Self-organization is a dynamic and lineage-intrinsic property of mammary epithelial cells

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Loss of organization is a principle feature of cancers; therefore it is important to understand how normal adult multilinage tissues, such as bilayered secretory epithelia, establish and maintain their architectures. The self-organization process that drives heterogeneous mixtures of cells to form organized tissues is well studied in embryology and with mammalian cell lines that were abnormal or engineered. Here we used a micropatterning approach that confined cells to a cylindrical geometry combined with an algorithm to quantify changes of cellular distribution over time to measure the ability of different cell types to self-organize relative to each other. Using normal human mammary epithelial cells enriched into pools of the two principal lineages, luminal and myoepithelial cells, we demonstrated that bilayered organization in mammary epithelium was driven mainly by lineage-specific differential E-cadherin expression, but that P-cadherin contributed specifically to organization of the myoepithelial layer. Disruption of the actomyosin network or of adherens junction proteins resulted in either prevention of bilayer formation or loss of preformed bilayers, consistent with continual sampling of the local microenvironment by cadherins. Together these data show that self-organization is an innate and reversible property of communities of normal adult human mammary epithelial cells.

Most mammalian adult tissues are replenished and repaired throughout life by reservoirs of stem cells. As new somatic cells replace old ones or build new tissue, organization and architecture must be maintained. The alternative, loss of organization in adult tissues, is associated with cancer and other diseases. Lineage-specific progenitors or their differentiated progeny must have a means to reach their ultimate site of residence within the adult tissue. The robust ability to organize cells into tissues is marked from conception: Heterogeneous aggregates of dissociated cells from embryonic tissues, suspended in gels or hanging droplets, self-organize into semblances of the original tissues (1–5). The mechanisms governing self-organization during developmental morphogenesis (6–10) are likely conserved in the maintenance of organization in adult tissues. Here we use normal human mammary epithelial cells (HMEC) as a model to determine how organized states are preserved in normal adult epithelia.

The mammary gland undergoes cycles of proliferation and involution, showing as much as a 10-fold expansion in preparation for lactation followed by return to normal size. During these processes, the precise bilayered branching organization throughout the gland is maintained; secretory luminal epithelial cells (LEPs) line the lumen, surrounded by a layer of contractile myoepithelial cells (MEPs) that are adjacent to the basement membrane. We hypothesized that mammary epithelial cells possessed lineage-specific intrinsic abilities to self-organize into domains of lineage specificity. Such a mechanism would help explain how, for instance, the mammary stem cell-enriched zone in the ducts (11) is maintained separately from the rank-and-file LEPs and MEPs, and how LEPs and MEPs form and maintain bilayers. The phenomenon of self-organization has not been well studied in humans, perhaps because of the challenges of working with primary materials and a paucity of tractable culture systems for maintaining cell types from normal adult tissues. To facilitate a quantitative understanding of those processes in an adult epithelial tissue, we used a robust cell culture system that enables culture of pre-stasis normal HMEC obtained from reduction mammaplasties for 40–60 population doublings while maintaining both the LEP and MEP lineages (12). Flow cytometry-enriched cells from both lineages were placed in arrays of micropatterned microwells, where their distributions were tracked over time to generate a dynamic understanding of lineage-specific self-organizing behavior.

Results and Discussion

Quantification of Self-Organizing Activity in Different Lineages of Normal Human Mammary Epithelial Cells. We first used a classical self-organization assay to determine whether different lineages of cultured HMEC derived from reduction mammaplasty possessed an intrinsic ability to form bilayered structures. Subpopulations of LEPs and MEPs, defined as CD227+/CD10−/keratin 19 (K19)−/keratin 14 (K14)− and CD227+/CD10+/K19+/K14+, respectively (11), were enriched by FACS from heterogeneous normal finite- life span (HMEC) (Fig. 1 C, A, and A′′). The two lineages were labeled with long-lasting fluorescent membrane dyes of different wavelengths, mixed together, and then were suspended in hanging droplets. The formation of cores of LEPs surrounded by MEPs (Fig. 1 B), similar to their organization in vivo, was observed over 48 h. However, the considerable variation in aggregate size, shape, and focal planes precluded a quantitative understanding of the phenomenon.

Therefore, a microwell culture platform was engineered that confined the HMEC mixtures to a 3D cylindrical geometry, which enabled quantification of lineage distributions over time (Fig. 1 C). Representative optical sections of mixed LEPs and MEPs in microwells, taken at middepth (~25 μm) at 0 and 48 h, suggest self-organization had occurred, as compared with mixtures of arbitrarily labeled HMEC cultures (Fig. 1 D). Time-lapse microscopy from one well demonstrates the dynamic nature of the organizing process (Fig. S1 and Movie S1). Heat maps showing the lineage distributions over time suggested that in a majority of microwells LEPs formed a ring surrounding cores of MEPs by 24 h, and last for at least 48 h (Fig. 1 E, Upper). A 1:1 ratio of LEPs to MEPs was determined empirically to provide the most clearly separable distributions, as compared with ratios of 1:2 or 1:3. Using relatively more LEPs than MEPs (e.g., in a ratio of 2:1 or 3:1) was difficult due to the paucity of LEPs. Arbitrarily labeled HMEC cultures, mixed at a 1:1 ratio, showed overlapping dis-
tutions of cells that did not resolve into distinct populations (Fig. 1E, Lower). Quantification of the heat maps (Fig S2) confirmed that a core of LEPs surrounded by MEPs was observable as early as 24 h, and showing as much as a fourfold difference in LEP:MEP ratios at the core versus the periphery by 48 h ($P < 0.001$) (Fig. 1E’, Upper). By contrast there was no difference in ratios at the core and the periphery of the arbitrarily labeled HMEC controls at any time point (Fig. 1E’, Lower). Infections in the graphs sometimes were observed toward the peripheral regions because of imperfect registration of the well images. Taken together, these results indicate that self-organizing is an innate property of the LEP and MEP HMEC lineages.

Levels of E-Cadherin Expression Are Lineage Specific. Self-organizing behavior has been ascribed to disparate adhesive properties among the participating cells in embryonic progenitors from the three germ layers, in cancer cell lines, and in fibroblasts engineered to express cell–cell adhesion molecules (the differential adhesion hypothesis, reviewed in ref. 6). Cadherin cell–cell adhesion molecules, particularly E-cadherin, play key roles in tissue morphogenesis during vertebrate gastrulation (13). Quantification of images of fluorescently immunostained tissue sections of normal mammary gland (Fig. 2A) from two individuals revealed that more E-cadherin protein was present at the borders between two LEPs than at the borders between a LEP and a MEP ($P < 0.001$) (Fig. 2B). Flow cytometry measurements of E-cadherin surface protein levels were made on LEPs and MEPs. In HMEC strains at fourth passage from six individuals, a reproducible pattern was observed, whereby more E-cadherin was detected on LEPs than on MEPs (Fig. 2C). The lineage-specific expression levels of E-cadherin made it an attractive candidate for further testing of the differential adhesion hypothesis as it pertains to self-organization among HMEC.

Functional Identification of Adhesion Molecules That Drive Tissue Self-Organization. To determine whether cadherins played a functional role in the self-organization of LEPs and MEPs, inhibitors of E-, P-, and VE-cadherin were added to the medium of the LEP and MEP markers K14 (red) and K19 (green), respectively, verified that (A′) CD227+ LEPs were K14+/K19−, and (A′′) CD10+ MEPs were K14−/K19+. Nuclei were counterstained with DAPI (blue). (B) Images of mixtures of fluorescently labeled LEPs (green) and MEPs (red) suspended in hanging droplets and imaged with a confocal microscope just after mixing at 0 h (Left) and at 48 h (Right). (Scale bars: 20 µm.) (C) Cartoon representation of the microwell self-organization assay. Fluorescently labeled LEPs (green) and MEPs (red) were mixed together and placed in arrays of microwells that did not support cell adhesion. Thirty wells were imaged with a confocal microscope just after the addition of cells (0 h) and again at 24 h and 48 h. Fluorescence from both green and red channels in one optical section per well was binarized and then combined and averaged to generate two gray-scale composite images that were overlaid to generate a single two-color composite distribution map for each condition, with LEP distributions in green and MEP distributions in red.

Distribution maps of LEP (green) and MEP (red) in four different microwells at 0 h and 48 h (Upper) and of controls, which were heterogeneous HMEC arbitrarily labeled with red or green fluorescent labels (Lower). (E) Distribution maps of LEP (green) and MEP (red) (Upper) or control mixtures at the 0-h, 24-h, and 48-h time points (Lower). (E′) Quantiﬁcation of heat maps in E showing changes in mean distribution of red and green pixels along the radius around 360° of arc at three time points. Green lines show 0 h, red lines show 24 h, and blue lines show 48 h. SD is shown by the lightly shaded regions of colors corresponding to each line.

Self-Organization Was Prevented After Negative Modulation of the Actomyosin Network. Previous studies of mammary epithelial morphogenesis have implicated profound roles for the actomy-
osin regulatory network in normal morphogenesis (15, 16). We therefore examined the impact on HMEC self-organization of the actomyosin network inhibitors ML-7, a myosin light-chain kinase (MLCK) inhibitor (17), and Y27632, a Rho kinase (ROCK) inhibitor that blocks both ROCK1 and ROCK2 (18). Inhibitors were added at the beginning of the experiment and were refreshed every 24 h with medium changes. Analysis of LEPs and MEPs distributions over 48 h revealed that both inhibitors prevented self-organization; there were no differences in lineage distribution between the core and peripheral regions (Fig. 3B and B'). Modulation of the actomyosin network also is known to cause changes in the elasticity and cortical tension of cells. Self-organization studies of germline progenitor cells dissociated from zebrafish embryos suggested that differential actomyosin-dependent cell-cortex tension was a crucial component of self-organization in that system (8). Stiffer cells organized to the inside and were surrounded by softer cells, and disruption of the stiffness relationship by actomyosin network inhibitors led to deficits in self-organization (8). Using an atomic force microscope, we measured the elasticity of untreated and inhibitor-treated MEPs and LEPs. Although untreated MEPs tended to be stiffer than LEPs, that difference became significant only in the presence of the inhibitors (P < 0.001) (Fig. 3C). MEP stiffness was unaffected by ML-7 and by Y27632, but those inhibitors caused softening of LEPs (Fig. 3C). In HMEC the actomyosin network inhibitors increased the magnitude of the difference in elasticity between LEPs and MEPs but did not alter their relative elasticity (i.e., LEPs always were softer than MEPs), suggesting that in this system self-organization was not driven by differential elasticity.

Perturbations of the Actomyosin Network in the Microwell Platform Revealed That Self-Organization Is Dynamic and Reversible. How did the actomyosin inhibitors upset the self-organizing mechanism? We investigated whether the actomyosin inhibitors affected expression or binding activities of E-cadherin in HMEC. Addition
of ML-7 or Y27632 to the HMEC culture medium did not change lineage-specific differences in E-cadherin expression as measured via flow cytometry; invariably, LEPs expressed more E-cadherin than did MEPs (Fig. 4A). The ability of recEcad simply to bind surfaces of HMEC suspension also was measured by flow cytometry. Binding of recEcad did not occur in Ca^{2+}-free medium or when HMEC were preincubated with an E-cadherin–blocking antibody (Fig. 4B). A 6-h pretreatment with Y27632 or ML-7 did not prevent recEcad binding (Fig. 4B). Therefore, neither differential expression levels of E-cadherin nor its ability simply to bind other E-cadherin molecules at the surface was impacted by ML-7 or Y27632.

Because of the central importance of the cytoskeleton in adherens junction regulation, we considered the possibility that the actomyosin inhibitors modulated the ability of E-cadherin junctions to mature or remodel (19), thereby impacting HMEC organization. Disruption of MLCK would prevent proper localization of myosin II A to the E-cadherin junction, disrupting E-cadherin clustering and decreasing homophilic adhesion (20). Conversely, mature adherens junctions were unable to break down and recycle in the presence of Y27632 in HCT116 and MDCK epithelial cell lines (21). Those reports predicted that ML-7 would ablate already-organized HMEC structures, whereas Y27632 would preserve them. Accordingly, ML-7 or Y27632 was added to mixtures of LEPs and MEPs in the microwell assay, either just after cells were added to wells at the start of the assay (0 h) or after 24 h, when the DMSO controls already started to show signs of organization. In contrast to the experiments shown in Fig. 3B, in which the inhibitors were refreshed every 24 h, in these experiments the inhibitors were added a single time, with the expectation, based on empirical findings, that the inhibitor’s activity would begin to weaken by 48 h. This protocol tested the reversibility of the system, because in one condition sorting would be prevented from the beginning and then gradually would be unleashed, and in the second condition sorting would be allowed to get underway before perturbation by the inhibitors after 24 h. Both inhibitors, when added at 0 h, prevented self-organization through the 24-h time point, but as the inhibitors’ activity diminished significant, differences in LEP:MEP ratios in the core and at the periphery were observed by 48 h (P < 0.01) (Fig. 4 C and C’). The unique phenotypes of each inhibitor were revealed when they were added after the assay had been underway for 24 h. Before addition of the inhibitors, the LEP were enriched at the core and were surrounded by peripheral MEPs (P < 0.001) (Fig. 4 C and C’). When measured at 48 h, addition of ML-7 had obliterated organization, eliminating any difference in the distributions of the lineages (P = ns), whereas Y27632 had preserved the self-organized LEP cores that were encircled by MEPs (P < 0.001) (Fig. 4 C and C’). As a whole, these observations are consistent with the following model: ML-7 prevented adherens junction formation or maturation, and breaking the adherens junctions prevented cells from self-organizing and caused dissolution of already organized structures. Conversely, Y27632 prevented adherens junction recycling, so the cells could not let go of...
one another to sample the surrounding microenvironment. Thus, both establishing and maintaining organized states are dynamic and reversible processes.

Here we demonstrated that self-organization of mammary epithelial cells is a lineage-specific process that is principally E-cadherin driven; however, P-cadherin also may play a role in organizing the MEP layer. Unaltered normal finite-lifespan HMEC and the microwell assay were used together with recombinant proteins and antibodies that blocked specific adherens junction proteins. The elegant proof-of-principal experiments, which showed differential levels of cell–cell adhesion molecules can drive self-organizing, were performed using fibroblasts and other immortal cell lines that were engineered to express different levels of cadherins and other nonadherens junction proteins. It is remarkable, given the undoubted complexity of the LEP and MEP cell surfaces, that E-cadherin plays so central a role in the process of self-organization in those cells. It has been hypothesized that self-organizing is not simply the result of differential levels of cadherin expression or of binding affinities, but rather that adhesion energy and the ability to remodel cell–cell junctions are crucial determinants (22). Dynamic analysis of HMEC in the microwell assay platform in the presence of actomyosin inhibitors provided support for this hypothesis in the context of mammary gland (Fig. 4 C–E). Elegant time-lapse imaging studies of mouse mammary organoid morphogenesis also revealed that the actomyosin inhibitors Y27632 and ML-7 disrupted the clean bilayered organization (15), but not to the catastrophic extent observed in the HMEC microwell assay. Because the mouse mammary organoids were developed in vivo, a number of additional cellular interconnectivities crucial for tissue stability may have formed that were absent in our recombined system. Although we focused on cell–cell E-cadherin junctions, other adhesive and physical interactions, such as desmosomal interactions between LEPs and MEPs (23), undoubtedly are important in maintaining mammary gland organization and bear further dissection. Cell–extracellular matrix (ECM) interactions also will likely affect sorting in vivo.

Because the microwell assay uses a nonfouling coating to prevent cell adhesion, the adherens junction proteins may have had a more pronounced effect on self-organizing than they would have had in the presence of ECM. Atomic force microscopy analysis of LEP and MEP on plastic dishes indicated that LEP tended to be softer than MEP. However, a cultured murine epithelial cell line became less stiff when in contact with laminin-1, a principal component of basement membrane, than when in contact with plastic (24). Therefore, MEPs in vivo may be less or equally as stiff as LEPs because of their direct contact with basement membrane. Future iterations of the microwell platform will help elucidate more of the factors involved in making stable and organized tissues.

Studying self-organizing behavior of a human epithelium generally is challenging because results cannot be extrapolated easily to in vivo conditions. However, observations of breast cancer pathogenesis suggest the basic mechanisms described here are important for maintaining mammary gland organization. E-cadherin expression and localization frequently are misregulated in breast cancers (25–27), and loss of E-cadherin is a hallmark of the epithelial-to-mesenchymal transition, which is associated with invasive and aggressive breast cancer (28). The mechanisms governing self-organization also are important in the context of regenerative tissue maintenance. As MEPs and LEPs are produced anew by mammary progenitor cells in vivo, they must adopt their appropriate place within the tissue, or, alternatively, the progenitors must be able to move to receive instructive microenvironments that direct cell-fate decisions (29). Understanding tissue self-organization mechanisms may help explain how stem cell differentiation and maintenance of tissue architecture in adults is controlled.

Materials and Methods

Cell Culture. HMEC strains were established and maintained according to previously reported methods (12, 30). Cells were maintained in M87A medium and used for assays at fourth and fifth passages; strain 240L was the only strain used for self-organizing and binding assays.

Microwell Self-Organization Assay. Micropatterned substrata were made according to Tan et al. (31). Polydimethylsiloxane (PDMS) microwell arrays were formed by curing prepolymer with base:cure ratio of 10:1 (Sylgard 184) against a prepatterned master. The arrays of wells were peeled away and were cut into 1-cm² pieces that were affixed with a few microliters of uncured PDMS to the bottom of a 24-well plate (Mitek). Plates with microwells were UV oxidized for 7 min (UV-Oxy-Cleaner 42; Jelgift Co.), blocked with 2 mg/mL BSA (Sigma) for 1 h under vacuum, and rinsed with PBS and M87A. All self-organizing experiments were conducted with HMEC strain 240L.

Flow Cytometry Sorting and Assays. HMEC at fourth or fifth passage were trypsinized and resuspended in medium. For enrichment of LEP and MEP images, anti-CD227-FITC (clone HMPV; BD) or anti-CD10-PE (clone HI10a; BioLegend) were introduced into the wells and were allowed to load for 30–60 min. Excess cells were washed away with medium; inhibitors then were added to the medium after excess cells were washed away and at every medium change. Anti–E-cadherin (100 μg/mL clone HECD-1; Invitrogen); anti–E-cadherin (100 μg/mL clone HECD-1; Invitrogen); anti–P-cadherin (100 μg/mL clone NCC-CAD-299; Abcam); recombinant human (rh)E-cadherin-Fc (recEcad, 100 μg/mL; R&D Systems); rhVE-cadherin (100 μg/mL; R&D Systems); Y27632 (10 μM; Calbiochem); or ML-7 at 3 × 10⁻⁶ M (Calbiochem), HMEC were imaged at 0, 24, or 48 h with a spinning disk confocal microscope (Carl Zeiss). Red and green fluorescence channels in images taken at the −25-μm z axis positions of 30 wells from each condition at each time point were binarized using the Threshold function, merged into a single channel, and then averaged using ImageJ software (National Institutes of Health). Gray-scaled average images corresponding to LEP and MEP were merged into a single image with red or green look-up tables applied to each average image.

Heat maps were normalized to the highest intensity value and were used to quantify sorting using the expression log(mean green pixel intensity/mean red pixel intensity). A script was written using MATLAB (Mathworks) to plot differential intensity as a function of the distance from the center and to compute the average plot from θ = 0–360° (Fig 52).

Flow Cytometry Sorting and Assays. HMEC at fourth or fifth passage were trypsinized and resuspended in medium. For enrichment of LEP and MEP images, anti-CD227-FITC (clone HMPV; BD) or anti-CD10-PE (clone HI10a; BioLegend) was added to the medium at 1:50 for 25 min on ice. HMEC then were washed in PBS and sorted on a FACs Vantage DIVA (BD) into their own medium.

E-cadherin expression on LEP and MEP was measured by addition of anti–E-cadherin-A647 (clone 67A4; Biolegend) to the above mixture at 1:50.

Atomic Force Microscopy Measurements of Stiffness. Once samples were equilibrated to 25 °C, cell deformity was measured, and stiffness was calculated as previously described (24). The resulting data were plotted using Prism (GraphPad Software) (n = 45).

Statistics. E-cadherin images and atomic force microscopy were analyzed using the Kruskal–Wallis test and Dunn’s test for multiple comparisons, using a 95% confidence interval. Differences between first and third/thirtieth log, (mean green fluorescence/mean red fluorescence) per pixel plotted as a function of distance from the center were analyzed by one-way ANOVA, using Bartlett’s test for equal variance and followed by a Tukey’s test for multiple comparison using a 99.9% confidence interval. Statistics were computed with Prism (GraphPad Software, Inc.).

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Supporting Information

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SI Materials and Methods

Cell Culture. Human mammary epithelial cell (HMEC) strains were established and maintained according to previously reported methods (1, 2). Finite-lifespan pretransit HMEC were obtained from reduction mammary tissue (strains 240L, 191, 172, and 456) or from surgically resected nontumor areas of mastectomy tissue (strains 335P and 451P). Cells were initiated as organoids in primary culture in M85 or M87A medium and were subjected to multiple partial trypsinizations as described. Cells were maintained in M87A medium and used for assays at fourth and fifth passage; strain 240L was the only strain used for self-organizing, E-cadherin expression, and binding assays.

Microwell Self-Organization Assay. Micropatterned substrata were made according to Tan et al. (3). Polydimethylsiloxane (PDMS) microwell arrays were formed by curing prepolymer with a base: cure ratio of 10:1 (Sylgard) against a prepatterned master. The arrays of wells were peeled away and were cut into 1-cm² pieces that were affixed with a few microliters of uncured PDMS to the bottom of a 24-well plate with good optical properties (Mitek). Plates were UV oxidized for 7 min (UVO-Cleaner 42; Jelight Co.), blocked with 2 mg/mL BSA (Sigma) for 1 h under vacuum, and rinsed with PBS and M87A. Flow cytometry-sorted HMEC were stained with CD-Dil, SP-DIIOC18(3), or DilC18(5)-DS (Invitrogen), used at 1:1,000 in PBS for 5 min at 37 °C followed by 15 min at 4 °C. Cells were washed extensively with medium after staining. Dye-stained HMEC were mixed at ratios of 1:1 [luminal epithelial cells (LEP):myoepithelial cells (MEP)], or 1:1 (randomly stained green:red HMEC cultures) and were resuspended in M87A medium at 1 million cells/mL. HMEC then were introduced into the wells and allowed to load for 30–60 min. Excess cells were washed away with medium. All self-organizing experiments were conducted with HMEC strain 240L. Inhibitors were added to the cell suspensions just before they were added to wells, and more inhibitor was added to the medium after cells outside the wells were washed away and at every medium change. Inhibitors used were anti–E-cadherin (100 µg/mL; clone HECD-1; Invitrogen), anti–P-cadherin (100 µg/mL; clone NC1-CAD-299; Abcam), rHE-cadherin-Fc (recombinant E-cadherin fused to the human IgG-Fc region, recEcad) (100 µg/mL; R&D Systems), rVE-cadherin (100 µg/mL; R&D Systems), Y27632 (10−5 M; Calbiochem), or ML-7 (3 × 10−6 M; Calbiochem). HMEC were imaged at 0, 24, or 48 h with a spinning disk confocal microscope (Carl Zeiss). Red and green fluorescence channels in images taken at the ~25-µm z axis positions of 30 wells from each condition at each time point were binarized using the Threshold function, merged into a Z-stack, and then averaged using ImageJ software (National Institutes of Health). Gray-scaled average images corresponding to LEP and MEP were merged into a single image with red or green look-up tables applied to each average image. After heat maps were generated, further analysis was required to quantify differences in self-organizing behavior. Briefly, heat maps were normalized to the highest intensity value and used to quantify sorting using the expression log−(mean green pixel intensity/mean red pixel intensity). A script was written in MATLAB (Mathworks) to plot differential intensity as a function of the distance from the center and to compute the average plot from θ = 0 to 360° (Fig. S2).

Flow Cytometry Sorting and Assays. HMEC at fourth or fifth passage were trypsinized and resuspended in their medium. For enrichment of LEP and MEP images, anti–CD227-FITC (clone HMPV; BD) or anti–CD10-PE (clone HI10a; BioLegend) was added to the medium at a ratio of 1:50 for 25 min on ice. Then HMEC were washed in PBS and sorted on a FACS Vantage Diva (BD) into their own medium.

E-cadherin expression was measured on LEP and MEP by addition of anti–E-cadherin-A647 (clone 67A4; Biolegend) to the above mixture at a ratio of 1:50.

To determine effects of inhibitors, Y27632 at 10−5 M (Calbiochem) or ML-7 at 3 × 10−6 M (Calbiochem) was added to HMEC medium for 6 h at 37 °C (5% CO2) before trypsinization and subsequent measurement of E-cadherin by FACS.

To measure the ability of recEcad to bind to HMEC in the presence of inhibitors, cells were suspended in their medium in Falcon tubes (to prevent adhesion to a culture surface) in the presence of Y27632 at 10−5 M (Calbiochem), ML-7 at 3 × 10−6 M (Calbiochem), or anti–E-cadherin (100 µg/mL; clone HEC1D-1; Invitrogen), or in calcium-free medium for 6 h at 37 °C (5% CO2). recEcad conjugated to human IgG Fc region was added at 100 µg/mL for 1 h on ice. HMEC then were washed with medium and incubated with anti-human IgG-A633 (1:500; Invitrogen) in their own medium.

Atomic Force Microscopy Stiffness Measurements. Once samples were equilibrated to 25 °C, cell deformity was measured, and stiffness was calculated as previously described (4). Briefly, a Bioscope Catalyst atomic force microscope (Veeco) was mounted onto a spinning disk confocal microscope (Carl Zeiss). Atomic force microscopy (AFM) images were taken using the Threshold function, merged into a Z-stack, and then analyzed using a custom MATLAB (Mathworks) script assuming a Hertz model. The resulting data were plotted using Prism (GraphPad Software) (n = 45).

Immunofluorescence Staining. FACS-sorted HMEC were allowed to adhere to methanol-cleaned coverslips for 2 h. Adherent cells were fixed in methanol:acetone (1:1) at −20 °C for 15 min, blocked with PBS/5% normal goat serum/0.1% Triton X-100, and incubated with anti-keratin 14 (anti-K14) (polyclonal; 1:1,000; Covance) and anti-keratin 19 (anti-K19) (1:20; clone Troma-III; Developmental Studies Hybridoma Bank) overnight at 4 °C. Goat anti-rabbit A568 and goat anti-rat A488 secondary antibodies (1:500; Invitrogen), and Hoechst 33422 (1:1,000; Sigma) were added for 2 h at room temp. Cells were imaged with a spinning disk confocal microscope (Carl Zeiss).

Sections of formaldehyde-fixed and paraffin-embedded (FFPE) normal human breast tissue (4 µm thick), affixed to slides, were purchased from ProSci (http://www.prosci-inc.com/). Slides were baked at 55 °C for 1 h to fix the tissue to the slide and to remove much of the paraffin. Complete deparaffinization was done according to the protocol published on the Abcam website. Antigen retrieval was done according to the citrate buffer (pH 6.0)-based protocol published on Abcam website. Slides were not allowed to dry out after deparaffinization and were stored in PBS at 4 °C if not stained immediately. Before reaction with primary antibodies, the slides were blocked for 1 h at room temperature or overnight at 4 °C in normal goat serum (NGS) blocking buffer: 5% NGS, 0.001% azide, 0.1% Triton X-100, and PBS. The unconjugated primary antibody recognizing human cytokeratin 19 was diluted 1:100 in NGS blocking buffer before incubation with the slides in a cold room on a rocker platform overnight, followed by three
consecutive 10-min washes in PBS at ambient temperature. Secondary antibodies (goat anti-mouse Alexa Fluor 568) were diluted 1:500 in PBS and incubated with the slides for 1 h at ambient temperature on a slow rocking platform, followed by three 10-min washes in PBS. The conjugated antibodies against K14 (Alexa Fluor 633) and against E-cadherin (Alexa Fluor 488) then were diluted 1:100 in NGS blocking buffer and incubated with the slides overnight in a cold room on a rocker platform, followed by three 10-min washes in PBS. DAPI diluted 1:4,000 in PBS was added to the slides for 5 min; then the slides were rinsed with PBS and destained overnight in PBS in the cold room. The next day slides were overlaid with Fluormount-G (Southern Biotech) and a #1 coverslip and allowed to dry in the dark at ambient temperature overnight before being sealed with clear nail polish. Imaging was accomplished on a Zeiss 510 spinning disk confocal microscope. For each area of interest, five focal planes of \( \sim 2 \mu m \) vertical separation were imaged. Images were processed using Image J software.

K14 purified rabbit polyclonal antisera MK14 (AF 64, catalog #PRB-155P; Covance), supplied in PBS, 0.03% thiomersal, was conjugated with Alexa Fluor 633 according to the protocol from Molecular Probes. Briefly, 1 mg Alexa Fluor 633 (Invitrogen/Molecular Probes) was resuspended at 5 mg/mL in acetonitrile and used at 10-fold molar excess to the IgG. Excess Alexa Fluor 633 was aliquoted, rotovapped to dryness, and then was stored in dark at \(-20^\circ C\). K19 Abcam ab7754 (mouse MAb IgG2A) used at 1:100, E-cadherin Ab, Alexa 488 conjugated #3199 from Cell Signaling. The specificity of anti-E-cadherin was determined using recombinant E-cadherin peptide conjugated to the Fc fragment of human IgG (blocking) (catalog #648-EC; R&D Systems) used at 1:5 dilution, equivalent to 50 \( \mu g/mL \).

**Statistics.** E-cadherin images and atomic force microscopy were analyzed using the Kruskal–Wallis test and Dunn’s test for multiple comparisons, using a 95% confidence interval. Differences between first and third thirtiles of log2 (mean green fluorescence/mean red fluorescence) per pixel plotted as a function of distance from the center were analyzed by one-way ANOVA, using Bartlett’s test for equal variance and followed by a Tukey’s test for multiple comparison using a 99.9% confidence interval. Statistics were computed with Prism (GraphPad Software, Inc.).


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**Fig. S1.** Frame from a time-lapse movie of HMEC self-organization. LEP (green) and MEP (red) enriched from strain 240LB were time-lapse imaged in a microwell every 30 min from +6 h to +48 h.
Movie S1. A time-lapse movie of HMEC self-organization. LEP (green) and MEP (red) enriched from strain 240LB were time-lapse imaged in a microwell every 30 min from +6 h to +48 h.

**Other Supporting Information Files**

*SI Appendix (PDF)*
clear all;

% Input maps for analysis
inputImageG = imread('labarge/rECAD/rE,green,0hAVG_0h.tif');
inputImageR = imread('labarge/rECAD/rE,red,0hAVG_0h.tif');

% Normalize each map to highest intensity
ng = im2double(inputImageG) ./ max(max(im2double(inputImageG)));
nr = im2double(inputImageR) ./ max(max(im2double(inputImageR)));

% Convert maps to express the separation of intensities
% Inf or NaN's can be avoided by giving zero values a small value
ng(ng==0)=0.01;
nr(nr==0)=0.01;
nt = log2(ng./nr);

% Determine center
imSize = length(inputImageG(1,:));
center = round(imSize/2 + 1);

% Replot with respect to the center
% Array of summed values
unwrap = zeros(360,round(imSize/2));

for theta = 0:359
    display(['Extracting column ' num2str(theta+1) ' of 360']);

    % Rotate the image by the desired amount
    rotated = imrotate(nt,theta,'bicubic','crop');

    % Extract the column from the center to the bottom
    unwrap(theta+1,:) = rotated(center:end,center);
end

% Determine average/standard deviations
for (i = 1:round(imSize/2))
    ntavg(i)=mean(unwrap(:,i));
    ntstd(i)=std(unwrap(:,i));
end

ntavg=ntavg';
ntstd=ntstd';