Published in final edited form as:

J Microsc. 2013 September ; 251(3): 212–223. doi:10.1111/jmi.12017.

Mammary ductal elongation and myoepithelial migration are regulated by the composition of the extracellular matrix

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Summary

Mammary branching morphogenesis occurs over a period of weeks deep inside an adipocyte-rich stroma. The adipocytes contain light-scattering lipid droplets that limit the depth of penetration of visible light. Organotypic culture methods were developed to enable high-resolution optical monitoring of branching morphogenesis *ex vivo*. A challenge has been to identify the best culture conditions to model specific developmental events. We recently demonstrated that collagen I induces protrusive invasion in both normal and neoplastic mammary epithelium. In this study, we observed that the abundance of collagen I fibrils correlated strongly with invasive behaviour, even when the collagen I concentration was identical. We found that the extent of fibril assembly was experimentally manipulable by varying the incubation time at 4°C following pH neutralization. We next tested the capacity of collagen I fibrils to induce invasive behaviour when presented in combination with basement membrane proteins (Matrigel). We found that epithelial organoids in mixed gels of collagen I and basement membrane proteins exhibited more extensive branching morphogenesis but did not initiate protrusions into the matrix. Organoids in pure Matrigel produced many small epithelial buds that were bare of myoepithelial cells. Surprisingly, organoids in mixed gels of collagen I and Matrigel produced fewer epithelial buds, the buds elongated further, and the elongating buds remained covered by myoepithelial cells. Our mixed gels therefore provide a more physiologically accurate model of mammary branching morphogenesis. Our results also suggest that changes in the composition of the extracellular matrix could induce migration of epithelial cells past myoepithelial coverage.

Keywords

Basement membrane; collagen I; extracellular matrix; mammary branching morphogenesis

Introduction

The mammary epithelium forms as a placode in the embryo and elaborates into a small branched network of ducts during fetal development (Hogg *et al.*, 1983). This ductal rudiment persists until the beginning of puberty, when increasing levels of steroid hormones induce a rapid phase of branching morphogenesis (Sternlicht *et al.*, 2006). Mammary ducts are organized before and after puberty as a bilayered tube (Hogg *et al.*, 1983), with an apically located luminal epithelial cell layer and a basally located myoepithelial cell layer

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(Deugnier *et al.*, 2002). However, ductal elongation and bifurcation are accomplished by a specialized structure at the end of the duct known as the terminal end bud (TEB) (Williams & Daniel, 1983; Hinck & Silberstein, 2005). Epithelial organization within the TEB is distinct from that of a bilayered duct, with multiple luminal cell layers and a single, basally positioned myoepithelial cell layer. Normal mammary morphogenesis therefore involves large changes in proliferation, apico-basal polarity and tissue architecture (Mailleux *et al.*, 2007; Ewald *et al.*, 2008; Ewald *et al.*, 2012).

The critical events of mouse mammary branching morphogenesis occur over a period of weeks during postnatal development, with the ducts embedded deep inside an adipocyte-rich stroma. These adipocytes, or fat cells, contain abundant lipid droplets that scatter, and therefore limit the depth of penetration of, visible wavelengths of light. It is possible to observe stromal cell dynamics around the TEB using advanced two-photon microscopy (Ingman *et al.*, 2006), but there are no published studies reporting how epithelial cell dynamics within the TEB *in vivo* accomplish ductal elongation.

The experimental and optical inaccessibility of mammary branching morphogenesis *in vivo* has led our group and others to develop a diverse array of three dimensional (3D) culture systems to model mammary epithelial morphogenesis (Simian *et al.*, 2001; Debnath *et al.*, 2003; Nelson *et al.*, 2006; Fata *et al.*, 2007; Ewald *et al.*, 2008; Nelson *et al.*, 2008; Provenzano *et al.*, 2009; Ewald, 2010). These assays have distinct cellular inputs and technical foundations but share the goal of developing more accurate models of epithelial morphogenesis than are possible in conventional 2D cell culture. We specifically optimised our culture methods to enable continuous optical monitoring of branching morphogenesis (Fata *et al.*, 2007; Ewald *et al.*, 2008; Ewald, 2010; Ewald *et al.*, 2012).

These diverse organotypic culture assays have in common the combination of epithelial cells and 3D gels of extracellular matrix (ECM) proteins. A major challenge is to identify the appropriate culture conditions to model specific developmental events, as the composition and organization of the ECM can induce profound changes in epithelial cell behaviour (Weaver & Roskelley, 1997; Hagios *et al.*, 1998; Nelson & Bissell, 2005; Provenzano *et al.*, 2008). We recently demonstrated that collagen I induces protrusive and disseminative behaviours in both normal and neoplastic mammary epithelium (Nguyen-Ngoc *et al.*, 2012). *In vivo* analysis of the TEB at different stages of branching morphogenesis revealed no epithelial protrusions or dissemination into the surrounding stromal ECM (Williams & Daniel, 1983; Mailleux *et al.*, 2007; Ewald *et al.*, 2008; Ewald *et al.*, 2012). Therefore, 3D culture in pure collagen I gels can transiently induce epithelial cell behaviours that are not observed during normal development *in vivo*. Our published imaging analyses of the cell dynamics underlying mammary branching morphogenesis instead relied on culture of mammary epithelium in Matrigel, a commercial matrix composed chiefly of laminin I, collagen IV, and entactin (Kleinman & Martin, 2005). Careful comparison of the epithelium in 3D culture and *in vivo* revealed a close correspondence in epithelial organization, especially in the proliferation, polarity, and cell-cell interactions within the multilayered luminal compartment (Ewald *et al.*, 2008; Ewald *et al.*, 2012). However, elongating epithelial buds in Matrigel frequently migrated free of myoepithelial coverage, a rare event *in vivo* (Ewald *et al.*, 2008).

In this study, we varied the composition of the ECM in organotypic cultures to answer two questions. First, we sought to identify features of collagen I gels that correlated with invasive behaviour. Second, we sought to test the capacity of collagen I to induce protrusions and dissemination in the presence of competing signals from basement membrane proteins.

Results

The supramolecular organization of collagen I correlated with the extent of epithelial invasion

Collagen I is a fibrillar collagen and has multiple hierarchical states of organization (Prockop & Kivirikko, 1995). Collagen I polymers can assemble into fibrils, and these fibrils can assemble into larger fibers. The organization and orientation of collagen I fibers correlate with the extent of breast cancer invasion and patient prognosis (Provenzano *et al.*, 2006; Wolf *et al.*, 2009; Conklin *et al.*, 2011). Native type I collagen is commercially available as an acid-solubilised solution purified from rat tails. The solution needs to be adjusted to a neutral pH for gellation to occur. We previously reported that direct contact between mammary epithelial cells and pure collagen I gels induced protrusive and disseminative behaviours (Nguyen-Ngoc *et al.*, 2012). However, we observed variation in the extent of invasion of epithelial cells into collagen I gels prepared according to different protocols. We hypothesized that the time interval between neutralization at 4°C and gellation at 37°C could influence fibril assembly and epithelial cell behaviour.

To test this hypothesis, we varied the time at 4°C between neutralization and gellation, which we termed as 'preincubation', in samples from the same batch of 3 mg mL⁻¹ collagen I (Fig. 1A). We then used second-harmonic generation (SHG) microscopy to visualize the supramolecular organization of collagen I in the resulting gels. With a brief preincubation period of 5 min at 4°C, we observed no detectable collagen fibrils by SHG (Fig. 1B). Small, sparse collagen fibrils became evident with a 30 min preincubation period (Fig. 1C), and the density of fibrils rapidly increased with a preincubation time of 1–2 h (Fig. 1D,E). The timing of fibril assembly in our study is similar to that observed in previous work analysing glioma invasion (Yang *et al.*, 2010).

We next tested whether these large differences in the size and abundance of collagen I fibrils affected epithelial cell behaviours. We compared the response of epithelial organoids derived from the same mouse to gels prepared from the same batch of neutralized 3 mg mL⁻¹ collagen I (Fig. 1A). We only varied the preincubation time of the collagen I at 4°C following pH neutralization. At the end of the preincubation interval, epithelial organoids were added to the collagen I solution and the mixture was allowed to gel at 37°C. We observed protrusive organoids in gels corresponding to all preincubation intervals tested. However, the frequency of subcellular protrusions into the ECM and the extent of epithelial growth correlated strongly with the preincubation time and the density of collagen fibrils (Fig. 1B'–E'). With a brief preincubation at 4°C, epithelial cells initiated fewer ECM-directed protrusions, and the extent of epithelial growth was reduced (Fig. 1B'). With longer preincubation times at 4°C (1–2 h), the number of ECM-directed protrusions (red arrows, Fig. 1) and the extent of epithelial growth increased (Figs. 1C'–E' and 2I).

Our data indicate that the supramolecular organization of collagen I regulates the protrusive and migratory response of epithelial cells, even at equal protein concentrations. Our data are consistent with past experiments comparing collagen I fibril organization following gellation at different temperatures (Raub *et al.*, 2007).

The extent of ECM-directed protrusions and epithelial migration were both independent of collagen I concentration

The protein concentration of collagen I can affect its supramolecular organization and the mechanical properties of the resulting gel (Provenzano *et al.*, 2009). Changes in collagen I concentration can influence mammary epithelial cell behaviour considerably in some assays (Provenzano *et al.*, 2008; Provenzano *et al.*, 2009). As such, we used SHG to compare

collagen I gels with the same preincubation time but gelled at different protein concentrations. We tested the effect of collagen I concentration via two strategies. First, we preincubated 3 mg mL⁻¹ collagen I for 1 h, then diluted aliquots to 2 and 1 mg mL⁻¹ and gelled each at 37°C (Fig. 2A). We observed a broadly similar organization of collagen I fibrils by SHG in each concentration (Fig. 2B–D), suggesting that much of the fibril assembly occurred during preincubation at 4°C. We next asked whether the protein concentration of collagen I during gelation influenced epithelial invasion. We cultured mammary organoids from the same mouse in collagen I gels preincubated at 3 mg mL⁻¹ for 1 h and adjusted to 2 mg mL⁻¹ or 1 mg mL⁻¹ before combining with organoids. The morphology (Fig. 2B'–D') and frequency (Fig. 2J) of protrusive invasion were similar at all tested concentrations.

We next tested whether the concentration of collagen I during preincubation had a significant impact on either the organization of collagen I as measured by SHG or on the epithelial response to the resulting gel. We neutralized the collagen I and adjusted the concentration to 1, 2, or 3 mg mL⁻¹, preincubated all samples for 1 h, and then created both empty gels for SHG and organoid-containing gels. We observed a broadly similar supramolecular organization of collagen I by SHG at each concentration when preincubated for 1 h at 4°C (Fig. 2F–H), though there appeared to be an increased density of fibrils at 3 mg mL⁻¹. However, the epithelial response to the collagen I was indistinguishable across the different concentrations tested (Fig. 2F'–H',K). We cannot exclude the possibility that the different concentrations would generate very different supramolecular organizations of collagen I with a different preincubation interval.

In summary, the duration of preincubation at 4°C had a stronger effect on epithelial behaviour than the protein concentration of the collagen I (Fig. 2I vs. Fig. 2J,K). With preincubation times longer than 1 h, the resulting gels were more opaque, limiting their usefulness for live imaging. We therefore chose a 1 h preincubation and a 3 mg mL⁻¹ concentration for the remaining experiments and termed this preparation 'preassembled collagen I'.

Mixed gels of collagen I and Matrigel did not induce protrusive invasion

We next sought to determine whether collagen I would still induce protrusive invasion when presented in the context of basement membrane proteins, such as laminin I and collagen IV. These proteins are in high concentration in the commercial ECM Matrigel. We first tested whether the organization of the collagen I fibrils would be maintained after we mixed the preassembled collagen I solution with Matrigel (Fig. 3A). SHG imaging revealed that collagen I fibrils were maintained in the mixed gels and that we could vary their abundance by varying the fractions of Matrigel and preassembled collagen I (Fig. 3B–F). The fact that collagen I fibrils were still detectable even when gelled in the presence of Matrigel was consistent with their preassembly in the preincubation interval. In the remaining experiments, we describe mixed gels based on the ratio of Matrigel and preassembled collagen I, for example, 3 parts Matrigel to 7 parts collagen I is referred to as 3M7C.

We next used these mixed gels to compare the behaviour of mammary organoids isolated from the same mouse and embedded in gels in which the only difference was the relative proportion of Matrigel and preassembled collagen I (Fig. 3G). Organoids in pure gels of collagen I (3 mg mL⁻¹) were highly protrusive (246/246 buds; Fig. 3L,L',M). In contrast, we observed essentially no protrusions into the matrix during epithelial bud elongation in mixed gels containing Matrigel (Fig. 3H–K and M). Epithelial buds in Matrigel and in mixed gels typically exhibited a smooth, nonprotrusive surface during collective migration (Fig. 3H'–K'), while epithelial buds in pure collagen I formed protrusions into the matrix (red arrows, Fig. 3L').

The extent of epithelial elongation correlated with collagen I density in mixed gels

Surprisingly, we observed that the pattern of branching morphogenesis and the extent of epithelial elongation depended strongly on the fraction of collagen I in the gel (Fig. 3H–L). The average length of epithelial buds increased from 58 μm in Matrigel to 104 μm and 95 μm in 5M5C and 3M7C (Fig. 3O). Similarly, only 8% of organoids in Matrigel had at least one bud longer than 100 μm (Fig. 3H',N), while we observed elongated buds in 63% of 7M3C organoids and 83% of 5M5C and 3M7C organoids (Fig. 3I'–K',N). These data suggested that the percentage of preassembled collagen I in the mixed ECMs required to induce extensive epithelial elongation was between 50% and 70%.

Mammary organoids undergo branching morphogenesis in Matrigel through the initiation and elongation of new epithelial buds (Ewald *et al.*, 2008). We next used time-lapse differential interference contrast (DIC) microscopy to compare branching morphogenesis in the mixed ECM gels (Fig. 4). We did not observe epithelial protrusions into the ECM during initiation or elongation in any of the mixed gels (three independent experiments, minimum of 30 movies per condition, Figs. 3M, 4A–C'). We observed a strong correlation between the persistence of elongation and the fraction of collagen I in the gel. In particular, organoids embedded within 3M7C gels typically displayed very extensive epithelial elongation (Fig. 4C,C').

The extent of myoepithelial coverage during epithelial elongation correlated with collagen I density

Mammary ducts *in vivo* are elongated by the TEB, a specialized region of mammary epithelium with multiple luminal cell layers enclosed by an essentially complete single layer of cells expressing myoepithelial markers (Williams & Daniel, 1983; Hinck & Silberstein, 2005). Organoids in Matrigel initiate many small epithelial buds, and these buds often elongate free of myoepithelial coverage (Fig. 5A and Ewald *et al.*, 2008). Since the extent of epithelial elongation varied in the mixed gels, we next asked whether the extent of myoepithelial coverage varied as well. We monitored the relative position of cell types in elongated buds using antibodies raised against the luminal epithelial cell marker keratin-8 and the myoepithelial cell markers keratin-14 and smooth muscle actin (SMA). We found that elongated buds in Matrigel, 7M3C and 5M5C gels were typically free of myoepithelial cells (Fig. 5A–C), while elongating buds in 3M7C and pure collagen I remained covered by a complete layer of myoepithelial cells (Fig. 5D–E). The fraction of myoepithelium-covered epithelial buds consistently correlated with the fraction of collagen I using three tested myoepithelial cell markers (Fig. 5F–H).

We next used time-lapse confocal microscopy to distinguish whether myoepithelial coverage was maintained during epithelial elongation or restored afterward. We utilized dual fluorescent transgenic reporter mice in which all cell membranes were labelled with a red fluorescent protein (mT/mG, Muzumdar *et al.*, 2007) and myoepithelial cells were labelled with a green fluorescent protein under the control of the myoepithelial-specific keratin-14 promoter (Vaezi *et al.*, 2002). We found that, similar to Matrigel, new epithelial buds in 7M3C and 5M5C gels initiated and elongated free of myoepithelial cells. However, myoepithelial cells occasionally caught up and restored full coverage by the end of imaging (Fig. 6A–C). Importantly, complete myoepithelial coverage was typically maintained during initiation and elongation in the majority of organoids in 3M7C gels (Fig. 6D). These data suggest that either the composition or supramolecular organization of the ECM regulates myoepithelial coverage of luminal epithelial cells.

Discussion

In this study, we used 3D organotypic culture to test the influence of collagen I on mammary epithelial cells in two contexts. We first explanted organoids from the same mouse into gels with equal concentrations of collagen I but varied polymerisation conditions. We found that the capacity of collagen I to induce protrusive invasion and dissemination (Nguyen-Ngoc *et al.*, 2012) depended strongly on the preincubation interval prior to gellation, with longer intervals correlating with more extensive fibril assembly. We next demonstrated that the preassembled collagen I fibrils were maintained in mixtures with Matrigel. We then used these mixed gels to test whether collagen I could still induce protrusive invasion in the context of basement membrane proteins. Epithelial organoids in mixed collagen I/Matrigel gels did not initiate protrusions or disseminate cells into the matrix. The extent of epithelial elongation correlated positively with the concentration of collagen I fibrils in the mixed gel. Surprisingly, the extent of myoepithelial coverage also correlated positively with the concentration of collagen I fibrils in mixed gels. To our knowledge this is the first report that ECM composition can regulate myoepithelial coverage. The observation of cancer cells beyond myoepithelial coverage defines invasive breast cancer in clinical samples (Sternlicht & Barsky, 1997; Leonard & Swain, 2004; Hu *et al.*, 2008; Polyak, 2010). Our data suggest that the composition of the ECM could directly regulate myoepithelial coverage of breast tumours and thereby impact the *in situ* to invasive transition.

Technical implications for organotypic models of epithelial morphogenesis

Many 3D culture models rely on collagen I to support cell survival and migration, and previous studies have identified differences in collagen I organization between pepsinized and acid-solubilised collagen I (Wolf *et al.*, 2009). We identified the time interval between neutralization of acid-solubilised collagen I and gelling as a critical parameter that determines the abundance of fibrils in the final gel. Unintended variation in this time interval could result in highly varying ECM microenvironments, even when the same source and final concentration of collagen I are used. In the specific context of mammary branching morphogenesis, our mixed 3M7C gels supported a more organotypic form of branching, with more extensive epithelial elongation and more complete myoepithelial coverage, similar to that of TEBs *in vivo* (Williams & Daniel, 1983; Hinck & Silberstein, 2005). The organisation and dynamics of the luminal cells were broadly similar in mixed gels and pure Matrigel. Matrigel cultures are technically easier to prepare and will suffice for many analyses. However, in future studies focused either on the pattern of branching morphogenesis or on the interactions between luminal and myoepithelial cells, our mixed 3M7C gels provide a more organotypic model than Matrigel alone. Future studies will be required to determine the relative contributions of matrix rigidity, ECM protein composition, and Matrigel-associated growth factors to epithelial phenotypes in the mixed gels.

Implications for understanding mammary branching morphogenesis

The stroma surrounding mammary ducts *in vivo* contains abundant collagen I fibers located just outside the basement membrane (William & Daniel, 1983). In our previous study, we demonstrated that direct contact with pure collagen I gels induced protrusive invasion in normal mammary epithelial cells (Nguyen-Ngoc *et al.*, 2012). However, mammary epithelial cells would not normally have direct access to the collagen I fibers as a complete basement membrane is maintained during TEB elongation *in vivo* (Williams & Daniel, 1983). Our data suggest that collagen I in the stroma could nonetheless influence the extent and pattern of branching morphogenesis.

Translational implications for breast cancer

Ductal carcinoma *in situ* (DCIS) describes neoplastic epithelial growths in the breast that remain confined within the basement membrane (Leonard & Swain, 2004). Clinically, invasive breast cancer is distinguished from DCIS by observation of cancer cells invading past myoepithelial coverage (Polyak & Hu, 2005; Polyak, 2010). In our previous study, we demonstrated that direct contact with pure collagen I was sufficient to induce protrusive invasion of both normal and neoplastic mammary epithelial cells (Nguyen-Ngoc *et al.*, 2012). In this study, we reveal that changes in the relative abundance of collagen I, collagen IV, and laminin I are sufficient to induce migration of epithelial cells past myoepithelial coverage. Taken together, our data suggest that both the protein composition and the supramolecular organization of the ECM can rapidly induce changes in epithelial behaviour similar to those observed during malignant progression. Importantly, these responses were observed in normal epithelial cells, suggesting that breast cancers may achieve important steps in malignant progression, such as invasion past myoepithelial coverage, through changes in cell–matrix interactions and/or changes in ECM composition.

Materials and methods

Mouse lines and breeding

All experiments were done in accordance with an approved protocol from the Johns Hopkins University Animal Care and Use Committee. Most experiments were done with Charles River FVB/N mice. For confocal time-lapse imaging, myoepithelial cells were visualized using keratin-14::actin-GFP transgenic mice (Vaezi *et al.*, 2002), in which the keratin-14 promoter drives cell-type specific expression of an actin-GFP fusion protein. Cell membranes were visualized using the membrane TdTomato signal in the mT/mG transgenic line (Muzumdar *et al.*, 2007). Keratin-14::actin-GFP were generously provided by Elaine Fuchs (Rockefeller University, NY, NY, USA), and mT/mG mice were obtained from Jackson Laboratory and back-crossed to FVB/N.

Isolation of primary mouse mammary organoids

We isolated organoids following previously described protocols (Ewald *et al.*, 2008; Nguyen-Ngoc *et al.*, 2012). In short, we collected mammary glands, minced the tissue with a sterile scalpel, and isolated the epithelial fraction following collagenase and trypsin treatment. Epithelial organoids were separated from single cells by differential centrifugation.

Collagen gel preparation

We used acid-solubilised rat tail collagen I (BD Bioscience #354326) as starting material. We combined 8 μL NaOH 1N, 25 μL 10X DMEM and 217 μL collagen I into 250 μL of solution. The volume was scaled up as necessary. Collagen solution was mixed until it had a stable, uniform colour. The pH was adjusted to 7.0–7.5. The concentration of collagen I varies between commercial lots and was always adjusted to 3 mg mL^{-1} by 1x DMEM. 10–20 μL of the collagen I solution was used to make a thin underlay for each well. The rest of the collagen I solution was preincubated at 4°C, on wet ice, from 5 min to 2 h (Fig. 1A). At the end of the specified preincubation interval, the collagen I solution was mixed with epithelial organoids, and the mixture was plated on top of the underlay and incubated at 37°C for 1 h to allow gelation before addition of culture medium. To generate the mixed gels, preassembled collagen I solutions (3 mg mL^{-1} , preincubated for 1 h at 4°C) were mixed with Matrigel (BD Biosciences #354230, Growth Factor Reduced) in 3:7, 5:5, and 7:3 (v/v) ratios. The mixtures were then combined with epithelial organoids plated, and

incubated at 37°C for 1 h for gellation. Organoids were combined at an approximate ratio of two organoids per microliter of ECM solution.

Antibody staining

Organoids cultured in all matrix conditions were fixed with 4% (v/v) paraformaldehyde in PBS for 20 min, washed twice with PBS for 10 min, permeabilised with 0.5% (v/v) Triton X-100 in PBS for 20 min, and washed twice for 10 min. Samples were then blocked with 10% FBS (v/v) in PBS for 2 h, incubated with primary antibody in 10% FBS for 2 h at RT, washed twice, and blocked another 1 h in 10% FBS in PBS. They were next stained with secondary antibody for 1 h and washed twice with 10% FBS in PBS before imaging. Primary antibodies were anti-keratin 8 (1:100) (TROMA-1, Developmental Studies Hybridoma Bank), anti-keratin 14 (1:500) (PRB-155P Covance) and anti smooth muscle actin-FITC conjugate (1:250) (F3777, Sigma).

Time-lapse imaging

Live DIC imaging was conducted using a Zeiss Cell Observer system with a Zeiss AxioObserver Z1 and an AxioCam MRM camera. Images were collected at 20 min intervals with exposure time of approximately 250 ms and analysed by Axiovision software. Live confocal imaging was performed using a custom spinning disc microscope (Ewald *et al.*, 2008; Ewald, 2010). Temperature was maintained at 37°C and CO₂ at 5%.

Image acquisition

Confocal images were acquired using a custom spinning disc microscope with 40× c-APO oil and 20× air objectives. Second harmonic generation (SHG) was conducted using a Zeiss LSM 710 laser-scanning microscope with a 25× water objective. SHG excitation was via a Coherent Inc. Chameleon laser tuned to 880 nm. For SHG imaging, laser power was set to 35, gain was in the range of 800–1000 depending on sample intensity, the digital offset was 1200, and the digital gain was 1.0. SHG images in the figures were collected with equivalent gain settings and are presented with equivalent digital contrast, brightness, and levels. Images were assembled using Imaris software.

Acknowledgments

We thank Jen Beck for technical support, Kevin Cheung for helpful discussions and assistance with second harmonic imaging, Ryan Gray for preliminary experiments, Eliah Shamir for critical comments on the manuscript, and the Johns Hopkins School of Medicine Microscope Facility for access to the Zeiss 710 two photon microscope. This study was funded by grants from the National Cancer Institute (U01 CA155758 and U54 CA151838). AJE was supported by a Research Scholar Grant, RSG-12-141-01-CSM from the American Cancer Society.

References

- Conklin MW, Eickhoff JC, Riching KM, Pehlke CA, Eliceiri KW, Provenzano PP, Friedl A, Keely PJ. Aligned collagen is a prognostic signature for survival in human breast carcinoma. *Am J Pathol.* 2011; 178:1221–1232. [PubMed: 21356373]
- Debnath J, Muthuswamy SK, Brugge JS. Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. *Methods.* 2003; 30:256–268. [PubMed: 12798140]
- Deugnier MA, Teuliere J, Faraldo MM, Thiery JP, Glukhova MA. The importance of being a myoepithelial cell. *Breast Cancer Res.* 2002; 4:224–230. [PubMed: 12473168]
- Ewald, AJ. Practical considerations for long-term time-lapse imaging of epithelial morphogenesis in three-dimensional organotypic cultures. In: Wong, R.; Sharpe, J., editors. *Imaging in Developmental Biology.* Cold Spring Harbor Laboratory Press; New York: 2010. p. 623-645.

- Ewald AJ, Brenot A, Duong M, Chan BS, Werb Z. Collective epithelial migration and cell rearrangements drive mammary branching morphogenesis. *Dev Cell*. 2008; 14:570–581. [PubMed: 18410732]
- Ewald AJ, Huebner RJ, Palsdottir H, Lee JK, Perez MJ, Jorgens DM, Tauscher AN, Cheung KJ, Werb Z, Auer M. Mammary collective cell migration involves transient loss of epithelial features and individual cell migration within the epithelium. *J Cell Sci*. 2012; 125:2638–2654. [PubMed: 22344263]
- Fata JE, Mori H, Ewald AJ, Zhang H, Yao E, Werb Z, Bissell MJ. The MAPK(ERK-1,2) pathway integrates distinct and antagonistic signals from TGF α and FGF7 in morphogenesis of mouse mammary epithelium. *Dev Biol*. 2007; 306:193–207. [PubMed: 17448457]
- Hagios C, Lochter A, Bissell MJ. Tissue architecture: the ultimate regulator of epithelial function? *Philos Trans R Soc Lond B Biol Sci*. 1998; 353:857–870. [PubMed: 9684283]
- Hinck L, Silberstein GB. Key stages in mammary gland development: the mammary end bud as a motile organ. *Breast Cancer Res*. 2005; 7:245–251. [PubMed: 16280048]
- Hogg NA, Harrison CJ, Tickle C. Lumen formation in the developing mouse mammary gland. *J Embryol Exp Morphol*. 1983; 73:39–57. [PubMed: 6875464]
- Hu M, Yao J, Carroll DK, Weremowicz S, et al. Regulation of in situ to invasive breast carcinoma transition. *Cancer Cell*. 2008; 13:394–406. [PubMed: 18455123]
- Ingman WV, Wyckoff J, Gouon-Evans V, Condeelis J, Pollard JW. Macrophages promote collagen fibrillogenesis around terminal end buds of the developing mammary gland. *Dev Dyn*. 2006; 235:3222–3229. [PubMed: 17029292]
- Kleinman HK, Martin GR. Matrigel: basement membrane matrix with biological activity. *Semin Cancer Biol*. 2005; 15:378–386. [PubMed: 15975825]
- Leonard GD, Swain SM. Ductal carcinoma in situ, complexities and challenges. *J Natl Cancer Inst*. 2004; 96:906–920. [PubMed: 15199110]
- Mailleux AA, Overholtzer M, Schmelzle T, Bouillet P, Strasser A, Brugge JS. BIM regulates apoptosis during mammary ductal morphogenesis, and its absence reveals alternative cell death mechanisms. *Dev Cell*. 2007; 12:221–234. [PubMed: 17276340]
- Muzumdar MD, Tasic B, Miyamichi K, Li L, Luo L. A global double-fluorescent Cre reporter mouse. *Genesis*. 2007; 45:593–605. [PubMed: 17868096]
- Nelson CM, Bissell MJ. Modeling dynamic reciprocity: engineering three-dimensional culture models of breast architecture, function, and neoplastic transformation. *Semin Cancer Biol*. 2005; 15:342–352. [PubMed: 15963732]
- Nelson CM, Inman JL, Bissell MJ. Three-dimensional lithographically defined organotypic tissue arrays for quantitative analysis of morphogenesis and neoplastic progression. *Nat Protoc*. 2008; 3:674–678. [PubMed: 18388950]
- Nelson CM, Vanduijn MM, Inman JL, Fletcher DA, Bissell MJ. Tissue geometry determines sites of mammary branching morphogenesis in organotypic cultures. *Science*. 2006; 314:298–300. [PubMed: 17038622]
- Nguyen-NGOC KV, Cheung KJ, Brenot A, et al. The ECM microenvironment regulates collective migration and local dissemination in normal and malignant mammary epithelium. *PNAS*. 2012; 109:E2595–E2604. [PubMed: 22923691]
- Polyak K. Molecular markers for the diagnosis and management of ductal carcinoma in situ. *J Natl Cancer Inst Monogr*. 2010; 2010:210–213. [PubMed: 20956832]
- Polyak K, Hu M. Do myoepithelial cells hold the key for breast tumor progression? *J Mammary Gland Biol Neoplasia*. 2005; 10:231–247. [PubMed: 16807803]
- Prockop DJ, Kivirikko KI. Collagens: molecular biology, diseases, and potentials for therapy. *Annu Rev Biochem*. 1995; 64:403–434. [PubMed: 7574488]
- Provenzano PP, Eliceiri KW, Campbell JM, Inman DR, White JG, Keely PJ. Collagen reorganization at the tumor-stromal interface facilitates local invasion. *BMC Med*. 2006; 4:38. [PubMed: 17190588]
- Provenzano PP, Inman DR, Eliceiri KW, Keely PJ. Matrix density-induced mechanoregulation of breast cell phenotype, signaling and gene expression through a FAK-ERK linkage. *Oncogene*. 2009; 28:4326–4343. [PubMed: 19826415]

- Provenzano PP, Inman DR, Eliceiri KW, Knittel JG, Yan L, Rueden CT, White JG, Keely PJ. Collagen density promotes mammary tumor initiation and progression. *BMC Med.* 2008; 6:11. [PubMed: 18442412]
- Raub CB, Suresh V, Krasieva T, Lyubovitsky J, Mih JD, Putnam AJ, Tromberg BJ, George SC. Noninvasive assessment of collagen gel microstructure and mechanics using multiphoton microscopy. *Biophys J.* 2007; 92:2212–2222. [PubMed: 17172303]
- Simian M, Hirai Y, Navre M, Werb Z, Lochter A, Bissell MJ. The interplay of matrix metalloproteinases, morphogens and growth factors is necessary for branching of mammary epithelial cells. *Development.* 2001; 128:3117–3131. [PubMed: 11688561]
- Sternlicht MD, Barsky SH. The myoepithelial defense: a host defense against cancer. *Med Hypotheses.* 1997; 48:37–46. [PubMed: 9049988]
- Sternlicht MD, Kouros-Mehr H, Lu P, Werb Z. Hormonal and local control of mammary branching morphogenesis. *Differentiation.* 2006; 74:365–381. [PubMed: 16916375]
- Vaezi A, Bauer C, Vasioukhin V, Fuchs E. Actin cable dynamics and Rho/Rock orchestrate a polarized cytoskeletal architecture in the early steps of assembling a stratified epithelium. *Dev Cell.* 2002; 3:367–381. [PubMed: 12361600]
- Weaver VM, Roskelley CD. Extracellular matrix: the central regulator of cell and tissue homeostasis. *Trends Cell Biol.* 1997; 7:40–42. [PubMed: 17708898]
- Williams JM, Daniel CW. Mammary ductal elongation: differentiation of myoepithelium and basal lamina during branching morphogenesis. *Dev Biol.* 1983; 97:274–290. [PubMed: 6852366]
- Wolf K, Alexander S, Schacht V, Coussens LM, von Andrian UH, van Rheenen J, Deryugina E, Friedl P. Collagen-based cell migration models in vitro and in vivo. *Semin Cell Dev Biol.* 2009; 20:931–941. [PubMed: 19682592]
- Yang YL, Motte S, Kaufman LJ. Pore size variable type I collagen gels and their interaction with glioma cells. *Biomaterials.* 2010; 31:5678–5688. [PubMed: 20430434]

Fig. 1. Supramolecular organization of collagen I correlated with the extent of epithelial invasion. (A) Schematic description of preparation of collagen I gels *in vitro*. We neutralized collagen I at 3 mg mL⁻¹ and preincubated at 4°C for varying time intervals. We both created empty gels for SHG analysis and mixed the preincubated collagen I solutions with epithelial organoids for analysis of cell behaviour. (B–E) SHG images of collagen I organization following preincubation for (B) 5 min, (C) 30 min, (D) 1 h, and (E) 2 h. (B'–E') DIC images of representative epithelial organoids cultured in collagen I gels with varying preincubation times (B–E, respectively). Red arrowheads indicate subcellular protrusions of epithelial cells into the ECM.

Fig. 2.

Preincubation time had a larger effect on epithelial behaviour than collagen I concentration. (A) Schematic description of preincubation of 3 mg mL⁻¹ collagen I at 4°C for 1 h, dilution to different concentrations, and gelation for SHG analysis or combination with epithelial organoids for cell behaviour analysis. (B–D) SHG images showing collagen I organization at (B) 1 mg mL⁻¹, (C) 2 mg mL⁻¹, and (D) 3 mg mL⁻¹. (B'–D') Representative DIC images of organoids cultured in gels of different collagen I concentration. (E) Schematic description of dilution of neutralized collagen I to 1, 2, and 3 mg mL⁻¹ prior to a 1 h preincubation. Collagen I solutions were then gelled for SHG analysis or combined with organoids for cell behavioural analysis. (F–H) SHG images of representative collagen I organization at (F) 1 mg mL⁻¹, (G) 2 mg mL⁻¹, or (H) 3 mg mL⁻¹. (F'–H') Representative DIC images of

organoids cultured in gels of different collagen I concentration. (I–K) Percent of organoids showing protrusive invasion into collagen gels with (I) different preincubation, times, (J) different collagen I protein concentrations after preincubation, and (K) different collagen I protein concentrations during preincubation (min. 3 independent experiments each, number of organoids analysed indicated in each bar graph). Red arrowheads indicate subcellular protrusion of epithelial cells into the ECM.

Fig. 3.

The extent of epithelial elongation varies with ECM composition. (A) Schematic description of preparation of mixed ECM gels. In each case collagen I was neutralized at 3 mg mL^{-1} and preincubated at 4°C for 1 h prior to mixing with Matrigel. The resulting mixed ECM solutions were either gelled for SHG analysis or were combined with epithelial organoids and gelled for cell behavioural analysis. (B–F) SHG images showing collagen fibrils in mixed ECM gels of different ratios of Matrigel (M) and collagen I (C): (B) Matrigel only, (C) 7M3C, (D) 5M5C, (E) 3M7C, and (F) collagen I only. (G) Schematic description of 3D organotypic culture in different mixed ECMs. (H–L) Representative DIC images of epithelial organoids after 100 h of culture in mixed gels with different ratios of Matrigel and

collagen I (B–F, respectively). Black arrowheads indicate the buds that will be shown at higher magnification. (H'–L') Selected buds showing the nonprotrusive leading front in (H') Matrigel only, (I') 7M3C, (J') 5M5C, and (K') 3M7C, and the protrusive leading front in (L') collagen I only. Red arrowheads indicate ECM-directed subcellular protrusions. Dashed lines indicate the length from the body of the organoid to the tip of the bud, quantified in N and O. (M) Percent of epithelial buds with at least one ECM-directed protrusion into the ECM observed during time-lapse imaging of elongation (three independent experiments, with the number of analysed buds indicated in each bar graph). (N) Percent of organoids with at least one epithelial bud greater than 100 μm cultured in different mixed ECMs (three independent experiments, with the number of analysed organoids indicated in each bar graph). (O) Distribution of epithelial bud length from organoids cultured in different mixed ECMs (three independent experiments, p -value determined by Student's t -test, two tailed, unequal variance).

Fig. 4. Epithelial elongation correlated with collagen I density (A–C) Representative frames from DIC time-lapse movies of organoids cultured in (A) 7M3C, (B) 5M5C and (C) 3M7C mixed ECM gels. (A'–C') Insets show bud elongation at higher magnification from organoids in (A–C), respectively.

Fig. 5. Myoepithelial coverage correlates with the fraction of collagen I in mixed ECMs. (A–E) Immunofluorescence staining of the myoepithelial cell marker keratin-14 and the luminal epithelial cell marker keratin-8 in epithelial buds of organoids cultured in (A) Matrigel, (B) 7M3C, (C) 5M5C, (D) 3M7C, and (E) collagen I gels. (F–H) Percent of epithelial buds covered by myoepithelial cells using three different markers: (F) smooth muscle actin (SMA) immunoreactivity, (G) keratin-14 immunoreactivity, and (H) GFP+ using a transgenic myoepithelial reporter mouse in which the keratin-14 promoter drives GFP expression. Minimum of three independent experiments, with the number of analysed epithelial buds indicated in each bar graph.

Fig. 6. Myoepithelial coverage is maintained during elongation in mixed ECM gels with a high fraction of collagen I. (A–D) Frames of confocal time-lapse movies showing myoepithelial cells (green, keratin-14 promoter transgenic GFP reporter mouse) and cell membranes (membrane TdTomato mT/mG reporter mouse) in elongating buds in (A) Matrigel, (B) 7M3C, (C) 5M5C, and (D) 3M7C.