Mechanical regulation of mammary epithelial branching morphogenesis

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Abstract

Spatial patterning of cell behaviors establishes the regional differences within tissues that collectively develop branched organs into their characteristic tree-like shapes. Although a variety of endocrine, paracrine, autocrine and extracellular matrix (ECM) signals have been implicated as global regulators of mammary epithelial branching morphogenesis, comprehensive understanding of the mechanisms of pattern formation during the process is lacking. Here we show that the pattern of branching morphogenesis of three-dimensional (3D) engineered mammary epithelial tissues is controlled in part by gradients of endogenous mechanical stress.

We used microfabrication to build model mammary epithelial tissues of defined geometry that branched in a stereotyped pattern when induced with growth factors. We combined continuum mechanics with computational modeling, atomic force microscopy and confocal reflectance microscopy to define the experimental parameters required to directly measure the mechanical stress profile of the tissues. We found that calculating stresses accurately in these settings required accounting for cell-induced mechanical heterogeneities within the ECM. Using this technique, we measured endogenous traction forces at the epithelial surface and resolved qualitative and quantitative patterns of mechanical stress throughout the tissue. We discovered that the mechanical profile of the tissues was dictated by the epithelial geometry, with cells within certain geometric features consistently experiencing higher forces. In addition to quantifying tissue-induced
forces, this method allowed us to define the parameters which govern epithelial force
generation and subsequently fabricate tissues with precisely tuned mechanical profiles.

Using this platform, we demonstrated that mammary epithelial branches initiated from
sites of high mechanical stress within the preexisting tissue; the extent of branching
correlated with the local magnitude of stress. Branch sites were defined by activation of
focal adhesion kinase (FAK), inhibition of which disrupted morphogenesis. Modulating
mechanical stress by manipulating cellular contractility, matrix stiffness, intercellular
cohesion and tissue geometry led to concomitant changes in both FAK activation and
branching.

We found further that mammary epithelial branch extension in collagenous matrices was
driven by tensile mechanical forces, which enhanced elongation by activating
mechanosensitive signaling with the invading cells and conditioning the obstructing ECM.
Specifically, cell-generated tension activated FAK and p130 Cas within the cells of the
leading edge, and induced the nuclear translocation of myocardin-related transcription
factor A (MRTF-A), which was required for branch extension. Blocking cellular tension
led to loss of MRTF-A activation and prevented branch extension. Further, tensile forces
at the leading edge facilitated invasion into the 3D matrix by remodeling and creating
aligned tracks within the ECM. Although in vivo confirmation is pending, these data
contribute to our understanding of the physical rules that guide the normal development
of the mammary gland and other branching epithelia, and may help unlock general
engineering strategies to build such organs ex vivo.
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Finally, as I am completing this highest academic degree, I cannot but think of my grandmother. Despite her utmost appreciation for education and thirst for knowledge (she wanted to be a schoolteacher, a truly extravagant dream for a girl in World War II rural Macedonia), she had to leave school after her fifth grade and contribute to the family by making flax threads. To me, she will always stand a reminder that having the opportunity to do science is a privilege, but also that education is not necessary, and certainly not sufficient, to make one an admirable human being.
To my grandmother.
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Chapter 1

Introduction

(Chapter adapted from Gjorevski & Nelson, Nat Rev Mol Cell Biol 2011)

1.1 Mammary gland development and branching morphogenesis

The mammary gland produces and delivers milk from mother to newborn. The only organ after which an entire class of animals has been named, the gland is thought to be responsible for the evolutionary success of mammals, primarily due to milk’s nutritional and antimicrobial content (1). Lactation is made possible by the architecture of the gland. Like other organs used for fluid transport, the mammary epithelium develops into an elaborate network of branched ducts that maximize surface area within a constrained volume. The mature bilayered mammary duct consists of an outer layer of myoepithelial cells and an inner layer of luminal epithelial cells, which surround a hollow lumen and differentiate into milk-producing alveoli; release of milk through the duct occurs upon hormonal triggers and contraction of the myoepithelium. The epithelial ductal tree is enveloped by a basement membrane (2) and embedded within a complex stroma, the mammary fat pad, which contains fibroblasts, adipocytes, blood vessels, nerves and a variety of immune cells, all of which are important for normal mammary gland development and function.
Figure 1.1: Stages of mammary gland development. In the mouse embryo, mammary development begins when five pairs of placodes form in the epithelium adjacent to the fat pad precursor. These placodes invaginate to form the mammary buds. By embryonic day 18.5 (E18.5), a rudimentary gland has formed that remains morphogenetically quiescent until puberty. During puberty, hormonal cues trigger the formation of the terminal end buds (inset). Through extensive elongation, bifurcation and lateral branching, the full epithelial tree is formed. Adapted from (3).

Mammary development occurs in three distinct and differentially regulated stages: embryonic, pubertal and adult (Fig. 1.1). In mice, embryonic mammary development begins mid-gestation with the formation of five pairs of placodes of the epithelial layer that invaginate into the underlying mesenchyme to form the mammary buds, or anlagen. The mammary bud proliferates and extends 10-20 sprouts (4), thus transforming into a rudimentary ductal structure, itself capable of producing milk (“witch’s milk”) upon birth. After birth, the rudimentary gland enters a phase of morphogenetic quiescence.
During puberty, the ends of the rudimentary ducts are prompted by elevated levels of ovarian hormones, including estrogen, to proliferate and swell into distinct multilayered epithelial structures known as terminal end buds (TEBs). These ductal structures then undergo successive rounds of elongation, bifurcation and lateral branching until reaching the limit of the fat pad, thus elaborating a full epithelial tree. During pregnancy, the luminal epithelium proliferates and differentiates into milk-producing secretory alveoli. Massive apoptosis then removes 80% of the epithelium during post-lactational involution. Remarkably, the mammary gland maintains its ability to perform this dramatic remodeling during the pregnancy-lactation-involution cycle for several decades in humans.

The study of mammary morphogenesis during the past century has implicated a long list of signals in the regulation of this process, including hormones, growth factors, receptor tyrosine kinases, extracellular matrix (ECM) molecules and proteases. Over the past five years, sophisticated genetic, real-time imaging, computational and culture studies along with large-scale gene profiling have revealed links between the various signals, cellular behaviors and physical phenomena that drive mammary development, and unveiled the integrated nature of these cues. We now understand that the morphodynamics of the mammary gland is dictated by signaling between several cell types, integrated dynamically over multiple length scales (cell, tissue, organ, and organism).
Puberty is perhaps the most striking stage of mammary gland development. Branching morphogenesis, the process which transforms the neonatal epithelial rudiment into a full ductal tree during the pubertal stage, is the focus of this dissertation. Branching morphogenesis is not specific to the mammary gland: it is used extensively across the animal kingdom to construct organs comprised of elaborate epithelial networks, including the *Drosophila* trachea and salivary gland, and the vertebrate lung, kidney and salivary gland. Nevertheless, although many of the signals that direct mammary development overlap with those involved in the morphogenesis of other branched epithelia, several features make the mammary gland unique. Whereas chemotactic gradients guide extending branches in systems including the *Drosophila* trachea (5-6), the mammalian lung (7) and the ureteric bud (8), there exists no evidence for chemotaxis as the guidance mechanism for mammary branching. Likewise, the large variation between mammary glands precludes the possibility for predetermined genetic control of its morphogenesis, as is the case with the largely stereotyped airways of the embryonic lung (9). The stochastic form of the gland suggests a dynamic control, dictated by microenvironmental context. Indeed, recent studies have unveiled the context-dependent interplay between mechanical factors and molecular signals derived from different cell types to induce the cellular behaviors and matrix remodeling that ultimately drive morphogenesis.

### 1.1.1 Regulation of branching morphogenesis by molecular signals: a brief overview

A plethora of molecular signals from cells of epithelial and stromal origin cooperate to carry out mammary branching mophogenesis (Fig. 1.2). This epithelial-stromal dialogue
Figure 1.2: Endocrine, paracrine, autocrine and ECM signals cooperate to regulate mammary development. (A) Global endocrine signals from the ovary and pituitary gland activate a plethora of paracrine signalling pathways to initiate mammary morphogenesis. Cellular crosstalk between the epithelial and stromal compartments is mediated by growth factors.
including insulin-like growth factor 1 (IGF1), hepatocyte growth factor (HGF) and the epidermal growth factor (EGF) and fibroblast growth factor (FGF) families, which bind to their cognate receptors to induce cell proliferation, survival and branching. Classic pathways such as WNT and Hedgehog, which are activated by signaling through primary cilia, are also emerging as indispensable regulators of the process. (B) Autocrine cues such as transforming growth factor-β (TGFβ) serve as negative regulators of mammary morphogenesis. (C) Mammary gland patterning is directed in part by matrix metalloproteinases (MMPs), which display spatially localized expression and activity during puberty and serve both to control cell migration and survival, and to degrade the ECM. Integrin-dependent ECM signalling and mechanical cues are emerging as additional regulators of mammary morphogenesis. ADAM17, a disintegrin and metalloproteinase domain-containing protein 17; AREG, amphiregulin; EGFR, EGF receptor; EPHA2, ephrin type-A receptor 2; ERα, oestrogen receptor-α; FAK, focal adhesion kinase; FGFR, FGF receptor; GHR, growth hormone receptor; MAPK, mitogen-activated protein kinase; TGFβRI/II, TGFβ receptor I and II. Adapted from (3).

is initiated by ovarian and pituitary hormones, including growth hormone (GH) and estrogen, which can signal to both types of cell. Knocking out estrogen receptor (ER)-α leads to hypoplastic development of the epithelial tree (10-11), whereas exogenous estrogen can rescue pubertal branching in ovariectomized mice (12). ERα is required in the stroma (13), which in response to estrogen produces hepatocyte growth factor (HGF) to induce epithelial branching (14). Estrogen also binds to ERα in the epithelium, thereby inducing the expression of amphiregulin (Areg) (15-19), which is subsequently cleaved from the epithelial surface and can signal back to stromal cells by binding to epidermal growth factor receptor (EGFR) on the stromal membrane. EGFR is required in the stromal compartment (20), and exogenous addition of EGFR ligands can rescue pubertal development of ovariectomized animals (15), consistent with an essential role for EGFR-mediated signalling downstream of estrogen.

Estrogens, however, are not sufficient, as they fail to rescue branching in hypophysectomized animals (21). Branching is restored by GH or insulin-like growth factor-1 (IGF1) (21), suggesting that pituitary GH is a master regulator of pubertal
mammary development. Transplantation experiments demonstrated that GH induces expression of IGF-1 in stromal cells (22), which signals to its receptor (IGFR1) in the epithelium (21). Several other receptor tyrosine kinases have profound effects on pubertal mammary development, including Ron (23), EphA2 (24), and fibroblast growth factor receptor (FGFR). FGF2 and FGF7 rescue growth and branching of EGFR-null mammary organoids in culture (18), suggesting that FGFR signaling occurs either downstream of, or in parallel to, signaling through EGFR. The compartmental localization and requirement of Areg, EGFR, IGF1 and IGFR1 highlights the critical importance of integrated paracrine signaling between the epithelium and stroma during pubertal development.

Sophisticated genetic approaches have recently offered a glimpse into the local roles of signaling cues, notably FGFs, during mammary morphogenesis. Mosaic inactivation and reversible attenuation of FGFR2 have demonstrated that FGFR function is more important locally within the TEBs than in the subtending ducts, and that FGFR contributes to mammary development by regulating proliferation of the luminal epithelial layer in the gland. Stat5a also shows a similar spatially restricted response to hormone signaling: it is expressed in response to estrogen and progesterone in the subtending ducts, but not the TEBs (25). Mice deficient for Stat5a show defects in lateral branching, but not ductal extension or TEB bifurcation (26). Global paracrine and endocrine signals can thus have varied local effects depending on whether the epithelial cells are in the TEBs or in the ducts.
In addition to growth factor receptors, matrix metalloproteinases (MMPs) have emerged as local regulators of mammary morphogenesis, through both their ability to mediate signaling and to clear paths in the surrounding ECM during branching. Distinct spatial patterns of MMPs have been detected in mammary tissue. MMP14 is elevated in and around the TEBs (27-28); MMP9 is expressed at homogeneously low levels by both the epithelium and the stroma (28); MMP3 is expressed throughout the stroma (28); MMP2 is reduced at sites of lateral branching (28). Knockout analyses have shown that the effects of MMPs on mammary development differ at particular stages. MMP2-null mice exhibit delayed ductal invasion during early puberty and increased lateral branching during late puberty (28). MMP3 does not affect ductal elongation, but instead induces lateral branching (28). Intriguingly, MMP2 and MMP3 seem to contribute to branching via different mechanisms. Increased apoptosis is observed in MMP2-knockout mice, which suggests that MMP2 influences branching by promoting cell survival (28). MMP3, on the other hand, probably promotes branching by driving clearance of the local ECM, as suggested by the degradation of collagen IV and laminin-111 specifically at sites of lateral branching (28).

Although intense investigation over the past decade has revealed many of the mechanisms which drive mammary branching morphogenesis, the answers to several key questions, especially those pertaining to pattern formation, remain elusive. What are the molecular differences between the epithelial cells within the simple, bilayered mature duct and those within the multilayered, migratory and proliferative TEB? What prompts and schedules the dichotomous branching of the TEBs instead of their forward
elongation? How are the sites and angles of lateral branches specified? What are the physical mechanisms whereby branches extend? How do they navigate the fat pad to avoid each other and stop once they have run out of available space? Pattern formation during mammary development can be attributed in part to spatial non-uniformities in the expression and activity of key regulators (including MMPs and signaling through FGFR, as discussed above). However, the question of how these non-uniformities arise still remains. At the start of puberty, the rudimentary mammary epithelium already has an anisotropic (non-spherical) branched geometry and patterning information may be encoded in the shape of this pre-existing structure. Tissue geometry can instruct morphogenesis by creating spatial gradients of key molecular regulators. Computational models of the secretion and diffusion of the autocrine morphogen transforming growth factor-β (TGFβ) have shown that its concentration profile is determined by tissue geometry (29). In microfabricated mammary tissues, branching is inhibited at sites of high TGFβ concentration (29). Accordingly, TGFβ gradients may specify sites of branch initiation and maintain proper ductal spacing in vivo, thus generating the characteristic open architecture of the gland (30). Indeed, overexpression of TGFβ1 leads to hypoplastic mammary development in vivo (31), whereas TGFβ-deficient mice exhibit elevated ductal proliferation and accelerated lateral branching (32-34).

In addition to biochemical signals, tissues and organs, including the mammary gland, are exposed to cues of physical nature. Mechanical cues, in particular, have been implicated in several aspects of mammary morphogenesis, homeostasis and disease. Mammary epithelial cells self-organize into tubules when cultured on floating (compliant) collagen
gels, but fail to form tubules on attached (stiff) gels (35). Matrix stiffness similarly governs the functional differentiation of mammary epithelial cells, which can synthesize milk proteins in soft but not stiff environments (36). Drastic perturbations in the normal mechanical environment of mammary tissue can lead to phenotypes characteristic of malignancy. Culturing mammary tissue within matrices of high, tumor-like stiffness disrupts tissue architecture and promotes invasiveness (37-38); tumorigenesis of the breast in vivo is accompanied and possibly driven by ECM crosslinking and stiffening (39). Despite evidence implicating mechanics as a regulator of mammary gland biology, comprehensive and rigorous studies of its role in mammary branching morphogenesis are lacking. Understanding the mechanical nature of mammary morphogenesis requires quantitative knowledge of key biomechanical parameters within the microenvironment of the gland, as well as their spatiotemporal variations. Furthermore, the molecular mechanisms whereby mammary epithelial cells sense, interpret and translate these physical parameters into a functional morphogenetic response must be defined.

1.2 Mechanical regulation of basic cellular and morphogenetic processes

1.2.1 Generation, transmission and concentration of mechanical stress in biological systems

The ability of biological systems – cells and tissues – to generate and transmit mechanical forces over a distance has long been recognized. Harris and co-workers visualized cell-generated forces nearly three decades ago by demonstrating that fibroblasts plated on silicone membranes pull on their substratum, creating wrinkles (40). Such forces have
since been extensively analyzed and measured (41-43). Endogenous forces arise from the
tendency of cells to contract. In response to various stimuli, non-muscle myosin II motors
undergo ATP-dependent activation and “walk” along actin filaments, thus creating
contractile, force-generating actomyosin bundles (44-45). The best-described regulators
of myosin II and the overall contractile machinery of the cell include myosin light chain
kinase (MLCK) and the Rho effector Rho-associated kinase (ROCK) (46-48). MLCK
directly phosphorylates the regulatory myosin light chain (MLC), whereas ROCK has a
dual role: it promotes myosin activation both by phosphorylating MLC and by
inactivating MLC phosphatase. These regulatory proteins thus form the machinery that
enables cells to contract and pull.

Cellular contraction alone is not sufficient for the generation of stress. Mechanical stress
necessitates cellular attachment and contraction against a substratum capable of resisting
deformation (49-50). The ability of a substratum to resist deformation, thus balancing
cytoskeletal forces and giving rise to stress, is quantified by its elastic modulus (stiffness),
a physical parameter implicated in the regulation of normal and pathologic processes (51-
52). How matrix stiffness affects the generation of mechanical stress has been tested by
plating cells on a substratum comprised of ECM-coated beads of submicrometer (i.e.
subcellular) size. The beads were not physically linked, allowing the cells to displace
them without encountering resistance. These experiments demonstrated that cells plated
on such substrata fail to produce stress, which suggests that substratum stiffness is
necessary for the generation of stress (50) (Fig. 1.3 A). In fact, matrix stiffness not only
maintains cell-generated mechanical stress, but also modulates it: stiffer two-
Figure 1.3: Cellular generation, transmission and concentration of mechanical stress. (A) Cells in suspension or attached to soft matrices incapable of resisting deformation fail to generate mechanical stress. (B) Cells attached to a stiff substratum contract isometrically, giving rise to mechanical stress locally. (C) When cells are connected into cohorts, mechanical stresses generated at the single cell level are transmitted over a distance via intercellular junctions. Transmission of stress across tissues of anisotropic geometry results in concentration of stress and formation of gradients; for example, maximum stress occurs at corners of square monolayers. Adapted from (53).

dimensional (2D) substrata and 3D matrices lead to activation of the Rho pathway, stronger cell-matrix adhesion and ultimately enhanced generation of force (51-52) (Fig. 1.3 B). Cell-generated stresses thus require both cytoskeletal contraction and attachment to ECM or neighboring cells.
Epithelial cells rarely function individually *in vivo*. Instead, they are connected to their neighbors via cell-cell junctions and to the ECM via cell-matrix adhesions, thus forming functional epithelial tissues. Importantly, the junctional proteins are directly or indirectly linked to the force-generating cytoskeletal machinery, thus creating a supra-structure capable of long-range transmission of mechanical stresses produced at the cellular level.

It is by now widely accepted that the actin cytoskeleton is physically linked to the ECM at focal adhesions, comprised of transmembrane integrin receptors and numerous scaffolding proteins (54-55). This link not only tethers the cell to the ECM, which resists deformation leading to mechanical stress, but also serves as a conduit for inside-out channeling of mechanical force. Techniques used to quantify cell-generated forces, such as 2D or 3D traction force microscopy, rely upon the transmission of force from cells to 2D substratum or 3D matrix (41, 56). Here, matrix deformations induced by cells are visualized, measured and, when possible, converted to mechanical stresses. Cells may transmit stress over long distances through compliant matrices in order to communicate mechanically with adjacent cells or tissues. Hammer and co-workers have demonstrated that endothelial cells in culture can detect and respond to substratum deformation due to stresses originating from neighboring cells (57). The extent of matrix deformation depends upon its stiffness, suggesting that ECM stiffness determines the maximum distance over which cells can communicate mechanical signals.

Cells can also transmit stresses directly to coherent neighbors. Adherens junctions are a type of intercellular junction maintained by calcium-dependent homophilic interactions
between cadherins. The engagement between the extracellular cadherin domains of adjacent cells triggers the recruitment of structural and signaling proteins on the cytoplasmic face, which anchor the junction to actin creating physical continuity between the cytoskeletons of adjacent cells (58). Actin cables which circumscribe wounds in epithelial sheets appear to be continuous from cell to cell and connected by clusters of E-cadherin at cell-cell contacts (59-60). The collective contraction of the interlinked actin cables generates force which is transmitted at ranges that are considerably longer than the length of a single cell and span the entire perimeter of the wound, driving wound closure (59-60).

Collective cellular contraction and transmission of the resulting stress within tissues of anisotropic (i.e. non-spherical) geometries leads to concentration of stress and formation of spatial stress gradients. The existence of mechanical gradients in cellular monolayers has been predicted computationally and confirmed experimentally (61-62). Here, lithography-based microfabrication methods were used to control the 2D geometry of the tissues, and maximum stress was observed at sharp corners and regions of high convex curvature (Fig. 1.3 C). As expected, preventing transmission of stress by disrupting the physical link between the cadherins and the actin cytoskeleton abrogated the gradients, rendering the mechanical stress spatially uniform. Tissue-level heterogeneities in the distribution of mechanical stress have also been demonstrated in amphibian embryos and correlated with morphological patterns and mechanochemical processes in vivo (63). Cellular contraction may thus be used as a microenvironmental cue to signal over large distances during development.
The role of the mechanical microenvironment in the regulation of morphogenesis and pathogenesis is becoming ever more recognized. Cellular geometry and spreading control the transition of cells between proliferation and apoptosis (64), as well as the ability of a cell to undergo epithelial-mesenchymal transition (EMT) (65). The mechanical properties of the microenvironment direct the differentiation of stem cells: the fate of human mesenchymal stem cells is determined by the stiffness of the underlying 2D substratum or the surrounding 3D matrix (66-67). These mechanically-regulated basic cellular processes (proliferation, differentiation, apoptosis, EMT) are finely orchestrated within the developing embryo to give rise to a complex multicellular organism. Thus, one could imagine a role for physical force in the control of development on a larger scale. In culture, mechanical gradients within 2D cellular monolayers serve to pattern cellular processes, including proliferation, stem cell differentiation and EMT, across fields of cells (61-62, 68). A number of recent studies implicate mechanics as a regulator of embryogenesis in vivo (reviewed in (69)). Mesoderm and notochord stiff enough to resist buckling are needed during *Xenopus laevis* gastrulation (70-71), and actomyosin contractility is required for dorsal closure in the *Drosophila melanogaster* embryo (72-73). Twist – a master regulator of cell shape changes during gastrulation – has been implicated as a major player in the mechanical control of embryogenesis. Applying both tensile and compressive force on *Drosophila* embryos results in increased Twist expression (74-75), whereas laser ablation of dorsal epithelium leads to reduced levels of Twist (74). Tissue contractility has also been implicated in organogenesis during embryonic development. Down-modulating Rho-mediated tension inhibits branching
morphogenesis of the embryonic lung (76) and kidney (77), whereas increasing tension promotes branching (78).

1.2.2 Mechanosensing and mechanotransduction

Endogenous and exogenous mechanical forces influence a variety of biological behaviors, as discussed above. However, we still have a poor grasp of cellular mechanosensing and mechanotransduction, the mechanisms whereby cells and tissues sense and interpret physical signals and convert them into a functional response.

A number of cellular structures are emerging as mechanosensors, including the focal adhesion machinery. Numerous proteins are recruited at focal adhesions and phosphorylated in a stress-dependent manner (49). Focal adhesion kinase (FAK) and Src, in particular, have been implicated as mechanosensors. FAK undergoes enhanced phosphorylation in response to mechanical stress (56, 79-80) and is required for sensing of substratum stiffness during fibroblast migration (81). Similarly, fluorescence-resonance energy transfer analysis has shown that Src is activated at adhesion sites in response to mechanical stress (82). Active FAK and Src direct a plethora of cellular processes including proliferation, differentiation, adhesion, motility and invasion (81, 83-84). It must be emphasized, however, that mechanosensitive pathways often feed back to regulate the generation of force, thus serving as more than passive sensors. This feedback complicates studies aimed at defining specific roles within the mechanobiological machinery of the cell (85-86).
Figure 1.4: Possible modes of cellular mechanotransduction. (A) Cell-generated mechanical stress can remodel the surrounding matrix, releasing ECM-bound regulatory molecules such as growth factors. Binding of these growth factors to their receptors under conditions of high tension allows increased growth factor signalling. (B) Cryptic binding sites in the ECM may be unable to access their receptors under conditions of low tension. In response to high tension, remodelling of the ECM may expose these binding sites, allowing them to engage more integrin receptors and trigger enhanced downstream signalling. (C) Mechanical stress can regulate the nuclear localization and activity of transcription factors by modulating the relative levels of globular (G)-actin and filamentous (F)-actin. Under high tension, reduced levels of G-actin may allow release of a transcription factor to the nucleus to increase gene expression. Adapted from (3).

How does mechanotransduction occur? That is, what are the molecular-level effects of force responsible for causing biochemical and functional response? One relatively well-
documented mechanism is force-induced changes in protein conformation. Studies in molecular mechanics report stress-triggered alteration in a number of protein structural motifs (reviewed in (87-88)). For instance, cellular contractile activity is sufficient to partially unfold fibronectin, exposing otherwise hidden (cryptic) regions (89) (Fig. 1.4 A). Stress-mediated matrix remodeling may also release ECM fragments that have particular biological activities or ECM-bound growth factors (Fig. 1.4 B). Physical forces also open ion channels tethered to the ECM or cytoskeletal filaments, and thus influence the flux of ions into or out of the cells (87-88). Mechanical stress also regulates transcription and may thereby alter the synthesis of various regulatory proteins (Fig. 1.4 C). For example, mechanical stress modulates the balance between monomeric and filamentous actin, which controls the nuclear localization of transcription factors of the myocardin family (90-92), and thus induces localized expression of mesenchymal markers within epithelial tissues in culture (91). Although intense study in the past decade has elucidated many mechanisms, identification of the mechanosensing and mechanotransduction machinery is very much an active area of investigation.

1.2.3 Techniques to study mechanobiology

Comprehensive understanding of how the mechanical environment regulates morphogenesis, including that of the mammary gland, requires methods to measure mechanical parameters quantitatively and perturb them reproducibly. Spatiotemporal patterns of mechanical stress must be defined and compared to patterns of morphogenesis. In addition, causative and mechanistic studies must be performed, wherein mechanical
perturbations are introduced in biological systems and their resulting morphogenetic effects and mechanisms thereof defined. The field, however, is hampered by the current lack of in vivo studies, which are complicated owing to the large plastic deformations, fragility and physical and optical inaccessibility of developing mammalian tissues. Indeed, the vast majority of in vivo studies have been limited to simple non-mammalian model organisms including Drosophila, C. elegans and Xenopus embryos. Mechanobiological aspects of mammalian cells and tissues have been almost exclusively studied in culture.

As discussed above, mechanical forces generated by human fibroblasts in culture were visualized for the first time nearly three decades ago, when Harris and coworkers demonstrated that fibroblasts locomoting on 2D silicon membranes pull on their substratum, creating wrinkles (93). Monitoring the distribution and size of the wrinkles allowed semi-quantitative characterization of endogenous cellular forces. Approximately two decades later, Pelham and Wang introduced the use of bioinert polyacrylamide (PAA) gels with tunable stiffnesses to study the effect of external mechanical perturbations on cellular behavior (94). These two techniques later evolved into traction force microscopy (TFM), which incorporates fluorescent beads into the PAA substrata to visualize displacements due to cell-generated traction forces. TFM allows study of both cellular response to external mechanical changes and measurement of endogenous forces, producing force maps with subcellular resolution (95-96). An alternative technique involves plating cells on top of an array of elastomeric micrometer-scale posts. Here, the
cell-generated forces are computed from the deflection of the elastic posts, which reduces the mathematical complexity of TFM (97).

Sophisticated lithography-based microfabrication methods developed in the past decade have enabled researchers to control the magnitudes of mechanical stress experienced by isolated single cells by restricting cell spreading (64). Furthermore, these methods were critical in demonstrating that tissue geometry controls the distribution of mechanical stress over cellular monolayers (98). This discovery inspired the use of microfabrication as a means to precisely control tissue geometry, and consequently spatial patterns of stress within cell monolayers, allowing further insight into the role of mechanics as a potential regulator of biological processes. Indeed, microfabrication was instrumental in demonstrating that mechanical stress spatially patterns important developmental processes such as apoptosis (64), proliferation (98), differentiation (99-100) and epithelial-mesenchymal transition (61, 65), and unveiling some of the underlying mechanisms of mechanotransduction (61).

Although 2D studies have furnished valuable information about the mechanical behavior of cells and have elucidated several modes of mechanotransduction, cells in vivo typically reside in a 3D environment and the overwhelming majority of developmental, physiological and pathological processes are inherently 3D. Several recently-developed techniques for microfabricating 3D epithelial tissues hold promise in addressing questions in developmental mechanobiology with a higher level of physiological and histological realism (101). We have developed an approach that relies upon replica
molding of biomimetic hydrogels around elastomeric stamps to generate cavities of micrometer-precision size and geometry (29, 102). Cells subsequently inserted into the cavities self-organize into 3D tissues. The main advantages of engineered 3D epithelial tissues include the accessibility and ease with which mechanical modulations can be performed and endogenous mechanical characteristics interrogated quantitatively. The concentration of the hydrogels or the extent of crosslinking can be varied to modulate matrix stiffness, thereby altering the mechanical signals originating outside the cells. Conversely, molecular or pharmacological approaches can be taken to perturb mechanical elements within the cells, including integrins, intercellular adhesion and cytoskeletal proteins. In a fashion similar to the microfabricated 2D tissues, the patterns of mechanical stress can be controlled by the 3D geometry, which would in this case allow researchers to draw correlative and causative links between the spatial profile and magnitudes of mechanical forces, on one hand, and sites and extents of 3D morphogenetic processes within the tissues, on the other. Furthermore, matrix deformations due to tissue morphogenesis can be readily measured and used to quantify morphogenetic forces in a spatially and temporally resolved manner.

We used the microfabrication methods described above in conjunction with computational modeling to explore the role of mechanical stress in the branching morphogenesis of model 3D mammary epithelial tissues. In particular, we adapted existing and developed new techniques to measure the endogenous mechanical stress profile of quiescent mammary tissue and monitor how it changes during morphogenesis. We used these techniques to define the role of endogenous mechanical gradients in the
patterning of branching morphogenesis, i.e. in the selection of sites of future branches within the preexisting tissue. Finally, we investigated the physical mechanisms and their molecular effects by which mammary branches extend and navigate the matrix.
Chapter 2

Endogenous mechanical gradients spatially pattern branching morphogenesis of engineered mammary tubules

(Chapter adapted from Gjorevski & Nelson, Integr Biol 2010)

2.1 Introduction

Branching morphogenesis is a striking example of complex tissue architecture arising from spatially patterned cell behavior. Subgroups of cells are instructed to form nascent branches and invade the surrounding stroma while neighboring cells remain quiescent. A number of biochemical signals – growth factors, proteases, ECM molecules and morphogens (103-104) – act as global regulators of branching morphogenesis in vivo and in culture. Nevertheless, the local regulators that determine branch initiation points and the spacing between ducts remain obscure (105). Advances in engineering 3D tissues have opened the possibility of using organotypic tissue mimetics to study such complex developmental programs quantitatively (106). To delineate the local regulators of branching, our group recently developed an engineered tissue model of the mammary duct comprised of mouse mammary epithelial tubules of precisely defined geometry surrounded by collagen gel (29, 102). Although it does not completely replicate the histology of the mammary gland, this technique generates thousands of engineered tissues of identical size, shape, and branching pattern, making it useful for identifying the signals that influence branch initiation sites. Branching of the engineered tubules was
inhibited by high concentrations of autocrine-secreted transforming growth factor (TGF)-
β (29), confirming a long-standing hypothesis in the field (107). Branch sites in vivo and
in culture thus appear to be controlled in part by concentration gradients that form in the
surrounding microenvironment.

In addition to biochemical cues, tissues are exposed to cues of biophysical nature,
including substratum stiffness and cytoskeletal tension, which control key morphogenetic
processes such as proliferation, apoptosis, and differentiation (64, 66, 98-99, 108). Recent
studies have also suggested a role for the mechanical environment in the development of
branched tissues (35, 37, 76, 78). Down-regulating contractility in the embryonic lung
results in decreased branching (76) whereas up-regulating contractility promotes
branching (78). Similarly, disrupting stress fiber formation and tissue contractility
inhibits branching in the kidney and results in a dysmorphic organ (77). Recently, a
contractility-mediated mechanochemical checkpoint was shown to regulate cleft
progression during branching of the mouse salivary gland (109). In the lung, salivary
gland, and mammary gland, the basement membrane thins out adjacent to emerging
branches (2, 76, 110), consistent with the hypothesis that mechanical stresses are
concentrated at future sites of branching and influence matrix turnover and
morphogenesis (111). Mechanical stresses arise from the isometric contraction of
individual cells, but become concentrated into patterns as a result of asymmetries in the
geometry of the tissue, as demonstrated in amphibian embryos (63) and in 2D cultured
epithelial sheets (98). We thus set out to test whether the pattern of branching is
templated by endogenous patterns of mechanical stress.
Here, we used numerical and engineered culture models to investigate the role of endogenous mechanical stress in the patterning of branching morphogenesis. We show experimentally that mechanical stress is distributed non-uniformly across 3D model epithelial tissues. Branching occurs at regions of high stress, and changes in the extent of branching correlate with changes in the magnitude of stress at branching sites. Endogenous stress activated FAK, inhibition of which prevented branching of the model tissues. These results suggest that mechanical stresses and biochemical signals from the microenvironment cooperate to determine sites of branching.

2.2 Results

2.2.1 Mechanical stress is distributed non-uniformly across model epithelial tissues

To investigate whether patterns of mechanical stress were present within morphogenetic epithelia, we used the finite element method (FEM) to solve a computational model of an epithelial tubule contracting within a compliant ECM gel (Fig. 2.1A). We simulated contraction of the epithelium by prescribing an isometric prestrain chosen to match the experimentally measured relative change in the dimensions of the tubule after detergent-mediated relaxation. The resulting maximum principal stress within the tissue was computed. The elastic moduli of the epithelial and ECM portions of the model were
Figure 2.1: Patterns of mechanical stress correspond to sites of branching in epithelial tissues. (A) FEM mesh, showing the cellular (epithelium) and ECM (matrix) portions of the stress model. (B, C) FEM stress profile of an epithelial tubule. (D) Fluorescent image of engineered mammary epithelial tubule showing cellular membranes (green) and microbeads (red) embedded in the surrounding collagen gel. (E) Map of bead displacements around a single engineered tubule and (F) average displacements around 15 tubules. (G) Phase contrast image of engineered tubule before induction of branching. (H) Phase contrast image, (I) fluorescent image of E-cadherin (red) and nuclei (blue), and (J) frequency map of engineered tubules after induction of branching. (K, L) Branching is quantified by measuring the pixel intensity (PI) at a fixed location away from the tip in the grayscale frequency map, and then calculating the percent of tubules that have extended branches. Scale bars, 50 μm; displacement scale bar (red), 3 μm. Adapted from (112).
chosen to match those of normal mammary cells and tissue (36-37).

For cylindrical tubules, the FEM model predicted that stress would be distributed non-uniformly and concentrated at the tips of the tubules (Fig. 2.1B,C). To verify the predicted stress experimentally, we took advantage of the fact that regions within an elastic medium that experience higher mechanical stress store more elastic strain energy. Relaxing the strain results in displacement of the matrix to its reference (undeformed) state; the magnitude of displacement correlates with the local magnitude of stress (96, 113). We used a microfabrication approach to engineer mammary epithelial tubules of size and geometry corresponding to those of the FEM model, embedded in a collagenous matrix (29, 102). Matrix displacement was monitored by incorporating fluorescent beads within the surrounding collagen gel (Fig. 2.1D). Cell relaxation resulted in non-uniform displacement of the beads around the tubules (Fig. 2.1E,F). Larger displacements were recorded near the tips than near the trunks, indicating that stress was concentrated at the tips of the tubules, consistent with the predictions of the FEM model. Adding hepatocyte growth factor to the engineered tubules induced multicellular branches to invade into the surrounding collagen in a highly patterned fashion from the tips of the tubules (Fig. 2.1G-I), as reported previously (29). The average pattern of branching was visualized by stacking and color-coding multiple (~50) images of tubules stained with DAPI (Fig 2.1J). Comparing the stress distribution and branching profiles reveals that branches emerge from regions that experience elevated stress.
Figure 2.2: Patterns of mechanical stress and branching morphogenesis are affected by cellular contractility. Mechanical stress profiles of tubules with increasing contractility: prestress ($p_0$) that yields prestrain ($\varepsilon_0$) of (A) 0.2 % (B) 0.6 % (C) 1% and (D) combined stress profiles. (E-H) Matrix displacement maps around control tubules and tubules treated with 10 μM Y27632 or 0.1 nM calyculin A. (I-K) Frequency maps of branching from control tubules and tubules treated with 10 μM Y27632 or 0.1 nM calyculin A. (L) Branching was also quantified by measuring the pixel intensity 12 μm away from the tubule tip. Branch frequency decreases when tubules are treated with Y27632, and increases when tubules are treated with calyculin A. (M-O) Frequency maps and (P) quantification of branching from tubules constructed of cells transduced with adenovirus encoding RhoA-N19, RhoA-L63, or control vector. *$p<0.05$; **$p<0.01$; Scale bars, 50 μm. Adapted from (112).
The extent of branching was also quantified by measuring the pixel intensity within frequency maps at a fixed location from the tips of the initial tubule for the three replicates of each condition. The averaged pixel intensities at these locations were divided by the pixel intensity at the trunk of the tubule (corresponding to cell frequency of 100%) to yield the percent of tubules that had extended branches (Fig 2.1K,L).

### 2.2.2 Cellular contractility regulates mechanical stress and branching morphogenesis

To define the role of mechanical stress in branching morphogenesis, we perturbed the stress experienced by the tissue by modulating cellular contractility. To confirm that altering myosin-mediated contractility at the cellular level successfully perturbs mechanical gradients at the tissue level, we simulated the effect of contractility using the computational model introduced above. Varying the contractility of the epithelium in the FEM model had no effect on the relative patterns of simulated stress, but increasing contractility led to a higher magnitude of stress at the tips of the tubules (Fig. 2.2A-D). Based on these results, we examined how modulating cellular contractility affected mechanical stress and branching morphogenesis of the engineered tubules. Contractility of the actomyosin cytoskeleton is regulated in part by signaling through RhoA, its effector ROCK, and MLCK. Reducing contractility with the ROCK inhibitor Y27632 (114) or increasing contractility with the myosin light chain phosphatase inhibitor calyculin A (115) had no effect on the pattern of stress or branching (Fig. 2.2E-K). However, as contractility increased the magnitude of
stress and extent of branching (the fraction of tubules that branched) from the tips increased (Fig. 2.2H,L). Branching also correlated with contractility when tubules were transduced with dominant negative (RhoA-N19) or constitutively active (RhoA-L63) RhoA (Fig. 2.2M-P). Similar results were obtained using the MLCK inhibitors ML-7 and BDM and the phospholipid mediator lysophosphatidic acid (LPA) (Fig. 2.3). These data are consistent with the FEM predictions: as cellular contractility increases, the patterns of mechanical stress and branching morphogenesis are affected by cellular contractility. (A-E) Frequency maps of branching from control tubules and tubules treated with 0.1 μg/ml cytochalasin D, 10 μM ML7, 20 mM BDM, or 10 μg/ml LPA. (F) Branching was also quantified by measuring the pixel intensity 12 μm away from the tubule tip. Branch frequency decreases when tubules are treated with contractility inhibitors, and increases when tubules are treated with LPA. *p<0.05; **p<0.01; Scale bars, 50 μm. Adapted from (112).
stress and branching remain unchanged, but the magnitudes of stress and branching both increase.

Proliferation and cell motility are required for branching in this system (29). To test whether the contractility manipulations affected branching simply by affecting cell proliferation within the tubules, we incubated the tissues with the thymidine analog EdU, under each pharmacological treatment. The extent or pattern of EdU incorporation was not affected by the contractility modulators (Fig. 2.4A-L). To test for effects on cell motility, we tracked movements of cells stably expressing a nuclear localization
Figure 2.5: Patterns of mechanical stress and branching morphogenesis are affected by matrix stiffness. Mechanical stress profiles of tubules surrounded by matrices of different stiffness: (A) \( E_{\text{matrix}} = 680 \) Pa, (B) \( E_{\text{matrix}} = 860 \) Pa and (C) combined stress profiles. Frequency maps of branching tubules embedded in collagen gel crosslinked with (D) 0 and (E) 50 mM D-ribose. (F) Branch frequency increases as the stiffness of the collagen gel increases (G). Rheological measurements of collagen gels crosslinked with D-ribose. *p<0.05; Scale bars, 50 \( \mu \)m. Adapted from (112).

sequence-YFP (NLS-YFP) fusion protein. Cellular speed was not significantly reduced by treatment with contractility inhibitors (Fig. 2.4M). Thus, proliferation and motility were excluded as potential cellular mechanisms by which contractility influences branching.
2.2.3 Matrix stiffness regulates mechanical stress and branching morphogenesis

The mechanical properties of a tissue are defined by the contractility of the cells as well as by the compliance of the surrounding ECM. Stiff ECM disrupts the tubulogenesis of mammary epithelial cells (35) and induces proliferation, invasion and loss of mammary epithelial architecture (37, 39, 116). Matrix elasticity has also been shown to control membrane protrusions by individual endothelial cells (117) and growth factor receptor expression and sprouting during blood vessel morphogenesis (118). To examine the effect of ECM compliance on the stress profile within the tissue, we performed simulations in which we varied the elastic modulus of the matrix portion of the FEM model. Increasing matrix stiffness increased the magnitude of stress but had no effect on the pattern (Fig. 2.5A-C). We examined the effect of ECM compliance on branching morphogenesis experimentally by varying the stiffness of the collagen surrounding the engineered tubules by crosslinking with D-ribose (119) (Fig. 2.5G). The elastic moduli of the crosslinked gels corresponded to the moduli of the matrix within the FEM model. Increasing the stiffness of the matrix had no effect on the pattern of branching but enhanced branching from the tips (Fig. 2.5D-F). Thus, as the matrix becomes stiffer the patterns of mechanical stress and branching remain unchanged, but the magnitudes of stress and branching both increase.
2.2.4 Intercellular cohesion governs mechanical stress and branching of epithelial tissues

Figure 2.6: Intercellular cohesion is required for patterning stress and branching morphogenesis. β-catenin immunofluorescence in (A) control mammary epithelial cells and (B) cells expressing EΔ. Matrix displacement maps following relaxation of (C) control tubules and (D) tubules expressing EΔ. Frequency maps of branching from (E) control tubules and (F) tubules expressing EΔ. (G) Immunofluorescence analysis of EdU incorporation in one control tubule or (H) one tubule transduced with EΔ adenovirus. (I-J) Quantification of EdU incorporation in 50 tubules. (K) Average speed of cells within control tubules and tubules transduced with EΔ adenovirus. Error bars indicate s.e.m. Scale bars, 50 μm. Adapted from (112).

Individual cells within tissues are mechanically coupled to their neighbors via cadherin-mediated intercellular adhesions (120), and perturbing these connections has been found to prevent the transmission and concentration of mechanical stress (98). To disrupt the transmission of stress between cells within the tubules, we transduced the cells with an
adenovirus encoding a dominant-negative mutant of E-cadherin (EΔ) that blocks connection to the actin cytoskeleton by inhibiting the junctional localization of β-catenin (121) (Fig. 2.6A,B). We constructed mammary epithelial tubules from the EΔ-expressing cells. Expression of EΔ resulted in a flattened stress profile across the tubules, as determined by evaluating the displacements of beads in the collagen surrounding the tubules (Fig. 2.6C,D). Expression of EΔ completely prevented branching (Fig. 2.6E,F) without inhibiting cell proliferation or motility (Fig 2.6G-K). These data suggest that branching requires transmission of mechanical stress.

**Figure 2.7: Levels of FAK pY397 at cell-matrix adhesions are affected by cellular contractility.** (A-E) Immunofluorescence analysis of cells treated with 10 μM Y27632, 0.1 μg/ml cytochalasin D, 20 mM BDM, 10 μM ML7 or 0.1 nM calyculin A. Levels of FAK pY397 at the matrix adhesions decrease when cells are treated with contractility inhibitors, and increase when cells are treated with calyculin A. Scale bars, 50 μm. Adapted from (112).
2.2.5 Focal adhesion kinase activity is non-uniform across epithelial tissues

Figure 2.8: FAK is activated at tips of tubules and required for branching morphogenesis. (A) FAK pY397 immunofluorescence and (B) frequency map in engineered tubules. FAK pY397 immunofluorescence in (C) control cells and (D) cells expressing FAK Dter. Frequency maps of FAK pY397 in (E) control tubules and (F) tubules expressing FAK Dter. Frequency map of branching from (G) control tubules and (H) tubules expressing FAK Dter. (I) Western blot analysis of FAK pY397 and total FAK in tubules treated with Y27632 or calyculin A. FAK pY397 immunofluorescence in (J) control tubules and (K) tubules constructed in collagen crosslinked using ribose. (L) Immunofluorescence analysis of EdU incorporation in one control tubule or (M) one tubule transduced with FAK Dter adenovirus. (N-O) Quantification of EdU incorporation in 50 tubules. Error bars indicate s.e.m.; *p<0.05; Scale bars, 50 μm. Adapted from (112).

Although the influence of the mechanical environment over biological processes is well-documented, the molecular mediators of the process are largely unclear. We thus set out
to define the molecular mechanisms by which mechanical stress is sensed by the microfabricated mammary tissues and translated into a cellular response. FAK integrates a number of extracellular cues to control ECM adhesion and cell migration (122). Deletion of FAK retards mammary ductal elongation (123) and causes aberrant branching morphogenesis (124).

These findings, along with evidence that mechanical stress activates FAK by phosphorylation at Y397 (79-80), inspired us to consider the potential role of FAK in the mechanical regulation of branching morphogenesis. Indeed, mammary epithelial cells used in this study expressed FAK, which, in its phosphorylated form, was present within punctate focal adhesions (Fig. 2.7A). Treatment with pharmacological or molecular inhibitors and promoters of contractility led to concomitant increase or decrease in the activation of FAK, confirming the role of cellular tension in the process (Fig. 2.7B-I). Immunofluorescence analysis of multicellular tissues revealed that FAK phosphorylation was increased at the tips of the tubules (Fig. 2.8A,B), consistent with our experimental and computational findings that cells in these regions also experienced the highest stress. To test directly whether mechanosensing through FAK is necessary for branching of the engineered tubules, we expressed FAK Dter, a dominant-negative mutant that lacks the kinase domain (125). Cells that expressed FAK Dter showed reduced phosphorylation of Y397 at cell-matrix adhesions (Fig. 2.8C,D) and a homogeneously low level of FAK phosphorylation across the tubules (Fig. 2.8E,F). Furthermore, FAK Dter-expressing tubules failed to undergo branching morphogenesis (Fig. 2.8G,H). However, expression of FAK Dter had no effect on cell proliferation within the tubules (Fig. 2.8L-O).
expected, treatment with pharmacological agents that inhibited actomyosin contractility and branching led to decreased phosphorylation of FAK Y397, whereas phosphorylation was increased upon treatment with agents that activated contractility and branching (Fig. 2.8I). Furthermore, stiffening the collagen matrix, which increased extent of branching (Fig. 2.5), enhanced the phosphorylation of FAK Y397 at the ends of the tubules (Fig. 2.8J,K). These data suggest that mammary tissue can sense mechanical stress in part by activating FAK; activation of signaling through FAK is required for branching morphogenesis.

2.2.6 Tissue geometry patterns mechanical stress and branching morphogenesis

Recent studies have established a relationship between the geometry of 2D epithelial sheets and their patterns of endogenous mechanical stress (61, 98). To determine how geometry affects mechanical stress and branching morphogenesis of 3D epithelial tissues we varied the shape of the initial structure. Changing the shape altered the predicted distribution of mechanical stress across the tissue, with regions of high convex curvature experiencing the highest stress (Fig. 2.9A-C, M-O). Immunofluorescence analysis of engineered tubules of the corresponding geometries showed that activated FAK was concentrated in the high stress regions (Fig. 2.9D-F). Branching assays revealed a partial match between the patterns of stress and branching (Fig. 2.9G-I): regions of low stress were prevented from branching, but not all high stress regions branched (asterisks in Fig. 2.9H,I). This apparent discrepancy can be explained by considering the concentration profile of TGFβ, an epithelial-secreted morphogen that inhibits branching morphogenesis in this system (29). An FEM model of TGFβ secretion and diffusion predicted that every
Figure 2.9: Patterns of mechanical stress and branching morphogenesis are affected by tissue geometry. (A-C) FEM stress profiles of tubules of various geometry. Frequency maps of (D-F) FAK pY397 immunofluorescence and (G-I) branching from tubules of the corresponding geometries. Arrows denote regions of high mechanical stress that branch, and asterisks denote regions of high mechanical stress that do not branch. (J-L) Predicted concentration profiles of TGFβ around tubules of various geometry. (M-O) Relative strength of the mechanical and biochemical inputs as a function of position within the tissue. Scale bars, 50 μm. Adapted from (112).
high stress region that failed to branch was exposed to high concentrations of TGFβ (Fig. 2.9J-O). These results suggest that high mechanical stress is necessary but not sufficient for branching: when cells within the engineered tissues are presented with opposing mechanical and biochemical cues, the biochemical signal (i.e. TGFβ) dominates the resulting phenotype.

2.4 Discussion

Spatial non-uniformities of mechanical stress have previously been reported in 2D epithelial sheets (61, 98). Here, we predicted computationally and demonstrated experimentally that 3D model epithelial tissues experience patterns of endogenous mechanical stress that are dictated by the initial shape of the tissue. Although cytomechanical factors have been implicated in the morphogenesis of the lung (76) and kidney (77), the regulation of branching pattern has been traditionally ascribed to biochemical and genetic factors (103, 126). Indeed, approaches developed by our group recently demonstrated that the concentration profile of the autocrine inhibitory morphogen TGFβ acts locally to control morphogenesis by preventing spurious branching (29). Here, we showed for the first time that mechanical stress is elevated at branch sites, which suggests that mechanical gradients may also play a role in the patterning of branching morphogenesis. It is important to note, however, that branching only occurred at locations where the biochemical and mechanical cues were predicted to reinforce each other. Cells were prevented from branching when provided with the stimulatory cue of high mechanical stress and the inhibitory cue of high TGFβ.
concentration. Conversely, low TGFβ concentration failed to permit branching when mechanical stress was removed pharmacologically. We found also that the magnitude of mechanical stress at branching sites correlated with the extent of branching: decreasing the magnitude of stress by decreasing cellular contractility or matrix stiffness resulted in decreased branching, whereas increasing the magnitude of stress resulted in increased branching.

How might mechanical stress regulate branching morphogenesis? Possible mechanisms include removal of steric hindrances by matrix degradation and enhanced cellular motility and invasiveness. Branch initiation in the lung (76), salivary gland (110), and mammary gland (2) is preceded by local thinning of the basement membrane, which is thought to remove a physical obstacle, thus allowing the adjacent epithelium to invade in a fashion analogous to a “run in a stocking” (76, 78). Matrix turnover at branching sites has been attributed to a stress-induced local increase in the production of ECM-degrading MMPs (76). This local increase in MMP activity, however, necessitates the pre-existence of spatial non-uniformities in mechanical stress. In the present study we demonstrated that such mechanical gradients can arise within a model epithelial tissue from actomyosin contraction of individual cells and subsequent intercellular transmission of stress along the anisotropic tissue geometry. Consistent with this model, MMP14 is elevated at the leading edge of mouse mammary and ureteric ducts (28, 127-128) and at the tips of engineered mammary tubules (129). Inactivation of the Rho pathway abolishes both elevated MMP14 (129) and branching from the tips. Stress-induced branching may thus proceed through local stimulation of MMP activity (130-131).
Figure 2.10: Snail1, Snail2, and E47 are necessary and sufficient for branching morphogenesis of mammary epithelial cells (A) Microfabricated mammary epithelial tubules were treated with No GF or EGF for 8 h and stained for Snail1, Snail2, or E47. (B) Frequency maps of 50 tubules stained for Snail1, Snail2, E47, or vimentin. (C) Mammary epithelial cells were transfected with shSnail1, shSnail2, shE47, Sc, or NT and used to generate microfabricated mammary epithelial tubules. Tubules were treated with No GF, EGF, or HGF for 24 h. Branching was quantified as described above. All results were confirmed with at least two independent shRNA constructs for each gene. (D) Mammary epithelial cells were transfected with Flag-tagged Snail1, Snail2, E47, YFP, or NT and used to generate microfabricated mammary epithelial tubules. Branching was quantified as described above; error bars indicate ±s.e.m. ($n=5$). **$P<0.01$ versus NT (No GF) (two-way ANOVA with Bonferroni comparison). Scale bars, 50 μm. Adapted from (132).

High local stress could also activate a mechanically-sensitive signaling pathway resulting in enhanced cellular motility and invasion. Our observation of elevated levels of active FAK at branch sites, along with the finding that FAK activity is necessary for branching, suggests that high mechanical stress might induce branching morphogenesis in part through activation of FAK. At the cellular level, FAK regulates migration and invasion
Figure 2.11: Spatial patterning of EMT correlates with endogenous gradients of cytoskeletal tension. (A) FEM-predicted distribution of mechanical stress in rectangular epithelial sheets treated with TGFβ. (B) Immunofluorescence staining and (C) frequency map for αSMA expression in rectangular epithelial sheets treated with TGFβ. (D) FEM-predicted distribution of mechanical stress in sinusoidal epithelial sheets treated with TGFβ. (E) Immunofluorescence staining and (F) frequency map for αSMA expression in sinusoidal epithelial sheets treated with TGFβ. Scale bars, 50 μm. Adapted from (61).

by causing membrane protrusions and focal adhesion turnover (reviewed in (122, 133)). At the tissue level, deletion of FAK disrupts normal development of the mammary gland (123-124) and lung bronchioles (134). Conversely, tissue stiffness was found recently to activate FAK and induce invasion of mammary epithelial cells in culture and in vivo (116). Activated FAK could thus regulate branching by promoting migratory and invasive behavior at the tips of the tubules.

High mechanical stress may also result in gene expression changes at nascent branch sites. Expression of mesenchymal markers such as Twist-1, Twist-2 and Snail has been
observed within the terminal end buds of mouse mammary ducts (135), which, as predicted by the FEM model described above, likely experience elevated mechanical stress compared with the subtending duct. Consistently, neo-expression of genes linked to EMT occurs at the tip regions of engineered mammary epithelial tissues (29, 132). The roles of these genes in branching morphogenesis were recently elucidated by our group. We found that the transcription factors Snail1, Snail2 and E47 are necessary and sufficient to induce branching, likely through partial down-regulation of E-cadherin, subsequent loosening of intercellular adhesions and increased cell motility (132) (Fig. 2.10). Furthermore, evidence is emerging to implicate tissue-level mechanical gradients as the patterning cue of EMT within multicellular tissues. Our group recently found that mechanical stress induces patterned EMT in 2D epithelial monolayers, by inducing the nuclear translocation of EMT-inducing myocardin-related transcription factor A (MRTF-A) (Fig. 2.11) (61). A similar phenomenon may occur in the context of 3D epithelial tissues undergoing morphogenesis into collagenous ECM. Further studies are required to define the gene expression changes at branch initiation sites, which may be the junction at which mechanical and biochemical signals are integrated during branching morphogenesis.

It is important to emphasize that TGFβ is not responsible for the expression of mesenchymal markers in this system. Although TGFβ is a potent promoter of EMT in mammary epithelium containing an oncogenic or disruptive stimulus (136-138), the overwhelming majority of normal mammary epithelial cells, including the murine EpH4 cells used in our studies, do not undergo EMT upon treatment with TGFβ (139-140).
Indeed, our group has previously demonstrated that the spatial profile of TGFβ correlates inversely with the locations at which mesenchymal markers are expressed within our model tissues (29).

It is well accepted that mechanical changes accompany and drive malignant transformation and progression within branched organs such as the breast and lung (141-142). To the best of our knowledge, the work presented here provides the first experimental confirmation that mechanical stress is altered at branch initiation sites in multicellular aggregates, and supports the hypothesis that mechanical stress also dynamically orchestrates the normal morphogenesis of tree-like tissues (105), most notably the mammary gland. Accordingly, comprehensive understanding of mammary epithelial branching morphogenesis requires knowledge of the mechanical profile of homeostatic mammary tissue and how it evolves in time and space during development and disease. Finally, the results of this study, implicating mechanical stress as a regulator of organogenesis, underscore the need to consider endogenous multicellular mechanics in tissue engineering strategies aimed at faithfully reconstructing these organs ex vivo.

2.4 Materials and Methods

Cell culture and reagents. Functionally normal EpH4 mouse mammary epithelial cells were cultured in 1:1 Dulbecco’s Modified Eagle’s Medium: Ham’s F12 nutrient mixture (DMEM:F12) (HyClone), 2% fetal bovine serum (Atlanta Biologicals), 5 μg/ml insulin (Sigma), 50 μg/ml gentamycin (Sigma). Tubules were treated upon induction of
branching with Y27632 (Tocris), calyculin A (Calbiochem), cytochalasin D (Tocris), ML7 (Calbiochem), BDM (Calbiochem), or LPA (Cayman Chemical) diluted to the concentrations indicated in the text. Recombinant adenoviruses encoding E-cadherin lacking the β-catenin-binding domain (EΔ) and mutant FAK lacking the autophosphorylation site (FAK Dter) were gifts from Christopher Chen (University of Pennsylvania) and Lewis Romer (Johns Hopkins University), respectively. Recombinant adenoviruses encoding RhoA-N19 and RhoA-L63 were obtained from Cell Biolabs. High titer preparations of recombinant adenoviruses were generated using the AdEasy Virus Purification Kit (Stratagene). Cells were transduced at an MOI resulting in >99% transduction efficiency.

**Microfabrication.** Elastomeric stamps of poly (dimethylsiloxane) (PDMS; Sylgard 184, Ellsworth Adhesives) containing the desired geometries in bas-relief were fabricated by a combination of photolithography and soft lithography (29, 102). Stamps were rendered non-adhesive by coating with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Modified stamps were placed atop a drop of neutralized liquid collagen (Koken, Japan) which was then gelled at 37°C. Stamps were removed and a suspension of mammary epithelial cells was allowed to settle within the molded collagen cavities. The extra cells were washed away with culture medium and a gelled collagen “lid” was placed on top of the pattern. The epithelial cells adopted the shape and size of the collagen cavities they occupied, forming tubules that remained quiescent until they were induced to branch by adding hepatocyte growth factor (5 ng/ml; Sigma). Within 24 h after growth factor addition, multicellular branches invaded the surrounding collagen.
matrix. To increase the stiffness of the collagen surrounding the tubules, the molded gels were crosslinked by incubation in D-ribose at 37°C for one week prior to adding cells.

**Measurement of matrix displacements.** To visualize displacements within the collagen matrix, 1-μm diameter fluorescent microbeads (Invitrogen) were mixed with the neutralized liquid collagen. The positions of the beads in a single plane were recorded before and after relaxing the tubules with 0.05% Triton X-100 in PBS. The displacement maps were generated using the Imaris tracking software (Bitplane). To validate that greater displacements corresponded to greater stress, we used the displacement maps to calculate the average strain (which is proportional to the average stress) at the midsection and ends of the tubules.

**Rheometry.** The rheological measurements of collagen gels hydrated with culture media were performed on a Physica MCR 501 rheometer (Anton Paar) with cone-and-plate geometry. The chamber was held at 37°C and 100% humidity using a Peltier plate and humidity chamber. All measurements were performed in the linear viscoelastic regime (0.01-3% strain, 0.1-25 rad/sec angular frequency). The measured storage modulus ($G'$) was used to compute Young’s modulus ($E$) using $E = 2G'(1+\nu)$, assuming a Poisson ratio $\nu$ of 0.2(143).

**Imaging and immunofluorescence analysis.** To quantify the magnitude and pattern of branching, samples were fixed, stained for nuclei with Hoechst 33258 (Invitrogen), and visualized using a Hamamatsu Orca 1394 camera attached to a Nikon Eclipse Ti
microscope at 10× magnification. The binarized images of ~50 tubules were stacked with Scion Image software to obtain a pixel frequency map, which was subsequently color-coded in Adobe Photoshop. All experiments were conducted at least three times. The extent of branching was also quantified by measuring the pixel intensity within frequency maps at a location 12 μm from the tips of the initial tubule for the three replicates of each condition. The averaged pixel intensities at these locations were divided by the pixel intensity at the trunk of the tubule (corresponding to cell frequency of 100%) to yield the percent of tubules that have extended branches. Values less than 5% were considered to be noise arising from misalignment during stacking.

For immunofluorescence analysis of β-catenin and E-cadherin, samples were washed in PBS and fixed in 4% paraformaldehyde for 15 min at room temperature. Samples were permeabilized with 0.1% Triton X-100 in PBS (PBS-T) for 15 min and blocked for 1 h at room temperature in 10% goat serum in PBS (PBS-S), followed by overnight incubation in primary antibody recognizing β-catenin (Sigma) or E-cadherin (Cell Signaling) at 1:100 dilution in PBS-S. Samples were washed extensively with PBS and incubated in secondary antibody cocktail at 1:1000 in PBS-S for 1 h at room temperature.

For immunofluorescence analysis of FAK pY397, samples were washed in PBS and fixed in 4% paraformaldehyde for 15 min at room temperature. Samples were permeabilized 2 x 10 min in 0.5% Igepal Ca-630 and incubated in PBS-T for 15 min. Samples were blocked overnight at 4°C in PBS-S, followed by overnight incubation at 4°C in primary antibody recognizing FAK pY397 (Invitrogen) at 1:100 dilution in PBS-S.
Samples were washed extensively with PBS and incubated in secondary antibody at 1:1000 in PBS-S overnight at 4°C. Frequency maps were constructed from fluorescence images as described above.

Proliferating cells were visualized with the Click-iT EdU Imaging Kit (Invitrogen) as previously described (129). Frequency maps of cell proliferation were constructed from fluorescence images as described above.

**Numerical modeling.** A 3D computational model of the engineered tubules was solved for the profile of mechanical stress using the finite element method (FEM) in Comsol Multiphysics 3.5a software (Comsol Inc). For conciseness, we report the maximum principal stress across the tissues. No shear stresses exist in the principal directions at any point of the tissues. Thus, the highest of the three principal stresses (all normal) summarizes the stress state at that point in a single quantity. The epithelial tubules of varying geometry were represented as a contractile shell 10 μm thick (144) with Young’s modulus of 500 Pa (36) and Poisson ratio of 0.499 (practically incompressible). An equilibrium state was assumed and a zero pressure drop across the shell was prescribed. The tubule was embedded in a passive compliant slab of cylindrical geometry (2 mm in height and diameter), Young’s modulus of 170 Pa and Poisson ratio of 0.2, representing the collagen matrix in the culture model (37, 143). The external boundaries of the collagen matrix were fixed by prescribing zero displacement. The strains measured experimentally did not exceed 5%, which is well under the limit for linear behavior of collagen (143). Accordingly, the gel was modeled as a linearly elastic solid.
We modeled isometric tension arising from cellular contractility by prescribing a prestress \( (p_t) \) to the epithelial portion. The prestress value was chosen to match the prestrain \( (\varepsilon_0 \sim 0.6\%) \) found experimentally by measuring the length of initial and detergent-relaxed tubules. Stress tensors were calculated throughout the system, and the maximum principal stress through the midplane of the tubules was visualized and reported.

We modeled changes in cellular contractility within the epithelial tubule by changing the magnitude of the imposed prestress. In particular, to model increased or decreased contractility, we increased or decreased \( p_t \) to yield \( \varepsilon_0 \) of 0.2% or 1%, respectively. These values were determined experimentally by measuring the length of tubules treated with Y27632 or calyculin A before and after relaxation. Similarly, to model a stiffer ECM, we increased the modulus of the matrix portion to match the stiffness of the crosslinked gels at 860 Pa, while keeping the modulus of the epithelial shell fixed at 500 Pa.

**Real-time microscopy.** For real-time imaging, tubules were constructed of mammary epithelial cells that stably expressed NLS-YFP(129). Time-lapse movies were collected using a Hamamatsu ECCD camera attached to a Nikon Ti-U inverted microscope customized with a spinning disk (BD Biosciences) and fitted with a humidified environmental chamber held at 37°C and 5% CO\(_2\). Confocal stacks of 20-25 images (2 \( \mu \)m thick) were acquired using a Plan Apo 20× 0.4 NA objective every 15 min beginning
at 24 hours after initial microfabrication for a total of 20 hours. Movies were assembled and cells tracked in 3D using ImarisTrack (Bitplane).

**Western blot analysis.** Samples were lysed using RIPA buffer, mixed with Laemmlli sample buffer, heated at 75°C for 5 min, resolved by SDS/PAGE, and transferred to nitrocellulose. Membranes were blocked in 5% milk and incubated overnight at 4°C in 5% milk containing antibodies specific to FAK pY397, total FAK (Invitrogen) or actin (Cell Signaling). Antibodies were visualized using the ECL Plus Western Blotting Detection System (GE Healthcare).
Chapter 3

Mapping of mechanical stresses within three-dimensional mammary epithelial tissues

(Adapted from Gjorevski & Nelson, *Biophys J* 2012)

3.1 Introduction

Mechanical signals regulate a variety of basic cellular processes including survival, proliferation, differentiation and epithelial plasticity (61, 66, 98-99). Mechanical forces also drive the cellular changes which sculpt tissues and organs during embryogenesis (145-146) and can feed back to direct the activation of key molecular regulators of morphogenesis (75). Abnormalities in the mechanical environment of epithelial tissues can contribute to their malignant transformation and progression (35, 37, 39). In the previous chapter we presented evidence suggesting that tissue-level mechanical gradients in part regulate mammary epithelial branching morphogenesis. Quantitative and qualitative information about the mechanical forces imparted and experienced by cells and tissues in a physiologically relevant context is thus required to understand the physical basis of development and disease.

Several techniques have been created to measure the forces generated by single cells and sheets of cells plated on 2D substrata. Traction force microscopy (TFM), introduced by
Dembo, Wang and colleagues, remains the most widely used approach (95). In TFM, individual cells are plated on synthetic hydrogels with tunable mechanical properties. Movement or contraction of the cells causes the substratum to deform and the resulting displacements of the free surface are converted into traction forces using the inverse Boussinesq formulation. The mathematical complexity of TFM can be circumvented using microfabricated arrays of elastomeric pillars, with which spatially resolved cellular forces are calculated by measuring the deflection of the individual pillars (97, 147). These techniques have demonstrated that cells exert forces that are directed inward, toward the centroid of the cell, and that are mostly tangential to, but can also be normal to the surface of the 2D substratum (148-149).

Studies using these 2D systems have furnished valuable information about the mechanical behavior of cells and have unveiled several modes of mechanotransduction (64, 150). However, cells in vivo typically reside in a 3D microenvironment and the overwhelming majority of developmental, physiological and pathological processes are inherently 3D. In an effort to assess cellular mechanics in a more physiological 3D context, Chen and colleagues developed a method to measure the traction forces imparted by single cells fully encapsulated within a synthetic polyethylene glycol hydrogel (151). In this study, a finite element mesh was generated to represent the cell and surrounding gel and a discretized Green’s function was used to convert the experimental displacements into tractions at each node. Cells in these synthetic 3D matrices exerted inward-directed, tangential forces near long membrane protrusions, and small inward-
directed normal forces near the cell body. The traction behavior of single cells in 3D was thus surprisingly analogous to the behavior of those attached on 2D surfaces.

Efforts in mechanobiology have thus focused on measuring the forces generated by single, usually fibroblastic or cancerous, cells. However, early development and organogenesis are largely epithelial phenomena and epithelial cells rarely function individually. Instead, these cells are connected to each other and the ECM to form 3D polarized epithelial tissues. Mammary epithelial branching morphogenesis, in particular, is a multicellular process during which the maintenance of intercellular junctions enables efficient long-range transmission of biochemical and biophysical signals, critical for sculpting the complex functional architecture of the mammary gland (3). Even force information about individual, metastatic cancer cells is only partially useful in defining the physical basis of cancer until placed within the mechanical context of the primary tumor or untransformed tissue. Indeed, cancer’s metastatic spread often proceeds through the collective migration of an interconnected group of cells (152-153). It is unlikely that the mechanical stress generated by cellular collectives is simply the sum of the stresses generated by their constituent cells. The interconnectivity and altered topology of cells within 3D multicellular tissues likely gives rise to emergent mechanical behavior. Quantitative methods to measure forces generated at the tissue level are therefore needed.

Here, we took advantage of the uniform nature of microfabricated tissues to uncover the experimental parameters required for precise calculation of mechanical forces exerted by 3D epithelial tissues. Using this approach, we measured the mechanical stresses exerted
by quiescent mammary epithelial tissues of arbitrary geometry, surrounded by a matrix of
native type I collagen. The use of native collagen matrices enabled us to recapitulate
physiologically relevant tissue-mediated changes in the local material properties, which
we found to have a significant effect on the mechanical profile of the tissues. Traction
forces at the epithelial surface and patterns of normal and shear stresses throughout the
surrounding matrix were quantified and found to be dictated by the geometry of the
epithelium. Although the cells within the tissues were contracting and thus generating
tension, the tissue itself could exert compressive forces when engineered into certain
physiologically relevant shapes.

3.2 Results

3.2.1 Mapping of mechanical stress within 3D epithelial tissues

To compare the mechanical behavior of mammary epithelial tissues to that of individual
cells, we first examined the contractile activity of single mammary epithelial cells seeded
on top of or embedded within collagen gels. We monitored the contractile activity of the
cells and the resulting matrix deformation by tracking fluorescent beads embedded within
the collagen. Single epithelial cells plated on top of collagen induced sharp displacement
gradients, with maximum displacements occurring near large membrane protrusions (Fig.
3.1A). Single epithelial cells fully embedded in collagen, on the other hand, induced
negligible displacements, with magnitudes under the threshold of
Figure 3.1: Matrix deformation within 3D mammary epithelial tissues. (A) Substratum deformation induced by a single epithelial cell plated on top of collagen gel. (B) Matrix deformation induced by a single epithelial cell fully embedded within collagen gel. (C) Diagram showing components of microfabricated tissues: multicellular epithelial duct is surrounded by type I collagen which contains embedded fluorescent beads. (D) Signal-to-noise ratio in one sample and average signal-to-noise ratio of 30 samples. (E) Average 3D matrix deformation induced by 30 epithelial tissues. Scale bars, 50 μm. Adapted from (Gjorevski and Nelson, Biophys J 2012).

experimental noise (Fig. 3.1B). Notably, cells embedded within collagen displayed a round morphology and lacked actin-rich protrusions.

To elucidate the mechanical behavior of quiescent epithelial tissues, we constructed multicellular mammary tissues of precisely controlled size and geometry using the microfabrication method described previously (102) (Fig. 3.1C). Each tissue was identical in size and geometry, allowing us to average the displacement data collected over multiple samples, thereby enhancing the signal-to-noise ratio (Fig. 3.1D). The
Figure 3.2: Mechanical characterization of collagen gels. (A) Plot of the Young’s modulus, calculated from the shear modulus $G'$ with a Poisson ratio of 0.2, as a function of applied strain. The tissue-induced strain values at most locations throughout the gel fell below 5%, and maximum strain of 7% was recorded. (B) Plot of the Young’s modulus as a function of frequency. Measurements were conducted at applied strain of 1%. The Young’s modulus of the collagen gels is not influenced significantly by applied strain magnitude or frequency. Plots show average ± s.e.m. Adapted from (Gjorevski and Nelson, *Biophys J* 2012).

epithelial tissues induced significant displacement of the beads (Fig. 3.1E, n=30). The displacements were notably larger than those around individual epithelial cells embedded within collagen, and formed a gradient that spanned the three dimensions of the tissue. However, in marked contrast to the cell-level gradients that arose when single cells lay on top of the gel, the displacements changed negligibly over the length of individual cells within the tissue. These data suggest that epithelial tissues contract as a continuum and thereby exhibit emergent mechanical behavior, starkly different from the individual behavior of their constituent cells in a 2D or 3D environment.

In the work described in Chapter 2, we inferred the spatial distribution of mechanical stress based on patterns of displacement in the collagen gel adjacent to the epithelium. To characterize the mechanical environment of the epithelial tissue rigorously, quantification
of the mechanical forces experienced by the cells of the epithelium and the stresses that accumulate within the matrix is required. In addition to deformation information, this problem requires knowledge of the material properties and constitutive behavior of the medium, i.e. the collagen gel. The bulk material properties of the gel were determined by rheometry and its constitutive behavior was ascertained by examining the stress-strain relation under experimentally relevant magnitudes and frequencies of forces. The stress-strain relation that was recorded remained linear throughout the strain regime (Fig. 3.2). Accordingly, Hooke’s law for isotropic materials was used to describe the constitutive behavior of the collagen gels during tissue-induced deformation. Strains throughout the collagen gel were calculated from the 3D displacement field using the displacement gradient matrix (Eq. 7). The Cauchy stress tensor throughout the gel was calculated directly from the strains, using Hooke’s law for isotropic materials (Eqs. 8-10). Traction forces at the epithelium-matrix interface were subsequently calculated (Eqs. 11-12). Owing to the complex 3D geometry, we reconstructed the epithelial surface and

Figure 3.3: Mechanical stress distribution over 3D mammary epithelial tissues. (A) Epithelial surface reconstruction from confocal stack of tissue stained for cell membranes and finite element mesh generation. (B) Average 3D traction forces over epithelial surface of n=30 tissues. (C) Validation of constitutive model and assumption of homogeneity for collagen gel. Adapted from (Gjorevski and Nelson, *Biophys J* 2012).
Figure 3.4: Effect of error in material properties and choice of constitutive model for collagen on calculation of stress. (A) Comparison of matrix deformation measured experimentally and deformation simulated in homogeneous, elastic material of varying material properties. (B) Comparison of matrix deformation measured experimentally and deformation simulated in homogeneous, viscoelastic material. Viscoelastic parameters were determined by fitting the two-mode Maxwell model to the experimental $G'$ vs frequency data obtained by bulk rheometry. Adapted from (Gjorevski and Nelson, *Biophys J* 2012).

computed the surface tractions using FEM (Fig. 3.3A-B). The magnitude of the traction vectors across the surface of the epithelial tissue was non-uniform; maximum traction was observed at the short ends of cylindrical tissues (Fig. 3.3B), as predicted computationally in Chapter 2. To test the validity of the constitutive model, we ascribed experimental displacements measured near the tissue as boundary conditions of a homogeneous, isotropic computational domain and simulated displacements away from the boundary into the domain. The simulated displacements were compared to those measured experimentally throughout the collagen gel (Fig. 3.3C). We found that the displacements measured experimentally were consistently higher and propagated further than those calculated from the model, suggesting that additional information is needed to accurately calculate tissue-generated forces. There are three possible explanations for the discrepancy: error in the material properties of collagen measured by bulk rheometry,
Figure 3.5 Visualization and quantification of tissue-induced mechanical heterogeneities within the matrix. (A) Confocal reflection image of collagen gel around single cell-free molded cavity. (B) Average collagen intensity around 20 cell-free cavities. (C) Confocal reflection image of collagen gel around single epithelial tissue. (D) Average collagen intensity around 20 epithelial tissues. (E) Quantification of collagen intensity in A and C. (F) Quantification of collagen intensity in B and D. (G) The elasticity of the collagen surrounding the tissue was probed using AFM. Shown is a representative plot of the collagen gel stiffness away from the side and the end of the tissue. Stiffness maps of the matrix surrounding the epithelium were generated for three separate tissues and averaged. Adapted from (Gjorevski and Nelson, *Biophys J* 2012).
inadequate constitutive model for collagen, or local variations in the mechanics of the gel. We tested the contributions of these potential sources of error computationally. Varying the bulk mechanical properties of the computational domain to account for possible inaccuracies in the rheometric analysis did not change the simulated displacement profile (Fig. 3.4A). Similarly, using a viscoelastic constitutive model to describe the computational domain did not affect the profile of the simulated displacements (Fig. 3.4B). Accordingly, we set out to test whether mechanical heterogeneities or anisotropies within the collagen gel were responsible for the discrepancy.

3.2.2 Epithelial tissues cause mechanical heterogeneities in the surrounding matrix

Cells remodel the ECM during development and disease progression (154). Mammary epithelial cells in vivo synthesize and deposit several ECM proteins, including laminins and collagens, thereby altering the local ECM density and possibly also the local mechanical properties (155). Conversely, cells of the mammary gland express several matrix-degrading enzymes including matrix metalloproteinases, which can locally degrade the ECM (18, 28, 129). Breast cancer cells have been shown to contract, remodel and align collagen matrices in culture (156). Cell-induced remodeling may give rise to local differences in the material properties of the ECM, rendering it mechanically heterogeneous or anisotropic. In such a case, the assumption of homogeneity and isotropy would likely be a source of inaccuracies in solving the inverse problem.
Figure 3.6: Mechanical heterogeneities significantly affect the mechanical profiles of mammary epithelial tissues. (A) Experimentally measured tissue-induced matrix displacements throughout a midsection of the tissue. (B) Matrix deformations recovered assuming a homogeneous material. (C) Matrix deformations recovered within a heterogeneous material. Mechanical variations were ascribed from experimental AFM data. (D) Matrix displacements in A, B and C along a line away from the end of the tissue. (E) and (F) Discrepancy between experimental deformation and deformation simulated in a homogeneous or heterogeneous matrix, respectively. (G) Traction at the epithelial surface assuming a mechanically homogeneous or heterogeneous matrix. (H) Average traction at the end of the tissue assuming a homogeneous or heterogeneous matrix. Adapted from (Gjorevski and Nelson, *Biophys J* 2012).

To determine whether the engineered epithelial tissues remodeled their surrounding ECM, we visualized the structure of the collagen gel using confocal reflectance microscopy (157-159). Imaging of cell-free gels revealed a spatially homogeneous distribution of collagen fibrils, indicating that the microfabrication process alone did not introduce heterogeneities in the structure or density of collagen (Fig. 3.5A,C,D,F). However, we found that the matrix surrounding engineered tissues displayed consistently higher signal intensities near the epithelium, suggesting a cell-mediated local increase in collagen.
density (Fig. 3.5B,C,E,F). These changes may be attributed to strain-induced matrix compaction, alignment, or de novo collagen synthesis, as discussed above.

To test whether the heterogeneities in collagen density led to locally altered mechanical properties, we used atomic force microscopy (AFM) to measure the micro-scale elasticity of the collagen matrix surrounding the epithelium. We probed the stiffness of the gel at various locations around the tissue and generated a stiffness map of the region (Fig. 3.5G). This approach revealed striking mechanical variations: the stiffness of the collagen increased near the epithelium. However, whereas a relatively shallow stiffness gradient (spanning approximately 1 kPa) was detected near the side of the epithelial tissue, a sharp gradient, which spanned approximately 4 kPa, was present near the short ends of the tissues (Fig. 3.5G). Curiously, the stiffness profile of the gel did not fully correlate with the map of collagen density as visualized by confocal reflectance microscopy. The stiffness map showed a similar profile as the maps of tissue-generated deformation of the gel (Fig. 3.1E), motivating us to speculate that the sharp increase of stiffness near the end of the epithelial tubes results from local compaction or alignment of the matrix.

3.2.3 Cell-induced matrix heterogeneities significantly affect the mechanical profile of epithelial tissues

To test the effect of variations in stiffness on the calculation of force, we simulated deformation of a gel which incorporated the mechanical heterogeneities measured by
AFM (Fig. 3.6A-C). The resulting displacements (Fig. 3.6C) were compared to the experimentally measured displacements (Fig. 3.6A) and to those simulated within a mechanically homogeneous gel (Fig. 3.6B). We discovered that accounting for mechanical variations dramatically reduced the discrepancy between the simulated and the measured displacements (Fig. 3.6D-F), which strongly suggests that assuming homogenous mechanical properties is not appropriate to capture the mechanical behavior of collagen in this context. Accordingly, we incorporated the stiffness variations of the gel, as measured by AFM, and recalculated the mechanical stress throughout the midsection of the epithelial tissue (Fig. 3.6G). Accounting for mechanical variations did not have a discernable effect on the pattern, but significantly altered the magnitude of the surface traction calculated. In particular, the average traction at the ends of the epithelium increased nearly two-fold (Fig. 3.6G-H).

We also used this approach to calculate the major components of the Cauchy stress tensor throughout the collagen gel surrounding the epithelium (Fig. 3.7). These were consistent...
with the tractions calculated at the epithelial surface. High positive (tensile) stresses were observed in the direction locally normal to the epithelial surface, whereas negative (compressive) stresses accumulated in the direction tangential to the surface at each location (Fig. 3.7A,B). The magnitude of the tensile stresses was significantly higher in the matrix regions surrounding the short ends of the epithelium, consistent with our finding that the epithelium at this region pulls with higher traction forces. Striking patterns of high shear stresses were also notable at these regions (Fig. 3.7C). There thus
appeared to exist a consistent relationship between types and magnitudes of mechanical stress, on one hand, and geometrical features within the tissue on the other.

3.2.4 Epithelial tissue geometry dictates the spatial distribution of mechanical stress

We showed above that multicellular mammary epithelial tissues exhibit emergent mechanical behavior that differs from that of individual epithelial cells. Cells of the mammary gland self-organize into a structure which contains a variety of geometrical features, including acute and obtuse angles (at bifurcations) and varying degrees of positive and negative curvature. To examine how the geometric boundary conditions of the tissue affected the resulting mechanical behavior, we constructed epithelial tissues that recapitulate geometrical aspects of the mammary ductal tree (Fig. 3.8A,G). The tissue-mediated increase in collagen density near the epithelium appeared to be independent of the boundary conditions and local geometry (Fig. 3.8B,H), which, by contrast, profoundly affected the extent of deformation of the adjacent matrix and the mechanical profile of the tissue. Large matrix deformations generally occurred in the directions along the long axes of the tissue, near acute angles and near regions of high curvature (Fig. 3.8C,I). Large traction forces were observed at angular regions of the epithelial boundary, with acute angles pulling on the matrix with higher inward force than obtuse angles (Fig. 3.8D). The mechanical stresses arising within the matrix adjacent to the epithelium also depended upon the tissue geometry. Tensile stresses arose in the direction locally normal to the epithelial surface, whereas compressive stresses...
Figure 3.9: Emergent compression in epithelial tissues is driven by actomyosin-mediated contractility. (A) Position of fluorescent marker beads around a single control epithelial tissue in its reference (relaxed) and deformed configuration. (B) A map of average matrix displacements around multiple (n=17) control tissues. (C) Position of fluorescent marker beads around a single tissue treated with blebbistatin (25 μM) in its reference (relaxed) and deformed configuration. (D) A map of average matrix displacements around multiple (n=17) tissues treated with blebbistatin. Adapted from (Gjorevski and Nelson, Biophys J 2012).

accumulated in the locally tangential direction (Fig. 3.8E,F). Tensile stresses were larger in the matrix adjacent to acute epithelial angles than in matrix regions near obtuse epithelial angles (Fig. 3.8E,F).

We observed unexpected geometry-dependent mechanical behavior in curved duct-like tissues (Fig. 3.8G). Although regions of high curvature consistently generated higher traction forces (Fig. 3.8J), the forces were not directed inward everywhere along the epithelial boundary. In particular, the forces generated at regions of concave curvature
were directed outward, suggesting that the epithelium at these regions pushes against the matrix (Fig. 3.8J). Notably, we did not observe cell proliferation localized to the regions experiencing compressive forces (data not shown). Furthermore, blocking myosin motor activity abolished both the tensile forces at the convex regions and the compressive forces at the concave regions of the tissue (Fig. 3.9). These data together strongly suggest that the compressive forces at the tissue-level are driven by the same molecular mechanisms that drive cellular contraction, and are not simply a product of expansive growth, as has been observed in the case of growing tumor spheroids (151, 160). This mechanical behavior of the epithelium is reflected by the distribution of stresses within the surrounding matrix: whereas compressive stresses typically arose in the direction locally tangential to the epithelial boundary, compressive stresses near concave boundaries accumulated in both the locally normal and tangential directions (Fig. 3.7K,L). This phenomenon further highlights the emergent mechanical behavior of multicellular epithelial tissues: whereas individual epithelial cells can only pull on the surrounding matrix in a myosin-dependent fashion, the collective myosin-mediated contraction of epithelial tissues can give rise to regions where the tissue effectively pushes against the matrix.

3.2.5 Mechanical interaction between adjacent epithelial tissues

It is by now well-established that biochemical and mechanical signals are communicated between multiple cell types to guide the development of the mammary gland and maintain its function (3). Exchange of signals within the tissue is critical to maintain the
open ductal architecture of the gland: mammary branches navigate the fat pad to maintain proper spacing and avoid aberrant crossing or collisions with adjacent ducts. The mechanisms underlying this phenomenon are unclear.

Communication between and within tissues and organs is mediated by the transmission of secreted autocrine and paracrine molecular signals, which can act at varying length scales.
In addition, long-range communication can be established through mechanical interaction (161). Endothelial cells plated on compliant substrata can sense each other by detecting strains generated by neighboring cells and channeled through the matrix (57). This mechanical interaction was shown to direct the migration of individual cells, and promote the establishment of cell-cell contacts, thus possibly fostering tissue development. Similarly, fibroblasts and human mesenchymal stem cells in 3D fibrin matrices can communicate position and orientation via long-range transmission of mechanical signals (162).

To test whether a homologous form of traction force-mediated mechanical communication can occur between mammary epithelial tissues, we varied the distance between epithelial tissues and measured their corresponding mechanical profiles. We used microfabrication to position the tissues relative to each other such that one end of the tissue (hereafter referred to as ‘distal’) was always far away from neighboring tissues, whereas ‘proximal’ ends of adjacent tissues were separated by 400, 200, 100 or 50 μm (Fig. 3.9). At a separation of 400 μm, there was virtually no difference between the matrix deformation recorded or the traction force calculated at the distal and proximal ends (Fig. 3.9A-C), suggesting that the tissue was mechanically “unaware” of the neighboring epithelium. Reducing the distance between the tissues to 200 μm resulted in a significant decrease in the matrix deformation at the proximal end and a moderate decrease in the corresponding traction forces (Fig. 3.9D-F). The difference in both matrix deformation and traction between the distal and proximal ends was further increased as the tissues were positioned 100 μm or 50 μm apart (Fig. 3.9G-L). These results indicate
that the mechanical profile of epithelial tissues is not fully determined by their constituent cells, the multicellular geometry and the material properties of the matrix immediately surrounding the tissue. The final mechanical landscape of the tissue is also affected by its broader mechanical environment – in this case, forces generated by neighboring tissues. The nature, extent and length scale of this mechanical interaction likely depends upon multiple factors including the stiffness of the matrix and the contractile activity of the participating tissues.

3.3 Discussion

Recent methodological advances have enabled measurement of 3D forces exerted by cells attached to 2D substrata (149) and fully embedded within synthetic hydrogels (151). Although these studies have dramatically improved our knowledge of how cells interact with their physical microenvironment, we are still far from understanding how these interactions are governed in a native, physiological context (163). In particular, previous studies fall short in recapitulating the physical complexity of the native ECM, which is chemically and mechanically heterogeneous, viscoelastic, nonlinear and often changes dynamically over time spans relevant to developmental and pathological processes. Further, they report forces generated by single cells, leaving the contribution of intercellular forces, which serve to not only maintain the integrity of epithelial tissues but also to regulate their morphogenesis (146, 164), as a major unknown (163). Here, we combined 3D tissue microfabrication approaches with TFM, confocal reflectance and atomic force microscopy to examine the mechanics of 3D epithelial tubules within a
native ECM. Although we initiated this study with the objective of defining the mechanical behavior of quiescent mammary tissue, the principles and methodology it yielded can be generalized to any multicellular epithelial tissue surrounded by ECM. In particular, our data reveal the existence of unexpected mechanical behaviors in multicellular tissues, and uncover several parameters to consider in future endeavors to mechanically map native tissues.

The microfabrication approaches allowed us to engineer epithelial tissues with architecture and structure reminiscent of those of the mammary ducts and numerous other ductal tissues within the body, such as secretory glands and the vascular system. By embedding the epithelium within type I collagen, a major component of the native mammary ECM, we made a step forward in capturing the complexity of the physiological environment. In particular, we recapitulated physiologically realistic (154-156) ECM remodeling processes in which the epithelial tissue introduced mechanical heterogeneities within the surrounding matrix and which are overlooked when using bioinert synthetic hydrogels. We visualized these non-uniformities and demonstrated that they lead to mechanical heterogeneities by measuring the local mechanical properties of the collagen gel directly. Our data strongly suggest that the accurate calculation of mechanical stress in these settings requires accounting for these mechanical variations and that this parameter should be considered in attempts to quantify mechanical stress in native tissues.

Whereas previous investigations have measured cell-matrix forces that arise due to the contraction of single cells, we calculate mechanical stresses exerted by multicellular
epithelial tissues, thereby defining their intrinsic mechanical ‘tone’. Although our study does not decouple the separate contributions of cell-cell and cell-matrix forces, it reports the overall tissue mechanical profile, which is sculpted by both and displays a number of emergent characteristics that are not observed in single cells. Indeed, the very ability of the cellular collective to exert force sufficient to deform the surrounding matrix seems to be directly dependent upon the existence of intercellular forces, as no deformation of the matrix around single epithelial cells was recorded. A notable difference between the mechanics of 3D multicellular tissues explored here and those of single fibroblastic cells in 3D is that the latter exert large inward-directed shear forces and small inward-directed normal forces (151), whereas wide regions of large inward-directed normal forces were observed at the ends of the duct-like tissues. Moreover, we discovered that the collective contraction of the interconnected group of cells can give rise to emergent outward-directed normal (compressive) forces, which are absent when a single cell interacts with a 3D matrix (151). Surprisingly little attention has been given to the biological roles of endogenous tensile (pulling) and compressive (pushing) forces, which might have distinct or even opposing effects. For example, whereas high tensile stresses in single cells and cellular sheets have been associated with proliferation (64, 98), accumulating compressive stresses are postulated to arrest the cell cycle during development of the Drosophila wing imaginal disc (165-166). The minimal attention given to the potentially separate roles of shear, tensile and compressive stresses might owe to the fact that current models and techniques for quantification fail to recapitulate them fully and reproducibly.
Further, we showed that the endogenous mechanical tone of mammary epithelial tissues is affected by nearby contractile tissues, which may offer a physical equivalent of paracrine chemical signaling as a mechanism for communication between neighboring cells and tissues. It is tempting to speculate that such a mechanism may be used by mammary epithelial branches in sensing nearby ducts and responding accordingly to navigate away and prevent collisions. Of course, this hypothesis must be tested rigorously \textit{in vivo}.

The microfabrication and 3D traction force microscopy methods presented here allow us to directly measure the mechanical stresses generated by 3D epithelial tissues, define the parameters which govern epithelial force generation and subsequently fabricate tissues with precisely tuned mechanical profiles. Tissue geometries can be designed to control not only the magnitude of stress at a certain location, but also the type of stress (tensile, compressive, shear). Such controlled application of force can help elucidate how cells and tissues sense and respond to quantitative and qualitative variations in force. The use of microfabrication enabled averaging of displacement data across multiple tissues of fixed size and geometry, thus increasing the signal-to-noise ratio and confidence in interpretation of the data, and removing artifacts. Finally, this method furnishes knowledge of the epithelial geometry, the material properties and the deformation of the matrix by simultaneous imaging of the cells, beads and ECM, and can thus provide a promising platform to explore the long-term spatiotemporal variations in the mechanical landscape of morphogenetic epithelial tissues.
3.4 Materials and Methods

Cell culture and reagents

Functionally normal EpH4 mouse mammary epithelial cells were cultured in 1:1 DMEM:F12 supplemented with 2% fetal bovine serum (Atlanta Biologicals), 5 μg/ml insulin, and 50 μg/ml gentamicin (Sigma).

Microfabricated tissues

3D epithelial tissues were constructed as described previously (102). Briefly, neutralized liquid type I collagen (4 mg/ml; Koken) was gelled at 37°C around stamps of poly(dimethylsiloxane) (PDMS; Sylgard 184, Ellsworth Adhesives) to generate micrometer-scale cavities of defined size and geometry. A concentrated suspension of mammary epithelial cells was allowed to settle within the cavities and a second layer of collagen was placed on top of the gel. Cells were initially randomly dispersed within the cavities; individual cells formed junctions with each other and the surrounding collagen, secreted a basement membrane, and organized into a 3D epithelial tissue within 24 hrs (29).

Scanning Electron Microscopy (SEM)

The internal structure of the collagen gels (formed as described above) was visualized using SEM. The gels were prepared for SEM by fixation, followed by serial dehydration. Specifically, the samples were fixed in 4% paraformaldehyde for 1 h at room temperature, and then washed with ddH2O twice for 10 min. The samples were then subjected to serial
dehydration using an ethanol/ddH₂O series: 30%, 50%, 70%, 90% and two 100% ethanol washes for 10 min each. The samples were subsequently washed using a graded ethanol/HMDS series: 33%, 50%, 66% and 100% HMDS washes for 15 min each. Finally, the gels were cut and dried overnight with their cross-section facing up. The dried samples were mounted on SEM sample stubs using carbon tape and coated with Pd/Au to a thickness of 6 nm using a Denton Vacuum Desk II sputter coater (Denton Vacuum). The samples were imaged at 5,000× and 10,000× magnification using a JEOL 840 SEM (JEOL Ltd) (Fig. 3.11). The average pore size within three separate 5 μm × 5 μm regions of the three different gels was measured manually in ImageJ (NIH). Keeping in mind that the average pore size of the gel (~100 nm) was significantly smaller than
both the beads (1 μm), the average distance between the beads (17±4 μm), and the smallest dimension of the force-applying epithelial tissues (50 μm), we modeled the collagen gel as a continuous medium.

**Measurement of Darcy permeability and pore size estimation**

Permeability was measured by monitoring the flow rate of water through microfabricated channels of collagen under a known hydrostatic pressure. Channels within PDMS elastomers (cross-sectional area of 1 mm² and length of 1 cm) were formed using standard lithography techniques and filled with collagen. Flow rates induced by a hydrostatic pressure of 1 cm H2O were measured. The Darcy permeability (K) of the sample was determined from the flow rates and the geometric features of the apparatus, using Darcy’s law:

\[
Q = K \frac{A \Delta P}{L \mu}
\]

where \( Q \) is the flow rate, \( A \) is the cross-sectional area of the channel, \( L \) is the length of the channel, \( \Delta P \) is the pressure drop and \( \mu \) is the viscosity of water. Our measurements yield a permeability constant of 0.070±0.016 μm² for the collagen gels used in this study.

We estimated the fiber diameter by using the empirical form of the Spielman-Goren fiber matrix model, which describes the transport properties of a random fibrous network (167):

\[
K = 0.31a^2 \phi^{-1.17}
\]
where $a$ is the fiber radius and $\phi$ is the solid fraction of the gel, obtained by multiplying the collagen concentration with the effective specific volume of collagen, reported previously as 1.89 ml/g (168). We estimated a fiber diameter of 54±6 nm, which is in reasonable agreement with the fiber sizes measured from the SEM images.

Finally, we used the Carman-Kozeny model (169-170) to estimate the pore size of the collagen gel:

$$K = \frac{\varepsilon a^2}{4k}$$  \hspace{1cm} (3)

This model treats the gels as a mesh of cylinders, randomly oriented in three dimensions and described by a geometric factor $k$:

$$k = (2k_1 + k_\parallel)$$  \hspace{1cm} (4)

where:

$$k_1 = \frac{2\varepsilon^3}{(1-\varepsilon) \left[ 2\ln\left(\frac{1}{1-\varepsilon}\right) - 3 + 4(1-\varepsilon) - (1-\varepsilon)^2 \right]}$$  \hspace{1cm} (5)

$$k_\parallel = \frac{2\varepsilon^3}{(1-\varepsilon) \left[ \ln\left(\frac{1}{1-\varepsilon}\right) - \frac{1-(1-\varepsilon)^2}{1+(1-\varepsilon)^2} \right]}$$  \hspace{1cm} (6)

The porosity of the gel $\varepsilon$ is related to the solid fraction by the equation $\varepsilon = 1 - \phi$. Using Eqs. 3-6, we estimated a pore size of 435±26 nm.

**Calculation of stress within epithelial tissues**

**Measurement of matrix displacements**
To visualize tissue-induced matrix deformations, 1-μm diameter fluorescent polystyrene beads (Invitrogen) were dispersed in the neutralized collagen solution at high density (\(\sim 4 \times 10^8\) beads/ml). Confocal stacks of 120 images (spaced 1 μm apart) were collected before and after relaxing the tissues with 0.05% Triton X-100 in PBS using a Hamamatsu ECCD camera attached to a Nikon Ti–U inverted microscope customized with a spinning disk (BioVision Technologies). After lysing the tissues, the retraction of the collagen gel was virtually instantaneous. 3D and in-plane bead displacements were extracted using the Autoregressive Motion tracking routine in Imaris (Bitplane). The tissue-induced strains within the collagen gel were calculated from the full 3D displacement field, using the displacement gradient matrix:

\[
\epsilon_{ij} = \frac{1}{2} \left( \frac{\partial u_i}{\partial x_j} + \frac{\partial u_j}{\partial x_i} \right)
\]

(7)

Where \(i=1,2,3\), \(\epsilon\) is the strain tensor, \(u_i\) is the displacement in direction \(i\), and \(x_i\) are rectangular spatial coordinates. The calculated strain values never exceeded 7%.

To quantify the experimental noise of displacement measurement, we monitored the positions of fluorescent markers in cell-free collagen gels. Averaging the displacement maps of 30 samples led to a nearly five-fold increase in the signal-to-noise ratio, expressed as the ratio between the maximum cell-induced displacement and maximum recorded noise.

The 3D collagen gel surrounding the tissue was prepared in a layer-by-layer fashion, as described above. To confirm that the two collagen layers were integrated mechanically,
Figure 3.12: Validation of physical continuity of collagen gels. The physical integrity of the collagen matrix was validated by loading the two layers with beads of different colors, and tracking the tissue-induced displacements of the layers separately. (A) In fully bound collagen gels, tissue-induced displacements propagated smoothly, with no apparent jumps or discontinuities at the boundary between the top and bottom layers. (B) Physical detachment of the top and bottom layers, which occurs but rarely (<5% of samples), is accompanied by a distinct cellular phenotype: cells fail to reorganize into quiescent epithelial tubules, and migrate as monolayers out of the wells, between the two collagen layers. (C) An asymmetric displacement profile was recorded within gels in which the top and bottom layers were detached. The bottom layer was displaced dramatically, whereas negligible displacements were observed in the top layer. Scale bars, 50 μm.
we embedded beads of different colors into each layer before microfabrication, which allowed us to distinguish between displacements induced by the tissue in each domain separately (Fig. 3.12). By visual inspection, the displacement field propagated smoothly and continuously across the boundary from the bottom to the top layer. Consistently, the x-, y- and z-displacements were a continuous function of depth, with no discontinuities or jumps apparent at the boundary between the layers (Fig. 3.12A). Moreover, the displacement profiles were symmetric about the midsection of the tissue in the z-direction, i.e. displacements around the interface between the two layers were similar to those at the same depth below the tissue, where the gel is truly continuous. In a small fraction (<5%) of all microfabrication experiments, the gels failed to bind fully, owing to a film of liquid trapped between the two layers. Such cases were readily identifiable, as the cells failed to form a quiescent epithelial tubule, and instead migrated as monolayers between the two gels (Fig. 3.12B). An asymmetric displacement profile was recorded within gels in which the top and bottom layers were detached. The bottom layer was displaced dramatically, whereas negligible displacements were observed in the top layer. (Fig. 3.12C).

**Mechanical properties, constitutive model of collagen gels and calculation of stress**

The material properties of the collagen gels were determined via bulk rheometry using the cone-and-plate setup on a Physica MCR 501 rheometer (Anton Paar). The chamber was held at 37°C and 100% humidity using a Peltier plate and humidity chamber. Oscillatory strains were imposed ranging between 0.01% and the maximum cell-induced strains of 7%. The stress-strain relationship that was recorded remained linear throughout
the strain regime. Accordingly, Hooke’s law for isotropic materials was used to describe the constitutive behavior of the collagen gels during tissue-induced deformation:

\[
T_{ij} = \frac{1}{2} [\lambda \varepsilon_{ik} \delta_{ij} + 2 \mu \varepsilon_{ij}] 
\]

(8)

\[
\lambda = \frac{\nu E}{(1+\nu)(1-2\nu)} 
\]

(9)

\[
\mu = \frac{E}{2(1+\nu)} 
\]

(10)

where \(\delta_{ij}\) is the Kronecker delta, \(T\) is the Cauchy stress tensor, \(\mu\) and \(\lambda\) are the Lamé parameters, \(E\) is the Young’s modulus and \(\nu=0.2\) is the Poisson ratio (143). The fully determined strain field allowed us to calculate the Cauchy stress directly (using Eqs. 8-10), in a forward fashion thus circumventing the need to make assumptions about the stress state and geometry and invoking ill-posed inverse formulations.

**Epithelial tissue surface reconstruction, mesh generation and calculation of surface tractions**

Cell membranes within the engineered epithelial tissues were visualized using the Vybrant DiO dye (Invitrogen) and confocal stacks of 30 images (spaced 2-\(\mu\)m apart in the z-direction) of the tissues were collected. Epithelial surfaces were rendered using Imaris and reconstructed using AutoDesk Inventor Professional. The components and magnitude of the surface traction vector were calculated from the Cauchy stress tensor as follows:

\[
t_i = \sum_j T_{ij} n_j 
\]

(11)
\[ |\vec{t}| = \sqrt{t_1^2 + t_2^2 + t_3^2} \]  \hspace{1cm} (12)

where \( t_i, i = 1,2,3, \) are the components of the stress vector; \( n_j, j = 1,2,3, \) are the components of the unit normal vector at a point on the epithelial surface; \( T_{ji} \) are the components of the Cauchy stress tensor, and \( |\vec{t}| \) is the magnitude of the traction vector.

To simplify solving Eq. 11 over a complex 3D geometry, we used the finite element method (FEM). Specifically, the reconstructed 3D surface was imported into the Comsol Multiphysics 3.5a modeling environment and enclosed within a second computational domain of cylindrical geometry (2 mm in height and diameter), representing the collagen gel. A quadratic tetrahedral finite element mesh of the epithelial surface and the surrounding gel was subsequently generated.

Validation of model for collagen

To validate our assumptions of homogeneity and isotropy, we took advantage of the fact that the strain and stress state of an elastic solid, along with its internal deformation field, is uniquely determined by a set of displacement boundary conditions and knowledge of the solid’s material properties and constitutive behavior. We thus used the experimentally measured displacements of the gel at the boundary of the epithelial tissue to simulate the corresponding internal displacements within an ideally elastic, homogeneous and isotropic medium. The simulated displacements were then compared to the experimentally measured internal displacements. More specifically, we first substituted Eq. 7 into Eq. 8 to obtain the stress in terms of displacement gradients, and then substituted the result into the equilibrium form of the equation of motion (Eq. 13) to
obtain three second-order partial differential equations for the three displacement components (Eq. 14):

\[
\frac{\partial T_{ji}}{\partial x_j} = 0
\]  

\[
(\lambda + \mu) \frac{\partial^2 u_k}{\partial x_i \partial x_k} + \mu \frac{\partial^2 u_i}{\partial x_k \partial x_k} = 0
\]  

The boundary conditions were as follows: three experimentally measured displacement components at the epithelium-matrix interface and three zero-displacement components at the outer boundaries of the collagen gels (zero displacement far away from the tissue). Given the complex 3D geometry of the tissue-matrix boundary, Eq. 14 was solved using FEM.

**Confocal reflection microscopy**

The structure of the collagen around the tissues was visualized using a Leica SP5 laser-scanning confocal microscope. Collagen matrices were illuminated with an Argon laser (488 nm) and imaged in reflection mode using a 20× oil-immersion objective. Images of the collagen surrounding 20 tissues or cell-free collagen cavities were taken at a z-position corresponding to the middle of the tissue or well. The raw intensities of the 20 images were averaged using ImageJ and color-coded using Adobe Photoshop to generate color maps of reflection signal intensity.

**Atomic force microscopy (AFM)**

AFM analyses to probe the local elasticity of collagen gels were performed on an MFP 3D system (Asylum Research) in a liquid environment to prevent drying of the gel. Force
measurements were conducted using cantilevers with low spring constants (k=0.05 N/m; MikroMasch). After initial contact with the collagen gel surface, force vs piezo displacement (F-z) data were collected at a speed of 1 Hz for a total z-distance of 4 μm. Since the tissues have two axes of symmetry, only the upper left quadrant was scanned with a resolution of ~10 μm. Three F-z curves were recorded at each location probed. Young’s moduli were computed using the Elastic algorithm for a pyramidal tip (Igor Pro; Wavemetrics). Indentation measurements to compare the elasticity of the gel near the side and the end of the tissue were performed on five separate samples, and full elasticity maps were generated for three separate samples. Magnitudes and distribution of elasticity were highly reproducible between samples.
Chapter 4

Physical mechanisms of mammary branch extension in 3D collagenous matrices

4.1 Introduction

The generation of a full and functional mammary epithelial ductal tree is dependent upon successive rounds of bifurcation and forward extension of mammary ducts. The mammary duct, led by the TEB, is a highly motile organ which elongates at rates as high as 0.5 mm per day during the process of branching morphogenesis (171).

It is clear that branching morphogenesis is triggered by endocrine hormones at the onset of puberty and propelled by dynamic and bidirectional epithelial-stromal crosstalk (3). Nevertheless, despite the fact that mammary ductal extension is regulated by molecular cues, it is ultimately a translocation process wherein epithelial cells penetrate *en masse* through a dense fibrous stroma containing a variety of cell types (adipocytes, contractile fibroblasts, blood vessels, immune cells). The dynamic deformation and remodeling of the stroma, which undoubtedly accompany branch elongation as a means to overcome the resistance to forward movement, are processes physical by nature. The physical character of this morphogenesis is underscored by studies demonstrating that TEBs can successfully sense and avoid mechanical obstacles, which are devoid of a chemical that
may diffuse and serve as a long-range signal to repel advancing branches (171). The physical mechanisms which drive mammary branch extension are poorly understood.

Several models of the physical mechanisms of mammary branch extension have been formulated, based upon observations of the ductal architecture and the surrounding ECM structures. Early histochemical and autoradiographic visualization approaches have shown that bundles of fibrillar type I collagen are deposited preferentially around the subtending duct (172). It has been hypothesized that these rigid collagen deposits serve as circumferential restraints which channel the expansive force generated by proliferation within the TEBs into forward extension (171). A recent study by Bissell and colleagues assumed a similar ‘pushing’ model to estimate the forces which must be exerted by growing mammary epithelial branches to overcome the resistance of the ECM (173). These researchers concluded that the conical morphology assumed by mammary epithelial branches in collagenous matrices is optimal for overcoming ECM resistance upon translocation. In addition, they suggested that branches elongate by following paths of lower resistance, created by proteolytically-mediated softening of the matrix.

The aforementioned studies relied upon static snapshots of mammary branches in vivo or in culture to draw conclusions about duct extension, despite the fact the process is dynamic by nature. Recent advances in time-lapse confocal microscopy have enabled visualizing the branching morphogenesis of Matrigel-embedded primary mammary organoids in real-time (174). Nevertheless, dynamic imaging of the elongating epithelium alone is not sufficient to rigorously define the physical mechanisms of ductal extension.
Concomitant physical changes within the stromal compartment must be elucidated at the same time.

We used a combination of time-lapse confocal microscopy and confocal reflection imaging to visualize both the epithelial and matrix compartments during branching morphogenesis of engineered mammary epithelial tissues. We discovered that mammary branches pull on the collagen matrix as they extend, generating tensile forces. In addition to playing a physical role during the translocation, these forces enhance the process by eliciting mechanosensitive signaling within the cells of the leading edge and by reorganizing the collagen matrix ahead of the extending branch. The results of this study provide insight into how mammary ducts elongate during branching morphogenesis to build the functional gland, but may also shed light on the general physical mechanisms that drive collective cell migration in fibrous extracellular matrices.

4.2 Results

4.2.1 Mammary epithelial branches extend by pulling on the surrounding ECM

To explore the physical mechanisms of mammary branch elongation, the culture model of mammary epithelial branching morphogenesis described in Chapter 2 was used. Briefly, a combination of photolithography and soft embossing was used to generate an organotypic array of mammary epithelial tissues embedded within a 3D matrix of type I collagen (102) (Fig. 4.1A, t=0 h). Upon stimulation with HGF, the tissues undergo
Figure 4.1: Mammary epithelial branches extend by exerting tensile forces on the 3D matrix. (A) Branch initiation and extension from 3D engineered mammary epithelial tissues. (B) Collagen matrix is displaced toward the tissue, as epithelial branches initiate and elongate. Confocal stacks of GFP-actin (C) were used to reconstruct the surface of the branched epithelial tissues (D). Matrix displacements due to branch extension (B) were used to calculate the forces experienced by the tissue at a snapshot during the process of branching. Scale bars, 50 μm. Adapted from (Gjorevski and Nelson, in preparation).
branching morphogenesis: multicellular branches initiate at the end of the cylindrical tubules and begin elongating through the collagenous matrix (Fig. 4.1A, t=4-24 h). This method generates hundreds of branching tissues positioned at the same z depth within the gels, allowing for facile simultaneous imaging of multiple tissues and thus enabling rigorous statistical analysis and increasing the confidence in interpretation of the data. To define the physical mechanisms of branch extension, we simultaneously imaged the deformation of the cellular and matrix portions of the tissues. The epithelium was visualized by transducing the cells with an adenovirus encoding a Lifeact-GFP fusion protein (175). The movements of the matrix were visualized by embedding fluorescent markers within the collagen gel. We discovered that, as branches initiated and extended through the matrix, the resident beads at adjacent locations moved toward the growing branch (Fig. 4.1B). This suggests that the epithelial branches elongate by using a pulling mechanism. To rigorously characterize the mechanical events underlying the process, we set out to calculate the forces generated.

Calculating the forces generated and experienced by the ECM closely surrounding the branching tissue required numerical accessibility to the complex and dynamically evolving 3D geometry. Accordingly, we reconstructed the epithelial surface from 3D confocal fluorescence imaging data, converted it into a solid domain and generated a tetrahedral mesh (Fig. 4.1C,D). We adapted the method for mapping of mechanical forces within quiescent epithelial tissues to calculate the forces over the branching tissue at a snapshot in time (Fig. 4.1E). As expected, the forces were highest at the leading edge of the growing branches. Importantly, the forces at these locations were directed inward and
Figure 4.2: Primary mammary and breast cancer cells collectively migrate through collagen matrices using a pulling mechanism. Clusters of EpH4 normal mouse mammary epithelial cells (A), primary mammary organoids (B) and clusters of MDA-MB-231 invasive breast cancer cells (C) embedded in 3D collagen gels. In each case, the cells collectively invaded by pulling on the matrix. Scale bars, 50 μm. Adapted from (Gjorevski and Nelson, in preparation).

were therefore tensile, confirming that branch extension was driven by pulling against the impeding matrix.

To ensure that the mechanism described above was not specific to the engineered model of branching morphogenesis, we measured matrix displacements which occur within two classic models of branching: mammary epithelial cell clusters and primary mouse mammary organoids embedded within type I collagen (Fig. 4.2A,B). We found that mammary clusters and organoids branched using an analogous physical mechanism. The collagen matrix preceding the leading edge was pulled toward the extending branch. The
resulting displacement fields were highly localized and directional and propagated several hundred micrometers ahead of the leading edge (Fig. 4.2D,E). Surprisingly, invasive breast cancer cells underwent collective migration into the collagen gels using the same pulling mechanism (Fig. 4.2C,F). However, in contrast to epithelial branches, cancer cell invasions induced significantly smaller and less spatially localized and directed matrix displacements.

4.2.2 Tensile forces at tips of branches enhance elongation by eliciting mechanosensitive signaling and transcription

To test whether the tensile forces that arose at the tips of the growing branches were required for extension, we blocked cytoskeletal tension by treating the tissues with blebbistatin, an inhibitor of actomyosin-mediated contractility. Keeping in mind that contractility and tension in this system are required for branch initiation from the preexisting tissue, the inhibitor was added after the branches had initiated. Blocking cell-generated tension prevented branch extension (Fig. 4.3A).

Mechanical forces are indispensable in biology at the physical level, driving the cellular deformations which sculpt developing tissues and organs. In addition to their physical role, the signaling and regulatory functions of mechanical forces have become apparent (176). To examine whether the tensile forces which arise at the tips of the extending branches serve a role beyond facilitating the physical translocation of the epithelium, we visualized mechanoresponsive signaling pathways within the tissues. FAK and p130
Figure 4.3: Tensile forces drive branch extension by activating mechanically sensitive intracellular signaling and transcription. (A) Branch extension from control tissues and tissues treated with blebbistatin (12.5 μM). (B) FAK pY397 and phospho-p130 Cas immunofluorescence in control and blebbistatin-treated branches. MRTF-A localization in (C) control and (D) blebbistatin-treated branches. (E) Branch extension from control tissues and tissues treated with CCG-1423 (10 μM). (F) Branch initiation and extension from control tissues and tissues transfected with shMRTF-A. Scale bars, 50 μm. Adapted from (Gjorevski and Nelson, in preparation).

Cas are well-documented mechanosensory proteins, which undergo enhanced activation in response to mechanical stimulation (80-81, 177). Both proteins signal downstream of integrins to direct a variety of cellular processes including cell survival, adhesion, motility and invasiveness (83, 122). Importantly, FAK has been implicated in mammary
gland development: deletion of FAK in the gland prevents ductal elongation in vivo (123). Immunofluorescence analysis revealed stronger activation of both FAK and p130 Cas within the growing branches, as evidenced by their localization to discrete cell-ECM adhesions (Fig. 4.3B). Consistently, disrupting cytoskeletal tension abolished the enhanced activation within the branches (Fig. 4.3B).

The collective migration of border cells during Drosophila oogenesis is driven by tension-mediated activation of serum response factor (SRF) and its cofactor MRTF-A (178). Cell stretching in response to tensile forces generated during collective invasion induces nuclear translocation of MRTF-A, its association with SRF and subsequent transcription of SRF-target genes, regulating differentiation, proliferation and motility. Notably, MRTF-A is also required for branching morphogenesis of the Drosophila trachea (179) and has recently been found to be subject to an analogous form of tensional regulation in mammary epithelial cells (61). To test whether mammary branch extension, itself a form of collective epithelial cell migration, is regulated by MRTF-A in a tension-dependent fashion, we stained the branching tissues for the transcription factor (Fig. 4.3C). We found that MRTF-A was mostly nuclearly localized within the extending branches, whereas localization within the initial tissue was both nuclear and cytoplasmic. Abolishing cellular tension within the tissue attenuated the nuclear translocation of MRTF-A, and retarded branch extension (Fig. 4.3D). To determine whether MRTF-A activity is required for branch extension, we treated the tissues with CCG-1423, which blocks the interaction between MRTF-A and SRF (180). Disrupting MRTF-A activity significantly impaired branch extension (Fig. 4.3E). We also confirmed the effect by
taking a molecular approach: shRNA-mediated depletion of MRTF-A within the tubules significantly reduced branch initiation and extension compared to the control tissues transfected with scrambled shRNA (Fig. 4.3F).

4.2.3 Tensile forces enhance branch extension through matrix reorganization at the leading edge

It has been recently suggested that, during mammary epithelial branching morphogenesis in collagenous matrices, branches extend by following paths of lower resistance, created by protease-mediated degradation and softening of the ECM (173). To test for such a scenario, we used confocal reflection microscopy to visualize the structure of the collagen matrix surrounding the branching epithelium. We discovered that matrix degradation indeed occurs (Fig. 4.4A). However, the degradation was pericellular, i.e. sufficient to accommodate the growing structure, with no apparent loss of collagen structure indicative of proteolysis ahead of the leading edge.

To resolve potential alterations within the fibrillar structure of collagen during branch extension, we embedded clusters of mammary epithelial cells within sparse (concentration of 2 mg/ml) collagen matrices and induced branching morphogenesis. In this case, we observed dramatic remodeling of the collagen matrix ahead of the leading edge of the branch: the collagen fibrils were compacted and aligned into parallel and highly directional tracks emanating from the tip of the branch (Fig. 4.4B). By contrast,
the collagen fibers far away or near non-branching regions of the epithelium were considerably sparser and randomly oriented. Visualizing the matrix around branching primary organoids revealed a similar form of reorganization, even in dense (6 mg/ml) collagen gels: the matrix was remodeled into dense and spatially localized bundles,
propagating far away (~300 μm) from the tip of the extending branch (Fig. 4.4C). Notably, treatment of the branching tissues with contractility inhibitors abolished the collagen reorganization ahead of the leading edge (Fig. 4.4D), indicating that the effect was mediated by tensile forces generated by the migrating cells.

4.3 Discussion

The physical mode by which epithelial ducts extend through the complex stroma during the process of branching morphogenesis is one of the major outstanding questions in mammary gland development. We used a combination of confocal time-lapse imaging, confocal reflection microscopy and continuum mechanics to demonstrate that mammary epithelial branches move through 3D collagenous matrices by using a pulling mechanism: the cells at the leading edge of the branch initiate actin-rich protrusions which attach to collagen fibers within the impeding matrix, deforming it as they pull themselves and the following cells forward.

We measured the matrix deformations which accompany branching morphogenesis and characterized the underlying mechanical forces for the first time, confirming that the process is physically driven by tensile forces generated by cytoskeletal contraction. Although the approach used to calculate the forces from knowledge of the epithelial geometry and the deformation of the matrix is qualitatively informative with regard to the spatial distribution and direction of force, there likely are significant errors in the magnitudes of force reported, owing mostly to the simplifying assumptions made while
solving the problem. Specifically, the matrix surrounding the epithelium was approximated as a linearly elastic, mechanically homogeneous and isotropic solid. In reality, the local strains experienced by the matrix adjacent to extending branches exceed 10% and are applied at low rates (~24 hrs), making an assumption of non-linear and viscoelastic behavior a more suitable model (143). Furthermore, the highly directional alignment and bundling of collagen fibers near the leading edge of the branches (Fig. 4.4) likely introduce anisotropies and heterogeneities, which are dynamic and evolve both spatially and temporally. Incorporating all of the levels of physical complexity into the reconstruction of stresses is a formidable task, owing to both technical and theoretical considerations. Nevertheless, some of the experimental tools detailed in this and the previous chapters can be used in conjunction with recent theoretical formulations to make advances toward untangling this complex problem. For instance, keeping in mind that confocal reflection microscopy allows visualization of the structure of collagen without cellular fixation or use of exogenous labeling agents, it can be used to monitor spatiotemporal structural variations that may affect the mechanical properties and constitutive behavior of the matrix. Indeed, theoretical frameworks have been recently devised to incorporate externally-introduced bulk-level fiber orientations into an anisotropic constitutive model for collagen (181-182). These could be adapted and applied specifically near the leading edge of extending branches to account for local reorganization and directionality of the ECM. Laser tweezer-based microrheology approaches could be used to spatially and temporally map the mechanical heterogeneities around the branching tissues without fatally interfering with the morphogenetic program (183-184).
The cells at branching locations thus extend protrusions, attach to the surrounding ECM and apply tensile forces on the adhesion in order to propel themselves forward and branch. However, fundamental principles of mechanics stipulate that the cells not only exert but also experience tension, and we presented evidence that this tension might not serve a merely physical, but also a regulatory role in the process of branch elongation. Specifically, the tensile stresses which accumulate at branch tips may elicit local mechanosensitive signaling and transcription changes which reinforce the collective migration potential of the cells. We found that the mechanosensory proteins FAK and p130 Cas, which are known to enhance motility and invasiveness (83, 122, 177), are preferentially activated within growing branches and that this activation was tension-dependent (Fig. 4.3). Furthermore, we showed that, in response to high local tension, the transcription factor MRTF-A undergoes nuclear translocation and activation within extending branches (Fig. 4.3). The target genes expressed include those encoding major cytoskeletal proteins and modifiers thereof (178-179), which are themselves involved in both generating tension and driving migration. These findings suggest that the leading edge of the extending epithelial branch might be a site of a positive mechanochemical feedback loop, wherein forces that drive translocation may trigger cellular responses which ultimately lead to additional force generation and migration. Sophisticated studies in which the temporal profile of signaling events is compared against the dynamics of force generation are required to investigate this hypothesis rigorously.
In addition to regulating branch extension through the induction of mechanosensitive intracellular signaling, tensile forces may facilitate collective migration by conditioning the matrix ahead of the leading edge. We observed the formation of highly aligned collagen fiber ‘tracks’, which propagated from the tip of the growing branch into the unpopulated regions of the 3D matrix (Fig. 4.4). It is possible that the formation of these tracks allows more efficient forward migration by removing randomly-oriented fibers that might otherwise physically hinder cellular movement. Indeed, distinct radial reorganization of collagen fibers surrounding the invasive regions of breast tumors in vivo has been previously observed (116, 185). Moreover, culture studies demonstrated that this reorganization was a result of cellular contractile activity and that it served to facilitate further invasion of breast cancer cells (156). Our data suggest that the directional alignment of ECM fibers is not a physical feature specific to cancer cell invasion, but might be a general strategy used by collectively migrating cohorts, including mammary epithelial branches, to overcome steric hindrances within dense 3D matrices. Keeping in mind the striking directionality of the collagen tracks formed ahead of the leading edge, it is tempting to speculate that they are used by the extending branch as means for spatial navigation through the 3D matrix.

In summary, this study revealed a previously unforeseen physical mechanism for mammary branch extension through dense collagenous matrices. Contrary to theories attributing forward movement to pushing forces (153, 171) or MMP-mediated ECM degradation (173), we discovered that mammary epithelial branch extension in culture was driven by tensile (pulling) forces. These forces served a role beyond the physical
one: they enhanced the migratory process by eliciting mechanosensitive signaling within the branching cells and by remodeling the ECM into aligned tracks. These data may help unlock strategies to use exogenous forces or their downstream cellular and ECM effects to stimulate the growth and extension of mammary branches \textit{ex vivo}.

\section*{2.4 Materials and methods}

\textbf{Cell culture and reagents.} Functionally normal EpH4 mouse mammary epithelial cells were cultured in 1:1 Dulbecco’s Modified Eagle’s Medium: Ham’s F12 nutrient mixture (DMEM:F12) (Hyclone), 2\% fetal bovine serum (Atlanta Biologicals), 5 \(\mu\text{g/ml}\) insulin (Sigma), 50 \(\mu\text{g/ml}\) gentamycin (Sigma). Growth medium was supplemented with HGF (Sigma, 5 ng/ml). Tubules were treated approximately 4 hrs after induction of branching with CCG-1423 (Cayman Chemical, 10 \(\mu\text{M}\)) or blebbistatin (Sigma, 25 \(\mu\text{M}\)). Invasive MDA-MB-231 cancer cells were cultured in DMEM supplemented with 10\% fetal bovine serum and 50 \(\mu\text{g/ml}\) gentamycin. Cells were visualized for time-lapse imaging by transduction with adenoviruses encoding H2B-mCherry or LifeAct-GFP.

\textbf{Microfabrication.} Elastomeric stamps of poly(dimethylsiloxane) (PDMS; Sylgard 184, Ellsworth Adhesives) containing the desired geometries in bas-relief were fabricated by a combination of photolithography and soft lithography (29, 102). Stamps were rendered non-adhesive by coating with 1\% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Modified stamps were placed atop a drop of neutralized liquid collagen mixture (Koken, Japan), containing carboxylate-modified fluorescent microspheres.
(Molecular Probes), which was then gelled at 37°C. Stamps were removed and a suspension of mammary epithelial cells was allowed to settle within the molded collagen cavities. The extra cells were washed away with culture medium and a gelled collagen “lid” was placed on top of the pattern. The epithelial cells adopted the shape and size of the collagen cavities they occupied, forming tubules that remained quiescent until they were induced to branch with HGF.

**Preparation and branching of cell clusters.** Clusters of mammary epithelial cells were prepared by overnight shaking (180 r.p.m. at 37°C for 15 h) in the presence of 0.083% (w/v) of pluronic F108 (BASF). Cell clusters of ~100 mm in diameter were collected by centrifugation (200 r.p.m. for 1 min) and embedded within 4 mg/ml of non-pepsinized native type I collagen (Koken). A cell-free layer of collagen was placed underneath the layer containing clusters. Growth media including no growth factor, EGF (4.2 nM, Invitrogen), or HGF (4.2 nM, Sigma) was added to the samples.

**Isolation and culture of primary mammary organoids.** Primary mammary epithelial organoids were prepared from 8-week-old CD1 mice (Charles River). Inguinal glands were removed aseptically, minced with razor blades, and incubated with agitation (350 rpm) at 37°C in DMEM/F12 supplemented with 0.2% collagenase (Roche), 0.2% trypsin (Sigma), 5% FBS, 5 μg/ml insulin, and 50 μg/ml gentamicin. The digested tissue was centrifuged at 1800 rpm for 10 min. The supernatant was removed, centrifuged three times, and the resulting cell pellet was resuspended in DMEM/F12 supplemented with 20 U/ml DNase I (Sigma). The organoids were separated from single cells (mainly
fibroblasts) using differential centrifugation. Organoids were resuspended in DMEM/F12 supplemented with 10% FBS, 5 ng/ml EGF, insulin/transferrin/sodium selenite (ITS; Sigma), penicillin/streptomycin (Sigma), and gentamicin, and immediately embedded in collagen gels.

**Immunofluorescence analysis.** Samples were washed in PBS and fixed in 4% paraformaldehyde for 15 min at room temperature. Samples were permeabilized 2 x 10 min in 0.5% Igepal Ca-630 and incubated in PBS-T for 15 min. Samples were blocked overnight at 4°C in PBS-S, followed by overnight incubation at 4°C in primary antibody recognizing FAK pY397 (Invitrogen), phospho-p130 Cas (Cell Signaling) and MRTF-A (Sigma) at 1:100 dilution in PBS-S. Samples were washed extensively with PBS and incubated in secondary antibody at 1:1000 in PBS-S overnight at 4°C.

**Transfections.** Mouse pLKO.1 lentiviral MKL1 shRNA (shMRTF-A#1) and shMRTF-A#2 were obtained from Open Biosystems. Mouse pLKO.1 lentiviral scrambled shRNA was obtained from Addgene. Cells were transfected with plasmids using the Fugene HD (Roche) delivery agent.

**Real-time microscopy and measurement of matrix displacements.** Time-lapse movies were collected using a Hamamatsu ECCD camera attached to a Nikon Ti-U inverted microscope customized with a spinning disk (BD Biosciences) and fitted with a humidified environmental chamber held at 37°C and 5% CO₂. The branching epithelium transduced with LifeAct-GFP and the fluorescent beads were imaged simultaneously in
the green and red channels, respectively. Confocal stacks (390×390×100 μm, spaced 1 μm in the z-direction) were acquired using a Plan Apo 20× 0.4 NA objective every 2 hrs beginning at 24 hours after initial microfabrication for a total of 20 hours. 3D and in-plane bead displacements were extracted using the Autoregressive Motion tracking algorithm in the image analysis and tracking software Imaris (Bitplane). The displacement gradient matrix was for finite strains used to calculate the tissue-induced strains within the collagen gel:

\[
\varepsilon_{ij} = \frac{1}{2} \left( \frac{\partial u_i}{\partial x_j} + \frac{\partial u_j}{\partial x_i} - \frac{\partial u_k}{\partial x_j} \frac{\partial u_k}{\partial x_i} \right)
\]

(1)

**Mechanical properties and constitutive model of collagen gels.** The material properties of the collagen gels were determined through bulk rheometry using the cone-and-plate setup on a Physica MCR 501 rheometer (Anton Paar). The chamber was held at 37°C and 100% humidity using a Peltier plate and humidity chamber to mimic experimental conditions of the engineered tissues and to prevent drying out of the collagen gel. Hooke’s law for isotropic materials was used to describe the constitutive behavior of the collagen gels during tissue-induced deformation:

\[
T_{ij} = \frac{1}{2} \left[ \lambda \varepsilon_{kk} \delta_{ij} + 2 \mu \varepsilon_{ij} \right]
\]

(2)

\[
\lambda = \frac{\nu E}{(1+\nu)(1-2\nu)}
\]

(3)

\[
\mu = \frac{E}{2(1+\nu)}
\]

(4)
where $\delta_{ij}$ is the Kronecker delta, $T$ is the Cauchy stress tensor, $\mu$ and $\lambda$ are the Lamé parameters, $E$ is Young’s modulus and $\nu=0.2$ is the Poisson ratio (143).

**Epithelial tissue surface reconstruction and mesh generation.** The surface of the branched epithelium at a snapshot in time (24 hrs after branch induction) was reconstructed from 3D confocal stacks showing actin-GFP within the cells. Image segmentation to define the cellular portion of the 3D stack was performed manually in ImageJ (NIH). A 3D surface was subsequently generated using Amira (Visage Imaging) and converted to a parasolid object using Mesh2Solid (Sycode). The solid was imported into Comsol Multiphysics 4.2a (Comsol Inc) and enclosed within a second computational domain of cylindrical geometry (2 mm in height and diameter), representing the collagen gel. A quadratic tetrahedral finite element mesh of the epithelial surface and the surrounding gel was generated.

**Calculation and reporting of mechanical stress.** The equations of motion, the displacement gradient matrix (Equation 1), and Hooke’s law for isotropic materials (Equations 2-4), were used to solve for the Cauchy stress tensor throughout the domain. The boundary conditions were as follows: displacements at the epithelium-matrix interface were interpolated from experimentally measured bead displacement values, and displacement of zero was assumed far away from the tissue, at the outer boundaries of the collagen gel. The components and the magnitude of the traction vector at a point on the epithelial surface were calculated as:

$$ t_i = \sum_j T_{ij} n_j $$ (5)
\[ |\mathbf{t}| = \sqrt{t_1^2 + t_2^2 + t_3^2} \]  

(6)

where \( t_i, i = 1,2,3, \) are the components of the stress vector; \( n_j, j = 1,2,3, \) are the components of the unit normal vector at a point on the epithelial surface; \( T_{ij} \) are the components of the Cauchy stress tensor, and \( |\mathbf{t}| \) is the magnitude of the traction vector.

**Confocal reflectance microscopy.** Collagen structure was visualized with a Leica SP5 laser-scanning confocal microscope (Leica Microsystems). Unlabeled collagen matrices were illuminated with an Argon laser (488 nm) and imaged in reflection mode using a 20× and 63× glycerin-immersion objectives.
Conclusions and future directions

The intense study of mammary gland biology over the past century has revealed that a plethora of endocrine, paracrine, autocrine and ECM signals cooperate to regulate mammary development. Nevertheless, key questions pertaining to pattern formation within the gland, including the selection of branch sites, have remained unanswered (3, 103, 105, 186). Motivated by reports underlining the potential role of physical signals in branching morphogenesis (77-78, 109), we set out to examine whether mechanical forces serve as instructive cues in mammary development.

Using a combination of microfabricated tissues, computational modeling and 3D traction force microscopy, we discovered that mechanical forces were distributed non-uniformly and formed gradients over mammary epithelial tissues of anisotropic (non-spherical) geometries. We demonstrated that these gradients served to template branching from the initial structure. There was striking spatial correlation between the distribution of mechanical stress and the pattern of branching: branches initiated from sites experiencing elevated mechanical tension. Modulating mechanical stress pharmacologically or molecularly led to concomitant changes in the extent of branching. The tissue-level mechanical gradients were required for patterned branching morphogenesis, as abolishing
these gradients by disrupting intercellular transmission of force inhibited branching. We discovered further that mammary epithelial tissues sensed high local mechanical tension through FAK, a well documented mechanosensory protein which was activated specifically at sites within the tissue experiencing high forces. Inhibiting mechanosensing by inactivation of FAK prevented branching from high-stress locations. We also defined the gene expression changes and resulting cellular behaviors whereby mechanical stress may promote branching. Our group has observed the acquisition of mesenchymal attributes within epithelial cells at branching sites (29, 132). We discovered that these phenotypic changes were necessary and sufficient for branching, and that they were driven by the localized induction of EMT-related transcription factors Snail1, Snail2 and E47 (132). Preliminary studies indicate that the patterned expression of these EMT-related genes is induced by the aforementioned mechanical gradients, and begin to spell out the underlying mechanism. Mechanical gradients promote patterned EMT in sheets of mammary epithelial cells by regulating the extent of nuclear translocation and resulting activation of the transcription factor MRTF-A (61). Consistently, down-modulating mechanical tension within branching mammary tissues led to the loss of nuclear MRTF-A within the branches, which ceased to elongate thereafter.

This work also defined the physical mechanism whereby mammary epithelial branches elongate in 3D fibrillar matrices. We found that the forward translocation is driven by tensile forces, which cells at the leading edge of the branch exert onto the impeding collagen fibers. These forces enhance collective movement by triggering mechanosignaling within the cells and by conditioning the matrix for further invasion.
This work has furnished novel tools and fundamental concepts, whose applicability and relevance may reach further than the field of mammary branching morphogenesis. To define the role of the mechanical environment in mammary development, we moved the existing methodologies for measuring mechanical parameters into the more physiological 3D, heterogeneous and multicellular realm. These tools can be readily used to address a range of mechanobiological questions in a variety of contexts. Furthermore, the modes of mechanotransduction that have emerged from this research may be quite general and utilized in processes other than mammary epithelial branching morphogenesis.

At the fundamental level, this work unveiled novel mechanisms for pattern formation during morphogenesis of 3D epithelial tissues. Concentration gradients of diffusible biochemicals (morphogens) forming over fields of cells are a textbook mechanism of morphogenetic pattern formation (187-189). We showed that forces generated by contraction at the single-cell level can be transmitted and concentrated into tissue-level mechanical gradients, which can also serve a patterning role during morphogenesis of 3D epithelial tissues. Collectively, our data support a long-standing hypothesis (190), underscoring the geometric control of morphogenesis, which only recently began receiving experimental confirmations (29, 98, 161, 191). This paradigm asserts that morphogenesis is self-referential, and that the patterning information which sculpts the future shape is encoded within the shape of the pre-existing structure.
It is important to emphasize that these data were generated using culture models of mammary epithelial branching morphogenesis and that these principles are yet to be confirmed in the native mammary environment. Owing mostly to poor physical and optical accessibility, however, the problem is challenging to investigate in vivo. Methods are needed to measure spatiotemporal variations in the physical properties and force landscape of the developing mammary gland, as well as to perturb these quantities reproducibly. In the absence of such techniques, mechanical stresses can be inferred indirectly by visualizing mechanically sensitive molecules such as phosphorylated myosin light chain, FAK or Src (49, 192). Indeed, immunohistochemical analyses have revealed that tenascin-C, the secretion of which is enhanced by mechanical tension (193), is present at high levels around the TEBs (194), consistent with our computational models and culture studies predicting elevated mechanical stress in these regions. Promising techniques used to directly explore mechanics during the development of other organs could also be adapted for the mammary gland. Morphometric analyses of tissue deformation during morphogenesis, performed by tracking fluorescent markers immobilized onto the epithelial surface, have quantified mechanical strain and stress during cardiac looping and head fold formation within the avian embryo (195-196). These techniques could be combined with emerging approaches for intravital imaging of the mammary gland (197-200), including optical coherence tomography, multiphoton microscopy, or mammary ‘window’ imaging (199) which permits long imaging sessions (up to 24 hours) over multiple days.
We thus propose that the pattern of the mammary epithelial tree is a product of a morphodynamic process integrating molecular and mechanical signals over multiple length and time scales. The specific roles of individual molecular regulators have been investigated for years and, in a separate effort, we dissected the contribution of mechanical signals using engineered culture models. Securing multi-scale and comprehensive knowledge of the process, however, will require taking systems-level approaches to synthesize data from separate sources into a ‘mammary branching regulatory network’. These efforts would couple spatiotemporal measurements of mechanical parameters with large-scale molecular studies, such as genome-wide transcript analyses, and monitoring of downstream phenotypic changes, including cell proliferation, apoptosis, shape change, migration and EMT. The resulting information would instruct better-targeted perturbation of molecular and mechanical components alike to examine both their effect on the branching phenotype and their mutual crosstalk. As these large amounts of data accumulate, the challenge will lie in interpretation and synthesis into plausible multiscale models and regulatory networks of branch formation. Sophisticated in silico simulation approaches will likely prove helpful, if not necessary to accomplish this formidable task.
Bibliography


