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Cellular Foundations of Mammary Tubulogenesis

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Abstract

The mammary gland is composed of a highly branched network of epithelial tubes, embedded within a complex stroma. The mammary epithelium originates during embryonic development from an epidermal placode. However, the majority of ductal elongation and bifurcation occurs postnatally, in response to steroid hormone and growth factor receptor signaling. The process of pubertal branching morphogenesis involves both elongation of the primary ducts across the length of the fat pad and a wave of secondary branching that elaborates the ductal network. Recent studies have revealed that mammary epithelial morphogenesis is accomplished by transitions between simple and stratified organization. During active morphogenesis, the epithelium is stratified, highly proliferative, has few intercellular junctions, and exhibits incomplete apico-basal polarity. In this review, we discuss recent advances in our understanding of the relationship between epithelial architecture, epithelial polarity, and ductal elongation.

Keywords

branching morphogenesis; breast cancer; mammary gland; apico-basal polarity; collective cell migration; tubulogenesis

1. Introduction

The mammary gland is composed of bilayered tubes embedded within an adipocyte rich stroma. The epithelial cells that constitute these tubes are connected to each other by extensive intercellular junctions and have a high degree of apico-basal polarity. This polarity is manifested both by morphological specializations, such as apically-localized microvilli, and by segregated membrane domains containing distinct apical and basal polarity complexes^{1,2,3}. These adhesive junctions must be remodeled for either individual or collective cell movement to occur. The strong apico-basal polarity also places constraints on tubulogenesis, as many of the molecular determinants of epithelial polarity serve distinct roles in migratory cells¹. In this review, we will focus on the cellular mechanisms used by the mammary epithelium to build and elaborate the ductal network during puberty. Other

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authors have reviewed the genetic requirements for mammary gland formation⁴ and the cellular steps in embryonic mammary development^{5,6}.

1.1 Embryonic origins of the mammary epithelium

Mammary ducts in the adult have a bilayered organization, with keratin-14+ (K14+) myoepithelial cells positioned basally relative to the K8+ luminal epithelial cells. In contrast, the embryonic mammary epithelium originates as an epidermal placode and invades into the mesenchyme with a stratified organization and without a morphologically evident lumen⁷. Both the cell type specific gene expression and relative positioning of myoepithelial and luminal epithelial cells are late features that are acquired progressively during fetal and early postnatal development^{8,9,10}. As the mammary bud initiates elongation, many of the epithelial cells are K8+K14+ and K14+ cells are detected in both basal and interior positions^{8,11}. It is only late in fetal development that a mature lumen forms, lined with K8+ luminal epithelial cells and surrounded by basally positioned K14+ cells^{7,11}. Myoepithelial cells are named for their contractile properties, which are regulated by genes such as smooth muscle actin (SMA). This contractile gene expression program is activated postnatally and so the mature myoepithelial phenotype of K8-K14+SMA+ is not established until the onset of puberty⁹. Taken together, the embryonic mammary epithelium during morphogenesis is stratified, lacks a contiguous lumen, and is composed of cells with combinations of “cell type specific” gene expression that are uncommon in adult ducts. While the result of embryonic mammary development is a small network of polarized simple ducts, the organization of the epithelium during morphogenesis is neither simple nor polarized⁷ (Figure 1A).

1.2 Postnatal branching morphogenesis of the mammary epithelium

The mammary epithelium finishes fetal development as fully polarized bilayered ducts⁷. These simple tubes are then essentially quiescent until the rise in steroid hormones that triggers the onset of puberty^{12,13}. Before puberty the mammary ducts are all simple (Figure 2A,A') and they then stratify to produce stratified terminal end buds^{14,15,16} (TEBs) (Figure 2B,B'). Terminal end buds have multiple layers of epithelial cells, referred to as body cells, surrounded by a basally positioned cap cell layer^{15,16}. Most body cells stain positive for luminal epithelial markers, such as K8 and E-cadherin¹⁷. However, there are basal marker positive body cells as well^{18,19}. Cap cells stain positive for basal cytokeratins and P-cadherin¹⁷. Consistent with the organization of the embryonic mammary epithelium, the body cells exhibit high levels of proliferation, have few intercellular junctions, and incomplete apico-basal polarity^{19,20}. Many body cells lack contact with either the main lumen or the basement membrane and isolated microlumens are observed within the body cell region^{19,20}, similar to the microlumens observed in the early fetal mammary ducts⁷ (Figure 1A vs. 2C'). TEBs form in response to iterative signals exchanged between the epithelium and surrounding mesenchymal cells, mediated through both steroid hormone receptors and growth factor receptors^{4,12,13}.

TEBs begin elongating at the onset of puberty, 3 weeks of age in mice. These stratified TEBs build centimeters of ducts as they cross the mammary fat pad and there is an abrupt transition from the stratified organization of the TEB to the simple organization of the

trailing duct (Figure 2C,C')^{16,19}. A secondary wave of side branching initiates a couple of weeks later and fills in the complexity of the network. The details of the branching pattern are highly variable and can be quite distinct even when comparing different glands in the same mouse. Common themes emerge at a more global level: TEBs stop elongating and revert to simple epithelial architecture when they reach the edges of the mammary fat pad. The result is a similar openness in the branching pattern across mice of the same strain. Classic experiments inferred a characteristic avoidance of TEBs from each other and also demonstrated that implanted beads soaked in TGF- β 1 would locally inhibit TEB elongation²¹. Taken together these data suggest the existence of a diffusible negative cue that locally represses branching and that the signal could be TGF- β 1 mediated. Modern investigations into the nature of intercellular communication among mammary epithelial cells revealed that their geometric organization predicted new sites of growth and that regions of repression of growth were dependent on TGF- β 1 signaling²². Genetic analysis of dozens of different alleles has demonstrated a rich molecular network regulating both the initiation and elongation of mammary ducts. We often conceptualize of cellular decisions being regulated by single “master regulators”. Both the pubertal and lactational differentiation of the mammary gland are regulated by both the levels and timing of hormones, including estrogen, progesterone, prolactin, and growth hormone^{23,24}. Genetic experiments have also demonstrated a requirement for FGFR1, FGFR2, ErbB1, ErbB2, ErbB3, Met receptor, and IGF-1R^{4,25}. It remains unclear exactly how mammary epithelial cells integrate signals from such diverse receptors or why so many seemingly similar receptors are needed to coordinate branching morphogenesis.

2. Cellular basis of mammary tubulogenesis

Careful histologic analysis revealed many of the key features of mammary epithelial development, such as the iterative transitions between simple polarized and stratified unpolarized architecture^{7,15,18,19,20}. Thorough in vivo genetic analysis has elaborated an impressive description of the molecular components required for normal mammary development and the utilization of these components in breast cancer^{4,26}. However, the optical inaccessibility of the mammary gland has typically limited the analysis of the genetic manipulations to histologic readouts, such as mitotic and apoptotic indices. This limitation has made it difficult to understand how the TEB functions as a multicellular machine for ductal elongation. It has also limited the precision of genetic analyses as many manipulations yield quantitatively similar reductions in, for example, ductal elongation.

2.1 Technical foundations for real-time analysis of mammary tubulogenesis

In the absence of real-time information about mammary ductal elongation, we rely heavily on analogies to other biological models. Our conceptual framework for understanding cell motility in general is founded on studies of mesenchymal and amoeboid cell migration^{27,28,29}. Migratory single cells polarize in a direction, extend actin based protrusions in response to that polarity, exert force on the substratum, and move in the direction of protrusion^{27,28} (Figure 3A). These concepts naturally led to the hypothesis that collective cell migration would involve a similar polarization of the front of the leading cells and actin-based protrusions into the surrounding environment (Figure 3B). This concept

works well to explain the migration of *Drosophila* border cells³⁰, *Drosophila* salivary gland elongation³¹, the zebrafish lateral line³², and multiple types of collective cancer invasion³³.

The most direct way to determine how TEBs elongate ducts would be to image the process in vivo. Since TEB elongation occurs over weeks, within an adipocyte-rich mammary fat pad, this requires long-term intravital imaging deep inside optically scattering tissue. Two-photon microscopy has enabled intravital imaging of stromal dynamics around TEBs³⁴ but no publications to date have imaged the cellular basis of TEB elongation in vivo. To model this process in a more experimentally and optically convenient format, diverse 3D culture assays have been developed. Mammary epithelial cells can be cultured on top of thin ECM gels^{35,36}, cultured within geometrically defined ECM environments²², or alternately pieces of primary mammary epithelium can be explanted within 3D ECM gels^{19,37,38}. To date, primary organoid assays have produced the most organotypic branching programs in either Matrigel¹⁹ or mixtures of Matrigel and collagen I³⁹. We focus on describing results obtained with these primary organoid assays as they model the in vivo events of stratification, initiation of new epithelial buds, duct elongation, and bifurcation^{14,19,20}.

2.2 Mammary epithelial buds initiate and elongate without ECM-directed protrusions

Mammary TEBs in vivo lack obvious cellular protrusions into the ECM even in high-resolution confocal images^{18,19} and are enclosed within an intact basement membrane¹⁵. However, it remained possible that the cap cells extended either fine or transient protrusions into the ECM that were difficult to detect in histologic sections. Accordingly, we optimized a 3D primary tissue organoid assay for long-term timelapse imaging^{19,40}. We did not observe ECM directed protrusions using cytoplasmic or actin-GFP based timelapse imaging, by phalloidin staining, or by transmission electron microscopy^{19,20} (Figure 3C-D'). The border between the epithelial cells and the ECM was strikingly smooth both in 3D culture and in vivo^{19,20} (Figure 3D,D'). Despite the robust localization of E-cadherin and β -catenin to points of cell-cell contact within the stratified epithelium, we observed few intercellular junctions connecting cells in the interior of the multilayer²⁰. We did observe abundant, interdigitating membrane protrusions, extending into the space between these interior cells²⁰. However, the lack of ECM-directed protrusions appears to limit the extent to which TEB cells can explore the space in front of them before migrating. This limitation suggests that TEBs may not migrate as directionally as, for example, growth cones on a neuron. Alternately, the TEB may sense soluble guidance cues collectively across a multicellular ensemble.

2.3 Asymmetric division of luminal cells initiates stratification and loss of polarity

Cells within the TEB exhibit fewer intercellular junctions and reduced apico-basal polarity when compared to the polarized mammary ducts they are generating^{19,20} (Figure 4A). At the molecular level, simple epithelial cells maintain segregated apical domains within the plasma membrane, enriched in the Par-3/Par-6/Cdc42 polarity complex⁴¹ (Figure 4B). This apical domain is separated by tight junctions from the basolateral domain, which is enriched in the Scribble/Lethal Giant Larvae/ Discs Large complex⁴¹ (Figure 4B). Immunofluorescent staining for these polarity proteins in vivo revealed that both simple organization and molecularly distinct membrane domains are absent in the TEB during

ductal elongation²⁰. We sought to determine the cellular mechanism that initiated the formation of the TEB and the relationship between stratification and loss of polarity.

Given that the starting point was a bilayered tube, with apically positioned luminal epithelial cells and basally positioned myoepithelial cells, we reasoned that stratification could initiate within either or both of these cellular populations. We analyzed the orientation of cell division during stratification in real-time in the organoid assay and the plane of mitosis in tissue sections collected during TEB formation in vivo¹⁴. In both cases, we exclusively observed stratification initiate from asymmetric divisions within the luminal population¹⁴ (Figure 4C), consistent with the emerging concept that ductal elongation during puberty is driven by unipotent stem and progenitor cells⁴². Our real-time analysis of the dynamics of ZO1-GFP revealed that tight junctions are maintained in the mother cell during this asymmetric cell division, while the daughter cell did not form tight junctions or segregated membrane domains (Figure 4D). This asymmetric cell division could therefore explain both stratification and loss of polarity, two early features of epithelial cancers.

We next demonstrated that expression of either constitutively activated ErbB2 or phosphomimetic MEK1DD was sufficient to induce stratification by this mechanism. Importantly, stratified epithelial tissues such as the epidermis have been shown to initiate stratification from basally positioned cells, in contact with a basement membrane⁴³. We believe our data are the first to report developmental stratification from the apical compartment, driven by cells out of contact with the basement membrane. Our data reveal a cellular mechanism for transient developmental stratification that can be readily co-opted during malignant progression through activation of receptor tyrosine kinase signaling.

2.4 Mammary tubulogenesis is driven by changes in epithelial organization and cell behavior

Synthesizing the real-time analysis in 3D culture with the histology of the TEB during elongation, the concept that emerges is of ductal elongation driven by changes in epithelial architecture (Figure 5A). The simple bilayered tube stratifies in response to steroid hormone and growth factor receptor signaling, creating a population of low polarity, highly proliferative, epithelial cells between the polarized luminal epithelial and myoepithelial cell layers. While the internal epithelial cells continue to express epithelial cytokeratins (e.g. K8) and cell adhesion molecules (e.g. E-cadherin), they have fewer intercellular junctions and incomplete apico-basal polarity. These internal epithelial cells then drive the elongation of the tube and ultimately resolve back to a simple tube.

A reasonable question is why are these transitions necessary? It is easy to conceive of mechanisms of tube elongation that don't require major changes in epithelial architecture. However, mammary development occurs iteratively and initiates from a highly polarized, junctionally connected epithelium. The question of epithelial motility and cell migration has been addressed by multiple labs. An early study used timelapse imaging to reveal active cell migration within non-invasive, stratified epithelial acini⁴⁴. We observed vigorous migration and dynamic cell rearrangements during branching of primary mammary organoids¹⁹. Mammary epithelial cells within geometrically defined matrix environments displayed MMP-14 dependent differential persistence of migration within the epithelium and these

differences determined their contribution to elongating branches⁴⁵. Finally, multiple groups have demonstrated coherent angular motions of mammary epithelial cells within multicellular groups⁴⁶ and have related these rotations to the organization of the basement membrane⁴⁷.

Despite differences in cellular inputs and culture formats, all of these studies support the idea that mammary epithelial cells within stratified groups are highly migratory. Indeed, when individual epithelial cells within an elongating duct are labeled with cytoplasmic GFP and imaged using timelapse confocal microscopy, they are actively migrating within the tissue²⁰ (Figure 5B). Without the Cell Tracker Red staining for tissue contrast, it would be easy to imagine that this was a mesenchymal cell migrating through the ECM. Instead, it is an epithelial cell migrating between other epithelial cells (Figure 5B). We speculate that this phenomenon of active migration confined within an epithelial multilayer represents an efficient solution to the dual need to enable dramatic remodeling of the postnatal mammary epithelium while still minimizing the likelihood of epithelial cells migrating to distant organs.

3. Relationship of normal mammary morphogenesis to breast cancer metastasis

The large majority of cancers arise in epithelial cells, and among epithelial malignancies both morbidity and mortality are driven by metastasis to distant organs⁴⁸. The resting configuration of a mammary duct is a highly polarized, highly adherent, essentially non-proliferative epithelium. With this quiescent state defining our sense of “normal” epithelial biology, it is tempting to view the problem of metastasis as a question of how the cancer “invents” epithelial motility from such stationary cells (Figure 6A). However, every real-time analysis has revealed that normal mammary epithelial cells have a high migratory potential, even when arranged in stratified epithelia and connected by intercellular junctions^{19,20,22,45,46,47}. The most important question might instead be: why don't these normal migratory epithelial cells leave (Figure 6B)? The key cell behavioral difference is that when normal cells reach the tissue compartment boundary they stop migrating, while metastatic cells ignore the boundary and migrate to distant organs.

We sought to understand the molecular regulation of tissue compartment boundaries and used mammary organoid as an observable, manipulable model system in which to explore this phenomenon. Our first surprise was when we explanted tumor organoids from genetically engineered mouse models of breast cancer. In vivo, the MMTV-PyMT mouse model will metastasize to the lungs in 95% of mice on the FVB background^{49,50}. Yet, when tumor organoids from this model were placed into Matrigel culture, essentially no cells disseminated into the ECM⁵¹. We repeated these experiments with primary human breast tumors and found the same result: even organoids isolated from tumors that had metastasized in the patient did not disseminate into Matrigel⁵¹. However, the microenvironment around a breast tumor is deficient in laminin I and collagen IV, the major protein constituents of Matrigel, and rich in fibrillar collagen I⁵². When organoids from the same tumor were explanted into different ECM gels, they were indolent in Matrigel and disseminative into collagen I⁵¹. This experiment demonstrated that the ECM is a potent

regulator of invasive and disseminative behavior in tumor tissue. We next tested the response of normal organoids to direct contact with collagen I. We observed the same initial response: protrusions and dissemination into the collagen I⁵¹, specifically into collagen I gels with a high fibrillar content³⁹. However, normal epithelium rapidly reestablished basement membrane coverage, ceased protrusive activity, and reinitiated a program of branching morphogenesis⁵¹. These experiments reveal a conserved response of invasion and dissemination following direct contact with fibrillar collagen I, and a robust capacity of normal epithelial cells to cease invasion and reestablish epithelial organization.

We next tested whether we could identify molecular interventions that could induce dissemination of normal epithelial cells into the ECM. We decided to contrast deletion of the cell adhesion gene, E-cadherin, with induced expression of the transcription factor, Twist1, a protein frequently associated with metastatic cell behaviors. We observed that deletion of E-cadherin induced loss of simple epithelial organization, apical movement of E-cadherin-cells into the lumen, basal invasion of E-cadherin- cells into the ECM, but not single cell dissemination⁵³. In contrast, expression of Twist1 induced dissemination of epithelial cells into the ECM within 24-72 hours⁵³. Twist1+ cells retained epithelial specific gene expression and membrane localized E-cadherin and β -catenin. Surprisingly, knockdown of E-cadherin in Twist1 expressing cells reduced single cell dissemination and resulted in a more collective form of invasion. RNA expression analysis revealed significant changes in cell-matrix adhesion and extracellular compartment genes, consistent with an epithelial motility program in which the cells optimize their gene expression to match the environment through which they will be migrating. We believe that this assay will allow us to identify the molecular programs that enable epithelial cells to bypass compartment boundaries and exit the epithelium. More generally, the molecular techniques developed in this study can be utilized to understand the consequences of genetic interventions on both cell behavior and tissue level function.

4. Conclusions and Future Directions

This is a tremendously exciting time in mammary epithelial biology. There are many groups bringing together innovative combinations of genetics, cell biology, and imaging to generate a cellular resolution understanding of epithelial structure and function. The iterative nature of mammary development throughout reproductive life provides the opportunity to study cycles of morphogenesis and quiescence in a normal context. There are many fascinating open questions that remain to be elucidated in the coming years. How do epithelial cells cooperate (or compete) to accomplish ductal elongation? What is the cellular mechanism by which the stratified TEB resolves back to simple epithelial organization? How do TEBS bifurcate? Are the cellular and molecular mechanisms employed to elongate TEBS recapitulated during side branching and lactational differentiation? These questions have fascinated generations of mammary gland biologists and the innovative tools available today should enable answers to each of these classic questions over the next decade.

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Abbreviations

ECM	Extracellular matrix
TEB	Terminal end bud

Highlights

- Mammary epithelium transitions between simple and stratified organization during development.
- Terminal end buds (TEBs) elongate mammary tubes during puberty without ECM directed protrusions.
- TEBs are stratified, have low apico-basal polarity, and high proliferation.
- Stratification is initiated by an asymmetric cell division with concomitant loss of polarity.
- Normal cells are protrusive and migratory within the epithelium but do not disseminate.

A. Embryonic Mammary Development

B. Postnatal Mammary Development

Fig. 1.

(A) Mammary development in the mouse begins at embryonic day 12 when an epidermal placode invades the mammary stroma. The stratified epithelium then elongates and hollows to form a rudimentary bilayered duct that remains essentially quiescent until puberty. Modified from Hogg et al, 1983. (B) Mammary ducts are elaborated during puberty to form the functional ductal network. Pubertal development initiates during postnatal week 3 with the formation of terminal end buds (TEBs), which are a stratified epithelium at the ductal tips. Over the next 7 weeks, primary ducts elongate across the mammary fat pad, and secondary branching completes the ductal tree.

Fig. 2.

(A) Mammary epithelium consists of a rudimentary ductal network prior to puberty. (A') Duct from a 2-week-old mouse stained for F-actin (red) and nuclei (green). All mammary ducts are bilayered before pubertal development. Modified from Huebner, Lechler, and Ewald, 2014. (B) TEBs form at ductal tips at the onset of puberty. (B') TEB from a 4-week-old mouse stained for F-actin (red) and nuclei (green). TEBs consist of a stratified epithelium with more than two cell layers. Modified from Huebner, Lechler and Ewald, 2014. (C) TEBs function as the elongation front of the mammary epithelium during pubertal development. (C') TEB stained for the basolateral polarity marker β -catenin (red), the tight junction protein zona occludins 1 (ZO-1), (green) and nuclei (blue). TEBs have incomplete apico-basal polarity with β -catenin present on nearly all cellular membranes and tight junctions present primarily at the apical-most membrane facing the luminal space. Aside from the primary luminal space, TEBs also have microlumens, highlighted with arrows, which are positive for tight junctions. Modified from Ewald et al, 2008.

Fig. 3.

(A) Migration of single cells involves front-rear polarization and actin-based protrusions at the leading edge. (B) A possible mechanism for ductal elongation is migration of a subset of polarized protrusive leader cells. (C) Organoid bud stained for F-actin (green) and nuclei (red). (C') Zoomed image of the elongation front of the bud shown in C. Actin-based protrusions are noticeably absent from the leading edge of elongating mammary branches. Modified from Ewald et al, 2008. (D) Electron micrograph of an elongating mammary bud. (D') Leading edge of the bud shown in D. Elongating mammary branches have a smooth leading edge when viewed at the ultrastructural level. Modified from Ewald et al, 2012.

Fig. 4.

(A) Model of mammary duct and TEB structure. Ducts are a bilayered epithelium with apical luminal epithelial cells (gray) and basal myoepithelial cells (brown). Luminal cells have tight junctions (green) at the apical-most point of cell-cell contact. Desmosomes (blue) are cell adhesion complexes that connect luminal cells to each other and to the myoepithelial cell layer. TEBs are composed of a low-polarity internal cell population (light blue) that is bounded by the basal myoepithelial layer and apical luminal cells. Internal cells have a diminished number of desmosomes and loose cell-cell contacts by electron microscopy. Tight junctions are absent from internal cells except at microlumens. Mammary ducts and TEBs are surrounded by basement membrane (red). (B) Schematic showing the apico-basal polarity of luminal epithelial cells. The polarity protein par-3 (orange) is localized to the apical membrane and scribble (red) is localized to the basolateral membrane. ZO-1 (green) marks the tight junction, which partitions the apical and basolateral membranes. (C) Model of the cellular mechanism of mammary duct stratification. Stratification is initiated by vertical proliferation of apically localized luminal epithelial cells, and the vertical divisions result in asymmetric localization of tight junctions and polarity loss for internal cells. Stratification is non-clonal, as multiple vertical divisions are observed within a single organoid. Low-polarity internal cells are proliferative, further driving stratification. Modified from Huebner, Lechler, and Ewald, 2014. (D) Vertical divisions resulted in asymmetric localization of par-3 (orange) ZO-1 (green) and scribble (red) in the apical mother cell and the internal daughter cell. This asymmetry directly results in polarity loss for the internal daughter cell. The cell fate marker numb (blue) is asymmetrically localized to the basal daughter cell during vertical divisions. Modified from Huebner, Lechler and Ewald, 2014.

Fig. 5.

(A) Schematic showing each stage of mammary branching morphogenesis. Prior to development, ducts are bilayered with apical luminal epithelial cells (gray) and basal myoepithelial cells (brown), and the entire duct is surrounded by basement membrane (red). The epithelium then stratifies, forming a low-polarity internal cell population (light blue). Low-polarity TEBs then elongate the epithelial ducts, and finally the epithelium polarizes back to a bilayer. (B) Still images from a movie of an organoid mosaically labeled with cytosolic GFP to visualize single cells within the epithelium. Cells are labeled green, the membranes are red, and the dashed line shows the elongation front. Cells were protrusive and migratory within the organoids. The arrow highlights a cell that moves to the basal surface and then back into the body of the elongating branch. Modified from Ewald et al, 2012.

A. How do metastatic tumors “invent” epithelial motility?

B. Why don't normal migratory cells leave?

Fig. 6.

(A) Cartoon depiction of epithelial cell dissemination past the basement membrane. (B) Schematic questioning why normal motile cells within the epithelium do not invade past the basement membrane.