

# Host epithelial geometry regulates breast cancer cell invasiveness

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Edited by Joan S. Brugge, Harvard Medical School, Boston, MA, and approved October 15, 2012 (received for review November 15, 2011)

**Breast tumor development is regulated in part by cues from the local microenvironment, including interactions with neighboring nontumor cells as well as the ECM. Studies using homogeneous populations of breast cancer cell lines cultured in 3D ECM have shown that increased ECM stiffness stimulates tumor cell invasion. However, at early stages of breast cancer development, malignant cells are surrounded by normal epithelial cells, which have been shown to exert a tumor-suppressive effect on cocultured cancer cells. Here we explored how the biophysical characteristics of the host microenvironment affect the proliferative and invasive tumor phenotype of the earliest stages of tumor development, by using a 3D microfabrication-based approach to engineer ducts composed of normal mammary epithelial cells that contained a single tumor cell. We found that the phenotype of the tumor cell was dictated by its position in the duct: proliferation and invasion were enhanced at the ends and blocked when the tumor cell was located elsewhere within the tissue. Regions of invasion correlated with high endogenous mechanical stress, as shown by finite element modeling and bead displacement experiments, and modulating the contractility of the host epithelium controlled the subsequent invasion of tumor cells. Combining microcomputed tomographic analysis with finite element modeling suggested that predicted regions of high mechanical stress correspond to regions of tumor formation in vivo. This work suggests that the mechanical tone of nontumorigenic host epithelium directs the phenotype of tumor cells and provides additional insight into the instructive role of the mechanical tumor microenvironment.**

host–tumor interactions | mechanotransduction | focal adhesion kinase | integrin clustering

More than 90% of all human mammary carcinomas originate in the epithelial ducts rather than the surrounding connective tissue (1), and the majority of these arise from the terminal ductal lobular unit (2). In mice, strains with robust lateral branching in the ductal tree show high incidence of spontaneous mammary tumors (3–5). Moreover, the specific location where a tumor develops in the breast can have a profound effect on the resulting breast cancer outcome: patients with tumors in the upper lateral quadrant of the breast have a better prognosis than those with tumors that develop in one of the other three quadrants (6, 7). These architecture-dependent variations in tumor development underscore the notion that the microenvironment of the tumor in vivo plays an important role in determining the phenotypic outcome of genetic mutations (8, 9).

In culture and in vivo, the tumor microenvironment can suppress or induce a malignant phenotype in cells with a preexisting malignant genotype (10–13). Indeed, many people harbor potentially malignant tumors that remain dormant for several years (14–16). Recent findings suggest that whereas the microenvironment surrounding a tumor normally provides tumor-suppressive signals (10, 17–19), the loss of tissue homeostasis can induce the development of an aberrant tumor microenvironment that can act as a potent tumor promoter (19–23). Thus, genetic mutations that have the potential to lead to the initiation of tumors can be restrained by a nonpermissive tissue microenvironment (19).

A developing tumor can affect the mechanical properties of its microenvironment in ways that drive tumor progression (24, 25).

Normally, cells exert contractile forces against their surrounding microenvironment (26, 27). Failure to maintain a balance between these exerted forces and the opposing forces from the microenvironment causes aberrations that promote the progression of disease, including cancer (28). Cells recognize an increase in ECM stiffness and respond by generating increased traction forces on their surroundings; this in turn enhances growth, survival, and invasion of tumor cells by promoting focal adhesion maturation and signaling through actomyosin contractility (29). Motility, invasion, and metastatic dissemination are assisted by the disruption of cell–cell junctional integrity and cytoskeletal remodeling (28–31). The signaling molecule Rho GTPase has been identified as a key effector of these processes: Rho activity is frequently elevated in tumors (32), and elevated signaling through Rho can regulate cell proliferation (32) and invasion (33–35). Increased Rho signaling can also affect the alignment of collagen fibers and prime the microenvironment for subsequent invasion by tumor cells (35).

Here we used a 3D microlithography-based approach to examine how the phenotype of tumor cells varies with their location within an engineered epithelial host tissue. Our results show that mammary tumor cells proliferate or invade preferentially in regions of endogenously generated mechanical stress. We show that the tumor cell phenotype can be controlled by altering the contractility of the host epithelial tissue through the expression of dominant-negative or constitutively active RhoA. We further show that at regions of high mechanical stress that can activate the tumor phenotype, tumor cells require signaling through focal adhesion kinase (FAK) to invade. In regions of lower mechanical stress that inhibit tumor cell invasion, artificially inducing the assembly of focal adhesions by promoting integrin clustering is sufficient to drive the invasive phenotype.

## Results

**Tumorigenic Phenotype Is Suppressed Within Engineered Mammary Ducts.** To explore whether the normal microenvironment of the host could alter the phenotype of tumor cells, we used a microlithography-based approach to engineer surrogate ducts composed of normal mammary epithelial cells that contained a single tumor cell (Fig. 1*A* and Table S1). We varied the initial position of the tumor cell within the engineered ducts and monitored its resulting phenotype (Fig. 1*B*). We found that the proliferation of several breast cancer cell lines depended on their initial location in the tissue. In particular, tumor cell lines previously shown to be non-invasive, including SKBR3, MDA-MB-361, and MDA-MB-453, proliferated at a significantly higher rate when they were located at the ends of the tissue surrogates, compared with when the tumor cells were located elsewhere within the duct (Fig. 1*C*).

Author contributions: E.B., D.C.R., and C.M.N. designed research; E.B., J.P.G., K.L., N.G., and D.C.R. performed research; and E.B. and C.M.N. wrote the paper.

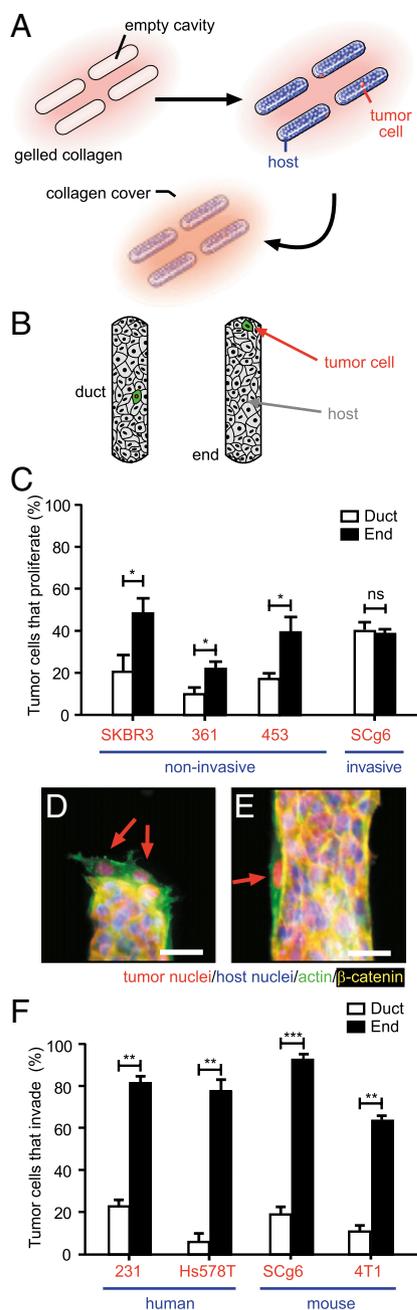
The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1118872109/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1118872109/-DCSupplemental).



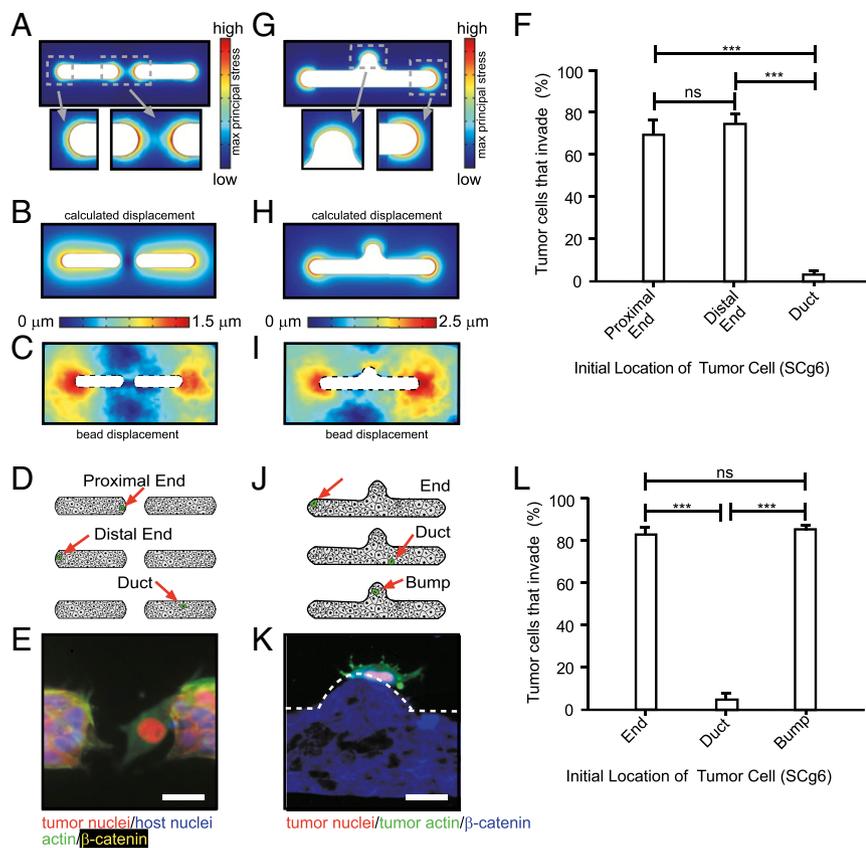
**Fig. 1.** Normal host microenvironment reduces tumor cell proliferation and suppresses invasion from the duct. (A) Schematic of 3D microlithography-based approach to engineer epithelial tissues. (B) Schematic depicting duct vs. end locations in engineered epithelial tissues. (C) Noninvasive tumor cells exhibit increased proliferation at the ends compared with the duct; invasive tumor cells show no preference as quantified by EdU (5-ethynyl-2'-deoxyuridine) incorporation. Tumor cells (nuclei in red) invading from the end of a tissue and (E) suppressed from invading from the duct. (Scale bars, 25  $\mu$ m.) (F) Quantification of invasion using different combinations of normal (host) cells and tumor cell lines reveals that the spatial regulation of invasive phenotype is not cell line-dependent. Error bars represent SEM ( $n = 3$ ). Paired  $t$  test: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

Conversely, whereas the proliferation rates of invasive breast tumor cell lines, including SCg6, 4T1, MDA-MB-231, and Hs578T, were independent of their initial location within the tissue (Fig. 1C and Fig. S1), these same cells showed dramatic differences in invasiveness depending on their location. When grown as homogeneous cultures, the cells invaded randomly into the surrounding

collagen gel, as would be expected for tumor cell lines characterized as invasive (Fig. S2). However, when individual tumor cells were incorporated into nonmalignant host tissue surrogates, they invaded much more readily when initially located at the end rather than in the duct (Fig. 1D–F and Fig. S3). Tumor cell invasion required signaling through epidermal growth factor receptor and the catalytic activity of matrix metalloproteinases, because inhibiting these with AG1478 and GM6001, respectively, blocked invasion (Fig. S4). Breast tumor cell lines previously shown to be noninvasive remained noninvasive when incorporated within the engineered ducts, regardless of their location (Fig. S5). The end region thus selectively promotes proliferation or invasion of mammary tumor cells, suggesting the presence of instructive cues in the host microenvironment.

**Tumor Phenotype Corresponds with Endogenous Mechanical Stress of the Host Tissue.** Tissue geometry can affect cellular phenotype by establishing regional differences in endogenous mechanical stress or concentration gradients of diffusible molecules, including TGF- $\beta$  (36, 37). We used the finite element method (FEM) to simulate two tissue geometries that could distinguish between these signals and reveal which regulated tumor cell invasion. As previously described (36, 38), we simulated contraction of an epithelial tissue within a collagenous matrix and used FEM to compute the resulting maximum principal stress within the tissue, which represents a coordinate-invariant description of the stress at each point (Fig. 2A); full details of the model can be found in *SI Methods*. For adjacent pairs of ducts, this model predicted that the ends of the tissue experience high mechanical stress, with higher stress predicted at the proximal ends than the distal ends. To test our simple computational model, calculated displacements (Fig. 2B) were compared with those measured experimentally (Fig. 2C) by tracking the movement of beads embedded within the surrounding collagen before and after relaxing the tissue. We found that our computational models generated displacement fields that were similar in magnitude to the experimental system. Similarly, our model predicted regions of high displacement at the tips and correctly predicted lower displacements at the proximal ends than at the distal ends (because the tissues are in opposition to each other and thus pulling on the matrix between them in opposite directions) but failed to capture some of the larger spatial patterns of displacement in the matrix. We then engineered pairs of adjacent ducts that contained a single tumor cell (Fig. 2D). Invasion occurred at regions of high mechanical stress, as tumor cells invaded from both the proximal and distal ends but were prevented from invading when in the duct region (Fig. 2E and F). Similarly, tissues with a central bump were predicted to have high mechanical stress and displacements at the ends and at the bump (Fig. 2G–I), and tumor cells invaded preferentially from these high-stress regions (Fig. 2J–L). Tumor cell invasion thus correlates with regions of high endogenous mechanical stress calculated within the engineered host epithelium. Surprisingly, the levels of invasion at the end and the bump regions were not significantly different, despite the distinct levels of predicted mechanical stress. This suggests that there is a threshold level of stress within the host at which invasion occurs.

**Altering Host Tissue Contractility Directs Tumor Cell Invasiveness.** Mechanical stress is generated by the actin cytoskeleton and transmitted between cells through cadherin-mediated intercellular adhesions (39). Reducing mechanical stress by inhibiting the Rho effector, Rho kinase (ROCK) blocked invasion of the tumor cells from the ends of the tissues (Fig. 3A, B, and L). Similarly, inhibiting actomyosin-mediated contractility using blebbistatin, a selective inhibitor of nonmuscle myosin II ATPase activity, blocked tumor cell invasion from the tissue ends (Fig. 3C and L). However, increasing cytoskeletal contractility and mechanical stress using the phosphatase inhibitor calyculin A did not further increase invasion from the ends (Fig. S6), suggesting that a maximum level of



**Fig. 2.** Regions of high endogenous mechanical stress are permissive to tumor cell invasion. (A and G) Predicted endogenous mechanical stress and (B and H) displacement. (C and I) Experimental bead displacement in culture. (D) Schematic showing proximal end, distal end, and duct regions of adjacent tissues. (E) Fluorescence image of SCg6 tumor cell invading from the proximal end of a tissue. (Scale bar, 25  $\mu\text{m}$ .) (F) Quantification of invasion from adjacent tissues. Error bars represent SEM ( $n = 3$ ). One-way ANOVA and Tukey-Kramer posttest:  $***P < 0.001$ . (J) Schematic showing end, duct, and bump regions of a tissue. (K) Fluorescence image of SCg6 tumor cell invading from the bump region of a tissue. (Scale bar, 25  $\mu\text{m}$ .) (L) Quantification of invasion from ducts with a bump. Error bars represent SEM ( $n = 3$ ). One-way ANOVA and Tukey-Kramer posttest:  $***P < 0.001$ .

invasion was already induced by the microenvironment of this region of the surrogate tissues. This finding is again consistent with a threshold level of stress required for invasion.

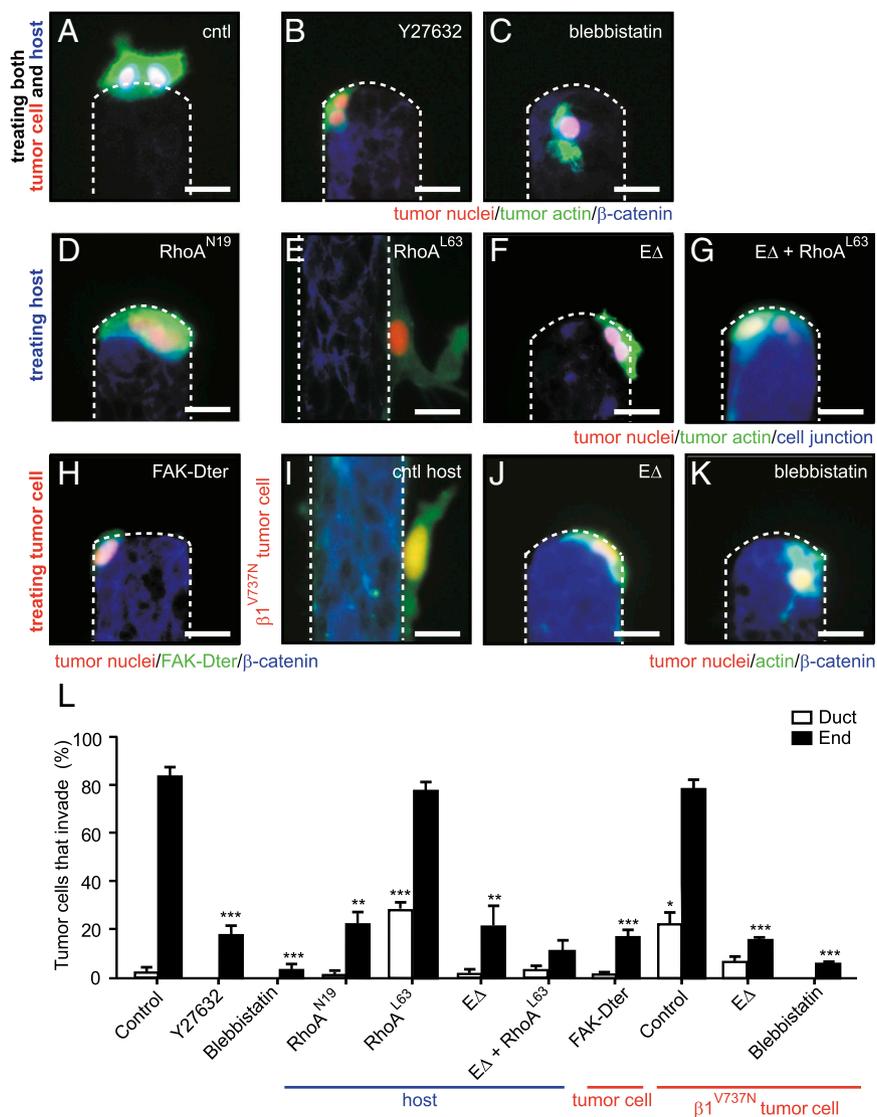
We next selectively modulated the contractility of the non-malignant host epithelial tissue without altering that of the embedded tumor cell. Low levels of Rho activity prevent myosin light chain phosphorylation and cytoskeletal contraction (40). Transducing host epithelial cells with an adenovirus encoding dominant-negative RhoA (RhoA<sup>N19</sup>) reduced the levels of endogenous contractility generated within the host tissue (Fig. S7A) without causing the tissues to dissociate (Fig. S7B). Host expression of RhoA<sup>N19</sup> also reduced the subsequent invasion of tumor cells from the ends of the tissue (Fig. 3D and L). Conversely, transducing host epithelial cells with an adenovirus encoding constitutively active RhoA (RhoA<sup>L63</sup>) resulted in an increase in the levels of endogenous contractility generated by the tissues (Fig. S7A), which again remained intact (Fig. S7B). We found that when embedded within RhoA<sup>L63</sup>-expressing host tissue surrogates, tumor cells were able to invade from all locations (Fig. 3E and L). Intercellular transmission of mechanical stress can be disrupted by expressing a dominant-negative mutant of E-cadherin that lacks the  $\beta$ -catenin-binding domain (E $\Delta$ ) and thereby prevents coupling to the actin cytoskeleton (36, 41, 42). Disrupting intercellular transmission of mechanical stress in the host epithelial cells using an adenovirus encoding E $\Delta$  (Fig. S7A and B) significantly reduced invasion of tumor cells (Fig. 3F and L) and prevented preferential proliferation of noninvasive tumor cell lines (Fig. S8). The effects of the adenovirus encoding E $\Delta$  did not result from a loss of apicobasal polarity within the host, as shown by staining for ZO-1 (Fig. S7C), and could not be rescued by simultaneously expressing RhoA<sup>L63</sup> (Fig. 3G and L). These data suggest that the tumorigenic phenotype is modulated by variations in mechanical stress that are generated by actomyosin-mediated contractility of the normal host epithelium and transmitted through intercellular adhesions within the host tissue.

### Tumor Cell Invasion Is Regulated Through Focal Adhesions and Integrin Clustering.

It has been shown that mechanical stress results in increased integrin expression, activation, and focal adhesion formation, ultimately leading to tumor cell invasion (29, 43). Furthermore, the phosphorylation and activation of FAK has been found to be enhanced in high-stress regions of mammary epithelial tissues (36). To determine whether FAK regulates the response of tumor cells to host tissue contractility, we expressed a dominant-negative mutant that lacks the kinase domain (FAK-Dter) (44) (Fig. S7D). Transducing tumor cells with an adenovirus encoding FAK-Dter significantly inhibited their ability to invade (Fig. 3H and L). Conversely, forcing the assembly of focal adhesions specifically in tumor cells by selectively expressing an auto-clustering mutant of  $\beta$ 1-integrin ( $\beta$ 1<sup>V737N</sup>) (Fig. S7E-G) induced invasion from all locations within the tissue surrogates (Fig. 3I and L). However, inducing focal adhesion assembly in tumor cells did not rescue the inhibition of invasion resulting from expression of E $\Delta$  in the host cells (Fig. 3J and L) or treatment with blebbistatin (Fig. 3K and L). These data suggest that integrin clustering and FAK activation are required, but not sufficient, for invasion from the high-stress ends. Whereas promoting the assembly of focal adhesions can induce invasion in regions of lower mechanical stress, it does not induce tumor cells to invade when the intercellular transmission of mechanical stress between host cells is disrupted or when contractility is inhibited throughout the tissue. These data are consistent with the notion that tumor cell invasion requires a threshold level of mechanical stress within the host tissue, as inferred from the tissue geometry experiments (Fig. 2).

### Predictions of Mechanical Stress Correspond with Tumor Development in Vivo.

To determine whether regions of high mechanical stress correspond with those preferential for tumor development in vivo, we compared qualitatively the endogenous stress profiles in a simple contractile model of the mammary gland with the spatial patterns



**Fig. 3.** Endogenous contractility of the host epithelium regulates tumor cell invasion. Fluorescence images of EpH4 host tissue with SCG6 tumor cell (A) invading at the end or inhibited from invading when tissues are treated with (B) Y27632 or (C) blebbistatin. (D) Expression of dominant-negative RhoA (RhoA<sup>N19</sup>) in host epithelial cells inhibits invasion from the end. (E) Expression of constitutively active RhoA (RhoA<sup>L63</sup>) in host epithelial cells induces invasion from the duct. (F) Disrupting the intercellular transmission of mechanical stress by expressing EΔ in host epithelial cells inhibits invasion of tumor cells from the end, even when (G) constitutively active RhoA is expressed. (H) Expression of FAK-Dter in tumor cells inhibits invasion from the end. Autoclustering of β1-integrin through expression of β1<sup>V737N</sup> in tumor cells (I) induces invasion from the duct but fails to induce invasion in tissues (J) containing EΔ-expressing host cells or (K) treated with blebbistatin. (L) Quantification of invasion of tumor cells from experiments depicted in A–K. Error bars represent SEM (n = 3). Paired *t* test: \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.

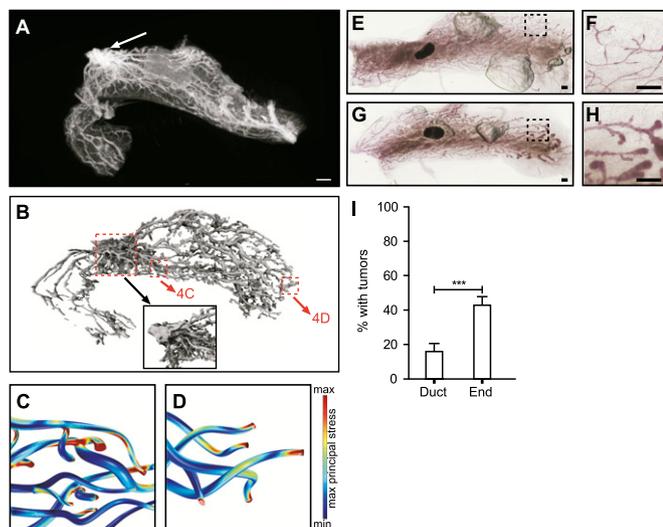
of tumor formation in an inducible transgenic mouse. Micro-computed tomography (μCT) was used to create a 3D rendering of the mouse mammary gland (Fig. 4A). μCT generated serial cross-sectional images of the 3D mammary epithelial ductal tree. The data generated by μCT were converted to a solid model that recapitulated the native architecture of the gland (Fig. 4B), consisting of a hollow epithelial tree embedded in a 3D ECM. As a first approximation, the ECM was modeled as a homogeneous linearly elastic solid with the material properties of collagen. We used FEM to calculate the maximum principal stress throughout this geometry. This qualitative model of endogenous contractility in the 3D mammary renderings predicted elevated mechanical stress at the ends of the epithelial tree compared with the ducts (Fig. 4C and D).

To investigate whether tumors develop preferentially at these predicted high-stress regions within the murine mammary gland, we created mice transgenic for an inducible form of the kRas oncogene, under the control of the mouse mammary tumor virus (MMTV)

promoter. Using an inducible oncogene allowed us to introduce expression of kRas in the mammary epithelium after the ductal tree had developed and thus to examine spatial variations in tumor formation in the context of the adult gland. When kRas was expressed in adult mammary glands, tumors developed preferentially at the ends of mammary epithelial ducts (Fig. 4G–I), whereas control mice failed to develop tumors (Fig. 4E and F). These data suggest that the phenotypic outcome of expression of the oncogene depends on the local microenvironment of the mammary gland. In particular, these data are consistent with a possible role for the mechanical environment of the host epithelium and suggest that low-stress regions such as the ducts may suppress the formation of tumors.

## Discussion

The cellular, chemical, and physical properties of the tumor microenvironment have a profound effect on tumor progression (10,



**Fig. 4.** Computational model shows that mechanical stress corresponds with regions of tumor development in vivo. (A)  $\mu$ CT unprocessed volume rendering of the inguinal mammary gland of an 8-wk-old CD-1 mouse. (B) 3D rendering of the network of mammary epithelial ducts. (Inset) Detailed view of the ductal network near the nipple region. Computational model of predicted maximum principal stress (C) along the ducts and (D) at the ends of a network of epithelial ducts embedded in collagen. (Scale bar, 500  $\mu$ m.) Whole-mount of inguinal mammary gland from (E and F) control mouse, (G and H) MMTV-rtta/tet-kRas mouse. (Scale bar, 500  $\mu$ m.) (I) Quantification of tumor location in kRas-expressing glands. Error bars represent SEM ( $n = 4$ ). Paired  $t$  test:  $***P < 0.001$ .

23, 29, 43, 45–48). Here we found that the mechanical environment of the host epithelium also plays a critical role in tumor cell phenotype. We determined that tumor cells embedded in nonmalignant tissues proliferate or invade preferentially when located in regions characterized by high endogenous mechanical stress. We further found that tuning the contractility of the host epithelium altered the dependence of tumor cell phenotype on location in the host tissue. We found similar behaviors for a variety of mouse and human mammary cancer cells; that these phenotypic effects are present even when using tumor cells containing a variety of genetic alterations illustrates the importance of these host-derived microenvironmental cues on tumor cell behavior.

It is becoming increasingly clear that mechanical cues from the tumor microenvironment alter tumor cell behavior. Tumors are appreciably stiffer than normal tissue (21, 49). During transformation of the mammary gland, the tumor epithelium and tumor-associated vasculature, as well as the surrounding ECM, undergo an increase in stiffness (49). The enzyme lysyl oxidase causes cross-linking and remodeling of collagen and stiffening of the ECM in mammary tumors; inhibiting lysyl oxidase was found to prevent tissue stiffening, reduce tumor incidence, and delay the progression of the tumors that did form (43). The associated changes in ECM stiffness can disrupt normal tissue architecture and induce a malignant phenotype through integrin clustering and activation of ERK (29, 43, 50). Indeed, alignment of collagen fibrils in the tumor stroma is an independent negative prognostic indicator for human breast carcinomas (51). In addition to these mechanical contributions from the ECM, our data suggest that the mechanical tone (that is, the intrinsic tissue-level contractility or tension) of the surrounding nonmalignant host epithelial tissue may be critical in establishing regions that modulate tumor phenotype. Remarkably, we found that changes in contractility of the surrounding host tissue control the subsequent invasion of tumor cells. Similar to mechanisms by which tumor cells sense changes in ECM stiffness, our data suggest that tumor cells sense alterations

from the host epithelial environment in part through integrin activation and focal adhesion assembly.

Our data show that tumors develop in regions of the epithelium that experience higher mechanical stress: FEM analysis of our 3D rendering predicted higher mechanical stress at the distal regions of the epithelial tree, which is where we found tumors developing in response to kRas expression (Fig. 4). However, the mechanism by which the neighboring cells transmit the mechanical information to the tumor cell is likely to be complex. Indeed, it is important to note that both mechanical stress and mechanical strain are predicted to correlate with the tumor phenotypes examined here. It is difficult to uncouple stress from strain, because they are intimately related through the constitutive response of the tissue, and furthermore, it is as yet unclear whether cells in a given context sense one or the other or both (52–54). Thus, although we refer to the regions that induce tumor cell proliferation and invasion as “high stress,” we do so without prejudice. From the perspective of the cell, it is probably more informative to focus on the molecular changes that result from spatial variations in mechanical tone, such as conformational changes in mechanically responsive proteins or activation of mechanically sensitive signaling cascades (26). Some insight into this can be gained from regional analyses of mouse mammary glands, which have found in distal growth regions increased expression of epithelial cell-specific factors that are critical for cancer development, including GATA3 (55) and FGFR2 (56). Another possibility is regional differences in the organization of ECM components, which could affect cell behavior in important ways (57).

Here we found that expression of constitutively active RhoA in the host tissue induced tumor cell invasion from regions that otherwise suppressed tumor phenotype, whereas dominant-negative RhoA inhibited invasion. Signaling through the RhoA pathway induces collagen alignment and matrix remodeling, which are dependent on contractility and can guide local tumor cell invasion in culture (35, 45) and in vivo (58). Collagen cross-linking and increasing tissue stiffness enhance integrin signaling and assembly of focal adhesions, thereby promoting a malignant phenotype (43). In the skin, expression of conditionally active ROCK has been found to increase collagen density and tissue stiffness, leading to activation of FAK and ultimately epidermal hyperplasia (47). We found here that FAK activity was required in the breast cancer cells for their invasion from regions of high mechanical stress, consistent with a possible role for RhoA-induced collagen remodeling and activation of FAK in the patterned behavior.

Regardless of the mechanism by which the host transmits this information to the tumor cell, it is clear from our data that the host contractility is a dominant feature. Although activation of FAK, and by inference focal adhesion formation, are required for tumor cell invasion, ectopic induction of integrin clustering was not sufficient to induce invasion of tumor cells residing in a flaccid or uncoupled host. Altogether, these data suggest that there is some minimum threshold of host contractility that is required for induction of an invasive phenotype in the tumor cell.

Conversely, we found that the duct region of mammary tissues has lower intrinsic mechanical tone and provides microenvironmental cues that are suppressive of tumor cell proliferation and invasion. These data add to our growing understanding that the microenvironment can provide important contextual cues that suppress tumorigenic behavior (10, 17–19). Indeed, occult tumors are surprisingly common (14–16); autopsies have revealed that 20% of young and middle-aged women have clinically occult breast tumors (16). However, it is unclear how these tumors are restrained from progressing to malignant cancers. Our results show that the mechanical properties of the normal host epithelium may be critical for suppressing tumorigenic phenotype in cells with a tumorigenic genotype. These differences in tumor cell behavior depending on location within the host epithelium strengthen the importance of studying tumorigenesis in the context of a tissue rather than in isolated individual cells (9) and reinforce the importance of using

3D culture models to recapitulate the natural architecture of tissues and complement studies in vivo (59).

## Methods

Three-dimensional tissues of precise geometry were generated using micro-lithography as previously described (37, 60). To generate tissues that contained a single tumor cell, labeled tumor cells and functionally normal host cells were mixed in a 1:100 ratio. Detailed methods are available in *SI Methods*.

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