RESEARCH ARTICLE

Mammary epithelial tubes elongate through MAPK-dependent coordination of cell migration

Robert J. Huebner, Neil M. Neumann and Andrew J. Ewald*

ABSTRACT

Mammary branching morphogenesis is regulated by receptor tyrosine kinases (RTKs). We sought to determine how these RTK signals alter proliferation and migration to accomplish tube elongation in mouse. Both behaviors occur but it has been difficult to determine their relative contribution to elongation in vivo, as mammary adipocytes scatter light and limit the depth of optical imaging. Accordingly, we utilized 3D culture to study elongation in an experimentally accessible setting. We first used antibodies to localize RTK signals and discovered that phosphorylated ERK1/2 (pERK) was spatially enriched in cells near the front of elongating ducts, whereas phosphorylated AKT was ubiquitous. We next observed a gradient of cell migration speeds from rear to front of elongating ducts, with the front characterized by both high pERK and the fastest cells. Furthermore, cells within elongating ducts oriented both their protrusions and their migration in the direction of tube elongation. By contrast, cells within the organoid body were isotropically protrusive. We next tested the requirement for proliferation and migration. Early inhibition of proliferation blocked the creation of migratory cells, whereas late inhibition of proliferation did not block continued duct elongation. By contrast, pharmacological inhibition of either MEK or Rac1 signaling acutely blocked both cell migration and duct elongation. Finally, conditional induction of MEK activity was sufficient to induce collective cell migration and ductal elongation. Our data suggest a model for ductal elongation in which RTK-dependent proliferation creates motile cells with high pERK, the collective migration of which acutely requires both MEK and Rac1 signaling.

KEY WORDS: Collective cell migration, MAPK signaling, Branching morphogenesis, Epithelial development, Tubulogenesis, Mammary gland

INTRODUCTION

Epithelial tubes are an essential building block of animal organs but the mechanisms that drive their elongation and branching remain incompletely understood (Andrew and Ewald, 2010). Since mammalian organs increase in size and structural complexity during development, researchers have sought to understand the relative contributions of proliferation, cell shape change and migration to lung, salivary gland and kidney tubulogenesis (Andrew and Ewald, 2010; Alescio and Di Michele, 1968; Goldin and Wessells, 1979; Lubarsky and Krasnow, 2003). Recent advances in organotypic culture and imaging have shed light on how these processes drive mammalian tube growth and development (Shamir and Ewald, 2014; Kim et al., 2013; Schnatwinkel and Niswander, 2013; Packard et al., 2013; Larsen et al., 2006; Hsu et al., 2013; Tang et al., 2011).

The mammary gland is an interesting model for the study of tubulogenesis due to its cyclical development; an epithelial rudiment is established in the embryo and elaborated during puberty and within each lactation cycle (Huebner and Ewald, 2014; Hennighausen and Robinson, 2005; Sternlicht, 2006). The majority of tubulogenesis occurs in the postnatal animal, triggered at the onset of puberty by steroid hormones (Sternlicht, 2006; Hinck and Silberstein, 2005). These circulating hormones interact with local signaling networks, primarily through receptor tyrosine kinases (RTKs) (Hennighausen and Robinson, 2005; Sternlicht et al., 2006). The genetic requirements for branching morphogenesis are increasingly clear (McNally and Martin, 2011) but it remains difficult to determine how these signals regulate cell behavior. The principal challenge to optical analysis in vivo is that mammary tubes are embedded within an adipocyte-rich stroma and light scattering from the lipid limits the depth of imaging. Novel three-dimensional (3D) culture methods permit mammary development to be recapitulated ex vivo and studied dynamically (Simian et al., 2001; Sternlicht, 2005; Fata et al., 2007; Ewald et al., 2008, 2012; Huebner et al., 2014).

Past real-time analyses from our laboratory revealed that the migratory cells within the tips of elongating mammary ducts exchange positions dynamically; the front is in essence a cell behavior and not a lineage (Ewald et al., 2008). Surprisingly, the basal surface of the mammary tube is smooth at the light microscopy and ultrastructural level and there are no actin-based protrusions extending into the extracellular matrix (ECM) (Ewald et al., 2012). This unit of migration is distinct from other epithelial tubes, such as the Drosophila trachea (Ghabrial and Krasnow, 2006) or zebrafish lateral line (Valentin et al., 2007), both of which exhibit highly proliferative leader cells (Lubarsky and Krasnow, 2003; Lecaudrey and Gilmour, 2006). However, live-cell imaging of the mouse salivary gland and kidney revealed a smooth elongation front similar to the mammary epithelium, supporting the general relevance of this organizational state (Larsen et al., 2006; Watanabe and Costantini, 2004; Chi et al., 2009). These studies collectively raise the question: how do epithelial tubes elongate when they lack commonly observed features of cell migration (Lauffenburger and Horwitz, 1996)?

In this study, we use fluorescent reporter mice, 3D time-lapse confocal microscopy, and quantitative image analysis to elucidate the cellular basis of mammary tube elongation in real time. Our data first reveal high levels of phosphorylated ERK1/2 (also known as MAPK3/1) in the most migratory cells at the tips of elongating ducts. We next observed that mammary epithelial cells generate protrusions anisotropically, and migrate collectively, in the direction of tube elongation. Conditional activation and pharmacological inhibitors enabled assessment of the role of distinct RTK signaling.
RESULTS

Cells in the tips of elongating ducts display high pERK levels

Fetal mammary development produces a rudimentary network of polarized epithelial ducts, consisting of bilayered tubes, with apically positioned luminal epithelial cells and basally positioned myoepithelial cells (Hogg et al., 1983). Morphogenesis chiefly occurs during puberty, with individual ducts elongating over distances of 2.5 cm or more in the mouse (Sternlicht, 2006; Hinck and Silberstein, 2005). While both the initial rudiment and the mature ductal network are composed of quiescent, polarized cells, elongation is accomplished by a multilayered group of proliferative, low-polarity cells located at the tip of the duct (Huebner and Ewald, 2014). We previously demonstrated that an asymmetric cell division within the polarized luminal layer initiates an RTK-dependent stratification and loss of apicobasal polarity (Ewald et al., 2012; Huebner et al., 2014). We now seek to understand how these low-polarity cells collectively accomplish ductal elongation. To study RTK-induced branching morphogenesis in real time, we utilize 3D culture (Nguyen-Ngoc et al., 2015) and 3D time-lapse confocal microscopy (Ewald, 2013). Briefly, the mammary gland is surgically removed and pieces of epithelial ducts (‘organoids’) are isolated through a combination of mechanical disruption, enzymatic digestion and differential centrifugation (Nguyen-Ngoc et al., 2015). These epithelial organoids are then embedded in ECM proteins characteristic of the basement membrane and the stromal matrix (1:1 Matrigel:collagen I) and induced with FGF2, conditions that support in vivo-like branching morphogenesis (Nguyen-Ngoc and Ewald, 2013).

FGF2 stimulates RTK signaling and activates two signaling modules; mitogen activated protein kinase (MAPK) and protein kinase B (AKT) (Fig. 1A) (Schlessinger, 2004). MAPK and AKT have both distinct and overlapping roles. MAPK signaling regulates proliferation, differentiation, apoptosis and cell migration (Dhillon et al., 2007), while AKT signaling regulates cell survival, proliferation, metabolism and cell migration (Manning and Cantley, 2007). The molecular constituents of these signaling pathways are understood. It is less clear how specific RTK modules regulate cell behaviors within tissues during development. We first determined the localization of MAPK signaling in elongating mammary ducts. Antibody staining against phosphorylated ERK1/2 (pERK), two downstream kinases in the MAPKERK1,2 signaling module, was used to mark active MAPK signaling (Dhillon et al., 2007). We observed strong enrichment of pERK staining in the final few cell layers of elongating ducts (43/51 enriched at front, four replicates; Fig. 1C). Conversely, total ERK1/2 was diffusely present in the cytosol of all cells, suggesting that the pattern of pERK reflected signaling activity, as opposed to protein levels (45 organoids, four replicates; Fig. 1D). Antibody staining against the phosphorylated form of AKT (pAKT) was used to determine the location of active AKT signaling. We observed pAKT in the cytosol of all cells of elongating ducts (0/10 enriched at front, three replicates; Fig. 1B). Our observation of spatially enriched MAPK signaling during elongation is consistent with elevated levels of ERK signaling in the tips of the Wolffian duct (Hoshi et al., 2012), lung (Liu et al., 2004) and the mammary terminal end bud (TEB) in vivo (Lutete et al., 1999).

Elongation correlates with an increase in cell motility

We next hypothesized that if cell migration were a major driver of ductal elongation then we should see increases in speed and persistence during this phase of branching morphogenesis. To test this hypothesis, we imaged organoids derived from transgenic mice with nuclear (H2B-GFP, green) (Hadjantonakis and Papaioannou, 2004) and membrane (tdTomato, red) (Muzumdar et al., 2007) labels. This combination of markers enabled us to track individual cell movements quantitatively using time-lapse confocal microscopy. We examined organoids cultured without growth factor (no GF) and in organoids cultured with FGF2 at several stages: before (day 2), during (day 4) or after (day 7) elongation (Fig. 1E-G). This approach allowed direct comparison of migration at each stage of morphogenesis. Nuclei were tracked for 16 h and 20 min, with frames every 10 min.

We utilized the nuclei trajectories to compare mean cell speed and cell persistence. Mean cell speed was calculated as the total track length divided by the time of tracking. Persistence measures the tendency of a cell to continue migrating in the same direction and was calculated as track displacement divided by total track length. Cells were minimally motile without FGF2 (Fig. 1H,I), whereas FGF2 induces a modest increase in cell motility even prior to ductal initiation (Fig. 1E,E′). Cell motility was highest during active elongation (Fig. 1F,F′) and decreased as ducts stopped elongating (Fig. 1G,G′). Mean cell speed was significantly increased during active elongation (day 4) compared with the other conditions (P<0.0001, no GF, day 2 or 7; Fig. 1H). There was also a significant increase in cellular persistence during active elongation (P<0.0001, Fig. 1I). We conclude that FGF2 increases cell motility and that there is a burst in speed and persistence during elongation.

Cells within elongating ducts display a rear-to-front increasing gradient of cell speed

Cells within elongating branches (day 4) displayed highly variable speeds (Fig. 1H,I). We therefore asked if differences in speed are related to the location of a cell within the epithelium. We tracked cells in an elongating branch and measured the distance from that cell to the leading edge (Fig. 1K). Cells were tracked for 16 h and 20 min, with a 10-min time interval between frames. We then compared the average distance from the leading edge and the mean cell speed (Fig. 1L). A gradient of speeds was observed, with the fastest cells located near the front (Spearman r=0.77 and P<0.0001). Similar regional differences in cell speed have been described in the mouse salivary gland, suggesting a conserved mechanism for epithelial tube elongation (Hsu et al., 2013). By contrast, despite variations within individual branches, persistence displayed no consistent correlation with distance from the front (Spearman r=−0.11 and a non-significant P-value) (Fig. 1M). Our data reveal that persistence increases globally during elongation but displays no spatial pattern, whereas speed increases both globally during elongation and in the cells within the high-pERK elongation front.

Cells exchange into and out of the high motility elongation front

We previously demonstrated that cells change relative positions during elongation (Ewald et al., 2008). Our identification of a high-pERK, highly motile cell compartment at the elongation front led us to test the extent to which cells exchange into and out
of this region. Cells were classified as at the elongation front if they were in the luminal cell layer closest to the ECM, at the tip of the branch. We tracked cells in 3D over time and counted the frequency of cells entering into or exiting from this position. We rarely observed branch cells that migrated from the interior into the elongation front (4/47, Fig. S1A,A',C). Movement of cells from the elongation front to the interior of the branch was also infrequent (5/51, Fig. S1B,B',C). Therefore, although cells can exchange between front and interior positions, most cells remained within their original compartment during the period of observation.

Cell protrusions are anisotropically oriented in the direction of branch elongation

Migratory mammary epithelial cells form protrusions between other cells within the epithelium but do not extend protrusions into the ECM (Ewald et al., 2008, 2012). We hypothesized that if elongation is fundamentally a cell migration process then the cells should orient their

Fig. 1. Elongating organoids have a gradient of cellular speeds. (A) Summary of receptor tyrosine kinase (RTK) signaling. (B-D) Confocal sections of organoid branches, all expressing membrane-targeted tdTomato (red) and stained for nuclei (DAPI, blue). Antibody staining for (B) pAKT (ten organoids, three replicates), (C) pERK (51 organoids, four replicates) and (D) total ERK (45 organoids, four replicates) is shown in green. (E,F,G,J) Maximum intensity projections from 3D confocal movies of organoids expressing H2B-GFP (green) and membrane-targeted tdTomato (red). A minimum of 32 nuclei from three replicates were tracked for each condition. (E,F,G,J) Nuclei trajectories are shown from their respective movies. Cells were tracked for 16 h 20 min (100 frames), with purple and red indicating the beginning and end of the movie, respectively. Track tails represent the previous 4 h and 20 min. (E,G,J) Nuclei were tracked before branch initiation (day 2, E,E'), during active branch elongation (day 4, F,F') and after completion of elongation (day 7, G,G'). (H) Mean cell speeds were calculated from nuclei trajectories as track length divided by time. (I) Persistence was calculated from nuclei trajectories as displacement divided by total track length. (J,J') Nuclei were tracked during active branch elongation, 4 days after addition of FGF2. (K) Schematic of organoid branch, illustrating the distance from the cell (green) to the front of the branch. (L,M) Mean cell speed (L) and persistence (M) plotted as a function of distance from the front. A minimum of 34 cells from four replicates were tracked for each condition. Comparisons of speed and persistence between conditions were made using a single-factor analysis of variance (ANOVA, ****P<0.0001), while correlation determinations were calculated using Spearman's test for comparing two independent variables. ns, not significant.
protrusions in the direction of branch elongation. An alternative possibility was that protrusions could be oriented perpendicular to the direction of elongation, as observed during convergent extension (Keller, 2002). To distinguish between these possibilities, we lowered the Adeno-GFP titer to mosaically label individual cells and collected 3D confocal movies during elongation.

We quantified the orientation of protrusions by placing a transparent circular chart over a labeled cell, with 0° defined as the direction of branch elongation. We then counted the number of cellular protrusions within each of eight equally divided bins (Fig. 2A). Protrusions within the 0-45° or 270-315° bin were in the direction of branch elongation, whereas protrusions within the 180-135° and 180-225° bins were opposing the direction of branch elongation. Our comparison was between cells within the main organoid body or within an actively elongating branch (Fig. 2A).

Cells within the organoid body were highly motile and protrusive (Fig. 2B,B') but the orientation of protrusions in these body cells was isotropic (Fig. 2C). Cells within elongating branches were also highly motile and protrusive (Fig. 2D,D') but the orientation of their protrusions was highly anisotropic in the direction of branch elongation (Fig. 2D,D',E). We conclude that branch cells coordinate both the orientation of their protrusive activity and the speed of their migration.

**Rac1 signaling is required for cell motility and branch elongation**

The Rho family of small GTPases has well-defined roles in cell migration (Ridley, 2003). Specifically, Rac1 is required for the establishment of front-rear polarity and in generating actin-based protrusions at the leading edge of migrating cells (Nobes and Hall, 1995). Given the extensive, coordinated protrusions that we observed during branch elongation, we hypothesized that Rac1 is required for mammary cell motility and branch elongation. We tested this hypothesis by analyzing nuclei trajectories in elongating branches in the presence of the commercially available Rac1 inhibitor InSolution (Calbiochem, iRac1).

To characterize baseline cell migration, time-lapse movies of nuclear GFP were collected for 4.5 h during a period of active elongation (Fig. S2A,A'). Following this baseline imaging, organoids were treated with Rac1 inhibitor and imaged for 16 h and 20 min (Fig. S2B,B'). Nuclei trajectories and cell speeds were collected and calculated from the same region of each organoid. Rac1 inhibition with FGF2 decreased mean cell speed to a level indistinguishable from that without growth factor (Fig. S2C versus Fig. 1H).

We conclude that Rac1 signaling is acutely required for cell motility in the mammary epithelium, but it remained unclear whether Rac1-mediated cell migration is required for branch elongation. To test the requirement for Rac1 signaling during branching morphogenesis, we treated organoids with either Rac1 inhibitor or vehicle control at day 1 of culture. Consistent with previous work, branch initiation was completely blocked by continuous Rac1 inhibition (Ewald et al., 2008). We next tested the requirement for Rac1 signaling during elongation by adding Rac1 inhibitor or vehicle control at day 4 of culture (Fig. S2D,E). Vehicle-treated control organoids continued elongating (Fig. S2D,F). By contrast, organoids treated with Rac1 inhibitor stopped elongating and the mammary branches partially to completely collapsed into the organoid (Fig. S2E,F). We conclude that Rac1-dependent cell migration is required for branch elongation.

**Exogenous Rac1 signaling is not sufficient to induce mammary branch elongation**

Rac activation has been shown to be sufficient to orient the collective migration of *Drosophila* border cells (Wang et al., 2010).
Accordingly, we investigated whether Rac1 activation is sufficient to induce the initiation and elongation of mammary tubes. We utilized a transgenic mouse that enabled Cre-regulated expression of an active mutant of Rac1 (RacV12) and of EGFP (Srinivasan et al., 2009) (Fig. S2G). We varied the titer of an adenovirus expressing Cre to modulate the fraction of labeled, RacV12-expressing cells (Fig. S2H). We examined the effect of RacV12 in organoids cultured without growth factors as a strict test of its sufficiency to induce tube formation. Cre was delivered at the onset of culture, with ~80% of cells transduced based on GFP expression. We then compared the growth of control tissue with FGF2 and the growth of RacV12-expressing tissue without growth factor. FGF2-treated control organoids underwent normal branching morphogenesis (Fig. S2I). By contrast, organoids expressing RacV12 did not branch or increase in size (Fig. S2J). We conclude that the experimental induction of Rac1 activity is not sufficient to drive mammary branching morphogenesis.

**Proliferation is not required during ductal elongation**

MAPK regulates cell proliferation (Seger and Krebs, 1995) and it is possible that proliferation was restricted to the high-pERK compartment. To test this hypothesis, we collected 3D time-lapse movies of organoids with a genetically encoded chromosomal marker (H2B-GFP; Hadjantonakis and Papaioannou, 2004) that allowed us to visualize mitosis in real time. Widespread proliferation was observed in elongating branches but it was not confined to the elongation front, suggesting that it was not strictly correlated with the current level of pERK in a cell. Furthermore, branches could initiate and elongate in the absence of local proliferation (n=4 organoids; Fig. 3A). From these data, we conclude that proliferation is neither specific to the region of elongation nor necessarily observed during the process of elongation.

We next tested the requirement for proliferation during ductal elongation using aphidicolin, a DNA polymerase α inhibitor that blocks entry into mitosis (Ikegami et al., 1978). We examined four conditions: vehicle or aphidicolin treatment at the start of culture or at day 4 of culture, during active elongation. Organoids branched vigorously in response to FGF2 regardless of the early or late addition of vehicle. Specifically, all organoids treated at day 4 continued to elongate (37 of 37 movies, three replicates; Fig. 3B). Consistent with our previous data (Ewald et al., 2008), inhibition of proliferation early in culture resulted in a complete block of branching morphogenesis, with organoids arresting as small cysts (Fig. 3C). Surprisingly, treatment with aphidicolin at day 4 was not sufficient to block branching morphogenesis; treated samples continued to elongate for up to 2 days after treatment (60 of 60 movies, three replicates; Fig. 3D). We verified that aphidicolin was inhibiting proliferation in our system by treating branching organoids with aphidicolin for 24 h and then staining for the mitosis marker phospho-histone H3 (pH-H3). Control organoids had an average of 4.3 cell divisions per organoid (22 organoids, three replicates; Fig. 3E), whereas aphidicolin-treated organoids had no pH-H3+ cells (22 organoids, three replicates; Fig. 3F). We conclude that elongation can continue in the absence of proliferation through the migration of existing cells (Fig. 3G). We expect that proliferation and migration normally occur concurrently, with
proliferation serving as a source of new migratory cells and migration as the mechanism of elongating the tube.

**MAPK signaling is required for cell motility and branch elongation**

Since the most migratory cells at the tips of elongating branches had high pERK staining, we hypothesized that MAPK signaling would be required both for epithelial cell migration and branch elongation. We utilized a well-studied, small-molecule MEK inhibitor (U0126, iMAPK) to test this hypothesis, in an analogous fashion to our Rac1 inhibitor experiments above. We first collected time-lapse movies of nuclear GFP for 4.5 h during active branch elongation (Fig. 4A,A') and then treated with U0126 and imaged for 16 h and 20 min (Fig. 4B,B'). Nuclei trajectories and cell speeds were collected and calculated from the same region of each organoid. Our analysis revealed that MAPK inhibition results in a significant decrease in mean cell speed, to a level indistinguishable from that in the absence of growth factor (Fig. 4C versus Fig. 1H).

We next treated organoids with U0126 at either day 1 or day 4 of culture and collected time-lapse movies of tissue growth and branching morphogenesis for both control and treated organoids. Control organoids branched as expected (Fig. 4D), whereas addition of U0126 at day 1 completely blocked branching morphogenesis, consistent with prior reports (Fata et al., 2007; Huebner et al., 2014). Strikingly, addition of U0126 during active branching at day 4 resulted in an acute arrest in elongation and partial to complete regression of existing branches back into the body of the organoid (Fig. 4E). Only 2% of organoids continued to elongate upon inhibition (E). The dashed line marks presence of vehicle (D) or MEK/ERK inhibitor (E). The area of eight organoids from four replicates were quantified. (D,E) Time-lapse movies in the presence of vehicle (D) or MEK/ERK inhibitor (E). The dashed line marks organoid area at the time of vehicle or inhibitor addition.

**Mosaic MAPK activity is sufficient to induce mammary branch morphogenesis**

Since ERK signaling was highest in the most migratory cells and MAPK signaling was required for both cell migration and branch elongation, we hypothesized that experimental activation of MAPK would be sufficient to induce the initiation and elongation of mammary branches. To test this hypothesis, we utilized a transgenic mouse line that allowed Cre-regulated conditional expression of MEK1DD, a phosphomimetic, constitutively active mutant of MEK1 (MAP2K1) (Fig. 5A) (Srinivasan et al., 2009) and of EGFP. We then used adenovirally delivered Cre to conditionally and mosaically express MEK1DD in organoids isolated from this transgenic line (Fig. 5B).

We cultured control organoids in the presence or absence of FGF2 and cultured MEK1DD-expressing organoids in the absence of growth factor. We observed no branching in control organoids without growth factor (Fig. 5C) and we observed branching in nearly all control organoids treated with FGF2 (0%, 156 organoids versus 90%, 173 organoids, three replicates). Strikingly, experimental expression of MEK1DD was sufficient to induce initiation and elongation of mammary branches even in the absence of growth factor stimulation (30%, 204 organoids, three replicates; Fig. 5D). Interestingly, although most MEK1DD-expressing organoids had either 0 or 1 branches, we occasionally observed vigorous branching similar to that of FGF2-treated control organoids (Fig. 5E,F). MEK1DD-induced branches displayed apically enriched F-actin (Fig. 5G) and Par3 (Pard3) (Fig. 5H) and basally located smooth muscle actin+ myoepithelial cells (Fig. 5I). We conclude that MEK1DD expression is sufficient to induce the formation of polarized ducts.

We next sought to determine the location of MEK1DD-expressing cells within these new branches, using the co-expressed EGFP as a reporter. We observed that MEK1DD-induced branches were elongated by distinct clusters of EGFP-expressing cells (Fig. 5J). Finally, we tested the requirement for proliferation in MEK1DD-induced branching, by treating with aphidicolin early in culture and quantifying branch formation (Fig. 5P). Vehicle-treated control organoids were dependent on FGF2 for branching and sensitive to aphidicolin treatment, as above (Fig. 5K-M,P). Consistent with our model, MEK1DD organoids were also sensitive to early aphidicolin treatment and could not induce new branches in the absence of proliferation (Fig. 5N-P). These data reveal that MAPK signaling is necessary and sufficient to induce mammary branch elongation by creating migratory cells in

---

**Fig. 4. MAPK signaling is required for cell motility and branch elongation.** (A,A') 3D reconstruction of the first time point (A) and nuclei trajectories for the 4 h and 30 min prior to MEK/ERK inhibition (A'). (B) 3D reconstruction of the first time point (B) and nuclei trajectories for 4 h and 30 min, starting 12 h after MEK/ERK inhibition (B'). (C) Mean cell speeds were quantified before and after inhibition of ERK signaling. Cell speed was significantly higher before MEK/ERK inhibition (Mann–Whitney test, ***P<0.0001); a total of 64 cells from eight organoids from four replicates were quantified. (D,E) Time-lapse movies in the presence of vehicle (D) or MEK/ERK inhibitor (E). The dashed line marks organoid area at the time of vehicle or inhibitor addition.
a proliferation-dependent manner. Additionally, both wild-type and MEK1DD-induced branches are led by clusters of migratory cells with high levels of MAPK signaling.

**DISCUSSION**

Our goal in this study was to establish a cellular and molecular framework for the process of branch initiation and elongation in the mouse mammary gland. We first showed that the tips of elongating ducts have increased MAPK signaling, as reported by pERK levels. This staining correlated with a gradient of cellular speeds, with the fastest cells located within the high-pERK compartment at the elongation front. We did observe occasional migration of cells into and out of the elongation front but most cells remained in their respective compartments. Importantly, although proliferation was required to initiate both FGF2- and MEK1DD-induced branching, proliferation was not acutely required during elongation. We next showed that cells within elongating branches exhibited a striking alignment of their protrusions in the direction of branch elongation. This coordination of protrusions and migration led us to demonstrate that Rac1 activity is required for both cell migration and branch elongation. However, experimental activation of Rac1 was not sufficient to induce branching. MAPK signaling was also required for collective cell migration and branch elongation and, strikingly, mosaic expression of MEK1DD was sufficient to induce the initiation and elongation of polarized ducts in the absence of exogenous growth factors.

The cell behavioral basis of tube elongation

From first principles, it is possible to conceptualize the process of tube elongation operating through essentially any combination of proliferation, migration, and cell shape change (Andrew and Ewald, 2010). Two well-studied examples are the *Drosophila* trachea and salivary gland. In the *Drosophila* trachea, there is no proliferation during branching morphogenesis and most of the elongation and ramifications of the tubular network is accomplished by cell shape change (Lubarsky and Krasnow, 2003; Kerman and Andrew, 2010). In the *Drosophila* salivary gland there is a period of proliferation during the establishment and specification of the salivary gland (Kerman and Andrew, 2010). The elongation of the tube is then accomplished by a combination of cell migration and cell shape change, with no proliferation observed during tube elongation (Andrew and Ewald, 2010; Kerman and Andrew, 2010). The fact that proliferation does not occur during elongation in the *Drosophila* trachea or salivary gland has enabled exquisite genetic and time-lapse analyses of the cellular movements driving the development of these organs without the complication of increasing cell number (Ribeiro et al., 2002; Kato et al., 2004; Cheshire et al., 2008; Affolter et al., 2009; Lebreton and Casanova, 2014). This
proliferation followed by migration mechanism of tube elongation has also been observed during zebrafish kidney morphogenesis and so is utilized in vertebrates as well (Vasilyev et al., 2009). However, mammalian tubular organs exhibit a profound proliferation-driven increase in size during development and so it had appeared that the principles uncovered in model systems might not be sufficient to explain mammalian branching morphogenesis. In particular, mammary epithelial tubes elongate over centimeters in mice and much longer distances in large mammals and their elongation is clearly accompanied by extensive proliferation. Furthermore, genetic manipulations that reduce the rate of proliferation in the TEB slow the rate of tube elongation (McNally and Martin, 2011). Proliferation has been shown to play different roles during mammalian tubulogenesis, including the regulation of lung tube shape (Tang et al., 2011), driving the dispersal of cells in the ureteric bud (Packard et al., 2013), and inducing stratification of the mammary epithelium (Huebner et al., 2014). It remained unclear how exactly proliferation was contributing to mammary tube elongation.

It was reasonable to hypothesize that mammary branches elongate predominantly through localized proliferation. We excluded this possibility by observing spontaneous branch initiation in the absence of proliferation and by observing continued branch elongation following aphidicolin treatment. Instead, we demonstrated that mammary tube elongation is fundamentally accomplished by cell migration. We speculate that proliferation is utilized as a mechanism to produce migratory cells with lower apicobasal polarity and fewer intercellular junctions (Ewald et al., 2012; Huebner et al., 2014). Inhibiting proliferation limits the further generation of these migratory cells and therefore sets a limit on additional tube elongation, but does not prevent existing cells from elongating the tissue. Seen from this perspective, the model systems and mammalian organs differ in their timing of proliferation but share common mechanisms of cell migration and cell shape change as fundamental drivers of the elongation process.

Region-specific cell migration within the epithelium

A surprising feature of mammary branching morphogenesis is that the epithelial cells are highly protrusive and motile within the epithelium but do not protrude into the ECM (Ewald et al., 2008, 2012). Individualistic cell motility has also been observed in the mouse salivary gland (Larsen et al., 2006) and the mouse ureteric bud (Chi et al., 2009). In the present study, we demonstrated that mammary epithelial cells within active branches are selectively protrusive in the direction of elongation, which suggested that coordinated cell migration is both required and potentially sufficient for branch formation. Consistent with this conceptual model, we identified a gradient of cell speeds running from slow in the body of the organoid to fast in the high-pERK tips of elongating ducts. High levels of cell motility in the elongation front have also been observed in the mouse salivary gland (Hsu et al., 2013). We next demonstrated that both cell migration and branch elongation require Rac1 and MAPKs signaling. Strikingly, treatment with inhibitors of either pathway during duct elongation led to regression of existing mammary branches. Integrating our new data with published observations, we propose that RTK signaling induces the proliferation-dependent formation of highly motile epithelial cell clusters that elongate the epithelial tube but are unable to invade into the ECM, potentially owing to the very limited ECM-binding ability of luminal epithelial cells (Cerchiari et al., 2015). Importantly, during mammary migration, cells orient their protrusions and migration in the direction of tissue elongation, in contrast to convergent extension, in which protrusions and migrations are perpendicular to tissue elongation (Keller, 2002).

Localized MAPK signaling is sufficient to induce branch initiation and elongation

MAPK signaling is required for branching morphogenesis in the mouse submandibular gland (Kashimata et al., 2000), mouse kidney (Fisher et al., 2001), rat lung (Kling et al., 2002) and mouse mammary gland (Fata et al., 2007). In each of these tissues, general inhibition of MAPK signaling results in a decrease in tissue size, branch number and proliferation. These previous studies demonstrated that MAPK regulates proliferation during tube development and that proliferation is required for mammalian branching morphogenesis. Using time-lapse imaging and MAPK inhibition during active branching, we have shown here that MAPK signaling regulates cell motility during branch elongation at a time when proliferation is dispensable. Our conclusion is consistent with results from Madin-Darby canine kidney (MDCK) acini (O’Brien et al., 2004) and MCF-10A mammary acini (Pearson and Hunter, 2007), both of which experience increased cell motility in response to MAPK signaling. The relationship between cell migration, MAPK signaling and tubulogenesis appears to be deeply conserved as branchless is a master regulator of cell migration during Drosophila trachea formation and encodes a FGF ligand (Sutherland et al., 1996).

The key conceptual advances embodied in our paper are the dual demonstration that normal mammary elongation is led by clusters of motile, protrusive cells with high pERK levels, and that expression of MEK1DD is sufficient to induce the initiation and elongation of apicobasally polarized ducts through the generation of these migratory cell clusters. In particular, the sufficiency of MEK1DD to induce branch initiation and elongation is highly surprising because RTK signaling activates multiple pathways other than MEK, and genetics has implicated members of the EGFR, FGFR and MET receptor pathways in mammary branching morphogenesis (McNally and Martin, 2011). Our data lead us to speculate that MAPK is the fundamental signaling node for mammary tube elongation and to suggest that spatial control of pERK represents the key to generating directional growth despite a uniform concentration of growth factors. This perspective is consistent with the frequent activation of MAPK signaling in cancer (Whyte et al., 2009) and suggests that high MAPK activity might be sufficient to initiate collective cell invasion during metastasis. It will be crucial for future studies to elucidate the spatiotemporal dynamics of signaling in real time and to elucidate mechanisms of localized restriction of pERK.

MATERIALS AND METHODS

Transgenic animals

A transgenic mouse line expressing tdTomato was used to label cell membranes (Muzumdar et al., 2007) (Jackson Laboratory, #007676). A transgenic mouse line expressing H2B-GFP was used to label nuclei and was a kind gift from A. K. Hadjantonakis, Memorial Sloan Kettering (Hadjantonakis and Papaioannou, 2004). Transgenic mouse lines enabling conditional expression of RacG12V (RacV12) and MEK1DD were used (Srinivasan et al., 2009) (Jackson Laboratory, #012361 and #012352, respectively). Animal experiments were conducted in accordance with protocols approved by the JHU Medicine Institutional Animal Care and Use Committee.

Organotypic culture

The organotypic culture methods used here are described in detail by Nguyen-Ngoc et al. (2015). In brief, mammary glands were isolated from 8- to 12-week-old female mice and minced with a scalpel. The epithelium
was separated from fat and stromal tissue by collagenase-trypsin digestion, DNase treatment, and differential centrifugation. Organoids were suspended in a 1:1 mixture of growth factor-reduced Matrigel (BD Biosciences) and rat tail collagen I (Corning) and plated at 400 µl drops containing an average of one organoid/µl. The organoid suspensions were plated on 24-well glass-bottom plates (Greiner Bio One) sitting on a 37°C hotplate to promote gel polymerization, as described by Nguyen-Ngoc et al. (2015). Plates were incubated for 20 min at 37°C to ensure gelation before addition of culture medium. Where indicated, organoids were treated with 2.5 nM FGF2 (F0291, Sigma-Aldrich) to stimulate branching morphogenesis. For each set of experiments, a minimum of three independent replicates were performed, using either mammary organoids or individual cells within organoids as the unit of measurement for statistical analysis. The organoids were randomly assigned to each condition for measurement and analysis.

**Organoid antibody staining**

Our antibody staining protocols are described in detail by Nguyen-Ngoc et al. (2015). Briefly, organoid-containing ECM gels were fixed in 4% paraformaldehyde for 20 min and washed three times in PBS. Organoids were permeabilized in 0.5% Triton X-100 in PBS for 30 min. Blocking was for 3 h in 10% fetal bovine serum (FBS) in PBS. Following block, organoids were incubated with primary antibodies for 2 h in 10% FBS in PBS. Phospho-histone H3 (pH-H3, #9706), phospho-AKT (#42965), phospho-ERK (#2101) and total-ERK (#2102) antibodies were used at 1:500 (all Cell Signaling Technology). Par3 antibody was purchased from Millipore (07-330; 1:500) and smooth muscle actin antibody was purchased from Sigma (F3777; 1:500). After incubation with primary antibodies, organoids were washed three times in blocking solution. Organoids were then incubated with Alexa Fluor-conjugated secondary antibodies (Molecular Probes; 1:500), phalloidin (Invitrogen, A12380; 1:500) and DAPI (Invitrogen, #62248; 1:1000) for 1 h in blocking solution. Stained organoids were washed three times with PBS and analyzed by confocal microscopy.

**Adenoviral gene delivery**

Adeno-Cre or Adeno-GFP (Vector BioLabs) was added after organoid isolation prior to suspension in ECM. Isolated organoids were centrifuged in an Eppendorf tube at 520 g for 10 min. The supernatant was removed and organoids were resuspended in 100 µl DMEM-F12 (Gibco). Adenovirus was added at 5000-10,000 plaque-forming units per organoid to achieve gene expression in 50-80% of cells. Organoids were incubated with virus for 1 h at 37°C and then washed twice with DMEM-F12 and suspended in ECM for plating.

**Time-lapse differential interference contrast (DIC) microscopy**

DIC movies were collected on a Zeiss Cell Observer with an AxioObserver Z1 and an AxioCam MRM camera (as in Ewald, 2013). Organoids were imaged every 20 min for 5-7 days, with 100-200 movies collected in parallel. The environment was maintained at 37°C and 5% CO2 throughout imaging.

**Confocal microscopy**

Fixed samples were imaged using a Zeiss LSM 780 confocal microscope with a 40× LD-LCI C-Apochromat objective using ZEN imaging software. Time-lapse images were acquired on a spinning disk confocal microscope (Solamere Technology Group) using a 40× LD-LCI C-Apochromat lens (Ewald, 2013). The spinning disk microscope used µManager (Edelstein et al., 2010) and Piper (Stanford Photonics) to acquire images. Organoids were imaged from 4-16 h with a 10-15 min interval, with the temperature maintained at 37°C and CO2 at 5%. Brightness and contrast were adjusted across the entire image using Adobe Photoshop and Imaris (Bitplane) to maximize image clarity.

**Proliferation inhibition assay**

Organoids were cultured with FGF2 and imaged using time-lapse DIC microscopy. Aphidicolin was used at 0.625 µM (Fig. 5M,O) or 1.25 µM (Fig. 3C,D,F) and added at the start of culture or during active elongation (day 4); the lower dose because MEK1DD-expressing organoids showed greater cell death at 1.25 µM. Time-lapse movies were collected for 2 days from the time of inhibitor addition and analyzed to determine ducts that continued elongating. To verify inhibition of proliferation, organoids were treated with aphidicolin at day 4, fixed 24 h later, and stained for the proliferation marker pH-H3. No proliferation was observed in aphidicolin-treated samples.

**Nuclei tracking**

Nuclei were tracked semi-manually using the Imaris Spots function (Bitplane). Images were first smoothed using a 0.35 µm Gaussian filter, then the center of the nucleus was marked with a spot within each frame, and the spots were connected over time by the software. The Imaris OrthoSlicer function was used to enable accurate tracking in 3D. Mean cell speed was quantified as the total track length divided by time of tracking, and persistence was measured as the displacement divided by total track length. A single-factor ANOVA was used to test statistical significance. The gradient of cellular speeds in an elongating branch was identified using the Imaris Spots function by relating nuclear position to a spot identifying the tube front at every time point. The average distance from the elongation front for each nucleus was calculated and then compared with the mean cell speed and persistence for that cell. Spearman’s rank correlation coefficient was used to determine the statistical significance of distance from elongation front with mean cellular speed or persistence. Organoids were included in the analysis if they were actively elongating.

**Analysis of cellular protrusions**

Organoids were isolated and cultured from mice expressing membrane-targeted tdTomato in all cells. Cell morphology was highlighted using adenovirally delivered GFP, expressed in a mosaic subset of cells. Protrusions were analyzed based on 3D reconstructions of GFP expression. Images were captured every 15-20 min for 12-24 h and analyzed using Imaris (Bitplane). Protrusion data were collected using an eight-section pie with deviations every 45° on a transparent sheet. The 0 to 180° axis was aligned with the direction of branch elongation and the center of the pie was placed over the center of each analyzed cell. The number of protrusions per bin per cell was counted every 15-20 min for a minimum of 6 h. Protrusion data were plotted in sector charts using Mathematica (Wolfram), using the mean values for each bin and equally weighting each angle. Statistical analyses were performed using Oriana (Kovach Computing). Hotelling’s test was used to determine the significance of a weighted mean direction. The null hypothesis is that there is no mean direction. Hotelling’s paired test was used to determine the significance of the difference in the weighted mean direction between circular data. The null hypothesis is that there is no difference in the mean direction. Organoids were included in the analysis if they were actively elongating.

**Rac1 and ERK/MEK inhibition assays**

InSolution Rac1 inhibitor (Calbiochem) was used at 50 µM and the MEK/ERK inhibitor U0126 (Cell Signaling) was used at 10 µM. Organoids were cultured in FGF2 to induce branching morphogenesis. To assess inhibition of cell motility, elongating organoids expressing H2B-GFP were imaged for 4.5 h prior to inhibition and for 16 h and 20 min following inhibition. Mean speeds were quantified and then analyzed with a Student’s t-test, using a Mann-Whitney correction. To determine if Rac1 or MEK/ERK was required for branching, we collected DIC images every 20 min from 24-144 h. Inhibitors were added during active branching (day 4) and movies were analyzed to determine whether ducts continued elongating.

**Induction of activated Rac1 and MEK expression**

Active Rac1 (RacV12) and MEK1 (MEK1DD) are expressed under the Rosa26 promoter, downstream of a floxed STOP cassette and upstream of an internal ribosomal entry site and EGFP (Srinivasan et al., 2009). Adeno-Cre was used to induce mosaic expression of mutant proteins; control organoids from the same animal were treated with Adeno-GFP. Control organoids were cultured in the presence or absence of FGF2 (positive and negative controls, respectively). RacV12-expressing or MEK1DD-expressing organoids were cultured without FGF2 and assessed for branching in time-lapse movies and at day 7.


