Collective Epithelial Migration and Cell Rearrangements Drive Mammary Branching Morphogenesis

Andrew J. Ewald,1,* Audrey Brenot,1 Myhanh Duong,1 Bianca S. Chan,1 and Zena Werb1,*

1Department of Anatomy and Program in Cell Biology, University of California, San Francisco, San Francisco, CA 94143, USA
*Correspondence: andrew.ewald@ucsf.edu (A.J.E.), zena.werb@ucsf.edu (Z.W.)
DOI 10.1016/j.devcel.2008.03.003

SUMMARY

Epithelial organs are built through the movement of groups of interconnected cells. We observed cells in elongating mammary ducts reorganize into a multilayered epithelium, migrate collectively, and rearrange dynamically, all without forming leading cellular extensions. Duct initiation required proliferation, Rac, and myosin light-chain kinase, whereas repolarization to a bilayer depended on Rho kinase. We observed that branching morphogenesis results from the active motility of both luminal and myoepithelial cells. Luminal epithelial cells advanced collectively, whereas myoepithelial cells appeared to restrain elongating ducts. Significantly, we observed that normal epithelium and neoplastic hyperplasias are organized similarly during morphogenesis, suggesting common mechanisms of epithelial growth.

INTRODUCTION

A fundamental question in biology is, “how do cells collaborate to build and change the structure of epithelial tissues, while maintaining characteristically epithelial features such as cell-cell contact and barrier function”? (Pilot and Lecuit, 2005)?

We chose to study branching morphogenesis in the mammary gland as a model to understand how the cells of a postnatal epithelial tissue collectively remodel the architecture of a tubular network. The mammary gland is particularly suited to such studies, as it develops in an ongoing, hormonally driven process throughout reproductive life (Sternlicht, 2006; Woodward et al., 2001; Sternlicht et al., 2005; Wiseman et al., 2003). We cultured fragments of freshly isolated mammary epithelium (organisms) in 3D Matrigel gels and collected over 200 bright-field movies and over 400 3D confocal movies to determine the basic sequence of events involved in branching morphogenesis. In minimal medium, the epithelial pieces all formed “simple cysts” with a bilayered organization and a clear lumen (Hennighausen and Robinson, 2005). Quiescent mammary ducts are bilayered (Figure 1B; Figure S1; see the Supplemental Data available with this article online) and consist of a luminal and myoepithelial layer in both mice and humans. Hyperplasias and carcinomas have a very different architecture, with many cell layers (Figure 1C), increased proliferation, partial loss of cell polarity, and reduced intercellular adhesion (Bilder, 2004). However, during puberty, terminal end buds (TEBs) form transiently at the end of each primary duct. TEBs are characteristically different from quiescent ducts: they have multiple luminal cell layers, are enriched in mammary stem cells, and have high levels of proliferation (Figures 1D and 1E; Figure S1) (Hinck and Silberstein, 2005; Williams and Daniel, 1983). The multilayered TEB gives rise to bilayered ducts, with myoepithelial (ME) cells surrounding luminal epithelial (LE) cells (Figures S1A and S1A). Despite advances in dissecting the genetic regulation of mammary development (Howlin et al., 2006), the cellular basis of ductal elongation and bifurcation remains obscure.

We sought to observe mammary epithelial cells during the initiation, elongation, and bifurcation of new ducts and to identify molecular pathways involved in regulating each of these processes. We used primary, organotypic three-dimensional (3D) culture and long-term confocal time-lapse analysis to show that mammary ducts elongate through a different type of collective epithelial migration, with no leading cell extensions or leading actin-rich protrusions. We then used pharmacologic perturbations to identify molecular regulators of ductal morphogenesis. Finally, we showed that, during morphogenesis, mammary epithelium is similarly organized in organotypic cultures, during ductal invasion in vivo, and in mouse mammary hyperplasias.

RESULTS

Modeling Mammary Branching Morphogenesis in Organotypic Culture

Mammary ductal elongation in vivo occurs over the course of weeks, making it difficult to monitor directly. Accordingly, we imaged individual cells over time in an organotypic culture model of mammary branching morphogenesis (Fata et al., 2007; Simian et al., 2001; Sternlicht et al., 2005; Wiseman et al., 2003). We cultured fragments of freshly isolated mammary epithelium (organisms) in 3D Matrigel gels and collected over 200 bright-field movies and over 400 3D confocal movies to determine the basic sequence of events involved in branching morphogenesis. In minimal medium, the epithelial pieces all formed “simple cysts” with a bilayered organization and a clear lumen (Figure 1H). Addition of FGF2 to the medium induced initiation and elongation of new ducts (Movie S1). We considered an organoid branched if it had three ducts, as previously established (Fata et al., 2007). There was no correlation between the initial structure of the organoid and the sites of ductal initiation in culture.

Keratin-14-GFP-Actin Mice Enable Dynamic Visualization of Myoepithelial Cell Behavior

In vivo, the mammary epithelium consists of keratin-8- and -18-positive LE cells and smooth muscle α-actin (SMA)- and...
Collective Cell Migration in Mammary Morphogenesis

New Ducts Initiate from a Multilayered Epithelium

Ductal initiation from complex cysts was a transition from an essentially filled lumen to a progressively clearing lumen, with new ducts initiating and elongating at gaps in ME coverage (Figures 2A and 2B; Movies S4 and S5). The fronts of the elongating ducts were always multilayered, but there was often thinning and reversion to a bilayer at adjacent sites. LE cells appeared adherently aligned with each other, yet they moved chaotically in the direction of reversion to a bilayer at adjacent sites. ME cells and K14-GFP-actin+ cells at sites of bifurcation (Figures 2C–2G). We could not distinguish whether ME cells induced bifurcations or responded to a separate decision to bifurcate, but ME motility closely correlated with changes in the shape of elongating ducts (Movie S5).

Cessation of elongation was accompanied by a transition in the tip of the duct from a multilayered epithelium to a single or bilayered epithelium, shown in an organoid derived from a transgenic reporter mouse with GFP expressed in both LE and ME (β-actin-EGFP; Figure 3A; Movie S6). The tips of ducts in growth-arrested organoids were often free of SMA+ cells (Figure 2G). The timing and extent of ductal elongation varied among organoids, but each eventually stopped elongating and reverted to a bilayered organization.

Cells with Within Elongating Ducts Migrate Collectively, but Remain Epithelial

Since the multilayered tips of elongating ducts were organized quite differently from quiescent ducts, we sought to determine if they remained epithelial during morphogenesis. On the basis of close cellular association and the consistent localization of E-cadherin and β-catenin to surfaces of intercellular contact throughout branching (Figures 3B, 3E, and 3F), we conclude that ducts remained epithelial. Epithelia also characteristically have basement membranes at their basal surface. We chose to stain for laminin 332, as it is a component of mammary epithelial basement membrane, but not of Matrigel. Unbranched structures, whether simple or complex cysts, displayed complete coverage with laminin 332 (Figure 3C), whereas the tips of actively elongating ducts were generally free of laminin 332 (Figure 3D). We could not distinguish whether laminin 332 was proteolytically degraded, displaced, or simply not synthesized at these sites.

During Collective Epithelial Migration, Cells Are Incompletely Polarized

To determine whether apicobasal polarity was retained during elongation, we used four molecular markers: β-catenin to mark basolateral cell surfaces, APKC-ζ to mark apical cell surfaces, β1-integrin to mark cell-matrix interactions, and ZO-1 to mark luminal surfaces.

At the cellular level, APKC-ζ and β-catenin were both detected at all lateral cell surfaces in the multilayered region during the complex cyst phase and during active elongation. APKC-ζ colocalized with ZO-1, whereas β-catenin was distinct from ZO-1. We conclude that cells in the multilayered region are incompletely polarized and do not have distinct apical and basolateral domains (Figures 3E–3H).

At the tissue level, we observed β1-integrin localized to the cell-matrix interface (Figures 3I and 3J) and sometimes also to lateral sites of cell-cell contact (Figure 3I, yellow). ZO-1 localized to luminal surfaces and to small pockets and channels within the multilayered region. We conclude that the multilayered epithelium is polarized at the tissue level, but its constituent cells are
Figure 1. Mammary Epithelia Enter a Multilayered Epithelial State during Morphogenesis

(A) Mouse mammary ducts elongate greatly during puberty (carmine red-stained whole mounts). Terminal end buds (TEBs) are indicated (black arrows).
incompletely polarized relative to cells within quiescent regions of mammary epithelium.

Proliferation Is Required to Form Complex Cysts

Previous work showed that growth factor-induced proliferation is highest just before ductal initiation (Fata et al., 2007). We confirmed those results by staining for mitotic cells with phospho-histone H3 (H3-P) and observed frequent mitotic cells in the complex cyst and early branching stages (Figures 3K and 3L); there were fewer mitotic cells in simple cysts or late branching organoids. H3-P+ cells were present in all regions of organoids and showed no obvious correlation between the location of proliferation and the direction of extension.

Proliferation could be required to initiate branching, or to provide new cells to enlarge a branched structure. To distinguish whether inhibition of proliferation would produce arrest at the simple cyst stage or produce small, branched organoids, we tested whether inhibition of proliferation would produce arrest at the simple cyst stage (Figure S2); therefore, proliferation is required to form the multilayered, complex cyst.

Ductal Elongation Is Accomplished by a Multilayered Epithelium

We sought to determine the cellular mechanism of ductal initiation from complex cysts. To observe the cellular basis of ductal initiation, we collected 127 confocal time-lapse movies of initiating and elongating ducts. We observed that new ducts always initiated as multilayered epithelial structures (N = 394/394 ducts in 127 movies; Figure 4A; Movies S4–S8). Significantly, we never observed basally directed protrusions, cell movement out of the luminal epithelium, or initiation and elongation of fully polarized epithelial ducts (N = 0/394 ducts), ruling out chain migration as the mechanism for tubulogenesis in this system. Instead, ductal elongation was accomplished by a collectively migrating, dynamically rearranging, multilayered epithelium at the ductal tip. This structure reverted to a bilayered epithelium surrounding a simple lumen in the trailing duct (Figure 4A'). The thinning of the trailing duct from multiple to a single luminal layer also contributed to ductal elongation.

Ductal Elongation Occurs without Leading Cellular Extensions

Leading cell extensions or specialized actin-rich protrusions, such as filopodia and lamellipodia, are typically observed at the leading edge of individual migratory cells (Mitchison and Cramer, 1996) and of collectively migrating groups of cells, ranging from Drosophila border cells (Fulga and Rorth, 2002; Prasad and Montell, 2007) to zebrafish neural precursors (Lecaudey and Gilmour, 2006). In contrast to these previous studies, we never observed cellular extensions or actin-rich protrusions at the front of advancing mammary ducts (Figures 4B–4B'). Instead, F-actin was enriched along lateral cell surfaces and colocalized with ZO-1 at luminal surfaces.

Cells Dynamically Rearrange during Collective Epithelial Migration

We next asked if a constant group of cells remained at the invasion front or if cells exchanged positions during invasion (Figures 4C–4C'). To distinguish these possibilities, we followed individual cells during ductal extension by exploiting the mosaic expression of EGFP within the mammary epithelium of mice in which GFP was knocked into the Sca-1 locus (Sca-1-EGFP). To provide context, all cells were stained with CellTracker Red.

Cells in the multilayered region of the extending duct continuously exchanged positions (N = 394 ducts in 127 movies; see Movies S7 and S8). This cell rearrangement was characteristic of both newly initiated ducts (Figures 2A and 2B) and later ducts with well-defined lumens (Figures 3A, 4A’, and 4C’). We verified this conclusion by using three different GFP reporter lines: K14-GFP-actin (Figures 2A and 2B), Sca-1-EGFP (Figures 4A’ and 4C’), and β-actin-EGFP transgenic mice (Figure 3A). In all cases, the cells at the tip of the extending ducts dynamically rearranged.

Duct Initiation Requires Rac-1 and Myosin Light Chain Kinase

What are the molecular regulators of ductal initiation? Previous studies in mammary epithelium (Vargo-Gogola et al., 2006), ureteric bud (Meyer et al., 2006), 3D MDCK morphogenesis (Yu et al., 2003), and epidermis (Vaezi et al., 2002) identified Rac- and Rho-type GTPases as critical regulators of epithelial morphogenesis. Accordingly, we tested the role of these pathways in mammary epithelial branching morphogenesis, by using soluble inhibitors: Rac-1 Inhibitor (NSC23766) for Rac, Y27632 for Rho kinase (ROCK), and ML7 for myosin light chain kinase (MLCK).

Rac inhibition resulted in a strong dose-dependent inhibition of ductal initiation (Figure 5A). Rac-inhibited cysts were covered externally by ME cells, but they also had convoluted internal tubules, with associated internal ME cells (Figure 5D) and differed markedly from the normal complex cysts (Figures 1I–1K). These internal tubules were not observed in control...
Figure 2. Myoepithelial Cells Move Actively during Ductal Elongation and Bifurcation

(A) Single-section frames from a time-lapse movie of ductal initiation and elongation in a K14-GFP-actin/Ce1Tracker Red-labeled organoid (Movie S4).

(A') Schematic view of (A); ME is green, and the lumen is gray. An ME cell (yellow) moves from a basal to an interior position.

(B–B') 3D reconstructions of optical sections from a time-lapse movie of (B) ductal elongation and bifurcation (Movie S5) and, at higher magnification, (B') ductal elongation and (B'') ductal bifurcation in a K14-GFP-actin/Ce1Tracker Red-labeled organoid.

(C and E–G) 3D reconstructions of optical sections and (D) single optical sections of SMA-labeled ME in an (C) organoid, an (D) elongating duct, a (E) growth-arrested organoid, and (F and G) bifurcating ducts at higher magnification.

All images are from FGF2-treated organoids. Scale bars are 20 μm.
organoids (Figure 5C). Movies of organoids from the K14-GFP-actin (Figure 5G; Movie S9; 24 movies total) or β-actin-EGFP (not shown) transgenic mice revealed that the cysts were relatively static and had extensive ME coverage, internal ME cells, and persistently filled lumens.

MLCK inhibition also prevented ductal initiation in a dose-dependent fashion (Figures 5B, 5E, and 5F). Maximal inhibition was achieved at doses ranging from 4 to 10 μM, depending on the experiment. We did not observe internal tubules in MLCK-inhibited cysts. Long-term confocal movies of K14-GFP-actin organoids (Figure 5H; Movie S10) and β-actin-EGFP organoids (not shown) revealed that MLCK-inhibited organoids transiently formed complex cysts, but then gradually cleared their lumens and restored a largely bilayered epithelial structure (19 movies total). MLCK-inhibited organoids remained persistently enclosed in ME cells.

ROCK Is Required to Restore Bilayered Epithelial Architecture

As ducts ceased elongating, they transitioned from a multilayered epithelium to a single luminal layer (Figure 3A). We sought to understand the molecular regulation of this transition. Recent work identified p190-B Rho-GTPase as a regulator of mammary epithelial architecture, with its inactivation resulting in hyperplasia and disorganized myoepithelial coverage (Vargo-Gogola et al., 2006). Accordingly, we used Y-27632 (Uehata et al., 1997) to inhibit the related molecules ROCK1 and ROCK2. ROCK inhibitor-treated organoids branched at equal or higher rates than controls, but they were heterogeneous and poorly patterned (Figure 6; Figure S3; Movies S12 and S14–S17). The effects were acutely reversible when ROCK inhibitor was washed out (Figure S3E; Movie S17).

Our data indicate that ME location, behavior, and migration reflect, and appear to influence, the pattern of branching morphogenesis (Figures 1 and 2). ROCK inhibition resulted in disorganized, incomplete coverage by ME cells. After ROCK inhibition, ME cells were often spindle shaped, with no obvious correlation between ME position and final organoid structure (Figures 6B and 6C).

In control organoids, there was a sharp organizational difference between the multilayered, elongating end and the trailing duct behind it (Figure 4A). The trailing ducts of control organoids had clear, ZO-1-lined lumens, apically enriched F-actin, and a simple, bilayered structure. ROCK-inhibited organoids had no central lumen and instead localized ZO-1 and F-actin to small foci and had a persistent, disorganized, multilayered structure. No ROCK inhibitor-treated organoids were observed to revert to a bilayered architecture. LE cell shapes were also rounder, but E-cadherin remained localized to lateral LE surfaces in ROCK inhibitor-treated organoids, suggesting that the cells remained adherent and epithelial (Figure 6G). ROCK-inhibited organoids had abnormally positioned ME cells, abnormal LE and ME cells shapes, no central lumens, and a persistent failure to revert from a multilayered to a bilayered architecture.

To characterize the kinetics and cell behavioral basis of the ROCK inhibitor phenotype, we collected 67 long-term confocal movies from K14-GFP-actin (Figure 6M; Movie S12), β-actin-EGFP...
The effect of ROCK inhibition was acute, with changes in ME coverage and LE organization observable within 12 hr. A major question was the degree to which the abnormal architecture of the LE related to the loss of ME coverage (Figures 6B and 6C). ME cells appear to play a restraining role in normal organoids and seem to limit and pattern LE mobility (Figures 2A–2B; Movies S4 and S5). We examined the acute effects of ROCK inhibition on ME behavior by filming in parallel control and ROCK inhibitor-treated organoids from the same K14-GFP-actin+ mouse 1 hr after ROCK inhibition (Figures 6L and 6M; Movies S11 and S12). In control organoids, ducts initiated through gaps in ME coverage, elongated through the combined motility of LE cells and ME cells, and reverted to a bilayered architecture. Conversely, in ROCK-inhibited organoids, ME cells quickly changed shape, and their motility had little relationship to the vigorous growth of the largely LE organoid.

We next sought to distinguish whether LE behavior was altered in ROCK-inhibited organoids, by using GFP reporters that highlighted LE behavior. In control organoids, LE cells rearrange vigorously in the multilayered ends of ducts, but they are more restrained in the trailing duct region (Figure 4). In ROCK-inhibited organoids, LE cells rearranged vigorously throughout the multilayered epithelium (Sca-1-EGFP, Figure S3D; Movie S16; β-actin-EGFP, Figure S3; Movie S15). Taken together, these findings demonstrate that ROCK inhibition induces acute changes in LE and ME cell shape and cell behavior, abnormal epithelial architecture, and collapse of the lumen. ROCK inhibition results in a hyperbranched epithelium with disorganized ME coverage and prevents restoration of bilayered epithelial architecture.

Normal and Neoplastic Mammary Epithelial Morphogenesis In Vivo Occurs via a Multilayered Epithelial State

Does the multilayered epithelium we observe in culture resemble the in vivo epithelial organization of mammary ducts during morphogenesis? We selected two examples of in vivo epithelial morphogenesis: (1) TEBs during normal pubertal morphogenesis and (2) hyperplasias from a pathologically validated mouse model of luminal-type breast cancer (Herschkowitz et al., 2007; Lin et al., 2003), in which polyoma virus middle T oncogene is expressed under the control of the mouse mammary tumor virus promoter (MMTV-PymT) (Guy et al., 1992).

We first compared TEBs and quiescent ducts during normal morphogenesis in vivo to the multilayered epithelial organization of elongating ducts in culture (Figure S1). TEBs were multilayered and were enclosed in a layer of SMA+ cap cells. In the multilayered body cell region of TEBs, β-catenin, APKC-ζ, and β1-integrin all localized to lateral cell surfaces. ZO-1 localized apically, but largely to finger-like projections and isolated pockets within the multilayered body cell region. The lumens of quiescent ducts in vivo were simple and were lined by one single
luminal epithelial layer, with apically localized ZO-1 and APKC-ζ, basolaterally localized β-catenin, and basally localized β1-integrin. TEBs were also hyperproliferative relative to quiescent ducts. The most notable differences between TEBs in vivo and elongating ducts of organoids in culture were that TEBs were composed of significantly more cells and encountered a much more diverse cellular stroma. Therefore, the epithelial organization of TEBs in vivo and elongating ducts in culture was similar.

In early MMTV-PymT hyperplasias, we observed a progressive loss of SMA+ ME cells, with fewer ME cells correlating with less normal ductal morphology (Figure 7A), consistent with previous reports (Lin et al., 2003). Regions with little or no ME coverage were always multilayered. ZO-1 in multilayered hyperplasias was localized in small pockets (Figures 7A and 7B). In later lesions, SMA+ cells were rare or absent. In multilayered hyperplasias, β-catenin localized to all sites of cell-cell contact, indicating incomplete apicobasal polarity (Figure 7B). Therefore, normal and neoplastic epithelia during morphogenesis share a similar multilayered epithelial organization (Figures 7A–7D).

**DISCUSSION**

Our study has provided new insights into mammary branching morphogenesis and has identified a different type of collective epithelial migration without leading cellular extensions or protrusions. We dissected the process of branching morphogenesis into discrete subprocesses by using time-lapse confocal microcopy, and we identified molecular pathways regulating each subprocess (Figure 7). We showed that mammary epithelium first reorganizes from a quiescent, bilayered epithelium to a preinvasive, highly proliferative, incompletely polarized, multilayered epithelium. We identified Rac and MLCK as molecular regulators of the transition from this preinvasive state to the initiation of new ducts. We observed that cells within extending ducts dynamically rearrange, but remain adherent and localize E-cadherin and β-catenin to cell-cell contacts. During this collective epithelial migration, the tissue has distinct luminal- and basement membrane-contacting surfaces, but individual cells within the multilayered region are incompletely polarized. Finally, ROCK signaling is required to restore simple, bilayered epithelial architecture.

**Models of Collective Cell Movement**

Recent time-lapse imaging studies have established models for the collective movement of groups of cells, including neuronal precursors in the zebrafish lateral line (Lecaudey and Gilmour, 2006), epithelial cells during *Drosophila* dorsal closure (Jacinto et al., 2000), and border cell migration in *Drosophila* (Bianco et al., 2007; Prasad and Montell, 2007). In each of these examples, cells at the front of the migrating group extended cellular extensions or protrusions in the direction of movement. By contrast, cells at the front of elongating mammary ducts did not have leading cellular extensions or actin-rich protrusions. As protrusive activity can function to guide cells, how elongating mammary ducts move directionally remains an open question.

**The Role of Myoepithelial Cells in Branching Morphogenesis**

ME cells are implicated in normal development and neoplastic progression in the mammary gland (Gudjonsson et al., 2005;
Polyak and Hu, 2005) and have been identified as cellular tumor suppressors (Sternlicht et al., 1997). However, because most cell culture models of mammary morphogenesis contain only one cell type, often of mixed myoepithelial and luminal character, ME function has often been overlooked.

We directly monitored ME cell behavior during branching morphogenesis and observed ductal initiation at breaks in ME coverage, active migration of ME cells over the surface of LE in ducts, and a close relationship between ME localization, ME motility, and the resulting pattern of branching morphogenesis. In time-lapse movies, ME migration precede bifurcations. However, our results do not formally distinguish whether ME cells actively regulate ductal initiation and patterning or are early responders to another signal. In each of our perturbations, the localization of ME cells marked the resulting phenotype. Rac or MLCK inhibition blocked duct initiation and resulted in cysts with extensive ME coverage. ROCK inhibition resulted in a disorganized hyperbranching phenotype and disorganized, incomplete ME coverage. Our results suggest an important role for ME cells as cellular regulators of tissue architecture.

Do other branched epithelial organs rely on a similar balance between different cell types to produce branched ductal networks? Recent work in the vascular system has shown that perivascular smooth muscle cells or pericytes influence the patterning and function of endothelial networks in normal development and cancer (Hall, 2006; Song et al., 2005).

**Epithelial Morphogenesis during Development**

We demonstrated that epithelial architecture and polarity can be dramatically regulated in time and space within a group of actively rearranging cells. Our study followed live mammary epithelial cells through a reversible conversion from a highly polarized bilayered organization to a low-polarity, multilayered intermediate. Our observations build on genetic studies identifying cell adhesion proteins as critical regulators of mammary development and breast cancer (Hinck and Silberstein, 2005; Katz and Streuli, 2007; Knudsen and Wheelock, 2005).

Most work on epithelial morphogenesis has focused on embryonic tissues (e.g., lung [Liu et al., 2004], ureteric bud [Costantini, 2006; Meyer et al., 2004; Pohl et al., 2000], and salivary gland [Patel et al., 2006]) or immortalized cell lines such as MDCK cells (Zegers et al., 2003). Active cell rearrangements within a multilayered epithelium have been noted during...
Collective Cell Migration in Mammary Morphogenesis

Embryonic branching morphogenesis of the salivary gland in organ culture, but these movements declined with age and were not observed in neonatal tissue (Larsen et al., 2006).

Although the polarity of mature epithelia is well understood and recent work has illuminated how polarity emerges after initial cell-cell contact (Nejsum and Nelson, 2007; Yeaman et al., 1999), less attention has been paid to changes in apicobasal polarity during morphogenesis and tissue remodeling in adult tissues. Intriguingly, apicobasal polarity is also downmodulated during morphogenesis in other systems, including embryonic neurüctoderm (Aaku-Saraste et al., 1996; Lee et al., 2007) and HGF-induced morphogenesis in other systems, including embryonic neurüctoderm (Aaku-Saraste et al., 1996; Lee et al., 2007) and HGF-induced tubulogenesis in 3D MDCK cultures (Zegers et al., 2003).

We propose that the properties of stable epithelial polarity, distinct apicobasal membrane domains, and a single luminal cell layer used to define simple epithelia might instead be characteristic only of morphogenetically inactive epithelia. It is of interest to determine whether all vertebrate epithelial tissues must enter into an incompletely polarized, multilayered intermediate to remodel or repair their structure as well as what signaling pathways regulate entry into and exit from the multilayered state.

The Morphogenetically Active Epithelial State

We suggest that this reversible conversion of epithelial organization represents a regulated transition between different states of the same epithelium, rather than a transformation of epithelial type (metaplasia). As such, the shared low-adhesion, low-polarity, multilayered epithelial state that we observe in normal and neoplastic epithelium during morphogenesis may be an epithelial state specialized to allow growth and remodeling of the epithelium. We suggest the term “morphogenetically active epithelial state” to describe the organizational state of mammmary epithelium during morphogenesis. We hypothesize that similar transitions between different states of epithelial organization may be a general developmental mechanism for remodeling and repairing epithelial tissues.

Alternate Strategies for Collective Cell Movement

Collective epithelial migration lacks many of the features classically associated with an invasive or migratory phenotype, such as forward-oriented, cellular extensions or actin-rich protrusions (Mitchison and Cramer, 1996). It has been noted in clinical specimens and in explanted human 3D cultures that tumors can invade singly or as connected groups of cells (Friedl et al., 2004; Hegerfeldt et al., 2002). Clearly, there is more than one possible underlying cellular mechanism for tissue invasion, and additional work is needed to determine the relative importance of collective epithelial migration versus classic protrusive invasion in human tissue repair and in diseases such as breast cancer. It is also of critical importance to determine whether the cellular mechanisms of tissue invasion are cell type specific, or whether cellular invasion mechanisms may depend on the interplay between cell intrinsic factors and tissue microenvironmental context.

Epithelial Morphogenesis during Cancer Progression

We propose that a primary defect in early cancerous lesions may be aberrant entry into, or failure to exit from, this morphogenetically active state may facilitate tumor initiation or progression. During normal morphogenesis, mammary epithelium transiently reorganizes into a highly proliferative, low-polarity, multilayered state and then returns to a quiescent, single epithelial state. Aberrant entry into, or failure to exit from, this morphogenetically active state may facilitate tumor initiation or progression.

EXPERIMENTAL PROCEDURES

GFP Reporter Lines

Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at University of California, San Francisco. We used transgenic mice in which a GFP-actin fusion protein was expressed under the control of the keratin-14 promoter (K14-GFP-actin), acquired from E. Fuchs, Rockefeller University (Vaezi et al., 2002). The second mouse model expressed...
EGFP, knocked into the Sca-1 locus (Sca-1-EGFP), mosaically throughout the mammary epithelium (provided by T. Graubert, Washington University; Hanson et al., 2003). Transgenic mice expressing EGFP under the control of the β-actin promoter (β-actin-EGFP; Hadjantonakis et al., 1998) were obtained from Jackson Laboratory. FVB/N mice were purchased from Charles River.

Isolation of Primary Mammary Organoids

 Cultures were prepared as previously described (Fata et al., 2007). Briefly, glands were minced and tissue was shaken for 30 min at 37°C in a 50 ml collagenase/trypsin solution in DMEM/F12 (GIBCO-BRL), 0.1 g collagenase (Sigma C5138), 5 ml fetal calf serum, 250 μg of 1 μg/ml insulin, and 50 μl of 50 μg/ml gentamicin (all University of California, San Francisco Cell Culture Facility). The collagenase solution was centrifuged at 1500 rpm for 10 min, dispersed through 10 ml DMEM/F12, centrifuged at 1500 rpm for 10 min, and then resuspended in 4 ml DMEM/F12 + 40 μl DNase (2U/μl) (Sigma). The DNase solution was shaken by hand for 2–5 min, then centrifuged at 1500 rpm for 10 min. Organoids were separated from single cells through four differential centrifugations (pulse to 1500 rpm in 10 ml DMEM/F12). The final pellet was resuspended in the desired amount of Growth Factor Reduced Matrigel (BD Biosciences).

Organoid Assay

Organoid assays were performed in 24-well coverslip bottom plates (E&K Scientific EK-42892) for confocal time-lapse imaging and in 4-well coverslip bottom chambers (Nunc 155383) for routine culture. Typically, a 50 μl suspension of organoids in Matrigel was added to each well, followed by 30 min at 37°C. We then added minimal media (DMEM/F12, 1% v/v insulin, transferrin, selenium [Sigma] and 1% v/v penicillin/streptomycin [100x stock] or FGF2/branching medium (minimal medium + 2.5 nM FGF2 [Sigma F2921]). Inhibitors were used in a minimum of three separate experiments with similar results. Representative single experiments are shown. ROCK inhibitor (Y27632; Chemicon), Rac-1 inhibitor (NSC23766, Calbiochem), ML-7 (Calbiochem), and aphidicolin (Sigma) were used as indicated. All inhibitor experiments discussed in the text were done in FGF2 medium. ROCK inhibitor treatment in minimal medium resulted in simple cysts, which were indistinguishable from control organoids.

Antibody Staining

Organoids were equivalently in 25% sucrose in PBS for 1 hr., fixed in cold 1:1 methanol/acetone overnight at −20°C, then re-equilibrated in 25% sucrose in PBS for 1 hr. Samples were blocked for 1 hr with 5% serum, incubated with primary antibody (all 1:250 in PBS) for 2 hr to overnight, and rinsed three times in PBS. Secondary antibodies (Molecular Probes Alexa series, all 1:250 in PBS) were incubated with the organoids for 1–4 hr. F-actin was stained with Alexa 580 and Alexa 488 or 568 Phalloidin (1:40–1:1000), and nuclei were stained with To-Pro-3, DAPI, or propidium iodide (all 1:1000, Molecular Probes). Antibody stains were done at least 3 independent times, and a minimum of 25 organoids was inspected in each condition each time. Primary antibodies were E-cadherin (Zymed, 13-1900), ZO-1 (Chemicon, MAB1520), SMA (Sigma, F3777 or Zymed, 13-1900), E-cadherin (Becton Dickinson), ZO-1 (Chemicon, MAB1520), SMA (Sigma, F3777 or Chemicon), Rac-1 inhibitor (NSC23766, Calbiochem), ML-7 (Calbiochem), and aphidicolin (Sigma) were used as indicated. All inhibitor experiments discussed in the text were done in FGF2 medium. ROCK inhibitor treatment in minimal medium resulted in simple cysts, which were indistinguishable from control organoids.

REFERENCES


Collective Cell Migration in Mammary Morphogenesis


