells treated with tamoxifen (**Fig. 5F**). These data suggest that soft microenvironments upregulate formation of autophagosomes in ER $\alpha$ <sup>+</sup> breast cancer cells, which renders them resistant to tamoxifen. However, depleting Atg7 or Beclin1 does not alter the expression levels of ER $\alpha$  (**Fig. 5G**). Impairing autophagosome nucleation, therefore, enhances the response of MCF7 cells to tamoxifen without affecting ER $\alpha$  expression.

#### Autophagy is increased downstream of integrin-linked kinase

We then investigated the mechanisms by which substratum stiffness regulates autophagy. Mechanical signals received by  $\beta$ 1-integrin are transduced through integrin-linked kinase (ILK) to the actin cytoskeleton and downstream pathways (44). Consistent with previously reported findings in other breast cancer cell lines (29), we found that

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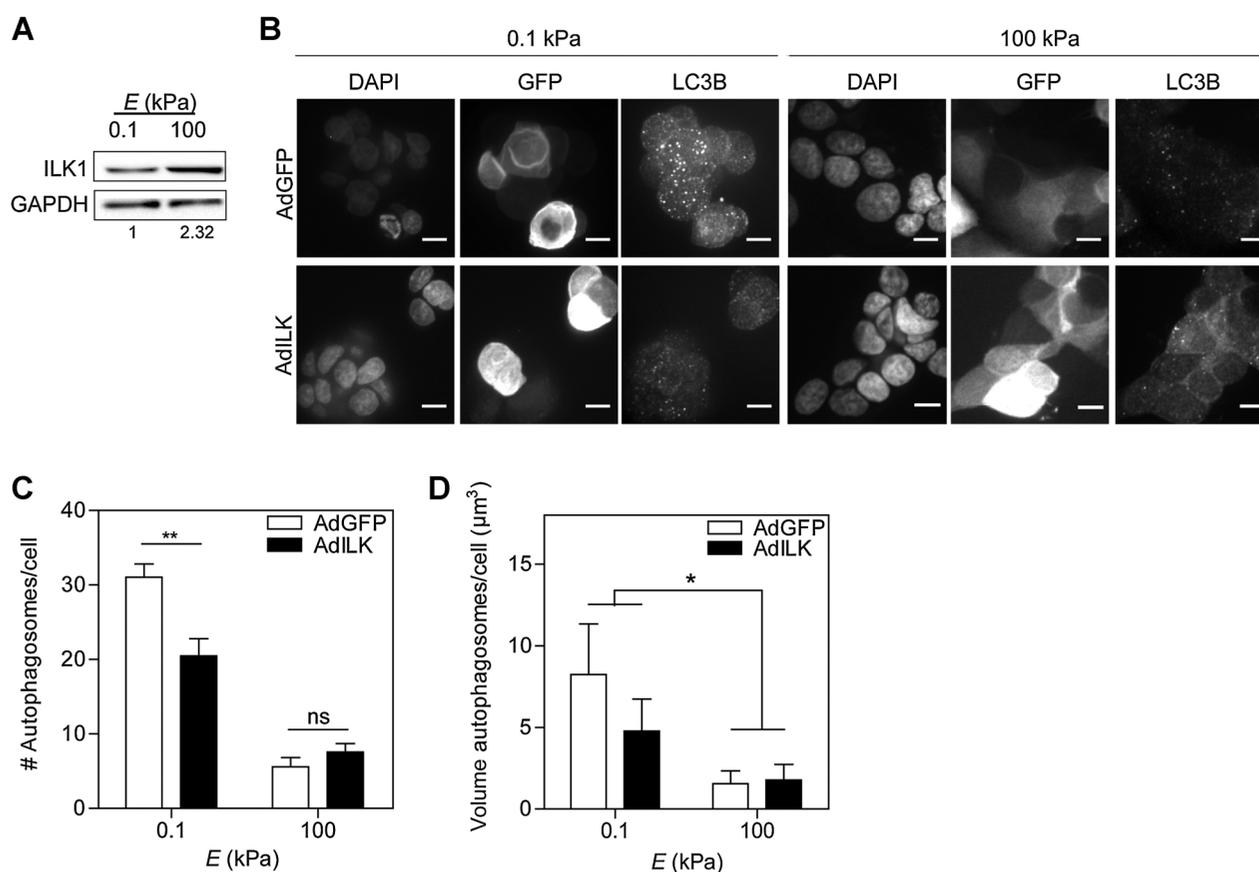
**Figure 5.**

Blocking autophagosome nucleation by siRNA-mediated knockdown renders MCF7 cells on soft substrata sensitive to tamoxifen. **A**, Stages of autophagosome assembly and degradation. **B**, Immunoblotting analysis for Atg7, Beclin1, or GAPDH in MCF7 cells cultured on soft or stiff substrata. Immunoblotting analysis for Atg7 (**C**) and Beclin1 (**D**) in MCF7 cells transfected with siRNA against Atg7 or Beclin1. **E** and **F**, Quantification of EdU incorporation (**E**) or TUNEL staining (**F**) in MCF7 cells transfected with siAtg7 or siBeclin1 and treated with or without tamoxifen (Tam) on soft or stiff substrata. **G**, Immunoblotting analysis for ERα or GAPDH in MCF7 cells transfected with siAtg7 or siBeclin1. Shown are mean ± SEM for three independent experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ , two-way ANOVA; ns, nonsignificant.

MCF7 cells cultured on stiff substrata have elevated expression of ILK (**Fig. 6A**). To determine whether integrin signaling through ILK regulates autophagy, we used an adenoviral approach to ectopically express ILK in cells cultured on soft or stiff substrata (**Fig. 6B**). Immunofluorescence analysis for LC3B (**Fig. 6B**) revealed that ectopic expression of ILK downregulates the number (**Fig. 6C**; Supplementary Fig. S3G) and volume (**Fig. 6D**; Supplementary Fig. S3H) of autophagosomes in MCF7 or ZR-75-1 cells on soft substrata. Our data therefore suggest that substratum stiffness regulates autophagosome formation, in part, by signaling through ILK.

## Discussion

The tendency of a tumor to metastasize to different tissues reflects how well it can adapt to the microenvironment of a secondary site. More than a century ago, Paget's seed and soil hypothesis posited that disseminated tumor cells will grow only in hospitable niches (45). Consistently, breast cancer metastases are most often detected in tissues such as the brain, lung, liver, and bone marrow, which are softer than both the normal mammary gland as well as the primary breast tumor. Secondary sites harbor dormant tumors that have been

**Figure 6.**

ILK regulates autophagy in MCF7 cells. **A**, Immunoblotting analysis for ILK or GAPDH in MCF7 cells cultured on soft or stiff substrata. **B**, Immunofluorescence analysis for LC3B in MCF7 cells after ectopic expression of ILK on soft or stiff substrata. **C** and **D**, Quantification of the number (**C**) and total volume (**D**) of autophagosomes in MCF7 cells after ectopic expression of ILK on soft or stiff substrata. Scale bars, 10 μm. Shown are mean ± SEM for three independent experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , two-way ANOVA; ns, nonsignificant.

implicated in breast cancer relapse (5, 8, 9); however, the survival mechanisms used by dormant cancer cells to perdure at soft metastatic sites have remained elusive. Here, we demonstrate that breast cancer cells cultured on soft microenvironments upregulate autophagy, increasing their ability to survive for prolonged periods of time. In addition, we show that breast cancer cells that are cultured on soft microenvironments downregulate expression of ER $\alpha$  and become impervious to treatment with tamoxifen (Fig. 7).

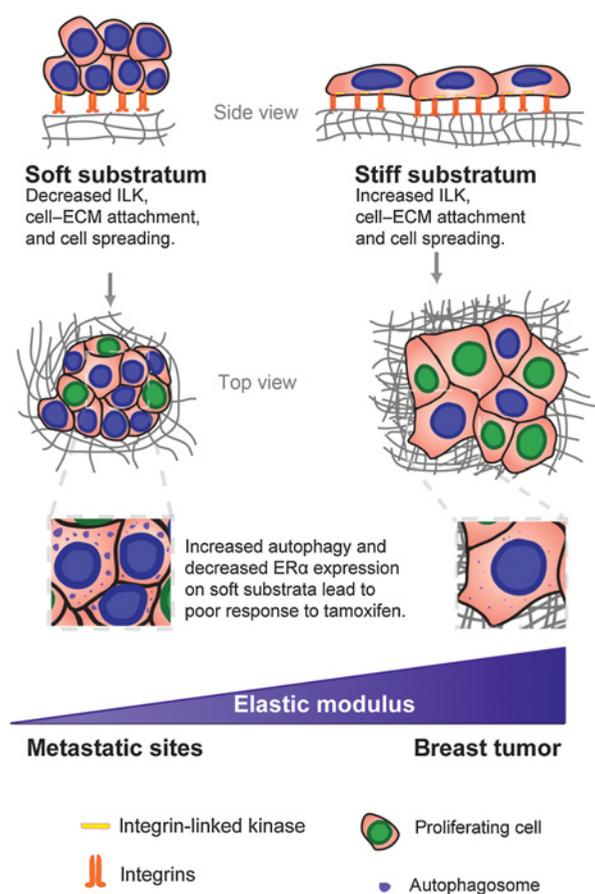
The majority of breast cancers are ER $^+$  and can therefore be targeted with endocrine therapies such as tamoxifen. However, many patients eventually develop resistance to these therapies due to decreased ER expression (46). Our data suggest that the mechanical microenvironment regulates the expression of ER $\alpha$ , thereby modulating the response to tamoxifen treatment. Novaro and colleagues previously reported that decreasing cell–ECM attachments in normal mammary epithelial cells by blocking  $\beta$ 1-integrin decreases ER $\alpha$  expression (47). Concordant with this finding, our data suggest that ER $\alpha$  expression is decreased in cells on soft microenvironments (Fig. 2E), which have weaker integrin-mediated cell–matrix adhesions (Fig. 6A). These observations could partially explain why secondary lesions are resistant to hormonal therapy and remain asymptomatic at soft metastatic sites.

Our data also demonstrate that on soft substrata, ER $\alpha^+$  breast cancer cells activate autophagy, which enhances their survival and promotes resistance to antiestrogen therapy (Figs. 4 and 5). Autophagy mobilizes intracellular energy stores to meet cellular metabolic demands (48) and can protect cancer cells from apoptosis (49, 50).

Consequently, preclinical studies have investigated combining autophagy inhibitors with chemotherapeutics (51), and it has been suggested that inhibiting autophagy leads to apoptosis of gastrointestinal stromal tumor cells (27). Autophagy has also been shown to play a role in the maintenance of dormant tumors (40). In an ovarian carcinoma model, the formation of dormant tumors was found to correlate with increased formation of autophagosomes (26). It is therefore possible that breast cancer cells activate autophagy pathways to survive in soft microenvironments and that this contributes to resistance to tamoxifen. Consistently, we found that inhibiting autophagy pharmacologically or by knocking down the expression of Atg7 or Beclin1 sensitizes breast cancer cells to tamoxifen when they are on soft substrata (Figs. 4F and 5G). Conversely, we found a decrease in ER $\alpha$  expression under conditions that increase autophagy, but it is unclear whether these two processes are directly coupled. Cook and colleagues demonstrated that knockdown of ER $\alpha$  leads to resistance to antiestrogen therapy while causing an increase in autophagy (52); however, it remains unclear whether ER $\alpha$  regulates induction of autophagy or if the increase in autophagy is solely a stress response caused by a decrease in ER $\alpha$  levels.

The effects of the mechanical microenvironment on therapeutic resistance are not limited to hormone therapies. We found that the

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**Figure 7.**

Substratum stiffness regulates autophagy, in part, by signaling through ILK. Breast cancer cells become resistant to tamoxifen on soft microenvironments, reminiscent of metastatic sites, in part due to increased autophagy and decreased expression of ER $\alpha$ .

response of breast cancer cells to 5-fluorouracil (5-FU) is similarly regulated by substratum stiffness (Supplementary Fig. S4A–S4D), and that breast cancer cells residing on soft substrata are sensitized to treatment with 5-FU upon pharmacologic inhibition of autophagy (Supplementary Fig. S5A–S5E). It was recently reported that autophagy is critical for the maintenance of dormant breast cancer cells, and that once dormant cells have transitioned to proliferation, they no longer require autophagy for survival (40). Our data also indicate that autophagy is increased in breast cancer cells that become less proliferative in soft microenvironments, and that response to chemotherapeutic agents could be ameliorated by impairing autophagy.

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The ability of a cell to proliferate depends on cell-ECM adhesion as well as exposure to growth factors and cytokines (53). A lack of ECM attachment in anchorage-dependent cells, as well as other microenvironmental stress factors, activates autophagy. Autophagy has been implicated in developmental processes that involve luminal clearing such as mammary morphogenesis (54), as well as in disease, where its role in cancer has been shown to be context-dependent. Although previous studies have not explored the regulation of autophagy by the mechanical microenvironment, several of them have emphasized the role of integrin-ECM interactions in autophagy regulation. During dissemination, detachment from the ECM can induce autophagy to give cancer cells time to establish cell-ECM contacts necessary to survive at secondary sites (53). The travel of cancer cells from a stiff tumor microenvironment to a soft secondary site could similarly result in a decrease in integrin-ECM interactions. Considering its role in the transmission of mechanical signals from  $\beta$ 1-integrin to downstream signaling pathways, we predicted that ILK could be involved in the regulation of autophagy. Consistently, we found that ectopically expressing ILK, which has previously been shown to strengthen cell-ECM adhesion (55), leads to a decrease in autophagy (Fig. 6; Supplementary Fig. S3G and S3H). Weaker cell-ECM attachments induced by soft substrata also lead to decreased proliferation and growth arrest, which could enable the long-term survival of breast cancer cells in soft microenvironments. These findings provide a potential connection between increased autophagy and tumor dormancy, as suggested recently (40), and implicate a role for the mechanical microenvironment in regulating cell-survival mechanisms in breast cancer. The role of ILK and its downstream cytoskeletal and signaling targets in autophagosome assembly will be the subject of a future study.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Authors' Contributions

A.A. Anlaş: Conceptualization, investigation, writing-original draft. C.M. Nelson: Conceptualization, supervision, funding acquisition, project administration, writing-review and editing.

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## Soft Substrata Activate Autophagy in Breast Cancer Cells

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## Soft Microenvironments Induce Chemoresistance by Increasing Autophagy Downstream of Integrin-Linked Kinase

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