Coherent angular motion in the establishment of multicellular architecture of glandular tissues

Kandice Tanner1, Hidetoshi Mori, Rana Mroue, Alexandre Bruni-Cardoso, and Mina J. Bissell1

Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720

Contributed by Mina J. Bissell, November 30, 2011 (sent for review November 9, 2011)

Glandular tissues form ducts (tubes) and acini (spheres) in multicellular organisms. This process is best demonstrated in the organization of the ductal tree of the mammary gland and in 3D models of morphogenesis in culture. Here, we asked a fundamental question: How do single adult epithelial cells generate polarized acini when placed in a surrogate basement membrane 3D gel? Using human breast epithelial cells from either reduction mammoplasty or nonmalignant breast cell lines, we observed a unique cellular movement where single cells undergo multiple rotations and then maintain it cohesively as they divide to assemble into acini. This coherent angular motion (CAMo) was observed in both primary cells and breast cell lines. If CAMo was disrupted, the final geometry was not a sphere. The malignant counterparts of the human breast cell lines in 3D were randomly motile, did not display CAMo, and did not form spheres. Upon “phenotypic reversion” of malignant cells, both CAMo and spherical architecture were restored. We show that cell-cell adhesion and tissue polarity are essential for the formation of acini and link the functional relevance of CAMo to the establishment of spherical architecture rather than to multicellular aggregation or growth. We propose that CAMo is an integral step in the formation of the tissue architecture and that its disruption is involved in malignant transformation.

actin dynamics | cancer | cell migration | multicellular assembly | cellular rotation

Epithelial organs form elaborate architectures consisting of ducts and acini in kidney, lung, salivary- and mammary- glands in vertebrates and invertebrates alike (1, 2). In tissues, loss of polarity markers is one of the earliest changes detected during malignant transformation (3, 4). The terminal unit of the arboreal tree, the acinus, is robustly recapitulated in 3D cultures of the mammary epithelial in laminin-rich gels (lrECM), where cells divide and organize into growth-arrested polarized spheres with basolateral and apical membrane domains surrounding a central lumen (5, 6). These models have shown the differential response of preinvasive and malignant epithelial cells to lrECM, where cells no longer form acini but aggregate into nonpolarized geometries similar to neoplastic lesions and tumors in vivo (6–11). To understand how tissue polarity and architecture are lost during transformation, one needs to first understand how the normal cells are able to form acini. However, the processes by which a single epithelial cell is able to recapitulate polarized structures that resemble structural units of function in 3D gels and in vivo are not known. We hypothesized that adult cells undergo a specific morphogenetic program to form and maintain quiescent acini, and that this program is corrupted in malignant transformation.

Using real-time imaging and lrECM gels, we uncovered a unique cellular movement where single breast epithelial cells completed multiple rotations that were maintained as they divided to assemble into acini. This movement is reminiscent of cellular behavior governing organ formation in early development stages of Xenopus and Helisoma embryos (12, 13), described more than two decades ago. That this embryonic process could be maintained in adult human cells in 3D gels is both surprising and exciting, and may provide a possible explanation for how the adult mammary gland after each pregnancy and involution can reorganize the epithelial tree (14).

On the other hand, malignant cells were randomly motile in lrECM gels on their way to form disorganized structures. Upon “phenotypic reversion” (11), however, they reentered the morphogenetic program to regain basally polarized structures. This switch suggested that there is a differential activation of motility signaling programs when malignant cells are phenotypically “normalized.”

In this article, we functionally link the coherent angular motion (CAMo) to the establishment of multicellular polarized spheres. We describe the relationship between tissue polarity, cell-cell adhesion, and cell movement, and determine the role of actomyosin structures and myosin light-chain–regulated forces on the establishment of the acinar structures. We also show the disintegration of these pathways in the malignant behavior of breast cancer cells, thus reinforcing the notion that coherent cellular movement within an ECM cocoon guides formation of structural units of tissues important for quiescence and homeostasis.

Results

To address the question of how single mammary cells can reestablish polarized acini in 3D lrECM, we used human mammary epithelial cells (HMECs) from both reduction mammoplasties and nonmalignant breast cell lines (S1-HMT3522 and MCF10A), as previously described (6, 7, 15). Single HMECs were imaged with confocal fluorescence microscopy continuously for 4 d; they underwent multiple rotations (~0.5–1 revolution per hour), and then continued to rotate cohesively as they and their progenies divided (Fig. 1A and Movies S1, S2, and S3). The observed chirality was random, suggesting that there were no directional preference for spherical formation under culture conditions used here. Individual nuclei (Fig. 1B), as well as the entire cluster, rotated with the angular velocity increasing as a function of cell size up to the four-cell stage (Fig. 1C). We named this global movement where cells coupled with the nearest neighbors rotated as a cohesive unit CAMo.

To determine whether CAMo was a driver of acinar morphogenesis or was simply a consequence of 3D multicellular aggregation, we compared the cellular movements of acini vs. aggregates. Acini grown from freshly excised single primary HMEC cells coherently rotated (Fig. 1E, Left, Fig. S1, and Movie S4), whereas cells preaggregated into clusters of ~20 cells before culturing in 3D were randomly motile (Fig. 1E, Right, and Movie S5) (16). Our finding that CAMo was observed only when acini evolved from a single cell suggests that self-generated centripetal forces are integral to the establishment of spherical geometry in lrECM gels.

Author contributions: K.T. and M.J.B. designed research; K.T., H.M., R.M., and A.B.-C. performed research; K.T. analyzed data; and K.T. and M.J.B. wrote the paper.

The authors declare no conflict of interest.

FREELY AVAILABLE ONLINE THROUGH THE PNAS OPEN ACCESS OPTION.

To whom correspondence may be addressed. E-mail: ktanner@lbl.gov or mjbissell@lbl.gov.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1119578109/-/DCSupplemental.
If indeed CAMo were central to formation of acini, it would follow that this morphogenetic program would be compromised in malignant cells because these cells do not form polarized acini (6, 11). Following the logic through, when malignant cells are phenotypically reverted to form acini-like structures (11; reviewed in 17), CAMo would have to be restored to allow formation of acini. To test the above hypothesis, we used an isogenic model of breast cancer progression, the HMT3522 series, where cells from reduction mammoplasty, were grown in defined medium and were spontaneously immortalized but remained nonmalignant (S1); upon removal of the sole growth factor, EGF, a malignant population (T4-2) emerged after injection into mice without addition of exogenous oncogenes (9, 18). We showed previously that these cells can be phenotypically reverted in 3D IrECM, where spherical structure is regained, even though these cells are “genotypically malignant” (9, 11). When seeded in IrECM, both single nonmalignant and malignant cells rotated, but malignant cells displayed higher centripetal velocities (Fig. 2Ai). The differential motility we observed between nonmalignant and malignant cells suggests that individual cell dynamics could be predictive of neoplastic transformation, even when cells are removed from the tissue.

The temporal window wherein phenotypic reversion of the malignant cells is obtained is restricted to the first 36–48 h in 3D cultures independently of the pharmacological agents used for reversion. This motivated the present studies whereby the
cellular reprogramming commitment required for reversion to acini-like structures has to be activated within this window. S1 and reverted-T4-2 (T4-2 Rev) cells exhibited staggered divisions with a delay between the second and third mitoses (Fig. 2Aii, Left and Right, and Table S1). On the other hand, T4-2 cells displayed no such delay (Fig. 2Aii, Center, Table S1, and Movie S6). This temporal difference suggested that the nonmalignant cells might use this delay to assemble adhesions required for the scaffold that allows them to form polarized acini.

We observed a shift in cell adhesion as measured by both sphe-
ricity and cellular movements after the first cell division during this “reversion window” (Fig. 2B). The S1 cells continued to exhibit CAMo as they formed spheres (Movie S7), whereas the T4-2 cells were loosely tethered, lacked CAMo, and became randomly motile (Fig. 2B and Movie S8), supporting the notion that the observed delays in timing of division may be important in establishment of functional cell-cell adhesions in S1 and T4-2 Rev cells (Movie S9). Both S1 and T4-2 Rev cells continued CAMo past this critical window, and T4-2 cells continued to be randomly motile (Fig. S2, and Movies S10, S11, S12, and S13). Thus, the type of cellular motion that cells undergo during early morphogenesis drives the final structure of the tissue. Using the MCF10a series, we confirmed that both cell adhesion and CAMo were maintained also for the nonmalignant MCF10a after the first cell division, but that these features were lost as a function of progression to malignancy (Fig. S3, Table S2, and Movies S14 and S15). Thus, the correlation between CAMo and normal morphogenesis and its loss as cells become malignant is conserved from normal primary cells to HMT3522 and MCF10a breast cancer series.

Tissue polarity is established via a temporally modulated multistage process regulated by the mammary microenvironment (17). There is evidence to indicate that growth and tissue polarity are separable in formation of polarized acini in 3D (19–21). Specifically, we showed that Akt and Rac1 act as downstream effectors of PI3K and function as separate pathways for cellular proliferation and tissue polarity, respectively (20). Partitioning deficient 3 homolog (PAR3), a key component of tight junctions (22, 23), is expressed differentially at both gene (PAR3) and protein (PAR3) levels in S1 and T4-2 cells (Fig. 3A and Fig. S4).

To determine the relationship between loss of CAMo and disruption of acinar polarity, we silenced PAR3 using shRNA in nonmalignant cells. Both CAMo and acinar structures were compromised where cells formed nonpolarized, grape-like structures, supporting the notion that angular motility and tissue polarity are reciprocally connected (Fig. 3B and C, and Movie S16). Similarly, interrogation of the role of E-Cadherin via a function-blocking antibody (24) compromised CAMo and, hence, acinar structure, emphasizing again the importance of cell-cell adhesion for maintenance of CAMo (Fig. 3A iv and v, B, and C).

The dynamic properties of actomyosin networks and microtubules have been shown to regulate cell motility and balance cellular forces (25). Pharmacological destabilization of microtubules did not inhibit single-cell centripetal motion (Fig. S5); thus, the switch from CAMo to random motility would predict differential spatiotemporal regulation of actomyosin. Were first-visualized actin dynamics using HMT3522 series transduced with mCherry LifeAct lentivirus (26). As early as 4 h postseedin in lreECM, a crescent-shaped cortical actin was established as if directing the angular motion (Fig. 4A and Movie S17). After the first cell division, the polarized cortical actin was retained in the S1 and T4-2 Rev cells (Fig. 4B i and iii), but became randomly distributed around the membranes in T4-2 cells (Fig. 4Bii), supporting a predictable connection between actin and CAMo.

To further identify the molecular components of the actomyo-
sin network that contribute to CAMo, we targeted the networks’ effectors, Myosin II, myosin light-chain kinase (MLCK), and Rho-associated kinase (ROCK) using pharmacological inhibitors (27–30). Because Rho-regulated contractile forces influence cell proliferation (31), we determined the appropriate titer of the inhibitor where cell proliferation was not—or minimally—affected. This determination allowed us to modulate the activity of myosin light chain without affecting growth. It is known that phosphorylation of myosin light chain-2 (MLC2) at threonine 18 and serine 19 (16) is involved in the motility of several cancer cell lines (32). We reasoned that by modulating MLC2 in nonmalignant S1 cells, we might be able to mimic motility of malignant T4-2 cells. Treatment with vehicle control confirmed that CAMo was unaffected (Fig. 4C Top, and Movie S18), whereas all three inhibitors disrupted CAMo. Addition of Blebbistatin, an inhibitor of Myosin II (33), led to random motility and resulted in formation of stellate structures (Fig. 4C, 2nd Panel, and Movies S19 and S20). The MLCK inhibitor, ML-7, also caused random motility, but cells formed aggregates rather than stellates (Fig. 4C, 3rd Panel, and Movies S21 and S22). ROCK inhibition, using Y-27632 (34), strongly reduced motility leading to formation of flattened discs (Fig. 4C, Bottom, and Movies S23 and S24). These data suggested that all three pathways are involved in regulation of CAMo. However, inhibition of each component led to formation of different structures and activity levels of MLC2 (Fig. 4D, ii and iii), further linking final structure to the type of motility determined by which components of the actomyosin program are active in cells forming the organs.

Discussion

Using 4D live imaging, we report a unique cellular movement, CAMo, by which acinar morphogenesis is made possible in 3D lreECM. Single adult human breast epithelial cells undergo multiple rotations, and then continue to divide cohesively to achieve
assembly into acini. Malignant cells do not exhibit CAMo and form disorganized structures in 3D. A unique and possibly related morphogenetic movement and program recently described by Haigo and Bilder (35) was shown to facilitate the transition of the geometry of the Drosophila follicle from that of a sphere to an ellipse. The description of the embryonic movement reinforces the notion that under appropriate contexts, adult cells may engage the embryonic morphogenetic programs to reestablish their tissue structure with the mammary gland as a prime example.

Fig. 3. The role of cell-cell adhesion and polarity proteins in CAMo. (A, i and ii) Immunofluorescence of actin, Par3, and DNA for S1 scramble and S1PAR3 shRNA. (Solid scale bar, 10 μm.) (iii) Immunoblots comparing protein levels. (iv and v) E-Cadherin immunofluorescence for S1 IgG and S1s cells anti-E-Cadherin. (Dashed scale bar, 20 μm.) (B) Micrographs show S1 Scramble (i), S1 PAR3 (ii), S1 IgG (iii), and S1 anti-E-Cadherin (iv), color coded for time: blue (0 h) to red (12 h); at the two-cell level, red is the endoplasmic reticulum. (C, Upper) Graph shows comparison of sphericity of the cell aggregates. ***P < 0.0001. (Lower) Graph depicting different types of motility for S1 scramble and S1IgG, which still rotate although S1 PAR3 shRNA and S1 anti-E-Cadherin undergo a combination of linear velocity as well as random motion. Student t test, where ***P < 0.0001.

Fig. 4. Initial cellular motion dictates final structure where only cells that undergo and maintain CAMo eventually form an acinus. (A) Micrographs of peripheral actin front, denoted by the white asterisk. (Scale bars, 10 μm.) (B, i) Micrographs show peripheral actin at the two-cell stage. (Scale bars, 20 μm.) (ii) Schematic of the method of quantification of the spatial distribution of actin. (iii) Average spatial actin distribution at the two-cell stage. Solid line, Gaussian approximation. (C) Pharmacological treatment of S1 shown with time stamped micrographs. (i) Cell tracking, color-coded (blue to red denotes increasing time). Red, endoplasmic reticulum. (Scale bars, 10 μm.) Trajectories in i show final architecture in ii. (iii) Median cross-section for actin (red), pMLC (green), and DAPI (blue). (D, i) Graph comparing sphericity, ***P < 0.0001. (ii) Comparison of motilities: ****P < 0.00001, ***P < 0.0001, and **P < 0.001. (iii) Immunoblots of pMLC, MLC, and Lamin A/C (internal control).
In our assay, nonmalignant and reverted malignant cells divided with a significant delay between the second and third mitoses, whereas loosely tethered malignant cells showed no such delay, suggesting that the timing delay may be needed to facilitate formation of cell-cell adhesions and the scaffold on which the tissue structure would be built. That “timing” has important functional consequences was described by Wong et al., who showed that if the duration of mitosis during human blastocyst formation was altered in any direction, the resultant embryo would be abnormal (36).

Not surprisingly, blocking E-Cadherin function disrupted cell-cell and to a lesser extent, cell-ECM adhesion, in turn impairing CAMo (Fig. 3). E-Cadherin plays a role in establishing and maintaining epithelia during development, from early embryogenesis through the later stages of organogenesis, as shown in Drosophila and E-Cadherin knockout mouse models (37–39). CAMo was also blocked by down-regulating PAR3, required in establishing polarity of epithelial tissues (22, 23). These data, coupled with the observation that malignant cells are randomly motile during formation of disorganized structures but perform CAMo upon phenotypic reversion, suggest that CAMo drives, or at least contributes, to tissue polarity.

Real-time visualization of actin dynamics revealed that crescent-shaped cortexal actin may direct centripetal motion. Such localization differs from anterior-posterior asymmetry of actin polarization studied on other surfaces (40). However, all underlie the importance of the actomyosin structures in generating motion. We observed here that single epithelial cells are able to rotate with little translocation in ImECM, thus expanding the known repertoire of cell motilities. Tumor cells can switch between mesenchymal (16, 41) and ameoboid (16, 42) movements, regulated by ROCK and Rac, respectively, and proteolytic activity for single-cell invasion in collagen gels (42). In our system, additional experiments are required to determine if there is localized ECM degradation or rapid turnover of cell-ECM adhesions to facilitate rotation within ImECM.

Cells can modulate their force generation as a response to local extracellular stiffness, where the malignant transformation is regulated in vitro and in vivo by stiffening the ECM (43, 44). Using sustained pharmacological inhibition of Myosin II, ROCK, and MLCK, we modulated activity of MLC2 in nonmalignant cells. This process disrupted the generation of centripetal forces, resulting in establishment of nonpolar structures, reinforcing the bidirectional relationship between physical and biochemical control of malignancy. Thus, myosin-regulated centripetal force can be added to the repertoire of known mechanisms by which cells can navigate their 3D microenvironment.

Previous studies in adult cell lines have shown rotating nuclei within static cells in 2D (45–47). A mathematical method was used to distinguish between random and centripetal motility during real-time visualization of lumen initiation in Madin-Darby canine kidney cell aggregates on the order of minutes (48), and inducible activation of ERK1/2 of nonmalignant epithelial cells within mature acini was shown to promote cell motility for several hours, disrupting epithelial architecture (49). These exciting findings reveal the potency of direct visualization to unravel and decipher novel cellular dynamics. Moreover, our visualization of the full acinar morphogenesis revealed the functional link between type of motility and realized structure. Continued visualization of the evolution of other tissues and forms may reveal an evolutionarily conserved mechanism by which cellular architecture is established and governed.

Materials and Methods

HMT3522 and MCF10A series (6–8), primary HMEC, kindly provided by William Curt Hines (Lawrence Berkeley National Laboratory, Berkeley, CA) and obtained from the University of California at San Francisco Cancer Center and the Cooperative Human Tissue Network, were cultured as previously described, and 3D samples gelled on glass chambers (Nalge Nunc) (7, 8, 15). Stable cell lines expressing mCherry LifeAct and H2B-GFP, and PAR3 KD cells were created (51). ER-tracker-mCherry (Invitrogen) was added to the medium (1:1,000) for 30 min and replaced before imaging. Before imaging, 5 μM Y-27632 (Calbiochem), 25 μM Blebbistatin, 0.5 μM ML-7 (Sigma), 200 μg/mL mouse anti-E-Cadherin, or anti-IGu Human (Invitrogen), respectively, were supplemented 5% ER/ECM/media (vol for 2 h). Media mixture was refreshed at day 3. At day 5, samples were fixed and stained as previously described (8).

Images were acquired at one frame per second with an upright Zeiss LSM 710. Images were of 134.9 × 134.9 μm² lateral dimensions, axial displacement of 75 μm (step size 0.5 μm) imaged with a 1.4 NA 63× oil-immersion objective, sequentially using 405 nm and 488 nm (respectively) lines from an argon ion laser and 546 nm from a solid-state laser. Emission BP filters were set for 450–465 nm, 505–525 nm, and 560–575 nm at a gain of 400. Four-dimensional images of dimensions 701 × 701 μm² lateral dimensions, axial displacement of 150 μm (axial step size, 2 μm), were imaged with a 0.8 NA 20× air objective at ~one frame per second. A time interval of 20 min was programmed after one z stack for 4 d. Samples were maintained at 37 °C and 5% CO2.

Image Processing. Images were exported using Zen 2009 software and ImageJ for display. Three-dimensional volume rendering and cell tracking were performed using object tracking and surface rendering algorithms in Bitplane Imaris software. For multicellular structures, individual cell traces excluding mitosis were used to calculate the angular velocity and mean square displacement (MSD). The sphericity, $\Psi$, of a three-dimensional particle with volume $V_p$ and surface area $A_p$ is:

$$\Psi = \frac{\sqrt{15} V_p^2}{A_p^{2/3}}$$  \hspace{1cm} [1]$$

where $V_p$ is the volume of the particle and $A_p$ is the surface area of the particle. Single cell centroid trajectories were analyzed as previously described (51). MSD (r) was calculated for r of one-quarter of total data points:

$$MSD(r) = \langle (x(t) - x(t + r))^2 + (y(t) - y(t + r))^2 + (z(t) - z(t + r))^2 \rangle$$  \hspace{1cm} [2]$$

following fitting parameters to the trajectories (51).

$$MSD(r) = 6Dr$$  \hspace{1cm} [3]$$

$$MSD(r) = 6Dr + \langle v^2 \rangle$$  \hspace{1cm} [4]$$

D is the diffusion coefficient and v is velocity.

Statistical Analysis. Bar graphs are shown as mean with SEs for trajectory analysis and a Student t test (unpaired, two-tailed, 95% confidence interval) was used to determine statistical significance. For sphericity analysis, distributions were not Gaussian (as confirmed by the Bartlett’s test); using nonparametric methods, we employed a two-sided Mann–Whitney test.

Acknowledgments. We thank Daniel Fletcher, Ramray Bhat, Alexander Borovsky, Jamie Bascom, Irene Kuhn, Joni Mott, and Mandana Vesieh for critical reading of the manuscript; Jamie Inman, Aaron Boudreau, Eva H. Lee, and Myan Do for initial assistance; William Curt Hines for kindly providing human tissue; and Douglas Brownfield, Ana Coreia, Cyrus Ghaier, Eileen Koh, Alvin T. Lo, Michelle Scott, and Damir Sudar for their helpful comments. This work was supported in part by postdoctoral Fellowship W81XWH-09-1-0666 (to K.T.) and predoctoral Fellowship W81XWH-08-1-0481 (to R.M.) from the US Department of Defense Breast Cancer Research Program. The work conducted for M.I.B.’s laboratory is supported by grants from the US Department of Energy, Office of Biological and Environmental Research, a Distinguished Fellow Award, and Low Dose Radiation Program Contract DE-AC02-05CH11213; National Cancer Institute Awards R37CA064786, U54CA126552, U54CA112970, U01CA143233, and NCI U54CA143836 (Bay Area Physical Sciences–Oncology Center, University of California, Berkeley, CA); and the US Department of Defense (W81XWH-B10736).


**Supporting Information**

**Tanner et al. 10.1073/pnas.1119578109**

**SI Materials and Methods**

**Molecular Cloning and Lentiviral Expression.** Human mammary epithelial cell (HMEC) lines S1 and T4-2 (HMT3522) (1-3), and MCF10A (4) were cultured as previously described. H2B-GFP (5) plasmid was purchased from Addgene (Cat#11680) and the insert was cleaved and cloned into pLentiCMV/TO-Neo, kindly provided by Alvin T. Lo (Lawrence Berkeley National Laboratory, Berkeley, CA). For lentiviral production, pLenti-CMV/TO-Neo H2B-GFP was cotransfected with pLP1, pLP2, and pL-PVSVG helper plasmids (Invitrogen) into 293FT host cells using Fugene6 transfection reagent (Roche) and the supernatant collected for viral transduction. S1, T4-2, and MCF10A cells were infected with viral supernatants in the presence of 4 mg/mL polybrene, and cell lines were stably selected with neomycin, subsequently infected with mCherry LifeAct (6) viral supernatant, and stably selected with puromycin.

Partitioning deficient 3 homolog (PAR3) knock-down was performed with PARD3 shRNA (Santa Cruz Biotechnologies), and cells with stably expressing shRNA were selected with puromycin.

**Cell Culture and Sample Preparation.** Normal primary human breast epithelial cells (N120, passage 1) were a kind gift from William Curt Hines, (Lawrence Berkeley National Laboratory, Berkeley, CA) obtained from the University of California at San Francisco Cancer Center and the Cooperative Human Tissue Network. N120 cells were isolated from tissue obtained from reduction mammoplasties as previously described (7, 8). HMT3522 and MCF10a series (10a, 10aT, and CA1) cell lines were cultured in 3D on covered glass chambers with a coverslip-bottomed no. 1.0 (Nalge Nunc) according to previously described methods (2-4). Phenotypic reversion of T4-2 cells was achieved by either using β1 integrin function-blocking (AIIB2) or EGFR inhibitor (Tyr- phostin, AG1478), as previously described (3, 9, 10).

After isolation, primary HMEC were either (i) embedded as single cells in lrECM (BD Biosciences Matrigel) and allowed to form acini (8 d) or (ii) preaggregated by centrifuging at 200 × g and resuspended without dissociation in laminin-rich gels (lrECM). Each preparation was then immediately placed at 37 °C to facilitate gelation for 30 min. Complete medium was then added to the cultures and replenished every 2 d. For live imaging, cell dye ER-tracker-mCherry (Invitrogen) specific for the endoplasmic reticulum was added to the medium at the final concentration of 1:1,000 for 30 min. Samples were replenished with fresh media before imaging. Dye was maintained as cells divided, resulting in the presence of dye in progeny.

**Pharmacological and Antibody Treatment.** The 3D cultures were pretreated with Blebbistatin (0.5 μM Y-27632 (Calbiochem), 25 μM Blebbistatin, 0.5 μM ML-7 (Sigma), 200 μM/mL mouse anti-E-Cadherin, or anti-IG (Human; Invitrogen), respectively, for 2 h before imaging by supplementing lrECM/medium suspension, where the final concentration was 5% lrECM (vol/vol). This treatment was repeated when media was refreshed at day 3 of 3D culture. At day 5, samples were fixed and stained according to previously described methodology (11, 12) for F-actin (Alexa 594 phalloidin), phospho-myosin light chain 2 (pMLC), Thr18/Ser19 (Antibody #3674, Cell Signaling), or E-Cadherin (BD Transduction Lab), secondary antibody Alexa 488, and DNA (DAPI). Cells were harvested also from lrECM for immunoprecipitation as previously described (10). Immunoblots of cell lysates were probed for pMLC, MLC (Antibodies cat. #3674 and #3672; Cell Signaling), PARD3 (Abcam; ab64840) and internal control, Lamin A/C. For live imaging, the cell dye was ER-tracker-mCherry (Invitrogen).

**Imaging Parameters. Immunofluorescence.** Images were acquired at a frame-rate of ~one per second with an upright Zeiss LSM 710 Meta confocal microscope. One-photon, confocal 3D dimensional images of 512 x 512 pixels (lateral dimensions), where the maximum axial displacement measured was 75 μm, were acquired with a 1.4 NA 63x oil-immersion objective corresponding to an area of 134.9 x 134.9 μm². Images were acquired in incremental steps of 0.5 μm in the axial direction. Samples were imaged sequentially with the 405 nm and 488 nm (respectively) lines from an argon ion laser with a power of <3% (total power 30 mW) and 546 nm from a solid-state laser (power of <10%). Band-pass filters were set in the emission pathway for blue (band-pass filter 450–465), green (band-pass filters 505–525 nm), and red (560–575 nm), and channels at a gain of 400 on the amplifier. For each channel, the pinhole was set to 1 Airy unit.

**Live cell imaging.** Three-dimensional images as a function of time were also obtained with a Zeiss LSM 710 Meta confocal microscope. Images of dimensions, 512 x 512 pixels (lateral dimensions) with a maximum axial displacement of 75 μm (axial step size, 2 μm) were acquired using a 0.8 NA 20x air objective at digital zoom of 0.6, corresponding to an area of 701 x 701 μm² at a rate of ~one frame per second. A time interval of 20 min was programmed between successive frames for 4 d. Settings were tuned to simultaneous excitation of the 488-nm line from an argon ion laser with a power of <3% (total power 30 mW) and 546 nm from a solid-state laser (power of <10%). A secondary dichroic mirror, SDM 560, was used in the emission pathway to separate the red (band-pass filters 560–575 nm) and green (band-pass filters 505–525 nm) channels, at a gain of 400 on the amplifier an image acquisition rate of one frame per second. The laser power for the 543-nm setting was set at <3% of the maximum power and the gain on the detectors was set to 450. Samples were maintained at 37 °C and 5% CO₂.

**Image processing.** Immunofluorescence images for each focal plane were exported using Zen 2009 (Zeiss) software and ImageJ for display. Three-dimensional volume rendering and individual cell tracking were performed using object-tracking and volume-rendering algorithms in Bitplane Imaris software. For multicellular structures, individual cell traces were exported for periods excluding mitosis and the angular velocity and mean square displacement were calculated.

**Sphericity.** Sphericity is a measure of how spherical an object is; thus, it provides a measure of compactness for a given shape (13). The sphericity, Ψ, of a particle is the ratio of the surface area of a sphere (with the same volume as the given particle) to the surface area of the particle.

\[ Ψ = \frac{\pi^2(6V_p)^{3/2}}{A_p} \]  

where \( V_p \) is volume of the particle and \( A_p \) is the surface area of the particle.

**Mean Square Displacement and Cell Tracking.** Trajectories of single-cell and geometric center of multicellular structures were analyzed in a similar manner to that described by Saxton and Jacobson (14), where the particle motion was extended to three
dimensions \((x, y, z)\). We calculated the mean-square displacement (MSD) as a function of a lag time \((\tau)\) for lags corresponding to one-quarter of the total datapoints:

\[
\text{MSD}(\tau) = \left\langle \left( (x(t) - x(t + \tau))^2 + (y(t) - y(t + \tau))^2 + (z(t) - z(t + \tau))^2 \right) \right\rangle.
\]

To quantitatively analyze the motion of the particles, we apply the following fitting parameters to the trajectories (14):

\[
\text{MSD}(\tau) = 6D\tau \quad \text{[S2]}
\]

\[
\text{MSD}(\tau) = 6D\tau + \langle v^2 \rangle \quad \text{[S3]}
\]

where \(D\) is the diffusion coefficient and \(v\) is velocity.

These equations consider normal random diffusion (Eq. \[S3\]), and directed motion with diffusion (Eq. \[S4\]) in three dimensions. These models were fitted to the experimental data, and the best-fitting model provides the parameters \(D\) and \(v\), for the cellular motion.

**Statistical Analysis.** Statistical comparisons were performed using the statistical toolbox of Graphpad Prism.

Bar graphs are shown as mean with SEs for trajectory analysis as the distributions were Gaussian and a Student’s t test (unpaired, two-tailed, 95% confidence interval) was used to determine if these distributions were statistically significantly different.

For sphericity analysis, distributions were not Gaussian (as confirmed by the Bartlett’s test); we compared the distributions using nonparametric methods. We compared whether the median values of the two samples were significantly different \((P < 0.01)\) using the two-sided Mann–Whitney test.

---


---

**Micrographs of representative images for acini evolved from single primary epithelial cells.**

Fig. S1. Acinar structures complete with lumen are observed after 10 d of culture for normal human primary epithelial cells. Micrographs of representative images for acini evolved from single normal primary epithelial cells seeded as single cells isolated from tissue excised during a reduction mammoplasty. Immunofluorescence showing spatial localization of the nuclei (DAPI-gray), α-6 integrin (basal polarity-red), ZO-1 (tight junctions-green), Vinculin (blue), and the combined channels.
A) Differential cell-cell adhesion and motilities after the first cell division for the MCF10a series

B) Global rotation is observed for non malignant MCF10a acini but not for MCF10aT and MCF10a-CA1 clusters

Fig. S2. Differential cell-cell adhesion and motilities after the first cell division for the human mammary epithelial cell MCF10a series. (A, i) Comparison of cell adhesion measured by sphericity of the cell aggregates for MCF10a series showing the relative changes of shape as a function of malignancy after the first cell division (two-cell stage) in early-stage 3D culture. Sphericity of ~1 indicates that cells have strong cell-cell adhesion; decrease in this value reflects that the cells are not tightly bound and the aggregate is no longer spherical. ***P < 0.0001 and *P < 0.01. (ii) Graphs showing the quantification as early as the first cell division during acinar morphogenesis; the distinction between nonmalignant and malignant is evident in the rotation of the cells: MCF10a multiple rotations whereas the premalignant MCF10aT T4-2 cells no longer rotate but instead show a combination of linear velocity as well as random motion (<v> and D respectively). Asterisks indicate P value as obtained using Student t test (unpaired, two-tailed, 95% confidence interval), where *P < 0.01. (B) Global rotation is observed for nonmalignant MCF10as but not for MCF10aT and MCF10a-CA1 clusters. Polar graph shows the coherent angular motion as demonstrated by tracking the geometric center as a function of time for MCF10a, MCF10aT, and MCF10a-CA1.

Fig. S3. Structural and migration differences observed after the first cell division for malignant T4-2, compared with the nonmalignant S1, are restored upon phenotypic reversion of T4-2 and is maintained for successive cell divisions. Micrographs of relative positions of nuclei as a function of time for S1 (i), T4-2 (ii), and T4-2 Rev (iii). The “a” and “b” are used to denote cells that were tracked for polar plots. Lower panel for each shows the pseudocolored nuclei.
Fig. S4. Expression levels of PARD3 for the HMT3522 human cancer progression series. (A) Graph showing the relative gene expression of the PARD3 levels for the HMT3522 series. (B) Micrographs showing the spatial localization using immunofluorescence of actin (red), PAR3 (green), and DNA (DAPI-blue) for the HMT3522 series. (Scale bar, 10 μm.)

Fig. S5. Pharmacological destabilization of microtubules does not prevent single cell rotation for nonmalignant human mammary epithelial cells cells. (Upper) Schematic of experimental protocol. (Lower) Graph shows comparison of rotation for S1s during and after pharmacological treatment with 5 μm Nocodazole of the nonmalignant HMT3522-S1 cells in 3D culture.
Table S1. Mitotic dynamics for HMT3522 series, where ± sign is SEM

<table>
<thead>
<tr>
<th>Cell type</th>
<th>First mitosis (1–2 cells)</th>
<th>Second mitosis (2–3 cells)</th>
<th>Third mitosis (3–4 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>1343 (±35)</td>
<td>230 (±50)</td>
<td>237 (±48)</td>
</tr>
<tr>
<td>T4-2</td>
<td>437 (±38)</td>
<td>224 (±82)</td>
<td>57 (±25)</td>
</tr>
<tr>
<td>T4-2 AIIB2</td>
<td>1318 (±64)</td>
<td>613 (±74)</td>
<td>352 (±187)</td>
</tr>
<tr>
<td>T4-2 Tyrphostin</td>
<td>1830 (±201)</td>
<td>800 (±100)</td>
<td>600 (±150)</td>
</tr>
</tbody>
</table>

Table S2. Percentage of rotating structures after 5-d seeded in culture

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Percentage of rotating clusters</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>76 (38/50)</td>
</tr>
<tr>
<td>T4-2</td>
<td>8 (4/50)</td>
</tr>
<tr>
<td>T4-2 tyrphostin</td>
<td>65 (33/50)</td>
</tr>
<tr>
<td>T4-2 AIIB2</td>
<td>62 (31/50)</td>
</tr>
<tr>
<td>MCF10a</td>
<td>70 (18/26)</td>
</tr>
<tr>
<td>MCF10aT</td>
<td>56 (18/32)</td>
</tr>
<tr>
<td>MCF10a-CA1</td>
<td>10 (2/21)</td>
</tr>
</tbody>
</table>

Movie S1. Acinar morphogenesis of HMT3522-S1, a non-malignant human breast epithelial cell line derived from reduction mammoplasty. Acinar morphogenesis in 3D laminin-rich gels (lrECM) was visualized with real-time fluorescence microscopy for 4 d. Coherent angular motion (CAMo) is observed as cells divide to form acini. For all movies, images were acquired at a rate of 1 frame/s with a time interval of 20 min between frames using real time confocal fluorescence microscopy. Playback rate is 30× the rate of acquisition.

Movie S1
Movie S2. Acinar morphogenesis of MCF10A, a nonmalignant human breast epithelial cell line derived from reduction mammoplasty. Cell division of MCF10a cells during acinar morphogenesis in 3D lrECM shown for 12 hr.

Movie S2

Movie S3. Single cell rotation of normal primary human breast epithelial cell. Wide view of single and small cell clusters at day 0 in 3D lrECM shown for 24 hr.

Movie S3
Movie S4. Acini derived from normal primary human breast epithelial cells undergo multiple rotations. CAMo continues to be present even after acini are formed; normal primary human breast epithelial cells visualized for 12 hr.

Movie S5. Aggregates derived from normal primary human breast epithelial cells display random motility. CAMo is not observed for multicellular aggregates of normal primary human breast epithelial cells shown for a period of 12 hr. These aggregates display random motility.
Movie S6. Evolution of malignant human epithelial cell, T4-2 in 3D-lrECM. Spatio-temporal evolution of T4-2 cells embedded in lrECM for 1 d shows symmetric division.

Movie S6

Movie S7. Acinar morphogenesis of non-malignant human epithelial cell, S1. Acinar morphogenesis of S1 cells acquired for 2 d showing CAMo is maintained as cells divide.

Movie S7
Movie S8. Malignant human breast epithelial cells, T4-2 show random motility after the first cell division. CAMo is no longer observed for T4-2 cells; image acquired for 12 hr. Instead, they show random, lateral motility.

Movie S8

Movie S9. Phenotypically reverted- malignant T4-2 human epithelial cells (T4-2REV) undergo CAMo when placed in IrECM. CAMo is restored in T4-2 Rev cells; image acquired for 12 hr.

Movie S9
Movie S10. Acinar morphogenesis of non-malignant human epithelial cell, S1. Acinar morphogenesis of S1 cells acquired for 1 d showing CAMo is maintained as cells divide.

Movie S11. Malignant human breast epithelial cells, T4-2 show random motility during tumor formation. CAMo is no longer observed for T4-2 cells; image acquired for 2 d. Instead, they show random, lateral motility.
Movie S12. Acinar morphogenesis of T4-2 Rev-Tyrphostin. Acinar morphogenesis in 3D laminin-rich gels (lRECM) was visualized with real-time fluorescence microscopy for 4 d. Coherent angular motion (CAMo) is observed as cells divide to form acini.

Movie S12

Movie S13. Acinar morphogenesis of T4-2 Rev-AIIB2. Acinar morphogenesis in 3D laminin-rich gels (lRECM) was visualized with real-time fluorescence microscopy for 4 d. Coherent angular motion (CAMo) is observed as cells divide to form acini.

Movie S13

Movie S14. Clusters of Pre-Malignant human breast epithelial cells, MCF10aT show both CAMo and random motility. Mixed behavior is observed for MCF10aT where some acini show CAMo and others show random, lateral motility as observed at day 4 for 24 hr.

Movie S14
Movie S15. Clusters of Malignant human breast epithelial cells, MCF10a-CA1 show random motility. MCF10a-CA1 clusters show random, lateral motility as observed at day 4 for 24 hr.

Movie S15

Movie S16. PAR3 knock-down in S1 cells display compromised CAMo motility. Compromised CAMo motility is observed for S1 PAR3 knock-down cells at two cells stage during establishment of multicellular structures in 3D cultures, shown for 12 hr.

Movie S16
Movie S17. Single cell rotation showing circularly polarized peripheral actin. Single T4-2 cell at day 0 in 3D IrECM shown for 6 hr.

Movie S18. S1 control cells display CAMo motility. CAMo motility is observed for S1-untreated cells at the two cells stage during establishment of multicellular structure in 3D cultures shown for 12 hr.
Movie S19. Loss of CAMo motility induced by Myosin II inhibition of S1 cells. CAMo motility is lost at the two cells stage during establishment of multicellular structure in 3D cultures when S1 cells are treated with 25 μM Blebbistatin shown for 12 hr.

Movie S19

Movie S20. Loss of CAMo motility induced by Myosin II inhibition of S1 cells. Evolution of structures when S1 cells are treated with 25 μM Blebbistatin shown for 2 d where CAMo motility is lost at the two cells stage and maintained during establishment of multicellular structure in 3D.

Movie S20
Movie S21. Loss of CAMo motility induced by MLCK inhibition of S1 cells. CAMo motility is lost at the two cells stage during establishment of multicellular structure in 3D cultures when S1 cells are treated with 0.5 μM ML-7 shown for 12 hr.

Movie S22. Loss of CAMo motility induced by MLCK inhibition of S1 cells. Evolution of structures when S1 cells are treated with 0.5 μM ML-7 shown for 2 d where CAMo motility is lost at the two cells stage and maintained during establishment of multicellular structure in 3D.
Movie S23. Loss of CAMo motility induced by ROCK inhibition of S1 cells. CAMo motility is lost at the two cells stage during establishment of multicellular structure in 3D cultures when S1 cells are treated with 5 μM Y-27632 shown for 12 hr.

Movie S23

Movie S24. Loss of CAMo motility induced by ROCK inhibition of S1 cells. Evolution of structures when S1 cells are treated with 5 μM Y-27632 shown for 2 d where CAMo motility is lost at the two cells stage and maintained during establishment of multicellular structure in 3D.

Movie S24